ADAM-8, a metalloproteinase, drives acute allergen-induced airway inflammation

Geneviève PAULISSEN, Natacha ROCKS, Maud M. GUEDERS, Denis BEDORET, Céline CRAHAY, Florence QUESADA-CALVO, Jonathan HACHA, Sandrine BEKAERT, Christophe DESMET, Jean-Michel FOIDART, Fabrice BUREAU, Agnès NOEL, Didier D. CATALDO

Laboratory of Tumor and Development Biology, GIGA-Research (GIGA-I³ and GIGA-cancer), University of Liege and CHU of Liege, Sart-Tilman, Belgium.

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Correspondence and reprint requests should be addressed to:

Professor Didier D. CATALDO
Tower of Pathology (B23)
GIGA-Research, University of Liege
4000 Liege, Belgium
Fax : +3243662939
Phone: +3243662521
E-mail: didier.cataldo@ulg.ac.be

List of abbreviations:

ADAM: A Disintegrin And Metalloproteinase

ADAM-8$^{-/-}$: ADAM-8 deficient mice

ADAM-8$^{+/+}$: ADAM-8 wild-type mice

sADAM-8: soluble form of ADAM-8

Summary

Asthma is a complex disease linked to various pathophysiological events including the activity of proteinases. The multifunctional **A D**isintegrin **A**nd **M**etalloproteinases (ADAMs) displaying the ability to cleave membrane-bound mediators or cytokines appear to be key mediators in various inflammatory processes. In the present study, we have investigated ADAM-8 expression and production in a mouse model of allergen-induced airway inflammation. In allergen-exposed animals, increased expression of ADAM-8 was found in the lung parenchyma and in dendritic cells purified from the lungs. The potential role of ADAM-8 in the development of allergen-induced airway inflammation was further investigated by the use of an anti-ADAM-8 antibody and ADAM-8 knock-out animals. We observed a decrease in allergen-induced acute inflammation both in BALF and the peribronchial area in anti-ADAM-8 antibody-treated mice and in ADAM-8 deficient mice (ADAM-$8^{-/-}$) after allergen exposure. ADAM-8 depletion led to a significant decrease of the $CD11c^{+}$ lung dendritic cells. We also report lower levels of CCL11 and CCL22 production in antibody-treated mice and ADAM-$8^{-/-}$ mice that might be explained by decreased eosinophilic inflammation and lower numbers of dendritic cells, respectively. In conclusion, ADAM-8 appears to favour allergen-induced acute airway inflammation by promoting dendritic cell recruitment and CCL11 and CCL22 production.

Introduction

Asthma is mainly characterized by chronic bronchial inflammation and hyperresponsiveness [1, 2]. The disease phenotype in humans encompasses generally repeated episodes of non permanent airway obstruction along with wheezing, breathlessness and cough. Mouse models of asthma mimic most of these human asthma features and provide valuable tools for deciphering disease mechanisms [3]. Such mouse models have proven valuable for elucidating the implication of specific gene products, such as matrix proteinases. Therefore, several research groups have studied the asthmatic phenotype by using knock-out animals and reported that MMP-9 depleted mice display a lower cell infiltration and bronchial responsiveness upon allergen exposure [4]. In sharp contrast, MMP-8 deficiency promotes a neutrophilic inflammation in the airways [5] and MMP-2 deficiency induces inflammatory cell accumulation in lung parenchyma responsible for asphyxia in some animals [6].

ADAMs (**A D**isintegrin **A**nd **M**etalloproteinases) belong to the metzincin superfamily of proteinases. The typical structure of ADAM proteinases consists of metalloproteinase, disintegrin, and cytoplasmic domains which endow the protein with catalytic activity, adhesion properties, and potentially signaling functions [7-10]. Light was shed on the potential role of ADAM proteinases in asthma by a genetic association study demonstrating a link between increased risk of asthma development and polymorphisms in the ADAM-33 gene [11]. It was also reported that ADAM-33 gene expression correlates with asthma severity [12]. ADAM-8 is another member of the ADAM family displaying some structural similarities with ADAM-33. ADAM-8 has been first cloned from mouse macrophages [13] and is expressed at baseline or after stimulation by a wide range of cell types including neurons,

osteoclasts, bronchial epithelial cells and cells of the immune system (eosinophils, polymorphonuclear leukocytes (PMN), monocytes, macrophages, dendritic cells and B lymphocytes) [13-17]. ADAM-8 is implicated in various complex biological processes such as neurodegenerative inflammation and osteoclastogenesis [18, 19]. Moreover, two distinct studies based on a wide genome microarray have shown that ADAM-8 mRNA expression appears to be increased in asthma [20, 21]. More recently, transgenic mice overexpressing a soluble form of ADAM-8 (sADAM-8) in liver tissue showed less cellular infiltration in an allergen-induced model of lung inflammation, suggesting that sADAM-8 might play a role as inflammation inhibitor [22]. Known biological functions of ADAM-8 are to date restricted to cell fusion and adhesion [23]. ADAM-8 also appears to be a sheddase and cleaves CD23, neural cell adhesion molecule close homologue of L1 (CHL1), beta-amyloid precursor protein (APP), L-selectin, vascular cell adhesion molecule-1 (VCAM-1), and myelin basic protein (MBP) while there is also evidence for a possible cleavage of pro-tumor necrosis factor (TNF) α and CD16 [24, 25] rendering plausible a role as inflammation modulator for this proteinase [25-27]. However, the question of whether ADAM-8 is involved in the development of asthma phenotype remains unanswered.

