

Soluble ADAM33 initiates airway remodeling to promote susceptibility for allergic asthma in early life

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Abstract

Asthma is a chronic inflammatory airways disease that usually begins in early life and involves gene-environment interactions. Although most asthma exhibits allergic inflammation, many allergic individuals do not have asthma. Here, we report how the asthma gene *A Disintegrin and Metalloprotease (ADAM)33*, acts as local tissue susceptibility gene that promotes allergic asthma. We show that enzymatically active soluble (s)ADAM33 is increased in asthmatic airways and plays a role in airway remodeling, independent of inflammation. Furthermore, remodeling and inflammation are both suppressed in *Adam33* null mice after allergen challenge. When induced in utero or added ex vivo, sADAM33 causes structural remodeling of the airways, which enhances post-natal airway eosinophilia and bronchial hyperresponsiveness following sub-threshold challenge with an aeroallergen. This substantial gene-environment interaction helps to explain the end-organ expression of allergic asthma in genetically susceptible individuals. Finally, we show that sADAM33-induced airway remodeling is reversible, highlighting the therapeutic potential of targeting ADAM33 in asthma.

Introduction

Even though our insight in the pathophysiology of asthma has increased, it still remains a substantial health and economic burden with 300 million patients suffering from asthma worldwide. It is a complex disease involving gene-environment interactions and most individuals with asthma exhibit allergen-induced T_H2-type inflammatory responses associated with increased expression of cytokines from the IL4 gene cluster linked to atopy (1). Asthma is also characterized by structural remodeling and thickening of the inner airway wall involving an increase in bronchial smooth muscle, collagen deposition and neo-angiogenesis. The classical paradigm of allergic asthma (2) suggests that T_H2-type driven airway inflammation plays a causal role in airway remodeling (i.e. inflammation precedes remodeling) (3) to result in the typical asthmatic phenotype (4-6). However, the early use of inhaled corticosteroids for wheezing in preschool children does not affect the natural history of asthma or wheeze later in childhood (7). Similarly, although treatment of mild-to-moderate asthmatic children with anti-inflammatory drugs provides asthma control and reduces airway responsiveness to methacholine, this fails to improve lung function when compared with placebo (8). Since airway remodeling (9) and bronchial smooth muscle (10) are increased in the airways of young children who subsequently progress to develop asthma, these studies suggest distinct mechanisms that affect remodeling early in the disease course, but the causal factors are unknown (11).

While the genetic susceptibility to atopy alone cannot explain asthma (12), the functional contribution of other asthma susceptibility genes to disease

pathogenesis is poorly understood (13). *A Disintegrin and Metalloprotease (ADAM) 33* is a susceptibility gene associated with asthma and bronchial hyper-responsiveness (BHR) (14, 15), findings that have been replicated in multiple populations (16). *ADAM33* alleles are also associated with an increased risk for COPD (17), accelerated lung function decline across the life course (18, 19) and impaired lung function early in life (20) suggesting a role in airway remodeling. *ADAM33* is mainly expressed in airway smooth muscle, fibroblasts and mesenchymal progenitor cells (21, 22), with each playing a role in asthma-related airway remodeling (23, 24). Although it is a membrane-anchored protein, *ADAM33* can be detected as a soluble protein (s*ADAM33*) in asthmatic airways where high levels of s*ADAM33* are correlated with reduced lung function (25). Little is understood about the contribution of s*ADAM33* to the pathogenesis of asthma, although it is known that s*ADAM33* protein is induced *in utero* by maternal allergy (26) and the enzymatically active recombinant protein is pro-angiogenic (27). Herein, we provide new insight into how the disease-related s*ADAM33* protein promotes airway remodeling without inflammation; we also demonstrate a significant gene-environment interaction in which s*ADAM33*-induced airway remodeling in early life affects the susceptibility of the airway tissue to environmental allergens to promote allergic airway inflammation and BHR. Finally, we show that *ADAM33*-driven airway remodeling is reversible highlighting the potential for s*ADAM33* as a target for disease modifying therapy.

Results

Soluble ADAM33 is increased in asthma. Since ADAM33 contains a metalloprotease (MP) domain, we tested if sADAM33 in BALF is enzymatically active. Consistent with a previous report (25), immunoreactive sADAM33 was detected in BALF using an antibody against the metalloprotease (MP) domain, with a strong band at ~25kDa (MP domain), another at ~52kDa (unprocessed MP domain, ie. Pro-MP) and other minor bands of higher molecular weight (ectodomain fragments containing the MP domain) (**Figure 1A**). The 52kDa band was confirmed as the unprocessed Pro-MP domains using an antibody against the Pro domain (**Supplemental Figure 1A**). The bands were similar to those previously characterized in BALF from sarcoid patients (28) and were significantly increased in asthma (**Figure 1B** and **Supplemental Figure 1B**). Neither ADAM33 antibody cross-reacted with recombinant ADAM8 and ADAM12 (**Supplementary Figure 2A,B,C**), which have been associated with asthma (29-31) and show high homology with ADAM33. Furthermore, using an ADAM33 specific FRET peptide cleavage assay, ADAM33 enzymatic activity was increased in asthma (**Figure 1C**). The presence of active sADAM33 in BALF was independent of corticosteroid treatment or the airway inflammatory cell profile (**Supplemental Table 1**). We also analyzed BALF from inbred mice after sensitization and challenge with house dust mite (HDM) extract allergen (**Supplemental Figure 3**), which induces features of asthma in murine lungs. Western blotting using an antibody to murine ADAM33 antibody (26) revealed distinct protein bands similar to the human ADAM33 at ~52 - 76kDa (**Figure 1D** and **Figure 1E**), smaller than the processed form of full-length mouse ADAM33

(~110kDa) and consistent with its proteolytic cleavage from its membrane-bound form to release sADAM33 ectodomain into the airways (32). Similar to the findings in human BALF, enzymatic activity of murine sADAM33 was significantly increased in the BALF of lungs from HDM-challenged mice when compared to the equivalent control mice (**Figure 1F**).

Soluble ADAM33 causes airway remodeling. To assess directly the function of sADAM33 *in vivo*, a doxycycline (Dox)-inducible human sADAM33 transgenic mouse model was generated by injecting a linearized *TRES-human-ADAM33-SS-PRO-MP-3Flag* construct (**Supplemental Figure 4A-H**) into FVB/N mouse pro-nuclei. By crossing the founder mice with *Ccsp-rtTA*(line 2)(33) mice, we obtained expression of human sADAM33 mRNA (**Figure 2A**) in the lungs of Dox fed double-transgenic (DTg) (*Ccsp/ADAM33*) mice but not in single-transgenic (STg) litter-mate controls. Epithelial expression of human sADAM33 protein in Dox-induced DTg *Ccsp/ADAM33* mice was confirmed by immunofluorescence staining (**Figure 2B,C**) and enzymatically-active sADAM33 was demonstrated in BALF (**Figure 2D,E**).

When 6–8 week old mice were fed a diet containing Dox for 4 or 8 weeks to induce transgene expression of human sADAM33 (**Supplemental Figure 5A**), there were no significant changes in the airways' expression of inflammatory (*Ccl11* (also known as *Eotaxin*), *Interleukin (Il)5*, *Il13*, *Cxcl1* (also known as *Kc*)) or goblet cell (*Muc5ac*) markers (**Figure 2F-J**) and no evidence of inflammatory cells in the BALF of these mice (**Supplemental Figure 5B**). In contrast, *alpha-smooth muscle actin* (*Acta2*; also known as *α -Sma*) mRNA and other genes

linked to airway remodeling, including *Collagen 1* and *3* (*Col1a1* and *Col3a1*), *Fibronectin* (*Fn1*) and *platelet/endothelial cell adhesion molecule 1* (*Pecam1*; also known as *CD31*) were all increased in whole lungs of DTg *Ccsp/ADAM33* mice compared with STg litter-mate controls (**Figure 2K-O**). Immunofluorescence staining for ACTA2 and PECAM1 in DTg *Ccsp/ADAM33* mice revealed increased smooth muscle surrounding the airways and airway vessels (**Figure 2Q and S**) compared to STg litter-mate controls (**Figure 2P and R**). Together these data support pro-myogenic and pro-angiogenic functions (27) for sADAM33. However, these remodeling changes failed to elicit BHR in response to inhaled methacholine (**Supplemental Figure 5C**).

Soluble ADAM33 causes airway 'premodeling' in developing lung. Since sADAM33 protein can be induced *in utero* by maternal allergy (26), we also determined the effect of inducing human sADAM33 during lung development by feeding transgenic mice a Dox-diet during pregnancy and for up to 4 weeks after birth (**Supplemental Figure 5D**). This caused sADAM33 to be expressed in the fetal lungs from around embryonic day (ED) 14/15 (corresponding to the time when the CCSP promoter becomes active (34)), until the mice were euthanized for analysis. From as early as ED17.5, the airway remodeling genes *Acta2*, *Col1a*, *Col3a1*, *Fn1*, *Pecam1* were all increased in whole lungs of DTg *Ccsp/ADAM33* mice compared with STg litter-mate controls (**Figure 3A-E**) while the inflammatory markers *Ccl11*, *Il5*, *Il13*, *Cxcl1* and goblet cell marker *Muc5ac* were not affected (**Figure 3F-J**), as was the case in the adult transgenic mice (**Figure 2F-O**). Immunofluorescence histochemistry in lungs of 4 week old

Ccsp/ADAM33 mice confirmed significant induction of airway remodeling involving increased airway and vascular smooth muscle compared to control mice (**Figure 3K,L,M,N**). As observed with the adult transgenic mice, these remodeling changes failed to elicit inflammation (**Supplemental Figure 5E**) or BHR in response to inhaled methacholine (**Supplemental Figure 5F**).

