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 with littermate (LM) 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 possibly other IgE-dependent diseases.

Article:

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 effects 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, CD23 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 IgE-independent mouse models [10]. One study [10] additionally examined CD23 transgenics with respect to experimental asthma induction and found decreased eosinophils in the bronchoaveolar lavage fluid (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 study, 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 ovalbumin (OVA). The mechanism is potentially because of a reduction in IgE and/or in the Th2 response.

Material and methods

Reagents

Chicken OVA and Imject alum adjuvant were purchased from Sigma (St. Louis, MO, USA) and Pierce (Rockford, IL, USA), respectively. Cytokines were measured using multiplex kits from Biorad (Hercules, CA, USA) 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 generations 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, USA) and were used in the inhibitor studies. Female mice aged 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 Fig. 1. Model A was developed by Williams and Galli [18] and was shown to be mast cell as well as IgE dependent. For Figs 2 and 3 this model was slightly modified mice were only injected four times with 50 μg OVA, then they were challenged starting on day 22. Galli has shown this modified model is also IgE/mast cell dependent (private communication). 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).
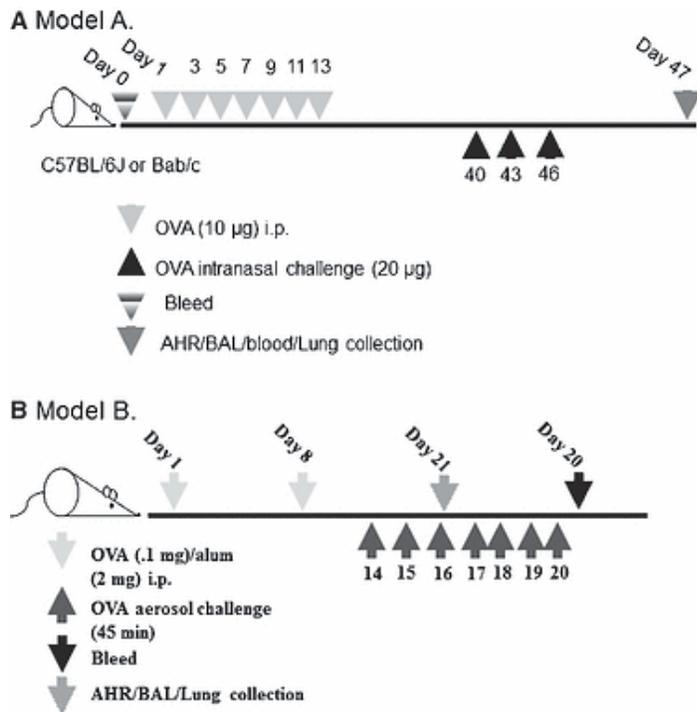
