A Potential New Target for Asthma Therapy: A Disintegrin and Metalloprotease 10 (ADAM10) involvement in murine experimental asthma

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Abstract

Background—Elevated levels of CD23, a natural regulator of IgE production, have been shown to decrease the signs of lung inflammation in mice. The aim of this study was to study the involvement of ADAM10, the primary CD23 sheddase, in experimental asthma.

Methods—ADAM10 was blocked either by using mice with a B cell specific deletion of the protease or pharmacologically by intranasal administration of selective ADAM10 inhibitors. Airway hypersensitivity (AHR) and bronchoaveolar lavage fluid (BALF) eosinophilia and select BALF cytokine/chemokine levels were then determined.

Results—Using an IgE and mast cell dependent mouse model, B cell specific ADAM10 $^{-/-}$ mice (C57B/6 background) exhibited decreased eosinophilia and AHR when compared to littermate controls. Treatment of C57B/6 mice with selective inhibitors of ADAM10 resulted in an even further decrease in BALF eosinophilia, as compared with the ADAM10$^{-/-}$ animals. Even in the Th2 selective strain, Balb/c, BALF eosinophilia was reduced from 60 to 23% respectively. In contrast when an IgE/mast cell independent model of lung inflammation was used, the B cell ADAM10$^{-/-}$ animals and ADAM10 inhibitor treated animals had lung inflammation levels that were similar to the controls.

Conclusions—These results thus show that ADAM10 is important in the progression of IgE dependent lung inflammation. The use of the inhibitor further suggested that ADAM10 was important for maintaining Th2 levels in the lung. These results thus suggest that decreasing ADAM10 activity could be beneficial in controlling asthma and possible other IgE dependent diseases.

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Keywords

ADAM10; Asthma; CD23; IgE; Th2

Introduction

The contribution of IgE to allergy was first established in the late 1960s (1) and with the development of anti-IgE therapy its role in asthma has become better appreciated. In experimental asthma models in mice the therapy leads to decreases in lung eosinophil infiltration, reduced airway hypersensitivity (AHR) and decreased IL-5 and IL-13 production (2), which changes are also seen in human patients(3). Although clearly beneficial, its cost-effectiveness in severe asthmatics has been questioned (4). In addition, anti-IgE therapy is only approved for those whose IgE levels fall in the 30 and 700 IU/ml range (5). Thus, additional therapies, especially for patients with IgE levels in excess of this range are needed.

CD23, the low affinity-receptor of IgE, is a type II integral transmembrane protein. It is predominantly expressed as a trimer at the cell surface (6) and it is in this oligomerized state that CD23 bound to IgE can decrease the synthesis of IgE (7). CD23 is a member of the C-type lectin family, making it unique among Fc receptors. IgE is bound by the lectin head of CD23 and the trimer has an effective affinity for IgE in the nM range. In addition to being a membrane bound protein, CD23 after cleavage, is also found as a soluble factor (sCD23). In humans sCD23 has been shown to have the opposite role of membrane CD23, as it increases B cell proliferation and IgE synthesis(8).

When administered in conjunction with antigen, agonist antibodies specific for the IgE binding site of CD23 cause decreased levels of IgE while having no effects on other isotypes (9). Anti-CD23 also decreased signs of experimental asthma in both IgE dependent and independent mouse models (10). One study (10) additionally examined CD23 transgenics with respect to experimental asthma induction and found decreased eosinophils in the BALF as well as reduced AHR (by penh); however, no mechanism was presented. Finally, when anti-CD23 therapy was used in a clinical trial there was a 40% drop in free serum IgE (11). While not sufficient for clinical efficacy, this trial did show that CD23 is a useful target to decrease IgE levels in humans.

The identification of A Disintegrin and Metalloproteinase 10 (ADAM10) as the primary CD23 sheddase (12) suggests an alternative method to promote the CD23 regulatory activity of IgE, namely by modulating B cell ADAM10 levels and/or activity. The ADAM10 mediated cleavage of CD23 primarily takes place after CD23 is sorted into exosomes in an ADAM10 dependent manner (13). While global ADAM10$^{-/-}$ mice die at embryonic day 9.5, ADAM10 B cell conditional knock outs (called ADAM10$^{-/-}$ from this point) have a large increase in surface CD23 and a corresponding decrease in sCD23 (14). Additionally, we have recently shown that the inhibition of ADAM10 in human B cell *in vitro* cultures decreases the synthesis of IgE (15). In this paper we show that when surface levels of CD23 are increased, the features of IgE dependent experimental asthma are reduced. We show that CD23Tg mice and ADAM10 B cell specific knockouts, which both have high levels of surface CD23, are less susceptible to IgE dependent asthma. Furthermore, we show that treatment of mice intranasally with ADAM10 inhibitors show substantially reduced responses to OVA. The mechanism is potentially due to a reduction in IgE and/or in the Th2 response.