In this study, we demonstrate through both genetic and antibody-based approaches that ADAM-8 is a key mediator for the development of asthma-linked inflammation. We describe that ADAM-8 depletion (transgenic mice deficient for ADAM-8) and injected antibodies raised against ADAM-8 impair the development of airway inflammation after allergen exposure. We also explored the mechanisms implicating ADAM-8 and report that ADAM-8 depletion modifies dendritic cell recruitment and production of key cytokines contributing to the network of interactions leading to eosinophilic inflammation.

Results

ADAM-8 expression and production in lungs of mice acutely exposed to allergens

In order to investigate ADAM-8 expression in an experimental model of asthma, male Balb/c mice were sensitized and subsequently exposed to ovalbumin (OVA) for 5 days. As expected, mice exposed to OVA showed higher BALF eosinophil percentages and peribronchial inflammation as compared to mice exposed to PBS (eosinophil percentage: 1.700±1.361 in sham-exposed mice *versus* 56.187±6.255 in allergen-exposed mice, $p<0.0001$; inflammation score: 0.325±0.067 in sham-exposed mice *versus* 1.161±0.084 in allergen-exposed mice, $p<0.0001$). ADAM-8 was significantly upregulated at mRNA level in lung parenchyma of mice acutely exposed to allergens as compared to controls (figure 1A and C). Moreover, ADAM-8 protein production was also significantly increased in OVA-exposed mice as assessed by Western blotting and immunohistochemistry (figure 1B, D and E). Interestingly, the immunohistochemical analysis revealed that ADAM-8 stained cells mostly correspond to inflammatory cells such as eosinophils and macrophages located in the peribronchial area (figure 1E).

ADAM-8 expression and production in chronic allergen-induced remodeling

In order to further investigate ADAM-8 expression and production in a chronic remodeling model of asthma, male Balb/c mice were sensitized and subsequently exposed to OVA for 5 days per week (odd weeks) from days 22 to 96 (eosinophil percentage: 4.033±0.704 in sham-exposed mice *versus* 24.600±1.613 in allergen-exposed mice, $p<0.0001$; inflammation score: 0.593±0.096 in sham-exposed mice *versus* 1.129±0.061 in allergen-exposed mice, $p<0.05$; glandular hyperplasia expressed in positive cells percentage: 0.062±0.062 in sham-exposed mice *versus*

33.640±4.363 in allergen-exposed mice, $p<0.0001$; basement membrane collagen: 0.654±0.046 in sham-exposed mice *versus* 1.444±0.070 in allergen-exposed mice, $p<0.0001$). In this long-term model, mRNA levels of ADAM-8 were only poorly detectable (figure 1A) and the ADAM-8 protein production was very weak (figure 1B). Moreover, no significant difference between mice exposed to allergen or not was observed by RT-PCR and Western blotting.

ADAM-8 blockade by antibody treatment

In order to assess the implication of ADAM-8 in asthma-related acute inflammation, Balb/c mice were treated with an anti-ADAM-8 antibody for 3 days while a control isotype was injected to control Balb/c mice. Two hours after antibody injection, mice were exposed to OVA by aerosols. As shown in table 1 and figure 2A, eosinophil percentages in BALF were significantly decreased upon anti-ADAM-8 antibody treatment as compared to control mice treated with control isotype ($p<0.05$). In peribronchial areas, acute allergen-induced inflammation was also decreased in anti-ADAM-8-treated mice (inflammation score: 1.080±0.160 in mice treated by control isotype *versus* 0.500±0.150 after anti-ADAM-8 antibody). Peribronchial eosinophilic inflammation was also significantly lower in anti-ADAM-8-treated mice ($p< 0.0005$) (figure 2B). Amounts of detectable ADAM-8 protein were strongly decreased in lung parenchyma of anti-ADAM-8-treated mice as assessed by Western blot (figure 2C). Moreover, decrease of eosinophilia BALF was assessed in both Th_2-prone mice (Balb/c) and non Th_2-prone (C57Bl/6) and found to be similar (figure 2D).

Acute allergen-induced inflammation in ADAM-8$^{-/-}$ animals

We also applied the allergen-induced acute inflammation model to ADAM-8 deficient mice (ADAM-8$^{-/-}$) and wild-type counterparts (ADAM-8$^{+/+}$). After allergen exposure,

BAL displayed an increase of eosinophil counts in ADAM-$8^{+/+}$ while allergen-induced eosinophil BAL infiltration expressed as absolute value or percentage was lower in ADAM-$8^{-/-}$ mice ($p<0.0005$) (table 2). Bronchial inflammation was also studied by histology. Upon allergen exposure, ADAM-$8^{-/-}$ mice displayed far less peribronchial inflammation than ADAM-$8^{+/+}$ mice ($p<0.0005$) (figure 2 E-H and K). Peribronchial tissue was specifically evaluated regarding eosinophil infiltration by histology and specific staining. After allergen exposure, eosinophil counts in airway walls were 5 times lower in ADAM-$8^{-/-}$ mice as compared to ADAM-$8^{+/+}$ ($p<0.0005$) (figure 2 I-J and L).

