

Acidic Mammalian Chitinase Is Not a Critical Target for Allergic Airway Disease

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The expression of acidic mammalian chitinase (AMCase) is associated with Th2-driven respiratory disorders. To investigate the potentially pathological role of AMCase in allergic airway disease (AAD), we sensitized and challenged mice with ovalbumin or a combination of house dust mite (HDM) plus cockroach allergen. These mice were treated or not treated with small molecule inhibitors of AMCase, which significantly reduced allergen-induced chitinolytic activity in the airways, but exerted no apparent effect on pulmonary inflammation *per se*. Transgenic and AMCase-deficient mice were also submitted to protocols of allergen sensitization and challenge, yet we found little or no difference in the pattern of AAD between mutant mice and wild-type (WT) control mice. In a separate model, where mice were challenged only with intratracheal instillations of HDM without adjuvant, total bronchoalveolar lavage (BAL) cellularity, inflammatory infiltrates in lung tissues, and lung mechanics remained comparable between AMCase-deficient mice and WT control mice. However BAL neutrophil and lymphocyte counts were significantly increased in AMCase-deficient mice, whereas concentrations in BAL of IL-13 were significantly decreased compared with WT control mice. These results indicate that, although exposure to allergen stimulates the expression of AMCase and increased chitinolytic activity in murine airways, the overexpression or inhibition of AMCase exerts only a subtle impact on AAD. Conversely, the increased numbers of neutrophils and lymphocytes in BAL and the decreased concentrations of IL-13 in AMCase-deficient mice challenged intratracheally with HDM indicate that AMCase contributes to the Th1/Th2 balance in the lungs. This finding may be of particular relevance to patients with asthma and increased airway neutrophilia.

Keywords: chitinase; house dust mite; ovalbumin; airway; asthma

The 18-glycosyl hydrolase protein, acidic mammalian chitinase (AMCase), hydrolyzes chitin, a molecule found abundantly in the environment as a component of fungal cell walls, exoskeletons of crustaceans and insects, and the microfilarial sheaths and eggs of parasitic nematodes (1–3). The accumulation of chitin is regulated by the balance between chitin biosynthesis and its degradation by chitinases. Although mammals do not express chitin synthase genes, several human chitinase family members were described, including chitotriosidase and AMCase (1, 4), although their biological importance remains poorly understood. With regard to AMCase, a role in host defense mechanisms may be

hypothesized, because AMCase is a potent inhibitor of dermatopathogenic fungal growth (2), and as such may contribute to the expulsion of invading parasites. In support of this hypothesis, concentrations of AMCase mRNA were found to be increased in the lungs of mice infected by parasites such as *Schistosoma mansoni* and *Nippostrongylus brasiliensis* (5–7). More recently, in a model of allergic airway disease (AAD), the pretreatment of chitin with AMCase or the administration of chitin to mice overexpressing AMCase resulted in an attenuated disease phenotype (8), indicating that AMCase plays a protective role in chitin-induced pulmonary inflammation.

Allergic asthma is a chronic pulmonary disorder caused by a combination of genetic factors, a history of respiratory infections, and exposure to airborne allergens or pollutants (9). The disease is thought to be largely mediated by Th2 cytokines, including IL-4, IL-5, and IL-13, and is characterized by an influx of eosinophils into the lung tissue, mucus metaplasia, and airway remodeling, as well as airway hyperresponsiveness (AHR) after allergen challenge. In humans, AMCase is increased in lung tissues of allergen-challenged patients with asthma, and in cases of fatal asthma, alveolar macrophages were shown to produce high concentrations of AMCase (10). Consistent with this observation, the expression of AMCase was induced via an IL-13/STAT-6-dependent pathway in a murine model of allergic pulmonary inflammation and in IL-13-overexpressing mice (10–12). However, in contrast to models of chitin-induced pulmonary inflammation, the inhibition of AMCase ameliorated pulmonary inflammation and AHR in a Th2-dependent model of allergic airway disease inhibition (10). Moreover, several polymorphisms and haplotypes of AMCase are associated with bronchial asthma (13), whereas a variant form of human AMCase with increased catalytic activity is linked to protection from asthma (14).

Contrasting data from various animal models, as well as clinical observations from patients with asthma, thus underline a complex role for AMCase in the pathophysiology of pulmonary inflammation. To explore the role of AMCase in allergic asthma, we evaluated the effects of AMCase gene expression and the perturbation of its chitinolytic activity in murine models of AAD. Two protocols used systemic sensitization to an allergen combined with aluminum hydroxide (alum) as adjuvant, which are thought to stimulate a largely T-cell-biased response. One protocol focused on house dust mite (HDM) administered intratracheally without adjuvant, which is thought to involve a robust innate immune component in addition to the expected Type 2 immune response (15).

Our results show that, although AMCase gene expression was highly up-regulated after various allergen exposure protocols, the pharmacologic or genetic modifications produced by the expression or enzymatic activity of AMCase exerted limited or no impact on disease phenotype. Conversely, the modulation of BAL neutrophil and lymphocyte numbers as well as of concentrations of IL-13 in AMCase-deficient mice intratracheally challenged with HDM suggests that AMCase contributes to the Th1/

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Th2 balance in the airways, which may be of particular relevance to the enhanced neutrophilia observed in severe forms of asthma.

MATERIALS AND METHODS

Reagents and Animals

Recombinant human AMCase and allosamidin (ALLO) were used as previously described (16). Age-matched 6- to 12-week-old female C57BL/6 wild-type (WT) mice were obtained from Taconic Farms (Hudson, NY). AMCase transgenic and gene-deficient mice were derived as described in the online supplement. All *in vivo* experiments were performed in accordance with protocols approved by Pfizer's Institutional Animal Care and Use Committee.