Material and Methods

Reagents

Chicken Ovalbumin (OVA) and Imject Alum Adjuvant were purchased from Sigma (St. Louis, MO) and Pierce (Rockford, IL), respectively. Cytokines were measured using multiplex kits from Biorad (Hercules, CA) according to the manufacturer's instructions. The ADAM10 hydroxamate inhibitors, INC008765 and INC009588 (16), were synthesized by the Incyte Corporation. These inhibitors are very selective for ADAM10 as shown by both cell free as well as cell based assays requiring at least 5 fold higher concentrations to inhibit MMP12 and at least 20 fold to inhibit any other enzymes including ADAM17 (16).

Mice

CD23 transgenics were described previously (17) and have been backcrossed 12 generation onto a Balb/c background. Littermates that were negative for the transgene were used as controls. B cell selective ADAM10$^{-/-}$ mice were also described previously (14) and are on a C57B/6 background. Mice negative for the cre gene were used as littermate (LM) controls. Female C57BL/6J and Balb/c mice were purchased from Jackson laboratory (Bar Harbor, ME) and were used in the inhibitor studies. Female mice ages 8-12 weeks were used in the experiments. All mouse protocols were approved by the VCU Institutional Animal Care and Use Committee.

Asthma models

Two asthma models were used and are shown in Figure 1. Model A was developed by *Williams et al.* and was shown to be mast cell as well as IgE dependent (18). Model B was developed here at VCU and was shown to be independent of IgE and mast cells through the use of IgE$^{-/-}$ (19) and mast cell deficient mice (W/Wsh), in both cases the eosinophil levels in the BALF and lung tissue were similar to wild type mice (data not shown).

FlexiVent

In some experiments, 18 hours after the final OVA challenge, airway resistance was measured using the Flexivent system (Scireq, Montreal, Canada) (19). Mice were anesthetized by intraperitoneal (IP) injection of 206.7 mg/kg of ketamine and 41.7 mg/kg of xylazine. Following cannulation and paralyzation mice were ventilated and measurements of baseline lung function were taken, mice were exposed to aerosols containing increased doses (10, 25, 50 mg/ml for C57B/6 or 2.5, 5, 10, 25 for BALB/c) of Acetyl-β-methylcholine chloride (Methacholine) (Sigma). Newtonian resistance (Rn), tissue damping (G) and tissue elastance (H) were all measured at baseline (PBS) and 50 mg/ml of methacholine using the Flexivent software version 5.3.

BALF/lung collection

Following the Flexivent analysis, BALF was collected by lavaging the lungs with 1 ml PBS. The BALF was centrifuged and supernatant fluids were saved for further analysis. Pelleted cells were resuspended in 100 μl PBS, counted, cytospun onto slides and stained with Diff-Quik (Siemens Healthcare Diagnostics, Deerfield, IL). Percentages were determined by counting of at least 100 leukocyte cells per cytospin. Additionally, lung and heart tissue were fixed in 10% formulin (Fisher) and embedded in paraffin by the VCU pathology core. 5 μm sections were cut onto microscope slides and stained with hemotoxin and eosin (H&E). A Nikon eclipse with a SPOT Flex Shifting Pixel Color Mosaic (Diagnostic Instrumental inc., Sterling Heights, MI) camera was used to take pictures of the sections.

ELISAs

Total mouse IgE was measured as previously described (20). For OVA specific IgE, R1E4 (rat anti-mouse IgE) was used as the capture antibody and OVA-DNP-Biotin in combination with Streptavidin-alkaline phosphatase (AP) was used for detection. Mouse IgE anti-DNP was used as a standard. For OVA-specific IgG1, OVA was used to capture the antibody. Samples and standard (serum from a mouse hyper immunized with OVA was used as an internal control) were detected with an AP-goat anti mouse IgG1. The liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured as previously described (21).

Real time PCR

Total RNA was extracted from disrupted lung tissue and purified as explained previously(14). For analysis the following previously published primers were used: GATA3 (22), T bet(22), IL- 25 (23), and IL-33 (23).