Importantly, we also found that exposure of human embryonic lung explants to highly purified enzymatically active recombinant sADAM33-Pro-MP *in vitro* caused increased bronchial smooth muscle in developing airways; this effect was dependent on the catalytic activity of the enzyme (**Figure 3P,R**) since mutation of the active site (E346A) was inactive and failed to reproduce this effect (**Figure 3O,Q**).

Allergen-induced asthma-like changes are inhibited in Adam33^{-/-} mice. To determine whether ADAM33 is required for allergen-induced airway remodeling, we evaluated *Adam33* null (*Adam33^{-/-}*) mice (**Supplemental Figure 6**) after intra-peritoneal sensitization and intra-tracheal challenge with HDM allergen (**Supplemental Figure 3**). Although a previous report had failed to observe changes in ovalbumin-induced airway responses in *Adam33^{-/-}* mice (35), we investigated effects of HDM extract as a common human aeroallergen to model more closely human asthma. In wild-type (WT) mice, HDM challenge caused an increase in sADAM33 enzyme activity in BALF, whereas there was no significant effect in *Adam33^{-/-}* mice (**Supplemental Figure 7A**), confirming the specificity of the ADAM33 FRET assay. Similarly, after HDM challenge, *Acta2*, *Col1a1*, *Col3a1* and *Fn1* mRNAs were significantly increased in whole lungs of WT mice (**Figure**

4A-D), but these were suppressed in the *Adam33*-deleted mice in a gene dosage-dependent fashion. Compared to saline treated control mice (**Figure 4E,I,G,K**), the HDM-induced increase in smooth muscle around the airways and vessels of WT mice (**Figure 4F and H**) was suppressed in the lungs of HDM challenged *Adam33*^{-/-} mice (**Figure 4J and L**) and paralleled by reduced methacholine-induced BHR (**Figure 5 and Supplemental Figure 7B**). Of note, mRNAs encoding the T_H2-type inflammatory genes, *Ccl11*, *Il5* and *Il13*, but not *Cxcl1*, were also suppressed in the HDM-challenged *Adam33*^{-/-} mice (**Figure 6A-D**) and were associated with a decrease in CCL11, IL-5 (**Figure 6E and F**), and eosinophils (**Figure 6G**) in BALF. These data suggest a previously unappreciated role for sADAM33 in regulating T_H2-type inflammation.

Early life induction of sADAM33 enhances allergen-induced asthma-like changes. To test the hypothesis that sADAM33 enhances asthma-like responses in the presence of a T_H2 stimulus, we treated *Ccsp/ADAM33* and STg litter-mate control mice with recombinant IL13 (**Supplemental Figure 8A**) and found a significantly increase in mRNA expression of the fibroblast-derived chemokine, *Ccl11* in the sADAM33-expressing mice (**Figure 7A**). This amplification effect led us to consider the potential for early-life interactions between sADAM33 and T_H2-type inflammation in response to low concentrations of allergen. We first performed a concentration-response experiment with HDM extract with a standard sensitization protocol (**Supplemental Figure 3**) to determine the amount of HDM extract (6.25µg), which elicited minimal BHR, eosinophilia, *Ccl11* and *Muc5ac* mRNA (**Supplemental Figure 8B-E**) in WT mice. This low-dose

allergen challenge protocol was then applied to DTg *Ccsp/ADAM33* and STg litter-mate control mice in which transgene expression was induced during lung development and early life (**Supplemental Figure 9A**). In response to HDM challenge, expression of genes mediating airway remodeling, *Acta2*, *Col1a*, *Col3a1*, *Fn1*, *Pecam1* (**Figure 7B-F**), T_H2 inflammatory cytokines, *Ccl11*, *Il5*, *Il13* (**Figure 7G-I**) and mucus production *Muc5ac* (**Figure 7K**), but not *Cxcl1* (**Figure 7J**) were increased in DTg *Ccsp/ADAM33* mice compared to equivalently treated STg litter-mate controls. Enhanced expression of inflammation and remodeling genes in the *Ccsp/ADAM33* mice was accompanied by increased airway resistance as measured by BHR in response to methacholine (**Figure 7L** and **Supplemental Figure 9B and C**) and accompanied with BALF eosinophilia (**Figure 7M**).

Airway remodeling induced by soluble ADAM33 is reversible. As a susceptibility gene close to the origin of asthma, ADAM33 is a potential therapeutic target. However, as airway remodeling is considered to be a relatively irreversible process, therapeutic intervention depends on demonstration that sADAM33 induced airway remodeling can be reversed after sADAM33 expression is blocked. This concept was tested using the DTg *Ccsp/ADAM33* transgenic mice in which sADAM33 expression can be regulated by provision, or removal, of Dox from the diet. In these experiments, sADAM33 expression was commenced in utero and continued for 28 days post-partum to promote a robust remodeling response; this was followed by 28 days without Dox feed to assess reversibility of the remodeling response (**Supplemental Figure 10A**). At day 56 when the

analyses were performed, ADAM33 expression in the lungs of DTg *Ccsp/ADAM33* mice was completely ablated as a result of removing the Dox diet (**Supplemental Figure 10B**). At the same time all airway remodeling genes *Acta2*, *Col1a*, *Col3a1*, *Fn1*, *Pecam1* had returned to baseline in whole lungs of DTg *Ccsp/ADAM33* mice and were similar to the levels in STg litter-mate controls (**Figure 8A-E**). Inflammatory markers *Ccl11*, *Il5*, *Il13*, *Cxcl1* and goblet cell marker *Muc5ac* were not affected by the presence or absence of sADAM33 expression (**Figure 8F-J**). Immunofluorescence histochemistry applied to lungs from 28 and 56 day old *Ccsp/ADAM33* mice that were maintained on Dox from in utero for the whole experiment confirmed significant induction of airway remodeling involving increased airway and vascular smooth muscle at both time points (**Figure 8L,N**) compared to control mice (**Figure 8K**). However the lungs of DTg *Ccsp/ADAM33* mice on Dox diet for 28 days and then without Dox for 28days showed a reduction in airway remodeling with decreased staining for airway and vascular smooth muscle (**Figure 8M**), similar to the STg litter-mate control mice (**Figure 8K**).

Discussion

In this report, we have defined the function of ADAM33 as a local tissue susceptibility gene for asthma and have uncovered a significant interaction between sADAM33-mediated airway remodeling and sensitivity to allergen exposure leading to allergic inflammation and BHR in early life. Ectopic expression of human sADAM33 in adult or fetal murine airways caused airway remodeling, which was completely reversible if expression of human sADAM33 was switched off. Almost identical structural changes (premodeling) were observed in human fetal lung in the presence of exogenous, enzymatically active sADAM33, but not the mutated, inactive enzyme. While on its own airway remodeling did not trigger inflammation or BHR in murine lungs, the remodeled lungs of mice expressing sADAM33 in utero were more susceptible to low amounts of HDM allergen exposure resulting in augmented BHR and eosinophilia in early post-natal life. Since sADAM33 enhances the effects of low amounts of allergen to produce asthma-like features, sADAM33 –induced airway remodeling could explain the difference in susceptibility of asthmatic individuals to environmental allergens, compared with atopic non-asthmatic subjects. This may be because sADAM33-induced airway remodeling results in increased numbers of fibroblast and smooth muscle cells that can produce mediators such as CCL11 (36, 37) which amplifies inflammation. Since sADAM33 is also pro-angiogenic (27), it may facilitate ingress of eosinophils or other inflammatory cells into the tissue in response to these chemoattractants.

The importance of ADAM33 for allergic airway responses was further highlighted using *Adam33*^{-/-} mice where HDM-induced airway inflammation, remodeling and

BHR were all significantly reduced in the absence of ADAM33. These data indicate an obligatory role for ADAM33 in the pathobiology of allergic airways disease via a mechanism involving loss of its membrane anchor to produce a dysregulated soluble protein (ie. a gain-of-function). Consistent with this, we found that sADAM33-Pro-MP protein and enzymatic activity was increased in human asthma and that levels were not affected by corticosteroid treatment.

The ability of sADAM33 to promote airway remodeling is consistent with the association of *ADAM33* polymorphism with BHR (14) and reduced lung function (18-20). Furthermore, our observation that levels of sADAM33 in human asthma are unaffected by corticosteroids would help explain the inability of corticosteroid treatment to affect lung function in young children (8) or the natural history of the disease (7). Present findings challenge the classical paradigm of allergic asthma in which T_H2 driven airway inflammation has primacy over airway remodeling to result in the typical asthmatic phenotype (2) (4-6). Both inflammation and remodeling seem to be of importance and mutually cooperative at different stages of the life-course. Since sADAM33 is induced *in utero* by maternal allergy (26), this may explain why airway remodeling (9) and bronchial smooth muscle (10) are increased in the airways of young children who subsequently develop asthma. These remodeled airways may provide the 'soil' that supports exaggerated responses to allergens in the airways of susceptible individuals leading to T_H2 -type inflammation and BHR that characterizes asthma. Such a paradigm might explain the association of ADAM33 polymorphism with progression of preschool wheeze into childhood asthma (38) (**Figure 9**).

