

Antigen-induced mast cell expansion and bronchoconstriction in a mouse model of asthma

Shannon Li,¹ Minara Aliyeva,¹ Nirav Daphtary,¹ Rebecca A. Martin,¹ Matthew E. Poynter,¹ Shannon F. Kostin,¹ Jos L. van der Velden,¹ Alexandra M. Hyman,¹ Christopher S. Stevenson,² Jonathan E. Phillips,² and Lennart K. A. Lundblad¹

¹The University of Vermont, Vermont Lung Center, Burlington, Vermont; and ²Hoffmann-La Roche Incorporated, Nutley, New Jersey

Submitted 25 February 2013; accepted in final form 21 November 2013

Li S, Aliyeva M, Daphtary N, Martin RA, Poynter ME, Kostin SF, van der Velden JL, Hyman AM, Stevenson CS, Phillips JE, Lundblad LKA. Antigen-induced mast cell expansion and bronchoconstriction in a mouse model of asthma. *Am J Physiol Lung Cell Mol Physiol* 306: L196–L206, 2014. First published November 27, 2013; doi:10.1152/ajplung.00055.2013.—Lung mastocytosis and antigen-induced bronchoconstriction are common features in allergic asthmatics. It is therefore important that animal models of asthma show similar features of mast cell inflammation and reactivity to inhaled allergen. We hypothesized that house dust mite (HDM) would induce mastocytosis in the lung and that inhalation of HDM would trigger bronchoconstriction. Mice were sensitized with intranasal HDM extract, and the acute response to nebulized HDM or the mast cell degranulating compound 48/80 was measured with respiratory input impedance. Using the constant-phase model we calculated Newtonian resistance (R_n) reflecting the conducting airways, tissue dampening (G), and lung elastance (H). Bronchoalveolar lavage fluid was analyzed for mouse mast cell protease-1 (mMCP-1). Lung tissue was analyzed for cytokines, histamine, and α -smooth muscle actin (α -SMA), and histological slides were stained for mast cells. HDM significantly increased R_n , but H and G remained unchanged. HDM significantly expanded mast cells compared with control mice; at the same time mMCP-1, α -SMA, Th2 cytokines, and histamine were significantly increased. Compound 48/80 inhalation caused bronchoconstriction and mMCP-1 elevation similarly to HDM inhalation. Bronchoconstriction was eliminated in mast cell-deficient mice. We found that antigen-induced acute bronchoconstriction has a distinct phenotype in mice. HDM sensitization caused lung mastocytosis, and we conclude that inhalation of HDM caused degranulation of mast cells leading to an acute bronchoconstriction without affecting the lung periphery and that mast cell-derived mediators are responsible for the development of the HDM-induced bronchoconstriction in this model.

mast cell protease-1; histamine; input impedance

MUCH OF OUR UNDERSTANDING of inflammatory diseases such as asthma is derived from studies in animal models. It is therefore important that the models used accurately replicate critical aspects of the human condition. Allergic asthmatics frequently suffer from acute bronchoconstriction upon antigen inhalation, and this acute phase is then often followed by an extended period of airways hyperresponsiveness (AHR) to, e.g., methacholine (MCh) challenge. The physiological phenotype of asthma can thus be divided into two phases. Mouse models, however, typically only exhibit the second AHR phase. Indeed, published reports show that an often-used ovalbumin (OVA)

mouse model of asthma does not exhibit any measurable bronchoconstriction to inhaled OVA (50, 54). Mice are clearly capable of generating an adequate allergic immune response with cellular inflammation following OVA sensitization and challenge, so why they do not also exhibit acute antigen-induced bronchoconstriction is enigmatic.

One possible explanation is that the relatively short OVA sensitization and challenge protocols typically used with mice do not allow sufficient time for mast cell numbers to increase in the lung (37), so that antigen inhalation does not lead to enough mediator release to cause functionally significant shortening of the bronchial smooth muscle. The increase in mast cell numbers in the airway tissues is a key aspect of the asthmatic phenotype in humans (2, 3, 38), preempting the critical roles played by histamine and mucus secretion in causing airway obstruction (17). Several studies have shown that this also applies in animal models. For example, intraperitoneal injection of crude extracts of house dust mite (HDM) from *Dermatophagoides pteronyssinus* in guinea pigs elicits Th2 sensitization and antigen challenge-induced bronchoconstriction (23), whereas bronchial anaphylactic reactions have been observed in sensitized Sprague Dawley rats following intravenous challenge with OVA (14). We recently demonstrated similar effects in mice that were subjected to sensitization inhalation exposure to HDM (39). In addition, allergic sheep exposed to inhaled tryptase, a mast cell serine protease released during mast cell activation, exhibit both bronchoconstriction and AHR, further implicating a role for mast cell-derived mediators (34).

Given the importance of the mouse in asthma research, it is an important goal to recapitulate mast cell expansion in this species when generating allergic models of asthma. Based on preliminary data from our laboratory (29), we hypothesized that HDM sensitization and challenge would induce lung mast cell expansion in mice and that reexposure to inhaled HDM would cause acute bronchoconstriction. We tested this hypothesis by measuring acute bronchoconstriction, the numbers of mast cells in the lung, and the quantities of mediators released from activated mast cells in HDM-sensitized and HDM-challenged wild-type mice and mice deficient in mast cells.

MATERIALS AND METHODS

Animals. Female BALB/cJ and C57BL/6J, 7–8 wk old, were purchased from Jackson Laboratory (Bar Harbor, ME). Mast cell-deficient mice Kit^{W-sh}/HNihrJaeBsmJ (Kit^{W-sh}) breeding pairs were purchased from Jackson Laboratory, and offspring were used at 7–8 wk of age. Housed in a pathogen-free environment, the mice had free access to standard laboratory chow and water ad libitum. All exper-

Address for reprint requests and other correspondence: L. K. A. Lundblad, Dept. of Medicine, The Univ. of Vermont, Vermont Lung Center, HSRF, Rm. 230, 149 Beaumont Ave., Burlington, VT 05405-0075 (e-mail: lennart.lundblad@uvm.edu).

imental procedures were approved by the University of Vermont Institutional Animal Care and Use Committee.

Sensitization with HDM. HDM *D. pteronyssinus* extract (Greer Laboratories, Lenoir, NC) was resuspended in $1\times$ Dulbecco's PBS (Invitrogen, Carlsbad, CA) at a concentration of 2.5 mg/ml. Mice were divided into two groups, experimental and control. Both groups of mice were anesthetized with isoflurane. Whereas the experimental group was sensitized with intranasal instillation of 50 μ l of the HDM suspension for 15 days over three consecutive weeks, the control group was sham sensitized with PBS. The animals showed no visible issues relating to the HDM administration. The amount of lipopolysaccharide (LPS) in the HDM extract varied from batch to batch, and we have calculated that the dose of LPS delivered to the mice varied from 1.36 to 4.39 EU at each administration.

Assessment of acute bronchoconstriction and AHR. After the last HDM intranasal exposure (72 h), the mice were anaesthetized intraperitoneally with pentobarbital sodium (90 mg/kg), and the trachea was cannulated and connected to a computer-controlled small animal ventilator (flexiVent; SCIREQ, Montreal, Canada) and ventilated at 200 breaths/min. Next the mice were paralyzed with pancuronium bromide (0.8 μ g/kg ip). The depth of anesthesia was monitored with EKG throughout the experiment as previously described (27, 28, 30). The animals were stabilized over about 10 min of regular ventilation at a positive end-expiratory pressure (PEEP) of 3 cmH₂O. A standard

lung volume history was then established by delivering two total lung capacity maneuvers to a pressure limit of 25 cmH₂O and holding for 3 s. Next, two baseline measurements of respiratory input impedance (Z_{rs}) were obtained. The mice were then exposed to either of the substances described as follows.

Assessment of HDM-induced bronchoconstriction. Seventy-two hours after the last HDM intranasal exposure, the mice were connected to the flexiVent as described above. Following stabilization and standard lung volume history the mice were either exposed to an inhalation of vehicle control dose of PBS over 40 s followed by a Z_{rs} measurement every 10 s for 3 min, or the mice received 40 s of HDM (2.5 mg/ml). The inhalation was immediately followed by Z_{rs} measurements every 10 s for 3 min. A Z_{rs} measurement takes 3 s, 1 s for exhalation to PEEP and 2 s to deliver the measurement oscillation, leaving 7 s for regular ventilation before the next measurement takes place.

Assessment of AHR. Mice were sensitized with HDM and prepared as described for HDM-induced bronchoconstriction above. The mice were tested for AHR using the following approach. Following stabilization and standard lung volume history, mice were exposed to an inhalation of aerosolized PBS (vehicle control) for 10 s. Z_{rs} was then measured every 10 s for 3 min. This complete sequence of maneuvers and measurements was then repeated for aerosol exposures to three incremental doses of MCh (3.125, 12.5, and 25 mg/ml) (41).