Murine Models of Allergic Airway Disease

C57BL/6 mice were sensitized intraperitoneally twice with either Grade V ovalbumin (OVA, 20 μ g, Sigma-Aldrich, St. Louis, MO) or a mixture of HDM (20 μ g, *Dermatophagoides pteronyssinus* extract; Greer Labs, Lenoir, NC) plus American cockroach extract (CRA, 20 μ g, *Periplaneta americana*) emulsified with alum (Inject Alum; Pierce, Rockford, IL). For OVA studies, mice were challenged for 30 minutes with aerosols of PBS or 5% OVA on Days 26–28, as previously described (17). For HDM/CRA studies, mice were lightly anesthetized with isoflurane and challenged intranasally with either 25 μ l PBS or 25 μ g (20 μ l) of HDM/CRA mixture on Days 14–16. Mice were treated orally with methylallosamidin (50 mg/kg, twice daily) dissolved in 0.5% methylcellulose and 2% Tween-80 as vehicle, or were injected intraperitoneally with dexamethasone (Dex, 10 mg/kg every day) on days of allergen challenge. When indicated, mice were challenged intratracheally with 100 μ g HDM on Days 0, 7, and 14 of the protocol. Mice were lightly anesthetized with isoflurane. A 1.5-inch polyethylene catheter mounted on a 30-gauge needle was introduced between the animal's vocal cords into the trachea, using an otoscope terminated with a 2-mm speculum (Welch Allyn, Skaneateles Falls, NY). On Days 0, 7, and 14 of the protocol, mice received 100 μ g HDM extract (Greer Laboratories) diluted in 40 μ l saline and administered intratracheally using a 100- μ l Hamilton glass syringe (VWR, West Chester, PA). For all protocols, lung function was evaluated 24–48 hours after allergen challenge in response to escalating doses of methacholine, using the flexiVent system (Scireq, Montreal, PQ, Canada) as described in the online supplement. All mice were killed by CO₂ asphyxiation to collect bronchoalveolar lavage (BAL) and various tissue samples for analysis. BAL cellularity and the preparation of lung tissue for histopathology were performed as previously described (17).

Statistical Analysis

Results represent the mean $\pm$ SEM of individual animals. Statistical significance for all results except lung mechanics was determined using a Mann-Whitney U test. Variations of lung mechanics were first analyzed with two-way ANOVA for group and dose of methacholine (MCh), followed by a Bonferroni *post hoc* test. Results were considered significant at $P < 0.05$.

Online Supplement

The online supplement describes immunoblotting methods, physiologic lung mechanics, and the generation of AMCase transgenic and gene-deficient mice. Figures E1–E6 comprise dot plots of pooled data for the experiments described in Figures 1–6. Figure E7 shows the strategy to generate AMCase transgenic mice and AMCase protein and chitinase activity. Figure E8 describes the strategy to delete the murine AMCase gene by Southern blotting.

RESULTS

Allergic Airway Disease in AMCase Transgenic Mice Sensitized and Challenged with OVA

AMCase was shown to be strongly up-regulated in several models of pulmonary inflammation, although its proinflammatory role remains to be established. Therefore, we modulated the

expression of AMCase, using transgenic mice, pharmacologic inhibition, and AMCase gene-deficient mice to further understand its role in AAD. We initially tested whether the constitutively increased expression of AMCase stimulates the development of pulmonary inflammation, or if high amounts of AMCase would exaggerate the lung response to an allergen challenge. We generated transgenic mice that overexpressed AMCase in the lung, using the Clara cell-specific promoter CC10 (Figure E1) (18). Two independent founder lines were initially examined for the expression of AMCase, and were found to be equivalent (Figure E7). Histopathological evaluation of these AMCase transgenic mice showed that the constitutive overexpression of AMCase in the lung did not result in any gross tissue abnormalities (data not shown). Figure 1A shows that naive AMCase transgenic mice, compared with WT control mice, exhibited levels of chitinase activity 6-fold higher in BAL samples (135 U/ml versus 23 U/ml, respectively) and 2-fold higher in serum samples (50 U/ml versus 26 U/ml, respectively). When sensitized with OVA emulsified in alum and exposed to challenges with OVA aerosols, AMCase transgenic mice developed airway inflammation, as evidenced by increased total cell numbers in BAL samples as well as individual counts of eosinophils, lymphocytes, and neutrophils (Figure 1B). AMCase transgenic mice manifested significantly lower airway eosinophilia in response to the OVA challenge compared with WT mice. Thus, these initial studies indicate that high constitutive amounts of AMCase expression and enzymatic activity do not cause spontaneous pulmonary inflammation, nor do they exaggerate OVA-induced allergic airway disease.

Inhibition of AMCase Activity in Mice Sensitized and Challenged with Various Allergens

Next, we evaluated the therapeutic potential of two small molecule inhibitors of AMCase activity in models of AAD. Chitinase

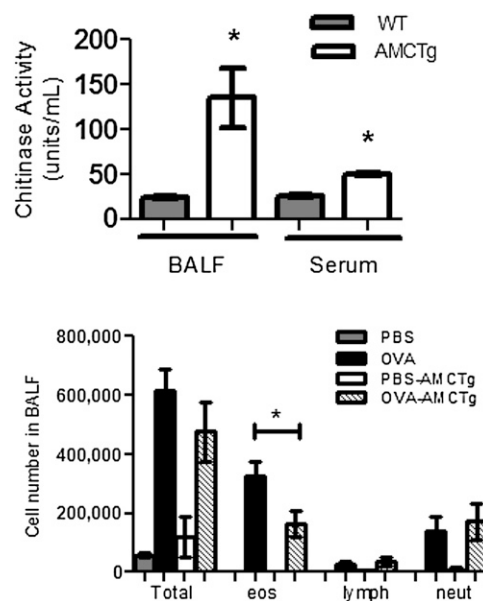


Figure 1. Pulmonary inflammation in ovalbumin (OVA)-challenged acidic mammalian chitinase (AMCase) transgenic (Tg) mice. (Top) Chitinase activity in WT and AMCase Tg (AMCTg) mice. (Bottom) Cellular inflammation in bronchoalveolar lavage fluid (BALF) from WT and AMCase Tg mice sensitized and challenged with PBS or OVA. Data were derived from one of two independent experiments in which both showed the same significant differences. Values represent means $\pm$ SEM, with 7–13 mice per group. * $P < 0.036$.

activity was significantly increased in BAL samples from mice that were OVA-sensitized and challenged. Treating these mice with Dex (10 mg/kg every day) or the chitinase inhibitor ALLO (50 mg/kg, twice daily) reduced OVA-induced AMCase enzymatic activity in BAL samples by 43% and 45%, respectively, as well as overall chitinase activity in lung tissues and serum samples (Figure 2A). As expected from this model, mice sensitized and challenged with OVA developed robust pulmonary inflammation, involving increased numbers of eosinophils, lymphocytes, and neutrophils in BAL samples (Figure 2B). Treatment with Dex largely abrogated the OVA-induced increase of total BAL cellularity ($P = 0.0001$), airway eosinophilia ($P = 0.001$), lymphocytes ($P = 0.0403$), and neutrophils ($P = 0.0332$). Conversely, treatment with ALLO failed to modulate total BAL cellularity and airway eosinophilia, compared with vehicle-treated animals (Figure 2B).

