Concurrent dual allergen exposure and its effects on airway hyperresponsiveness, inflammation and remodeling in mice

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SUMMARY

Experimental mouse models of asthma have broadened our understanding of the mechanisms behind allergen-induced asthma. Typically, mouse models of allergic asthma explore responses to a single allergen; however, patients with asthma are frequently exposed to, and tend to be allergic to, more than one allergen. The aim of the current study was to develop a new and more relevant mouse model of asthma by measuring the functional, inflammatory and structural consequences of chronic exposure to a combination of two different allergens, ovalbumin (OVA) and house dust mite (HDM), in comparison with either allergen alone. BALB/c mice were sensitized and exposed to OVA, HDM or the combination of HDM and OVA for a period of 10 weeks. Following allergen exposure, airway responsiveness was measured using the flexiVent small animal ventilator, and mice were assessed for indices of airway inflammation and remodeling at both 24 hours and 4 weeks after the final allergen exposure. Mice exposed to the HDM-OVA combination exhibited increased numbers of inflammatory cells in the bronchoalveolar lavage (BAL) when compared with mice exposed to a single allergen. Mice exposed to HDM-OVA also exhibited an elevated level of lung tissue mast cells compared with mice exposed to a single allergen. Following the resolution of inflammatory events, mice exposed to the allergen combination displayed an elevation in the maximal degree of total respiratory resistance (Max R_{RS}) compared with mice exposed to a single allergen. Furthermore, trends for increases in indices of airway remodeling were observed in mice exposed to the allergen combination compared with a single allergen. Although concurrent exposure to HDM and OVA resulted in increased aspects of airway hyperresponsiveness, airway inflammation and airway remodeling when compared with exposure to each allergen alone, concurrent exposure did not result in a substantially more robust mouse model of allergic asthma than exposure to either allergen alone.

INTRODUCTION

Asthma is a chronic disease of the respiratory tract, characterized by variable degrees of airflow obstruction, and is associated with airway inflammation, airway remodeling and airway hyperresponsiveness (AHR) (O'Byrne and Inman, 2003). The initiation and persistence of various aspects of asthma can often be attributed to exposure to one or more allergens (Pomes, 2002). Inhaled allergens are capable of inducing an inflammatory response in sensitized individuals and consequently cause, or exacerbate, respiratory symptoms that are characteristic of asthma (Wills-Karp, 1999). How allergens contribute to allergic sensitization and, in turn, airway inflammation and airway dysfunction remains unclear. An ongoing hypothesis is that the inflammatory response resulting from inhaled allergen might drive AHR directly, or induce structural changes in the airway leading to persistent AHR (Laprise et al., 1999; O'Byrne and Inman, 2003).

Experimental mouse models of asthma have offered important insights into the mechanisms of allergen-induced asthma (Epstein, 2004; Hellings and Ceuppens, 2004; Taube et al., 2004). Our lab uses two mouse models of allergic asthma involving chronic exposure to either (1) ovalbumin (OVA), an innocuous antigen capable of inducing airway inflammation, airway remodeling and AHR, but only if administered systemically when conjugated with an aluminum-based adjuvant prior to intrapulmonary challenge

(Kheradmand et al., 2002), or (2) house dust mite (HDM), a naturally occurring allergen that, when given via the airway, readily induces an asthmatic phenotype without the need for adjuvant or systemic sensitization. Both allergen exposure protocols result in immune-mediated airway inflammation defined by: elevated levels of IgE, the T-helper cell 2 (T_H2) cytokines interleukin (IL)-4, -5 and -13, and eosinophils; airway remodeling defined by increases in airway smooth muscle, collagen deposition and goblet cell hyperplasia; and AHR that is sustained after the resolution of eosinophilic inflammation (Inman et al., 1999; Leigh et al., 2002; Leigh et al., 2004c; Leigh et al., 2004a; Leigh et al., 2004b; Southam et al., 2007).

Most published models of allergic asthma typically explore responses to a single allergen. However, it is unlikely that humans are only exposed to a single allergen. In fact, natural exposure to a combination of two or more common aeroallergens has been linked with an increased risk for asthmatic symptoms (Gehring et al., 2001). This provokes the question: will concurrent exposure to more than one allergen in a mouse model worsen the asthmatic phenotype? The concept that the interaction between more than one allergen might lead to worsened disease is supported by a mouse model of brief allergen exposure (Sarpong et al., 2003), in which, compared with a single allergen, the combination of two allergens resulted in enhanced levels of airway inflammation and epithelial damage, but not airway reactivity. The sustained effects of concurrent exposure to multiple allergens in a chronic mouse model of asthma remain unknown. Given the central role that inflammation is believed to play in asthmatic pathophysiology, we

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hypothesized that concurrent chronic exposure to more than one allergen will increase the extent of airway remodeling and sustained AHR in a mouse model of chronic allergen exposure.

To investigate the structural and functional consequences of chronic exposure to a combination of two different allergens, we combined our individual OVA and HDM mouse models of chronic allergen exposure. We hypothesized that concurrent exposure to two different allergens would result in an increased inflammatory component during allergen exposure, which would be associated with a greater degree of AHR when compared with exposure to a single allergen. We further hypothesized that concurrent exposure to two different allergens, compared with a single allergen, would result in increased structural changes in the airway and an associated greater degree of sustained AHR, which would be present after the resolution of inflammation.