Statistics

All statistics were done using the student two-tailed T test, with bonferroni correction when multiple comparisons were being made. All bars represent standard error.

Results

ADAM10^{-/-} and CD23Tg mice have decreased signs of allergic lung inflammation in an IgE dependent model

To test the importance of CD23 and ADAM10 in asthma induction we used the murine models of allergic lung inflammation shown in figure 1. In agreement with Haczku *et al.* (10), we found that CD23Tg mice (Balb/c background), which have suppressed IgE (17) and have high expression of CD23 on lymphocytes, exhibited decreased eosinophilia (Fig 2a) when tested in an IgE-dependent asthma model (model A). We also observed decreased levels of the chemokine IL-5 (Fig 2c) which may explain the decreased eosinophilia seen. In contrast to the IgE-dependent model (model A) no differences in BALF eosinophilia was seen (Fig. 2b) when model B was used. This indicates that the decrease in asthma severity observed in the CD23Tg occurs because of decreased IgE in these animals. Given that ADAM10 is the CD23 sheddase and that mice which lack B cell ADAM10 have greatly increased CD23 expression (14) we next tested whether these mice would have reduced inflammation with model A. Lung AHR in ADAM10^{-/-} mice (C57B/6 background), as determined by total lung resistance (R_L) (Fig 3a) and forced oscillation technique (Fig 3b) was significantly lower than in the LM sensitized mice. Fig 3c shows that the ADAM10^{-/-} mice also had significantly reduced eosinophilia in the BALF. Total BALF cell numbers (data not shown) and percentages of other cell types in the ADAM10^{-/-} mice closely resembled the unsensitized animals. ADAM10^{-/-} mice also had reduced serum antigen-specific IgE levels (Fig 3d). In addition, significant decreases in both Th1 and Th2 cytokines were observed (Fig 3e). In contrast, when asthma was induced with model B, the ADAM10^{-/-} (data not shown) like the CD23Tg (Fig 2b) was not significantly different from the LM sensitized animals with respect to AHR or BALF eosinophilia (data not shown). Additionally, the Th1 cytokines IL-12 and MIP1-α were still significantly decreased in the IgE independent model, when comparing the OVA sensitized ADAM10^{-/-} mice to LM controls (data not shown). Over all these data show that increasing CD23 by inhibiting ADAM10 on B cells has the potential for strongly inhibiting the IgE dependent, but not the IgE independent lung inflammatory response by reducing IgE levels.

ADAM10 inhibition blocks the induction of experimental asthma

Given the above finding that mice lacking B cell ADAM10 have minimal pathology in IgE dependent lung inflammation, we sought to determine if inhibiting ADAM10 activity in WT mice would have the same affect. Experiments were conducted with two hydroxamate inhibitors of ADAM10 (both were found to act similar, thus the data from both were combined). They were dissolved in DMSO and administered intranasally during the induction of the asthma model. The inhibitors were administered intranasally at a concentration of 1 mg/kg (16), a dose where ADAM10 should selectively be inhibited, in DMSO/PBS every three days throughout the experiment. As a control, the ADAM10 inhibitors were also administered to unsensitized mice and found to have no effect (Fig 4 b, c). Using the IgE-dependent model (model A) we found that the inhibitors significantly decreased the signs of allergic airway inflammation, while having no toxic effects as measured by weight loss as well as the levels of liver enzymes (ALT, AST) in the serum (Table 1). The carrier (5% DMSO in PBS) was well tolerated; responses of the LM controls in Fig 3c were similar to Fig 4c. Thus, any reduction in asthma severity was due to inhibition of ADAM10 activity. We also found that similar to the ADAM10$^{-/-}$ (Fig 3), AHR as measured by forced oscillation technique (Fig 4b), as well as R_L (Fig 4a) was decreased by the administration of the ADAM10 inhibitor. The eosinophilia in the BALF was also found to be significantly reduced (Fig 4c); this decrease was even more drastic than in the ADAM10$^{-/-}$, from ~50% to 20% in the ADAM10$^{-/-}$ (Fig 3c) and from ~50% to 5% (Fig 4c) in WT mice treated with the inhibitor. Thus, this shows that ADAM10 expression by other cells, in addition to the B cell, is important in the progression of experimental asthma. The inhibitors are also possible blocking ADAM10 activity on epithelial cells as they have been shown to control the allergic response (reviewed in (24)) and have high expression of ADAM10 (25). Of note although the percentage of macrophages was increased, there were actually less macrophages in the BALF (data not shown) after inhibitor treatment. To determine if this effect was through an inhibition of IgE synthesis, as it was with the ADAM10$^{-/-}$, we measured the levels of total IgE in the serum (Fig 4d) but found it was unchanged from the LM. However, local production was decreased, as total IgE in the BALF was significantly decreased (Fig 4e). Hydroxamate inhibitors are known to have a short half-life in serum (26), however as clearly shown by our data when administered intranasally the inhibitory activity is sufficient for at least a 3 day period. The effects on IgE suggest local activity, however, blocking only the local production of IgE is advantageous as it has been shown that local but not systemic, production of IgE (27) is important for disease progression.