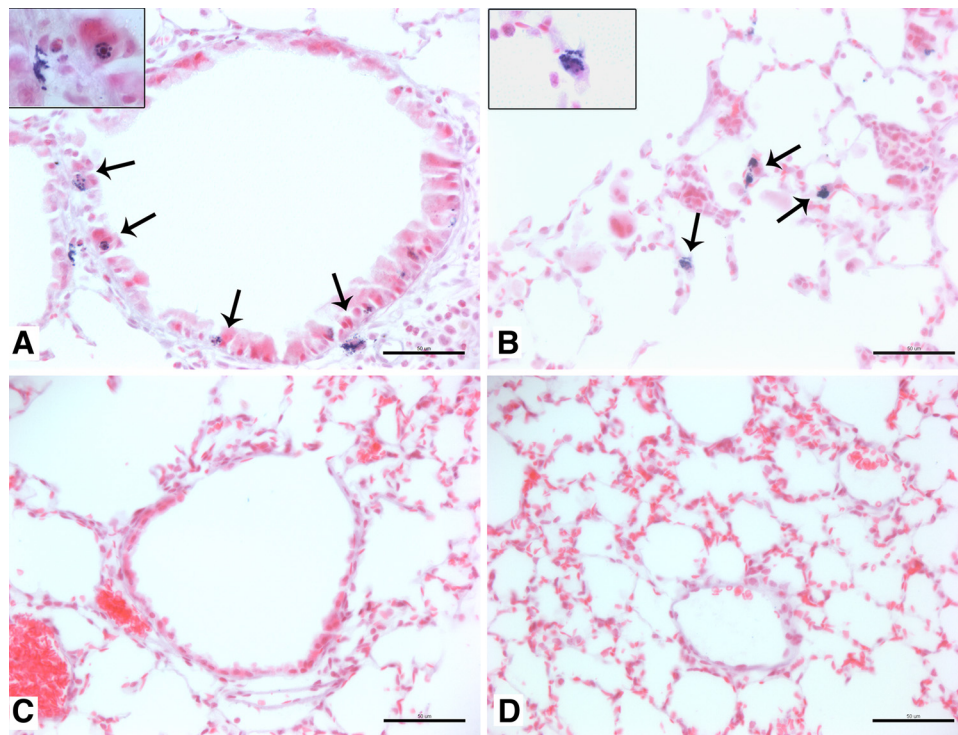
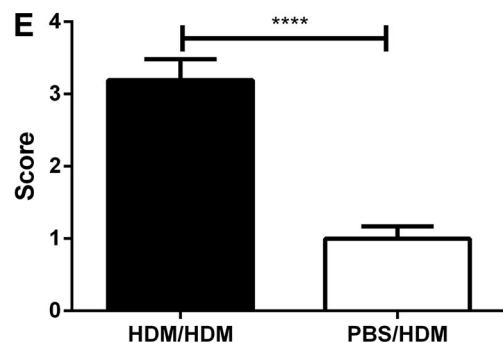


Fig. 1. Toluidene blue staining for mast cells in mouse lungs and histology score. HDM/HDM mice ($n = 8$, filled bar) were sensitized and challenged with house dust mite (HDM). PBS/HDM mice ($n = 7$, open bar) were sham sensitized with PBS but challenged with HDM. In the HDM/HDM group, mast cells (indicated by black arrows) were observed in both the central airway (A) and the parenchyma (B) at $\times 40$; the scale bar is 50 μ m. The image of a magnified mast cell is located in the top left corner of A and B at $\times 100$. In contrast, the PBS/HDM group showed very few mast cells, if any, in the central airway (C) and parenchyma (D). The toluidene blue-stained mast cells were blind scored and showed significant difference in mast cell population between the HDM/HDM and PBS/HDM groups (E) (**** $P < 0.0001$).



Compound 48/80-induced bronchoconstriction. Mice were sensitized with HDM and prepared as described for HDM-induced bronchoconstriction above. Following stabilization and standard lung volume history, mice were exposed to an aerosol of the mast cell degranulating compound 48/80 (1 mg/ml) or PBS (vehicle control) for 40 s, and Z_{rs} was then measured every 10 s for 3 min.

Respiratory mechanics. Respiratory impedance was determined using the forced oscillation technique described previously (30, 41). Briefly, Z_{rs} over the frequency range 1–20.5 Hz was determined using a 2-s broadband perturbation in volume applied by the flexiVent. Each determination of Z_{rs} was fit with the constant-phase model of impedance (44) given by

$$Z_{rs}(f) = R_n + i2\pi fI + \frac{G - iH}{(2\pi f)^\alpha}$$

where R_n is a frequency-independent Newtonian resistance reflecting that of the conducting airways, I is airway gas inertance, G characterizes tissue dampening, H characterizes tissue stiffness or elastance, i is the imaginary unit, and f is frequency in Hertz (20, 44). While the entire time course of the responses are shown in Figs. 1–9, for statistical purposes airways responses were quantified in terms of the average of the 18 measurements obtained at each MCh dose or following HDM inhalation (41).

Collection of bronchoalveolar lavage and lung specimens. Lungs were lavaged with 1 ml of $1\times$ Dulbecco's PBS, and the bronchoalveolar lavage fluid (BALF) fluid volume was measured for each sample. The cell pellet was separated from the supernatant, resuspended in 400 μ l of PBS, and counted. Differential cell counts were determined by spinning the cells to glass slides with a cytospin and staining with hematoxylin and eosin. The supernatant was saved for later analysis.

Enzymatic digestion and mast cell enumeration. Lungs were harvested from HDM-sensitized mice 72 h post the last intranasal HDM administration. Lung cells in freshly prepared enzyme mix (Miltenyi Biotec, Auburn, CA) were mechanically digested using a GentleMACs dissociator (Miltenyi Biotec): four rounds of program *m_lung_01* (8 s and 300 total rounds/run) followed by a 15-m incubation at 37°, then two rounds of program "B," a second 15-m incubation at 37° (38 s and 2,083 total rounds/run), and, finally, a single round of *m_lung_02* (31 s and 553 total rounds/run). A short centrifugation was performed to collect cells, which were then resuspended in PBS and filtered through a 70- μ m filter. After the cells were washed in PBS, red blood cells were removed with ACK lysing solution (8,024 mg/l NH_4Cl , 1,001 mg/l KHCO_3 , and 7.722 mg/l $\text{EDTA}\cdot\text{Na}_2\cdot 2\text{H}_2\text{O}$). Cells were washed, counted with an Advia 120 (Siemens, Munich, Germany), and resuspended in PBS in preparation for antibody staining and flow cytometric analysis.

Surface staining and flow cytometric analysis. Lung cells (2×10^6 cells/ml) in 1 ml PBS were stained with live/dead (Invitrogen, Grand Island, NY) staining, which was followed by Fc blocking (anti-CD16/CD32; BD Pharmingen) and surface staining with anti-CD3-FITC (Biolegend, San Diego, CA), anti-CD45-Pacific Orange (Invitrogen), anti-ckit-APC, and anti-Fc ϵ RI-PE (ebioscience, San Diego, CA) in FACS buffer (DPBS with 5% FBS) and fixed in 1% paraformaldehyde. Analysis was performed on 500,000–2,000,000 events using a Becton Dickinson LSR II FACS, BD FACSDIVA (Becton Dickinson, Franklin Lakes, NJ), and FlowJo (Tree Star, Ashland, OR). Mast cells were defined as $\text{CD3}^-\text{ckit}^+\text{Fc}\epsilon\text{RI}^+$ cells (35). Backgating demonstrated that these cells were of intermediate side scatter. The calculated number of live events analyzed did not differ between experimental groups ($P = 0.149$).

Toluidine blue staining of mast cells and histology. Lung tissue was inflated fixed at 30 cmH_2O with 10% formalin for toluidine blue (Electron Microscopy Sciences, Hartfield, PA) staining, embedded in paraffin, cut into 5- μ m sections, and mounted on slides. Slides were deparaffinized and rehydrated in xylene (three changes for 12 min

each), 100% ethanol (two changes for 5 min each), 95% ethanol (two changes for 5 min each), 70% ethanol, 50% ethanol, and distilled water for 5 min each. The slides were then stained in 0.5% toluidine blue in 0.5 N HCl (pH 0.5) overnight, rinsed in distilled water, counterstained in eosin-Y for 30 s, and dehydrated in 95% ethanol, 100% ethanol, and xylene for two changes each. Mast cells were examined under light microscopy. Differences between mast cell populations in experimental and control groups were determined by masked scoring (0 for the least presence of mast cells and 4 for the highest presence of mast cells) of the slides by three independent persons. The scores were averaged and presented as means $\pm$ SE.

Protein extraction protocol. Frozen lung tissue was pulverized to a fine powder using a liquid nitrogen-chilled mortar and pestle, transferred to the 100- μ l mark of a liquid nitrogen-chilled 1.5-ml Eppendorf tube, and lysed by adding 400 μ l of cell extraction buffer [PBS containing 0.5% Triton X-100 and $1\times$ protease inhibitor cocktail (Sigma, St. Louis, MO)] and allowing the tubes to warm to the temperature of wet ice. The mixture was vortexed and incubated on ice for 30 min with occasional vortexing. The cell lysates were clarified by centrifugation at 13,000 g at 4°C for 10 min. The clarified cell extracts were transferred to clean microcentrifuge tubes, which were stored at -80°C until ready for analysis. Protein concentration was determined using Bradford assay (Bio-Rad, Hercules, CA), protein concentration was equilibrated using cell extraction buffer, and cell extracts were diluted at least 1:10 in Standard Diluent Buffer before analysis by ELISA or Bio-Plex.

