

The Metalloprotease-Disintegrin ADAM8 Is Essential for the Development of Experimental Asthma

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Rationale: Expression of the metalloprotease ADAM8 is increased in patients with asthma, but the functional significance of elevated ADAM8 expression in the context of asthma pathogenesis remains elusive.

Objectives: To study development of asthma in ADAM8-deficient mice.

Methods: Ovalbumin-induced asthma was studied in wild-type, ADAM8-deficient, and ADAM8-chimeric mice. Lung inflammation was assessed by histology, analysis of bronchoalveolar lavage, and airway hyperresponsiveness.

Measurements and Main Results: ADAM8-deficient mice are highly resistant to the development of ovalbumin-induced airway inflammation and hyperresponsiveness. ADAM8 expression was induced in both hematopoietic cells and the nonhematopoietic microenvironment after induction of asthma, and ADAM8 expression in both cell populations was required for the full manifestation of asthma. Interestingly, loss of ADAM8 on T cells alone was sufficient to significantly decrease the asthma response. The attenuated response was not due to an intrinsic defect in antigen presentation or cytokine production but reflected an impaired migration of T cells, eosinophils, CD11b⁺ CD11c⁺, and CD11c⁺ cells from blood vessels to the lung and alveolar space, suggesting a general hematopoietic cell deficiency in the absence of ADAM8.

Conclusions: The results show that ADAM8 plays a proinflammatory role in airway inflammation. The milder disease outcome in the absence of ADAM8 suggests that this protein might be an interesting new target in treatment of this, and potentially other, inflammatory diseases in which recruitment of inflammatory cells is an essential part of pathogenesis.

Keywords: asthma; metalloprotease-disintegrin ADAM8; cell recruitment

Asthma is characterized by pulmonary eosinophilia, an imbalance toward a Th2 cytokine response, and an exaggerated increase in airway resistance in response to methacholine (1). Several studies demonstrated involvement of metalloproteases in asthma, suggesting roles in infiltration of lymphocytes and

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The migration of immune cells including T cells, dendritic cells, macrophages and eosinophils into the lung is central to the development of chronic inflammation in asthma. Cell migration is known to be dependent on the activity of many proteases, including ADAM8, a member of the ADAM family of metalloproteases, which is highly expressed in patients with asthma.

What This Study Adds to the Field

Loss of ADAM8 results in protection from airway inflammation and airway hyperresponsiveness in asthma. We show that this metalloprotease is essential for efficient recruitment of inflammatory cells involved in asthma pathogenesis, such as T cells, eosinophils, CD11b⁺ CD11c⁺, and CD11c⁺ cells. Adoptive transfer experiments showed that ADAM8 on hematopoietic cells as well as ADAM8 on nonhematopoietic cells was required for full asthma response, making this enzyme a potential target for pharmaceutical blockage of inflammation in asthma.

eosinophils into airway mucosa (2), in recruitment of Th2 cells into the lung airways (3), and in differentiation of Th2 cells (4). Two members of a disintegrin and metalloprotease family (ADAM) have also been associated with asthma. Increased expression of ADAM8 was observed in mouse models of asthma (5) and patients with asthma (6), and ADAM33 has been identified as a putative asthma susceptibility gene by positional cloning (7).

The ADAMs form a widely expressed family of transmembrane proteins, with 40 members found in various species. They contain multiple functional domains, including a zinc-dependent metalloprotease domain, a disintegrin/cysteine-rich domain, and a cytoplasmic domain with SH3-binding motifs (8). ADAM8, a protease (9–11) activated by an autocatalytic mechanism (11), has been detected in several myeloid lineages, lymphocytes (12–16), and other nonhematopoietic tissues (5, 17–23). Several studies propose a functional involvement of ADAM8 in cell migration (18, 22, 23). Involvement of ADAM8 in immune responses was proposed on the basis of its inducibility by inflammatory stimuli such as IFN- γ , LPS (24), and TNF- α (11). Indeed, ADAM8 expression has been associated with inflammatory diseases such as neurodegeneration (21, 25), tissue inflammation around loosened hip transplants (20), rheumatoid arthritis (15), and pulmonary disease (5, 26–28). Under normal conditions, ADAM8 expression in murine lungs is restricted to epithelial cells of distinct respiratory bronchioles (19). In mouse models of asthma, ADAM8 mRNA expression is strongly induced in the lung (5). Similarly, increased ADAM8

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mRNA levels are found in lung biopsies from patients with both mild and severe asthma (6). However, it remains unclear whether loss of ADAM8 has a pathological or beneficial affect in asthma, and whether it promotes or dampens inflammation in the asthmatic lung (29). ADAM8-deficient mice (ADAM8^{-/-}) have no obvious pathological phenotypes. It was, however, suggested that ADAM8^{-/-} mice might be affected under inflammatory conditions (19).

Here we have analyzed the role of ADAM8 in asthma by inducing disease in ADAM8^{-/-} mice, and found that these mice are highly resistant to asthma. Using bone marrow chimeric mice, we found that ADAM8 expression on both hematopoietic and nonhematopoietic cells contributed to the inflammatory response. Moreover, ADAM8 appears to be required for normal migration of inflammatory cells in the development of asthma.

METHODS

Mice

C57Bl/6 (CD45.2, CD45.1) and IL7R^{-/-} were purchased from Jackson Laboratory (Bar Harbor, ME). IL7R^{-/-} and ADAM8^{-/-} mice (19) have been backcrossed onto C57Bl/6 background. Mice were maintained and bred at the specific pathogen-free animal facility at The Biomedical Research Centre. All animal experiments were performed according to institutional guidelines and approved by the Animal Care Committee of UBC.

Induction and Analysis of Asthma

Asthma was induced and analyzed as previously described (1, 30) via two intraperitoneal injections of 0.2% ovalbumin (OVA) with Al(OH)₃ (both from Sigma-Aldrich; St. Louis, MO) on Days 1 and 8 and intranasal challenges with 0.2% OVA on Days 22, 23, 24, 26, and 28. After mice were killed (Day 29), bronchoalveolar lavage (BAL) was performed by three aspirations of 1.0 ml PBS. For airway hyperresponsiveness analysis, OVA-challenged mice were anesthetized with ketamine/xylazine, tracheotomized, and airway resistance was measured with a Flexivent apparatus (SCIREQ, Montreal, PQ, Canada).

Histology

Formalin-fixed lung tissue was embedded in paraffin, sectioned, and stained with hematoxylin and eosin. A histologic score from 0–5 was blindly attributed (0 = no sign of disease, 5 = most sign of disease) as previously described (1) for perivascular infiltration, peribronchial infiltration, epithelium damage, and parenchymal infiltration, for a potential maximum score of 20.

Flow Cytometry and Cell Sorting

Lungs were digested with 200 U/ml collagenase IV (Sigma-Aldrich) and lung inflammatory cells enriched by Percoll separation. Peripheral blood was drawn from saphenous veins and subjected to red blood cell lysis. Cell suspensions were stained with titrated amounts of antibodies: CD3 (145-2C11; Hybridoma Bank, Iowa City, IA), CD4 (RM4-5; eBioscience; San Diego, CA), CD8 (53-6.7; eBioscience), CD11b (M1/70; Hybridoma Bank), CD11c (N418; Hybridoma Bank), B220 (RA3-6B2; Hybridoma Bank, Iowa City, IA), CD45.1 (A20; eBioscience), CD45.2 (104; eBioscience), IL-4 (11B11; eBioscience, San Diego, CA), CCR3 (83101; R&D Systems, Minneapolis, MN), Gr-1 (RB6-8C5; Hybridoma Bank), EpCAM (G8.8; eBioscience), and IFN- γ (XMG1.2; eBioscience). For intracellular staining, cells were fixed with 1% paraformaldehyde, followed by permeabilization and staining in 0.1% Triton X-100 and 1% fetal calf serum in PBS. β -galactosidase activity was detected using the FluoReporter *lacZ* Flow Cytometry Kit (Molecular Probes, Eugene, OR) according to manufacturer's instructions. Where required, a fixed number of reference beads (Interfacial Dynamics, Invitrogen, Carlsbad, CA) were added to each sample for calculation of cell numbers. Samples were analyzed on a Calibur or LSRII flow cytometer and data analyzed with FlowJo software

(Treestar, Ashland, OR). Cell sorting (eosinophils, CCR3⁺CD3⁺B220⁻; CD11b⁺CD11c⁻ cells; CD11c⁺ cells; T cells, CD4⁺ and CD8⁺; epithelial cells, EpCAM⁺CD45⁻; purities > 95%) was performed on a FACS Vantage or FACS Aria (all from BD Biosciences; San Jose, CA).

Quantitative Real-Time PCR

RNA was extracted using TRIzol solution (Invitrogen, Carlsbad, CA). One microgram of total RNA was reverse transcribed using cDNA Archive Kit (Applied Biosystems, Carlsbad, CA). Gene expression for ADAM8 was quantified using a Duplex PCR analyzing ADAM8 (forward 5'-GGATGCTACTGTCCTGAACCA-3', reverse 5'-GAATATCCTGGGGAAGTGGGA-3') (Probe#83FAM, Universal Probe Library; Roche, Basel, Switzerland) and the endogenous control GAPDH (probe VIC labeled #4352339E; Applied Biosystems) in the same reaction. Reactions were performed using TaqMan master mix. IL-4, IL-5, IL-13 CCL5 and CCL11 expression was performed as described earlier (31, 32) using SYBR green master mix for detection (Fermentas). All reactions were run on a HT7900 real-time PCR system. Relative mRNA expression was calculated using 7000 SDS relative quantification software (all from Applied Biosystems).