To test if ADAM10 inhibitors had further effects beyond that of inhibiting IgE, we isolated total lung mRNA from treated and control mice and using qPCR, measured the level of several components known to be important in asthma progression, including the Th2 response. In the ADAM10$^{-/-}$ mice we found that the decrease in serum OVA-specific IgE correlated with a decrease in Th2 cytokines (Fig 3e). To test what effect administration of the inhibitor was having on T cell responses, mRNA levels of the dominate T cell transcription factors were measured by qPCR. Analysis demonstrated that GATA3 message levels were significantly decreased, while Tbet was not (Fig 4f). Recently two cytokines, IL-25 and 33 (reviewed in (28)), have been implicated in the induction of a Th2 response. While minimal IL-25 levels were detected, message for IL-33 was significantly decreased in mice treated with the ADAM10 inhibitor. These results show that ADAM10 is also important in the initiation of the Th2 response in the lung. Interestingly, when cytokines were analyzed in the BALF, IL-13 levels were not decreased (Fig 4g) when given the inhibitor, in spite of the known association of IL-13 and AHR (29). Alternative cell sources, not affected by ADAM10 inhibition, of IL-13 production (30) could potentially explain this finding. It is interesting to speculate that blockade of ADAM10 decreases IL-13 ability (29)

to act on epithelial cells in the lung. The decreased eosinophilia (Fig 3c) observed after ADAM10 inhibition can be explained by the strong inhibition of IL-5, the primary chemotactic factor in the recruitment of eosinophils, as well as by significant decreases in eotaxin and KC which are also involved in eosinophil recruitment (Fig 4g). Finally, IL-9 was also found to be decreased by the addition of the ADAM10 inhibitor. IL-9 is produced both by Th2 cells as well as the new subclass of T helper cells, Th9, and is known to enhance airway inflammation (31). Overall, the multiple changes help explain how the inhibition of ADAM10 suppresses IgE-dependent lung inflammation.

In order to confirm our findings in the BALF lung section from both the ADAM10$^{-/-}$ and inhibitor treated as well as their respective controls were stained with H&E. As is seen by the representative pictures in Fig 5(a, c) that when ADAM10 was blocked either genetically or with an inhibitor there was a decrease in inflammation compared to their controls (Fig 5b, d), including the infiltration of eosinophils. These results thus show that ADAM10 is important not only in the recruitment to the BALF of eosinophils but also to the lung tissue.

When the ADAM10 inhibitor administration was repeated in BALB/c mice using model A, similar results were seen expect the eosinophilia decrease in the BALF was not as drastic (60% to 23% in the Balb/c compared to 50% to 5% in the C57B/6 after inhibitor treatment). Finally, when the ADAM10 inhibitor was administered every three days during the induction of model B (IgE independent), no effect was seen. Overall these results show that regardless of the mouse background, the administration of an ADAM10 inhibitor is able to block the induction of experimental asthma.

Discussion

Although, it has previously been shown that if the cleavage of CD23 is blocked with a stabilizing antibody or if CD23 is overexpressed that the signs of lung inflammation were decreased (10), it was not known what role ADAM10 played in this or how CD23 was able to control lung inflammation. Here in we showed that overexpression of CD23 or blocking ADAM10 decreases the Th2 response as well as the other signs of lung inflammation, including eosinophilia, AHR and IgE production.