RESULTS

Outcome measurements were made at two different time points, 24 hours and 4 weeks following the final allergen exposures. Mice were sensitized and exposed to OVA alone, HDM alone or a combination of the two allergens (HDM-OVA) according to the allergen exposure protocols illustrated in Fig. 1.

Splenocyte recall

In mice from the OVA exposure group, there was a significant increase in IL-4 in the supernatant of splenocytes following recall with OVA compared with saline (SAL) recall ($P<0.05$) (Fig. 2). There was also a significant increase in IL-4 in the supernatant of splenocytes from the HDM exposure group when recalled with HDM compared with SAL recall ($P<0.05$). The supernatant of splenocytes from the HDM-OVA group showed significant increases in IL-4 following recall with either HDM or OVA compared with SAL recall ($P<0.05$).

Airway inflammation in bronchoalveolar lavage (BAL)

Differential BAL cell counts were assessed 24 hours after chronic allergen exposure and, with the exception of macrophages, were increased in mice that underwent chronic exposure to OVA alone, HDM alone or the combination of HDM and OVA when compared
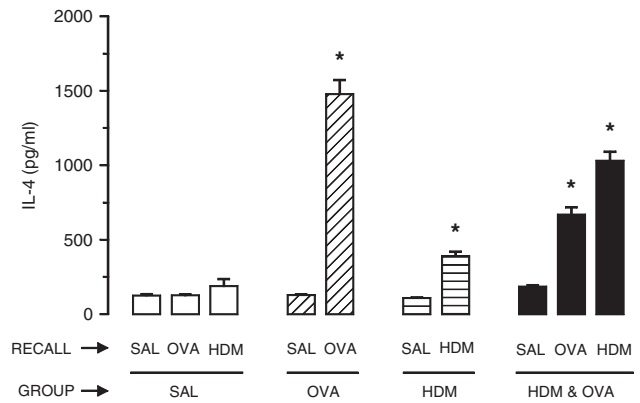
Fig. 2. In vitro expression of the T$_H$2-associated cytokine IL-4 through splenocyte recall. Splenocytes were isolated and cultured for 5 days in medium supplemented with SAL, OVA or HDM, as indicated under each bar. Values are mean±s.e.m. *$P<0.05$ compared with splenocytes cultured in medium supplemented with SAL.

with mice exposed to SAL (Fig. 3A). Mice exposed to the HDM-OVA combination exhibited a significant increase in the number of eosinophils and lymphocytes when compared with mice exposed to OVA alone ($P<0.05$), and showed a significant increase in the number of neutrophils and lymphocytes when compared with mice exposed to HDM alone ($P<0.05$). There were no significant differences in the number of macrophages between the four groups of mice.

At 4 weeks following chronic allergen exposure, there was a significant reduction in the number of BAL eosinophils compared with at 24 hours after exposure. Although there was a substantial reduction in the number of eosinophils in the BAL at the 4-week time point, the eosinophil numbers actually remained elevated in mice that underwent chronic exposure to HDM alone, or the combination of HDM and OVA, when compared with mice exposed to SAL (Fig. 3B). Mice exposed to the HDM-OVA combination exhibited a significant elevation in the number of eosinophils, neutrophils, lymphocytes and macrophages when
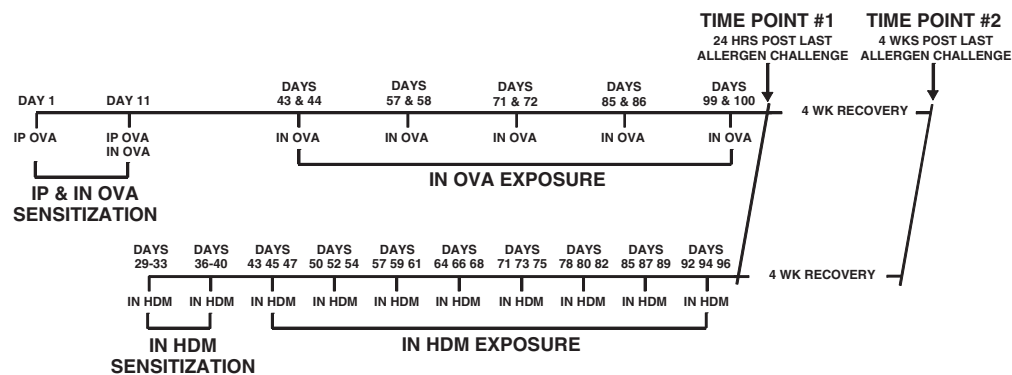
Fig. 1. Allergen sensitization and exposure protocols. OVA mice received OVA according to the OVA exposure protocol and received SAL according to the HDM exposure protocol. HDM mice received HDM according to the HDM exposure protocol and received SAL according to the OVA exposure protocol. The dual allergen group received both HDM and OVA, combining both allergen exposure protocols. Control mice received SAL according to both allergen exposure protocols. IN=intranasal; IP=intraperitoneal.
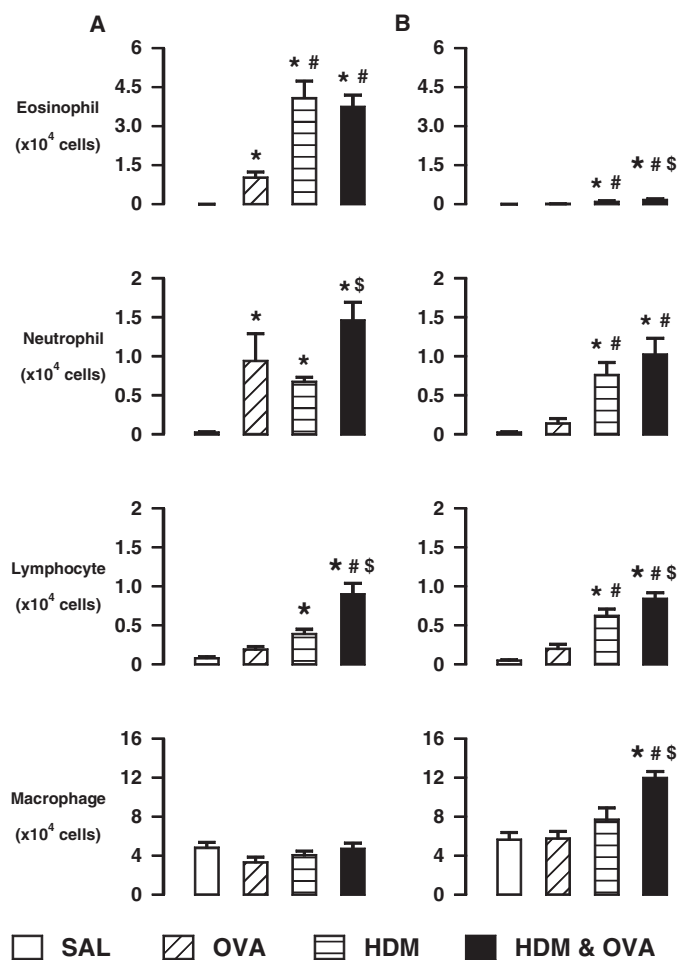
Fig. 3. Total and differential BAL fluid cell counts after chronic exposure to SAL, OVA, HDM or a combination of HDM and OVA. BAL cell counts were performed at 24 hours (A) and 4 weeks (B) after chronic exposure. Values are mean±s.e.m. *$P<0.05$ compared with SAL; #$P<0.05$ compared with OVA; $P<0.05$ compared with HDM.