Bone Marrow Transfer and Adoptive Transfer

A quantity of 5×10^6 bone marrow cells was injected intravenously into lethally irradiated recipient mice. Twelve weeks after reconstitution, asthma was induced in mice with blood chimerism greater than 80%. A quantity of 1×10^6 enriched T cells from lymph nodes (Easy Sep Mouse T Cell Enrichment Kit, purities >97%; StemCell Technologies; Vancouver BC Canada) was injected intravenously into recipient mice. Four weeks after adoptive transfer, asthma was induced.

In Vitro T Cell Differentiation Assays

A quantity of 2×10^5 splenocytes was activated with anti-CD3 (145-2C11; eBioscience) and anti-CD28 (37.51; eBioscience) (1 μ g/ml each). Th1 polarization: IL-12 (10 ng/ml; eBioscience) + anti-IL-4 (10 μ g/ml, 11B11; eBioscience); Th2 polarization: IL-4 (40 ng/ml; eBioscience) + anti-IFN- γ (10 μ g/ml, XMG1.2; eBioscience). After 3 days, cells were stained for CD4, intracellular IFN- γ , and IL-4 and analyzed by flow cytometry. Cell-free supernatants were analyzed for cytokine secretion with cytometric bead assays (BD Biosciences) or sandwich ELISA (eBioscience).

OVA Recall Assays

A quantity of 5×10^5 total inflammatory lung cells from OVA-challenged mice was stimulated with increasing doses of OVA. After 24 hours, cell-free supernatants were harvested and analyzed for cytokine secretion with cytometric bead assays (BD Biosciences) according to manufacturer's instructions.

Detection of OVA-specific IgG1, IgG2a, and IgE

For detection of OVA-specific serum IgG1 and IgG2a, plates were coated with 2% OVA in sodium carbonate 0.1 M, pH 9.5. Serum was added to the plates for 2 hours at 37°C, rinsed, and the OVA-specific IgGs were detected using biotinylated rat antibody specific for mouse IgG1 and IgG2a (BD Biosciences) respectively, followed by streptavidin coupled to horseradish peroxidase (HRP) and ABTS substrate (Sigma-Aldrich). For detection of OVA-specific IgE, plates were coated with rat anti-mouse IgE (BD Biosciences) and sera were added to wells. Captured OVA-specific IgE was detected using biotinylated OVA and subsequently streptavidin-HRP and ABTS substrate.

Migration Assays

Mice were injected intraperitoneally with chitin and eosinophils from peritoneal lavage counted 40 hours after injections by flow cytometry (33). A quantity of 2×10^5 bone marrow-derived dendritic cells, stimulated overnight with 200 ng/ml LPS (Sigma-Aldrich), or T cells from spleens activated as described above were seeded into the upper chambers of Matrigel (BD Biosciences)-coated 24-well Transwell

chambers (Costar, Lowell, MA). Chemoattractant—100 nM SDF-1 (in-house) and 25 ng/mL CCL5 (Peprotech, Rocky Hill, NJ), respectively—was added to the lower chambers. After 6 (T cells) to 20 (dendritic cells) hours, cell numbers in upper and lower chambers were determined by flow cytometry. Migration efficiencies were calculated by dividing numbers of cells in the lower chamber by the total number of cells in lower and upper chamber. This migration efficiency was divided by the migration efficiency in a control chamber lacking Matrigel and chemoattractant (spontaneous migration). Bone marrow-derived macrophages, unstimulated or previously activated with 250 ng/ml LPS (Sigma-Aldrich) or 100 U/ml TNF- α (Peprotech), were seeded in the upper chambers with medium with higher serum content added to the lower chambers (Biocoat Matrigel Invasion Chambers; BD Biosciences). After 20 hours, invaded cells were fixed with methanol, stained with toluidine blue, and counted.

Statistics

Data are presented as mean values and error bars as SEM. Statistical significance was assessed using unpaired, two-tailed, Student's *t* test with $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ (Figures 1, 3 and 6). Statistics were calculated using statistic software SPSS 16 for Mac (SPSS Inc., Chicago, IL).

RESULTS

Absence of ADAM8 Attenuates Airway Inflammation

A well-described OVA-induced mouse model of asthma (1, 30) was used to verify the role of ADAM8 in the development of asthma. Asthma was induced in wild-type (wt), heterozygous, and ADAM8^{-/-} mice by sensitization with intraperitoneal injections of OVA followed by intranasal OVA challenges. The lung inflammatory response was assessed 1 day after the last intranasal challenge by analysis of BAL content and histologic studies. In ADAM8^{-/-} mice, the total alveolar cell infiltrate was significantly reduced compared with wt mice (Figure 1A), whereas ADAM8 heterozygous mice showed an intermediate phenotype indicating that the number of infiltrating cells correlates with the ADAM8 gene dosage (Figure 1A) (19).

In BAL of OVA-challenged wt mice, approximately half of the infiltrating cells were eosinophils, followed by equal frequencies of macrophages, lymphocytes, and neutrophils. In BAL of OVA-challenged ADAM8^{-/-} mice, all cell types were reduced by at least 50% (Figure 1B). The heterozygous mice showed again an intermediate phenotype that extended to all hematopoietic subsets analyzed with eosinophils reaching significance ($P < 0.05$) and the other cell types showing a trend. These data indicate that airway inflammation induced by OVA is largely attenuated in ADAM8^{-/-} mice compared with wt mice. Viability of recovered cells was verified using Hoechst/PI staining, and was found to be similar in both groups (data not shown). The amount of OVA-specific IgE was approximately 30% lower in the serum of ADAM8^{-/-} mice (Figure 1C), but reached levels that suggested a good sensitization to OVA. Moreover, OVA-specific IgG1 was found to be similar in wt and ADAM8^{-/-} mice, further suggesting that the sensitization to OVA is not deficient in these mice. Finally, serum levels of OVA-specific IgG2a were low in both mouse strains, which is indicative of a normal Th2 response by ADAM8^{-/-} mice.

To confirm that these mice developed less severe tissue inflammation, lungs from naive and OVA-challenged wt and ADAM8^{-/-} mice were sectioned and stained with hematoxylin/eosin and a histologic inflammatory score was blindly attributed (Figure 1D). No significant differences were observed between wt and ADAM8^{-/-} lungs isolated from naive mice. Administration of OVA in wt mice induced vigorous tissue inflammation,

characterized by severe perivascular (PV), peribronchial (PB), and parenchymal (PA) infiltration and damage to the bronchial epithelium (ED), resulting in a mean total histologic score of 14.6 ± 0.94 . ADAM8^{-/-} mice had less severe perivascular and peribronchial infiltration, and an almost complete lack of parenchymal infiltration, resulting in a significantly lower histologic score of 8.7 ± 1.4 ($P = 0.01$; $n = 4$) (Figure 1E). These data clearly demonstrate that ADAM8 deficiency confers protection from asthma in mice.

ADAM8^{-/-} Mice Do Not Develop Airway Hyperresponsiveness

Airway hyperresponsiveness (AHR) is a major hallmark of asthma and known to be linked to the degree and type of airway inflammation induced by allergens and to airway remodeling (34, 35). As ADAM8^{-/-} mice exhibited a major decrease in their inflammatory response to OVA, we tested whether lack of ADAM8 leads to alterations in the AHR by measuring airway resistance to increasing doses of intravenous methacholine (1, 30). As expected, OVA-challenged wt mice had a significantly higher airway resistance than naive mice. However, OVA-challenged ADAM8^{-/-} mice had only a moderate response to methacholine that was comparable to that of unchallenged wt mice (Figure 1F). This lack of enhanced airway responsiveness correlates with the decreased inflammation observed in ADAM8^{-/-} mice.

ADAM8 mRNA Is Increased in Several Cell Populations in the Asthmatic Lung

Increased ADAM8 expression was previously reported in asthmatic lungs in total perivascular and peribronchial inflammatory cells and in the inflamed respiratory epithelium (5). Accordingly, we detected 5.5-fold increased mRNA expression levels in total lung tissue of OVA-challenged wt mice as compared with lung tissue of naive wt mice (Figure 2A). In particular, qRT-PCR on FACS-sorted cells (purities >95%) from OVA-challenged wt mice revealed strongest mRNA induction in eosinophils (CCR3⁺CD3⁻B220⁺) and CD11b⁺CD11c⁻ cells followed by CD11c⁺ cells, CD4 T cells, and epithelial cells (EpCAM⁺CD45⁻). ADAM8 expression in CD8⁺ T cells was only slightly increased compared with naive splenocytes (Figure 2B). This is, to our knowledge, the first time that ADAM8 mRNA has been detected in T cells. The targeted allele of ADAM8 contains a *lacZ* gene with an internal ribosomal entry site, providing an opportunity to extend the expression analysis of ADAM8 (19) through a β -galactosidase activity test. Strong β -galactosidase activity was detected in activated T cells but not in naive T cells, confirming the mRNA data (see Figure E1 in the online supplement). The data suggest that the phenotype observed in the ADAM8^{-/-} mice could be due to lack of ADAM8 in any of the analyzed inflammatory cells or structural cells of the microenvironment.

