

Enhanced allergic airway disease in old mice is associated with a Th17 response

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Summary

Background The prevalence of asthma in the elderly is increasing and associated with higher mortality than in children or young adults. However, the effects of age on the development and character of allergic asthma have been understudied. It has been suggested that mixed Th2/Th17 responses cause more severe forms of asthma, but the role of Th17 response in allergic airway disease and aging is not well understood.

Objective To investigate age-dependent characteristics and Th17 immune response in allergic airway disease in a murine house dust mite (HDM)-allergen model.

Methods Twelve-week-old and 15-month-old male BALB/c mice were sensitized and challenged with HDM. Bronchoalveolar lavage fluid (BALF), airway inflammation and hyper-responsiveness (AHR), serum immunoglobulin and splenic T cells were assessed. Age-related T cell activation was analyzed in a co-culture with bone marrow-derived dendritic cells (BMDC) and splenic CD4⁺T cells from young and old mice.

Results Features of allergic airway disease such as mucous cell hyperplasia, infiltration of airway eosinophils and lymphocytes, Th2 cytokine expression and serum IgG1 levels were greater in old compared to young mice. In contrast to the more marked inflammatory/immune responses to HDM in old mice, AHR was greater in young HDM-treated mice.

Only the old mice developed airway neutrophil infiltration and a Th17 immune response upon HDM exposure, with increases in BALF cytokines IL-17A and KC, and Th17 cytokine producing T cells in the spleen. Stimulation of CD4⁺T cells and BMDC co-cultures with HDM, resulted in an enhanced Th17 cytokine response in cells isolated from old mice.

Conclusions and Clinical Relevance Our findings in mice suggest that the severity and character of allergic airway disease are age dependent, with a bias towards a Th17 immune response with aging. Elderly, asthmatics may be prone to develop severe allergic airway inflammation with a mixed Th2/Th17 immune response.

Keywords allergic asthma, aging, house dust mite, mice, Th17

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Introduction

Although allergic asthma is more prevalent in children and young adults, the incidence of allergic airway disease in the elderly (> 65 years of age) is growing and associated with high mortality [1]. Symptoms of asthma are heterogeneous and often under-diagnosed in the elderly due to similarities with other chronic pulmonary diseases such as chronic obstructive pulmonary disease [2]. In comparison to children and young adults, the pathogenesis of asthma in the elderly is characterized by more frequent, irreversible airway obstruction and less by an atopic response [3]. Older asthmatics, however,

are often excluded from research protocols, with the consequence that less is known about asthma in the elderly compared to children [3].

The aging process affects the performance of innate and adaptive immunity, a phenomenon known as immunosenescence or inflamm-aging [4]. The impact of immunosenescence on the development or progression of allergic airway disease, however, is not well understood [5]. Clinical studies in elderly asthmatics resulted in varied outcomes. Some of these studies suggest a continued allergic CD4⁺ T-helper type 2 cells (Th2) response with aging, while others suggest a reduced immunoglobulin IgE production and less atopic asthma

in elderly patients [6, 7]. In murine ovalbumin (OVA) models of allergic airway disease, however, old mice (12–18 months) were shown to have a higher Th2 response compared to young mice (6–12 weeks) [8–10].

In addition to allergic Th2 response, other factors play a role in the development of the chronic airway disease. Greater pulmonary neutrophilic inflammation has been reported in elderly asthmatics compared to the inflammatory response in children [11]. Neutrophilic inflammation can augment the severity of allergic airway disease and has been linked to a CD4⁺ T-helper type 17 cell (Th17) response [12]. Mixed Th2/Th17 inflammatory responses were associated with more severe forms of asthma [13]. It has also been suggested that Th17 response might play a prominent role in airway inflammatory response with aging [5]. The implication of Th17 response in allergic airway disease with aging, however, has not been further investigated.

House dust mite (HDM) is one of the most prevalent aeroallergens worldwide, with up to 85% of asthmatics showing allergic reactions to HDM and affecting people of all ages [14]. In the current study, we investigate allergic airway inflammation to HDM in young (12 weeks) and old mice (15 months) to test our hypothesis that age-related changes during experimental asthma are associated with enhanced Th17 response. Young and old mice were intranasally sensitized and challenged with HDM, and the magnitude of histopathological and immunological features of allergic airway disease was assessed in the lungs, serum and spleen. We furthermore investigated age-dependent HDM response in a co-culture system consisting of bone marrow-derived dendritic cells (BMDC) and splenic CD4⁺ T cells isolated from young and old mice, to investigate which cell type plays a key role in determining age-dependent HDM allergic response.

Materials and methods

Murine asthma model

Male BALB/c mice were obtained from Harlan Laboratories (Indianapolis, IN, USA). Young mice were purchased at an age of 6–8 weeks and used for experiments 1 week after arrival. Old mice were purchased at an age of 9–10 month (retired breeders) and aged for another 5 months at Michigan State University animal housing facilities until experimental use. All animal procedures and experimental protocols were approved by the Institutional Animal Care and Use Committee.

The experimental antigen, HDM, was obtained from *Dermatophagoides pteronyssinus* whole body extract (Greer, Lenoir, NC; Lot # 165197). The animals were intranasally sensitized on days 1, 4 and 7 with 0 (vehicle control) or 10 µg HDM protein in a total volume of 30 µL of saline. Mice were anesthetized with 4% isoflu-

rane in O₂ for each intranasal instillation. On day 21, HDM-sensitized mice were challenged intranasally with 20 µg of HDM protein. All animals were sacrificed 48 h post challenge on day 23. Animal sacrifice and necropsy were performed as previously described in detail [15].

Dendritic cell and T cell co-culture

Bone marrow-derived dendritic cells were isolated and differentiated as described previously [16]. CD4⁺ T cells were isolated from spleens of young and old mice using the murine MACS CD4⁺ T cell isolation kit (Miltenyi Biotec, CA, USA). BMDC and CD4⁺ T cells were established in a co-culture at a ratio of 1 : 10 in RPMI 1640 cell culture medium plus 10% FBS, 1% Pen/Strep and 5 ng/mL GM-CSF for 3 days and co-exposed with or without 20 µg/mL HDM. Cell proliferation was measured using CountBright™ absolute counting beads for flow cytometry (Molecular Probes, Eugene, OR, USA), and cytokine expression in the supernatant was analyzed as described for bronchoalveolar lavage fluid (BALF) cytokines.

BALF cytometry and inflammatory cytokine analysis

Bronchoalveolar lavage fluid cells were characterized as described previously [15]. BALF cytokines such as interleukins (IL)-1β, IL-4, IL-5, IL-6, IL-13, IL-17A, tumour necrosis factor-α (TNFα) and interferon-γ (IFNγ) were measured with the enhanced sensitivity FLEX SET (200 fg to 200 pg/mL) Cytometric Bead Array (BD Biosciences, San Jose, CA, USA). Keratinocyte-derived chemokine (KC, CXCL1) and cytokines in cell culture supernatant were measured with the standard FLEX SET (2.5–2000 pg/mL). Cytokine analysis was performed using a FACSCalibur flow cytometer (BD Biosciences) and according to the FLEX SET manual.

Analysis of IgE/IgG1

Mouse IgE Ready-SET-Go! ELISA kit (eBioscience, San Diego, CA, USA) was used according to the manufacturer's protocol to determine the total levels of IgE in blood serum. An indirect ELISA was developed for determination of HDM-specific IgG1 in serum and BALF. This procedure is described in detail in the supplementary information. The optical density (OD) was read at 450 and 405 nm for IgE and IgG1, respectively, and IgE serum concentrations were calculated from obtained standard curves.