Figure 1. Models of asthma used. (A) Mast cell/IgE-dependent model. This model [18] was used normally in Figs 4 and 5C,D or with modification as explained in Materials and methods in Figs 2A,C and 3. Called Model A in text. (B) Mast cell/IgE-independent model. Called Model B in text.
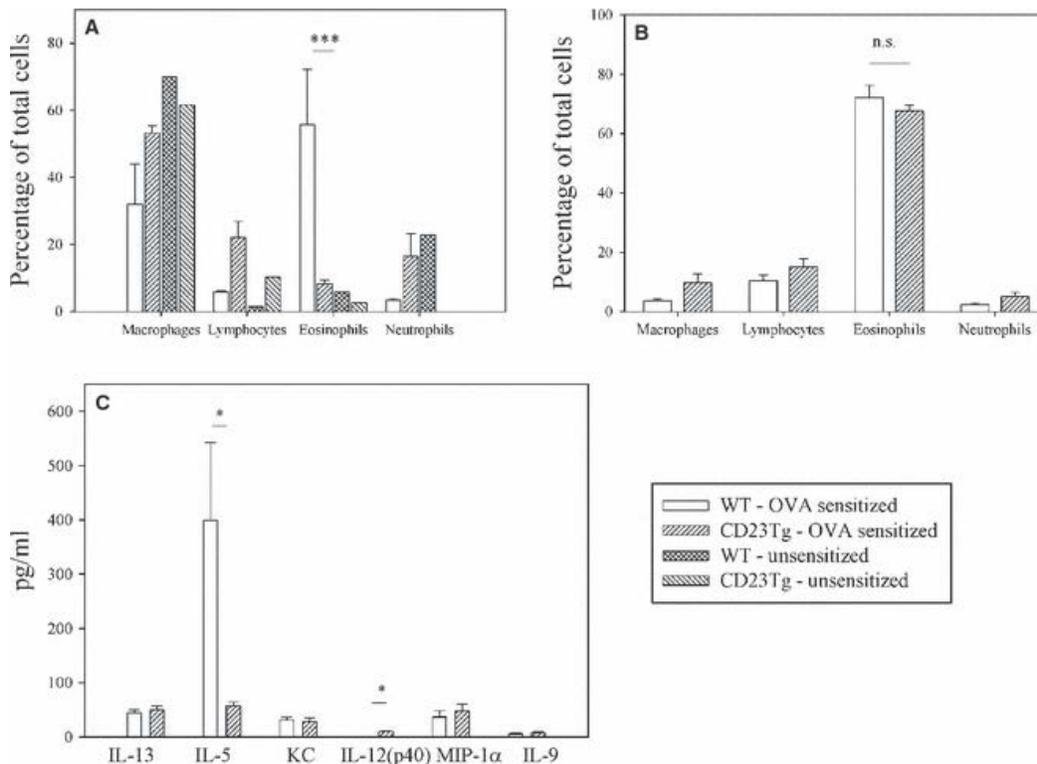
Figure 2. Overexpression of CD23 in CD23Tg can control eosinophilia in an IgE-dependent model but not an IgE-independent model. (A) Asthma was induced in Balb/c littermates (LM) and CD23Tg using model A. Bronchoaveolar lavage fluid (BALF) were collected and the percentages of cells were determined as explained in the Material and methods by counting at least 100 cells. Shown is the average of four mice. (B) Asthma was induced in Balb/c LM and CD23Tg using model B. The percentages of cells were determined as in A. Shown in the average of at least four 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 < 0.05 and **P value < 0.001.
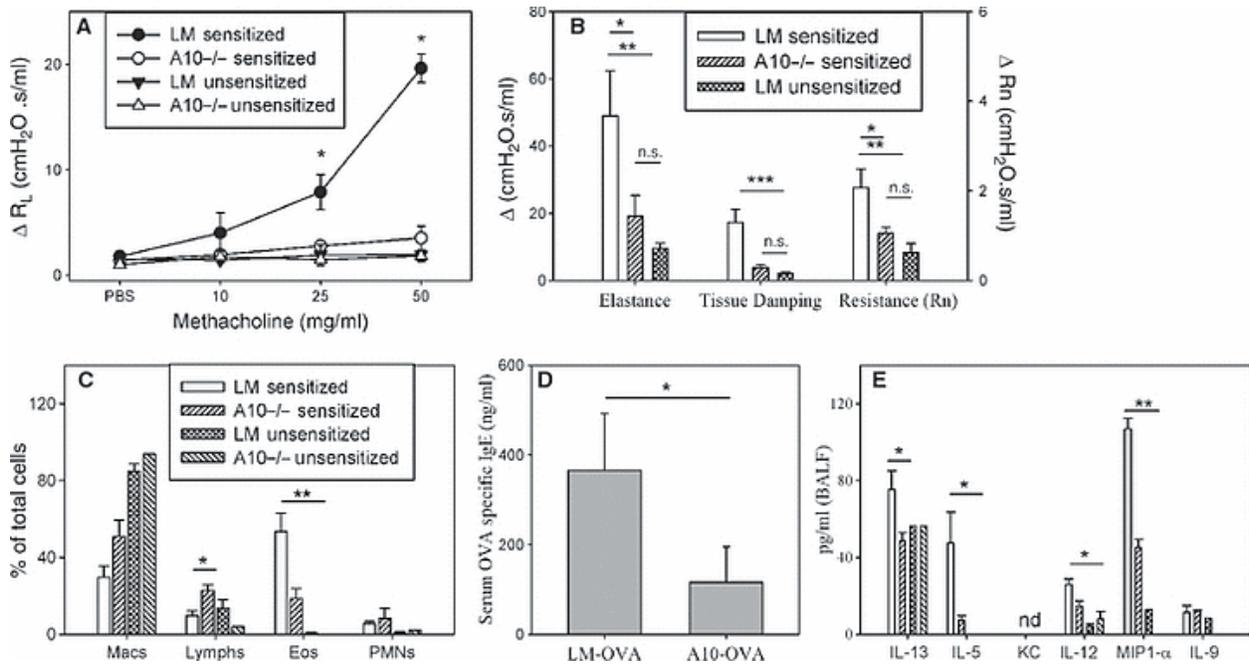
Figure 3. Conditional removal of ADAM10 decreases the severity of disease in a IgE/mast cell–dependent model. (A) Mice were sensitized and challenged using model A (mice are on a C57B/6 background). On day 29, lung function was assessed by measuring total lung resistance as explained in the Material and methods; shown is the average of at least 4 mice per treated group. Significance is between ADAM10$^{-/-}$ and littermate. (B) Rn (Newton Resistance), Tissue Dampening and 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 bronchoaveolar lavage fluid (BALF) were counted and stained as explained in the Material and methods. Percentages of cells were then determined in the BALF by counting of at least one hundred cells, and numbers presented are 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 <0.05, **P value < 0.01 and ***P value < 0.001.

