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Adam8 Limits the Development of Allergic Airway Inflammation in Mice

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To determine whether a disintegrin and metalloproteinase-8 (Adam8) regulates allergic airway inflammation (AAI) and airway hyperresponsiveness (AHR), we compared AAI and AHR in wild-type (WT) versus Adam8−/− mice in different genetic backgrounds sensitized and challenged with OVA or house dust mite protein extract. OVA- and house dust mite–treated Adam8−/− mice had higher lung leukocyte counts, more airway mucus metaplasia, greater lung levels of some Th2 cytokines, and higher methacholine-induced increases in central airway resistance than allergen-treated WT mice. Studies of OVA-treated Adam8 bone marrow chimeric mice confirmed that leukocyte-derived Adam8 predominantly mediated Adam8’s anti-inflammatory activities in murine airways. Airway eosinophils and macrophages both expressed Adam8 in WT mice with AAI. Adam8 limited AAI and AHR in mice by reducing leukocyte survival because: 1) Adam8−/− mice with AAI had fewer apoptotic eosinophils and macrophages in their airways than WT mice with AAI; and 2) Adam8−/− macrophages and eosinophils had reduced rates of apoptosis compared with WT leukocytes when the intrinsic (but not the extrinsic) apoptosis pathway was triggered in the cells in vitro. Adam8 was robustly expressed by airway granulocytes in lung sections from human asthma patients, but, surprisingly, airway macrophages had less Adam8 staining than airway eosinophils. Thus, Adam8 has anti-inflammatory activities during AAI in mice by activating the intrinsic apoptosis pathway in myeloid leukocytes. Strategies that increase ADAM8 levels in myeloid leukocytes may have therapeutic efficacy in asthma. The Journal of Immunology, 2013, 190: 000–000.

Asthma is a chronic disease characterized by airway inflammation, airway hyperresponsiveness (AHR), and intermittent bronchoconstriction. It affects 300 million people worldwide and causes an estimated 180,000 deaths per year (1). Asthma is caused by complex interactions between environmental and genetic factors, but its pathogenesis is not completely understood.

Matrix metalloproteinases (Mmps) regulate allergic airway inflammation (AAI) and AHR in mice by modulating airway inflammation (2–5), subepithelial fibrosis (6–8), and airway smooth muscle cell proliferation (9). For example, Mmp-2, -7, and -9 regulate AAI in mice by proteolytically cleaving proinflammatory mediators to regulate the biologic activities of these mediators (3, 5, 10). Mmp-8 reduces AAI in mice by increasing granulocyte apoptosis (4). However, little is known about the activities of other metalloproteinase (MP) subfamilies in asthma pathogenesis. The a disintegrin and MP (ADAM) subfamily of MPs was first linked to asthma in 2002, when ADAM33 was identified as the first asthma susceptibility gene (11). Although Adam33 does not regulate AAI in mice (12), it may promote chronic remodeling processes in asthmatic airways (13). ADAMs are zinc-dependent transmembrane MPs and are characterized by a multidomain structure, which can include pro-, MP, disintegrin, cysteine-rich, transmembrane, and intracellular domains (14). The MP domain proteolytically cleaves and releases signaling molecules and their receptors from cell surfaces to regulate the biological activities of these molecules (15). The disintegrin domain of some ADAMs binds to integrins to regulate cell adhesion and migration (16). The cysteine-rich and epidermal growth factor (EGF)-like domains of these molecules (15). The disintegrin and metalloproteinase-8; sL-selectin, soluble L-selectin; SNP, single nucleotide polymorphism; TBST, TBS containing 0.05% (v/v) Triton; TSLP, thymic stromal lymphopoietin; WT, wild-type.