compared with mice exposed to OVA alone ($P<0.05$); furthermore, they showed a significant elevation in the number of eosinophils, lymphocytes and macrophages when compared with mice exposed to HDM alone ($P<0.05$).

Mast cells (MCs)

At 24 hours following chronic allergen exposure, there was a significant increase in the number of MCs/mm^2 of lung tissue in mice exposed to either HDM alone or the combination of HDM and OVA when compared with mice exposed to SAL ($P<0.05$) (Fig. 4A). However, mice exposed to OVA alone did not exhibit a significant difference in the number of MCs/mm^2 of lung tissue when compared with mice exposed to SAL ($P<0.05$) (Fig. 4A). Mice exposed to the HDM-OVA combination exhibited a significant increase in the number of MCs/mm^2 of lung tissue when compared with mice exposed to OVA alone, but showed no significant difference when compared with mice exposed to HDM alone ($P<0.05$).
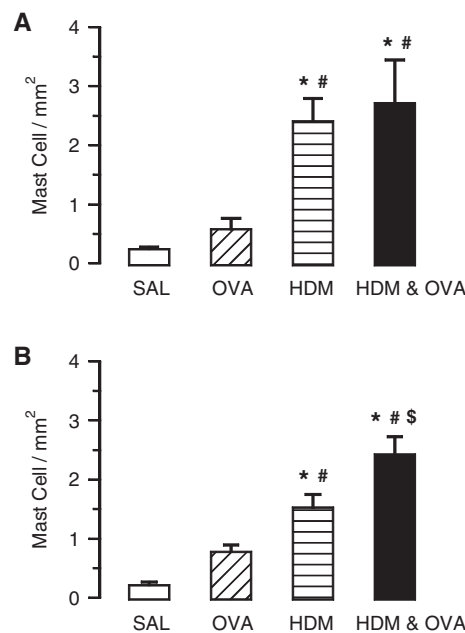

Fig. 4. Morphometric quantification of MCs in the lung tissue of mice after chronic exposure to allergen. MCs were quantified at 24 hours (A) and 4 weeks (B) after chronic exposure to SAL, OVA, HDM or a combination of OVA and HDM. Data is expressed as the number of MCs per area of lung tissue (MCs/mm^2). Values are mean±s.e.m. *$P<0.05$ compared with SAL; $P<0.05$ compared with HDM; #$P<0.05$ compared with OVA.

At 4 weeks following chronic allergen exposure, there was a significant increase in the number of MCs/mm^2 of lung tissue in mice exposed to either HDM alone or the combination of HDM and OVA when compared with mice exposed to SAL ($P<0.05$) (Fig. 4B). However, mice exposed to OVA alone did not exhibit a significant difference in the number of MCs/mm^2 of lung tissue when compared with mice exposed to SAL ($P<0.05$) (Fig. 4B). Mice exposed to the HDM-OVA combination exhibited a significant increase in the number of MCs/mm^2 of lung tissue when compared with either mice exposed to OVA alone or mice exposed to HDM alone ($P<0.05$).