The mechanism of the reduced signs of experimental asthma after ADAM10 inhibition is currently under investigation. Based on the findings reported we propose two possibilities. First, as we found that administration of the inhibitor blocked IgE production (Fig 3e), this decrease in IgE would be reflected in decreased mediators released from mast cells after antigen challenge, thus resulting in decreased signs of experimental asthma, including AHR. One of the reasons for decreased IgE production is reduced cleavage of CD23, as we have previously shown that inhibitors of ADAM10 (15) and loss of ADAM10 (14) reduce CD23 cleavage both *in vitro* and *in vivo* respectively. Additionally, using a hu-PBL model in SCID mice, the inhibition of CD23 cleaveage was previously shown to correlate with decreased IgE synthesis (32). Secondly, the stimulatory activity of IgE complexes that bind to CD23 and enhance antigen presentation has been well documented by the Heyman laboratory (reviewed in (33)). In a recent paper we showed that ADAM10 is not only the sheddase of CD23, but also sorts CD23 into exosomes (13). Once released from the cell, the CD23 containing exosomes could bind IgE complexes and cause increased antigen presentation and T cell responses. The ADAM10 B cell conditional knockouts do not have these CD23 containing exosomes (13), and the lack of these CD23 containing exosomes could possibly explain part of the inhibition of the Th1, as well as the Th2, responses. Such exosomes, containing bound IgE complexes, would be anticipated to enhance dendritic cell activation of T cells.

Overall, the combination of using B cell ADAM10 knockouts as well as hydroxamate inhibitors of ADAM10, clearly indicates an important role for ADAM10, and CD23, in Th2-induced asthmatic disease, and suggests that hydroxamate inhibitors of ADAM10, directly administered to the airway, may have utility to modulate this disease.

Acknowledgments

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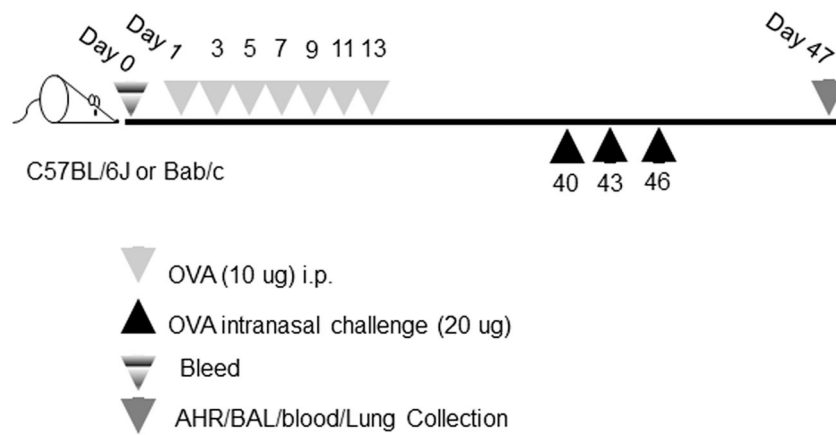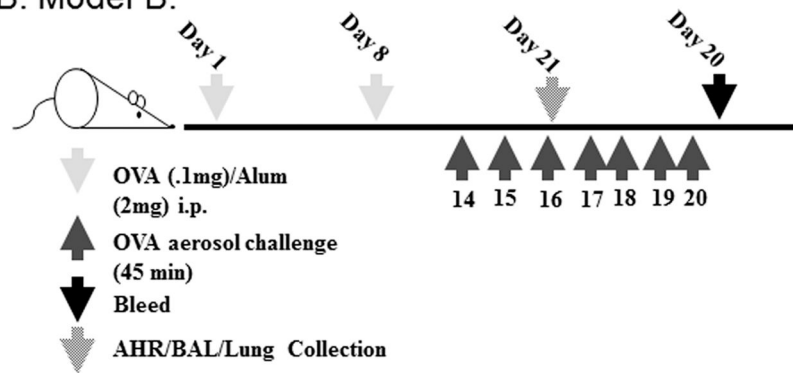
Figure 1. Models of asthma used
(A) Mast cell/IgE dependent model. This model (18) was used in Figures 2(a, c), 3 and 4 and 5. Called Model A in text. (B) Mast cell/IgE independent model. Called Model B in text.
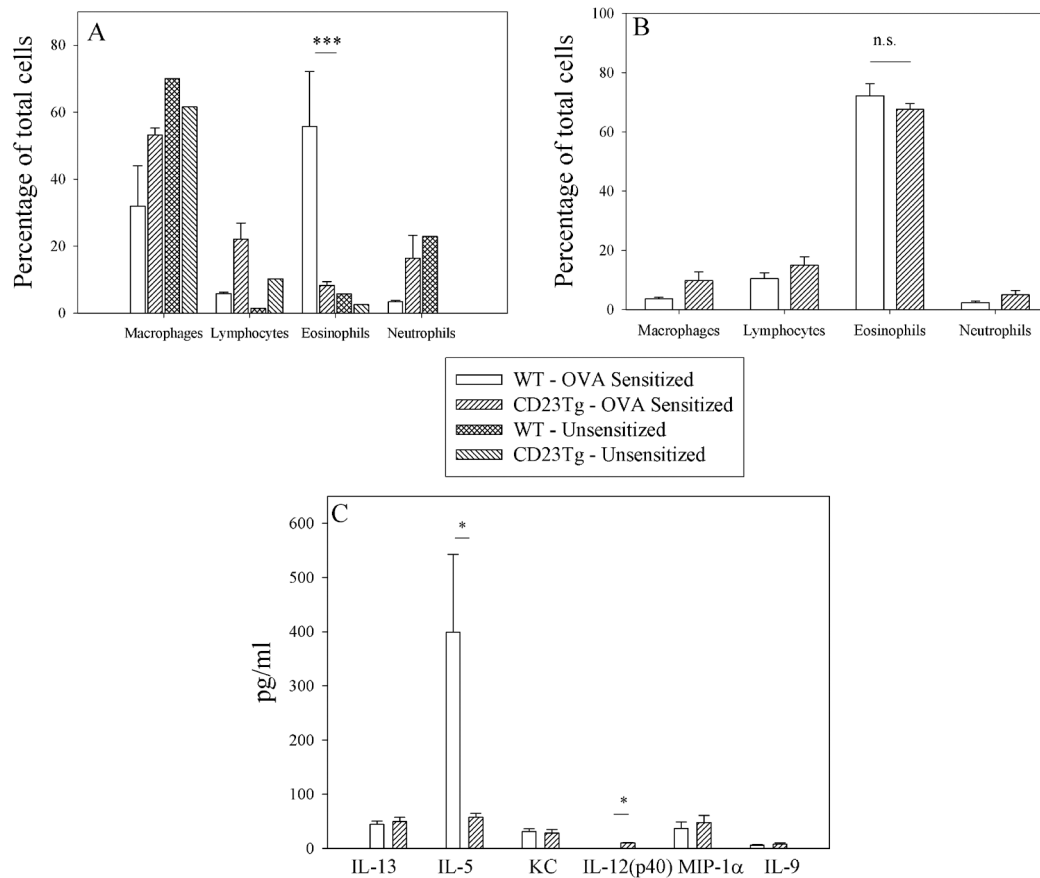
Figure 2. Overexpression of CD23 in CD23Tg can control eosinophilia in an IgE dependent model but not an IgE independent model