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Abbreviations used in this article: AAI, allergic airway inflammation; ADAM, a disintegrin and metalloproteinase; AHR, airway hyperresponsiveness; alu, aluminum hydroxide; BAL, bronchoalveolar lavage; BM, bone marrow; EGF, epidermal growth factor; HDM, house dust mite; IC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; LMVEC, lung microvascular endothelial cell; LTB4, leukotriene B4; M1, classically activated; M2, alternatively activated; MBF, major basic protein; Mmp, matrix metalloproteinase; MP, metalloproteinase; PAS, periodic acid-Schiff; PMN, polymorphonuclear neutrophil; rADAM8, recombinant human a disintegrin and metalloproteinase-8; Rn, central airway resistance; sADAM8, soluble a disintegrin and metalloproteinase-8; s-lectin, soluble L-lectin; SNP, single nucleotide polymorphism; TBST, TBS containing 0.05% (v/v) Triton; TSLP, thymic stromal lymphopoietin; WT, wild-type.

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structure of the ADAM family. Its MP domain cleaves CD23, CD40L, 1-selectin, r-selectin, VCAM-1, and pro–TNF-α in vitro (22–24), but it is not clear whether these proteins are substrates for Adam8 in vivo. Little is known about the functions of the other domains of Adam8.

Adam8 expression is upregulated in the lungs of mice with AAI and human subjects with asthma (19, 25, 26). However, recent studies of mice over- or underexpressing Adam8 in the OVA model of AAI and AHR have reported conflicting results on the activities of Adam8 in regulating AAI and AHR. OVA-sensitized and -challenged transgenic mice that constitutively overexpress a soluble form of Adam8 had decreased peribronchial inflammation when compared with non-transgenic mice (27), suggesting that Adam8 has anti-inflammatory properties in the airways. In contrast, two more recent studies of mice lacking Adam8 both reported that Adam8 promoted OVA-induced AAI in mice (28, 29). However, one of these studies reported that Adam8 promoted chemotaxis of T cells, eosinophils, and macrophages (28), whereas the other study reported that Adam8 increased dendritic cell accumulation in the airways of mice (29). Thus, additional studies are needed to better understand the activities of Adam8 in regulating AAI and AHR in mice.

To address the uncertainties about the activities of Adam8 in regulating AAI and AHR, we compared AAI and AHR in Adam8−/− and wild-type (WT) mice in two different genetic backgrounds (mixed SvEv129 × C57BL/6 and BALB/c) sensitized and challenged with two different allergens using different sensitization protocols. We sensitized mice with high- versus low-dose OVA and an adjuvant by the i.p. route, or with a more clinically relevant allergen (house dust mite [HDM] protein extract) via the respiratory mucosal route in the absence of an adjuvant. We then challenged the mice with the same allergen by the inhaled route. Irrespective of the strain of mouse, allergen, or dose of allergen studied, Adam8−/− mice had greater AAI and AHR than WT mice. Additionally, leukocyte-derived Adam8 reduced AAI by increasing airway myeloid leukocyte cell death rates, thereby reducing the accumulation of eosinophils and macrophages in the airways of mice with AAI. Studies of Adam8 levels in lung sections from human subjects with or without asthma revealed that in asthma patients, airway eosinophils had robust staining for ADAM8, but patients, airway eosinophils had robust staining for ADAM8, but in asthma patients, airway eosinophils had robust staining for Adam8, but airway macrophages had minimal Adam8 staining. Thus, our study identifies leukocyte-derived Adam8 as a potent anti-inflammatory protein in the airways of the mice. Additionally, we report that a member of the Adam family promotes activation of the intrinsic apoptosis pathway in myeloid leukocytes to thereby limit airway inflammation. Our results identify Adam8 as a potential therapeutic target for human asthma.