Real-time PCR of pulmonary tissues

Total RNA was isolated from right lung lobes using RNeasy Mini Kit (Qiagen, Boston, MA, USA) according to the manufacturer's instructions and as described pre-

viously [15]. A list of analyzed genes and primers is provided in the supplementary information.

Histology and airway morphometry

The left lung lobe of each animal was inflation fixed with 4% paraformaldehyde, cut in random oriented serial sections and embedded in paraffin. Paraffin sections were then stained with haematoxylin and eosin (H & E) and with Alcian Blue (pH 2.5)/Periodic Acid-Schiff (AB/PAS). AB/PAS-stained mucosubstance in pulmonary bronchioles was quantified as a volume density per length of basal lamina and in accordance with the official guidelines for lung stereology [17] (see supplementary information).

Airway hyperresponsiveness (AHR) measurements

Airway hyperresponsiveness was measured by forced oscillation techniques, using the flexiVent system (flexiVent, Scireq, Montreal, Canada). A more detailed description of the procedure is provided in the supplementary information. In brief, incremental concentrations (0, 1.25, 2.5, 5, 10, 20 and 40 mg/mL) of the bronchoconstrictor acetyl- β -methacholine (MCh, Sigma-Aldrich St. Louis, MO, USA) were intratracheally delivered via an attached nebulizer. Pulmonary resistance and compliance were measured within a total of 12 perturbation manoeuvres. Total lung resistance (R) along with airway resistance (R_n) was then calculated using the flexiVent software, based on a single compartment model [18].

Splenocyte immunophenotyping and activation

Splenocytes were isolated from control and HDM mice and labelled with anti-mouse CD4-FITC, anti-mouse CD8-APC, anti-mouse CD25-APC, anti-mouse CD44-PECy7 and anti-mouse CD62L-PE as recommended by the supplier (Biolegend, CA). CD8⁺ and CD4⁺ T cell populations as well as naïve (CD4⁺, CD25⁻, CD44^{lo}, CD62L^{hi}), central memory (CD4⁺, CD25⁻, CD44^{hi}, CD62L^{hi}), effector memory (CD4⁺, CD25⁻, CD44^{hi}, CD62L^{lo}) and regulatory T cells (T-reg) (CD4⁺, CD25⁺) were assessed by flow cytometry. Splenocytes were further activated with anti-CD3 and anti-CD28 (Biolegend San Diego, CA, USA) for 72 h, and mRNA expression was measured as described for lung tissue.

Statistics

Each experimental group consisted of 6–7 mice. Data were reported as group means $\pm$ standard error of the mean (SEM). Grubbs outlier test was performed, and significant outliers were excluded from the data set. Differences among groups were analyzed by a two-way

analysis of variance (ANOVA) followed by a pairwise comparison with Student-Newman-Keuls test. Significance was assigned to *P* values ≤ 0.05 . All analyses were conducted using SigmaPlot software (SYSTAT Software Inc; San Jose, CA, USA).

Results

HDM-Induced allergic airway inflammation is enhanced in old mice

Bronchoalveolar lavage fluid eosinophils and lymphocytes were elevated in both young and old mice exposed to HDM (HDM mice) compared to control mice (saline mice) (Fig. 1a). The number of BALF eosinophils, however, was 10-fold higher in old compared to

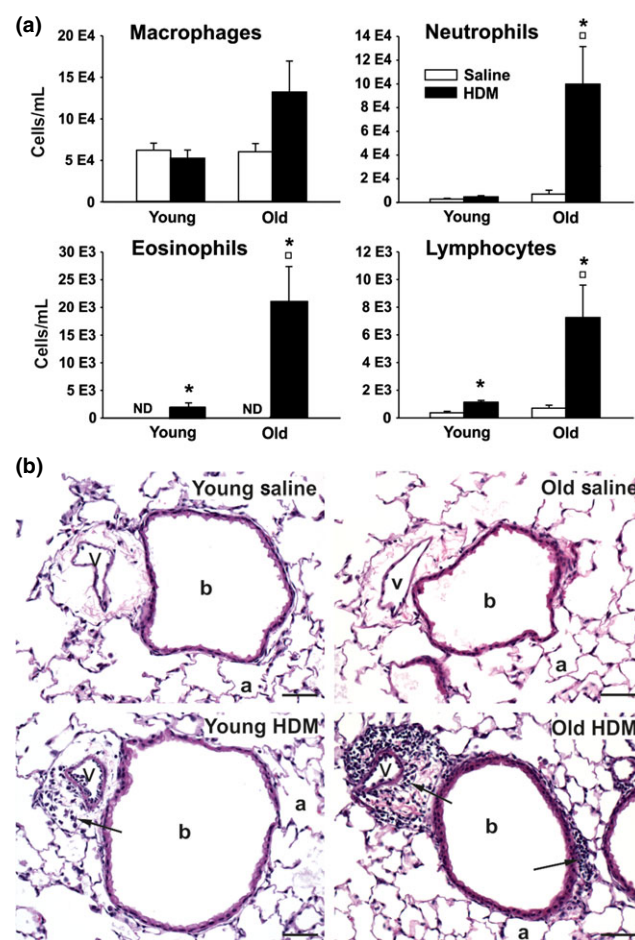


Fig. 1. Bronchoalveolar lavage fluid cytometry and pulmonary histopathology. (a) Amounts of macrophages, neutrophils, eosinophils and lymphocytes in BALF. *Significant changes (*P* < 0.05, *n* = 6) when compared to saline mice, □ significant changes when compared to young mice of the same exposure group. (b) Representative light photomicrographs of lung tissue sections stained with haematoxylin and eosin, showing a peribronchiolar and perivascular mixed inflammatory cell infiltration in HDM mice (arrows). b: bronchiolar airspace, a: alveoli, v: blood vessel, scale bar: 50 μ m.

young HDM mice, and lymphocytes were 6-fold higher. Neutrophils were only significantly elevated in old HDM mice (14-fold), but not in young HDM mice. There were no significant changes in macrophage numbers in regards to age or HDM-treatment. Likewise, there were no differences in BALF cells between young and old saline mice.

In both age groups of mice, intranasal sensitization and challenge with HDM caused a peribronchiolar and a perivascular influx of inflammatory cells that was principally located in the proximal aspects of the lung lobe (Fig. 1b). Inflammatory cells were mainly mononuclear cells (lymphocytes and plasma cells) with lesser numbers of granulocytes and were more abundant in old compared to young HDM mice.

HDM-treatment also caused airway epithelial remodelling characterized by mucous cell metaplasia, as identified with AB/PAS staining for intraepithelial neutral and acidic mucosubstances in bronchiolar epithelium (Fig. 2a). This metaplastic epithelial response was restricted primarily to large-diameter pre-terminal bronchioles of HDM mice and was more conspicuous in old mice (Fig. 2a). Morphometrically, the volume density of AB/PAS-stained mucosubstances in the bronchiolar epithelium was significantly greater in HDM mice. Intraepithelial mucosubstances in the lungs of old HDM mice were 1.9-fold greater than in young mice that were similarly treated (Fig. 2b). Accordingly, gene expression levels of genes related to mucus production and secretion (*Clca3*, *Muc5ac*, *Spdef*; Figs 2c–e) were also significantly induced in HDM mice. The mRNA expression levels of these genes, except for *Clca3*, were significantly higher in old compared to young HDM mice.

AHR was assessed in young and old saline and HDM mice by measuring total lung (R) and airway (R_n) resistances after exposure to increasing doses of methacholine (Fig. 3a). In general, old mice were less responsive to methacholine compared to young mice as suggested previously [8, 19]. However, both, young and old mice developed increased AHR after HDM-treatment, and the relative response after HDM treatment was similar in both age groups.