Biomarker measurements. Frozen lung tissue was pulverized to a fine powder using a liquid nitrogen-chilled mortar and pestle, transferred to the 100- μ l mark of a liquid nitrogen-chilled 1.5-ml Eppendorf tube, and lysed by adding 400 μ l of PBS and allowing the tubes to warm to the temperature of wet ice. The mixture was vortexed and incubated on ice for 30 min with occasional vortexing. The cell lysates were clarified by centrifugation at 13,000 g at 4°C for 10 min. The clarified cell extracts were transferred to clean microcentrifuge tubes that were stored at -80°C until ready for analysis. Protein concentration was determined using Bradford assay (Bio-Rad). Protein concentration was equilibrated using cell extraction buffer, and cell extracts were diluted 1:10 in Standard Diluent Buffer before analysis by ELISA for IL-4, IL-5, IL-13, IL-17, and IFN- γ . The concentration of mouse mast cell protease-1 (mMCP-1) in BALF was determined by enzyme-linked immunosorbent assay (mouse MCP-1 ELISA Ready-SET-go kit; eBioscience). The concentration of histamine, extracted from frozen lung tissues, was

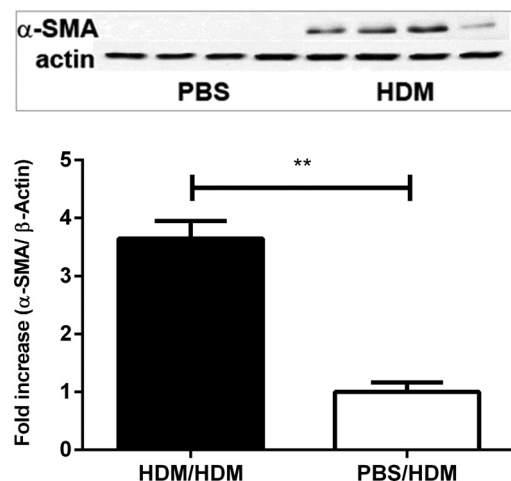


Fig. 2. HDM-induced α -smooth muscle actin (α -SMA) expression, analyzed by Western blotting. An amplification of α -SMA in the lung tissues was observed in the HDM/HDM ($n = 4$, filled bar)-treated group, whereas no α -SMA expression was observed in the control PBS/HDM ($n = 4$, open bar) group ($P < 0.01$).

measured using a Histamine Enzyme Immunoassay kit validated by SPI-BIO (Cayman Chemical, Ann Arbor, MI).

α -Smooth muscle actin and β -actin. Protein lysates were prepared by mincing fresh lung tissue in cold lysis buffer immediately followed by homogenization. Lysates were incubated on ice for 30 min, followed by 30 min of centrifugation at 16,000 g . A portion of the supernatant was saved for protein determination, before the addition of Laemmli sample buffer. Total protein was assessed by the Bio-Rad DC Protein Assay kit (Bio-Rad) according to the manufacturer's instructions. α -Smooth muscle actin (α -SMA) and β -actin (both Sigma) protein abundance was evaluated by Western blotting.

Data analysis. Origin 8.0 (OriginLab, Northampton, MA) and Graph-Pad Prism 5 were used for analysis, and data are expressed as means $\pm$ SE. Respiratory mechanics data following a challenge were collected for 3 min, and the whole measurement epoch including 18 measurements was treated as one and analyzed using one-way ANOVA. Respiratory effects with 48/80 were analyzed with Kruskal-Wallis test for unequal variances. Cells, cytokines, α -SMA, and flow cytometric data were analyzed using two-tailed t -tests. Histamine, mMCP-1, and histology scores were analyzed using Mann-Whitney's nonparametric test. Differences were considered statistically significant at $P < 0.05$.

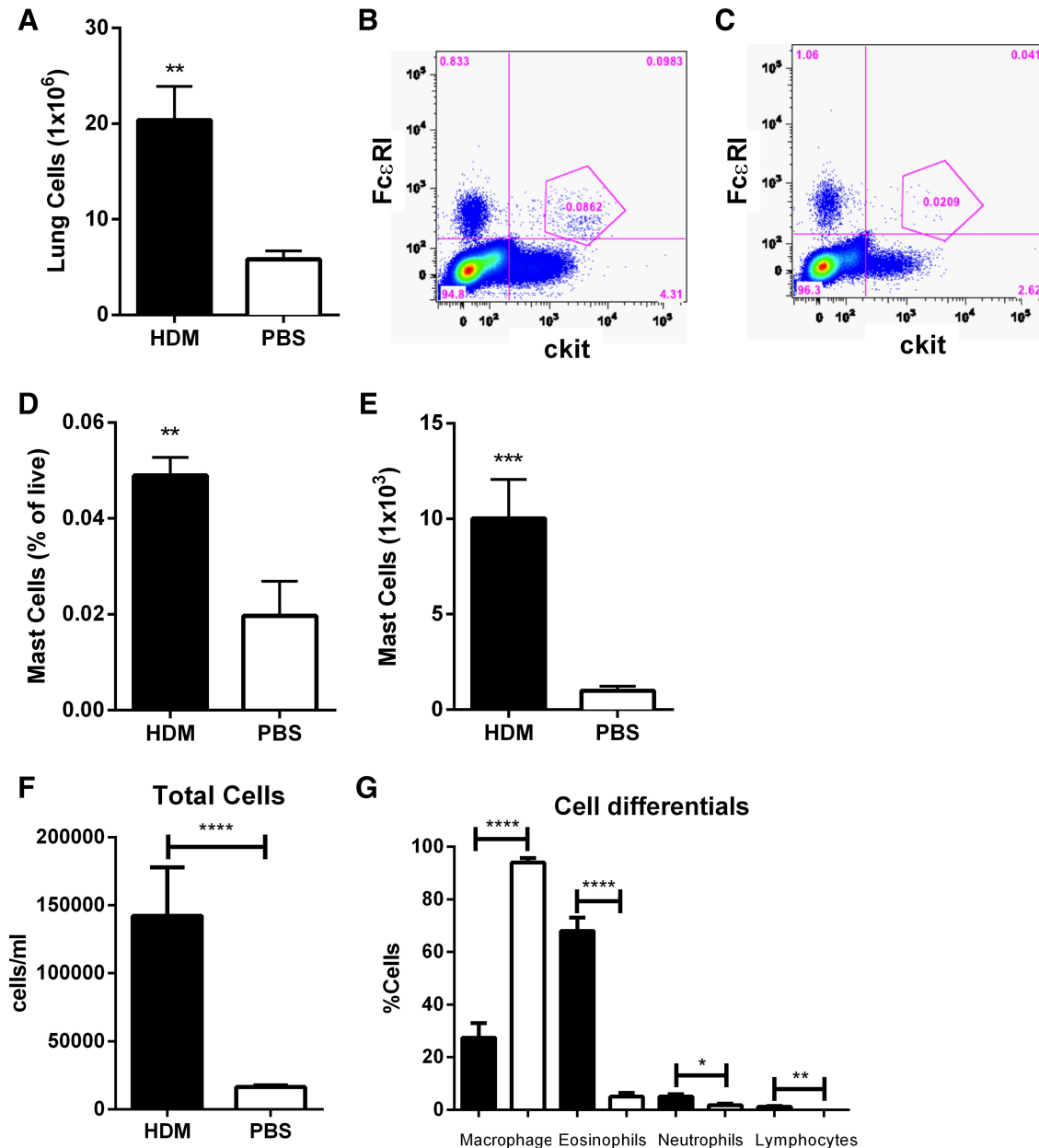


Fig. 3. HDM-induced increase in inflammatory cells. Following enzymatic digestion of lungs from HDM-sensitized (solid bars; $n = 7$) or sham-sensitized (open bars; $n = 7$) PBS mice, single cell suspensions were enumerated (A) and stained with live/dead, anti-CD3-FITC, anti-ckit-APC, and anti-Fc ϵ RI-PE. Live cells that were fitc/CD3 $^{-}$ were evaluated for ckit and Fc ϵ RI expression. A representative sample of ckit $^{+}$ Fc ϵ RI $^{+}$ cells is shown for HDM (B)- and PBS (C)-treated mice. Total mast cells were enumerated by applying the percent mast cells within the live population (D) to total lung cell counts (E). Total cell count was increased in bronchoalveolar lavage fluid (BALF; *** $P < 0.001$ and **** $P < 0.00001$) (F). Macrophages dominated in the control mice BALF (**** $P < 0.00001$), whereas eosinophils (**** $P < 0.00001$), neutrophils (* $P < 0.05$), and lymphocytes (** $P < 0.01$) dominated in the HDM mice (G).

RESULTS

HDM sensitization leads to mast cell expansion. First, we explored if HDM sensitization would lead to mast cell expansion in the lung. As evidenced by histological scoring (Fig. 1), we found significant expansion of mast cells in BALB/cJ, sensitized with HDM, not only in the conducting airways but also in the parenchyma. In contrast, in control lungs few mast cells were seen in the airways and almost none in the parenchyma. The histology of the airways showed general inflammation with thickened epithelium (Fig. 1). In addition, we discovered that α -SMA increased about seven times in the lungs from HDM-sensitized mice, further supporting that airway remodeling could be ongoing (Fig. 2).