Materials and Methods

Materials

Aluminum hydroxide (alum), Cadexa buffer, and PBS were purchased from Thermofisher Scientific (Pittsburgh, PA). HDM protein extract was obtained from Greer Laboratories (Lenoir, NC). Recombinant Adam8, recombinant caspase-3, murine and goat Abs to human Adam8 and CCL3, goat anti-murine TNF-α IgG, and ELISA kits for measuring MIP-1α, TSLP, CCL17, CCL22, eotaxin, TGF-β, and 1-selectin were purchased from R&D Systems (Minneapolis, MN). Abs to active caspase-3 and isotype-matched control Abs were obtained from Cell Signaling Technology (Danvers, MA). Rhodamine-conjugated donkey anti-mouse IgG, rhodamine-conjugated donkey anti-rabbit IgG, and fluorescein-conjugated donkey anti-goat IgG were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). The rabbit anti-human CD68 IgG was obtained from Abcam (Cambridge, MA), and the murine anti-human major basic protein (MBP) IgG was obtained from US Biologicals (Marblehead, MA). The ELISA kit for murine Adam8 was purchased from Ab-Online (Atlanta, GA), and the total protein assay kit was obtained from Bio-Rad (Hercules, CA). All other Abs were obtained from BD Biosciences (San Jose, CA).

Recombinant cytokines and chemokines were purchased from PeproTech (Rocky Hill, NJ). ELISA kits for measuring lung levels of IL-4, -5, -12, and -13, TNF-α, GM-CSF, and IL-10 were obtained from eBioscience (San Diego, CA). An ELISA kit for measuring OVA-specific IgE levels in serum samples was purchased from MD Biosciences (Minneapolis, MN). An ELISA kit for quantifying RANTES levels in lung samples was obtained from Enzyme (Huntington Station, NY). The Vector Red staining kit, a 3,3′-diaminobenzidine staining kit, Ag retrieval solution, peroxidase blocking solution, and ABC kit were purchased from Vector Laboratories (Burlington, CA). Kits for periodic acid–Schiff (PAS) and nonspecific esterase staining, murine anti-human smooth muscle α actin IgG2b, and chicken egg OVA were obtained from Sigma-Aldrich. The Mitobright staining kit was obtained from Invitrogen (Carlsbad, CA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Animals

All procedures performed on mice were approved by the Harvard Medical School Institutional Animal Care and Use Committee. Mice were housed in a barrier facility under specific pathogen-free conditions. Adam8−/− mice were generated and initially studied in a mixed SvEv129 × C57BL/6 strain, and WT littermate mixed SvEv129 × C57BL/6 strain mice were studied as controls. Adam8−/− mice were obtained from Andrew Docherty (UCB Cell Tech, Slough, U.K.) and Carl Blobel (Hospital for Special Surgery, New York, NY). Adam8−/− mice have normal lifespan, fertility, and long-term survival when compared with parental WT littermate controls, and no abnormalities have been identified in unchallenged mice (20).

The Adam8−/− SvEv129 × C57BL/6 strain mice were backcrossed eight generations into a BALB/c genetic background. Initial experiments confirmed that F8 BALB/c WT littermate controls had similar responses to allergens as parental WT littermate control mice. Thus, we studied age- and sex-matched F8 BALB/c WT parental littermate mice as controls in subsequent experiments. The genotypes of the mice were confirmed using PCR-based protocols performed on genomic DNA extracted from tail biopsies.