IgG1 response to HDM is markedly greater in old mice

IgG1 and IgE immunoglobulin (sub-) classes (Fig. 3b) are associated with Th2 responses. We measured elevated serum levels of HDM-specific IgG1 in young and old mice after sensitization and challenge with HDM. The IgG1 serum levels were 3-times greater in old compared to young HDM mice. Similarly, HDM-specific IgG1 in BALF was markedly increased by HDM exposure and was substantially higher in old compared to young mice. No increase in serum levels of total IgE

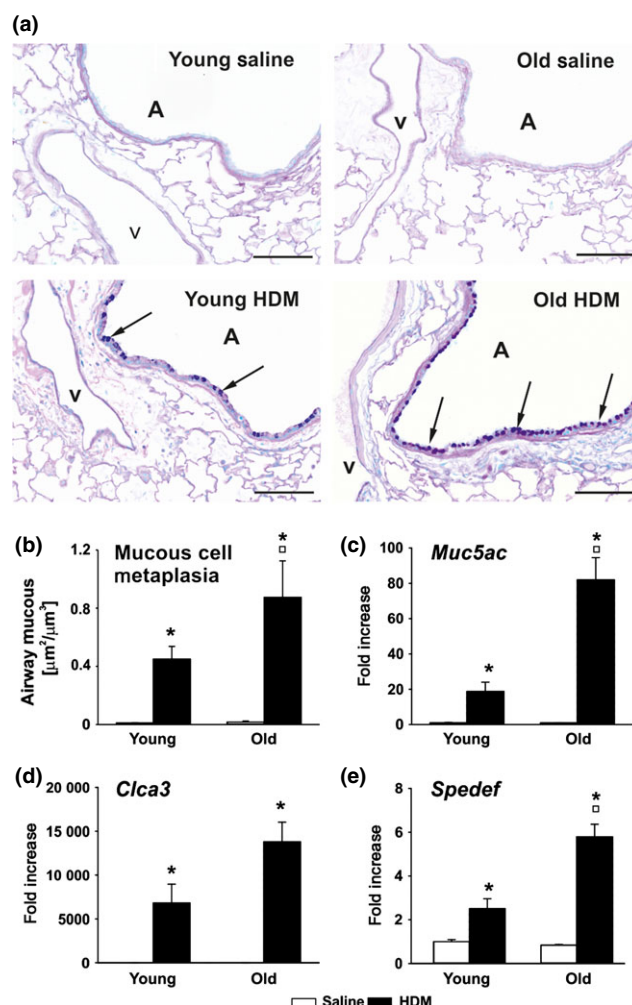


Fig. 2. Airway epithelial mucus cell metaplasia. (a) Increase in airway epithelial mucus, as a feature of allergic airway disease, was analyzed on histological lung sections, stained with AB/PAS. Intraepithelial mucosubstances (arrows) are present in young and old HDM mice. A: airway, v: vessel; scale bar: 100 μm . (b) Morphometric measurements of intraepithelial AB/PAS mucosubstances in serial sections of the left lung show a significant increase in mucosubstances per length of basal lamina with HDM-treatment. Gene expression levels of genes related to mucus production and secretion (c) *Clca3*, (d) *Muc5ac* and (e) *Spdef* are induced as well in lungs of HDM-treated mice. *Significant changes ($P < 0.05$, $n = 6$) when compared to saline mice of the same age group, □ significant changes when compared to young mice of the same exposure group.

was detectable in young and old HDM mice. Attempts to measure HDM-specific IgE in the serum or BALF were not successful.

Lungs of old HDM mice have stronger Th2/Th17 cytokine response

HDM sensitization and challenge induced lung gene expression (Table 1) of cytokines and BALF proteins (Fig. 4) associated with an allergic Th2 response.

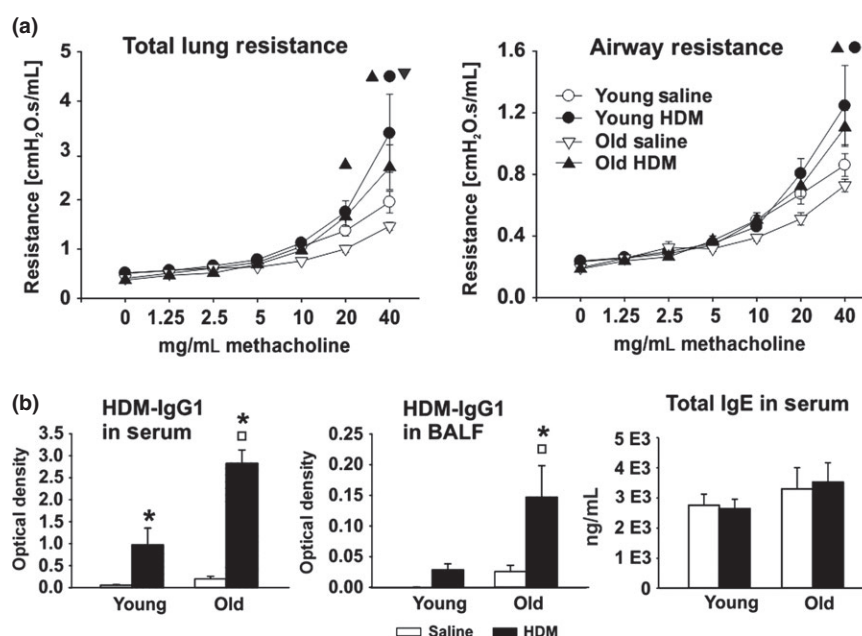


Fig. 3. Airway hyperresponsiveness and serum and BALF immunoglobulin expression. (a) Dose response and increasing total lung and airway resistance with increasing methacholine challenge in all exposure groups. ▲: significant ($P < 0.05$, $n = 6-7$) HDM effect in old mice, ●: significant HDM effect in young mice, ▼: significant age-dependent difference in HDM mice. (b) HDM-specific IgG1 levels are measured as optical density in serum and BALF of all experimental groups. Serum levels of total IgE are detected by an ELISA and presented as total serum concentration. *Significant changes ($P < 0.05$, $n = 6$) when compared to saline mice of the same age group, □: significant changes when compared to young mice of the same exposure group.

Table 1. Gene expression profile in lung tissue of young and old mice sensitized and challenged with HDM. Relative induction in young HDM mice, normalized to young saline mice (Young HDM); relative induction in old saline mice, normalized to young saline mice (Old Saline); relative induction in old HDM mice normalized to old saline mice (Old HDM). *: Significant changes ($P < 0.05$, $n = 6$) in HDM-compared to saline mice of same age group, □: significant age-related changes within the same treatment group