ADAM8 of Hematopoietic and Nonhematopoietic Origin Contributes to Pulmonary Inflammation

To test for the respective contributions of hematopoietic cell-derived and microenvironment-derived ADAM8 in pulmonary inflammation, asthma was induced in bone marrow chimeric mice. We observed a significant decrease in total BAL cells in both wt mice reconstituted with ADAM8^{-/-} bone marrow (Figure 3A) and ADAM8^{-/-} mice reconstituted with wt bone marrow (Figure 3B), suggesting that ADAM8 from hematopoietic as well as from nonhematopoietic cells contributes to asthma. Furthermore, differential cell counts revealed a de-

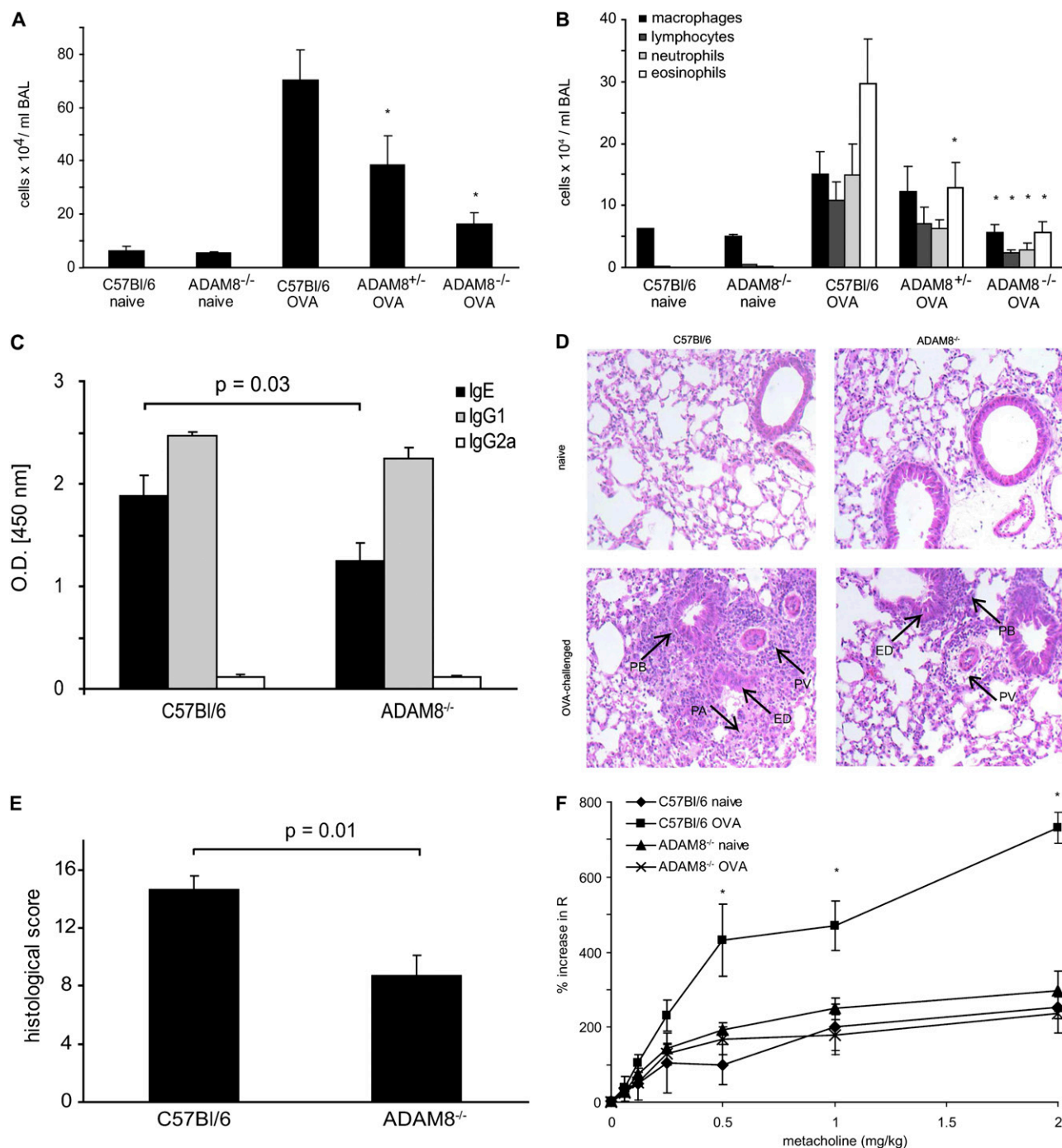


Figure 1. Airway inflammation induced by ovalbumin (OVA) is attenuated in ADAM8^{-/-} mice. (A) Total cell counts and (B) differential cell counts in bronchoalveolar lavage (BAL) from naive and OVA-challenged wild-type (wt), heterozygous, and ADAM8^{-/-} mice. Asterisks indicate statistical significance between respective cell types of OVA-challenged wt type and heterozygous or ADAM8^{-/-} mice, respectively. (C) OVA-specific IgE, IgG1, and IgG2a levels in serum OVA-challenged wt and ADAM8^{-/-} mice. (D) Lung sections from naive and OVA-challenged wt and ADAM8^{-/-} mice stained with hematoxylin/eosin. Arrows: PV = perivascular infiltration; PB = peribronchial infiltration; ED = epithelium damage; PA = parenchymal infiltration. (E) Histologic inflammatory scores for each parameter: perivascular infiltration, peribronchial infiltration, parenchymal infiltration, and epithelial damage (0 = no sign of disease, 5 = profound inflammation; with a total maximal score of 20). (F) Airway resistance in response to increasing doses of intravenous methacholine in naive and OVA-challenged wt and ADAM8^{-/-} mice. Asterisks indicate statistical significance between OVA-challenged wt and ADAM8^{-/-} mice. Data in A, B, and C are representative of three independent experiments, with $n \geq 4$ age-matched female mice per group tested. Data in D, E, and F are representative of 4–5 female mice per group. Data are presented as mean values and error bars as SEM. * $P < 0.05$.

crease in all hematopoietic subsets in the alveoli (Figures 3C and 3D). These data indicate that ADAM8 expressed on both inflammatory cells and the microenvironment contributes to asthma. Accordingly, the AHR was significantly decreased in

both wt mice reconstituted with ADAM8^{-/-} bone marrow and ADAM8^{-/-} mice reconstituted with wt bone marrow as compared with wt mice reconstituted with wt bone marrow (Figure 3E).

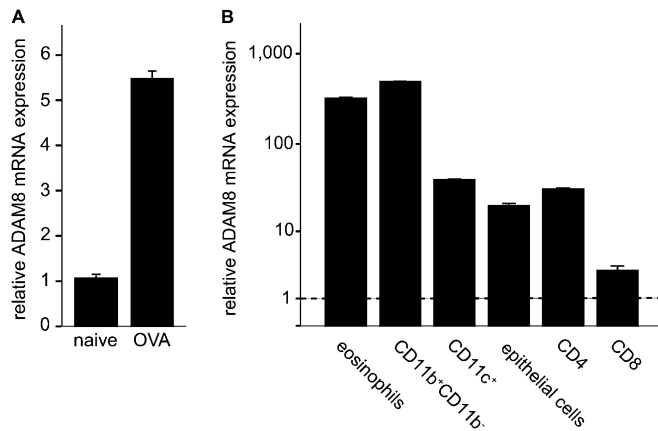


Figure 2. ADAM8 expression in asthmatic lung tissues. (A) ADAM8 mRNA expression in total lung tissue of naive and OVA-induced wt mice ($n = 5$). (B) ADAM8 mRNA expression in highly purified eosinophils, CD11b⁺CD11c⁻ cells, epithelial cells, CD11c⁺ cells, CD4⁺ T cells, and CD8⁺ T cells from digested lung tissues of five OVA-induced wt mice (purities > 95%). mRNA levels were normalized using the reference gene GAPDH and expressed relative to naive splenocytes (= 1). Data are presented as mean values and error bars as SEM.

ADAM8 on T Cells Contributes to Development of Airway Inflammation

Previous studies have shown that mice that lack T and B cells fail to develop experimental asthma, and that adoptive transfer of T cells is sufficient to restore the disease in these mice (36). To determine whether T cell-expressed ADAM8 was required for the development of asthma, we used IL-7 α -chain receptor-deficient mice (IL7R^{-/-}), which have a defect in T and B cell development (37), and mice double deficient for the IL-7 α -chain receptor and ADAM8 (ADAM8^{-/-}/IL7R^{-/-}). Susceptibility of IL7R^{-/-} and ADAM8^{-/-}/IL7R^{-/-} mice to experimental asthma was significantly reduced, as indicated by a five- to eightfold decrease of cells in BAL when compared with wt mice (Figures 4A and 4B).

Equal numbers of purified T cells from peripheral lymph nodes (with equal frequencies of CD4 and CD8 T cells; data not shown) of either wt or ADAM8^{-/-} mice were adoptively transferred into IL7R^{-/-} and ADAM8^{-/-}/IL7R^{-/-} mice, and experimental asthma was induced 4 weeks after transfer. Transfer of wt or ADAM8^{-/-} cells led to similar donor T cell frequencies in the respective recipients, suggesting that ADAM8 on donor cells did not interfere with T cell reconstitution efficiency (data not shown). Wt T cells transferred into either IL7R^{-/-} (Figure 4A) or ADAM8^{-/-}/IL7R^{-/-} mice (Figure 4B) restored lung inflammatory cell infiltration to two-thirds of the value of wt mice. In contrast, cell infiltration into lungs of IL7R^{-/-} (Figure 4A) and ADAM8^{-/-}/IL7R^{-/-} mice (Figure 4B) that had received ADAM8^{-/-} T cells remained at levels comparable to those of IL7R^{-/-} and ADAM8^{-/-}/IL7R^{-/-} mice that had not received a transplant. This suggests that although other cells expressing ADAM8 are required for the full development of allergy, ADAM8 expression by T cells alone is sufficient to significantly induce an allergic phenotype.

Cytokine Production Is Normal in Activated ADAM8^{-/-} T Cells

To test whether the attenuated disease in ADAM8^{-/-} mice was due to failure of ADAM8^{-/-} T cells to produce Th2 cytokines,

splenocytes from wt and ADAM8^{-/-} mice were activated *in vitro* under neutral, Th1, or Th2 polarizing conditions. The frequencies of IL-4- and IFN- γ -producing cells (Figure 5A) and Th1 and Th2 cytokine secretion (Figures 5B–5D) were not significantly different in wt and ADAM8^{-/-} mice.