Because ALLO exerted a broad inhibitory effect on AMCase and other chitinases, the specific inhibition of AMCase activity was further evaluated using a novel small molecule inhibitor identified from an in-house chemical library screening. This compound, called “AMCase inhibitor (1)” in the present study, has a structure distinct from that of ALLO, and was previously described in detail by Cole and colleagues (19). Moreover, considering the possibility that AMCase inhibitors are more active in models using chitin-rich allergens, we sensitized and challenged mice with a combination of HDM and CRA. Systemic sensitization, followed by intranasal challenges with HDM/CRA, induced an increase in chitinase activity compared with naive mice (199 U/ml versus 26 U/ml, respectively), which was significantly decreased by 63% and 43% via Dex and the AMCase inhibitor (1), respectively (Figure 3A). As expected, HDM/CRA also induced a robust increase of total BAL cellularity, mainly driven by an influx of eosinophils, lymphocytes,

and neutrophils in the airways. Here, amounts of total BAL cellularity, eosinophils, lymphocytes, and neutrophils were also reduced when HDM/CRA-challenged mice were treated with Dex (Figure 3B, $P < 0.05$). However, despite the inhibition of BAL chitinase activity that was comparable to that with Dex, treatment with “AMCase inhibitor (1)” (50 mg/kg, twice daily) failed to modulate allergen-induced pulmonary inflammation in this model.

These results, and in particular those obtained with chitin-rich HDM/CRA and an AMCase-specific inhibitor compound, indicate that the significant reduction of AMCase hydrolytic activity *in vivo* exerts little or no effect on the modulation of AAD.

Generation and Characterization of AMCase-Deficient Mice

The previous experiments focused on the inhibition of AMCase catalytic activity and its impact on AAD by means of small molecule inhibitors. These compounds did not totally block allergen-induced chitinase activity in the airways, which leaves the possibility that the remaining AMCase enzyme activity or another possible function of AMCase contributed to allergen-induced pulmonary inflammation. To address this issue, we generated AMCase-deficient mice by the deletion of exon 5 of the gene, which contains the catalytic region of AMCase (Figure E8). Quantitative RT-PCR experiments, using pairs of primers that spanned exons 1 and 2 (Figure 4A) or exons 8 and 9 (data not shown), showed that full-length or partial transcripts for the *AMCase* gene were absent in these mice. However, the expression of the chromosomally proximal and related gene *YMI* was unchanged (Figure 4B).

No detectable chitinase activity was evident in BAL samples from AMCase-deficient mice, even when these animals were OVA-sensitized and challenged, which indicated that not only that no AMCase enzymatic activity was occurring, but also that challenge with OVA did not induce any other chitinase activity in the lungs of AMCase-deficient mice (Figure 4C). As expected, increased chitinolytic activity was evident in OVA-challenged WT mice (Figure 4C). The absence of AMCase protein expression in BAL samples was also confirmed by immunoblot, using an antibody that recognizes the C-terminal domain of the enzyme (Figure 4D). In contrast to BAL samples, both WT and AMCase-deficient mice manifested detectable chitinolytic activity in serum samples (Figure 4E), although at lower concentrations in AMCase-deficient mice compared with WT control mice (8 U/ml versus 26 U/ml, respectively). This low chitinolytic activity in the serum of AMCase-deficient mice was attributed to the chitinase family member, chitotriosidase.

AAD in AMCase-Deficient Mice Sensitized and Challenged with OVA

To explore whether the complete absence of AMCase expression and activity would alleviate symptoms of AAD, we compared AMCase-deficient mice with their WT counterparts in a model of OVA-induced pulmonary inflammation. OVA-sensitized and challenged WT mice developed robust pulmonary inflammation, characterized by an influx of eosinophils into the airways (Figure 5A). AMCase-deficient mice undergoing the same protocol did not differ from WT animals in terms of total BAL cellularity or eosinophil, lymphocyte, neutrophil, and macrophage counts. Similarly, AMCase deficiency did not reduce allergen-induced AHR in response to escalating doses of the muscarinic agonist MCh, which remained comparable to the AHR in OVA-challenged WT control mice (Figure 5B).

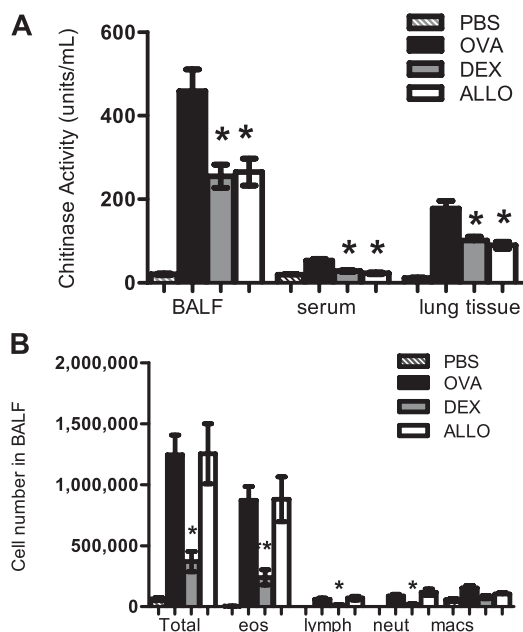


Figure 2. The inhibition of chitinase does not block OVA-induced pulmonary inflammation. (A) Chitinase activity in tissue collected from mice sensitized and challenged with PBS or OVA, and treated with dexamethasone (Dex) or allosamidin (ALLO). (B) Cellular inflammation in mice treated as already described. * $P < 0.01$. Data were derived from one of three independent experiments, in which both showed the same significant differences. Values represent means $\pm$ SEM with 10 mice per group.

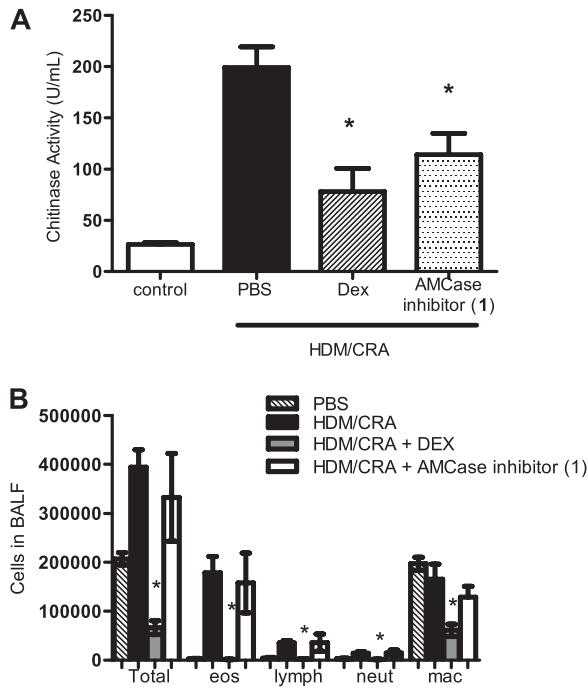


Figure 3. No effect of novel chitinase inhibitor house dust mite (HDM)/cockroach extract (CRA)-induced pulmonary inflammation. (A) Chitinase activity in tissue collected from mice sensitized and challenged with a combination of HDM/CRA and either untreated (black bars), treated with Dex (gray bars), or treated with “AMCase inhibitor (1)” (hatched bars), as indicated in MATERIALS AND METHODS. (B) BAL inflammatory cell counts in mice treated as already described. $*P < 0.05$. Values represent means $\pm$ SEM, with 9–10 mice per group.