To further confirm that HDM sensitization leads to an increased number of mast cells in the lungs, we performed a flow cytometric analysis of lung single cell suspensions from either HDM-allergically sensitized mice or mice sham sensitized with PBS. HDM-treated mice had a significantly greater number of lung cells than PBS-treated mice (Fig. 3A). Following surface staining, we enumerated lung mast cells by gating on fitc/CD3^- live cells that were $\text{ckit}^+\text{Fc}\epsilon\text{RI}^+$ (35). We observed that HDM-treated mice had increased numbers of mast cells compared with PBS-treated mice (Fig. 3, B and D). Because HDM-sensitized mice had a statistically higher percent of live cells ($P < 0.0001$), but not fitc/CD3^- cells ($P = 0.195$), than PBS-treated mice, $\text{ckit}^+\text{Fc}\epsilon\text{RI}^+$ were calculated as a percent of live cells (Fig. 3D). By multiplying the total number of lung cells (Fig. 3A) by the percentage of mast cells (Fig. 3D), we found that HDM-treated mice had significantly higher absolute number of $\text{ckit}^+\text{Fc}\epsilon\text{RI}^+$ mast cells compared with PBS-treated mice ($P < 0.001$, Fig. 2E). We also discovered a $\text{ckit}^-\text{Fc}\epsilon\text{RI}^+$ population that we expect to be basophils or possibly dendritic cells (10, 35, 40). The viability following lung digestion was typically 98–100%. The BALF in sensitized mice was dominated by eosinophils (Fig. 3, F and G).

Inhalation of HDM induced bronchoconstriction. After validating the expansion of mast cells, we progressed to investigate if mastocytosis would lead to consequences on respiratory mechanics; in particular, we were interested in determining if HDM inhalation by sensitized mice would trigger a bronchoconstriction. The HDM-sensitized mice showed an immediate and significant increase in R_n upon HDM inhalation challenge, whereas G and H values remained unchanged, suggesting that the immediate effects of HDM inhalation were restricted to the conducting airways (Fig. 4). Administration of PBS vehicle did not cause any significant response. To elucidate whether mast cells were activated by inhaled HDM, we analyzed mMCP-1 and histamine in BALF and lung tissue, respectively. Levels of both mediators were significantly elevated, and we interpret these findings as indicative of mast cell activation and degranulation (Fig. 5). Lung wet weight was significantly increased by HDM, suggestive of lung edema and possibly also remodeling and infiltration of inflammatory cells (Fig. 5C). We attempted to detect Th2 cytokines in the lung tissue at the time of HDM-induced bronchoconstriction, which was 72 h post last intranasal HDM. This likely was not the optimal time point to detect elevated levels of cytokines (53); nevertheless, we discovered a characteristic Th2 profile with significant increases in IL-4, IL-5, IL13, and IL-17, but no significant change in INF- γ (Fig. 6). Naïve mice were found to have

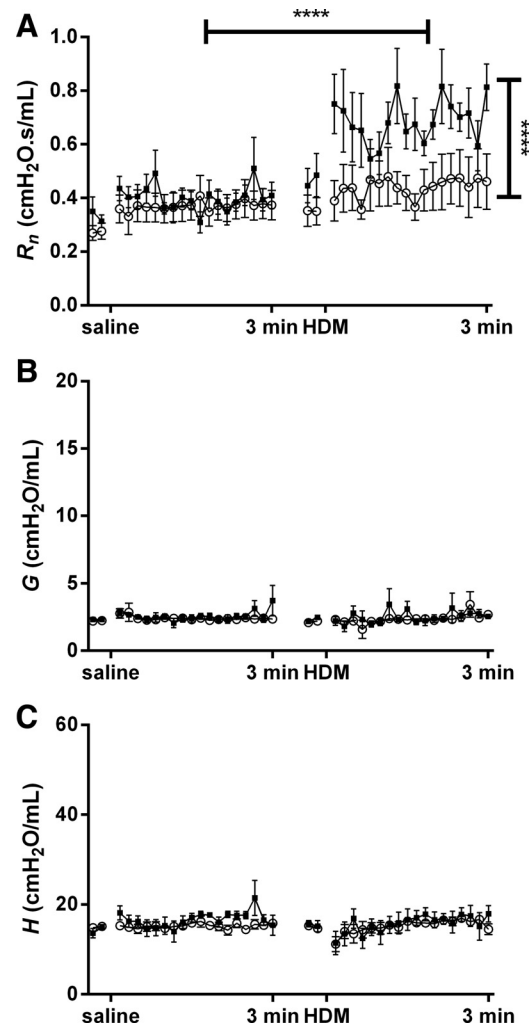


Fig. 4. Effect of saline and HDM inhalation on respiratory mechanics. HDM/HDM mice ($n = 10$, solid black squares) were sensitized and challenged with HDM. PBS/HDM mice ($n = 10$, open circles) were sham sensitized with PBS but challenged with HDM. Respiratory input impedance (Z_{rs}) was measured every 10 s for 3 min after saline and HDM (2.5 mg/ml) aerosol challenge. When HDM-sensitized mice were challenged with nebulized HDM, Newtonian resistance (R_n) was significantly increased compared with baseline (**** $P < 0.0001$). In contrast, sham-sensitized mice showed no significant increase in R_n when challenged with HDM. No significant changes were observed in tissue dampening (G) and lung elastance (H), suggesting that bronchoconstriction was mainly localized to the central airways, without affecting the periphery of the lung.

similar levels of cytokines as control PBS mice (data not shown).

HDM-induced bronchoconstriction is absent in mast cell-deficient mice. In sensitized Kit^{W-sh} mice we found no evidence of HDM-induced bronchoconstriction, which was not different from the sham-sensitized control group. By contrast, the HDM-sensitized positive control C57Bl/6J mice had a significant bronchoconstriction response although not as pronounced as in the BALB/cJ. The BALF cell numbers and differentials were similarly and significantly increased in the sensitized Kit^{W-sh} and C57Bl/6J mice compared with sham-sensitized Kit^{W-sh} (Fig. 7). This shows that the allergic inflammatory response in the Kit^{W-sh} mice was comparable to that of the wild-type C56Bl/6J mice.

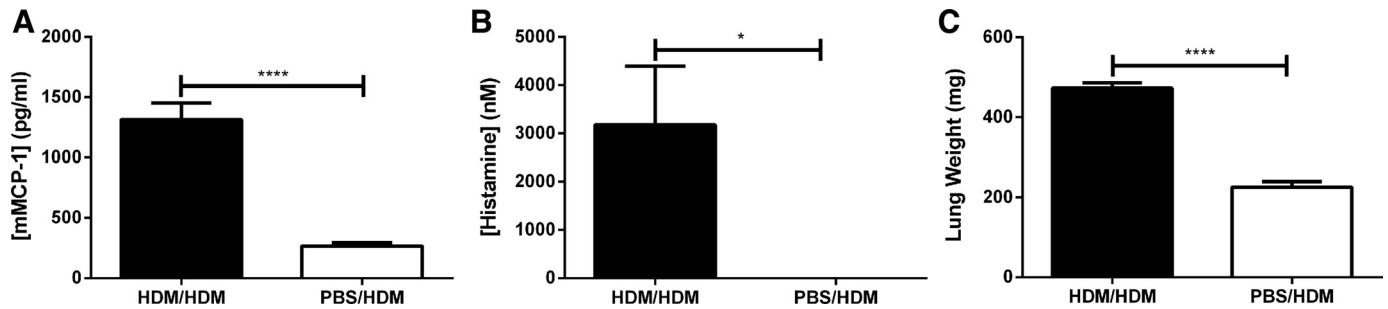


Fig. 5. Lung weights, mouse mast cell protease-1 (mMCP-1) in BALF, and histamine concentration in lung tissue. HDM/HDM mice ($n = 8$, black bars) were sensitized and challenged with HDM. PBS/HDM mice ($n = 7$, open bars) were sham sensitized with PBS but challenged with HDM. HDM/HDM mice had significantly heavier lungs than the controls, and ELISA data revealed significantly elevated mMCP-1 and histamine levels in the HDM/HDM mice, indicating degranulation of mast cells (* $P < 0.05$ and **** $P < 0.0001$).

HDM-induced AHR. In contrast to the effects seen on bronchoconstriction by HDM inhalation, when HDM-sensitized mice were tested for AHR with inhaled MCh they responded primarily with significant increases in G and H (Fig. 8), indicating that the AHR was localized primarily in the peripheral airways similarly to what we have found previously with HDM and OVA (25, 41).

Compound 48/80 inhalation induced mast cell degranulation. The mast cell population was expanded in the murine lung by HDM sensitization, and we then hypothesized that antibody-independent activation of mast cells would cause degranulation and trigger a similar response as HDM inhalation, i.e., a bronchoconstriction. Compound 48/80 is a mast cell degranulating agent that has been used previously in several animal studies and shown to trigger the release of several mast cell mediators, some of which are acutely bronchoactive (17, 24, 42, 46). We used HDM-sensitized mice with an expanded pool of lung mast cells (as seen in Figs. 1 and 3) and then administered nebulized compound 48/80 while measuring respiratory mechanics. A significant increase in R_n was observed following compound 48/80 inhalation (Fig. 9) indicative of bronchoconstriction. Moreover, the mMCP-1 titre in the BALF was significantly higher than in PBS control mice (Fig. 9), and was found to be at the same level as in HDM-challenged mice (Fig. 5).