Airway remodeling

Comparisons with SAL control mice

At 4 weeks following chronic allergen exposure, we observed a significant increase in collagen deposition, as determined by Picrosirius Red (PSRed) staining, in mice that underwent chronic exposure to OVA alone, HDM alone or the combination of HDM and OVA when compared with mice exposed to SAL ($P<0.05$) (Fig. 5). Mice exposed to the HDM-OVA combination showed a significant increase in the quantity of α-smooth muscle actin (α-SMA) staining when compared with mice exposed to SAL ($P<0.05$). Surprisingly, mice exposed to HDM alone or OVA alone showed no significant difference in the quantity of α-SMA staining when compared with mice exposed to SAL. In addition, there was a significant increase in the number of periodic acid-Schiff (PAS)-positive goblet cells in mice exposed to OVA alone, HDM alone or the combination of HDM and OVA when compared with mice exposed to SAL ($P<0.05$) (Fig. 5).
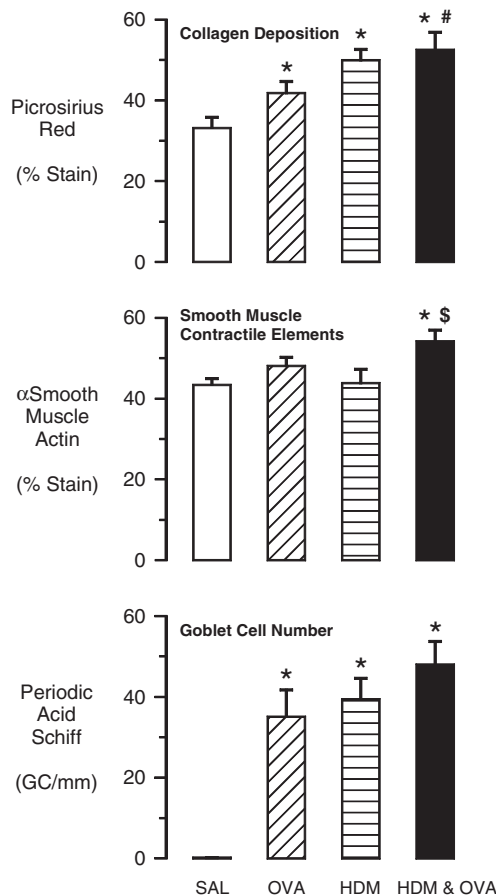
Fig. 5. Morphometric quantification of goblet cells, subepithelial collagen deposition and smooth muscle contractile elements in the airways of mice after chronic exposure to allergen. Morphometric quantification was performed at 4 weeks following chronic exposure with SAL, OVA, HDM or a combination of OVA and HDM. Subeithelial collagen deposition (top) was quantified using a PSRed stain, smooth muscle contractile elements (center) were detected by immunostaining for α-SMA and goblets cells (GC, bottom) were quantified using a PAS stain. Data is expressed as the percentage of positively stained tissue in the region of interest in the PSRed and α-SMA stained sections, and as the number of goblet cells (PAS-positive cells) expressed per length of airway wall [GC/millimeter (mm)]. Values are mean±s.e.m. *$P<0.05$ compared with SAL; $^{\$}P<0.05$ compared with HDM; $^{\#}P<0.05$ compared with OVA.

Comparisons between single and dual allergen-exposed mice
PSRed stain. Mice exposed to the HDM-OVA combination exhibited a significantly greater amount of PSRed staining for collagen deposition when compared with mice exposed to OVA alone ($P<0.05$), but there was no significant difference when compared with mice exposed to HDM alone.
α-SMA immunostain. Mice exposed to the HDM-OVA combination exhibited a significantly greater amount of α-SMA staining when compared with mice exposed to HDM alone ($P<0.05$), but showed no significant difference when compared with mice exposed to OVA alone.
PAS stain. There was no significant difference in the number of PAS-positive goblet cells in mice exposed to the combination of

HDM and OVA when compared with mice exposed to either HDM or OVA alone.

Airway physiology
At 24 hours after chronic allergen exposure, mice that underwent exposure to OVA alone, HDM alone or the combination of HDM and OVA exhibited a significantly elevated level of airway responsiveness when compared with mice exposed to SAL (Fig. 6A). Mice receiving the HDM-OVA combination exhibited significantly elevated maximal methacholine (MCh)-induced bronchoconstriction (Max R_{RS}) compared with mice exposed to OVA alone ($P<0.05$), but showed no significant difference when compared with mice exposed to HDM alone (Fig. 6B).

At 4 weeks after chronic exposure, mice receiving OVA alone, HDM alone or the combination of HDM and OVA exhibited a significantly greater degree of airway responsiveness when compared with mice exposed to SAL (Fig. 6C). Mice receiving the HDM-OVA combination exhibited a significantly elevated Max R_{RS} compared with mice exposed to HDM alone ($P<0.05$) and an elevated, although not significant, Max R_{RS} when compared with mice exposed to OVA alone (Fig. 6D) ($P=0.06$).