To assess the capacity of lung inflammatory cells to respond to OVA and to present antigen, total inflammatory lung cells from OVA-challenged wt and ADAM8^{-/-} mice were isolated and re-stimulated with increasing doses of antigen to evaluate the cytokine response. The secretion of IL-5 in response to increasing doses of OVA was similar in wt and ADAM8^{-/-} cells (Figure 5E). As expected for the Th2 asthma response, IFN- γ secretion was below detection limits in wt and ADAM8^{-/-} cells (data not shown). Thus, our data show that there is no abnormal skewing of the cytokine responses and no defect in antigen presentation in ADAM8^{-/-} mice, and we therefore conclude that the attenuated asthma response observed in ADAM8^{-/-} mice is not due to inability of ADAM8^{-/-} T cells to develop into Th2 cells in response to stimulation by antigen-presenting cells. However, levels of pro-asthmatic chemokines and cytokines measured by qRT-PCR in the lung tissue of OVA-sensitized mice (Figure 5F) were significantly lower in ADAM8^{-/-} mice, indicating that the overall inflammatory state of the ADAM8^{-/-} lungs was lower than that of wt lungs.

Tissue Infiltration by ADAM8^{-/-} Inflammatory Cells Is Impaired

Cell infiltration into the lung during airway inflammation is one of a cascade of events required to develop asthma. In response to chemotactic signals released after antigen challenge, hematopoietic cells egress from the vasculature and enter the airway mucosa and bronchoalveolar space, which is dependent on effective cell migration and extracellular matrix degradation (38). The catalytic activity of ADAM8 has been reported to be important for the migration of tumor cells (23). To test whether ADAM8 is also required for inflammatory cell entry into the lung, we determined the numbers of cells (eosinophils, CD11b⁺CD11c⁻, CD11c⁺ and T cells) in blood, lung tissue, and alveoli of asthma-induced wt and ADAM8^{-/-} mice (Figures 6A–6E, and Figure E2). Although there was no significant difference in the numbers or frequencies of cell subsets in the blood, ADAM8^{-/-} mice had significantly lower levels of all cell subsets in the lung tissue and an even more pronounced decrease in the BAL, suggesting that cell recruitment is impaired in ADAM8^{-/-} mice.

To examine whether lack of ADAM8 generally affects the capacity of inflammatory cells to migrate, a series of *in vivo* and *in vitro* migration assays were set up to examine the effect of loss of ADAM8 on the cell types indicated above. Eosinophils have been reported to express ADAM8 on podosome structures that are necessary for invasion (14). Consistent with a role for ADAM8 on these structures, we found in a pilot experiment that ADAM8^{-/-} eosinophils migrate up to 50% less effectively through Matrigel in response to eotaxin than corresponding wt eosinophils (Figure E3). Since eosinophils are potently recruited to the peritoneum in response to chitin injection (33), we used an assay to further confirm an eosinophil trafficking defect. Consistent with the Matrigel assay, significantly lower numbers of eosinophils were recruited to the peritoneum in response to injected chitin in ADAM8^{-/-} mice than in wt mice (Figure 6F).

Similarly, we found that loss of ADAM8 from macrophages, which are known to normally express it (13) and to play a role in asthma (39), led to a 50% decrease in migratory activity *in vitro* (Figure 6G). Treatment of macrophages with

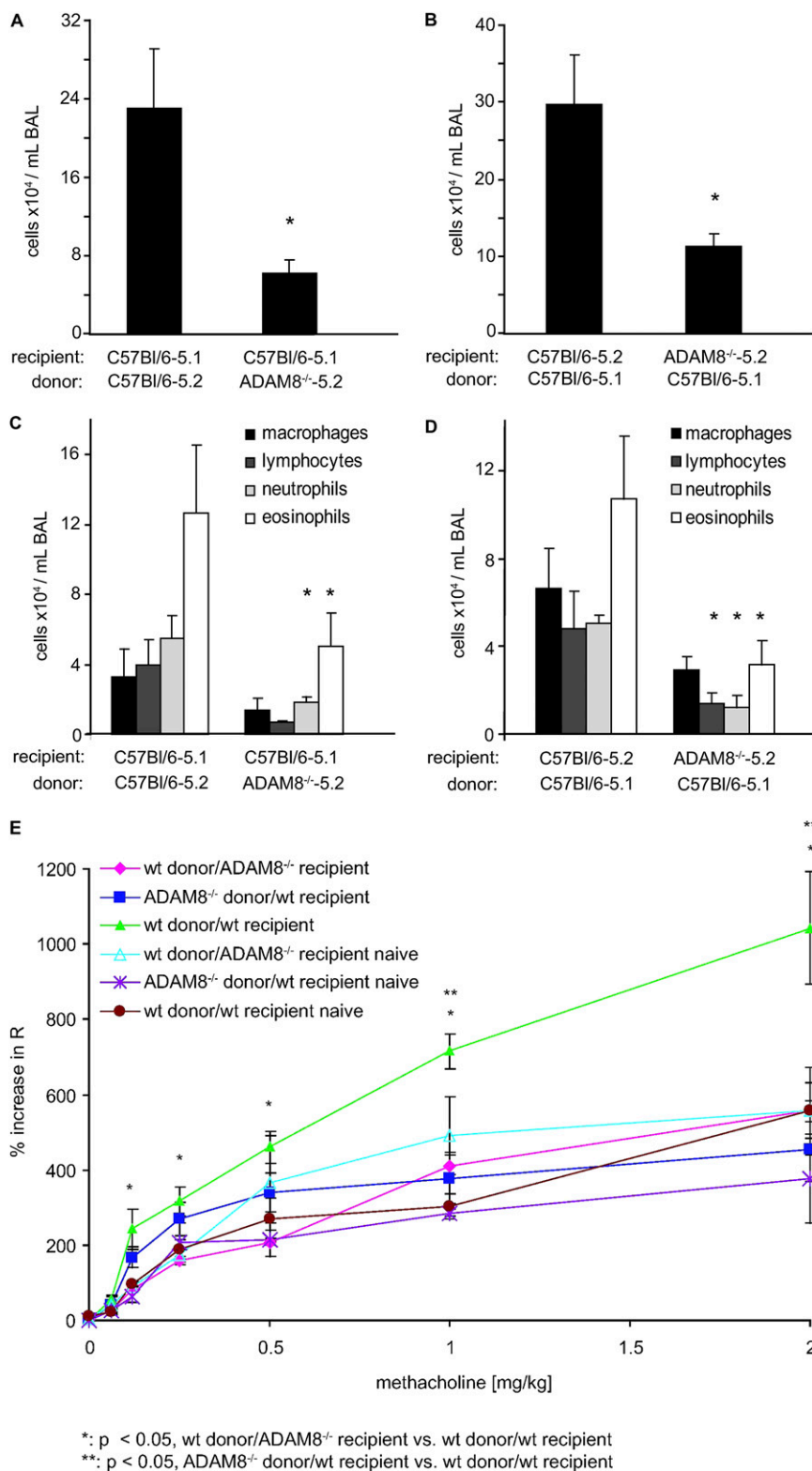


Figure 3. ADAM8 on hematopoietic cells and the nonhematopoietic microenvironment contributes to experimental asthma. Total cell count in BAL of OVA-challenged (A) wt mice reconstituted with ADAM8^{-/-} bone marrow cells and (B) ADAM8^{-/-} mice reconstituted with wt bone marrow cells. Differential cell counts in BAL of OVA-challenged (C) wt mice reconstituted with ADAM8^{-/-} bone marrow cells and (D) ADAM8^{-/-} mice reconstituted with wt bone marrow cells. Asterisks indicate statistical significance between respective cell types from wt or ADAM8^{-/-} mice in BAL of reconstituted mice. (E) Airway resistance in response to increasing doses of intravenous methacholine in naive and OVA-challenged wt mice reconstituted with ADAM8^{-/-} bone marrow cells, ADAM8^{-/-} mice reconstituted with wt bone marrow cells, and wt mice reconstituted with congenic wt cells. Asterisks indicate statistical significance as indicated in the figure. Data from each reconstitution experiment are representative of two independent experiments with $n \geq 4$ age-matched female mice per recipient group. Data are presented as mean values and error bars as SEM. * $P < 0.05$, ** $P < 0.01$.

LPS or TNF- α is known to up-regulate ADAM8 expression (21, 24). Consistent with this up-regulation, we found that stimulated macrophages from wt mice had a two- to threefold increased migratory activity, compared with unstimulated wt macrophages, whereas stimulation of ADAM8^{-/-} macrophages did not result in an increased migration. Thus, consistent with our observations with eosinophils, we found

ADAM8 to play a cell autonomous role in enhancing macrophage migratory ability (Figure 6G).

Dendritic cells like macrophages up-regulate ADAM8 upon stimulation (16) and are important in the development of asthma (40). Again, we found that bone marrow-derived dendritic cells from ADAM8^{-/-} mice migrated less efficiently in response to SDF-1 than their wt counterparts (Figure 6H).