OVA-induced AAI using high and low doses of OVA for sensitization

Age- and sex-matched 8–12-wk-old WT and Adam8−/− mice were used for all studies. Initially, cohorts of SvEv129 × C57BL/6 WT and Adam8−/− mice were sensitized using a high dose of OVA (200 μg dissolved in 200 μl endotoxin-free PBS along with 1 mg alum) on days 0 and 7 by the i.p. route and then challenged for 20 min with an aerosolized 6% solution of OVA in endotoxin-free PBS on days 14–17. In all subsequent experiments, SvEv129 × C57BL/6 Adam8−/− mice versus WT littermate SvEv129 × C57BL/6 WT mice and BALB/c Adam8−/− mice versus BALB/c parental littermate WT mice were sensitized with a low dose (10 μg) of OVA diluted in 200 μl endotoxin-free PBS along with 1 mg alum by the i.p. route on days 0 and 7. Mice were then challenged with an aerosolized 6% solution of endotoxin-free OVA in PBS on days 14–17. All studies were performed on sex-matched F8 BALB/c WT parental littermate WT mice were sensitized, bronchoalveolar lavage (BAL) was performed, and total numbers of BAL leukocytes were counted. The percentage of each leukocyte subset in BAL samples was counted on modified Wright-stained cytocentrifuge preparations, and the absolute numbers of each leukocyte subset were calculated (31). Eosinophils were enumerated on Wright-Giemsa–stained cytocentrifuge preparations. We also immunostained BAL leukocyte preparations with a PE-conjugated mAb to Ccr3 and analyzed the cells by flow cytometry. We gated on the granulocyte population of cells identified by their forward and side scatter characteristics and quantified the percentage of eosinophils (granulocytes that were Ccr3 positive). In other cohorts of mice, mice were euthanized 24 h after the last OVA or PBS challenge, lungs were inflated to 25 cm H2O pressure, fixed in formalin, embedded in paraffin, and midsagittal lung sections were stained with H&E or PAS.

HDM protein–induced AAI

Mice were sensitized and challenged with HDM protein extract, as described previously (32). Briefly, three doses of HDM protein extract (100 μg in 50 μl endotoxin-free PBS or PBS alone) were delivered by the intranasal route at weekly intervals, and 72 h after the last HDM challenge, AAI was measured as outlined above. Preliminary experiments confirmed that prior reports (32) that 72 h after the last HDM challenge was optimal for measuring AAI and AHR in the mice.

AHR to methacholine challenges

Twenty-four hours after the last OVA challenge or 72 h after the last HDM challenge, mice were anesthetized with pentobarbital (100 mg/kg) and
a tracheostomy was performed. Central airway resistance (Rn) was measured in unchallenged or OVA-treated WT or Adam8<sup>−/−</sup> mice using a computer-controlled small animal ventilator (Flexivent; SCIREQ, Montreal, Canada) set at a tidal volume of 10 ml/kg, 120 breaths per min, and 2 cm H<sub>2</sub>O positive end expiratory pressure. Mice were challenged with PBS alone or a solution of 1–45 mg/ml methacholine in PBS.

**Lung dendritic cell quantification**

Lung dendritic cells were quantified as previously described (33). Briefly, lungs were removed from mice and enzymatically digested using 1 mg/ml collagenase IV and 0.5 mg/ml DNase from bovine pancreas in RPMI 1640 medium. Cells were then stained with allophycocyanin-conjugated hamster anti-mouse CD11c IgG or nonimmune allophycocyanin-conjugated hamster IgG as a control. Flow cytometry was performed using a FACSCanto II (BD Biosciences). Leukocyte subpopulations were identified by their forward and side scatter characteristics and positive immunostaining to quantify autofluorescence high CD11c<sup>bright</sup> cells (macrophages) versus auto-fluorescence low CD11c<sup>bright</sup> cells (dendritic cells).

**Quantification of cytokines and chemokine levels in lung samples from PBS- versus OVA-treated mice**

Lungs were harvested 24 h after the last OVA or PBS challenge, homogenized in PBS containing 0.5% (v/v) Triton, 1 mM PMSF, 1 mM 1,10-o-phenanthroline, Sigma Mammalian Protease Inhibitor Cocktail, and 2 mM sodium fluoride. Cytokine levels were analyzed using a cytokine bead array (Pierce Sichellight Technology; Thermo Fisher Scientific, Woburn, MA), or using commercially available ELISA kits.

**Measurement of OVA-specific IgE, soluble L-selectin, and soluble VCAM-1 levels in serum samples from PBS- versus OVA-treated mice**

 Serum was obtained from PBS- and OVA-treated WT and Adam8<sup>−/−</sup> mice by performing right ventricular puncture 24 h after the last allergen challenge. OVA-specific IgE, soluble L-selectin (s-L-selectin), and soluble VCAM-1 levels were quantified using commercial ELISA kits.