The lung response to OVA in AMCase-deficient mice was further evaluated by a quantitative real-time PCR survey of several inflammatory genes. Several genes related to AAD were highly up-regulated in both AMCase-deficient and WT mice after OVA challenge, and no significant differences of expression were evident between the groups (Figure 5C). These genes included those specific for eosinophils, epithelial mucous metaplasia, and chemokines. Moreover, after challenge with OVA, the chitinase family member *YMI/Chi3L3* was strongly induced in WT as well as AMCase-deficient mice, although the expression of the *AMCase* gene was confirmed to be null in AMCase-deficient animals (Figure 5C).

These studies of AMCase-deficient mice thus confirmed that the complete absence of AMCase activity does not modulate AAD parameters such as BAL cellular inflammation, proinflammatory gene expression, or AHR.

AAD in AMCase-Deficient Mice Intratracheally Challenged with HDM

In the experiments already described, we sought to characterize the potentially proinflammatory role of AMCase in AAD. The common denominator in these studies using allergens that were or were not chitin-rich (e.g., HDM/CRA versus OVA), and eliciting the release of large amounts of AMCase in the airways, involved a focus on “classic” protocols of systemic allergen sensitization with an adjuvant (alum), followed by acute allergen challenges. Considering that such protocols may stimulate AAD through a Th2-dominated adaptive immune response, we examined AMCase-deficient mice in a separate model of AAD where a chitin-rich, natural airborne allergen (HDM) was

repeatedly instilled into the airways without adjuvant or preliminary systemic sensitization.

HDM crude extract was administered intratracheally on Days 0, 7, and 14 of the protocol. Lung mechanics were evaluated on Day 15, and tissue samples were harvested on Day 17, as previously described (15). Figure 6A shows how this protocol significantly up-regulated AMCase gene expression in the lungs of WT mice 12 hours after challenge with HDM. Increased AMCase enzymatic activity was also detected in BAL and serum samples 72 hours after challenge with HDM. Hematoxylin and eosin-stained lung-tissue sections were scored by a board-certified pathologist. For both WT and AMCase-deficient mice, the challenge with HDM resulted in bronchovascular inflammation dominated by lymphocytic and neutrophilic infiltrates, with fewer eosinophils present (Figure 6B). In contrast, inflammatory infiltrates within the alveolar regions were primarily histiocytic and lymphocytic, with fewer neutrophils and occasional eosinophils. The challenge with HDM also resulted in an increased formation of bronchiolar intraepithelial mucus, along with bronchiolar epithelial hyperplasia and hypertrophy in both WT and AMCase-deficient animals. In addition, an increased number of Type II pneumocytes and robust peribronchiolar smooth muscle hypertrophy was evident in WT and AMCase-deficient mice exposed to HDM. Masson’s Trichrome staining showed that challenge with HDM also induced a slight increase in collagen deposition in areas of peribronchiolar and perivascular inflammation, again with no apparent difference between

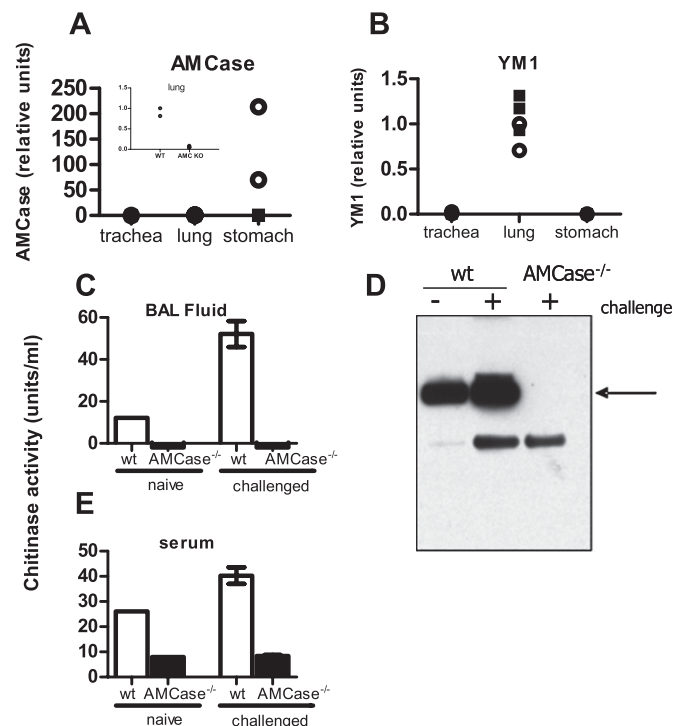


Figure 4. AMCase-deficient mice exhibit no chitinase airway activity, but retain normal expression of YMI. Gene expression of (A) AMCase and (B) YMI from tissue of wild-type (wt) mice (solid squares) and AMCase gene-deficient mice (open circles). (C) Chitinolytic activity and (D) immunoblot of AMCase in BAL samples from wt mice or AMCase-deficient mice. Arrow indicates AMCase. (E) Serum samples of wt and AMCase-deficient mice, as indicated. Data were derived from one of two independent experiments in which both showed the same significant differences. Values represent individual relative expression units for the expression of mRNA (A and B) and means $\pm$ SEM, with 4–10 mice per group for chitinase activity (C and E).