FlexiVent

In some experiments, 18 h after the final OVA challenge, airway resistance was measured using the Flexivent system (Scireq, Montreal, QC, 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, and tissue elastance 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, USA). Percentages were determined by counting of at least 100 leukocyte cells per cytospin. Additionally, lung and heart tissue were fixed in 10% formalin (Fisher) and embedded in paraffin by the VCU pathology core. Five micrometer 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, USA) 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 Fig. 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 with LM controls (data not shown). Overall, these data show that increasing CD23 by inhibiting ADAM10 on B cells has the potential for strongly inhibiting the IgE-dependent lung inflammatory response, but not the IgE-independent lung inflammatory response by reducing IgE levels.

ADAM10 inhibition blocks the induction of experimental asthma

Given the aforesaid finding that mice lacking B-cell ADAM10 have minimal pathology in IgE-dependent lung inflammation, we sought to determine whether 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 3 days throughout the experiment. As a control, the ADAM10 inhibitors were also administered to unsensitized mice and found to have no effect (Fig. 4B,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 because of 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 approximately 50% to 20% in the ADAM10$^{-/-}$ (Fig. 3C) and from approximately 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 similar amounts of macrophages in the BALF (data not shown) after inhibitor treatment. To determine whether 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 DMSO treated mice. 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.
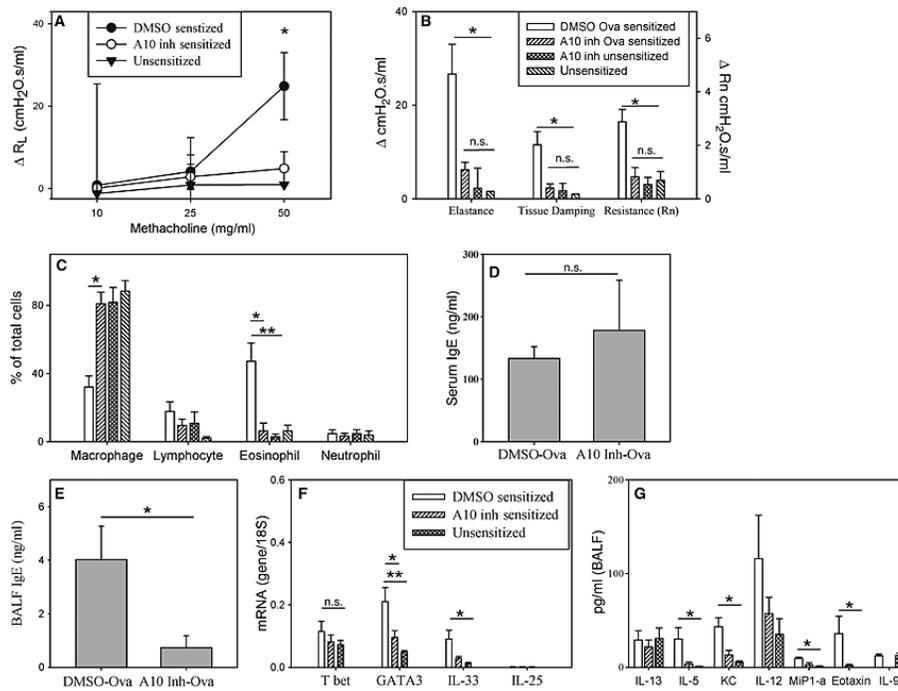
Figure 4. ADAM10 inhibitor blocks the induction of an IgE/mast cell–dependent model potentially through blocking the Th2 response. Asthma was induced using the IgE-dependent model (Model A). 1 mg/kg in a 20:1 mixture of PBS/DMSO of an ADAM10 inhibitor (called A10 Inh in figure) was injected intranasally every 3 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, airway hypersensitivity was measured using total lung resistance (significance is between ADAM10 inhibitor and DMSO treated) and (B) forced oscillation measurements as explained in Fig. 3B. Data represent the average of data from at least four sensitized mice and two unsensitized mice. (C) Percentages of bronchoaveolar lavage fluid (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) qRT-PCR 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 were 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 < 0.05 and **Pvalue < 0.01.