Individual approaches used in the current study have their inherent limitations. While specific expression of human sADAM33 might have been preferred in pulmonary mesenchymal cells which are the main site of ADAM33 expression (14, 21), the advantage of lung epithelial expression was that sADAM33 was released into the airway lumen as observed in human asthma. The relevance of the transgenic model was further confirmed using wild type mice where murine sADAM33 was observed in BALF after allergen challenge, and was further supported by data from *Adam33* null mice demonstrating that ADAM33 plays an essential role in development of inflammation and remodeling in this model. One unresolved question is how sADAM33 is released and transferred into the lumen of the airways in asthma. Future work should focus on the role of disease associated single nucleotide polymorphisms (SNPs), especially those in the transmembrane and cytoplasmic domains (14), which might result in increased susceptibility for release of sADAM33 and, as a consequence, result in stimulation of airway remodeling.

Currently, most asthma treatments are directed against T_H2 inflammation (3, 39), with little impact on airway remodeling. The rationale for targeting the metalloprotease (MP) of ADAM33 are strongly supported by our human and mouse data, which both provide strong evidence for a key role of sADAM33 as an initiator of remodeling, independent of inflammation, that is prevented by a mutant inactive MP and is reversible when sADAM33 expression is arrested.

Although historically targeting metalloproteases for diseases such as cancer have been problematic due to off-target effects (40), this problem may be overcome by development of specific small molecule inhibitors of the sADAM33

metalloprotease based on the unique ADAM33 crystal structure (41, 42).

Alternatively the development of monoclonal antibodies, antisense nucleic acids or specific microRNAs (43) may enable development of highly specific sADAM33 therapeutic approaches. Any of these strategies would be amenable to proof of concept testing using the murine models described herein, prior to translation into human studies. Development of any such agents that successfully inhibit sADAM33-MP would be anticipated to have potential as disease-modifying asthma therapies close to the origin of asthma (11).

Methods

Human samples. Bronchoalveolar lavage fluid (BALF) was obtained from healthy and asthmatic donors (**Supplemental Table 1**) by fiberoptic bronchoscopy performed in accordance to the British Thoracic Society (BTS) guidelines (44) and standard operating procedures of the National Institute for Health Research (NIHR) Wellcome Trust Clinical Research Facility and NIHR Southampton Respiratory Biomedical Research Unit at University Hospital Southampton NHS Foundation Trust, UK. BAL was performed by instilling 6 x 20 mL aliquots of pre-warmed normal saline into a sub-segmental bronchus of the anterior segment of the right upper lobe followed by gentle suction. Prior to processing, recovered BALF was filtered (BD Falcon cell strainer, Marathon Laboratory Supplies, UK) then centrifuged at 1,300 g for 10 mins at 4°C. In order to prepare samples for cytospin and supernatant storage, the cell pellet was resuspended in phosphate buffered saline (PBS) following removal of the supernatant, which was stored at –80 °C for later analysis. Cytocentrifuged (Thermo Shandon Ltd, UK) cells were stained with rapid Romanowsky stain (Raymond Lamb Ltd, UK) for differential cell counts of macrophages, neutrophils, eosinophils, lymphocytes and epithelial cells. A total of 400 cells were counted on coded samples by an operator unaware of the participant's clinical characterisation.

Human embryonic tissue was collected, staged, and processed as described previously(45). Gestational age was between 8 and 10 weeks. Human embryonic lungs were dissected into 1 to 2 mm pieces and cultured in transwells in Matrigel (BD Bioscience, Erembodegem, Belgium) in the presence of 60 ng/well of active

ADAM33 Pro-Metalloprotease or inactive mutant E346A ADAM33 Pro-Metalloprotease for 12 days, replenishing media and ADAM33 protein every second day as previously described(27). The tissue was harvested and processed for immunohistochemistry at day 12.

Mice. The animals were maintained in a pathogen-free environment and food and water were provided *ad libitum* in temperature-controlled rooms on a 14 hours light/10 hours dark cycle and animals of mixed sex were used for experiments.

A pTRES-hADAM33-SS-PRO-MP-3Flag plasmid (human ADAM33_ NCBI Reference Sequence: NM_025220.3) was generated as demonstrated in **Supplemental Figure 4A-G**. The plasmid was digested with the restriction endonucleases *AatII* and *SapI* to obtain a linearized construct (**Supplemental Figure 4F**). This was purified and used for microinjection into pro-nuclei from FVB/N mice in the Transgenic Animal and Genome Editing Core Facility at Cincinnati Children's Hospital Medical Center, Ohio, US. DNA was extracted from tails or ear tips of weanlings and used for PCR based genotyping in order to find *ADAM33* expressing founder mice (**Supplemental Figure 4H**). Founder mice were bred with *Ccsp-rtTA* (line 2) (33) to generate double transgenic (DTg) *Ccsp/ADAM33* and single transgenic (STg) litter-mate control mice that were used for phenotyping.

Expression of ADAM33 was induced in 6-8 week old double transgenic mice (*Ccsp/ADAM33*) by provision of Dox (Lab Diet, 5LOS W/625 ppm doxycycline; TestDiet, St. Louis, US) in the food *ad libitum* for 4 and 8 weeks until the point of

sacrifice. Single transgenic litter controls were fed Dox for the same time period (**Supplemental Figure 5A**).

Ccsp/ADAM33 pregnant dams were given Dox in their diet during pregnancy, weaning and to their offspring until 4 weeks after birth. DTg *Ccsp/ADAM33* and STg litter-mate control offspring were sacrificed at embryonic day 17.5 (ED17.5), 10 days postpartum (PD10) and PD28 for further analysis (**Supplemental Figure 5D**).

For studies of the reversibility of airway remodeling, *Ccsp/ADAM33* pregnant dams were given Dox in their diet during pregnancy, weaning and to their offspring until 28 and 56 days after birth. One group was given Dox for 28 days and then was fed without Dox for another 28 days. DTg *Ccsp/ADAM33* and STg litter-mate control offspring were sacrificed 28 and 56 days postpartum for further analysis (**Supplemental Figure 10A**).

For the ADAM33 knock out experiments, sperm were obtained from the Mouse Biology Program at the University of California, Davis, USA (a generous gift from Dean Sheppard and Chun Chen, Lung Biology Center, Department of Medicine, University of California, San Francisco, USA) (35). This was used for *in vitro* fertilization to reconstitute *Adam33^{-/-}* mice in the Transgenic Animal and Genome Editing Core facility at Cincinnati Children's Hospital Medical Center, Ohio, US. Sperm from homozygote *Adam33^{-/-}* 129/SVJae mice were added to oocyte-cumulus complexes from superovulated FVB/N female mice for *in vitro* fertilization. Mixed background mice heterozygote for *Adam33* were bred in order to get a mix of *Adam33^{-/-}*, *Adam33^{+/-}* and WT mice, which were genotyped as previously reported(35). 6–8 week old offspring of mixed sex were used for the

HDM extract challenge experiments according to the protocol described below (**Supplemental Figure 3**). The number of animals used for analysis was based on 3–5 animals per treatment group and were repeated up to three times. This was based on power calculations (<http://www.stat.ubc.ca>) for invasive airway resistance measurements from a pilot experiment of *Adam33*^{-/-} and wild-type mice challenged with HDM. Assuming an 80% power, a 5% significance level, a two-sided test, and that a difference in the mean airway resistance of 2.2–3.3 cm H₂O/s/ml between double transgenic and control mice is likely to be of scientific interest and a common standard deviation of 1.0–1.5, we estimated that sample sizes of about 2–8 mice would be required when testing concentrations of Methacholine at 0, 25, 50 & 100 mg/ml).

Genotyping was performed before litters were weaned and split by gender. At the same time animals were tagged with a metal clip or ear punches and given each a unique number. Animals with the appropriate genotypes were randomly included in the different experimental groups. The investigators were blinded during further analysis of the animals and processing of samples using only the unique identification number.

Genotyping PCR. Primers used for PCR amplification of the conditional *Ccsp/ADAM33* mice were as follows: *ADAM33-PRO-MP* forward 5'- CAG CTT CTC AGG ACT CTG GAC ATT C-3' and reverse 5'-CGG GAT CAC TAC TTG TCA TCG TC-3'. About 100 ng of genomic DNA was PCR amplified using the following conditions: *Ccsp*: 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s, 72 °C for 7 min, hold at 4 °C; *ADAM33-PRO-MP*: 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, 72 °C for

7 min, hold at 4 °C, and we analyzed the PCR products on 1.5% agarose gel with ethidium bromide to examine the presence of the ~440 bp *Ccsp* and ~ 636 bp *ADAM33-PRO-MP* allele band. For genotyping of the *Adam33* mutant alleles, primers and conditions were used that had been described previously (35).