Cytokine measurements by protein arrays and ELISA

As cytokines are key mediators in the development of the asthmatic phenotype, a cytokine array was performed on lung tissues in ADAM-$8^{-/-}$ mice and WT mice after acute allergen exposure. Among measured cytokines, CCL11 and CCL22 levels were found to be significantly lower after allergen exposure in ADAM-$8^{-/-}$ as compared to ADAM-$8^{+/+}$ (data not shown). These data were confirmed by ELISA ($p<0.0005$) (figure 3A-B). In mice exposed to OVA, CCL22 levels were positively correlated with eosinophil counts in the lung tissues ($r=0.7598$ and $p=0.0004$, (figure3E)). The modulation of CCL11 and CCL22 by ADAM-8 depletion has also been observed in our study based on ADAM-8 blockade by antibody injection ($p<0.05$) (figure 3C-D). Prototypical Th_2 cytokines IL-4, IL-5 and IL-13 have been assessed by ELISA in whole lungs extracts and did not display significant changes upon ADAM-8 depletion (figure 3 F-H).

Determination of dendritic cells recruitment in lung parenchyma in ADAM-8$^{-/-}$ animals

As CCL22 is mainly secreted by dendritic cells and macrophages, an immunohistochemistry against CD11c and F4/80, respectively was performed on lung tissues in ADAM-8$^{-/-}$ mice as compared to ADAM-8$^{+/+}$ after allergen exposure. Numbers of dendritic cells and alveolar macrophages positive for this staining were significantly decreased in ADAM-8$^{-/-}$ mice as compared to ADAM-8$^{+/+}$ after allergen exposure (figure 4 A-D). This suggests that lower levels of CCL22 are the consequence of a significant decrease of the two most important CCL22-producing cells in lung parenchyma from ADAM-8 deficient mice exposed to allergens.

ADAM-8 expression in lung dendritic cells

In order to verify the potential implication of ADAM-8 in immunological processes leading to asthma-related acute inflammation, we performed a RT-PCR on mRNA from dendritic cells extracted from lungs of mice acutely exposed to allergens. Levels of mRNA coding for ADAM-8 were higher in allergen-exposed mice as compared to control mice (figure 4E-F), suggesting that ADAM-8 might be a key regulator of dendritic cells behavior. Additionally, ADAM-8 expression was assessed by RT-PCR in other major cellular sources in the lung such as epithelial cells, eosinophils, alveolar macrophages and interstitial macrophages (Figure 4G).

Discussion

We designed the present study to investigate the potential role of the metalloproteinase ADAM-8 in allergen-induced airway diseases. Through genetic and pharmacological approaches in two different strains of mice (Balb/c, Th_2-prone and C57Bl/6, Th_1-prone), we report that ADAM-8 significantly contributes to the establishment of acute allergen-induced inflammation in asthma. This novel concept is based on the findings that anti-ADAM-8 antibody-treated mice and ADAM-8-deficient mice displayed significantly decreased levels of allergen-induced acute inflammation in BAL and in peribronchial areas. We also report for the first time that ADAM-8 is overexpressed in lung dendritic cells after allergen exposure. Moreover, we found increased levels of CCL11 and CCL22 after allergen exposure in ADAM-$8^{+/+}$ mice while these two cytokines failed to increase in ADAM-$8^{-/-}$ animals displaying therefore levels similar to sham-exposed mice for those two cytokines. We also reported a decrease of CCL11 and CCL22 production in anti-ADAM-8-treated mice. To explore the mechanism leading to a decrease of CCL22, we measured the numbers of dendritic cells and alveolar macrophages in the lung parenchyma and described a significant decrease of those two cell types in lungs of ADAM-$8^{-/-}$ animals. Moreover, CCL22 levels were highly correlated to eosinophilic inflammation in allergen-exposed animals. Finally, we also demonstrate that the expression and production of ADAM-8 proteinase were not chronically modulated in chronic asthma and therefore could not play significant roles in remodeling processes linked to asthma.

These novel findings are in accordance with emerging data on the implication of ADAM-8 in the pathogenesis of pulmonary diseases [28] and suggest that ADAM-8 might be a key regulator of dendritic cells and alveolar macrophages homeostasis.