Gene	Gene Alias	Function	Young HDM	Old Saline	Old HDM
<i>Ccl2</i>	MCP1	Monocyte and T cell chemokine	2.3 ± 0.4*	1.1 ± 0.1	12.1 ± 4.0 ^{□*}
<i>Ccl3</i>	Mip1a	Macrophage inflammatory protein	1.0 ± 0.1	1.0 ± 0.2	2.6 ± 0.9 ^{□*}
<i>Ccl8</i>	MCP2	Monocyte chemokine	94.2 ± 32.0*	3.3 ± 0.6 [□]	72.7 ± 9.2 ^{□*}
<i>Ccl11</i>	Eotaxin	Eosinophil chemokine	4.0 ± 1.0*	1.4 ± 0.2	7.9 ± 1.0 ^{□*}
<i>Chia</i>	AMCase	Acidic mamalian chitinase	2.2 ± 0.3*	-1.6 ± 0.1 [□]	6.6 ± 1.1 ^{□*}
<i>Chi3 l3</i>	YM1	Chitinase-like protein	2.0 ± 0.3*	1.2 ± 0.1	7.3 ± 2.3 ^{□*}
<i>Cxcl1</i>	KC	Neutrophil chemokine	1.3 ± 0.2	1.3 ± 0.1	5.5 ± 1.1 ^{□*}
<i>Saa3</i>	Saa3	Acute phase protein	2.8 ± 1.0*	1.4 ± 0.4	17.8 ± 1.6 ^{□*}
<i>Tnfα</i>	Tnfα	Acute phase response cytokine	-1.2 ± 0.1	1.8 ± 0.5 [□]	1.2 ± 0.1 [□]
<i>Il-1β</i>	IL-1β	Acute phase response cytokine	1.2 ± 0.1	2.3 ± 0.2 [□]	1.1 ± 0.1 [□]
<i>Il-6</i>	IL-6	Acute phase response cytokine	1.3 ± 0.2	1.5 ± 0.4	3.8 ± 1.5 ^{□*}
<i>Ifnγ</i>	Ifnγ	Th1 cytokine	1.7 ± 0.3*	1.7 ± 0.3	1.3 ± 0.3
<i>Il-2</i>	IL-2	Th1 cytokine	1.4 ± 0.3	1.7 ± 0.1 [□]	1.4 ± 0.2 [□]
<i>Tff2</i>	SP	Th1 suppressor, airway remodeling	9.9 ± 2.1*	-1.6 ± 0.3	22.7 ± 2.2*
<i>Il-4</i>	IL-4	Th2 cytokine	1.3 ± 0.1	2.4 ± 0.4 [□]	3.3 ± 0.3 ^{□*}
<i>Il-5</i>	IL-5	Th2 cytokine	3.6 ± 0.9*	-2.3 ± 0.4 [□]	23.7 ± 3.0 ^{□*}
<i>Il-13</i>	IL-13	Th2 cytokine	19.1 ± 5.1*	-2.2 ± 0.4 [□]	236.4 ± 31.6 ^{□*}
<i>Retnla</i>	Fizz-1	Th2 suppressor	37.1 ± 8.2*	1.8 ± 1.0	53.7 ± 4.2 ^{□*}
<i>Il-17a</i>	IL-17A	Th17 cytokine	1.9 ± 0.5	1.4 ± 1.0	25.2 ± 5.8 ^{□*}
<i>Il-17f</i>	IL-17F	Th17 cytokine	2.1 ± 0.5*	1.2 ± 0.3	4.4 ± 0.7 ^{□*}
<i>Il-21</i>	IL-21	Th17 cytokine	6.0 ± 1.5*	2.7 ± 0.9	6.3 ± 0.4 ^{□*}
<i>Il-10</i>	IL-10	T-reg cytokine	4.5 ± 1.3*	2.2 ± 0.5 [□]	6.8 ± 1.4 ^{□*}
<i>Foxp3</i>	Foxp3	T-reg transcription factor	1.4 ± 0.1	-1.1 ± 0.1	2.4 ± 0.4 ^{□*}

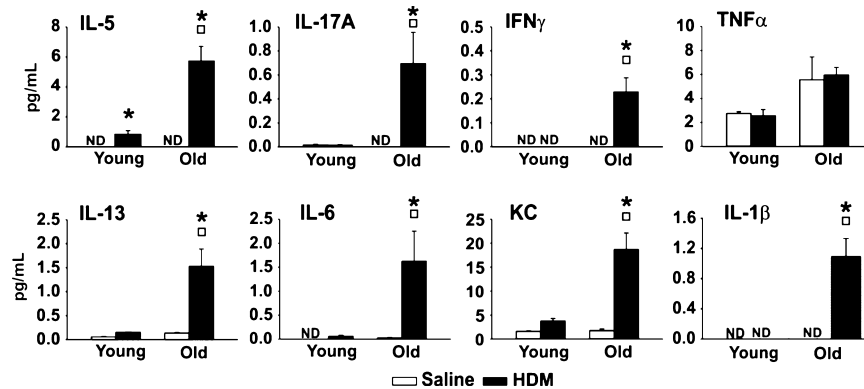


Fig. 4. Expression of BALF cytokines and chemokines. Different BALF cytokine concentrations were analyzed as for TNF α , KC, IFN γ , IL-1 β , IL-5, IL-6, IL-13, IL-17A. *Significant changes ($P < 0.05$, $n = 6$) when compared to saline mice of the same age group, $\square$ significant changes when compared to young mice of the same exposure group.

This included Th2 cytokines (IL-5, IL-13), eosinophil chemokine *Eotaxin* (*Ccl11*), as well as acidic mammalian chitinase (*AMCase*) and chitinase-like protein (*Chi3 l3*). In regards to BALF proteins, Th2 cytokine IL-5 was elevated in both young and old HDM mice. IL-5 concentrations were 7-fold higher in the old compared to young HDM mice, and Th2 cytokine IL-13 was only induced in the BALF of old HDM mice (12-fold of saline mice).

Th17 cytokine, IL-17A, was significantly induced in the BALF of old, but not of young HDM mice. The induction of IL-6 and KC was increased by 73-fold and 11-fold, respectively, in old HDM mice compared to the saline mice of same age. Gene expression of *Il-17a*, *Il-6* and *Cxcl1* (KC) was only induced in the lung tissue of old HDM mice (25-, 4- and 6-fold, respectively), whereas gene expression of other Th17 cytokines *Il-21*, and *Il-17f* was elevated in both, young and old HDM mice (*Il-17f*: each 2-fold; *Il-21*: 4- and 6-fold, respectively).

The expression of other cytokines such as Th1, T-reg and acute phase cytokines were also altered with age and HDM exposure. BALF levels of the Th1 cytokine, IFN γ , were increased as well in old HDM mice, but to a lesser extent than Th2 and Th17 cytokines. Similarly, this was also true for lung mRNA levels of the T-reg transcription factor, forkhead protein P3 (*Foxp3*) and *Il-10*. Gene expression of acute phase cytokines, *Tnf α* and *Il-1 β* , was not induced after HDM challenge, but slightly elevated in old compared to young saline animals. However, IL-1 β level in the BALF was significantly elevated in old HDM mice, whereas TNF α values did not significantly differ within age or treatment groups.

Age promotes a Th17 response upon HDM allergen exposure

We characterized T cell populations in the spleen of young and old, saline and HDM mice and found a

significant age-dependent decrease of naïve CD4 $^{+}$ T cells and an increase of memory effector CD4 $^{+}$ T cells (Fig. 5a). No age or treatment-related differences in splenic populations of CD4 $^{+}$ CD25 $^{+}$ T cells (which are predominately Tregs) were detected. The percentage of total CD4 $^{+}$ T cell in the spleen was the same in all experimental groups (Fig. S1). To assess function, isolated splenocytes ($n = 3-4$ per group) were activated with anti-CD3 and anti-CD28, and cytokine mRNA expression was measured (Fig. 5b). T cell activation in splenocytes of old, but not young HDM mice, resulted in significant increases in *Il-17a*, *Il-21* and *Il-22* expression compared to saline mice. No significant differences in Th2 cytokines (*Il-4*, *Il-5*, *Il-13*) were measured in young or old mice with HDM sensitization and challenge. Collectively, these results suggest that HDM exposure in old mice promotes Th17 cytokine responses in spleen in addition to lung, suggesting a systemic effect.