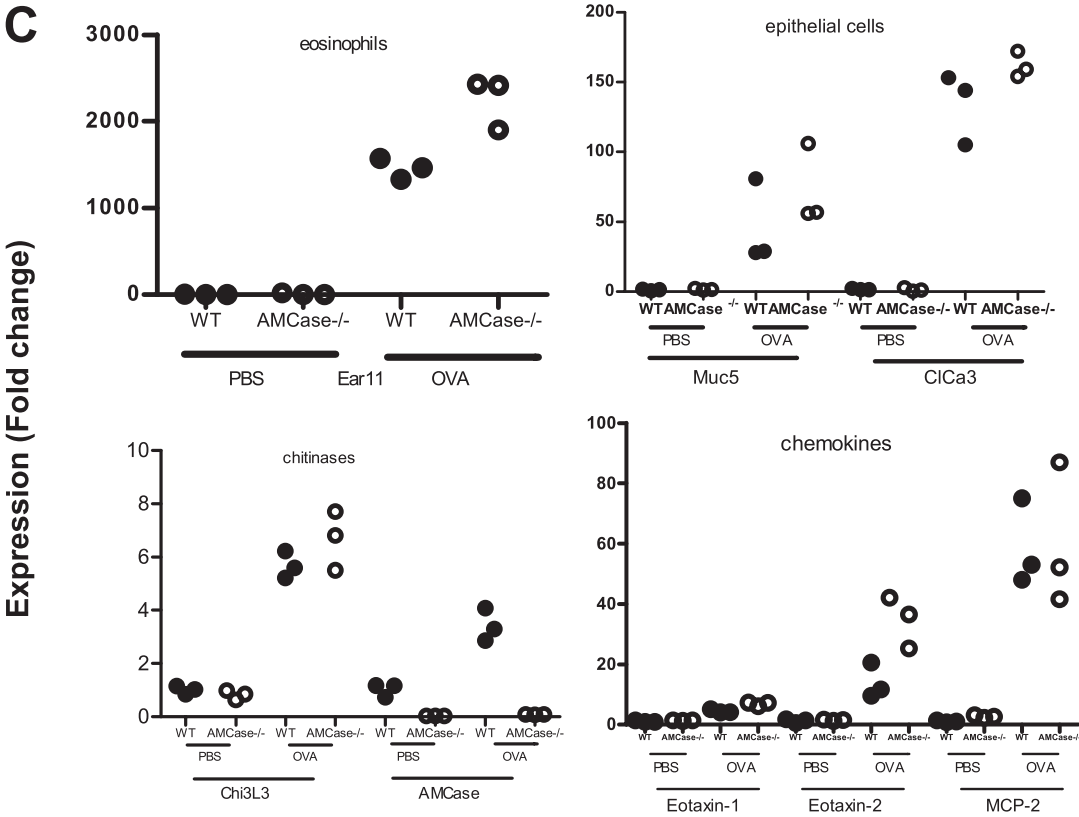
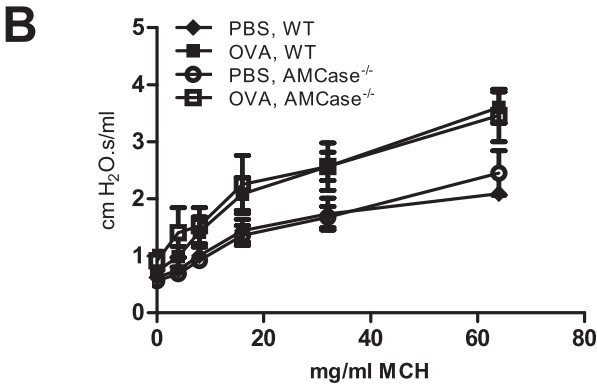
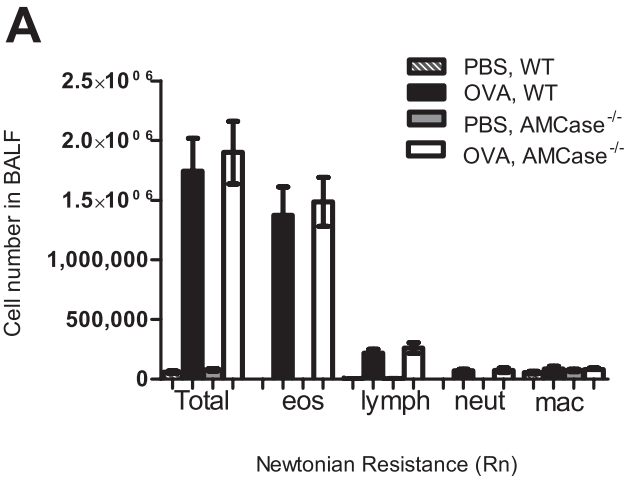


Figure 5. AMCase gene deficiency does not modulate OVA-induced allergic airway disease. (A) Total cellularity (Total) and numbers of eosinophils (eos), lymphocytes (lymph), neutrophils (neut), and macrophages (mac) in BAL samples, 24 hours after challenge with OVA. (B) Lung mechanics are expressed as Newtonian resistance (Rn). Data were derived from one of two independent experiments in which both showed the same significant differences. Values represent means $\pm$ SEM, with 8–9 mice per group. MCH, methacholine. (C) Genes representing the cellular influx of eosinophils (EAR11), epithelial mucous metaplasia, chemokines, and chitinases are shown for WT mice (solid circles) and AMCase gene-deficient mice (open circles) treated with PBS or challenged with OVA. Values represent data for individual animals.