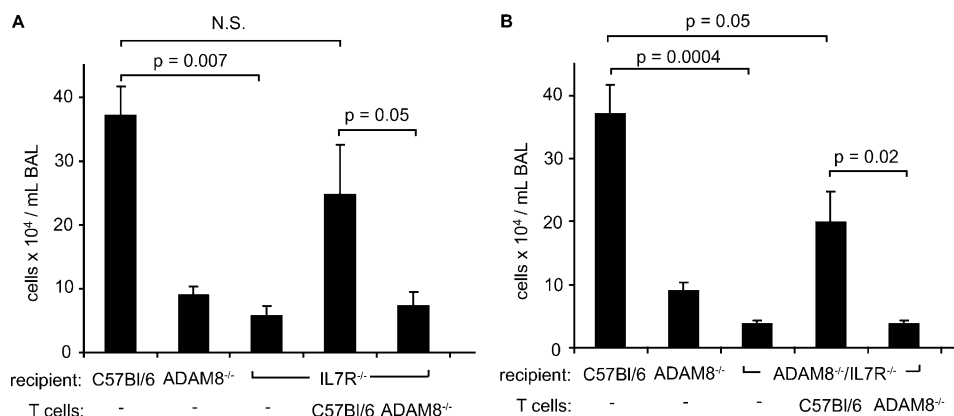


Figure 4. ADAM8 on T cells alone contributes to experimental asthma. (A) Total cell count in BAL of OVA-challenged wt, ADAM8^{-/-}, and IL7R^{-/-} mice. IL7R^{-/-} had received no transplant or a T cell transplant from wt or ADAM8^{-/-} mice, respectively. (B) Total cell counts in BAL of OVA-challenged wt, ADAM8^{-/-}, and ADAM8^{-/-}/IL7R^{-/-} mice. ADAM8^{-/-}/IL7R^{-/-} had received no transplant or a T cell transplant from wt or ADAM8^{-/-} mice, respectively. Data from each adoptive transfer experiment are representative of two independent experiments with $n \geq 4$ age-matched female mice per recipient group. Data are presented as mean values and error bars as SEM.

Finally, based on our findings that T cells from asthmatic lungs expressed ADAM8, we tested migration capacities of activated T cells from wt and ADAM8^{-/-} mice. Consistent with our observations for the other hematopoietic subsets, we found that activated ADAM8^{-/-} T cells migrated 17% (CD8) and 28% (CD4) less efficiently in response to CCL5 *in vitro* when compared with wt T cells (Figure 6I). In summary, these data suggest a cell-autonomous defect in the recruitment/migratory capacity of all inflammatory cell types lacking ADAM8^{-/-} and that this migratory impairment may be the underlying cause of the attenuated asthmatic response in ADAM8^{-/-} mice.

DISCUSSION

Several studies have reported an association between ADAM8 expression and the disease severity in patients with pulmonary disease (6, 27) and in mice with induced experimental asthma (5, 26). While it has, to date, been unclear whether lack of ADAM8 would promote or prevent development of airway inflammation, our data demonstrate conclusively that ADAM8 plays a nonredundant, proinflammatory role in a murine model of acute asthma.

Data from hematopoietic reconstitution experiments indicate that ADAM8 can act both in a cell-intrinsic and cell-extrinsic manner and that the contribution of ADAM8 from different cell types is required to mount a full and efficient asthmatic response. The ADAMs are multifunctional proteins with catalytic, adhesive, and signaling properties (8). Accordingly, ADAM8 has been reported to be involved in various phenomena, such as cleavage of extracellular matrix molecules (9–11, 25), homophilic interaction via the disintegrin domains (11) and integrin–disintegrin interaction (41). Whether a single ADAM8 function is sufficient or a combination of them is required for development of pulmonary lung inflammation is presently unclear.

Lung epithelial cells and infiltrating hematopoietic cells have been recognized as sources of ADAM8 (5, 6, 19). We confirm these reports and additionally show that in asthmatic lungs ADAM8 mRNA is expressed in lung epithelial cells, infiltrating eosinophils, CD11b⁺CD11c⁻ cells, CD11c⁺ cells and T cells. Our finding that T cells express ADAM8 is in contrast to previous studies (13, 16). Differential expression in murine and human T cells as well as the priming conditions and activation status of T cells may account for these discrepancies. In the previous studies, human T cell lines, or human peripheral blood T cells activated *in vitro* were used to verify ADAM8 expression. In

contrast, we used murine T cells that had been activated *in vivo* and had infiltrated the lungs under inflammatory conditions.

We were, furthermore, able to show that ADAM8 expression in T cells alone is sufficient to exacerbate experimental asthma in an otherwise ADAM8-deficient environment. Of note, however, is that there was a trend for more cell infiltration when wt T cells were transferred into an ADAM8-expressing environment as compared with transfer into an ADAM8-deficient environment. This suggests that ADAM8 expression on other cell lineages also contributes to development of experimental asthma consistent with our observations in bone marrow chimeric mice. The T cell transfer experiments, however, demonstrate that expression of ADAM8 by one cell type (T cell) is sufficient to induce pulmonary inflammation. The nature and extent of ADAM8 contributions to asthma when expressed in lineages other than T cells remains to be explored.

Since ADAM8 was detected in dendritic cells and T cells and the expression of ADAM8 in T cells alone was sufficient to induce disease, we considered the possibility that T cell priming might be affected in ADAM8^{-/-} mice. However, polarization experiments and antigen stimulation of isolated inflammatory lung cells showed that ADAM8^{-/-} T cells are fully capable of mounting an appropriate Th2 cytokine response, and that antigen presentation by alveolar macrophages and dendritic cells is functional. This is also supported by the fact that these mice produce normal IgG1 levels in response to OVA. The reduced infiltration of eosinophils, T cells, CD11b⁺CD11c⁻, and CD11c⁺ cells into lung parenchyma and BAL in ADAM8^{-/-} mice, however, point to a deficiency in recruitment of these cells under inflammatory conditions. Assays testing for the migratory capacities of individual inflammatory cell types contributing to the asthmatic response pointed to a consistent impairment in transmigration in the absence of ADAM8. This is in agreement with earlier studies reporting a role for ADAM8 in tumor cell migration (18, 22, 23). The fact that production of chemokines was reduced in the lungs of ADAM8^{-/-} mice in response to OVA challenges might have further contributed to the reduced migration of inflammatory cells into lung and BAL. It could also, however, simply be a reflection of the milder inflammation that developed in these mice. Our data also suggest that ADAM8 expressed on both hematopoietic and nonhematopoietic cells acts in cell recruitment, as nonhematopoietic cell-expressed ADAM8 contributed to migration of inflammatory cells to the lung. However, as alveolar macrophages express ADAM8 and are known to be radioresistant, there is a possibility that some of the protection observed in the ADAM8^{-/-} mice reconstituted

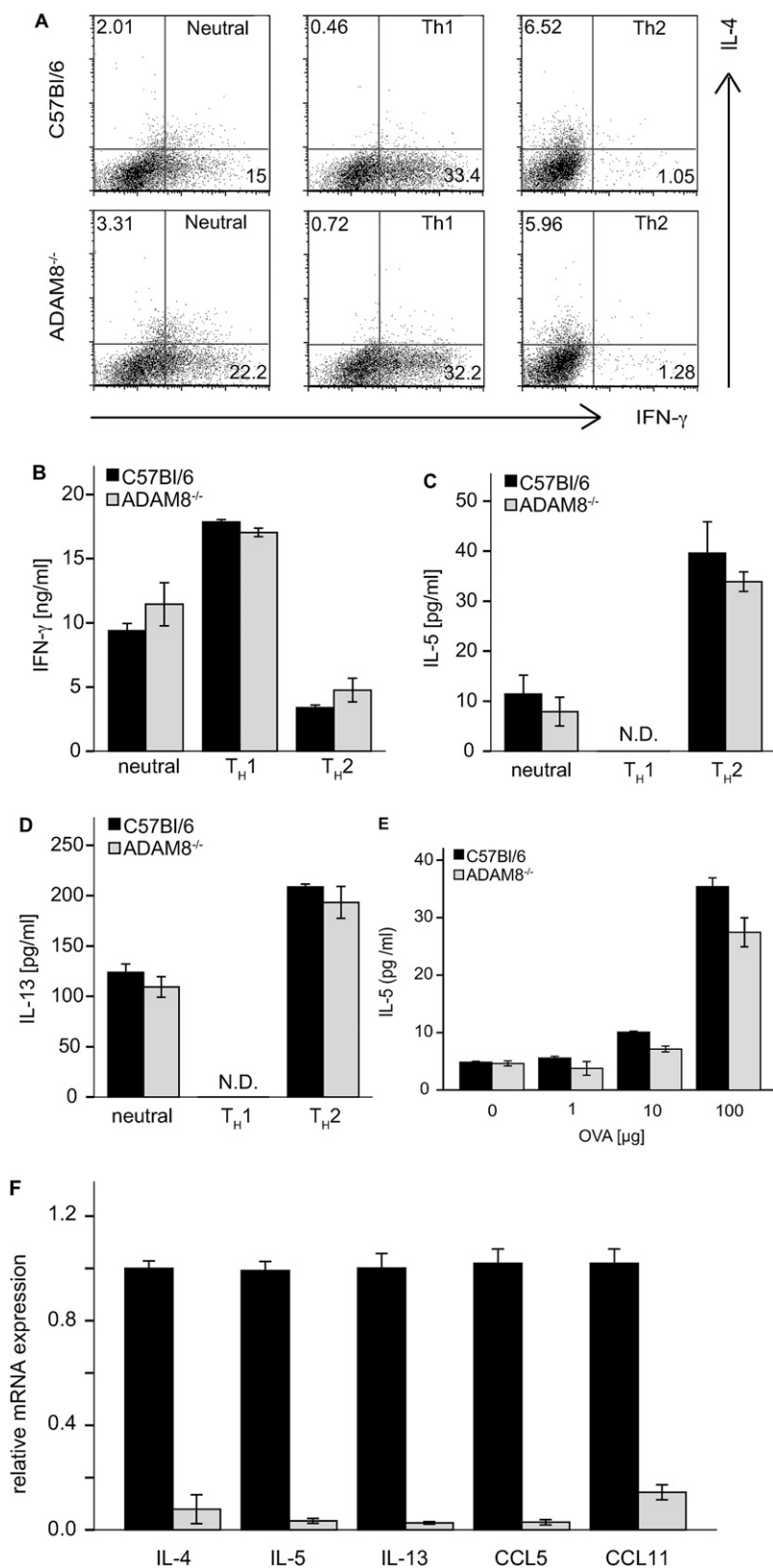


Figure 5. ADAM8^{-/-} inflammatory cells produce Th2 cytokines. (A–D) Splenocytes from naive wt and ADAM8^{-/-} mice were activated *in vitro*. (A) Frequencies of IFN- γ and IL-4-producing cells. (B) IFN- γ , (C) IL-5, and (D) IL-13 secretion under neutral, Th1, and Th2 polarizing conditions. (E) IL-5 release of inflammatory cells from lungs of OVA-challenged wt and ADAM8^{-/-} mice in response to increasing doses of OVA. (F) mRNA expression of indicated chemokines and cytokines in total lung tissue of OVA-challenged wt and ADAM8^{-/-} mice. mRNA levels were normalized using the reference gene GAPDH and presented relative to wt values (= 1). Data are representative of at least two independent experiments with $n \geq 4$ age-matched mice per group. Data are presented as mean values and error bars as SEM.