Age-dependent Th17 cytokine response to HDM is dependent on both CD4 $^{+}$ T cells and dendritic cells

We further tested whether BMDC, CD4 $^{+}$ T cells or both were involved in age-dependent allergic HDM response. We first analyzed expression of maturation (MHCII) and co-stimulatory molecules (CD80 and CD86) on BMDC from young and old mice exposed to 20 μ g/mL HDM for 16 h. HDM exposure induced the expression of CD86 in BMDC from young and old mice (Fig. 6a). The population of CD11c $^{+}$ expressing cells, however, was higher in BMDCs from old compared to young mice, and the old BMDC had significantly lower expression of MHCII. HDM exposure increased TNF α , KC, IL-1 β and IL-6 production in young and old BMDC (Fig. 6b). TNF α and KC levels were significantly greater in old compared to young HDM-exposed BMDC.

Co-cultures with primary CD4 $^{+}$ T cell and BMDC from young and old naïve mice were exposed for 72 h with HDM. We found that HDM exposure inhibited T

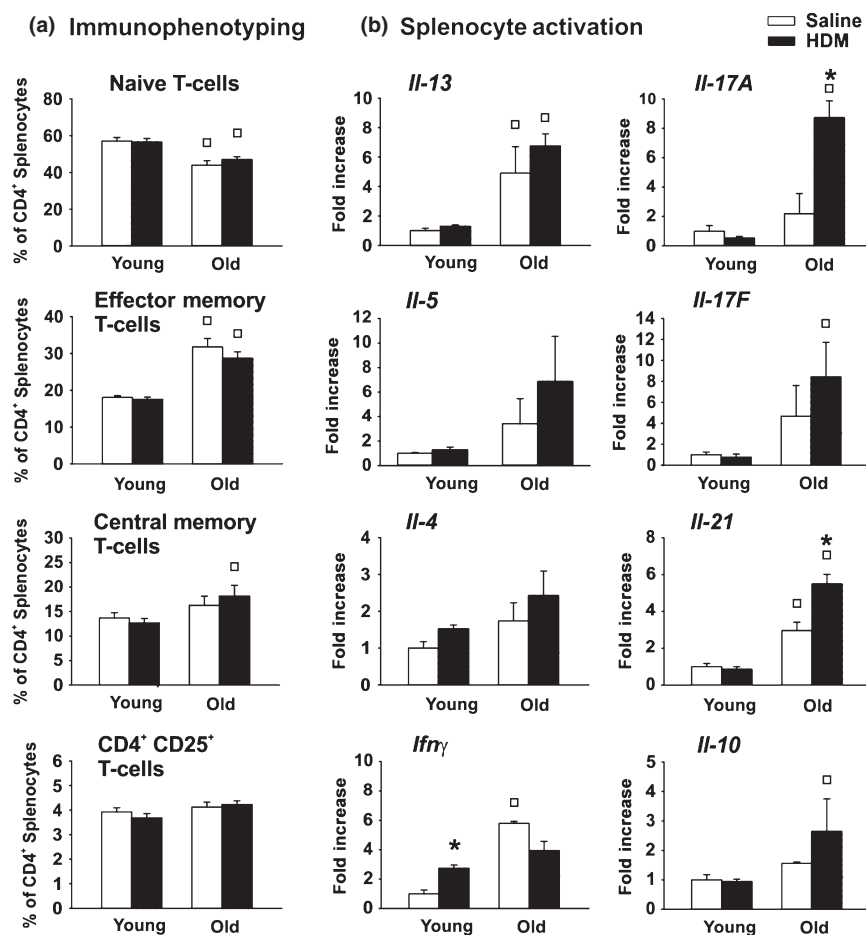


Fig. 5. Characterization and activation of T cells in the spleen. (a) CD4⁺ T cells in the spleen of all experimental groups were characterized by flow cytometry, and percentage of naïve (CD25⁻, CD44^{lo}, CD62L^{hi}), effector memory cells (CD25⁻, CD44^{hi}, CD62L^{lo}), central memory cells (CD25⁻, CD44^{hi}, CD62L^{hi}) and regulatory T cells (CD4⁺ CD25⁺) was assessed. (b) mRNA cytokine expression of activated splenocytes. *Significant changes when compared to saline mice of the same age group, □significant changes when compared to young mice of the same exposure group. ($P < 0.05$, $n = 3-4$).

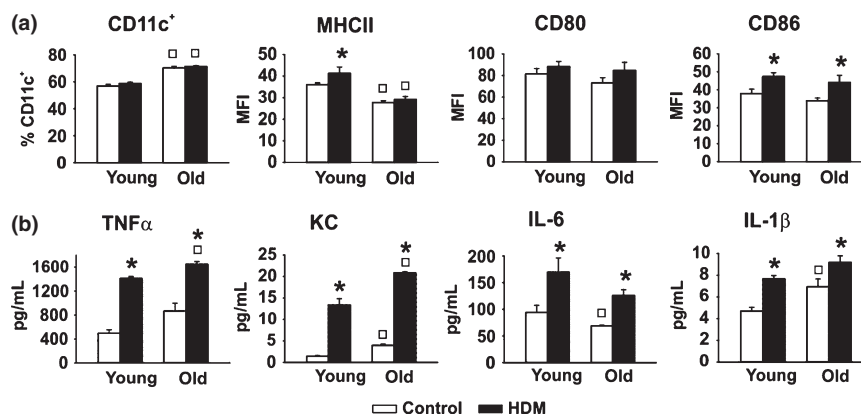


Fig. 6. HDM exposure of BMDC from young and old mice. (a) Expression of CD11c and co-stimulatory surface markers MHCII, CD80 and CD86 in controls and HDM-exposed BMDC from young and old mice. (b) Cyto- and chemokine TNF α , KC, IL-1 β and IL-6 in the cell culture supernatant of exposed BMDC. MFI: mean fluorescent intensity; *Significant changes ($P < 0.05$, $n = 3$) when compared to saline mice of the same age group, □significant changes when compared to young mice of the same exposure group.

cell proliferation or induced cell death in co-cultures with T cells isolated from old mice (old T cells), but not with T cells isolated from young mice (young T cells) (Fig. 7a). Age-related differences were also found in the cytokine expression profiles of T cells and BMDC. (Fig. 7b). In co-cultures with young T cells, HDM stimulation induced a significant increase in the Th2 cytokines, IL-5 and IL-13, which was not detected in co-cultures with old T cells. An increase in IL-17A and

IL-17F expression was found after HDM exposure. However, IL-17A expression was significantly higher in HDM-exposed co-cultures with old compared to young T cells (~5-fold), and IL-17F was significantly increased in co-cultures with old BMDCs. The IL-10 cytokine expression pattern was similar to IL-17A, but 500-fold lower. IFN γ was induced by HDM exposure in all co-culture groups, but the greatest induction was observed in the old T cells co-cultured with old BMDCs. Cytokine expressions of BMDC and T cells in single cultures were below the assay detection limit (data not shown).