(A) Asthma was induced in Balb/c LM and CD23Tg using model A. BALF were collected and the percentages of cells were determined as explained in the Material and Methods by counting at least 300 cells. Shown is the average of four mice. (B) Asthma was induced in Balb/c LM and CD23Tg using model B. The percentage of cells was determined as in A. Shown in the average of at least 4 mice per group. (C) The cytokines in the BALF of the mice used in A were determined using multiplex kits according to the manufactures' instructions. * = P value <.05 and ** = P value <.01
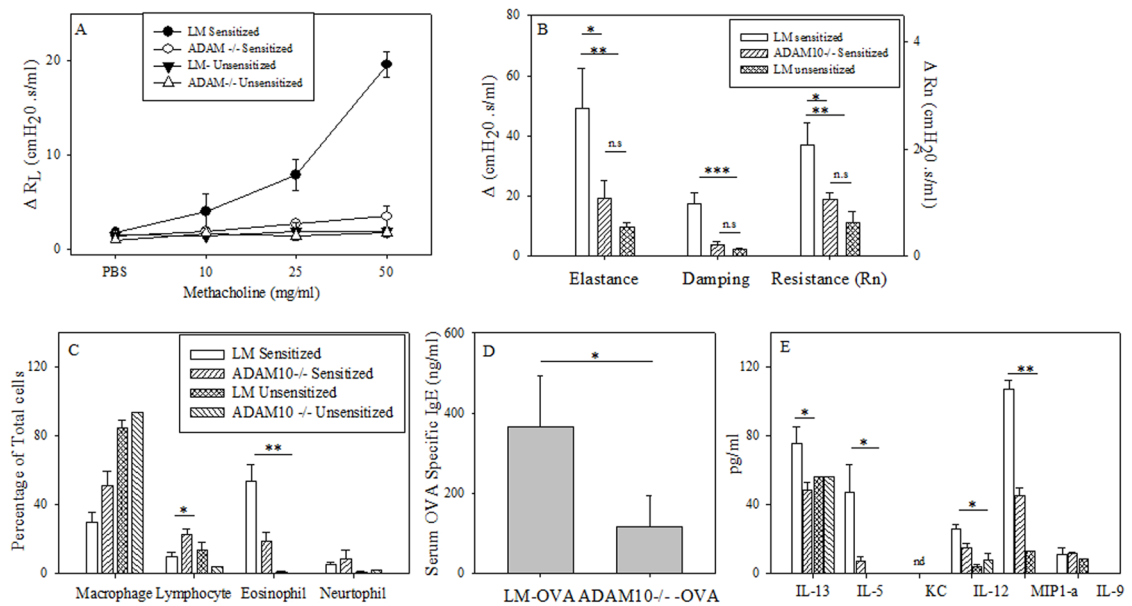
Figure 3. ADAM10$^{-/-}$ decreases the severity of disease in a mast cell dependent model
(A) Mice were sensitized and challenged using model A (mice are on a C57B/6 background). On day 47 lung function was assessed by measuring total lung resistance as explained in the Material and Methods; shown is the average of at least 4 mice per treated group. Significance is between ADAM10 $^{-/-}$ and LM. (B) Rn (Newton Resistance), G (Tissue Dampening) and H (Elastance) were determined at baseline (PBS) and after the addition of 50 mg/ml of methacholine, with the average of the three maximum points at each dose being used for each mouse. Shown is the average of at least 4 mice per group. (C) Cells from the BALF were counted and stained as explained in the materials and methods. Percentage of cells was then determined in the BALF by counting of at least one hundred cells and numbers presented is the average of at least four mice. (D) The levels of OVA specific IgE in the serum were determined by ELISA, shown is the average of at least five animals. (E) Cytokine levels in the BALF was determined by multiplex analysis, shown is the average of at least four animals (same key as in C). * = P value <.05, ** = P value <.01 and *** = P value < .001
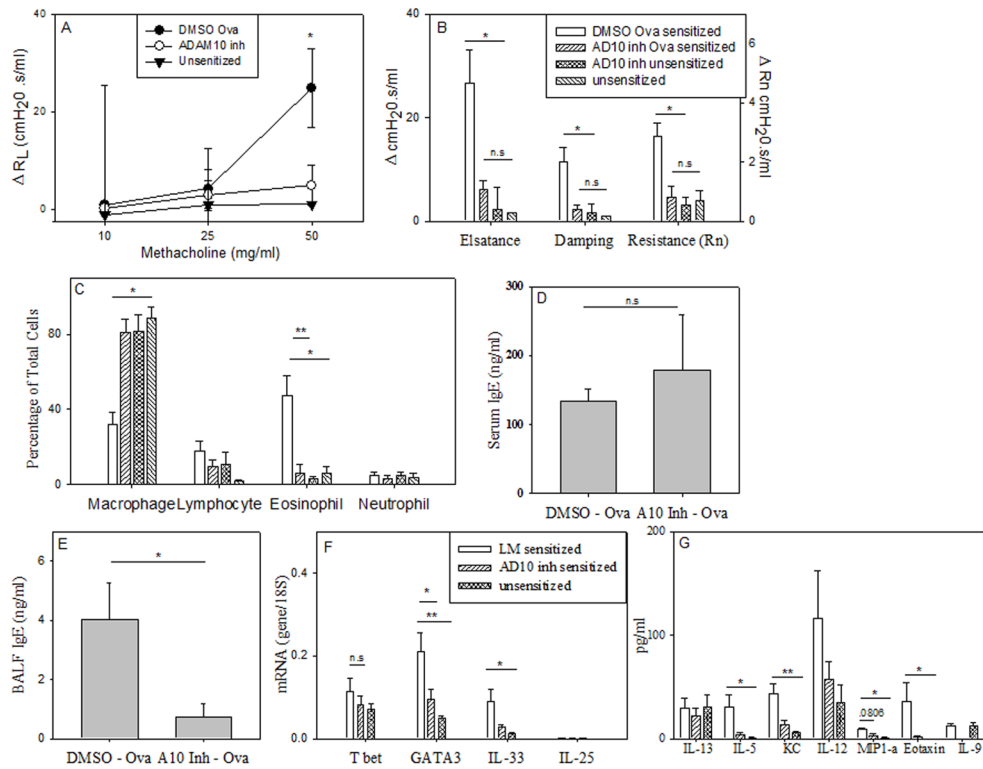
Figure 4. ADAM10 inhibitor blocks the induction of an IgE/mast cell dependent model potentially through blocking the Th2 response