with wt bone marrow could be due to continued presence of radioresistant ADAM8^{-/-} alveolar macrophages.

Development of AHR requires the migration of dendritic cells and T cells between lung and lymph nodes (42). Moreover, throughout the asthmatic response, inflammatory cells repeatedly egress from the vasculature into the airway mucosa and into the

bronchoalveolar space, which requires the capacity for migration and extracellular matrix degradation. Several studies have shown that eosinophils, T cells, and dendritic cells are essential to mount an asthma response, and that the absence of one individual cell population is sufficient to attenuate or block development of asthma (36, 40, 43). Our study suggests that all these cells are

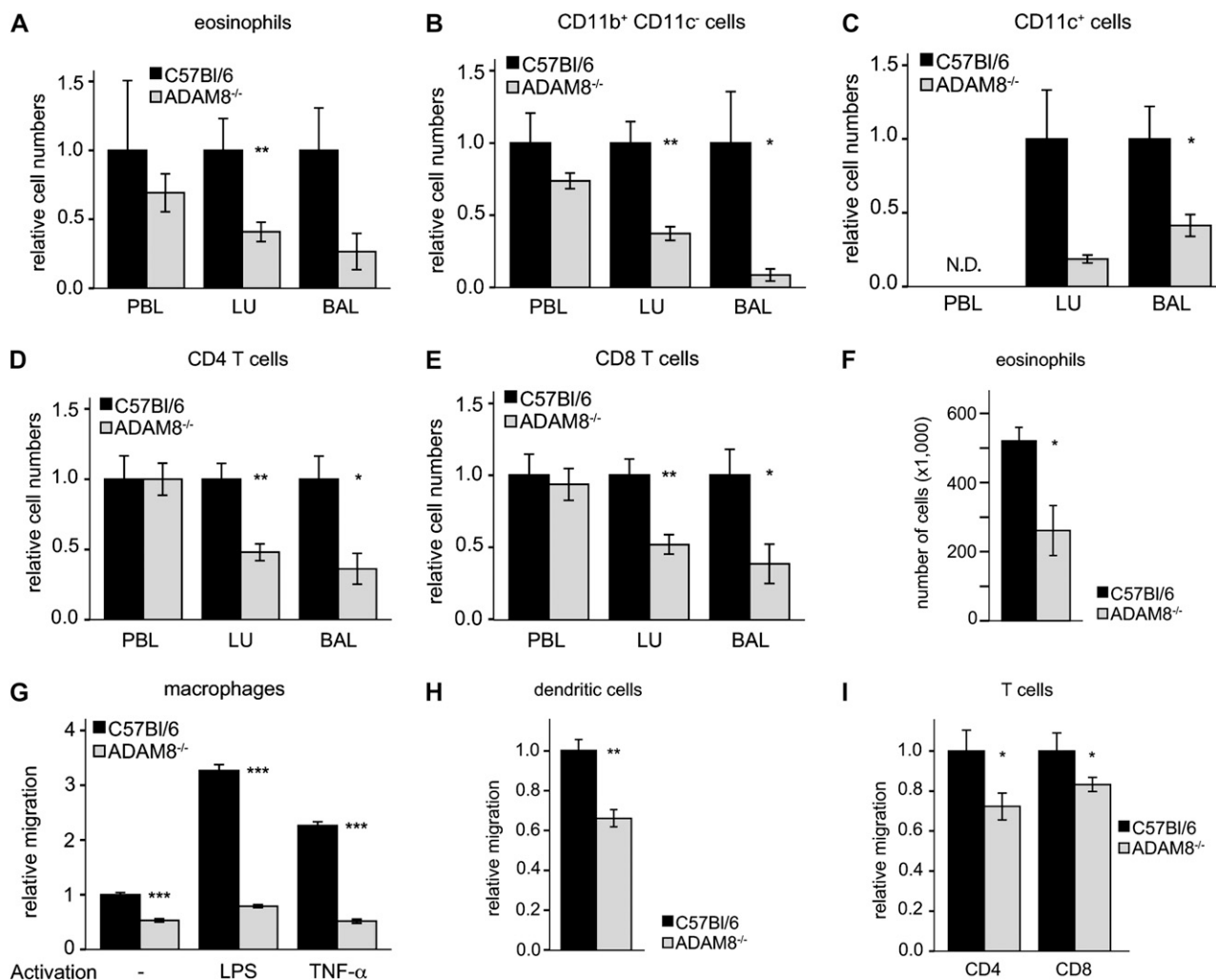


Figure 6. ADAM8 is important for cell recruitment in asthmatic mice and for cell migration *in vitro*. Relative numbers of (A) eosinophils, (B) CD11b⁺CD11c⁻ cells, (C) CD11c⁺ cells, (D) CD4, and (E) CD8 T cells in peripheral blood (PBL), lung (LU), and BAL of OVA-challenged wt and ADAM8^{-/-} mice. Cell numbers (in 1 μ l blood, 1 mg lung tissue or 1 ml BAL) were normalized to respective wt values (= 1). (F) Eosinophils harvested from intraperitoneal washes of chitin-injected wt and ADAM8^{-/-} mice. (G) Relative migration efficiencies of bone marrow-derived macrophages from wt and ADAM8^{-/-} mice with and without previous activation with LPS or TNF- α . Values were normalized to non-activated wt macrophages (= 1). (H) Relative migration efficiency of mature bone marrow-derived ADAM8^{-/-} dendritic cells compared with wt (= 1) in Matrigel migration assays in response to 100 nM SDF-1. (I) Relative migration efficiencies of activated ADAM8^{-/-} T cells compared with wt (= 1) in Matrigel migration assays in response to 25 ng/ml CCL5. Data in A–E are representative of three independent experiments with 4–7 age-matched mice per group. Data in F are representative of two independent experiments with 5 age-matched mice per group. Data in G were obtained from three independent cell preparations, with each experiment performed in triplicate. Data in H and I are representative of three independent cell preparations, with each experiment performed in triplicate. Data are presented as mean values and error bars as SEM. Asterisks indicate significant differences between values for wt and respective ADAM8^{-/-} cells. * P < 0.05, ** P < 0.01, *** P < 0.001.

affected in their capacity to migrate to the lung, and as these cells can bi-directionally influence each other and other cells (44–46). Lack of ADAM8 therefore would successively reduce (1) the priming of T cells in the lymph nodes via a defect on dendritic cell migration, and (2) the migration of all major inflammatory cells which are crucial to the development of asthma.

Based on recent observations we propose that the ADAM8 catalytic activity might be required to degrade extracellular matrix components such as fibronectin (47). Moreover, ADAM8 could also act indirectly as part of an activation cascade or a signaling pathway. Finally, our data do not exclude a role for the ADAM8 disintegrin/cysteine-rich domain in this process, through cell–ECM attachment, which may be regulated by integrins, affecting cell–cell contacts via the ADAM8 disintegrin domains.

Asthma had previously been induced in transgenic ATMS2 mice that overexpress the ectodomain of ADAM8. This soluble form may not correspond to the physiological soluble ADAM8 as it includes the disintegrin domain (48). ATMS2 mice had a phenotype similar to that we report here for ADAM8^{-/-} mice, (lower cell infiltrates, and reduced thickening of airway epithelium) and it was suggested that the soluble ADAM8 in ATMS2 mice could, via its disintegrin domain, interfere with the ability of membrane-bound ADAM8 to form cell–cell contacts and thereby decrease cell infiltration (28). In such a scenario soluble ADAM8 would act as a dominant-negative molecule inhibiting catalytic activity and binding ability of ADAM8. Therefore, “neutralization” of membrane ADAM8 by excess soluble ADAM8 in ATMS2 mice would result in a phenotype comparable to that of ADAM8^{-/-} mice.

The low level of ADAM8 expression at steady state and the fact that ADAM8-deficient mice do not have a pathologic phenotype at baseline highlight this particular protease as potential therapeutic target, and suggest that transient, specific inhibition of ADAM8 function *in vivo* might be possible without inducing major side effects (49). Moreover, data from bone marrow chimeras, T cell transfer experiments, and the fact that mice heterozygous for ADAM8 have an intermediate inflammatory response could indicate that the ADAM8 expression is limiting in asthma development. Partial inactivation of ADAM8 might thus be sufficient for treatment of asthma, again preventing possible side effects linked to the complete inhibition of this endogenous protein.