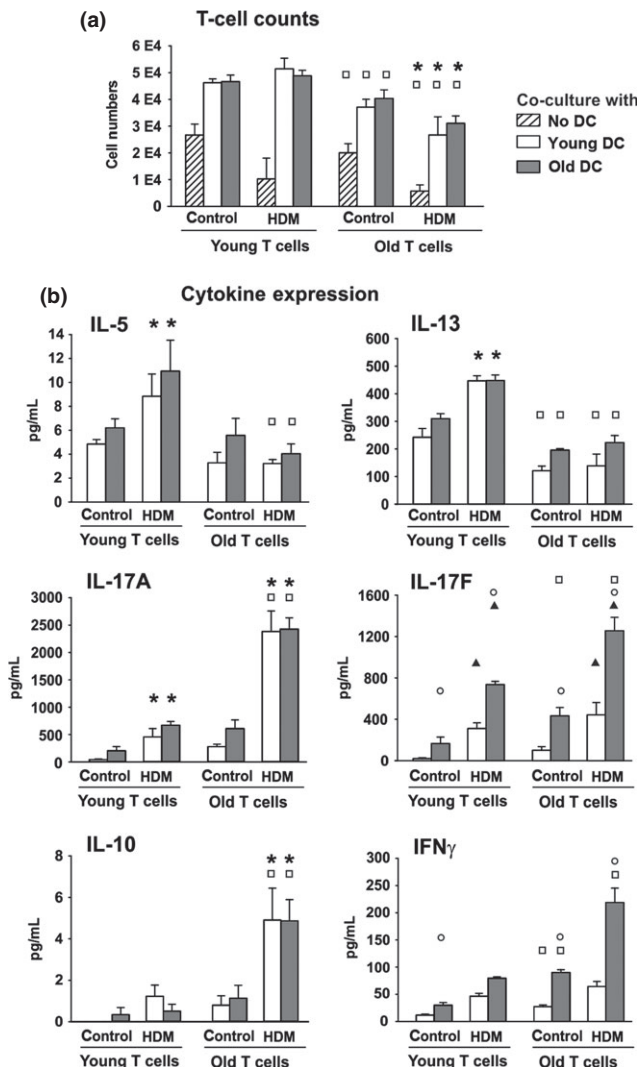


Fig. 7 Age-dependent T cell activation in co-culture with BMDC and HDM allergen exposure. CD4⁺ T cells were isolated from young and old mice and co-cultured with BMDC from young (white bars) and old (grey bars) mice with or without HDM allergen exposure. (a) T cell number and (b) IL-5, IL-13, IL-17A, IL-17F, IFN- γ and IL-10 cytokine expression in all exposure groups. A 3-way ANOVA was performed with a pairwise comparison (significance $P \leq 0.05$, $n = 3$, data shown as mean $\pm$ SEM). Group effects (interactions) are indicated with same level symbols. *: significant HDM effect in T cells, $\square$: significant age effect in T cells, $\blacktriangle$: significant HDM effect in BMDCs, $\circ$: significant age effect in BMDCs ($P > 0.05$, $n = 3$).

Discussion

Features of allergic airway disease such as mucous cell metaplasia, infiltration of airway eosinophils and lymphocytes, enhanced serum IgG1 levels and Th2 cytokine expression, were more pronounced in old compared to young HDM mice. Although young and old HDM mice had similar relative increases in AHR after methacholine challenge, young HDM mice showed greater sensitivity to methacholine compared to the old mice in the same treatment group, which is consistent with previous published findings [8, 19]. Furthermore, HDM exposure led to a Th17 immune response and airway neutrophil infiltration only in old mice. These results suggest an age-related shift towards an increased Th17 response upon exposure to HDM, which is consistent with the hypothesis that the elderly may be prone to develop severe allergic asthma with a mixed Th2/Th17 immune response.

Different murine models have been used to investigate mechanisms of allergic airway disease, but few address the aspect of allergic airway disease in aging. Two studies, comparing allergic airway disease in young (4–8 weeks) and older mice (approximately 10 months), found no age-dependent differences in allergic responses [20, 21]. These studies, however, were performed in mice that were relatively younger than the aged mice used in our study (15 months). This difference in age can have an impact on the study results as shown by Busse and colleagues [8, 9], who used a murine OVA model with advancing age – 6 weeks, 6 months, 12 months and 18 months. They found age-dependent increases of BALF eosinophils and IL-5, more airway epithelial mucus and elevated gene expression of Muc5AC [8–10], which concurs with our findings. Similarly, they also detected a greater AHR response in the young mice. In our study, we additionally found a pulmonary neutrophilic response in the old HDM mice and elevated levels of IL-17A and KC. The differences in results between the two studies could be related to the use of HDM as an allergen, because a prevalence for the development of an IL-17 response to HDM in late-phase allergen-induced inflammation has been

shown previously in humans [22]. HDM is furthermore one of the most prevalent aeroallergens worldwide and up to 85% of asthmatics are typically HDM allergic [14]. However, to the best of our knowledge, the allergic response with aging has not been addressed previously in an HDM allergen model.

This current study demonstrates elevated neutrophilic response in allergic airway disease of old mice, which is concordant with findings of clinical studies with young and elderly asthmatics. In the elderly, asthma is characterized by an neutrophilic phenotype [11, 23]. Sputum of older asthmatics has higher numbers of neutrophils and IL-8 and neutrophil elastase levels [11]. We found an increase in KC, MIP1 α , IL-6 and IL-17A in BALF protein and/or lung tissue mRNA in old HDM allergic mice. KC, a murine homologue of IL-8, and MIP1 α are both potent neutrophil chemoattractants and are partly regulated via IL-17A [24, 25]. Several murine models of allergic airway disease have demonstrated that IL-17A is an important mediator of neutrophilic airway inflammation and enhances Th2-associated eosinophils, AHR and airway MUC5AC expression [reviewed in [5]]. It has been suggested that neutrophilic asthma, with a mixed Th2/Th17 response, is associated with more severe asthmatic phenotypes [13, 26] and less responsive to corticosteroid treatment [26]. However, the potential of IL-17 to drive the allergic response in allergic airway disease of elderly has not yet been investigated. Using our murine model of HDM-induced allergic airway inflammation, we have documented for the first time that old mice develop a robust Th17 response compared to younger animals when sensitized and challenged with HDM.

By immunophenotyping the T cell population in the spleen, we found a decrease in naïve and an increase in memory T cells with age. This has been reported previously [27] and it has further been suggested that Th1 and Th2 populations decrease with age, while the Th17 population increases [28, 29]. In our study, T cell activation of isolated splenocytes resulted in higher overall cytokine expression with age. This might be due to the increased number of effector memory cells in the old animals. We also found an increased Th17 cytokine response (*Il-21*, *Il-17f*, *Il-17a*) in activated splenocytes from old HDM mice, suggesting that the Th17 response after HDM exposure in old mice was systemic. Conversely, no significant elevation of Th2 cytokines in activated splenocytes was measured after HDM exposure. In an *in vitro* co-culture model with BMDC and CD4⁺ T cells, isolated from young and old naïve mice, we found that T cell activation and cytokine responses upon HDM exposure was mostly dependent on the age of the T cell donor. T cells, isolated from young mice (young T cells), were more prone to develop a Th2 cytokine profile after HDM allergen exposure, and

conversely, T cells isolated from old mice (old T cells) were predisposed towards a Th17 response.

Several factors may contribute to the heightened Th17 response in old mice. It has been shown that Th17 cells are long-lived effector memory cells [30]. The increased percentage of effector memory T cells as observed in the spleen of old mice, may therefore contribute to the age-dependent increase in Th17 cytokine production. Due to relative low abundance of lymphocytes in BALF and lungs of naïve mice, we were not able to assess T cell sub-populations such as Th2 or Th17 cells in the lungs. However, the lungs of old compared to young HDM mice show significantly more lymphocytes and, accordingly, greater Th2 and Th17 cytokine expression. Th17 cytokine IL-17A (protein and gene expression) was only induced in lungs of old mice, whereas mRNA of *Il-17f* and *Il-21* was induced in both young and old HDM mice. Similarly, in our co-culture model and splenocyte activation experiments, we found a T cell age-dependent IL-17A, but not IL-17F expression after HDM exposure. IL-17A and F share 50% amino acid sequence identity and bind to the same receptor, but IL-17A was suggested to be mainly responsible for neutrophil influx in allergic airway disease [31, 32]. We therefore assume that the age-dependent Th17 response and neutrophil influx were predominately mediated by IL-17A.