IL13 and house dust mite extract (HDM) allergen challenges. Six to eight week-old DTg *Ccsp/ADAM33* and STg litter-mate control mice were challenged with 5µg of recombinant murine IL13 or saline by intra-tracheal installation and lungs were harvested for further analysis after 24hours (**Supplemental Figure 8A**). Six to eight week-old, age and gender-matched FVB/N, *Adam33* null, heterozygote and wild-type (WT) mice and DTg *Ccsp/ADAM33* and STg litter-mate control mice were sensitized intra-peritoneally with HDM extract (*Dermatophagoides pteronyssinus* extract protein 8.1mg/vial, Lot Nr. 218862; Greer, Lenoir, US) (20 µg/200 µl saline) on days 0 and 7. On days 14 and 19 the mice were challenged intra-tracheally with HDM 25 µg/100 µl saline to induce allergic airway inflammation or with saline alone as control (**Supplemental Figure 3**). In addition, a dose response experiment was performed with lower concentrations of HDM (6.25, 12.5 and 25 µg/100 ul saline) on days 14 and 19 in wild-type mice and the lowest dose was used for DTg *Ccsp/ADAM33* and STg litter-mate control mice that were on Dox from *in utero* (**Supplemental Figure 9A**). At day 21 after first sensitization, lung function was assessed (see below) and mice were harvested for BALF and lung tissue for further analysis (**Supplemental Figure 3**).

Western blotting. Human or murine BALF (from HDM challenged mice) samples were concentrated and diluted using 2x or 6x Laemmli sample buffer before loading equal protein concentrations on to the gels. Samples were run on 10% Tris/Glycine gels and transferred onto PVDF membranes. Where murine BALF samples were not concentrated, equal volumes of BALF were subjected to electrophoresis and transferred onto PVDF membranes. The transferred protein was assessed by Ponceau staining to ensure similar protein loading prior to blocking and Western blotting. Membranes were probed with polyclonal rabbit antibodies against the MP (ab39191, Abcam, Cambridge, UK; 1:5000) or Pro (ab39190, Abcam, Cambridge, UK; 1:5000) domains of ADAM33 (**Supplemental Figure 1**) or a polyclonal goat antibody against the ectodomain of mouse ADAM33 (AF2434, R&D, Abington, UK; 1:1000) as described previously (26, 28). Secondary antibodies were Rabbit TrueBlot® anti-rabbit IgG HRP antibody (18-8816, eBioscience, San Diego, CA, USA; 1: 5000) or rabbit anti-goat IgG HRP antibody (Calbiochem, Merck, Darmstadt, Germany; 1:10000). The blots were visualized using enhanced chemiluminescence (ECL+; GE Healthcare) with ImageQuant LS4000 or Amersham Imager 600 (GE Healthcare Life Sciences, Little Chalfont, UK). Semi-quantitative analysis was performed by densitometry. Quantity One Analysis software (Bio-Rad, Hercules, US) or ImageJ (National Institutes of Health, Bethesda, Maryland, USA) was used to determine the intensity of the bands by measuring the Integrated Density as a product of Area and Mean Gray value or by measuring Relative Density as result of the Area of the lane profile plot (<http://rsb.info.nih.gov/ij/docs/menus/analyze.html#gels>). All human and mouse BALF samples were run on the same gel with positive control samples in form of Cos-7 cell lysates transfected with human ADAM33-MP-Pro

and purified human recombinant ADAM33-MP-Pro protein or Cos-7 cell lysates transfected with full length mouse ADAM33 (Mouse cDNA clone MR217277-20; OriGene, Rockville, USA) on each gel.

Specificity testing of the human ADAM33 antibodies. Human recombinant ADAM33-Pro-MP (produced in-house), ADAM8-polyhistidine-tag (HIS) and ADAM12-polyhistidine-tag (HIS) (1031-AD-020 and 4416-AD-020; both from R&D, BioTechne, Oxford, UK) were applied in 2µl volumes containing 100, 75, 50, 25 or 0 ng of each protein onto pencil marked grids on PVDF membranes and air dried. After blocking, the membranes were incubated overnight with the polyclonal rabbit antibodies against the MP or Pro domains of ADAM33 (ab39191 and ab39190, Abcam, Cambridge, UK; 1:5000) or with Mouse anti-HIS antibody (372900, GE Healthcare, Little Chalfont, UK). After washing, bound antibody was detected using secondary antibodies: Donkey anti-Rabbit IgG HRP (NA9340V, GE Healthcare) or polyclonal Rabbit anti-mouse HRP (P0260, Dako, Ely UK) and visualized using enhanced chemiluminescence (ECL+; GE Healthcare) with an Amersham Imager 600 (GE Healthcare Life Sciences, Little Chalfont, UK) **(Supplemental Figure 2).**

Fluorescence resonance energy transfer (FRET) peptide cleavage assay.

A FRET peptide cleavage assay was performed using a peptide that was selective for ADAM33. Analyses were performed using a StepOnePlus (Applied Biosystems, Life Technologies, Paisley, UK) or a BioRad CFX96 (Bio-Rad) qPCR machine with a FAM (6-carboxyfluorescein) filter that measured fluorescence output every minute for 60 minutes. To assess enzymatic activity in BALF each reaction was incubated with 7 µl of neat BALF at 37°C with 4.4 µM (0.5 µl of 88.7

μM) FRET peptide (DABCYL-YRVAFQKLAE(FAM)K-NH₂)(41) (Severn Biotech, Kidderminster, UK), 10 μM (0.5 μl of 200 μM) ZnCl₂ in 2 μl of 5x assay buffer (100 mM HEPES pH 7.0, 2.5 M NaCl, 50 mM CaCl₂, and 1 mg/ml bovine serum albumin) in a total reaction volume of 10 μl . Enzymatic activity was determined by plotting the relative fluorescence units (RFU) against time after the background had been subtracted. The rate of the reaction (RFU/min) was determined from the line of best fit in the linear phase of the assay. Controls were recombinant soluble active ADAM33-PRO-MP and mutant ADAM33-PRO-MP (E356A) as previously published (27).

qRT-PCR. Lung tissue was either snap frozen or stored in RNAlater (Life Technologies) before homogenization and RNA extraction using Trizol[®] Reagent (Invitrogen, Life Technologies). Genomic DNA contamination was removed by digestion with DNase (Life Technologies). First-strand cDNA was generated by reverse transcription using the RT-Standard cDNA synthesis kit (PrimerDesign, Southampton, UK). qPCR was performed using a CFX96 qPCR machine (Bio-Rad) for 40 cycles 95 °C for 5 s and 60 °C for 20 s (fast protocol) or 40 cycles 95 °C for 15 s and 60 °C for 60 s (standard protocol) followed by a melt curve analysis for SYBR green based assays. All samples were run in duplicate. PCR product amplification was detected using a PCR or SYBR green mastermix (PrimerDesign) or TaqMan[®] Gene Expression Master Mix (Life Technologies) and primer sets without or with TaqMan/Perfect probes: *ADAM33-MP* forward 5'-CCT GGA ACT GTA CAT TGT GGC A-3', reverse 5'-GTC CAC GTA GTT GGC GAC TTC-3' and FAM-probe 5'-CCA CAC CCT GTT CTT GAC TCG GCA T-3'; *Acta2* forward 5'-TGA AGA GGA AGA CAG CAC AGC-3', reverse 5'-GGA GCA

TCA TCA CCA GCG AA-3' and FAM-probe 5'-CAG AGC CCA GAG CCA TTG TCG CAC-3'; *Col1a1* forward 5'-TCG TGG CTT CTC TGG TCT C-3', reverse 5'-CCG TTG AGT CCG TCT TTG C-3' and Perfect probe 5'-CAG GGT CCT CCT GGT TCT CCT GGT TCT CGA CCC TG-3'; *Col3a1* forward 5'-ATA TGC CCA CAG CCT TCT AC-3', reverse 5'-CAG GAA TGC CAG GAG GAC-3' and Perfect probe 5'-CTG CTC CTG TGC TTC CTG ATG GCC AGC AG-3'; *Fn1* forward 5'-AAG AGG ACG TTG CAG AGC TA-3' and reverse 5'-AGA CAC TGG AGA CAC TGA CTA A-3'; *Pecam1* forward 5'-TCC AAC AGA GCC AGC AGT AT-3' and reverse 5'-GCA GAG AGC AAT ACA GAG GAA-3' (PrimerDesign), *Ccl11*: Mm00441238_m1, *Il5*: Mm00439646_m1, *Il13*: Mm00434204_m1, *Cxcl1*: Mm04207460_m1, *Muc5ac*: Mm01276718_m1, *Gapdh*: Mm99999915_g1 (Life Technologies). Relative mRNA expression was quantified using $2^{-\Delta\Delta Ct}$ method (46).

Luminex multiplex analysis. Undiluted murine BALF samples were analyzed using a mouse Magnetic Luminex Screening assay containing a premixed multi-analyte kit for murine Ccl11 and IL5 according to manufacturer instructions (R&D Systems, Minneapolis, US) on a Luminex 200 (Luminex, Austin, US).