Table 1. Liver enzyme analysis indicates lack of toxicity for ADAM10 inhibitors

Parameter	DMSO – ovalbumin (OVA) sensitized	ADAM10 inh – OVA sensitized
Weight (time of sacrifice)	20.49 ± 0.50	20.43 ± 0.28
Aspartate aminotransferase (AST)	13.666 ± 6.81	15.558 ± 6.87
Alanine aminotransferase (ALT)	2.59 ± 0.93	3.64 ± 0.67

Just prior to measurement of airway hypersensitivity, the mice were weighed. Shown is the average of at least 8 mice per group. The serum isolated after the killing 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.

To test whether ADAM10 inhibitors had further effects beyond that of inhibiting IgE, we isolated total lung mRNA from treated and control mice, and using qRT-PCR, we 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 qRT-PCR. 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. 4C) 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 and by 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 sections from both the ADAM10$^{-/-}$ and inhibitor treated and their respective controls were stained with H&E. As is seen by the representative pictures in Fig. 5A,C that when ADAM10 was blocked either genetically or with an inhibitor, there was a decrease in inflammation compared with 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.
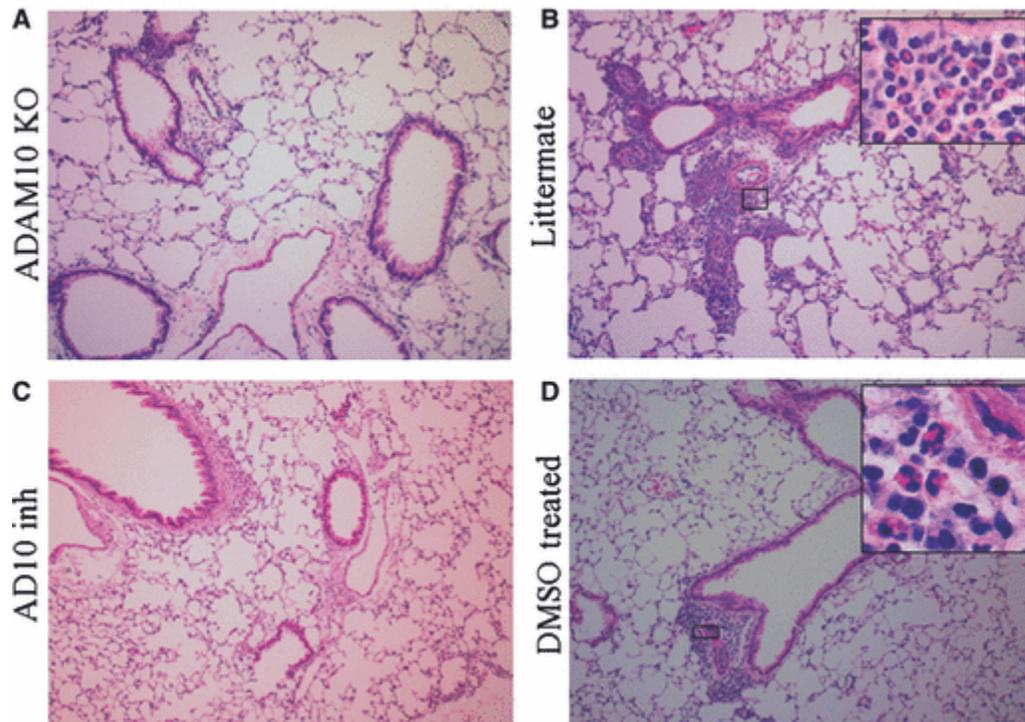
Figure 5. ADAM10 inhibition also blocks the inflammatory response in the lung tissue. The lungs from mice where asthma had been induced using Model A, were fixed and sectioned as explained in the Material and methods. The sections were then de-paraffinized and stained with

H&E. Larger photomicrographs are 10× 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 INC008765 throughout the induction of asthma or (D) DMSO control lungs. Photographs shown are representative from experiments using 4 mice per group.

When the ADAM10 inhibitor administration was repeated in BALB/c mice using model A, similar results were seen except the eosinophilia decrease in the BALF was not as drastic (60–23% in the Balb/c compared to 50–5% in the C57B/6 after inhibitor treatment). Finally, when the ADAM10 inhibitor was administered every 3 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. Herein, 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 cleavage was previously shown to correlate with decreased IgE synthesis [32]. Second, the stimulatory activity of IgE complexes that bind with CD23 and enhance antigen presentation has been well documented by the Heyman laboratory (reviewed in 33). In a recent study, 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.

Author contributions

JM contributed to each of the figures, JF contributed to part of Fig. 1, and SN contributed to the interpretation and understanding of the AHR. DG created the ADAM10$^{-/-}$ mice, AF and AK contributed to the creation of the asthma models, and finally DC gave guidance for the studies in his laboratory. AD and CK measured the liver enzymes in the serum. HM gave guidance on interpretation of inflammation in the lungs.

Conflict of interest

The authors have no conflicting financial interests.

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