DISCUSSION
In this study, we exposed a new mouse model of chronic allergen exposure to two different allergens concurrently. We have provided evidence that, compared with chronic exposure to either HDM or OVA alone, chronic simultaneous exposure to both allergens results in an elevated level of sustained AHR, which persists after the resolution of the ongoing inflammation from the allergen exposure period. Although we observed significant increases in aspects of airway inflammation, and increasing trends in the degree of airway remodeling, exhibited by mice exposed to the HDM-OVA combination compared with mice exposed to either allergen alone, we cannot completely attribute these changes to the increased level of sustained airway dysfunction. To confirm that mice were sensitized to the allergens that they were exposed to, an in vitro splenocyte recall was performed. When recalled with either OVA or HDM, splenocytes from mice exposed to the HDM-OVA combination exhibited an increase in IL-4 production, confirming sensitization to both allergens.

Consistent with a previous study of multiple allergen exposure (Sarpong et al., 2003), we observed increases in the total cell counts and the number of eosinophils, neutrophils and lymphocytes in mice exposed to the HDM-OVA combination, compared with mice exposed to either allergen alone, at 24 hours following the final allergen exposure. There were significant differences in the magnitude of BAL inflammatory cell responses between the OVA- and HDM-exposed mice. The eosinophilia was greater in the HDM mice and was, in fact, similar to the level seen in the HDM-OVA mice. This suggests that HDM might be the allergen driving this aspect of the inflammatory response at 24 hours following the final allergen exposure. A possible explanation for the greater degree of eosinophilic inflammation in the HDM-exposed mice, compared with the OVA-exposed mice, might be that the protease activity of the HDM extracts gives this aeroallergen its antigenic nature and prevents the development of tolerance (Fattouh et al., 2005; Johnson et al., 2004; Kheradmand et al., 2002), which is known to occur with chronic exposure to OVA (Swirski et al., 2002; Swirski et al., 2006).
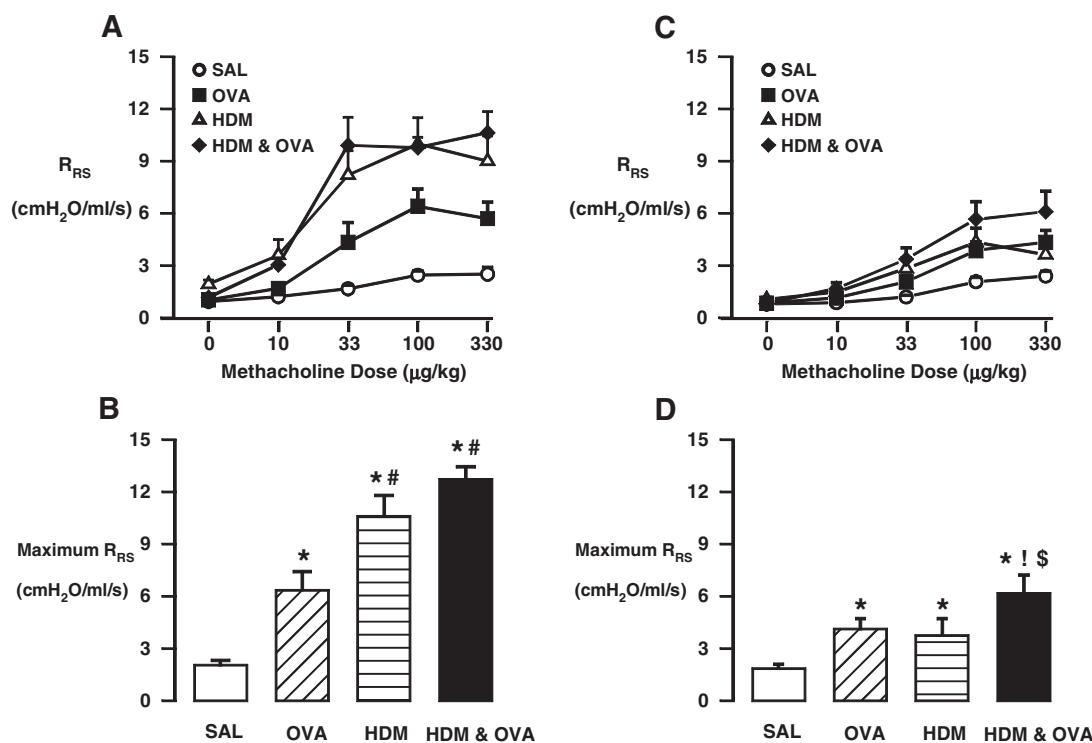
Fig. 6. Airway responsiveness by dose-response curves of the total respiratory system resistance (R_{RS}) against intravenous doses of MCh after chronic exposure to allergen. The R_{RS} was measured at 24 hours (A) and 4 weeks (C) after chronic exposure to SAL, OVA, HDM or a combination of HDM and OVA. Using the R_{RS}-MCh dose-response curves, the maximal degree of bronchoconstriction (Max R_{RS}) was calculated at 24 hours (B) and 4 weeks (D) after chronic exposure to SAL, OVA, HDM or the HDM-OVA combination. Values are mean±s.e.m. *$P<0.05$ compared with SAL; $^{\$}P<0.05$ compared with HDM; $^{\#}P<0.05$ compared with OVA; $^{!}P=0.06$ compared with OVA.