Inflammatory cell counts. Murine BALF samples were collected by washing the lungs three times with 800–1000 μ l sterile phosphate buffered saline (PBS). The total volume of the combined fluids was measured and centrifuged at 300 g for 5 min. The BALF supernatants were frozen for analysis of sADAM33. Red blood cells were lysed from the cell pellets, which were subsequently

resuspended in 300 μ l PBS. Cells were counted and 100,000 cells were loaded into a cytopsin funnel and centrifuged at 300 g for 5 min on to a glass slide.

Slides were air-dried and the cells stained using a Diff-Quick stain (Sigma, Poole, UK) followed by fixation and Hematoxylin and Eosin (H&E) staining. The different inflammatory cell types were counted to a total of 300 cells and expressed as the differential cell count in cells/ml BALF.

Assessment of lung function. Mice were anesthetized with 100 μ l of triple anesthetic containing a 4:1:1 mixture of ketamine, acepromazine, and xylazine by intra-peritoneal injection. A FlexiVent machine (Scireq, Montreal, Canada) was used to assess lung function in the form of airway resistance (R) after aerosolized methacholine challenge to provide a measure of BHR as described by the manufacturer's instructions. Airway resistance was measured by forced oscillation technique, increasing values indicating bronchoconstriction of the lungs. BHR measurements were obtained from individual animals using increasing stepwise concentrations of 0, 25, 50 and 100 mg/ml in Saline for the *Ccsp/ADAM33* and HDM extract challenged mice. On completion of lung function assessment, mice were sacrificed and BALF and lung tissue collected for further analyses.

Histochemistry and Immunofluorescence-histochemistry (IFHC). Lungs were inflation-fixed with 4% paraformaldehyde (PFA) or 10% neutral buffered formalin at 25 cm of water pressure for 5 minutes and then fixed overnight before embedding in paraffin wax. 5 μ m thick serial sections were cut for histological

staining using hematoxylin and eosin (H&E) or IFHC using standard protocols. A fluorescein isocyanate (FITC) conjugated mouse monoclonal antibody was used against ACTA2 (α SMA), 1:250 (F3777; Sigma, Poole, UK), a primary rabbit anti human ADAM33-PRO, 1:1000 (ab39190, Abcam, Cambridge, UK) with a secondary AlexaFluor 594 goat anti-rabbit IgG, 1:200 (Invitrogen) and a primary rat antibody against murine CD31 (PECAM1), 1:75 (DIA 310; Dianova, Stratech Scientific Ltd, Newmarket, UK) with a secondary Alexa Fluor 647 goat anti rat IgG, 1:500 (112-605-003; Jackson Immune Research, Stratech Scientific Ltd). Stained slides were mounted with a cover slip in ProLong Anti-Fade Gold (Life Technologies, Loughborough, UK), which contained 40,6-diamidino-2-phenylindole (DAPI) as nuclear counter stain. Human embryonic lungs were acetone-fixed and embedded in glycol methacrylate resin. Consecutive 2- μ m sections were cut and immunostained with a mouse monoclonal antibody against ACTA2 (α SMA), 1:40,000 (A2547, Sigma, Poole, UK) using standard protocols (21, 47). Lung sections were examined by light microscopy using a Zeiss Axioplan 2 (Carl Zeiss Microscopy, Peabody, MA, USA) or a Leica AF6000 LX (Leica Microsystems (UK) Ltd, Milton Keynes, UK) microscope equipped with AxioVision software (Zeiss) or LAS AF software (Leica).

Statistics. Normal distribution of the numeric data was evaluated and the appropriate parametric or non-parametric statistical tests were used. Statistical significance was assessed by using the two-tailed Student's t-test (parametric, unpaired data) with Welch's correction if standard deviations were not equal or Mann Whitney test (non-parametric, unpaired data) for comparisons between two

groups. For comparison of 3 or more groups a one-way analysis of variance (ANOVA) with Tukey's multiple comparison tests (parametric data) or Kruskal-Wallis test with Dunn's test for correction for multiple comparisons (non-parametric data) were used. For comparison of 2 or more groups with two independent variables a two-way ANOVA with Tukey's multiple comparison tests was used (Prism 6, version 6.0e, Graphpad, La Jolla, US). *P* values of ≤ 0.05 were considered significant. Data are presented as mean \pm s.d. or median with 25 and 75% interquartiles showing all points. Whiskers represent minimum and maximum values.

Study approvals. Collection and use of bronchoscopy samples was performed after approval from the Southampton and South West Hampshire Joint Local Research Ethics Committee and informed consent from the donors. Human embryonic lungs were harvested according to the Polkinghorne Committee guidelines and approval from the Southampton and South West Hampshire Joint Local Research Ethics Committee and informed consent from the donor. For all mouse experiments, the 3Rs (Replacement, Reduction and Refinement) principles were followed and experiments conducted according to the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines (48), the guidelines for the care and use of animals approved by the Institutional Animal Care and Use Committee at the Cincinnati Children's Hospital Research Foundation (Cincinnati, OH, USA) and the local Southampton University ethical committee under project and personal licenses from the Home Office, UK.

Author contributions

E.R.D helped design and carried out most experiments, analyzed data and contributed towards writing the manuscript. J.F.C.K. performed murine lung function and RNA expression studies. P.H.H provided human adult samples and clinical data and edited the manuscript. D.I.W provided human fetal lungs and edited the manuscript. S.T.H. acted as advisor and edited the manuscript. D.E.D. contributed to experimental design, interpretation of the data and co-wrote the manuscript. J.A.W supervised research, design of the ADAM33 transgenic mouse, provided critical reagents and laboratory space and edited the manuscript. H.M.H. supervised the project, designed and performed experiments, analyzed data and was the lead co-author of the manuscript.

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Figures and figure legends

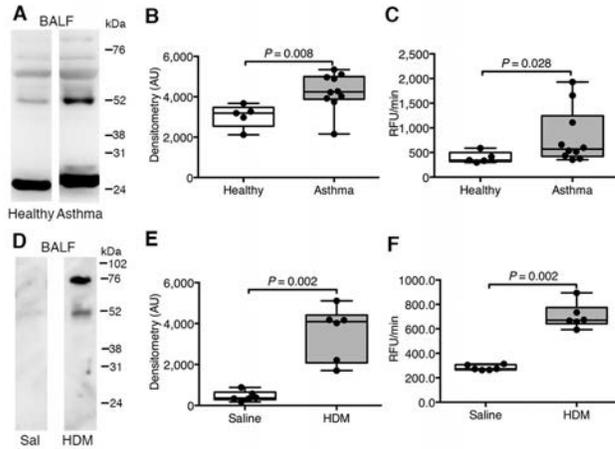


Figure 1

Figure 1. Increased soluble ADAM33 (sADAM33) enzymatic activity in bronchoalveolar lavage fluid (BALF) in human asthma and allergic mice. (A) Western blotting of BALF proteins from healthy (n=5) and asthmatic (n=10) subjects using an antibody recognizing the metalloprotease domain of human ADAM33; representative blots are shown. (B) Combined ADAM33 immunoreactive bands (at ~25kDa and between ~52 - 76kDa) were analyzed by densitometry in arbitrary units (AU) (Mann Whitney test). (C) Fluorescence resonance energy transfer (FRET) peptide cleavage assay for ADAM33-specific enzymatic activity in BALF from healthy (n=5) and asthmatic donors (n=10), expressed as relative fluorescence units per minute (RFU/min) (Mann Whitney test). (D) Immunoblotting of BALF protein from wild-type mice challenged with house dust mite (HDM) extract or saline (Sal) (n=6 per group) with an antibody against mouse ADAM33 extracellular domain. (E) Semi-quantitative analysis (bands at ~ 52 and 76kDa) by densitometry (Mann Whitney test). (F) FRET peptide cleavage assay using murine BALF from HDM challenged or saline controls (n=6 per group) (Mann Whitney test). Box plots show medians and 25th to 75th percentiles, and whiskers min to max/all points. Results are from 3 independent experiments (D,E,F).