DISCUSSION

Bronchoconstriction and AHR are the two prominent physiological phenotypes of allergic asthma. Many studies have been published on the pathogenesis of allergic asthma in human asthmatics. In attempting to emulate the development of chronic asthma in humans, studies have been conducted in various animal models ranging from guinea pigs to sheep typically studying AHR (14, 23, 34). In the present study, we were able to induce bronchoconstriction in mice sensitized with inhaled HDM showing that subsequent exposure to HDM leads to mast cell expansion in the mouse lung. This is a novel model of bronchoconstriction using a naturally occurring aeroallergen that frequently causes allergic asthma in humans (18, 31, 45).

By assessing the mechanical and physiological responses of the lung just after an inhalation exposure to HDM, we were able to demonstrate an acute increase in R_n that we interpret as the result of shortening of the airway smooth muscle. We base this conclusion on the observation shown in Fig. 4 that the response occurred exclusively in the conducting airways (R_n), whereas we did not find any response in the lung periphery (G or H) (4, 6, 9, 26, 30). Our conclusions about the localization and the nature of the respiratory responses following inhaled HDM and MCh are predicated on our understanding of and ability to infer mechanism from the relative changes of the impedance parameters R_n , G , and H . We have previously shown that R_n is a good reflection of the flow resistance of the

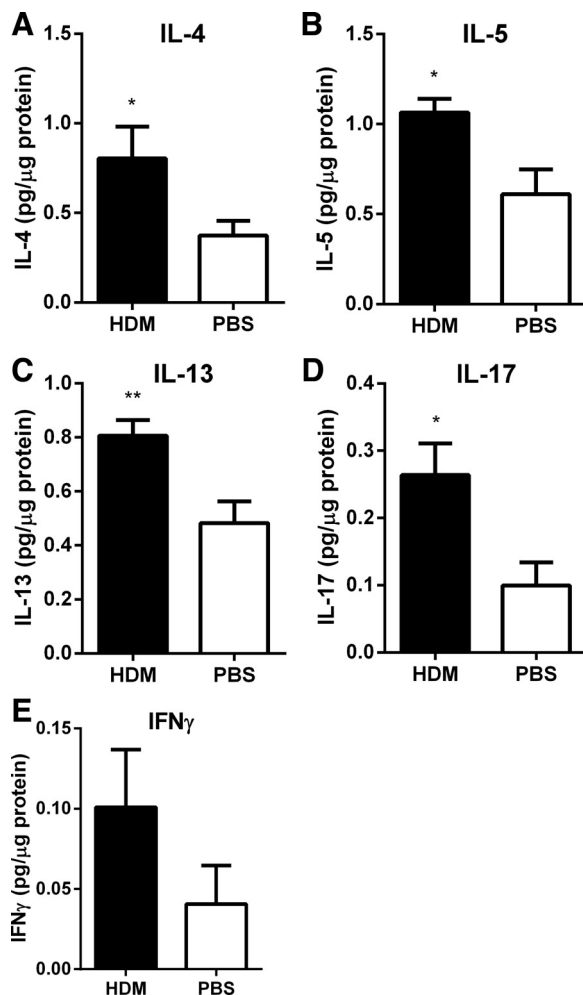


Fig. 6. Th2 cytokines extracted from snap-frozen lung tissues were analyzed by ELISA. HDM mice ($n = 8$, filled bars) were sensitized with HDM intranasally. PBS mice ($n = 8$, open bars) were sham sensitized with PBS intranasally. HDM mice had significantly higher levels of IL-4, IL-5, IL-13, and IL-17 compared with PBS controls (* $P < 0.05$ and ** $P < 0.01$).

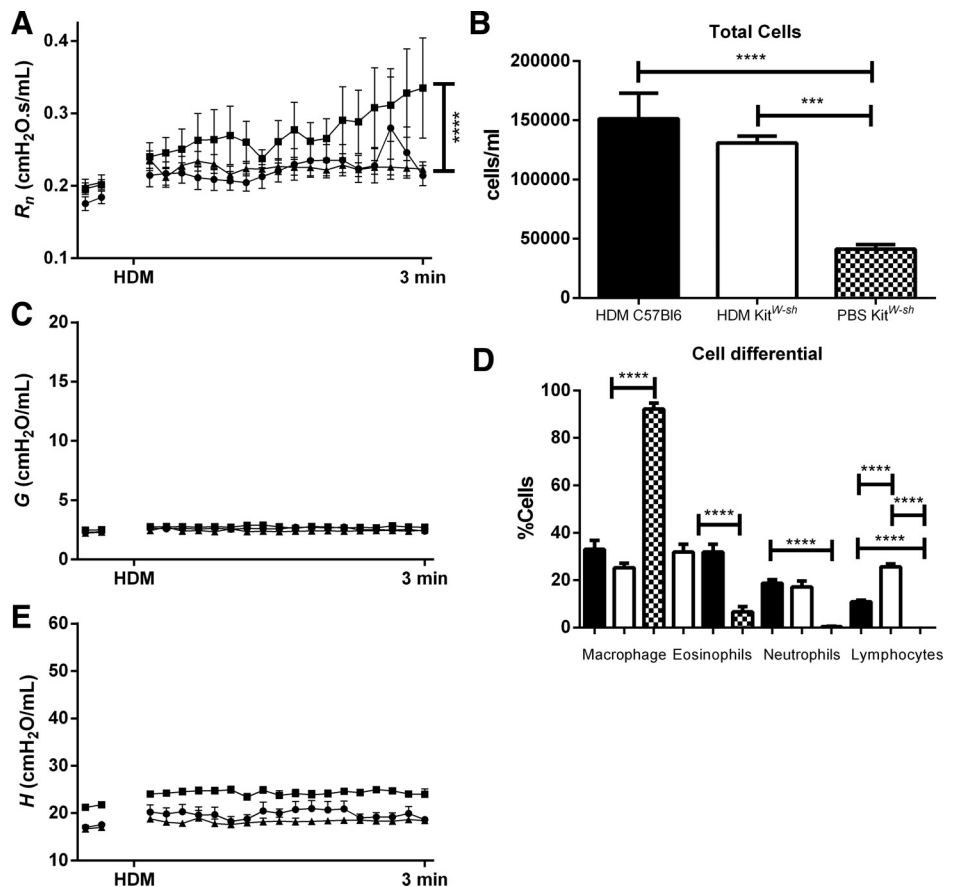


Fig. 7. Effect of HDM inhalation on respiratory mechanics and BALF cell differentials in mast cell-deficient HDM-sensitized mice (HDM Kit^{W-sh}, solid circles and open bars, $n = 8$), HDM-sensitized C57Bl6/J mice (HDM C57Bl6, solid squares and solid bars, $n = 7$), and PBS sham-sensitized mast cell-deficient mice (PBS Kit^{W-sh}, solid triangles and checkered bars, $n = 6$). Z_{rs} was measured every 10 s for 3 min after HDM challenge. HDM induced an acute bronchoconstriction (increase in R_n) in the HDM C57Bl6 group compared with the HDM Kit^{W-sh} and the control PBS Kit^{W-sh} (*** $P < 0.001$ and **** $P < 0.0001$). BALF cell numbers and differentials were significantly increased in the sensitized Kit^{W-sh} and C57Bl6/J mice compared with sham-sensitized PBS Kit^{W-sh}.

conducting airway tree (48) and that increases in G and H in the same proportion reflect closure of small airways (30, 51). These conclusions were further supported by in silico experimentation with anatomically based computational models of the mouse lung (7, 51, 52). Nevertheless, because these conclusions remain inferential, it is important to ask how the physiological findings correlate with measures of, e.g., inflammation and mediator release in the allergic lung.