Interestingly, in our *in vitro* co-culture model, we found a BMDC age-related effect for IL17-F and IFN γ , which occurred in both control and HDM-exposed co-cultures. BMDC of old mice had higher levels of CD11c⁺ expression and increased TNF α , IL-1 β and KC secretion. *Tnf α* and *Il-1 β* gene expression was also significantly greater in lungs of old compared to young naïve mice. IL-1 β was previously reported to stimulate Th17 development [33], but further studies will be needed to determine the individual contributions of these factors. In addition, we found that HDM exposure increased cytokine levels of TNF α , IL-1 β , IL-6 and KC in BMDC, and others have suggested that these cytokines may promote and/or complement the development of a Th17 response [33, 34]. Others have reported that HDM can induce toll-like receptor (TLR) 2 and TLR4 signaling, and TLR2 ligands in HDM can promote Th17-like responses [14]. Decreased function of TLR-signalling in inflamm-aging has been suggested previously as well as age-related reduction of TLR-induced CD80 and CD86 expression on monocytes from elderly (reviewed in [35]). The results of our study, however, do not give evidence of age-related loss of function in TLR signalling of antigen presenting cells.

Previous reports provide evidence that atopic asthma in elderly subjects declines [7], whereas in the current studies, we found a greater pulmonary allergic Th2 response and enhanced levels of serum IgG1 to HDM in old mice. However, splenocyte activation did not result

in an enhanced Th2 cytokine profile in HDM-treated animals, suggesting that the Th2 response may be more limited in scope than the Th17 response. The enhanced pulmonary Th2 allergic response in old HDM mice could also be related to an increase in IL-17A, because it has been shown that IL-17A, in combination with IL-5, can enhance eosinophilic cytolytic activity [36]. Various studies also indicate that the presence of IL-17A can enhance allergic eosinophilic airway inflammation [37], AHR [31, 38] or mucin gene expression [39]. More research is needed to verify this hypothesis, and our study was not designed to investigate synergistic mechanisms of IL-17A and Th2 response. However, future studies with IL-17 inhibitors or IL-17 knockout mice might help to understand the implications of Th17 response in age and allergy development.

It has to be noted that asthma is a heterogeneous disease, and animal models are limited in mimicking human asthma. Clinical studies are needed to determine if humans develop allergic asthma with an age-dependent Th17 response similar to the mice in our study. Also, recent studies have pointed out the importance of the gut microbiota in the development of allergies and asthma [40, 41]. The animals in our study were purchased from the same vendor, but housed in our own facility for 1 month or 6 months (young and old mice, respectively). Although the gut microbiota can adapt quickly [42], we cannot completely exclude the potential influence of the gut microbial community on the allergic response observed in this study. Another limitation of our study is that the old mice were only exposed to the allergen later in life, whereas elderly

asthmatics might develop allergic responses earlier in life. Despite these limitations, our murine HDM model of allergic airway disease reproduces a number of characteristics of late onset asthma in the elderly and might be helpful to study mechanisms of immunosenescence and the development of allergic airway disease in advanced age.

In conclusion, the results of our study in mice give evidence that the severity and character of allergic airway disease are age dependent and associated with an increased Th17 response. This suggests that the elderly may be more prone to develop severe allergic airway inflammation with a mixed Th2/Th17 allergic response. Further research is needed to fully understand the implications of Th17 immune response in aging and allergic airway disease.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Enhanced allergic airway disease in old mice is associated with a Th17 response

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 - c. HDM-specific IgG1 ELISA
 - d. Taqman primer source
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1. Material and Methods

a) Quantification of intraepithelial mucosubstance by means of morphometry

The left lung lobe of each animal was cut in serial sections (section thickness = 3 mm) with a randomly chosen orientation and start. The serial lung sections were embedded in paraffin and 5- to 6- μ m-thick sections were cut from the anterior surface. The sections were stained with hematoxylin and eosin (H&E) for routine light microscopic examination and with Alcian Blue (pH 2.5)/Periodic Acid–Schiff (AB/PAS) for identification of intraepithelial neutral and acidic mucosubstances in pulmonary bronchioles. For quantification of airway mucus, histological slides were scanned and digitalized with a slide scanner (VS110, Olympus) and quantitative evaluation of broncho-epithelial mucus was performed with the newCast system (Visiopharm, Denmark). Briefly, lung tissues on scanned slides were selected as region of interest and 20% of the lung tissue was captured at 20x magnification by systematic random sampling. A point intercept grid was superimposed over the sampled images to quantify the amount of mucus per basal lamina. The mucus density ($\hat{V}_M$) was estimated by counting the number of points falling on areas staining positively for ABPAS (P_M) multiplied by the area/point (a/p) and divided by the total number of point (n) in the reference area of the sampled images, as shown in equation 1 (EQ1).

$$\hat{V}_M = \frac{\sum P_M \times a / p}{n} \quad \text{EQ 1}$$

The surface density of the basal lamina ($\hat{S}_{BL}$) in the selected images was estimated by counting the number of intercepts (I) of the line probe with the basal lamina of conducting airways divided by the area per point (a/p) and

the number of points in the reference area of the sampled images (n), as described in EQ2. The mucus per basal lamina of conducting airways was then estimated by dividing $\hat{V}_M$ by $\hat{S}_{BL}$.

$$\hat{S}_{BL} = \frac{2 \times \sum I}{a / p \times n} \quad \text{EQ 2}$$

b) Airway Hyperresponsiveness (AHR) measurements

Mice were anesthetized by i.p. sodium pentobarbital (100 mg/kg body weight). A tracheostomy was performed and an 18 gauge cannula was inserted into the trachea and secured by suture. The animals were attached to a mechanical ventilator with a computer controlled piston pump (flexiVent, Scireq, Montreal, Canada). Mice were ventilated at a respiratory rate of 150 breaths/minute, tidal volume of 10 mL/kg, and positive end expiratory pressure (PEEP) of 2-3 cm H₂O. Incremental concentrations (0, 1.25, 2.5, 5, 10, 20 and 40 mg/mL) of a bronchoconstrictor, Acetyl- β -methacholine (MCh, Sigma-Aldrich) were delivered into the trachea via an attached nebulizer (Aeroneb). Prior to each MCh response curve, two deep inspirations were given by controlled ventilation. Following MCh administration, 12 perturbation maneuvers consisting of alternating measurements of sinusoidal, single frequency, oscillations (SnapShot) and broadband, multi-frequency, oscillations (Quick-prime) were performed. With the flexiVent software, the total lung resistance (R) along with airway resistance (Rn) was calculated from the collected data, using the single compartment model [19].

c) HDM-specific IgG1 ELISA

For determination of HDM-specific IgG1, 96 well plates (Costar, NY) were coated overnight with 10 μ g/mL HDM in PBS. Samples were loaded at a dilution of 1:2 in PBS and incubated overnight at 4°C. Detection antibody (biotin rat-anti-mouse IgG1, #553441, BD Pharmingen, CA), was loaded on the plate at a dilution of 1:1000 and incubated for 2 h at RT, followed by an incubation with avidin-horseradish peroxidase (Pharmingen #554058) at a dilution of 1:1000 for 30 min at RT. In a final step, plates were incubated with ABTS reaction substrate plus 0.1% H₂O₂ for 30 min at RT and optical density (OD) was read at 405 nm wave length with a Spectra Max Gemini plate reader (Molecular Devices, CA).

d) *Taqman Primer Souce***Table S1.** *TaqMan* gene expression analysis. Detailed list of all genes and primers used for gene expression analysis with the *TaqMan* gene expression assay from Applied Biosystems.