By contrast, if given strictly by inhalation in the laboratory, OVA is incapable of inducing a broad spectrum of allergic alterations in the lung (Kheradmand et al., 2002). In addition, continuous exposure to OVA for several weeks in animals that have been sensitized previously with an aluminum-based adjuvant conjugated to OVA causes a decline in, and complete cessation of, the airway inflammatory response rather than an increase in, or maintenance of, the inflammation (Swirski et al., 2002; Swirski et al., 2006). Although it appears that the inflammatory aspect of the response in dual challenged animals may be explained entirely by the observed response to HDM alone, it is important to point out that exposure to HDM followed by exposure to OVA has been shown to prevent the tolerance associated with OVA alone (Fattouh et al., 2005).

Following chronic allergen exposure, airway physiology measurements show similar trends to the eosinophil profiles at the 24-hour time point. The accumulation of eosinophils and their associated mediators in the airway has been found to correlate with the levels of AHR and disease severity in asthmatics (Bousquet et al., 1990). As with the eosinophilic profile and, in turn, the AHR observed at 24 hours following the final allergen challenge, the number of mast cells in the lung tissue of HDM-exposed mice was greater than in OVA-exposed mice, and was similar to the level seen in mice exposed to the dual allergen combination. The notion that mast cells might be the driving force behind AHR is consistent with the findings of Kobayashi et al. (Kobayashi et al., 2000), who observed that mast cell-deficient mice failed to show an increase in AHR after brief allergen exposure and that the reconstitution of bone marrow-derived mast cells in these mice restored the capacity to develop allergen-induced AHR.

The association between inflammatory cells and AHR suggests that ongoing airway inflammation might be a driving factor for AHR at the 24-hour time point. This is in agreement with our kinetic analyses of inflammation and AHR, which we reported previously (Southam et al., 2007). Compared with mice receiving HDM alone, we did not observe a further increase in AHR in mice receiving the HDM-OVA combination, this is consistent with the data presented by Sarpong et al. (Sarpong et al., 2003) who did not observe an additive effect from two allergens in terms of the AHR associated with inflammation.

At 4 weeks following chronic allergen exposure, there is a greater degree of sustained AHR in mice exposed to the HDM-OVA combination compared with mice exposed to either allergen alone. At this time point, eosinophilic inflammation in the BAL had subsided significantly, which is consistent with previous observations in our lab and by others (Johnson et al., 2004; Leigh et al., 2002; Leigh et al., 2004c; Leigh et al., 2004a; Leigh et al., 2004b; Southam et al., 2007). Of the inflammatory cells that we examined in the BAL, the main difference between the single and dual allergen-exposed groups was the number of lymphocytes at both the 24-hour and 4-week post allergen exposure time points. This illustrates an association between lymphocyte number and the magnitude of sustained AHR, with both being higher in response to the HDM-OVA combination than to either allergen alone. Although we were unable to quantify the levels of cytokines in the BAL fluid, as the HDM rendered these mediators undetectable, this observation supports the ongoing idea that lymphocytes, and the associated mediators that they release, might be responsible for the development and persistence of sustained AHR. In support of this concept, Foster et al. demonstrated the essential role of CD4^{+} T cells in regulating AHR in a mouse model of chronic asthma by depleting the CD4^{+} T cell population with an antibody and observing a decline in AHR (Foster et al., 2002).

At 4 weeks following chronic allergen exposure, the number of mast cells remains elevated in mice that have been exposed to the dual allergen combination compared with mice exposed to either

allergen alone. As discussed above, mast cells are required for the development of AHR and it may be that, as mice undergo prolonged exposure to multiple allergens in chronic models, the quantity of mast cells in the lung environment increases causing a corresponding increase in the degree of AHR.

Sustained AHR in asthma has been hypothesized to be the result of indices of airway remodeling (Bai et al., 2000; Bergeron and Boulet, 2006; Lambert et al., 1993; Wiggs et al., 1992), which may be secondary to prior periods of inflammation. Although some differences were observed in remodeling indices between the HDM-OVA mice and the single exposure groups, we have not observed a marked increase in any remodeling index in the dual exposure group when compared with either single allergen group.

In contrast to earlier studies, mice exposed to either HDM or OVA alone did not exhibit a significant increase in α-SMA compared with the SAL control mice (Johnson et al., 2004; Leigh et al., 2002; Leigh et al., 2004c; Leigh et al., 2004a; Leigh et al., 2004b; Southam et al., 2007). This failure to demonstrate an allergen-induced increase might have resulted from the apparent increase in the levels of α-SMA staining in the SAL control mice in this study compared with previous studies (Johnson et al., 2004; Leigh et al., 2002; Leigh et al., 2004c; Leigh et al., 2004a; Leigh et al., 2004b; Southam et al., 2007).

The elevated level of sustained AHR in mice exposed to the HDM-OVA combination cannot be completely attributed to the quantity of collagen, α-SMA or the number of goblet cells present. However, the quantity of airway remodeling may not be the influential factor; the quality of remodeling might have a more influential role. In vitro experiments have demonstrated that airway myocytes can exist in both a proliferative or contractile phenotype (Halayko et al., 1996; Halayko et al., 1999; Halayko and Solway, 2001; Owens, 1995). Additionally, in vitro data suggest that the different types of extracellular matrix (ECM) proteins might preferentially direct smooth muscle differentiation towards proliferative or contractile phenotypes (Hirst et al., 2000; Tran et al., 2006). We have not clearly defined the factors responsible for sustained AHR in our chronic mouse models of allergen challenge; however, further insight into the specific types of ECM proteins in the airway of allergen-exposed mice and how the deposition of these various ECM proteins might alter the smooth muscle phenotype in vivo should offer further understanding of how airway remodeling influences sustained AHR.