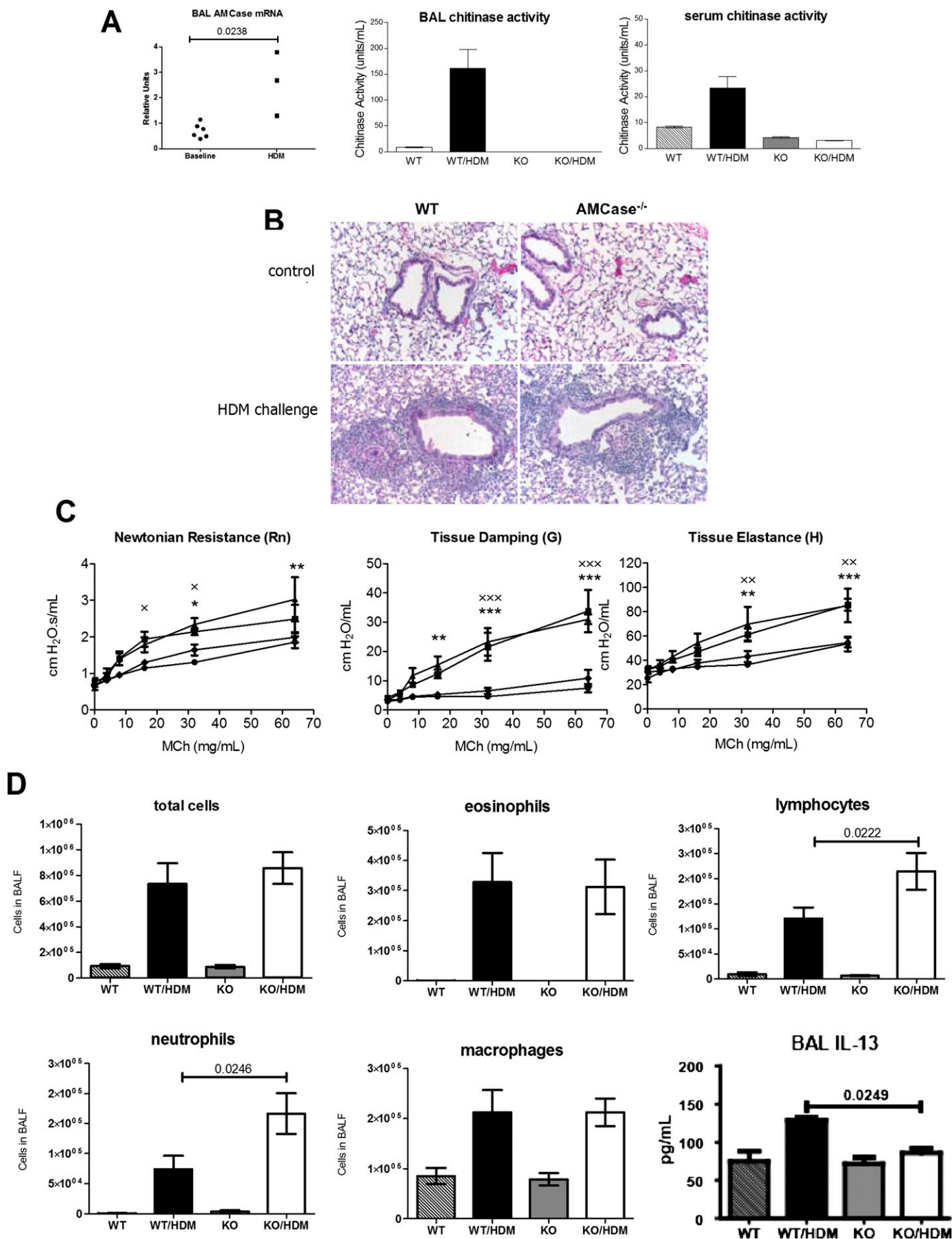


Figure 6. Modulation of HDM-induced allergic airway disease in AMCase-deficient mice. (A) AMCase mRNA expression in lung samples 12 hours after HDM challenge, and chitinase activity in BAL and serum samples 72 hours after challenge with HDM. MCh, methacholine. (B) Hematoxylin and eosin staining of lung sections. Magnification, $\times 40$. (C) Lung mechanics are expressed as Newtonian resistance (Rn), tissue damping (G), and tissue elastance (H). "X" indicates statistically significant differences between WT (saline/baseline) and WT (HDM) mice ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Asterisks indicate statistically significant differences between KO (saline/baseline) and WT (HDM) mice ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). (D) Total cellularity, numbers of macrophages, eosinophils, lymphocytes, and neutrophils, and concentrations of IL-13 in BAL samples, 72 hours after challenge with HDM. *P* values involve comparisons between HDM-challenged mice (AMCase-deficient versus WT). The data were pooled from two independent experiments. Values represent means $\pm$ SEM, with 7–15 mice per group.

WT and AMCase-deficient mice (not shown). Thus, histopathological findings remained overall similar in the lungs of WT mice and AMCase-deficient mice exposed to HDM.

Next, we evaluated the lung mechanics of AMCase-deficient mice and WT control mice challenged with escalating doses of MCh, 24 hours after exposure to HDM. Again, AMCase deficiency exerted little apparent impact on AHR, because the increase in Newtonian resistance, tissue damping, and tissue elastance remained altogether similar between AMCase-deficient mice and WT control mice (Figure 6C). Intratracheal challenge with HDM alone stimulated a robust increase in total BAL cellularity compared with naive animals (Figure 6D), although these numbers were similar between AMCase-deficient mice and WT control mice ($P < 0.0001$ and $P = 0.0013$ compared with naive control mice, respectively). Numbers of BAL eosinophils and macrophages were also comparable between AMCase-deficient mice and WT control mice. Intriguingly, numbers of BAL lymphocytes and neutrophils were significantly increased in AMCase-deficient mice compared with WT control mice ($P = 0.0366$ and $P = 0.0246$, respectively). BAL samples were then analyzed using a 22-plex assay for a Luminex platform (Invitrogen, Grand Island, NY), which indicated little difference between AMCase-deficient mice and their WT counterparts. Seemingly increased concentrations of CXCL1 (KC), IL-5, and IL-6 in BAL samples from AMCase-deficient mice indicated nonsignificant trends (not shown). Only concentrations of IL-13 levels were significantly modulated, and in fact, were lower in AMCase-deficient mice compared with WT control mice (Figure 6D). This finding is consistent with that in a previous report of decreased expression of IL-13 after the administration of AMCase-specific RNA inhibitory in a murine model of OVA-induced AAD (20).

These data indicate that repeated challenges of murine airways with HDM alone stimulate the release of high amounts of AMCase. AMCase deficiency overall did not modulate AAD, including total inflammatory cell influx into the lumen, tissue inflammation, and AHR. However, an interesting increase of lymphocyte and neutrophil numbers was evident in BAL samples, suggesting a regulatory rather than proinflammatory role for AMCase in response to HDM.

DISCUSSION

AMCase was previously identified as part of a distinct IL-13-dependent and STAT-6-dependent gene expression signature induced in the lungs of animals infected with helminthes or challenged with allergens (5–7, 11, 21). Contrasting reports suggest that AMCase either contributes to (10, 20, 22) or protects from (8) pulmonary inflammation. Indeed, in a "classic" model of AAD that included systemic sensitization to OVA/alum and acute OVA challenges, Zhu and colleagues (10) reported that AMCase is involved in the development of allergic pulmonary inflammation and AHR. We thus investigated whether the overexpression or inhibition of AMCase would modulate the parameters of AAD in murine models of allergen/alum sensitization and challenge, using allergens that were either chitin-rich (HDM/

CRA) or not (OVA). The HDM/CRA protocol also conveniently allowed for tests of whether chitin hydrolysis by AMCase contributes to the induction of pulmonary inflammation.

A previous report suggested that the overexpression of AMCase in mice prevented the chitin-induced pulmonary inflammation of eosinophils (8), seemingly in contrast with the proinflammatory effect of AMCase in OVA models of AAD (10, 22). We generated AMCase transgenic mice to evaluate whether the overexpression of AMCase in naive animals would lead to spontaneous signs of pulmonary inflammation, and whether allergen-induced AAD would be aggravated or diminished. OVA-challenged WT mice, in fact, expressed concentrations of AMCase similar to the maximum concentrations evident in AMCase transgenic mice. Naive AMCase transgenic mice did not show any signs of pulmonary inflammation. Moreover, after OVA/alum sensitization and acute OVA challenge, AMCase transgenic mice and WT control mice developed similar patterns of AAD, which demonstrated that high concentrations of AMCase in the airways do not trigger or potentiate allergic pulmonary inflammation, or attenuate an inflammatory response.