We determined mast cell expansion in histological lung specimens and confirmed that lung mast cells degranulate upon local exposure to HDM by causing significantly elevated levels of mMCP-1 and histamine in the BALF and tissue of allergic mice. Also, our histological data showed a significant mast cell increase following HDM sensitization, with mast cells apparent in the conducting airways and in the parenchyma. The expansion of mast cells in the lung tissue was further confirmed by the significant increase of ckit⁺FcεRI⁺ mast cells assessed by flow cytometry. There is a possibility that other cells expressing ckit and FcεRI could affect the results; however, we believe that the rigorous approach used in this study eliminated false positive signals and that the increased number of ckit⁺FcεRI⁺ cells actually reflects mast cells (Fig. 3). One limitation with our assessment of mast cell degranulation is the assay of histamine in the lung tissue. It is possible that the histamine in the lung homogenates could reflect both extra- as well as intracellular histamine; however, we were able to detect significant levels of mMCP-1 in the BALF that probably came from degranulating mast cells. Compound 48/80 was able to release mMCP-1 only in allergic airways, further supporting

that mast cell expansion and activation is necessary for degranulation products to appear. The appearance of mast cells coincided with the bronchoconstriction induced by inhaled HDM, suggesting that the mast cells may have been responsible. This is supported by the observation of a significant and immediate increase in R_n . In addition, we found that HDM significantly increased α -SMA in the lung tissue (Fig. 2). Although this is not proof that the smooth muscle proliferated, it nevertheless points to yet another mechanism involved in smooth muscle expansion being activated by HDM. We have also observed airway eosinophilic inflammation and Th2 sensitization in the HDM-sensitized and HDM-challenged mice, which could be explained by the action of, e.g., granulocyte macrophage colony-stimulating factor (GM-CSF) (11). Lung tissue Th2 cytokines were significantly elevated in HDM-sensitized mice. The relative differences were not as big as previously reported by, e.g., Wilson et al. in BALF using an OVA model (53); however, our cytokine levels were obtained 72 h after the last HDM intranasal sensitization dose, whereas Wilson et al. found that the cytokine titre peaked 4–24 h post an OVA challenge. Whereas there are a number of methodological differences between our model and the one used by Wilson et al., it is likely that the cytokine production in the tissue follows a different time course or that leakage of cytokines from tissue in the airway lumen is a temporary event. If that is the case, studying cytokine levels in the tissue could be more relevant to understand the inflammatory events during an allergic response. Indeed, in preliminary experiments we tried to quantify cytokines in BALF but found only near detection

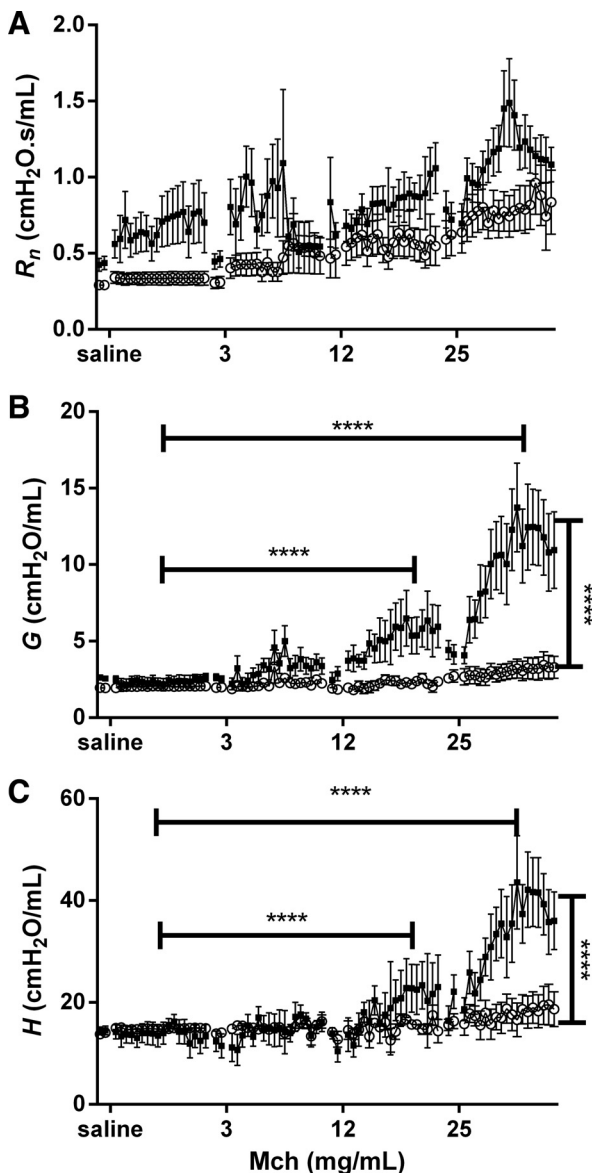


Fig. 8. Airways hyperresponsiveness assessed with methacholine (MCh). HDM/MCh mice ($n = 8$, black squares) were sensitized with HDM and challenged with incremental doses of nebulized MCh. PBS/MCh mice ($n = 7$, open circles) were sham sensitized with PBS and challenged with MCh. Z_{rs} was measured every 10 s for 3 min after saline and each dose of MCh. G and H values significantly increased with MCh challenge in HDM/MCh mice, suggesting the response was mainly localized to the lung periphery (**** $P < 0.0001$).

limit levels, and we then decided to use lung tissue instead where obviously the levels were higher. It is possible that cytokine activity in the tissue is more important for the lung pathology and, at least in this study, the titers of the Th2 cytokines are elevated in the tissue of sensitized mice.

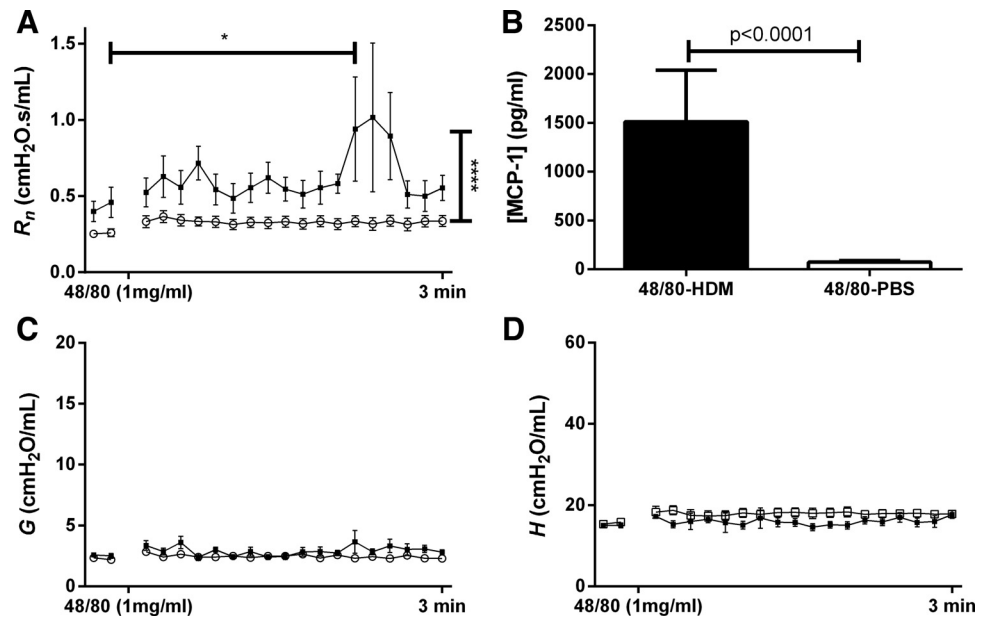
In addition to HDM-induced bronchoconstriction, our data show that sensitization with HDM leads to AHR affecting primarily the lung periphery as indicated by increases in G and H (Fig. 8). Thus, the acute bronchoconstriction to inhaled HDM (Fig. 4) is in stark contrast with the AHR (Fig. 8) triggered by inhaled MCh. Using OVA as allergen, we have previously shown that AHR is dominated by G and H , whereas

the contribution to the changes in impedance from R_n is relatively smaller. According to previously published computer simulations done by our group, the increases in G and H are the result of increased epithelial thickness and an increase in airway closure (25, 30, 41, 51). In a recent study we attempted to induce bronchoconstriction using an OVA model in mice. This was not successful in that inhaled OVA failed to induce any significant bronchoconstriction (27). In retrospect we did stain histological cuts from five OVA-sensitized mice for mast cells but found no expansion of mast cells (data not shown). The lack of mast cells in this commonly used OVA model of asthma was shown by Pae et al., but, and perhaps more interestingly, they also showed that regular exposures to OVA over 3 mo would increase numbers of mast cells in the airways (37). Because they did not assess lung mechanics we do not know if OVA would have elicited an airway response. At any rate, taken together the findings support the notion that mast cells need to be present for the lung to demonstrate antigen-induced bronchoconstriction. Using a sensitization protocol without adjuvant and assessing lung mechanics with the transfer impedance technique, Hessel et al. (21) showed that OVA inhalation increased respiratory resistance in addition to mast cell degranulation visualized by electron microscopy; however, they did not demonstrate an increase in lung mast cell numbers nor did they assess any mast cell mediator release. Because their model of transfer impedance in conscious mice involved applying pressure oscillations around the body generating flow through the nose, it was unavoidable that any restriction of air flow in the nose would affect the measurement of respiratory mechanics (16). Indeed, when Zosky et al. (54) used the same input impedance technique as we used in our present study they failed to find any OVA-induced bronchoconstriction, similar to what we found with OVA in a previous study (27). While it is possible that inhaled OVA triggered mast cell degranulation in the study by Hessel et al., it is, due to technical limitations of their study, impossible to resolve if the increase in respiratory resistance emanated from the nose or the lung.

We also need to acknowledge that there is a potential role for Toll-like receptor 4 (TLR4) signaling using HDM extract. Some of the signaling is likely via TLR4 because the Derp 2 allergen in HDM mimics a molecule that binds to TLR4 (MD-2) and allows for its activation, even in the absence of endotoxin contamination (49). Direct sensitization to HDM via inhalation is a phenomenon that happens in humans, and we believe our model captures some of the complex mechanisms that cause allergic airways disease; hence, the LPS in the HDM extract might be important to generate the full phenotype (43). On the other hand, it was recently reported that LPS can inhibit Th2 lung inflammation induced with HDM (13). The fact that the control mice in our study failed to respond to the HDM inhalation supports the notion that the acute bronchoconstriction was due to allergen effects and not direct effects of LPS.