Conclusion
In conclusion, concurrent chronic allergen exposure to the combination of HDM and OVA did not result in a substantially more robust mouse model of allergic asthma than exposure to either allergen alone. However, we did observe that concurrent exposure to HDM and OVA results in an elevated level of sustained AHR. Although we cannot specifically attribute the elevated level of sustained AHR to any particular outcome measurement made, this observation was associated with elevated numbers of lymphocytes and mast cells. Studies that use new allergen combinations and those that further investigate (1) the involvement of lymphocytes, and their associated mediators, in the development and persistence of AHR; (2) the role of mast cells in sustained AHR; and (3) the contribution of both the quantity and quality of allergen-induced airway remodeling to

sustained AHR should all offer additional insights into the elevated level of sustained AHR that results from multiple allergen exposure in mouse models of allergic asthma.

METHODS
Animals
Female BALB/c mice, aged 8-12 weeks (*n*=12 per group), were purchased from Charles River Laboratories (Saint-Constant, Quebec, Canada) and housed in environmentally controlled specific-pathogen-free conditions for 1 week prior to study, and throughout the experiments. All procedures were reviewed and approved by the Animal Research Ethics Board at McMaster University.

Allergen sensitization and exposure
Mice were sensitized and exposed to OVA, HDM or a combination of the two allergens (HDM and OVA) according to established chronic allergen exposure protocols (Fig. 1) (Inman et al., 1999; Leigh et al., 2002; Leigh et al., 2004c; Leigh et al., 2004a; Leigh et al., 2004b; Southam et al., 2007).

OVA exposure
All mice receiving OVA were sensitized to OVA through intraperitoneal (IP) injection on day 1 followed by both IP injection and intranasal (IN) installation on day 11. IP OVA injections involved precipitating 10% aluminum potassium sulfate with 0.05% OVA, adjusting the pH to 6.5, then centrifuging and resuspending the pellet in saline, followed by a 200 µl IP injection. This was followed by five periods of IN OVA exposure, each 2 weeks apart. During each 2-day period, OVA was administered on consecutive days at a concentration 100 µg/25 µl. Mice receiving OVA also received sham exposure to the HDM allergen (in the form of IN SAL), according to the HDM exposure protocol (Fig. 1).

HDM exposure
HDM extract from *Dermatophagoides pteronyssinus* (Greer Laboratories, Lenoir, NC) was resuspended with sterile phosphate buffered saline to reach a concentration of 15 µg/25 µl, which was subsequently aliquoted and frozen at −20°C. The HDM was thawed at 4°C overnight to be administered the next day. All mice receiving HDM were initially exposed to the allergen through a daily 25 µl (15 µg/25 µl) IN installation, for two periods of 5 days each (days 29-33 and 36-40). This was followed by an 8-week period of HDM exposure, during which mice were exposed to IN HDM three times per week, on every other day of the week (Mondays, Wednesdays and Fridays), starting on day 43. Mice receiving HDM also received sham exposure to the OVA allergen (in the form of IN SAL), according to the OVA exposure protocol (Fig. 1).

HDM-OVA exposure (combination)
Using the same methods of administration used for the single allergens alone, all mice receiving the combination of HDM and OVA were first sensitized to OVA and, following a 16-day rest period (days 12-28), were exposed to HDM on 5 days per week for a period of 2 weeks. In a pilot study conducted before the current study (data not shown), we attempted to sensitize mice to both allergens simultaneously. However, this resulted in poor health and death. Therefore, the 16-day rest period between sensitization to

each allergen was necessary to ensure that the mice remained healthy during the sensitization phase. Following sensitization, mice were exposed to both OVA and HDM, combining both allergen exposure protocols (Fig. 1).

Sham exposure (saline control mice)

Control mice were sensitized and exposed to SAL (25 µl IN) by following the same chronic allergen protocols illustrated in Fig. 1 and by using the same methods of administration.

Outcome measurements

Outcome measurements were made at two time points: 24 hours following the final allergen exposure, to investigate the association between airway inflammation and AHR, and at 4 weeks following the final allergen exposure, to investigate the association between airway remodeling and AHR at a time when airway inflammation had completely subsided (Fig. 1). Outcome measurements were assessed in eight groups of mice, consisting of four different groups of mice at each of the two time points. Group 1 was control mice exposed to SAL, group 2 was mice exposed to OVA, group 3 was mice exposed to HDM and group 4 was mice exposed to the combination of allergens (HDM and OVA) (n=12 mice per group). The primary outcome measurement at both time points was an in vivo assessment of airway responsiveness (R_{RS} to MCh). Additional outcome measurements at both time points included total and differential cell counts (eosinophils, neutrophils, lymphocytes and macrophages) in the BAL fluid, and airway morphometry using a computer-based image analysis system (Northern Eclipse, Version 7.0; Empix Imaging Inc., Mississauga, Ontario, Canada) to quantify the number of mast cells in a given area of lung tissue. Further outcome measurements made at 4 weeks following the final allergen exposure included splenocyte recall for the T_H2 cytokine IL-4, to confirm allergen sensitization, and airway morphometry using Northern Eclipse to quantify sustained structural changes in the airway.