**Generation of Adam8<sup>−/−</sup> bone marrow chimera mice**

Unchallenged BALB/c WT and Adam8<sup>−/−</sup> mice were lethally irradiated twice, 4 h apart, using a radiation dose of 450 cGy using a 137Cs source. Immediately after the second irradiation, mice received 2 million bone marrow (BM)–derived leukocytes i.v. in 200 μl PBS. We generated and studied four groups of mice by transplanting: 1) WT BM into WT recipients; 2) WT BM into Adam8<sup>−/−</sup> recipients; 3) Adam8<sup>−/−</sup> BM into WT recipients; and 4) Adam8<sup>−/−</sup> BM into Adam8<sup>−/−</sup> recipients. After 10 wk of BM engraftment, mice were sensitized and challenged with PBS or OVA (using a low dose of OVA along with alum via the i.p. route for allergen sensitization) as outlined above.

**Isolation of BM-derived murine monocytes**

Unchallenged BALB/c strain WT and Adam8<sup>−/−</sup> mice were euthanized, their femurs and tibias were dissected, and the BM was flushed out by injecting RPMI 1640 medium through the marrow cavities. Erythrocytes were removed using a hypotonic lysis step, and cells were cultured for 60 h in RPMI 1640 medium containing 10% FCS, 100 U/ml penicillin, 10 mM sodium fluoride. Cytokine levels were analyzed using a cytokine bead array (Pierce Sichellight Technology; Thermo Fisher Scientific, Woburn, MA), or using commercially available ELISA kits.

**Leukocyte adhesion assays**

BM-derived monocytes or eosinophils were isolated from unchallenged BALB/c WT and Adam8<sup>−/−</sup> mice and incubated at 37°C for up to 1 h in triplicate in fibronectin-coated tissue-culture wells using 2 × 10<sup>5</sup> cells/well for all assays (36, 37). Monocytes were incubated at 37°C in RPMI 1640 containing 1 mg/ml collagenase and 0.5 mg/ml DNase at 37°C for 30 min, and a positive selection for PECA-1 was performed using rat anti-murine PECA-1 Ab, anti-rat microbeads, and LS MACS columns (Miltenyi Biotec, Cambridge, MA). LMVECs were cultured until confluent. Macrophages and eosinophils were isolated from the airways of BALB/c mice using a tracheostomy. Central airway resistance (Rn) was measured in unchallenged or OVA-treated WT or Adam8<sup>−/−</sup> mice using a computer-controlled small animal ventilator (Flexivent; SCIREQ, Montreal, Canada) set at a tidal volume of 10 ml/kg, 120 breaths per min, and 2 cm H<sub>2</sub>O positive end expiratory pressure. Mice were challenged with PBS alone or a solution of 1–45 mg/ml methacholine in PBS.

**Quantification of Adam8 levels in airway eosinophils and macrophages from WT mice with AAI**

Macrophages and eosinophils were isolated from the airways of BALB/c WT mice sensitized and challenged with OVA 24 h after the last OVA challenge using BAL. Macrophages were removed by adherence to tissue culture plastic for 1 h at 37°C and removing nonadherent cells (>90% eosinophils as assessed by Wright-Giemsa stained cytocentrifuge preparations). Extracts of the eosinophils and macrophages were prepared in RIPA buffer containing 1 mM PMSF, 1 mM 1,10-o-phenanthroline, and Sigma mammalian protease inhibitor mixture. Adam8 protein levels and total protein levels were measured in cell extracts using a commercial ELISA kit and a Bradford dye-binding kit (Bio-Rad), respectively. Adam8 levels were normalized to total protein levels in all samples.