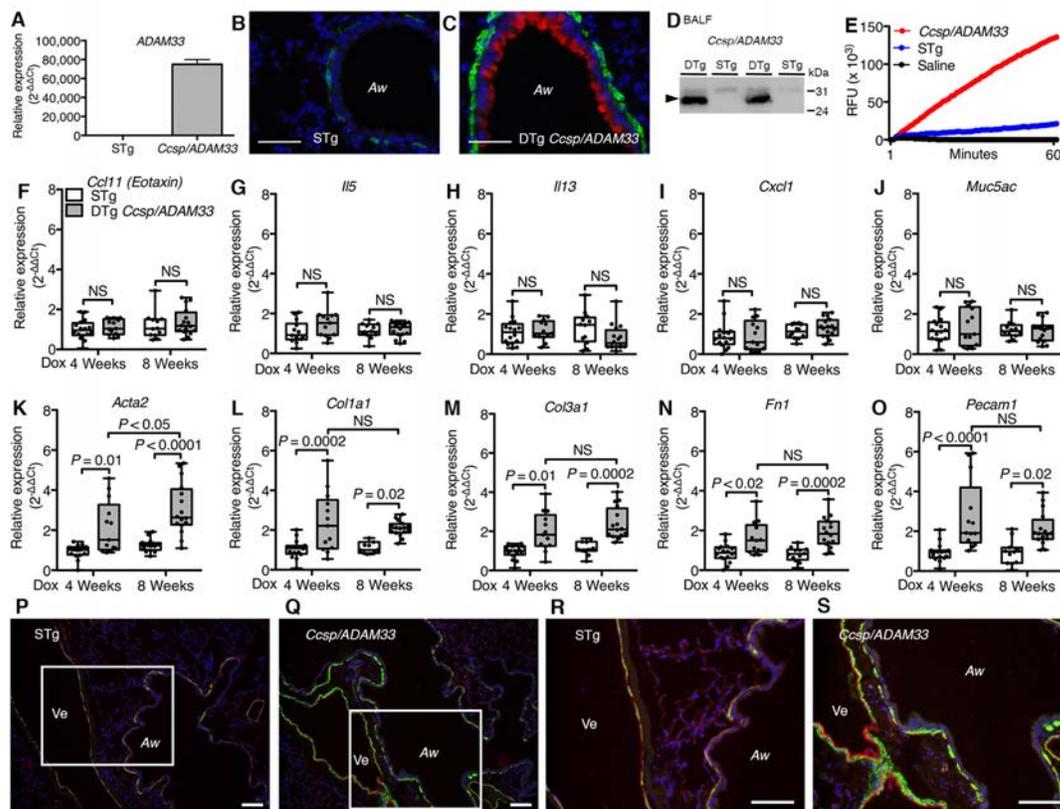


Figure 2

Figure 2. Transgenic expression of human soluble ADAM33 (sADAM33) causes airway remodeling. (A) Human *ADAM33* mRNA in single transgenic (STg) litter-mate control and double transgenic (DTg) *Ccsp/ADAM33* mouse lungs, (B,C) immunofluorescence staining for human ADAM33 (red), ACTA2/ α SMA (green), nuclei (blue) in lungs from (B) STg litter-mate control and (C) DTg *Ccsp/ADAM33* mice; representative images: scale bar, 50 μ m. (D) Western blotting for human ADAM33 in BALF from DTg *Ccsp/ADAM33* or STg control mice. (E) Fluorescence resonance energy transfer (FRET) peptide cleavage assay for ADAM33 enzymatic activity in bronchoalveolar lavage fluid (BALF) from DTg *Ccsp/ADAM33* (red) or STg (blue) mice, or saline control (black); representative traces. (F-O) Relative mRNA expression in whole lung lobe lysates from adult DTg *Ccsp/ADAM33* mice (grey bars) after induction of human ADAM33 for 4 (n=13) or 8 weeks (n=16) versus STg litter-mate controls (white bars) (n=16 or n=12, respectively): (F) *Ccl11/Eotaxin*, (G) *Il5*, (H) *Il13*, (I) *Cxcl1/Kc*, (J) *Muc5ac*, (K) *Acta2*, (L) *Col1a1*, (M) *Col3a1*, (N) *Fn1* (O) *Pecam1/CD31* (two-way ANOVA, Tukey's multiple comparison test). Box plots show medians and 25th to 75th percentiles, whiskers min to max/all points. Results are from 3 independent experiments (F-Q). (P-S). Representative immunofluorescence staining for ACTA2/ α SMA (green), PECAM1 (red), nuclei (blue) in lungs from (P,R) STg litter-mate control or (Q,S) DTg *Ccsp/ADAM33* mice after 8 weeks of transgene expression. White rectangles in (P,Q) are shown at higher magnification (R,S). Aw = airway, Ve = vessel. Scale bar, 100 μ m. Results are representative of 3 independent experiments (P-S).

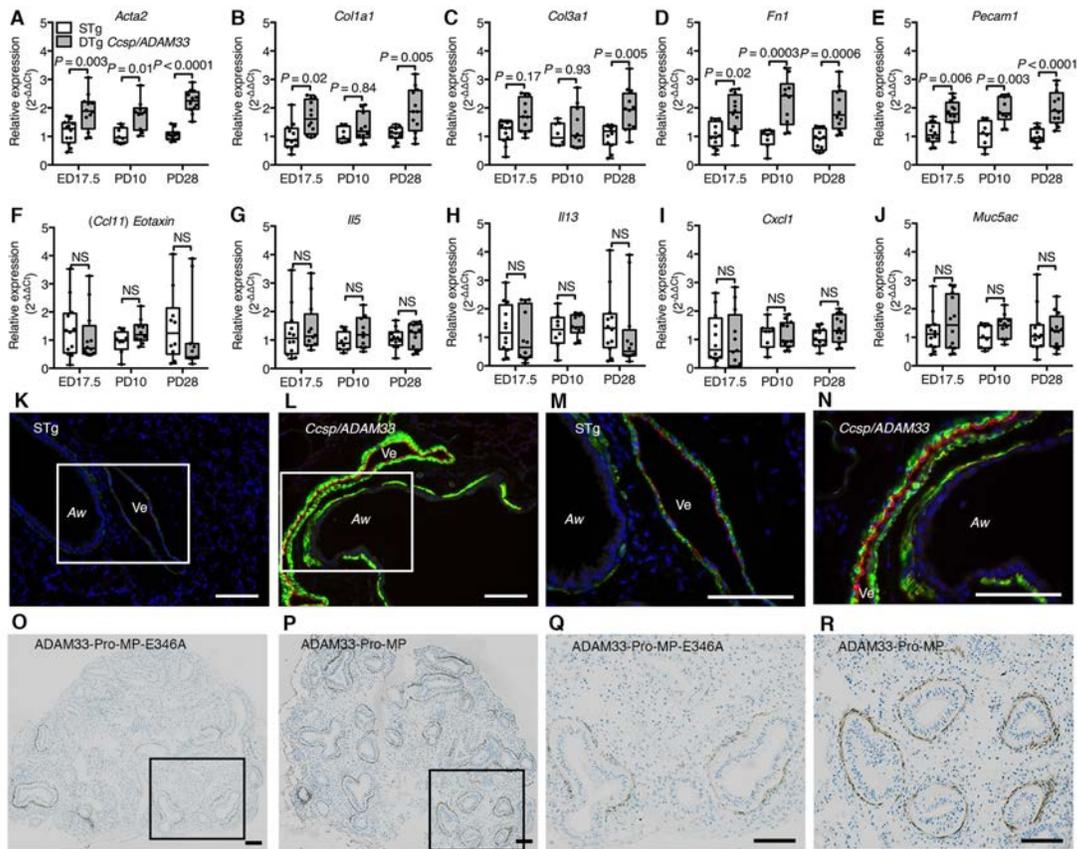


Figure 3

Figure 3. Human soluble ADAM33 (sADAM33) causes airway ‘premodeling’ in developing lungs. (A-J) Reverse transcription quantitative PCR (RT-qPCR) for remodeling and inflammatory gene mRNA expression in whole lung lobe lysates from double transgenic (DTg) *Ccsp/ADAM33* or single transgenic (STg) litter-mate control mice at embryonic day 17.5 (ED17.5) (n=12/12), 10 days (PD10) (n=8/10) and 28 days *post partum* (PD28) (n=12/12) in which transgene expression was induced by feeding Doxycycline during pregnancy and up to 4 weeks after birth: (A) *Acta2*, (B) *Col1a1*, (C) *Col3a1*, (D) *Fn1* (E) *Pecam1/CD31* (F) *Ccl11/Eotaxin*, (G) *Il5*, (H) *Il13*, (I) *Cxcl1/Kc*, (J) *Muc5ac*, (two-way ANOVA, Tukey’s multiple comparison test). Box plots show medians and 25th to 75th percentiles, whiskers min to max/all points. Results are from 3 independent experiments (A-J). (K-N). Representative immunofluorescence staining for ACTA2/αSMA (green), PECAM1 (red) and nuclei (blue) in lungs from (K, M) STg litter-mate control or (L, N) DTg *Ccsp/Adam33* mice after 4 weeks *post partum* of ADAM33 transgene expression is shown. White rectangles in (K, L) are shown at higher magnification (M, N). Aw = airway, Ve = vessel. Representative immunohistochemistry staining for ACTA2/αSMA (brown) of sections from 8-10 weeks post conception human embryonic lung explants cultures (n=3) in the presence of (O, Q) recombinant inactive mutant (E346A) ADAM33-Pro-Metalloprotease (ADAM33-Pro-MP) and (P, R) enzymatically active ADAM33-Pro-MP. Black rectangles in (O, P) are shown at higher magnification (Q, R). Scale bar, 100μm. Results are representative of 3 independent experiments (K-N and O-R).