Asthma was induced using the IgE-dependent model (Model A). 1 mg/kg in a 20:1 mixture of PBS/DMSO of an ADAM10 inhibitor was injected intranasal every three days throughout the experiment in wild type C57B/6. The control mice (wild type C57B/6) received just the PBS/DMSO mixture. (A) On day 47 AHR was measured using total lung resistance (Significance is between ADAM10 inhibitor treated and LM) and (B) forced oscillation measurements as explained in Fig 3b. Data represents the average of data from at least four sensitized mice and two unsensitized mice. (C) Percentage of BALF cells were determined as in Fig 2a. Shown is the average of at least three animals (same key as B). (D) Total IgE in the serum as determined by ELISA, average of at least four animals. (E) Total IgE in the BALF as determined by ELISA, average of at least three animals. (F) qPCR of total lung RNA was performed using the primers listed in material and methods. Shown is the average of at least five animals. The unsensitized groups were combined, as no difference where seen between them. (G) BALF cytokine levels as determined by multiplex ELISA. Shown is the average of at least five animals per group. Again unsensitized groups were combined. (same key as F). Number above comparison line equals P value, * = P value < .05 and ** = P value <.01
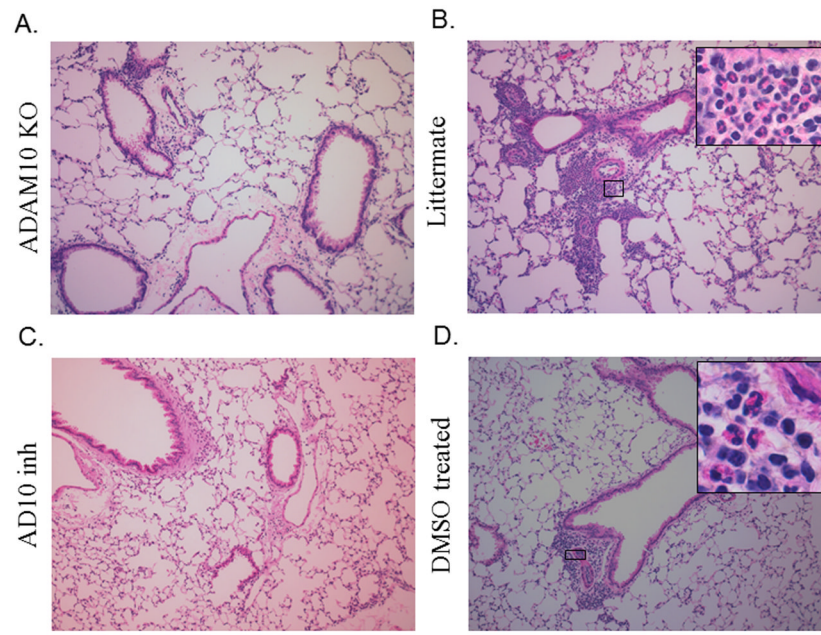
Figure 5. ADAM10 inhibition also blocks the inflammatory response in the lung tissue
The lungs from mice where asthma had been induced using Model A, where fixed and sectioned as explained in the Material and Methods. The sections were then de-paraffinized and stained with H&E. Larger photomicrographs are 10x with select inserts from areas showing obvious inflammation at 100x. (A) Photomicrographs using lungs from ADAM10 $^{-/-}$ mice or (B) their littermate controls. (C) Photomicrographs using lungs from C57B/6 that received INCB008765 throughout the induction of asthma or (D) DMSO control lungs. Photos shown are representative from experiments using 4 mice per group.

Table 1
Liver Enzyme analysis indicates lack of toxicity for ADAM10 inhibitors

Just prior to measurement of AHR the mice where weighed. Shown is the average of at least 8 mice per group. The serum isolated after sacrifice of three mice of similar treatment were pooled together and then AST and ALT levels were measured in the serum as explained in the Material and Methods. Shown is the average of at least two different pools.

Parameter	DMSO – OVA Sensitized	AD10 inh – OVA Sensitized
Weight (time of sacrifice)	20.48889 ± 0.503721	20.42727 ± 0.282872
AST (aspartate aminotransferase)	13.666 ± 6.81	15.558 ± 6.87
ALT (alanine aminotransferase)	2.589382 ± 0.931133	3.636805 ± 0.668527