From these data, one can speculate that ADAM-8 could play a significant role in asthma. This assumption is supported by the observation of increased levels of ADAM-8 mRNA expression in induced sputum cells and endobronchial biopsies from human asthmatics [12, 29] and previous authors have reported that ADAM-8 is overexpressed in mouse models of asthma. We demonstrate that ADAM-8 depletion modifies asthma phenotype. However, the precise mechanisms that could account for this ADAM-8 overexpression in asthma and its role as an effector are not yet completely dissected. Our data suggest that ADAM-8 might be essential for dendritic cells accumulation in lung parenchyma although other mechanisms such as a relationship between ADAM-8 and several interleukins (IL-4, IL-13) or related intracellular activation pathways (STAT-6) might also interfere with bronchial inflammation [21]. A genomic study recently reported a link between ADAM-8 single nucleotide polymorphisms and asthma in humans [30]. In addition, two separate genome-wide searches performed on mice subjected to allergen exposure discriminated ADAM-8 as a valid marker of allergen-induced inflammation [20, 21]. Interestingly, one of these studies compared different durations of exposure to allergens and reported that ADAM-8 was overexpressed only in acute models while ADAM-8 overexpression was not reported after chronic allergen exposures [20]. In line with this recent study of Di Valentin et *al* who performed microarray studies, we emphasized the role of ADAM-8 in acute inflammation and not in chronic remodeling associated to asthma. Moreover, this is in keeping with the findings of Matsuno et *al* who showed that ADAM-8 levels are increased in patients suffering from acute eosinophilic pneumonia but not chronic idiopathic eosinophilic pneumonia [31]. In our study, we report that there is less eosinophilia in the BAL in ADAM-8 KO mice. As a

consequence of these huge variations in eosinophil counts, we also found that total cell counts were significantly decreased in ADAM-8 KO animals.

Since there is no difference in features of chronic remodeling established between ADAM-8 deficient mice and wild-type mice exposed to allergens, we hypothesize that ADAM-8 is only implicated in acute airway inflammation. ADAM-8 cleaves various key molecules in inflammatory processes including CD23, pro-TNF-α and L-selectin [25, 26]. From this substrate specificity, it can be deduced that ADAM-8 might be an effector in immunological processes [22, 29]. However, ADAM-$8^{-/-}$ mice did not display any developmental abnormalities and their immune system was previously reported as not impaired by ADAM-8 gene deletion [32]. We verified that unchallenged ADAM-$8^{-/-}$ mice bear normal eosinophil percentages in their blood by studying blood smears (data not shown).

Our experiments using ADAM-$8^{-/-}$ animals and anti-ADAM-8 treatment demonstrate that ADAM-8 is *per se* responsible for a significantly increased allergen-induced acute inflammation both in lung tissue and BAL. In order to rule out the potential role of mice genetic background in our results, we compared the Th_2-prone strain Balb/c with Th_1-prone C57Bl/6 in the antibody-blocking experiment and we report similar decreases of lung inflammation. This validates our study on ADAM-8 genetically depleted mice performed on a Th_1-prone background (129/Ola; C57Bl/6 mixed genetic background as most of KO mice). These experiments allow us to demonstrate that the effects of ADAM-8 depletion reported in the present study are not restricted to a specific strain and probably correspond to a conserved mechanism. In a first approach, our results could seem in apparent discrepancy with those generated by Matsuno et *al* [22] using mice overexpressing soluble ADAM-8 in the liver. Indeed, these authors reported that sADAM-8 overexpressing mice were

protected from the development of histological inflammation when subjected to asthma induction (noteworthy, these authors did not find any variation of BAL inflammation) [22]. However, transgenic mice used by these previous authors express normal levels of membrane-bound ADAM-8 in their tissues and have an excess of sADAM-8 in their plasma, released from the liver, rendering the final picture far more complex than targeted gene deletion. Moreover, one cannot exclude the possibility that, in this setting, sADAM-8 might display some specific biological activities different from membrane-bound ADAM-8.

Mechanisms that could explain such a decrease in eosinophils as we observed in ADAM-$8^{-/-}$ and anti-ADAM-8-treated mice could be linked to a decreased production of CCL11 and CCL22, two chemo-attractants for eosinophils [33, 34]. CCL22, referred to as macrophage-derived chemokine (MDC), also takes part to Th2 inflammation. Interestingly, immunostainings performed in humans and mice suggested that cells producing ADAM-8 are mostly eosinophils [21, 29]. As CCL11 (eotaxin-1) is mainly secreted by eosinophils, a significant decrease of those cells, as reported in the present study might account *per se* for lower levels of CCL11 found after ADAM-8 inhibition. By contrast, CCL22 is mostly produced by macrophages and dendritic cells [35, 36] and appears to play a central role in allergen-induced inflammation since it is produced by Th_2-induced dendritic cells [37]. As demonstrated in this study, number of macrophages and dendritic cells present in lung tissue in allergen-exposed ADAM-$8^{-/-}$ mice is lower than ADAM-$8^{+/+}$ mice.

Very recently, other authors have reported that ADAM-8 is detectable in bronchial epithelium and smooth muscle cells [38]. In contrast with these observations, our own findings, in line with those of King et *al* who performed in situ hybridization, indicate that ADAM-8–expressing cells are mostly eosinophils and macrophages [21]. This

might be the consequence of technical issues (e.g. antibodies used by previous authors are different). However, one cannot exclude that ADAM-8 expressed by structural cells might play a significant role in the modulation of inflammation. One argument to state that stromal cells such as fibroblasts probably do not participate in the ADAM-8 production in an important proportion is that immunohistochemistry performed on lung sections do not display an important staining in these cells. In the present study, we measured the ADAM-8 expression by alveolar macrophages and interstitial macrophages since those two subtypes have recently been described to play very different roles in asthma pathology. We found that these two subtypes express ADAM-8 without obvious difference.