We then used the OVA and HDM/CRA models to test the therapeutic potential of inhibiting AMCase activity. Both protocols elicited elevated concentrations of AMCase in murine airways. The activity of AMCase hydrolase was also increased, and was significantly diminished after treating the mice with small molecule inhibitors of AMCase. Despite this reduction of AMCase chitinolytic activity, the two compounds did not modulate OVA-induced or HDM/CRA-induced AAD. We confirmed these results in AMCase-deficient mice, in which the complete absence of AMCase expression and activity also failed to alleviate the parameters of AAD.

Our data, using two separate allergen/alum models of AAD, contrast with previously published results, where a significant reduction in airway inflammation and AHR were produced by blocking AMCase (10, 20, 22). Arguably, such protocols, in which mice are sensitized systemically to an allergen emulsified with alum as adjuvant, are strongly biased toward a Type 2 immune response in three ways: (1) the adsorption of soluble antigens onto particulates delays their clearance and facilitates their presentation by APCs (23), (2) insoluble materials such as alum cannot be processed and thus enhance the production of antibodies as and the activation of antigen-presenting cells through the Nalp3 inflammasome (24), and (3) in contrast to molecules such as complete Freud's adjuvant or CpG oligodeoxynucleotides that mimic microbial stimuli, alum stimulates the proliferation of Th2 cells and the production of cytokines (25).

To address the possibility that the role of AMCase was potentially minimized in protocols of allergen sensitization with alum, we challenged WT and AMCase-deficient mice intranasally with HDM extract alone, in the absence of preliminary systemic sensitization with alum. Although this approach was expected to stimulate a sustained Type 2 adaptive immune response and airway eosinophilic inflammation (26), models of "real-life exposure to aeroallergens" may also elicit broader, host defense-like mechanisms (15, 27). For example, the intranasal administration of HDM successfully enhanced the immune response against an

otherwise weak or innocuous antigen such as OVA itself (28, 29). The protease Der p 2, which is a major allergen component of HDM, displays structural and functional homology with MD-2, a molecule associated with Toll-like receptor (TLR)-4 that facilitates the binding of LPS and the ensuing activation cascade (30). Moreover, chitin fragments such as those present in HDM extract were shown to stimulate the production of IL-10 by peritoneal macrophages in a TLR2-dependent manner (31). We thus speculate that a direct airway challenge with HDM extract partly results in the early activation of TLR-driven inflammatory pathways, followed by the development of a mixed Th1/Th17/Th2-driven adaptive immune response to the allergen (32). AMCase-deficient mice intratracheally challenged with HDM alone developed increased BAL total cellularity, lung histopathology, and AHR, similar to those observed in WT control mice. Most intriguingly, however, AMCase-deficient mice also exhibited an approximately 2-fold increase in BAL neutrophil and lymphocyte counts compared with WT animals, as well as a significant decrease in concentrations of IL-13, consistent with the reduced expression of IL-13 after the administration of RNA inhibitory (20).

A strong genetic association exists between AMCase and susceptibility to asthma (13, 33, 34). Conversely, a particular AMCase haplotype, resulting in the expression of variant proteins with increased enzymatic activity, was associated with protection from allergic asthma (14). This protective role of AMCase activity is consistent with our present results, where AMCase-deficient mice exposed to HDM developed a limited yet significant Th1/Th2 imbalance in their airways, with increased numbers of neutrophils and lymphocytes, whereas concentrations of IL-13 were decreased compared with those in WT mice. Considering that an elevated epithelial expression of AMCase was reported in cases of fatal asthma (10) but not mild to moderate asthma (14), and that increased airway neutrophilia is often a hallmark of severe, steroid-resistant asthma as well as sudden-onset fatal asthma (35), AMCase may, we speculate, play a regulatory rather than aggravating role in severe forms of asthma, perhaps through the breakdown of chitin-like substrates.