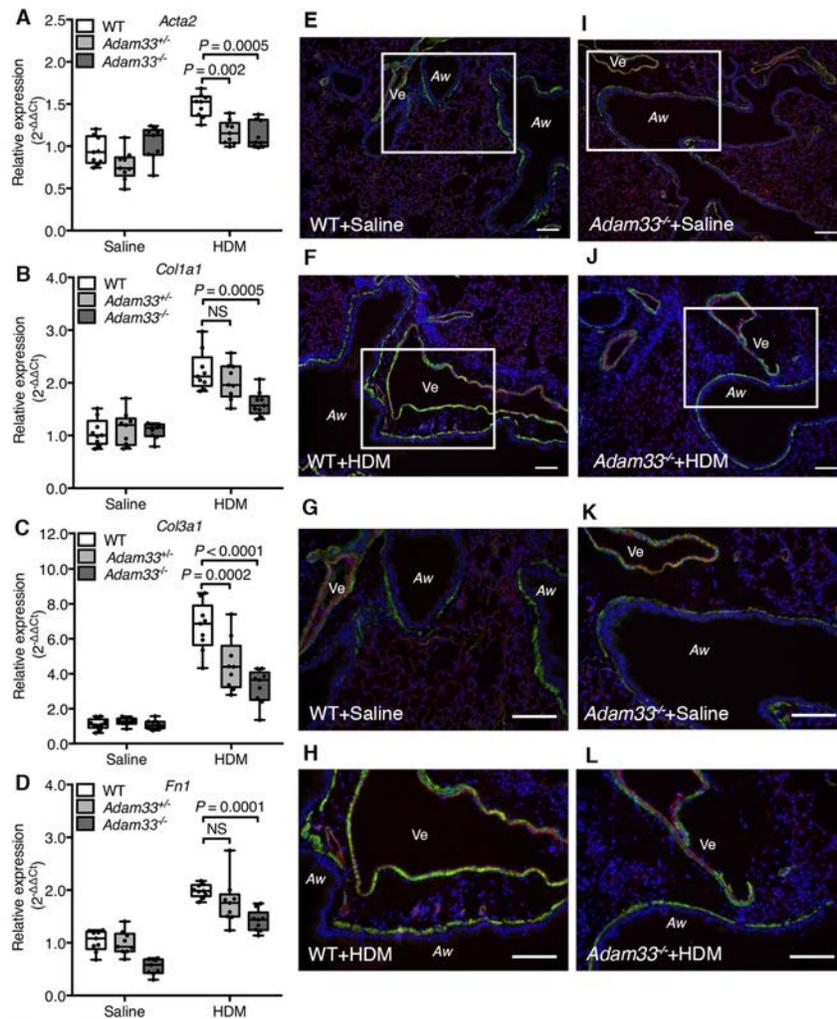


Figure 4 new

Figure 4. Suppression of house dust mite extract (HDM)-induced airway remodeling in *Adam33*^{-/-} mice. (A-D) Reverse transcription quantitative PCR (RT-qPCR) using whole lung lysates from wild-type (WT), heterozygote (*Adam33*^{+/-}) and *Adam33*^{-/-} mice challenged with Saline or HDM extract: (A) *Acta2/αSma*, (B) *Col1a1*, (C) *Col3a1*, (D) *Fn1*, (n=9 per group; two-way ANOVA Tukey's multiple comparison test). (E-L) Representative immunofluorescence staining of tissue sections for ACTA2/αSMA (green), PECAM1 (red), nuclei (blue) from mouse lungs after *in vivo* challenge with Saline or HDM extract: (E,G) WT+Saline and (F,H) WT+HDM (L,K) *Adam33*^{-/-}+Saline and (J,L) *Adam33*^{-/-}+HDM. White rectangles in E,F,L,J are shown (G,H,K,L) at higher magnification; scale bar, 100μm. Results are representative of 3 independent experiments (E-L).

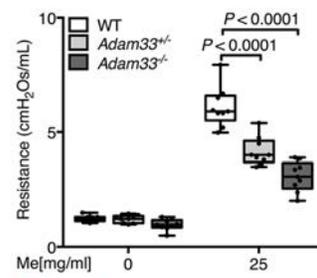


Figure 5 new

Figure 5. Suppression of house dust mite extract (HDM)-induced airway hyperresponsiveness in *Adam33*^{-/-} mice. Airway resistance in response to methacholine (Me) in wild-type (WT) (white box), *Adam33*^{+/-} (light gray box) and *Adam33*^{-/-} (dark gray box) mice following HDM exposure (n=9 per group; two-way ANOVA, Tukey's multiple comparison test). Results are representative of 3 independent experiments.

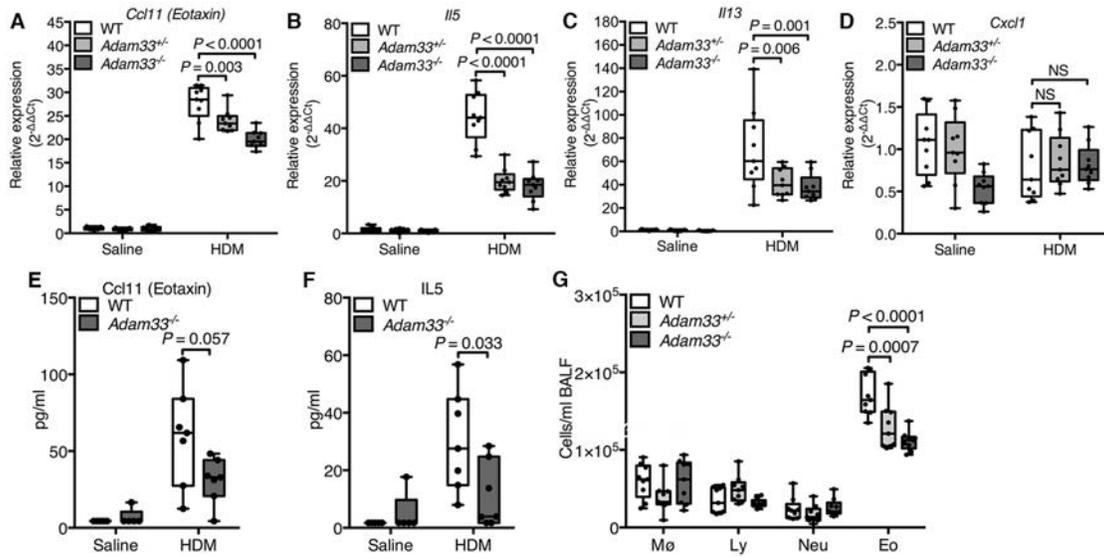


Figure 6 new

Figure 6. Suppression of house dust mite extract (HDM)-induced airway inflammation in *Adam33*^{-/-} mice. (A-D) Reverse transcription quantitative PCR (RT-qPCR) using whole lung lysates from wild-type (WT) (white box), heterozygote (*Adam33*^{+/-}) (light gray box) and *Adam33*^{-/-} (dark gray box) mice challenged with Saline or HDM extract: (A) *Ccl11/Eotaxin*, (B) *Il5*, (C) *Il13*, (D) *Cxcl1/Kc*; (n=9 per group; two-way ANOVA Tukey's multiple comparison test). (E and F) Multiplex assay for CCL11/Eotaxin and IL5 protein levels in bronchoalveolar lavage fluid (BALF) (n=5 or 7 per group; one-way ANOVA, Tukey's multi comparison test). (G) Differential inflammatory cell counts for macrophages (Mø), lymphocytes (Ly), neutrophils (Neu) and eosinophils (Eo) in BALF from WT, *Adam33*^{+/-} and *Adam33*^{-/-} mice challenged with HDM (n=9 per group; two-way ANOVA Tukey's multiple comparison test). Box plots show medians and 25th to 75th percentiles, whiskers min to max/all points. Results are from 3 independent experiments (A-G).