**Isolation of lung microvascular endothelial cells**

Lung microvascular endothelial cells (LMVECs) were isolated from unchallenged BALB/c WT mice as previously described (35). Lungs were digested in RPMI 1640 containing 1 mg/ml collagenase and 0.5 mg/ml DNase at 37°C for 30 min, and a positive selection for PECA-1 was performed using rat anti-murine PECA-1 Ab, anti-rat microbeads, and LS MACS columns (Miltenyi Biotec, Cambridge, MA). LMVECs were cultured until confluent. Macrophages and eosinophils were isolated from the airways of BALB/c mice using Boyden chambers. Buffer alone in the lower chambers was used as a control for both cell types.
Assessment of the catalytic activity of recombinant human ADAM8 against recombinant chemokines and cytokines in vitro

Human pro-ADAM8 (5 μM) was activated with 20 nM thermolysin for 40 min at 37°C in TBS containing 0.5% (v/v) Triton (TBST [pH 7.4]), and then thermolysin was inactivated by adding 50 μM phosphoramidin. To confirm efficient activation of pro-ADAM8, we incubated 200 nM thermolysin-activated ADAM8 or buffer alone with 10 μM (7-methoxy-coumarin-4-yl) acetyl-Pro-Leu-Ala-Gln-Val-(3-[2,4-DNP]-L2,3-diaminopropionyl)-Arg-Ser-Ser-Arg-NH2 [a quenched fluorogenic substrate that is cleaved by ADAM8 (22, 23)] for up to 18 h at 37°C in 200 μl in TBST and measured cleavage of the substrate using fluorometry (excitation λ 320 and emission λ 405). The purified ADAM8 preparation progressively cleaved this fluorogenic substrate over 18 h, confirming that the prodomain was cleaved as well. We then incubated 200 nM active human ADAM8 with or without 0.8 μM human IL-5, 5.5 μM human IL-4, 5.5 μM human MIP-1α, 5 μM human RANTES, 5 μM human IP-10, or 1 μM human TNF-α in 25 μl TBST. Note that preliminary experiments confirmed these concentrations of mediators were optimal for detecting noncleaved forms (as a control) for up to 24 h to trigger the extrinsic apoptosis pathway. Cells were plated at a density of 2 × 10⁶ cells/ml in chamber slides containing WT or Adam8−/− mice with AAI

BALB/c WT and BALB/c Adam8−/− mice were sensitized and challenged with OVA (using low-dose OVA and alum delivered by the i.p. route for the allergen sensitization step). Airway leukocytes were harvested from the mice 24 h after the last OVA challenge by performing BAL. BAL leukocytes were fixed in 2% paraformaldehyde in PBS, permeabilized in 100% methanol, and stained with an Alexa 488-conjugated rabbit anti-active caspase-3 (Asp302-Glu303) IgG or an Alexa 488-conjugated nonimmune rabbit IgG (5 μg of Ab/10⁶ cells). Leukocyte subpopulations were identified by their characteristic forward and side scatter using flow cytometry, and intracellular staining for active caspase-3 was quantified by gating on each leukocyte subpopulation. To measure loss of mitochondrial membrane potential in leukocytes, BAL leukocytes were stained with 2 mM 5,5′,6′,6′′-tetrachloro-1,1,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, a red fluorophore that accumulates in healthy mitochondria) in the presence and absence of 1 mM carbonyl cyanide m-chlorophenyl hydrazone (a membrane-depolarizing agent that reduces JC-1 accumulation in mitochondria, as a control) using a Mitoprobe kit (Invitrogen). BAL cell subsets were identified by their forward and side scatter characteristics and data acquired in the FL-1 and FL-2 channels using flow cytometry. Loss of signal in the FL-2 channel (loss of JC-1 accumulation in mitochondria, indicative of loss of mitochondrial membrane potential) was quantified for each leukocyte subset. We also quantified surface levels of apoptosis ligands and their receptors on BAL leukocytes from mice with AAI. Leukocytes were isolated from the airways of BALB/c WT and BALB/c Adam8−/− mice 24 h after the last OVA challenge by performing BAL and removing erythrocytes using a hypotonic lysis buffer step. BAL leukocytes were incubated in 200 μl PBS containing 2% FBS, 5 μg/ml CD16/32 blocking Ab, and 0.1% sodium azide for 30 min at 4°C. Cells were then incubated for 30 min at 4°C with 2 μg/ml hamster anti-murine Fas-ligand IgG, hamster anti-murine Fas IgG, hamster anti-murine TNF-R1 IgG, hamster anti-murine TRAIL IgG, or 0.4 μg/ml goat anti-murine TNF-α (or the same concentration of an isotype-matched nonreactive secondary Ab). Cells were washed twice in PBS, incubated for 20 min at 4°C with an Alexa 488-conjugated rabbit antihamster or rabbit anti-goat IgG secondary Abs, washed twice in PBS, and then fixed using 2% paraformaldehyde in PBS. Cell-surface staining was quantified using flow cytometry.