Our results are in accordance with a very recent paper published on-line during the revision period of this manuscript and reporting that genetic depletion of ADAM-8 is protective against main features of asthma [39].

We conclude that ADAM-8 is not only a marker of allergen-induced acute inflammation but probably also an effector since its depletion impairs the development of eosinophilic inflammation through decreasing lung dendritic cells and alveolar macrophages and modulating CCL11 and CCL22 production.

Material and Methods

Experimental asthma protocol

Six to 8 weeks old Balb/c male mice were provided by the animal house of the University of Liège (Belgium). ADAM-8 deficient mice and corresponding wild-type mice were kindly provided by Dr. Andrew Docherty (UCB-Celltech, Slough, UK). ADAM-8 knock-out (KO) mice display a 129/Ola; C57/BL/6 mixed genetic background and only brethren were used. We used only male ADAM-8 KO mice or controls in our experimental procedures. In order to perform a genotyping to ensure that animals were homozygous KO or homozygous WT before entering our experimental protocols, DNA was obtained from mice tails by "Machery-Nagel Nucleospin®Tissue" kit. Primer pair used to distinguish KO and WT is: CACTGTTGGACTGGCTAAGGTG and GACATCGGTAACATTGGTCAG. Protocols used in this study were approved by the Ethical Committee (University of Liège, Belgium). Concerning the acute inflammation model, male mice were sensitized by intraperitoneal injections of 10 µg OVA (Sigma Aldrich, Schnelldorf, Germany) complexed with aluminium hydroxide (Perbio, Erembodegem, Belgium) on days 0 and 7. From days 21 to 25, mice were challenged daily by OVA 1% aerosols for 30 minutes. In the control group, mice were exposed to PBS aerosols. Mice were sacrificed 24 hours after the last allergen exposure. For the remodeling model, mice were sensitized by intraperitoneal injections on days 0 and 11, and groups of mice were exposed to OVA or PBS aerosols 5 days per week (odd weeks) from days 22 to 96. As described in the "acute inflammation model", mice were sacrificed 24h after the last allergen exposure. We used OVA from one single batch that has been characterized regarding endotoxin content. We have performed endotoxin measurement (Lonza, Braine-l'Alleud, Belgium). Mean measured endotoxin levels in

the current batch of OVA are 0.236 ng of endotoxin/gr of OVA. We also ensured that PBS used in our experiment was endotoxin-free (manufacturer specifications indicate no detectable levels of endotoxin).

ADAM-8 blockade by antibody treatment

On days 0 and 7, male Balb/c and C57Bl/6 mice were sensitized by intraperitoneal injections of 10 µg OVA (Sigma Aldrich, Schnelldorf, Germany) complexed with aluminium hydroxide (Perbio, Erembodegem, Belgium). From day 21 and for 3 consecutive days, mice were exposed to OVA for 30 minutes. Two hours before each allergen challenge, intravenous injection was performed with either 25 µg anti-ADAM-8 antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) or control IgG antibody (R&D Systems, Minneapolis, MN, USA). Mice were sacrificed 24 h after the last allergen exposure.

Bronchoalveolar lavage fluid (BALF)

After sacrifice, a canula was inserted in the trachea of the mouse and BALF was performed manually by instillation of 4x1ml of PBS-EDTA 0.05mM (Calbiochem, Darmstadt, Germany). BAL was subjected to centrifugation for 10 minutes at 4°C and at 282 x *g*. Supernatants were stored at -80°C for future assessments while cell pellets were resuspended in 1ml PBS-EDTA 0.05mM in order to proceed with total and differential cell counts. Total cell number was measured by using a Z2 coulterX® particle count and size analyzer (Beckman Coulter, Analis, Namur, Belgium) and differential cell count was assessed by a skilled observer blinded to experimental details, based on morphological criteria. For this purpose, cells were centrifuged (cytospin) on a slide and stained with Diff Quick® (Dade, Brussels, Belgium).

Lung tissue processing

After recovering the BAL, thorax was opened and left lung was clamped. The right lung was cut and snap frozen in liquid nitrogen for RNA and protein extraction whereas the left lung was insufflated at constant pressure with 4% paraformaldehyde and then embedded in paraffin for histological analysis.

Histological analysis

Histological analyses were performed on 5 μm lung tissue sections. Sections were stained with hematoxylin and eosin and a peribronchial inflammation score was applied to each slide as previously reported [5]. Briefly, score 0 was assigned for bronchi with no mononuclear cell infiltration; score 1 corresponded to few mononuclear cells, score 2 was assigned if there were from 1 to 5 layer(s) of inflammatory cells around bronchi, and score 3 was chosen when thickness of inflammatory cells layer was > 5 cells and surrounded the entire bronchus. Six bronchi per mice were counted and statistical analysis was performed by using GraphPad Program. In order to visualize peribronchial eosinophilic infiltration, Congo Red staining was performed on slides. Peribronchial eosinophils were counted for 6 bronchi/mouse and those counts were reported to the perimeter of basal membrane epithelium measured with ImageJ Program. Statistical analysis was carried out using GraphPad software. Glandular cells were assessed using the Perodic Acid Shiff (PAS) staining. In order to detect peribronchial collagen deposition, Masson's Trichrome staining was carried out.