Conflict of Interest Statement: S.N. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.-R.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. C.Z. received \$1,001–\$5,000 from StemCell LLC in an unrestricted research grant. J.W.B.'s institution, King's College Business Ltd., holds \$5,001–\$10,000 in patents received or pending for an ADAM8 inhibitor. K.M.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. H.J.Z. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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References

- Blanchet MR, Israel-Assayag E, Cormier Y. Modulation of airway inflammation and resistance in mice by a nicotinic receptor agonist. *Eur Respir J* 2005;26:21–27.
- Kumagai K, Ohno I, Okada S, Ohkawara Y, Suzuki K, Shinya T, Nagase H, Iwata K, Shirato K. Inhibition of matrix metalloproteinases prevents allergen-induced airway inflammation in a murine model of asthma. *J Immunol* 1999;162:4212–4219.
- Jung YW, Zindl CL, Lai JF, Weaver CT, Chaplin DD. Mmp induced by gr-1+ cells are crucial for recruitment of Th cells into the airways. *Eur J Immunol* 2009;39:2281–2292.
- Goswami S, Angkasekwinai P, Shan M, Greenlee KJ, Barranco WT, Polikepahad S, Seryshev A, Song LZ, Redding D, Singh B, *et al.* Divergent functions for airway epithelial matrix metalloproteinase 7 and retinoic acid in experimental asthma. *Nat Immunol* 2009;10:496–503.
- King NE, Zimmermann N, Pope SM, Fulkerson PC, Nikolaidis NM, Mishra A, Witte DP, Rothenberg ME. Expression and regulation of a disintegrin and metalloproteinase (ADAM) 8 in experimental asthma. *Am J Respir Cell Mol Biol* 2004;31:257–265.
- Foley SC, Mogas AK, Olivenstein R, Fiset PO, Chakir J, Bourbeau J, Ernst P, Lemiere C, Martin JG, Hamid Q. Increased expression of ADAM33 and ADAM8 with disease progression in asthma. *J Allergy Clin Immunol* 2007;119:863–871.
- Van Eerdewegh P, Little RD, Dupuis J, Del Mastro RG, Falls K, Simon J, Torrey D, Pandit S, McKenny J, Braunschweiger K, *et al.* Association of the ADAM33 gene with asthma and bronchial hyperresponsiveness. *Nature* 2002;418:426–430.
- Edwards DR, Handsley MM, Pennington CJ. The ADAM metalloproteinases. *Mol Aspects Med* 2008;29:258–289.
- Fourie AM, Coles F, Moreno V, Karlsson L. Catalytic activity of ADAM8, ADAM15, and MDC-1 (ADAM28) on synthetic peptide substrates and in ectodomain cleavage of CD23. *J Biol Chem* 2003;278:30469–30477.
- Naus S, Reipschläger S, Wildeboer D, Lichtenthaler SF, Mitterreiter S, Guan Z, Moss ML, Bartsch JW. Identification of candidate substrates for ectodomain shedding by the metalloprotease-disintegrin ADAM8. *Biol Chem* 2006;387:337–346.
- Schlomann U, Wildeboer D, Webster A, Antropova O, Zeuschner D, Knight CG, Docherty AJ, Lambert M, Skelton L, Jockusch H, *et al.* The metalloprotease disintegrin ADAM8: processing by autocatalysis is required for proteolytic activity and cell adhesion. *J Biol Chem* 2002;277:48210–48219.
- Yoshida S, Setoguchi M, Higuchi Y, Akizuki S, Yamamoto S. Molecular cloning of cDNA encoding MS2 antigen, a novel cell surface antigen strongly expressed in murine monocytic lineage. *Int Immunol* 1990;2:585–591.
- Yoshiyama K, Higuchi Y, Kataoka M, Matsuura K, Yamamoto S. Cd156 (human adam8): expression, primary amino acid sequence, and gene location. *Genomics* 1997;41:56–62.
- Johansson MW, Lye MH, Barthel SR, Duffy AK, Annis DS, Mosher DF. Eosinophils adhere to vascular cell adhesion molecule-1 via podosomes. *Am J Respir Cell Mol Biol* 2004;31:413–422.
- Gomez-Gavito M, Dominguez-Luis M, Canchado J, Calafat J, Janssen H, Lara-Pezzi E, Fourie A, Tugores A, Valenzuela-Fernandez A, Mollinedo F, *et al.* Expression and regulation of the metalloproteinase ADAM-8 during human neutrophil pathophysiological activation and its catalytic activity on I-selectin shedding. *J Immunol* 2007;178:8053–8063.
- Richens J, Fairclough L, Ghaemmaghami AM, Mahdavi J, Shakib F, Sewell HF. The detection of ADAM8 protein on cells of the human immune system and the demonstration of its expression on peripheral blood B cells, dendritic cells and monocyte subsets. *Immunobiology* 2007;212:29–38.
- Choi SJ, Han JH, Roodman GD. ADAM8: a novel osteoclast stimulating factor. *J Bone Miner Res* 2001;16:814–822.
- Ishikawa N, Daigo Y, Yasui W, Inai K, Nishimura H, Tsuchiya E, Kohno N, Nakamura Y. ADAM8 as a novel serological and histochemical marker for lung cancer. *Clin Cancer Res* 2004;10:8363–8370.
- Kelly K, Hutchinson G, Nebenius-Oosthuizen D, Smith AJ, Bartsch JW, Horiuchi K, Rittger A, Manova K, Docherty AJ, Blobel CP. Metalloprotease-disintegrin ADAM8: expression analysis and targeted deletion in mice. *Dev Dyn* 2005;232:221–231.
- Mandelin J, Li TF, Hukkanen MV, Liljestrom M, Chen ZK, Santavirta S, Kitti U, Kontinen YT. Increased expression of a novel osteoclast-stimulating factor, ADAM8, in interface tissue around loosened hip prostheses. *J Rheumatol* 2003;30:2033–2038.
- Schlomann U, Rathke-Hartlieb S, Yamamoto S, Jockusch H, Bartsch JW. Tumor necrosis factor alpha induces a metalloprotease-disintegrin, ADAM8 (CD 156): implications for neuron-glia interactions during neurodegeneration. *J Neurosci* 2000;20:7964–7971.
- Valkovskaya N, Kaye H, Felix K, Hartmann D, Giese NA, Osinsky SP, Friess H, Kleeff J. ADAM8 expression is associated with increased invasiveness and reduced patient survival in pancreatic cancer. *J Cell Mol Med* 2007;11:1162–1174.
- Wildeboer D, Naus S, Amy Sang QX, Bartsch JW, Pagenstecher A. Metalloproteinase disintegrins ADAM8 and ADAM19 are highly regulated in human primary brain tumors and their expression levels and activities are associated with invasiveness. *J Neuropathol Exp Neurol* 2006;65:516–527.
- Kataoka M, Yoshiyama K, Matsuura K, Hijiya N, Higuchi Y, Yamamoto S. Structure of the murine CD156 gene, characterization of its promoter, and chromosomal location. *J Biol Chem* 1997;272:18209–18215.
- Naus S, Richter M, Wildeboer D, Moss M, Schachner M, Bartsch JW. Ectodomain shedding of the neural recognition molecule ch11 by the metalloprotease-disintegrin ADAM8 promotes neurite outgrowth and suppresses neuronal cell death. *J Biol Chem* 2004;279:16083–16090.
- Chiba Y, Onoda S, Hattori Y, Maitani Y, Sakai H, Misawa M. Upregulation of ADAM8 in the airways of mice with allergic bronchial asthma. *Lung* 2009;187:179–185.
- Matsuno O, Miyazaki E, Nureki S, Ueno T, Ando M, Ito K, Kumamoto T, Higuchi Y. Elevated soluble ADAM8 in bronchoalveolar lavage fluid in patients with eosinophilic pneumonia. *Int Arch Allergy Immunol* 2007;142:285–290.
- Matsuno O, Miyazaki E, Nureki S, Ueno T, Kumamoto T, Higuchi Y. Role of ADAM8 in experimental asthma. *Immunol Lett* 2006;102:67–73.
- Knolle MD, Owen CA. ADAM8: a new therapeutic target for asthma. *Expert Opin Ther Targets* 2009;13:523–540.
- Blanchet MR, Maltby S, Haddon DJ, Merckens H, Zbytniuk L, McNagny KM. CD34 facilitates the development of allergic asthma. *Blood* 2007;110:2005–2012.
- Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C. An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods* 2001;25:386–401.
- Liu C, Ueno T, Kuse S, Saito F, Nitta T, Piali L, Nakano H, Kakiuchi T, Lipp M, Hollander GA, *et al.* The role of ccl21 in recruitment of T-precursor cells to fetal thymus. *Blood* 2005;105:31–39.
- Reese TA, Liang HE, Tager AM, Luster AD, Van Rooijen N, Voehringer D, Locksley RM. Chitin induces accumulation in tissue of innate immune cells associated with allergy. *Nature* 2007;447:92–96.