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References

1. Boot RG, Blommaert EF, Swart E, Ghauharali-van der Vlugt K, Bijl N, Moe C, Place A, Aerts JM. Identification of a novel acidic mammalian chitinase distinct from chitotriosidase. *J Biol Chem* 2001;276:6770–6778.
2. Chen L, Shen Z, Wu J. Expression, purification and *in vitro* antifungal activity of acidic mammalian chitinase against *Candida albicans*, *Aspergillus fumigatus* and *Trichophyton rubrum* strains. *Clin Exp Dermatol* 2009;34:55–60.
3. Chou YT, Yao S, Czerwinski R, Fleming M, Krykbaev R, Xuan D, Zhou H, Brooks J, Fitz L, Strand J, *et al.* Kinetic characterization of recombinant human acidic mammalian chitinase. *Biochemistry* 2006;45:4444–4454.
4. Boot RG, Renkema GH, Strijland A, van Zonneveld AJ, Aerts JM. Cloning of a cDNA encoding chitotriosidase, a human chitinase produced by macrophages. *J Biol Chem* 1995;270:26252–26256.
5. Artis D, Wang ML, Keilbaugh SA, He W, Brenes M, Swain GP, Knight PA, Donaldson DD, Lazar MA, Miller HR, *et al.* RELMβ/FIZZ2 is a goblet cell-specific immune-effector molecule in the gastrointestinal tract. *Proc Natl Acad Sci USA* 2004;101:13596–13600.
6. Pesce J, Kaviratne M, Ramalingam TR, Thompson RW, Urban JF Jr, Cheever AW, Young DA, Collins M, Grusby MJ, Wynn TA. The IL-21 receptor augments Th2 effector function and alternative macrophage activation. *J Clin Invest* 2006;116:2044–2055.
7. Nair MG, Gallagher IJ, Taylor MD, Loke P, Coulson PS, Wilson RA, Maizels RM, Allen JE. Chitinase and Fizz family members are a generalized feature of nematode infection with selective upregulation of Ym1 and Fizz1 by antigen-presenting cells. *Infect Immun* 2005;73:385–394.
8. Reese TA, Liang HE, Tager AM, Luster AD, Van Rooijen N, Voehringer D, Locksley RM. Chitin induces accumulation in tissue of innate immune cells associated with allergy. *Nature* 2007;447:92–96.
9. Holgate ST, Arshad HS, Roberts GC, Howarth PH, Thurner P, Davies DE. A new look at the pathogenesis of asthma. *Clin Sci (Lond)* 2009;118:439–450.
10. Zhu Z, Zheng T, Homer RJ, Kim YK, Chen NY, Cohn L, Hamid Q, Elias JA. Acidic mammalian chitinase in asthmatic Th2 inflammation and IL-13 pathway activation. *Science* 2004;304:1678–1682.
11. Follettie MT, Ellis DK, Donaldson DD, Hill AA, Diesl V, DeClercq C, Sypek JP, Dörner AJ, Wills-Karp M. Gene expression analysis in a murine model of allergic asthma reveals overlapping disease and therapy dependent pathways in the lung. *Pharmacogenomics J* 2006;6:141–152.
12. Homer RJ, Zhu Z, Cohn L, Lee CG, White WI, Chen S, Elias JA. Differential expression of chitinases identify subsets of murine airway epithelial cells in allergic inflammation. *Am J Physiol Lung Cell Mol Physiol* 2006;291:L502–L511.
13. Bierbaum S, Nickel R, Koch A, Lau S, Deichmann KA, Wahn U, Superti-Furga A, Heinzmann A. Polymorphisms and haplotypes of acid mammalian chitinase are associated with bronchial asthma. *Am J Respir Crit Care Med* 2005;172:1505–1509.
14. Seibold MA, Reese TA, Choudhry S, Salam MT, Beckman K, Eng C, Atakilit A, Meade K, Lenoir M, Watson HG, *et al.* Differential enzymatic activity of common haplotypic versions of the human acidic mammalian chitinase protein. *J Biol Chem* 2009;284:19650–19658.
15. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat Med* 2009;15:410–416.
16. Olland AM, Strand J, Presman E, Czerwinski R, Joseph-McCarthy D, Krykbaev R, Schlingmann G, Chopra R, Lin L, Fleming M, *et al.* Triad of polar residues implicated in pH specificity of acidic mammalian chitinase. *Protein Sci* 2009;18:569–578.
17. Long AJ, Sypek JP, Askew R, Fish SC, Mason LE, Williams CM, Goldman SJ. Gob-5 contributes to goblet cell hyperplasia and modulates pulmonary tissue inflammation. *Am J Respir Cell Mol Biol* 2006;35:357–365.
18. Stripp BR, Sawaya PL, Luse DS, Wikenheiser KA, Wert SE, Huffman JA, Lattier DL, Singh G, Katyal SL, Whitsett JA. Cis-acting elements that confer lung epithelial cell expression of the CC10 gene. *J Biol Chem* 1992;267:14703–14712.
19. Cole DC, Olland AM, Jacob J, Brooks J, Bursavich MG, Czerwinski R, DeClercq C, Johnson M, Joseph-McCarthy D, Ellingboe JW, *et al.* Identification and characterization of acidic mammalian chitinase inhibitors. *J Med Chem* 2010;53:6122–6128.
20. Yang CJ, Liu YK, Liu CL, Shen CN, Kuo ML, Su CC, Tseng CP, Yen TC, Shen CR. Inhibition of acidic mammalian chitinase by RNA interference suppresses OVA-sensitized allergic asthma. *Hum Gene Ther* 2009;20:1597–1606.
21. Zimmermann N, Mishra A, King NE, Fulkerson PC, Doecker MP, Nikolaidis NM, Kindinger LE, Moulton EA, Aronow BJ, Rothenberg ME. Transcript signatures in experimental asthma: identification of STAT6-dependent and -independent pathways. *J Immunol* 2004;172:1815–1824.
22. Matsumoto T, Inoue H, Sato Y, Kita Y, Nakano T, Noda N, Eguchi-Tsuda M, Moriwaki A, Kan OK, Matsumoto K, *et al.* Demethylallosamidin, a chitinase inhibitor, suppresses airway inflammation and hyperresponsiveness. *Biochem Biophys Res Commun* 2009;390:103–108.
23. Brewer JM. (How) do aluminium adjuvants work? *Immunol Lett* 2006;102:10–15.

24. Eisenbarth SC, Colegio OR, O'Connor W, Sutterwala FS, Flavell RA. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature* 2008;453:1122–1126.
25. Kool M, Petrilli V, De Smedt T, Rolaz A, Hammad H, van Nimwegen M, Bergen IM, Castillo R, Lambrecht BN, Tschopp J. Cutting edge: alum adjuvant stimulates inflammatory dendritic cells through activation of the Nalp3 inflammasome. *J Immunol* 2008;181:3755–3759.
26. 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* 2004;173:6384–6392.
27. Phipps S, Lam CE, Kaiko GE, Foo SY, Collison A, Mattes J, Barry J, Davidson S, Oreo K, Smith L, *et al.* Toll/IL-1 signaling is critical for house dust mite-specific helper T cell type 2 and type 17 [corrected] responses. *Am J Respir Crit Care Med* 2009;179:883–893.
28. Fattouh R, Pouladi MA, Alvarez D, Johnson JR, Walker TD, Goncharova S, Inman MD, Jordana M. House dust mite facilitates ovalbumin-specific allergic sensitization and airway inflammation. *Am J Respir Crit Care Med* 2005;172:314–321.
29. Fuchs B, Braun A. Improved mouse models of allergy and allergic asthma: chances beyond ovalbumin. *Curr Drug Targets* 2008;9:495–502.
30. Trompette A, Divanovic S, Visintin A, Blanchard C, Hegde RS, Madan R, Thorne PS, Wills-Karp M, Gioannini TL, Weiss JP, *et al.* Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* 2009;457:585–588.
31. Da Silva CA, Hartl D, Liu W, Lee CG, Elias JA. TLR-2 and IL-17A in chitin-induced macrophage activation and acute inflammation. *J Immunol* 2008;181:4279–4286.
32. Maekawa A, Xing W, Austen KF, Kanaoka Y. GPR17 regulates immune pulmonary inflammation induced by house dust mites. *J Immunol* 2010;185:1846–1854.
33. Heinzmann A, Brugger M, Bierbaum S, Mailaparambil B, Kopp MV, Strauch K. Joint influences of acidic-mammalian-chitinase with interleukin-4 and Toll-like receptor-10 with interleukin-13 in the genetics of asthma. *Pediatr Allergy Immunol* 2010;21:e679–e686.
34. Chatterjee R, Batra J, Das S, Sharma SK, Ghosh B. Genetic association of acidic mammalian chitinase with atopic asthma and serum total IgE levels. *J Allergy Clin Immunol* 2008;122:e201–e207.
35. Wenzel SE. Severe asthma in adults. *Exp Lung Res* 2005;31:22.

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