For ADAM-8 protein detection, slides were deparaffinized and, after treatment with Target Retrieval Solution (Dako, Glostrup, Denmark), endogenous peroxidases were blocked with H_2O_2 3% (Merck, Darmstadt, Germany). Slides were then incubated with goat anti-ADAM-8 antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA,

USA) diluted at 1:500. After rinsing, rabbit anti-goat biotin coupled secondary antibody was applied on the slides followed by incubation with streptavidin/HRP (horse radish peroxydase) complex (Dako, Glostrup, Denmark). Peroxidase activity was revealed using the 3-3' diaminobenzidine hydrochlorid kit (DAB, Dako, Glostrup, Denmark).

For dendritic cells and alveolar macrophages detection, slides were deparaffinized and, after treatment with trypsin 0.1% (Sigma-Aldrich, Schnelldorf, Germany), endogenous peroxidases were blocked with H_2O_2 3% (Merck, Darmstadt, Germany). Slides were then incubated with hamster anti-CD11c (Abcam, Cambridge, UK) diluted 1:50 or rat anti-F4/80 antibody (Serotec, Düsseldorf, Germany) diluted at 1:100. After rinsing, goat anti-hamster or rabbit anti-rat biotin coupled secondary antibody was applied on the slides followed by incubation with streptavidin/HRP (horse radish peroxydase) complex (Dako, Glostrup, Denmark). Peroxidase activity was revealed using the 3-3' diaminobenzidine hydrochlorid kit (DAB, Dako, Glostrup, Denmark).

Lung RNA/protein extraction

Tissues were disrupted and completely homogenized forming a powder by a combination of turbulence and mechanical shearing and total RNA was extracted using RNeasy Mini kit following manufacturer's instructions (Qiagen, Gmbh D, Hilden, Germany). Total protein extracts were prepared by incubating crushed lung tissues in a 2M urea solution. Tissue lysates were centrifuged for 15 minutes at 16,100 x *g*.

Cell isolation

To obtain single-lung-cell suspensions, lungs were perfused with 5 ml PBS through the right ventricle, cut into small pieces, and digested for 1 hour at 37°C in 1 mg/ml collagenase A (Roche, Mannheim, Germany) and 0.05 mg/ml DNaseI (Roche, Mannheim, Germany) in HBSS. Lung DCs were isolated using criteria of $CD11c^{+}/F4/80^{-}$ cells, as previously described by Bedoret et *al* [40]. Cells were sorted by flow cytometry (FACSAria). The purity of isolated dendritic cells is about 91%. Alveolar macrophages (AM) and interstitial macrophages (IM) were isolated using following criteria: $CD11c^{+}/F4/80^{+}$ cells, and $CD11c^{-}/F4/80^{+}$ cells, respectively. Eosinophils were isolated using criteria $GR1^{+}/CCR3^{+}$ while epithelial cells were isolated using laser capture microdissection (LCM) system (Leica).

Semi-quantitative RT-PCR

ADAM-8 mRNA expression levels were investigated by semi-quantitative RT-PCR using the GeneAmp thermostable RNA RT-PCR kit (Applied Biosystems, Foster City, CA, USA). The design of oligonucleotides for ADAM-8 was based on the sequence available in the Genbank: 5'-ACATTGGTCAGGCAGCCTGTCT-3' (antisense) and 5'-CTGTGAATCAGGACCACTCCAA-3' (sense). The specificity of the selected sequences was verified using the NCBI BLASTN program (http://www.ncbi.nlm.nih.gov/BLAST/) and oligonucleotides were obtained from Eurogentec (Seraing, Belgium). RT was performed on 10 ng total RNA at 70°C during 15 minutes. PCR amplification conditions were optimized so that PCR products do not reach any saturation levels. Amplification started at 94°C for 15 seconds, 64°C for 20 seconds, and 72° for 10 seconds for 28 cycles, followed by 2 minutes at 72 °C. Products were then resolved on polyacrylamide gels (10%) and stained with Gel Star (Biowhittaker, Rockland, MD, USA). Analysis of the intensity of band was realized using Quantity One software (Biorad, Hercules, CA, USA). To

normalize mRNA levels in different samples, the value of the band corresponding to each mRNA level was divided by the intensity of the corresponding 28S rRNA band. In order to verify the specificity of amplification, PCR products were digested with appropriate restriction enzymes.

Western Blotting

Total protein extracts (20µg) were separated under reducing conditions on 12% polyacrylamide gels and transferred on PDVF membranes (Perkin Elmer Life Sciences, CA, USA). PVDF membranes were then blocked with PBS containing 10% milk and Tween 20 (0.1%) (Merck, Darmstadt, Germany). The primary antibody anti-ADAM-8 (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) diluted at 1:1500 was applied on membranes overnight at 4°C. After several washes, proteins were incubated with the secondary antibody conjugated with HRP (rabbit anti-goat) diluted at 1:2000 for 1 hour at room temperature (RT). The enhanced chemiluminescence (ECL) detection kit (Perkin Elmer Life Sciences, Boston, MA, USA) allowed visualization of immunoreactive proteins. Results were expressed as ratio between measured protein levels and corresponding actin levels used as loading control.