34. Bentley JK, Hershenovson MB. Airway smooth muscle growth in asthma: proliferation, hypertrophy, and migration. *Proc Am Thorac Soc* 2008; 5:89–96.
35. Hogg JC. The pathology of asthma. *APMIS* 1997;105:735–745.
36. Corry DB, Grunig G, Hadeiba H, Kurup VP, Warnock ML, Sheppard D, Rennick DM, Locksley RM. Requirements for allergen-induced airway hyperreactivity in T and B cell-deficient mice. *Mol Med* 1998;4: 344–355.
37. Peschon JJ, Morrissey PJ, Grabstein KH, Ramsdell FJ, Maraskovsky E, Gliniak BC, Park LS, Ziegler SF, Williams DE, Ware CB, *et al.* Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J Exp Med* 1994;180:1955–1960.
38. Blyth DI, Pedrick MS, Savage TJ, Hessel EM, Fattah D. Lung inflammation and epithelial changes in a murine model of atopic asthma. *Am J Respir Cell Mol Biol* 1996;14:425–438.
39. Careau E, Proulx LI, Pouliot P, Spahr A, Turmel V, Bissonnette EY. Antigen sensitization modulates alveolar macrophage functions in an asthma model. *Am J Physiol Lung Cell Mol Physiol* 2006;290:L871–L879.
40. van Rijt LS, Jung S, Kleinjan A, Vos N, Willart M, Duez C, Hoogsteden HC, Lambrecht BN. In vivo depletion of lung cd11c+ dendritic cells during allergen challenge abrogates the characteristic features of asthma. *J Exp Med* 2005;201:981–991.
41. Rao H, Lu G, Kajiya H, Garcia-Palacios V, Kurihara N, Anderson J, Patrene K, Sheppard D, Blair HC, Windle JJ, *et al.* Alpha9beta1: a novel osteoclast integrin that regulates osteoclast formation and function. *J Bone Miner Res* 2006;21:1657–1665.
42. Medoff BD, Thomas SY, Luster AD. T cell trafficking in allergic asthma: the ins and outs. *Annu Rev Immunol* 2008;26:205–232.
43. Lee JJ, Dimina D, Macias MP, Ochkur SI, McGarry MP, O'Neill KR, Protheroe C, Pero R, Nguyen T, Cormier SA, *et al.* Defining a link with asthma in mice congenitally deficient in eosinophils. *Science* 2004;305:1773–1776.
44. Jacobsen EA, Ochkur SI, Pero RS, Taranova AG, Protheroe CA, Colbert DC, Lee NA, Lee JJ. Allergic pulmonary inflammation in mice is dependent on eosinophil-induced recruitment of effector T cells. *J Exp Med* 2008;205:699–710.
45. Walsh ER, Sahu N, Kearley J, Benjamin E, Kang BH, Humbles A, August A. Strain-specific requirement for eosinophils in the recruitment of T cells to the lung during the development of allergic asthma. *J Exp Med* 2008;205:1285–1292.
46. Yang D, Chen Q, Su SB, Zhang P, Kurosaka K, Caspi RR, Michalek SM, Rosenberg HF, Zhang N, Oppenheim JJ. Eosinophil-derived neurotoxin acts as an alarmin to activate the tlr2-myd88 signal pathway in dendritic cells and enhances Th2 immune responses. *J Exp Med* 2008;205:79–90.
47. Zack MD, Malfait AM, Skepner AP, Yates MP, Griggs DW, Hall T, Hills RL, Alston JT, Nemirovskiy OV, Radabaugh MR, *et al.* AADAM-8 isolated from human osteoarthritic chondrocytes cleaves fibronectin at ala(271). *Arthritis Rheum* 2009;60:2704–2713.
48. Higuchi Y, Yasui A, Matsuura K, Yamamoto S. Cd156 transgenic mice: different responses between inflammatory types. *Pathobiology* 2002; 70:47–54.
49. Koller G, Schlomann U, Golfi P, Ferdous T, Naus S, Bartsch JW. ADAM8/ms2/cd156, an emerging drug target in the treatment of inflammatory and invasive pathologies. *Curr Pharm Des* 2009;15: 2272–2281.

**THE METALLOPROTEASE-DISINTEGRIN ADAM8 IS ESSENTIAL FOR THE
DEVELOPMENT OF EXPERIMENTAL ASTHMA**

Silvia Naus, Marie-Renée Blanchet, Klaus Gossens, Colby Zaph, Jörg W. Bartsch, Kelly
M. McNaghy and Hermann J. Ziltener.

Online data supplement

Supplemental Figure legends

Supplementary Figure 1

ADAM8 expression in naive and activated wt and ADAM8^{-/-} CD4 and CD8 T cells assessed by using the *lacZ* reporter gene that is inserted in the ADAM8 knockout allele. β -Galactosidase-mediated hydrolysis of the substrate fluorescein di- β -D-galactopyranoside (FDG) into fluorescent fluorescein results in increased fluorescence of activated ADAM8^{-/-} CD4 and CD8 T cells. Data are representative for three independent assays.

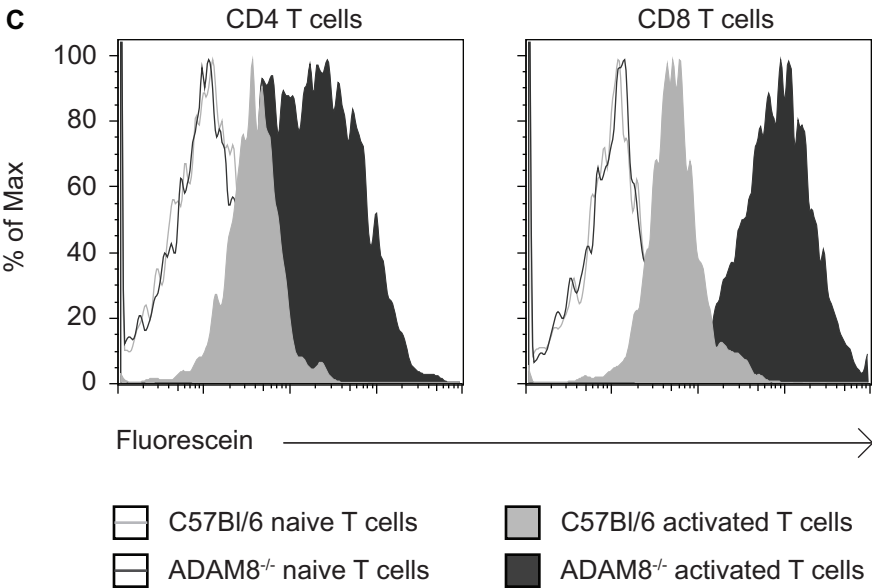
Supplementary Figure 2

Numbers of CD11b⁺CD11c⁻ cells, CD4 T cells, CD8 T cells and CCR3⁺ eosinophils in (A) peripheral blood (PBL; per μ l blood), (B) lung (LU; per mg tissue) and (C) BAL (per mL fluid) of OVA-challenged wt and ADAM8^{-/-} mice. Asterisks indicate significant differences between values for wt cells and respective ADAM8^{-/-} cells. Data are representative of three independent experiments with $n \geq 4$ age-matched mice per group.

Supplementary Figure 3

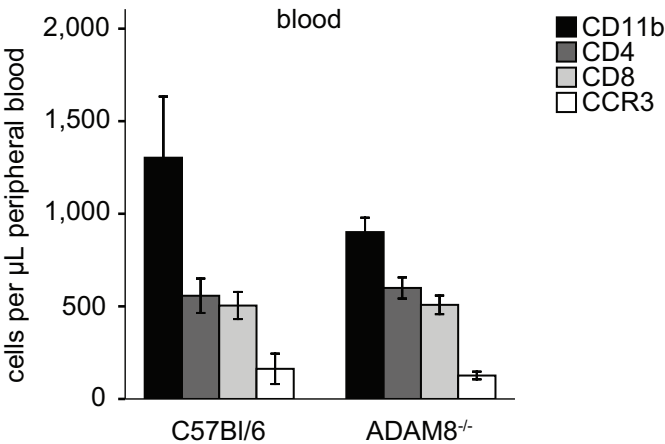
Relative migration efficiency of ADAM8^{-/-} compared to wt eosinophils (= 1) from lungs of respective OVA-challenged mice in Matrigel migration assays in response to 30 ng/mL eotaxin. Data are representative of two independent experiments with each condition containing cells pooled from 8-10 age-matched mice.

c

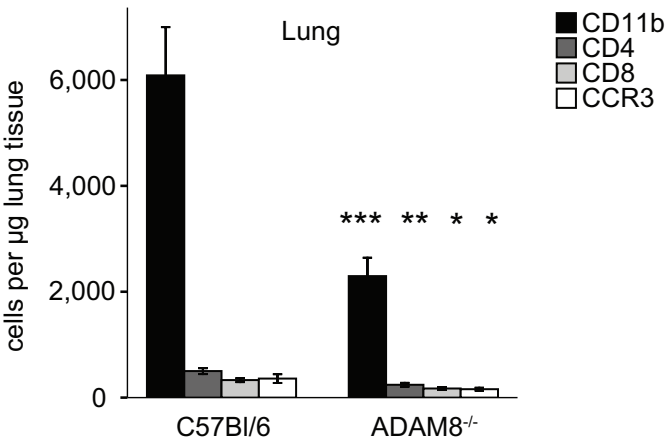


Supplementary Figure 2

A

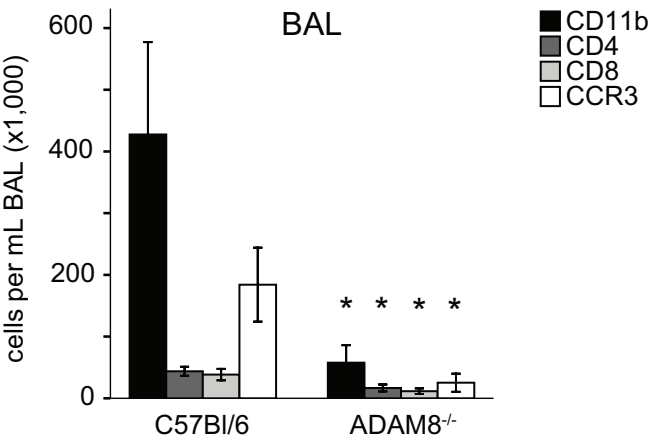


B



Supplementary Figure 2

c



Supplementary Figure 3