Airway responsiveness

Airway responsiveness was assessed by measuring the R_{RS} response to increasing doses of intravenous MCh using the flexiVent small animal ventilator (SCIREQ, Montreal, Canada), as described previously (Hirota et al., 2006).

Splenocyte recall

Splenocyte recall was performed as described previously (Johnson et al., 2004). Briefly, spleens were harvested, and splenocytes were isolated and diluted to a concentration of 8×10^6 cells/ml in complete RPMI. Splenocytes were cultured in complete RPMI alone, or in complete RPMI supplemented with either OVA (40 µg/ml) or HDM (40 µg/ml) in a flat-bottom, 96-well plate (Becton Dickinson) in quadruplicate. After 5 days of culture, supernatants were harvested and quadruplicates were pooled and frozen at –80°C until cytokine measurements were ready to be made.

Cytokine analysis

The levels of IL-4 in the supernatant of the splenocyte culture were measured using an ELISA kit for IL-4 (Quantikine; R&D Systems, Minneapolis, MN).

TRANSLATIONAL IMPACT

Clinical issue

Major characteristics of allergic asthma include airflow obstruction, inflammation of the airways and airway remodelling in response to inhaled allergens. Inhalation of environmental particles is harmless in some individuals; however, in allergic individuals, certain environmental particles are recognized as foreign allergens and cause an allergic airway reaction. This can result in increased sensitivity and reactivity of the airways, in which the airways of the asthmatic patient tighten and constrict in a process called airway hyperresponsiveness (AHR). Exposure to a multitude of indoor and outdoor allergens varies with the seasons and many people develop multiple allergies, such that concurrent exposure to more than one allergen can drastically enhance this allergic airway response. Most published models of allergic asthma explore responses to a single allergen. However, it is unlikely that humans are only exposed to a single allergen and the sustained asthmatic effects of concurrent exposure to multiple allergens are unknown.

Results

To determine the effect of simultaneous exposure to multiple allergens, this study combines two chronic mouse models of allergen challenge. The authors evaluate the structural and functional consequences to the airway after chronic exposure to ovalbumin (OVA) and proteins derived from house dust mites (HDM), either in combination or independently. They did not see a significant difference in AHR between animals exposed to HDM and OVA in combination, or to each allergen alone, after a transient exposure. Consistent with this, airway inflammation markers and airway remodelling in mice exposed to the combination of HDM and OVA did not significantly differ from mice exposed to HDM or OVA alone. However, following chronic exposure, they find that mice exposed to HDM and OVA in combination exhibit greater sustained AHR compared with either HDM or OVA alone.

Implications and future directions

There is a combinatorial effect of chronic exposure to multiple allergens that elevates inflammation and airway remodelling. A greater effort towards limiting allergen exposure, including to common house dust mites, may prevent subsequent allergen-induced airway disease. Further study of the combinatorial effects of allergens in animals and humans should offer insight into the mechanisms that cause the airway inflammation that leads to AHR.

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BAL fluid collection and analysis

BAL fluid was collected as described previously (Inman et al., 1999; Leigh et al., 2002; Leigh et al., 2004c; Leigh et al., 2004a; Leigh et al., 2004b; Southam et al., 2007). Differential cell counts were performed on 400 cells. Cells were classified, based on morphological criteria, as eosinophils, neutrophils and lymphocytes.

Lung histology and morphometry

Lung histology and morphometry were performed as described previously (Ellis et al., 2003; Inman et al., 1999; Leigh et al., 2002; Leigh et al., 2004c; Leigh et al., 2004a; Leigh et al., 2004b; Southam et al., 2007). Briefly, 3 µm-thick lung sections were cut and stained with: PSRed to quantify the presence of subepithelial collagen, PAS to demonstrate the presence of goblet cells, and Toluidine Blue to demonstrate the presence of mast cells. Additional sections were prepared for immunostaining using a monoclonal antibody against α-SMA (Clone 1A4, DAKO, Denmark) to quantify the amount of α-smooth muscle actin contractile proteins in the airway. Morphometric quantification of stained lung sections was performed using a customized digital image analysis system

(Northern Eclipse). Quantification of mast cells, which has not been described previously, was performed by collecting an image of the entire stained tissue section under a 1× objective to calculate the area of lung tissue. An individual blinded to the study codes then counted all of the mast cells under a 40× objective and the result was expressed as MCs/mm^2.

Statistical analysis

Values are expressed as mean±s.e.m. Student's *t*-tests were used to compare levels of IL-4, airway reactivity (the slope of the R_{RS}-MCh dose-response curve), Max R_{RS}, total and differential cell counts, and indices of airway remodeling between saline control mice and mice receiving allergen. Comparisons between the groups of mice receiving single allergens and the group of mice receiving the allergen combination were made using ANOVA (Statistica version 10.0). Post-hoc multiple-comparison testing was performed by using Duncan's test. All comparisons were two-tailed and *P* values less than 0.05 were considered to be statistically significant.

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