Cytokine Array

Detection of multiple cytokines in tissue lysates was achieved using the RayBiotech kit (RayBio Mouse Cytokine Antibody Array C series 1000, RayBiotech, Inc Norcross, GA, USA). Succinctly, array membranes were incubated with samples (pooled ADAM-8 KO OVA proteins (n=5) versus pooled ADAM-8 WT OVA proteins (n=5)). Then, a cocktail of biotin-labeled antibodies was applied on the membranes binding cytokines. Finally, array membranes were incubated with HRP-conjugated streptavidin. Signals were detected using ECL detection kit.

ELISA

ELISA measurement of CCL11 and CCL22 was performed on lung tissue lysates of anti-ADAM-8-treated mice and corresponding control mice but also ADAM-8 KO and corresponding wild-type mice. Briefly, microwell strips were coated with an anti-CCL11 or an anti-CCL22 murine monoclonal antibody (R&D Systems, Minneapolis, MN, USA) overnight. Samples were then added to the wells and incubated with the biotinylated antibody (R&D Systems, Minneapolis, MN, USA) for 2 hours at room temperature (RT). HRP-conjugated streptavidin was applied on samples before signal revelation.

Statistical analysis

Results were expressed as mean ± SEM. Statistical test was assessed on experimental groups using Mann-Whitney test. Correlations were sought by calculating Spearman's coefficient of correlation. These tests were performed using GraphPad InStat software (http://www.graphpad.com/instat). Graphs were obtained using GrahPad Prism software (http://www.graphpad.com/prism). P values < 0.05 were considered as significant.

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Conflict of interest

The authors declare that they have no competing interests.

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Figure legends and Tables

Figure 1: ADAM-8 expression and production in the lungs of mice acutely exposed to an allergen and after long-term allergen exposure in the chronic remodeling model.

(A) A representative example of semi-quantitative RT-PCR for ADAM-8 in whole lung homogenates in acute inflammation and chronic remodeling models (Balb/c mice) induced by OVA; PBS serves as a control. (B) A representative Western blot of ADAM-8 production in acute inflammation and chronic remodeling models (Balb/c mice). Actin serves as a loading control. (C) ADAM-8 mRNA levels in the lungs of mice exposed to OVA (n=15) and control PBS-treated mice (n=16) in an acute inflammation model. Results are expressed as mean ± SEM. *** $p < 0.0005$, Mann Whitney test. (D) ADAM-8 protein levels in the lungs of mice exposed to ovalbumin (OVA) (n=6) and control PBS-treated mice (n=6) in an acute inflammation model quantified by densitometrically scanning Western blots shown in (B). Results are expressed as mean ± SEM. * $p < 0.05$, Mann Whitney test. (E) Anti-ADAM-8 immunostaining in lung tissue after acute allergen exposure (magnification, X200). ADAM-8 expression is stained in brown and the peribronchial area is indicated by an arrow. Each experiment (acute inflammation and remodeling models) was performed twice with n=6 to 8 mice per group per experiment.

Figure 2: Impact of the anti-ADAM-8 antibody and depletion of ADAM8 on acute allergen-induced inflammation in mice.

(A-C) Balb/c or (D) Balb/c and C57Bl/6 mice were sensitized and exposed to OVA 2 hours after treatment with either IgG isotype control (n=6) or anti-ADAM-8 antibody (n=6). (A) Eosinophil counts presented as the percentage of eosinophils within 300 cells of the BALF of mice after treatment with anti-ADAM-8 antibody; mean ± SEM. *

p< 0.05, Mann Whitney test. (B) Peribronchial eosinophilic infiltration; mean ± SEM. *** p< 0.0005, Mann Whitney test. (C) A representative Western blot of ADAM-8 production in whole lung homogenates from mice treated with an anti-ADAM-8 antibody or an isotype control. (D) Relative inhibition of eosinophilia by anti-ADAM-8; mean ± SEM (A-D) Data are representative of two independent experiments.

(E-J) Histological analysis of lung tissues in ADAM-8-deficient (KO) mice acutely exposed to an allergen (OVA). (E-H) Representative paraffin sections after hematoxilin-eosin staining (magnification, 200x). (I-J) Representative paraffin section of lung tissue showing bronchi after congo red staining (magnification, 400x). The red staining (arrow) shows eosinophil cells. (K) Quantification of peribronchial inflammation score in the lung samples of WT and KO mice,acute inflammation model (n=8 mice per group); mean ± SEM. *** p< 0.0005, Mann Whitney test. (L) Quantification of eosinophil numbers normalised to bronchial perimeter expressed as mm basement membrane in lung samples of all in WT and KO mice, acute inflammation model (n=8 mice per group); mean ± SEM *** p< 0.0005, Mann Whitney test.

Each experiment of allergen-induced inflammation has been performed twice (n=8 mice per group in each experiment.