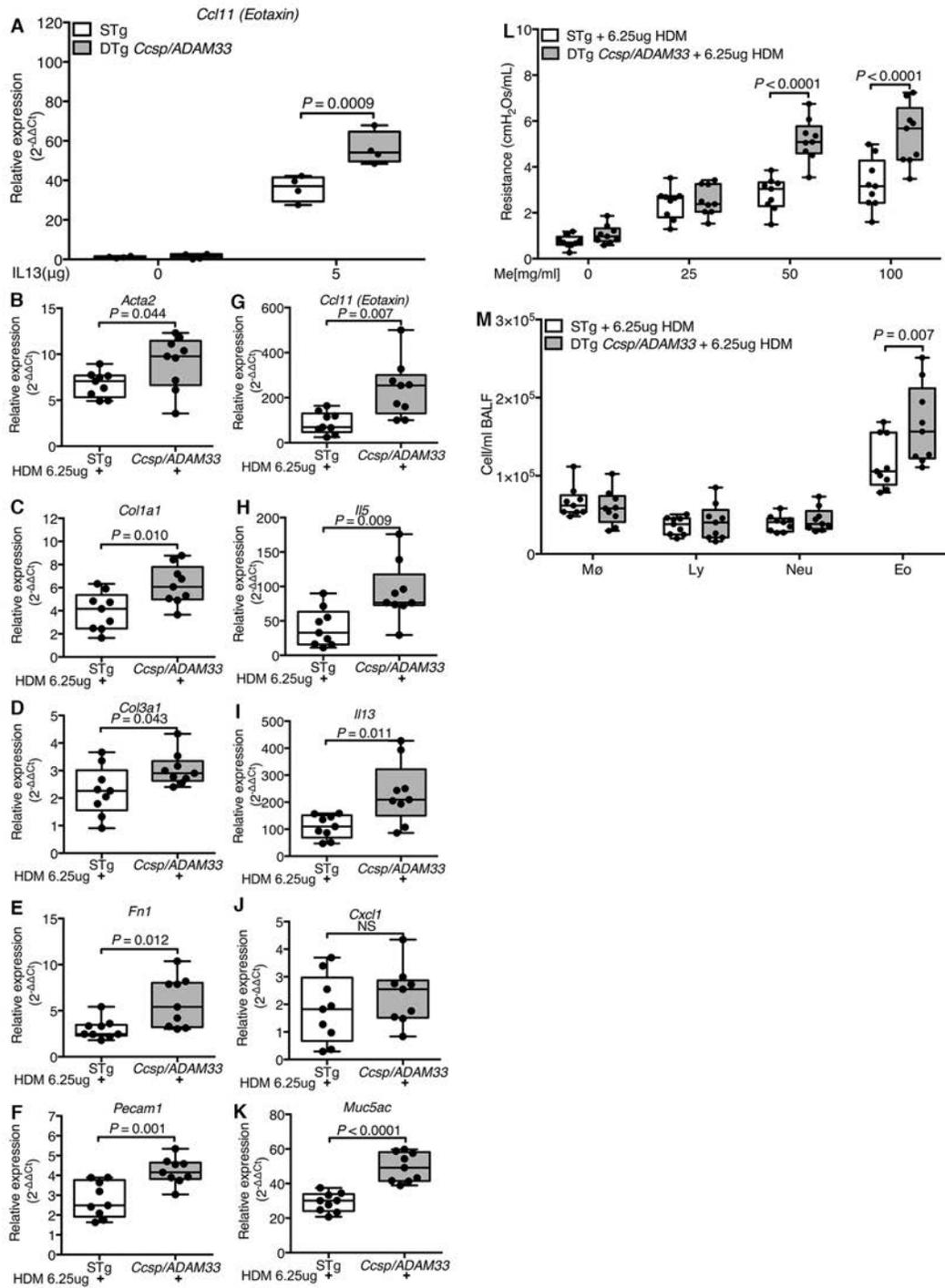


Figure 7

Figure 7. Soluble ADAM33 (sADAM33) augments airway responses to allergens. (A) Reverse transcription quantitative PCR (RT-qPCR) for *Ccl11/Eotaxin* mRNA expression in lung lobe lysates 24h after intra-tracheal installation of 5.0 μg murine IL13 in double transgenic (DTg) *Ccsp/ADAM33* (grey bars) and single transgenic (STg) litter-mate control mice (white bars) after transgene induction for 4 weeks (n=4/group; two-way ANOVA, Tukey's multiple comparison test). Results are from 1 experiment (**A**). (**B-M**) DTg *Ccsp/ADAM33* or STg control mice after transgene induction for 6 weeks were then sensitized and challenged with house dust mite (HDM) extract (6.5 μg) for analysis of gene expression, bronchial hyperresponsiveness (BHR) and inflammation: (**B-K**) RT-qPCR for relative mRNA expression (compared to saline challenged mice) in lung lobe lysates from DTg *Ccsp/ADAM33* or STg control mice: (**B**) *Acta2/αSma*, (**C**) *Col1a1*, (**D**) *Col3a1*, (**E**) *Fn1*, (**F**) *Pecam1/CD31*, (**G**) *Ccl11/Eotaxin*, (**H**) *Il5*, (**I**) *Il13*, (**J**) *Cxcl1/Kc*, (**K**) *Muc5ac* (all n=9 per group; unpaired Student's t-test or Mann Whitney test). (**L**) Airway

resistance in response to increasing concentrations of methacholine (Me) and **(M)** differential inflammatory cell counts for macrophages ($M\phi$), lymphocytes (Ly), neutrophils (Neu), eosinophils (Eo) in bronchoalveolar lavage fluid (BALF) after HDM or saline challenge (n=9 per group; two-way ANOVA, Tukey's multiple comparison test). Box plots show medians and 25th to 75th percentiles, whiskers min to max/all points. Results are from 3 independent experiments (**B-M**).

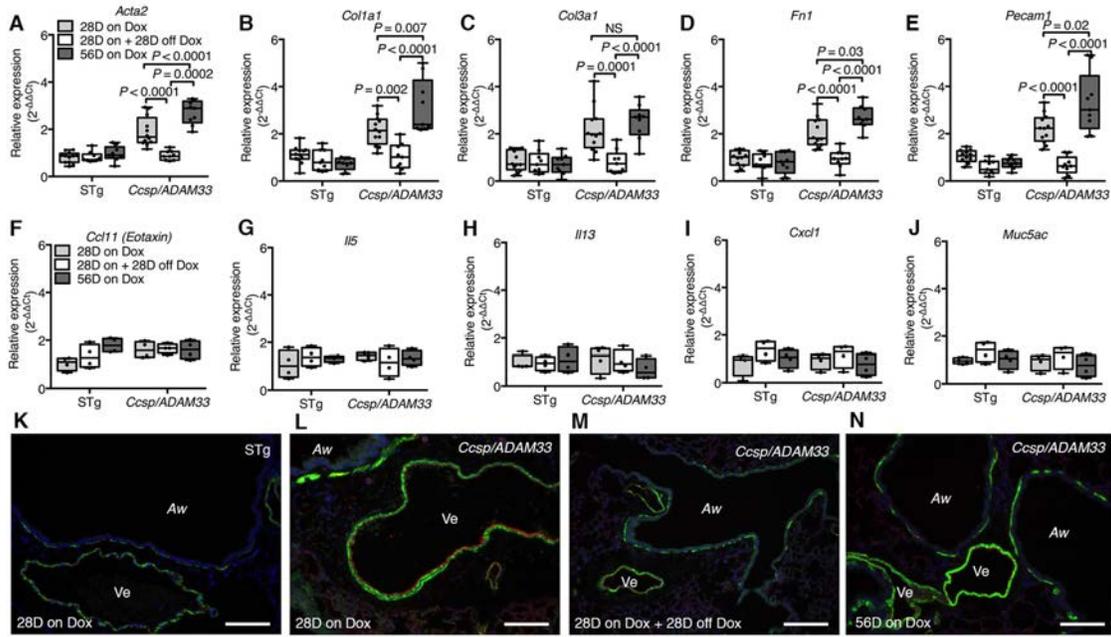


Figure 8

Figure 8. Airway remodeling induced by soluble ADAM33 (sADAM33) is reversible. (A-J) Reverse transcription quantitative PCR (RT-qPCR) for remodeling (n=9/group) and inflammatory (n=4/group) gene mRNA expression in whole lung lobe lysates from double transgenic (DTg) *Ccsp/ADAM33* or single transgenic (STg) litter-mate control mice in which transgene expression was induced by feeding Doxycycline (Dox) during pregnancy and for up to 28 days (28D on Dox) or 56 days (56D on Dox) after birth, or for 28 days after birth and then stopped for 28 days (28D on + 28D off Dox): **(A)** *Acta2*, **(B)** *Col1a1*, **(C)** *Col3a1*, **(D)** *Fn1* **(E)** *Pecam1/CD31* **(F)** *Ccl11/Eotaxin*, **(G)** *Il5*, **(H)** *Il13*, **(I)** *Cxcl1/Kc*, **(J)** *Muc5ac*, (two-way ANOVA, Tukey's multiple comparison test). Box plots show medians and 25th to 75th percentiles, whiskers min to max/all points. Results are from 3 independent experiments **(A-E)** and from 1 experiment **(F-J)**. **(K-N)**. Representative immunofluorescence staining for ACTA2/αSMA (green), PECAM1 (red) and nuclei (blue) in lungs from **(K)** STg litter-mate control or **(L)** DTg *Ccsp/Adam33* mice after 28days and **(N)** after 56 days *post partum* of ADAM33 transgene expression is shown. **(M)** DTg *Ccsp/Adam33* mice after 28days on Dox *post partum* of ADAM33 transgene expression and then 28 days off Dox. Aw = airway, Ve = vessel. Scale bar, 100 μm. Results are representative of 2 independent experiments **(K-N)**.

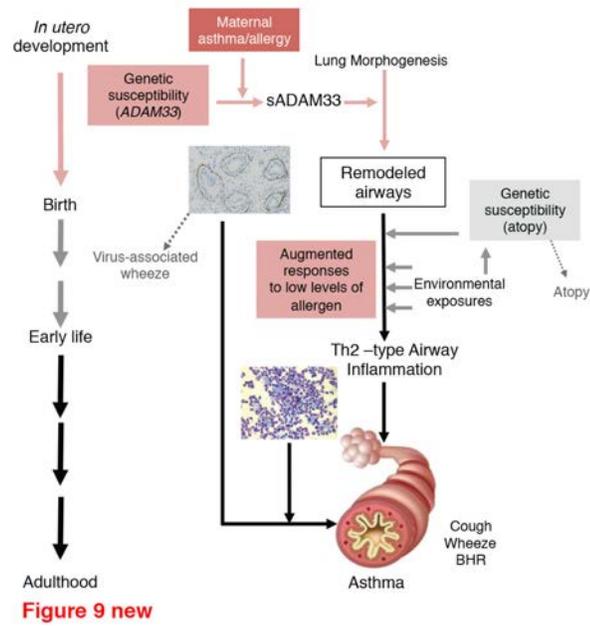


Figure 9. Schematic representation of the contribution of soluble ADAM33 (sADAM33) as a local tissue susceptibility gene in asthma pathobiology.