Gene Symbol	Gene name	Gene Alias	Array Group	Assay ID	NCBI Gene Reference
18S	18S rRNA		Endogenous	Mm03928990_g1	NR_003286.1
<i>Actb</i>	Actin, beta		Endogenous	Mm00607939_s1	NM_001101.3
<i>Arbp</i>	Acidic ribosomal phosphoprotein P0	ARBP (Endog)	Endogenous	Mm01974474_gH	NM_203736.1
<i>Gapd</i>	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Endogenous	Mm99999915_g1	NM_017008.3
<i>Gusb</i>	Glucuronidase, beta	Gus	Endogenous	Mm00446953_m1	NM_010368.1
<i>Hprt1</i>	Hypoxanthine guanine phosphoribosyl transferase	Hprt	Endogenous	Mm00446968_m1	NM_013556.2
<i>Ccl2</i>	Chemokine (C-C motif) ligand 2	MCP-1, Scya-2	Chemokine	Mm00441242_m1	NM_011333.3
<i>Ccl3</i>	Chemokine (C-C motif) ligand 3	MIP1-alpha	Chemokine	Mm00441258_m1	NM_011337.2
<i>Ccl8</i>	Chemokine (C-C motif) ligand 8	MCP-2	Chemokine	Mm01297183_m1	NM_021443.2
<i>Ccl11</i>	Chemokine (C-C motif) ligand 11	Eotaxin, Scya11	Chemokine	Mm00441238_m1	NM_011330.3
<i>Cxcl1</i>	Chemokine (C-X-C motif) ligand 1	KC/CINC-1/ IL8	Chemokine	Mm00433859_m1	NM_008176.3
<i>Ifnγ</i>	Interferon gamma	IFN-g	Cytokine	Mm00801778_m1	NM_008337.3
<i>Il-1</i>	Interleukin-1 beta		Cytokine	Mm00434228_m1	NM_008361.3
<i>Il-2</i>	Interleukin-2		Cytokine	Mm00434256_m1	NM_008366.2
<i>Il-4</i>	Interleukin-4		Cytokine	Mm00445259_m1	NM_021283.2
<i>Il-5</i>	Interleukin-5		Cytokine	Mm00439646_m1	NM_010558.1
<i>Il-6</i>	Interleukin-6		Cytokine	Mm00446190_m1	NM_031168.1
<i>Il-10</i>	Interleukin -0		Cytokine	Mm00439616_m1	NM_010548.1
<i>Il-13</i>	Interleukin-13		Cytokine	Mm00434204_m1	NM_008355.3
<i>Il-17a</i>	Interleukin-17A	Ctla8	Cytokine	Mm00439618_m1	NM_010552.3
<i>Il17f</i>	Interleukin-17F	C87042	Cytokine	Mm00521423_m1	NM_145856.2
<i>Il21</i>	Interleukin-21		Cytokine	Mm00517640_m1	NM_021782.2
<i>Tnf~</i>	Tumor necrosis factor alpha	TNF alpha	Cytokine	Mm00443258_m1	NM_013693.2
<i>Timp1</i>	Tissue inhibitor of metalloproteinase 1		Cytokine receptor	Mm00441818_m1	NM_001044384.1
<i>Retnla</i>	Resistin like alpha	Fizz-1	Immune resp.	Mm00445109_m1	NM_020509.3
<i>Saa3</i>	Serum amyloid A3	Saa-3	Immune resp.	Mm00441203_m1	NM_011315.3
<i>Chi3l3</i>	Chitinase 3-like 3	YM1,ECF-L	Chitinase	Mm00657889_mH	NM_009892.2
<i>Chia</i>	Chitinase, acidic	AMCase	Chitinase	Mm00458221_m1	NM_023186.3
<i>Tff2</i>	Trefoil factor 2 (spasmolytic protein 1)	SP, mSP	Growth factor	Mm00447491_m1	NM_009363.3
<i>Clca2</i>	Chloride channel calcium activated 2		Ion channel	Mm00661630_m1	NM_030601.2
<i>Clca3</i>	Chloride channel calcium activated 3	Gob-5	Ion channel	Mm00489959_m1	NM_017474.1
<i>Nqo1</i>	NAD(P)H dehydrogenase, quinone 1	Nmo-1, Ox-1	Oxidoreductase	Mm00500821_m1	NM_008706.5
<i>Muc5ac</i>	Mucin 5, subtypes A & C		Secretory	Mm01276725_g1	NM_010844.1
<i>Spdef</i>	SAM pointed domain containing ets transcription factor	PDEF, Pse	Transcription factor	Mm00600221_m1	NM_013891.4
<i>Foxp3</i>	Forkhead box P3	JM2	Transcription factor	Mm00475165_m1	NM_054039.1

2. Splenocyte Immunophenotyping

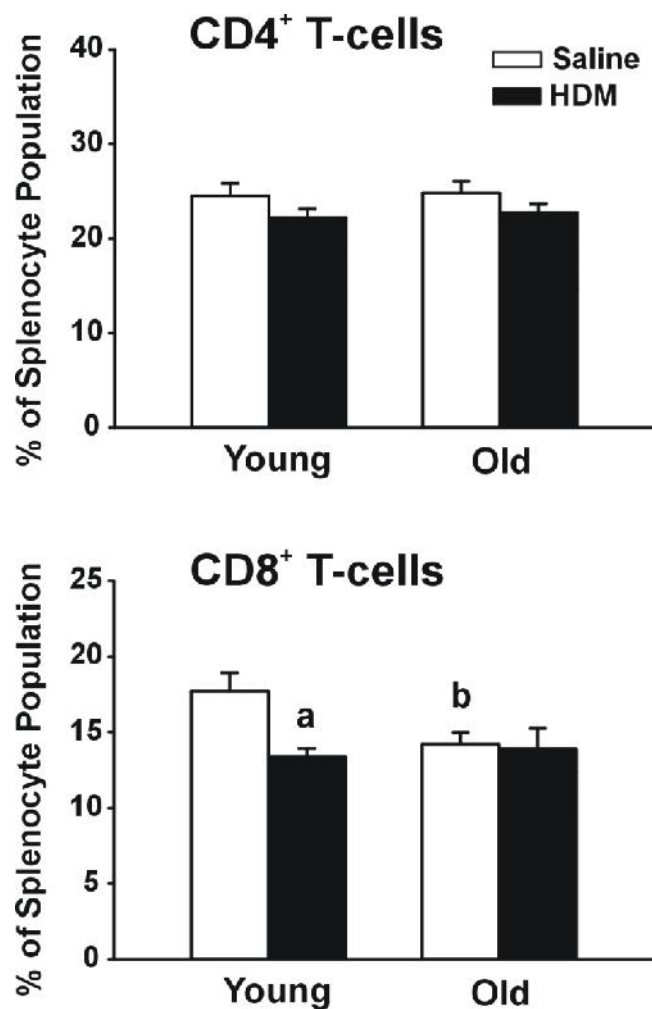


Figure S1. CD4⁺ and CD8⁺ T-cell population in spleens of young and old saline- and HDM-mice. The percentage of CD4⁺-T and CD8⁺-T-cells in the spleen of all experimental groups were characterized by flow cytometry. **a:** Significant changes ($p < 0.05$) compared to saline-mice of the same age group, **b:** significant changes compared to young mice of the same exposure group.