Figure 3: Measurements of CCL11 and CCL22 levels in ADAM-$8^{+/+}$ and ADAM-$8^{-/-}$ mice. (A-D) CCL11 and CCL22 levels in whole lung homogenates in ADAM-8 KO mice (n=8 mice per group) and anti-ADAM-8-treated mice (n=6 mice per group) acutely challenged with OVA as determined by ELISA.; mean ± SEM, * p< 0.05; ** p<0.005; *** p<0.0005, Mann Whitney test. (E) Relationship between lung tissue CCL22 levels and lung eosinophilic infiltration in ADAM-$8^{+/+}$ and ADAM-$8^{-/-}$ mice

exposed to ovalbumin; r=0.7598 p=0.0004 (Spearman correlation coefficient). (F-H) Levels of the main Th_2 cytokines are not modulated in whole lung homogenates in ADAM-8 KO animals. Results are expressed as mean ± SEM (Mann Whitney test).

Each experiment has been performed in duplicate.

Figure 4: Recruitment of dendritic cells and alveolar macrophages in lung parenchyma. (A) A representative staining against F4/80 (magnification, 400x) and two representative examples of staining against CD11c (magnification, 400x) in alveolar (left) and peribronchial (right) areas in ADAM-$8^{+/+}$ mice exposed to OVA. Positive staining is shown in brown. (B, C) Quantification of dendritic cell and macrophage numbers in lung parenchyma expressed in cell number per field (n=8 in each group); Results are expressed as mean ± SEM, * p< 0.05; ** p< 0.005, Mann Whitney test. (D) The percentage of dendritic cells among the total cell population recovered in the whole lung homogenates as determined by flow cytometry (right panel). Data are mean ± SEM, * p< 0.05; ** p< 0.005, Mann Whitney test (n=8). Representative flow cytometry plots of $CD11c^{+}/F4/80^{-}$ cells for ADAM-8 KO mice after allergen exposure (left panel, percentages indicated). (E) Representative ADAM-8 mRNA expression as determined by RT-PCR using dendritic cells from the lungs of mice acutely exposed to an allergen (OVA). (F) ADAM-8 mRNA expression in lung dendritic cells in acute inflammation model (n=4 mice). Results are expressed as mean ± SEM. ** p< 0.005, Mann Whitney test. (G) ADAM-8 mRNA expression in EC (epithelial cells), EO (eosinophils), AM (alveolar macrophages) and IM (interstitial macrophages) isolated from the lung of Balb/c mice.

Two independent experiments have been performed for every experimental condition.

Table : Cell counts in bronchoalveolar lavages in anti-ADAM-8-treated mice (1) and in ADAM-8 deficient mice (2).

1

	Isotype control	*Anti-ADAM-8*
epithelial cells (%)	10.250±1.438	11.114±1.962
eosinophils (%)	23.133±3.234	13.400±1.807*
neutrophils (%)	0.483±0.158	0.542±1.125
lymphocytes (%)	0.483±0.253	0.257±0.069
macrophages (%)	65.517±3.164	74.543±2.122*
total cells (10^3/ml)	41.855±5.844	32.065±9.626
epithelial cells (10^3/ml)	4.407±0.922	5.301±2.618
eosinophils (10^3/ml)	11.078±2.455	4.982±1.189
neutrophils (10^3/ml)	0.248±0.081	0.166±0.046
lymphocytes (10^3/ml)	0.232±0.156	0.163±0.070
macrophages (10^3/ml)	25.835±3.493	25.947±7.081

Results were expressed as mean ± SEM. * p< 0.05

2

	WT PBS	*WT OVA*	*KO PBS*	*KO OVA*
epithelial cells (%)	12.000±1.872	1.860±0.504***	15.575±1.388	7.589±1.075$^{***, \Delta}$
eosinophils (%)	0.578±0.434	69.750±2.323***	1.525±0.584	20.939±4.886$^{***, \Delta}$
neutrophils (%)	0.001±0.001	0.850±0.432	0.037±0.026	0.672±0.339
lymphocytes (%)	0.033±0.033	0.260±0.137	0.001±0.001	0.211±0.091
macrophages (%)	87.311±1.822	27.180±2.132***	82.394±1.531	70.439±4.416$^{*, \Delta}$
total cells (10^3/ml)	21.872±3.820	145.820±30.394***	44.464±13.122	27.816±2.930***
epithelial cells (10^3/ml)	2.230±0.269	2.919±1.376	5.714±0.953†	2.239±0.397**
eosinophils (10^3/ml)	0.133±0.104	103.660±22.198***	1.100±0.579	6.095±2.089$^{***, \Delta}$
neutrophils (10^3/ml)	0.001±0.001	0.547±0.251	0.049±0.043	0.111±0.050
lymphocytes (10^3/ml)	0.007±0.006	0.362±0.242	0.001±0.003	0.050±0.024
macrophages (10^3/ml)	14.621±3.701	29.594±7.305	30.908±9.810	16.529±2.310

Results were expressed as mean ± SEM. * p< 0.05 *versus* mice exposed to PBS. ** p<0.005 *versus* mice exposed to PBS. *** p<0.0005 *versus* mice exposed to PBS. † P<0.05 *versus* wild-type mice exposed to PBS. $^{\Delta}$ p<0.0005 *versus* wild-type mice exposed to OVA.