The ability of our HDM model to generate two distinct phenotypes, antigen-induced bronchoconstriction and AHR, sets this model apart from the classical OVA model in several important ways. For example, long-term passive exposure to OVA without adjuvants can lead to immunological tolerance (22, 36, 47). One has to challenge mice with OVA over a long period of time (months) to expand the mast cell population in the lung (37). In contrast, the HDM model produces robust immunological and physiological responses after only 3 wk of

Fig. 9. 48/80 induced bronchoconstriction, mMCP-1 quantified in BALF. HDM-48/80 mice ($n = 8$, black squares) were sensitized with HDM and then challenged with inhaled nebulized compound 48/80 while respiratory mechanics was measured. PBS-48/80 mice ($n = 8$, open circles) were sham sensitized with PBS and challenged with inhaled nebulized compound 48/80. Z_{rs} was measured every 10 s for 3 min after 48/80 challenge. R_n , indicative of bronchoconstriction, and mMCP-1 levels, indicative of mast cell degranulation, of the HDM-48/80 group were significantly higher than controls ($*P < 0.05$, and $****P < 0.0001$).



chronic exposure to the allergen, as reflected by our histological, immunological, and mechanical data.

Allergic asthma in humans is defined as a chronic inflammatory disease of the airways, usually resulting from long-term repeated exposure to allergen via inhalation (36). For the OVA model of allergic asthma, intraperitoneal sensitization with OVA/aluminum hydroxide is frequently used (36). In contrast, intranasal sensitization with HDM works without using adjuvants and probably better mimics the exposure mechanism by which humans are exposed to allergen. Intranasal delivery of HDM also results in Th2 sensitization and airway eosinophilic inflammation that appear to be mediated by endogenous GM-CSF production (11). In addition OVA is seldom associated with human asthma (36). HDM allergen extract, on the other hand, is composed of a mixture of mite protein, fecal pellets, and proteolytic enzymes capable of triggering both the innate and Th2-driven pathways of the immune response (19), and therefore may be more clinically relevant. On the other hand, it was reported that intermittent exposure to OVA over several weeks can generate mast cell expansion, although it is not known if this will translate into OVA-inducible bronchoconstriction (37).

We confirmed the role of mast cells by sensitizing and challenging *Kit^{W-sh}* mice and showing no responses. Also, when specifically degranulating mast cells with inhaled 48/80 in HDM allergic mice, we observed significant acute bronchoconstriction and elevated mMCP-1 similar to what we found with HDM inhalation. An increased number of mast cells were obviously needed for this response to take place since the control mice did not respond (Fig. 9). Also, while antigens such as HDM initiate mast cell degranulation via aggregation and crosslinking with FcεRI and monomeric IgE, compound 48/80 induces mast cell degranulation by directly activating G proteins via the interaction between its positively charged domain and the COOH-terminal domain of the G protein (12, 17). Furthermore, the connective tissue mast cell can be activated by FcεRI aggregation, compound 48/80, and substance

P, whereas the mucosal mast cell can be activated by FcεRI aggregation, but not by compound 48/80 or substance P (33). Also, while the mucosal mast cell exhibits strong T cell dependence in development (32, 33), the connective tissue mast cells exhibit little to no T cell dependence in development (1, 33). Hence, while it is possible that HDM primarily activated the mucosal mast cells in this study, the question still remains as to whether HDM protein or some other component in the HDM extract is capable of activating some FcεRI-independent pathway that would yield a similar response as direct mast cell activation with 48/80.

In conclusion, using our state-of-art technology for determining Z_{rs} (4–6, 8, 9), we have demonstrated an antigen-induced acute phenotype expressed as an airway constriction in mice allergic to HDM. This response was absent in *Kit^{W-sh}* mice, and, together with histological observations, flow cytometry, and mast cell mediator analysis, we conclude that the bronchoconstriction was caused by mast cell expansion and degranulation. We conclude that the combination of increased numbers of mast cells and mast cell mediator release, and possibly also an increase in airway smooth muscle in the conducting airways, are responsible for the antigen-induced bronchoconstriction.

ACKNOWLEDGMENTS

We acknowledge the expertise assistance with histology imaging by Dr. Douglas J. Taatjes and Nicole Bouffard at the Microscopy and Imaging Facility at the University of Vermont. We thank Dr. Cory Teuscher for supplying antibodies and Dr. Jonathan Boyson and Roxana Del Rio Guerra for expert help with the flow analysis. We are also indebted to Dr. Jason Bates for input into data interpretation and manuscript review.

GRANTS

The study was supported by grants from Hoffman-La Roche and National Institutes of Health Grants P30-GM-103532, RO1-HL-089177, and RO1-HL-085464.

DISCLOSURES

Dr. Lundblad received a research grant from Hoffmann-La Roche, Drs. Stevenson and Phillips were employees of Hoffmann-La Roche at the time of the study.

AUTHOR CONTRIBUTIONS

Author contributions: S.L., C.S.S., J.E.P., and L.K.L. conception and design of research; S.L., M.A., N.D., R.A.M., M.E.P., S.F.K., J.L.J.v.d.V., A.M.H., and L.K.L. performed experiments; S.L., M.A., N.D., R.A.M., M.E.P., S.F.K., J.L.J.v.d.V., A.M.H., and L.K.L. analyzed data; S.L., R.A.M., M.E.P., J.L.J.v.d.V., C.S.S., J.E.P., and L.K.L. interpreted results of experiments; S.L., N.D., and L.K.L. prepared figures; S.L. drafted manuscript; S.L., M.E.P., and L.K.L. edited and revised manuscript; S.L., M.A., N.D., R.A.M., M.E.P., S.F.K., J.L.J.v.d.V., A.M.H., C.S.S., J.E.P., and L.K.L. approved final version of manuscript.

REFERENCES

- Aldenberg F, Enerback L. Thymus dependence of connective tissue mast cells: a quantitative cytofluorometric study of the growth of peritoneal mast cells in normal and athymic rats. *Int Arch Allergy Appl Immunol* 78: 277–282, 1985.
- Alkhouri H, Hollins F, Moir LM, Brightling CE, Armour CL, Hughes JM. Human lung mast cells modulate the functions of airway smooth muscle cells in asthma. *Allergy* 66: 1231–1241, 2011.
- Amin K. The role of mast cells in allergic inflammation. *Respir Med* 106: 9–14, 2012.
- Bates JH, Allen GB. The estimation of lung mechanics parameters in the presence of pathology: a theoretical analysis. *Ann Biomed Engineering* 34: 384–392, 2006.
- Bates JH, Lauzon AM. Modeling the oscillation dynamics of activated airway smooth muscle strips. *Am J Physiol Lung Cell Mol Physiol* 289: L849–L855, 2005.
- Bates JH, Suki B. Assessment of peripheral lung mechanics. *Respir Physiol Neurobiol* 163: 54–63, 2008.
- Bates JH, Wagers SS, Norton RJ, Rinaldi LM, Irvin CG. Exaggerated airway narrowing in mice treated with intratracheal cationic protein. *J Appl Physiol* 100: 500–506, 2006.
- Bates JHT, Lauzon AM. Parenchymal tethering, airway wall stiffness, and the dynamics of bronchoconstriction. *J Appl Physiol* 102: 1912–1920, 2007.
- Bates JHT, Lutchen KR. The interface between measurement and modeling of peripheral lung mechanics. *Respir Physiol Neurobiol* 148: 153–164, 2005.
- Burton OT, Oettgen HC. Beyond immediate hypersensitivity: evolving roles for IgE antibodies in immune homeostasis and allergic diseases. *Immunol Rev* 242: 128–143, 2011.
- Cates EC, Fattouh R, Wattie J, Inman MD, Goncharova S, Coyle AJ, Gutierrez-Ramos JC, Jordana M. Intranasal exposure of mice to house dust mite elicits allergic airway inflammation via a GM-CSF-mediated mechanism. *J Immunol* 173: 6384–6392, 2004.
- Chahdi A, Fraundorfer PF, Beaven MA. Compound 48/80 activates mast cell phospholipase D via heterotrimeric GTP-binding proteins. *J Pharmacol Exp Ther* 292: 122–130, 2000.
- Daan de Boer J, Roelofs JJ, de Vos AF, de Beer R, Schouten M, Hommes TJ, Hoogendijk AJ, de Boer OJ, Stroo I, van der Zee JS, Veer CV, van der Poll T. Lipopolysaccharide inhibits Th2 lung inflammation induced by house dust mite allergens in mice. *Am J Respir Cell Mol Biol* 48: 382–389, 2013.
- Dahlback M, Brattsand R. Antigen-induced bronchial anaphylaxis in actively sensitized SD rats. Effects of local treatment with anti-asthmatic drugs. *Allergy* 41: 594–602, 1986.
- Finney MJ, Forsberg KI. Quantification of nasal involvement in a guinea pig plethysmograph. *J Appl Physiol* 76: 1432–1438, 1994.
- Gillfillan AM, Austin SJ, Metcalfe DD. Mast cell biology: introduction and overview. *Adv Exp Med Biol* 716: 2–12, 2011.
- Gotzsche PC, Johansen HK, Burr ML, Hammarquist C. House dust mite control measures for asthma. *Cochrane Database Syst Rev* CD001187, 2001.
- Gregory LG, Lloyd CM. Orchestrating house dust mite-associated allergy in the lung. *Trends Immunol* 32: 402–411, 2011.
- Hantos Z, Daroczy B, Suki B, Nagy S, Fredberg JJ. Input impedance and peripheral inhomogeneity of dog lungs. *J Appl Physiol* 72: 168–178, 1992.
- Hessel EM, Van Oosterhout AJ, Hofstra CL, De Bie JJ, Garssen J, Van Loveren H, Verheyen AK, Savelkoul HF, Nijkamp FP. Bronchoconstriction and airway hyperresponsiveness after ovalbumin inhalation in sensitized mice. *Eur J Pharmacol* 293: 401–412, 1995.
- Holt PG, Batty JE, Turner KJ. Inhibition of specific IgE responses in mice by pre-exposure to inhaled antigen. *Immunology* 42: 409–417, 1981.
- Hsue TR, Lei HY, Hsieh AL, Wang TY, Chang HY, Chen CR. Mite-induced allergic airway inflammation in guinea pigs. *Int Arch Allergy Immunol* 112: 295–302, 1997.
- Lagunoff D, Martin TW, Read G. Agents that release histamine from mast cells. *Annu Rev Pharmacol Toxicol* 23: 331–351, 1983.
- Llop-Guevara A, Colangelo M, Chu DK, Moore CL, Stieber NA, Walker TD, Goncharova S, Coyle AJ, Lundblad LK, O'Byrne PM, Lovric M, Jordana M. In vivo-to-in silico iterations to investigate aeroallergen-host interactions. *PLoS ONE* 3: e2426, 2008.
- Lundblad LK, Allen G, Thompson-Figueroa J, Bates JHT. Airway closure during bronchoconstriction in mice confirmed with micro-CT (Abstract). *Proc Am Thoracic Soc* 2: A564, 2005.
- Lundblad LK, Rinaldi LM, Poynter ME, Riesenfeld EP, Wu M, Aimi S, Barone LM, Bates JH, Irvin CG. Detrimental effects of albuterol on airway responsiveness requires airway inflammation and is independent of beta-receptor affinity in murine models of asthma (Abstract). *Respir Res* 12: 27, 2011.
- Lundblad LKA, Irvin CG, Adler A, Bates JH. A reevaluation of the validity of unrestrained plethysmography in mice. *J Appl Physiol* 93: 1198–1207, 2002.
- Lundblad LKA, Li S, Aliyeva M, Daphtary M, Van der Velden J, Hoffman MS, Lahue BS, Janssen-Heininger YMV, Poynter ME, Bates JHT, Stevenson CS, Phillips JE. Bronchoconstriction in mice allergic to house dust mite: a role for mast cells? (Abstract) *Am J Respir Crit Care Med* 185: A2405, 2012.
- Lundblad LKA, Thompson-Figueroa J, Allen GB, Rinaldi L, Norton RJ, Irvin CG, Bates JH. Airway hyperresponsiveness in allergically inflamed mice: the role of airway closure. *Am J Respir Crit Care Med* 175: 768–774, 2007.
- Marks GB. House dust mite exposure as a risk factor for asthma: benefits of avoidance. *Allergy* 53: 108–114, 1998.
- Mayrhofer G, Fisher R. Mast cells in severely T-cell depleted rats and the response to infestation with *Nippostrongylus brasiliensis*. *Immunology* 37: 145–155, 1979.
- Metcalfe DD, Baram D, Mekori YA. Mast cells. *Physiol Rev* 77: 1033–1079, 1997.
- Molinari JF, Scuri M, Moore WR, Clark J, Tanaka R, Abraham WM. Inhaled tryptase causes bronchoconstriction in sheep via histamine release. *Am J Respir Crit Care Med* 154: 649–653, 1996.
- Moore ML, Newcomb DC, Parekh VV, Van Kaer L, Collins RD, Zhou W, Goleniewska K, Chi MH, Mitchell D, Boyce JA, Durbin JE, Sturkie C, Peebles RS Jr. STAT1 negatively regulates lung basophil IL-4 expression induced by respiratory syncytial virus infection. *J Immunol* 183: 2016–2026, 2009.
- Nials AT, Uddin S. Mouse models of allergic asthma: acute and chronic allergen challenge. *Dis Models Mechanisms* 1: 213–220, 2008.
- Pae S, Cho JY, Dayan S, Miller M, Pemberton AD, Broide DH. Chronic allergen challenge induces bronchial mast cell accumulation in BALB/c but not C57BL/6 mice and is independent of IL-9. *Immunogenetics* 62: 499–506, 2010.
- Page S, Ammit AJ, Black JL, Armour CL. Human mast cell and airway smooth muscle cell interactions: implications for asthma. *Am J Physiol Lung Cell Mol Physiol* 281: L1313–L1323, 2001.
- Phillips JE, Peng R, Harris P, Burns L, Renteria L, Lundblad LK, Fine JS, Bauer CM, Stevenson CS. House dust mite models: will they translate clinically as a superior model of asthma? *J Allergy Clin Immunol* 132: 242–244, 2013.
- Ray P, Krishnamoorthy N, Ray A. Emerging functions of c-kit and its ligand stem cell factor in dendritic cells: regulators of T cell differentiation. *Cell Cycle* 7: 2826–2832, 2008.
- Riesenfeld E, Allen GB, Bates JH, Poynter ME, Wu M, Aimi S, Lundblad LK. The temporal evolution of airways hyperresponsiveness and inflammation. *J Allergy Ther* 1: 1–7, 2012.
- Rothschild AM. Mechanisms of histamine release by compound 48–80. *Br J Pharmacol* 38: 253–262, 1970.
- Ryu JH, Yoo JY, Kim MJ, Hwang SG, Ahn KC, Ryu JC, Choi MK, Joo JH, Kim CH, Lee SN, Lee WJ, Kim J, Shin DM, Kwon MN, Bae YS, Yoon JH. Distinct TLR-mediated pathways regulate house dust mite-induced allergic disease in the upper and lower airways. *J Allergy Clin Immunol* 131: 549–561, 2013.

44. **Schuessler T, Bates J.** A computer-controlled research ventilator for small animals: design and evaluation. *IEEE Trans Bio-medical Engineering* 42: 860–866, 1995.
45. **Shirai T, Yasueda H, Saito A, Taniguchi M, Akiyama K, Tsuchiya T, Suda T, Chida K.** Effect of exposure and sensitization to indoor allergens on asthma control level. *Allergology Int* 61: 51–56, 2012.
46. **Stevens RL, Austen KF.** Recent advances in the cellular and molecular biology of mast cells. *Immunol Today* 10: 381–386, 1989.
47. **Swirski FK, Gajewska BU, Alvarez D, Ritz SA, Cundall MJ, Cates EC, Coyle AJ, Gutierrez-Ramos JC, Inman MD, Jordana M, Stampfli MR.** Inhalation of a harmless antigen (ovalbumin) elicits immune activation but divergent immunoglobulin and cytokine activities in mice. *Clin Exp Allergy* 32: 411–421, 2002.
48. **Tomioka S, Bates JH, Irvin CG.** Airway and tissue mechanics in a murine model of asthma: alveolar capsule vs. forced oscillations. *J Appl Physiol* 93: 263–270, 2002.
49. **Trompette A, Divanovic S, Visintin A, Blanchard C, Hegde RS, Madan R, Thorne PS, Wills-Karp M, Giovannini TL, Weiss JP, Karp CL.** Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* 457: 585–588, 2009.
50. **van den Berge M, Kerstjens HA, Postma DS.** Provocation with adenosine 5'-monophosphate as a marker of inflammation in asthma, allergic rhinitis and chronic obstructive pulmonary disease. *Clin Exp Allergy* 32: 824–830, 2002.
51. **Wagers S, Lundblad LKA, Ekman M, Irvin CG, Bates JHT.** The allergic mouse model of asthma: normal smooth muscle in an abnormal lung? *J Appl Physiol* 96: 2019–2027, 2004.
52. **Wagers SS, Haverkamp HC, Bates JH, Norton RJ, Thompson-Figueroa JA, Sullivan MJ, Irvin CG.** Intrinsic and antigen-induced airway hyperresponsiveness are the result of diverse physiological mechanisms. *J Appl Physiol* 102: 221–230, 2007.
53. **Wilson RH, Whitehead GS, Nakano H, Free ME, Kolls JK, Cook DN.** Allergic sensitization through the airway primes th17-dependent neutrophilia and airway hyperresponsiveness. *Am J Respir Crit Care Med* 180: 720–730, 2009.
54. **Zosky GR, Lacombe AN, White OJ, Burchell JT, Janosi TZ, Hantos Z, Holt PG, Sly PD, Turner DJ.** Ovalbumin-sensitized mice are good models for airway hyperresponsiveness but not acute physiological responses to allergen inhalation. *Clin Exp Allergy* 38: 829–838, 2008.