Apoptosis of WT versus Adam8−/− mice and macrophages induced to undergo cell death in vitro

We induced apoptosis of BM-derived eosinophils or macrophages isolated from unchallenged BALB/c WT versus BALB/c Adam8−/− mice by incubating them at 37°C either: 1) in the absence of serum, agonists, or survival factors for up to 48 h to activate the intrinsic apoptosis pathway; or 2) with 10 μg/ml hamster anti-murine FAS (CD95) IgG; cross-linking Ab [clone Jo 2, which activates CD95 (40)] or 0.1 μg/ml hamster IgG Ab (as a control) for up to 24 h to trigger the extrinsic apoptosis pathway. Cells were stained with Annexin V conjugated to Alexa 488 and propidium iodide and analyzed using flow cytometry as described previously (31).

Surface levels of apoptosis ligands and receptors were quantified on WT and Adam8−/− macrophages and eosinophils using immunostaining and flow cytometry, as described above.

Intracellular active caspase-3, -8, and -9 levels

We measured levels of intracellular active caspases-3, -8, and -9 in macrophages and eosinophils that had been induced to undergo apoptosis as outlined above. Extracts of the cells were all prepared at 5 × 10⁶/ml in caspase lysis buffer [10 nM HEPES containing 0.1% 3-(3-cholamidopropyl)dimethylammonio-2-hydroxy-1-propanesulfonate, 2 mM EDTA, 5 μM DTT, 1 mM PMSF, 10 μg/ml pepstatin, 20 μg/ml leupeptin, and 0.1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride] as described previously (31). Intracellular levels of active caspase-3 were quantified in macrophage and eosinophil extracts by incubating them in triplicate with a quenched fluorogenic substrate that is specific for active caspase-3 (16 μM Ac-DEVD-7-amino-4-methylcoumarin) in the presence and absence of a specific caspase-3 inhibitor (20 μM Z-DEVD-fluoromethyl ketone) for 30 min at 37°C protected from light. A standard curve was generated by incubating the substrate with recombinant active caspase-3 assays standards (0–100 ng/ml), and cleavage of the substrate by leukocyte extracts or active caspase-3 assay standards was measured using fluorometry (F2500 fluorescence spectrophotometer; Hitachi, Tokyo, Japan; excitation λ 400 and emission λ 490). Substrate cleavage in the presence of the caspase-3 inhibitor was subtracted from the substrate cleavage in the absence of the caspase-3 inhibitor. Levels of each caspase-3 activity in cell extracts was determined by interpolation from the standard curve. Similarly, intracellular levels of active caspase-8 and -9 were measured by incubating cell extracts and assay standards of recombinant active caspase-8 or active caspase-9 (both at 0.5–5 μM) in triplicate with a quenched fluorogenic substrate that is specific for either active caspase-8 (14 μM IETD-7-amino-4-trifluoromethylcoumarin) or caspase-9 (50 μM LEHD-7-amino-4-trifluoromethylcoumarin), both in the presence and absence of an inhibitor of caspase-8 (20 μM Ac-IETD-aldehyde) or caspase-9 (20 μM Z-LEHD-fluoromethyl ketone), respectively. Cleavage of the substrates by standards and cell extracts was measured using fluorometry (excitation 400 λ and emission λ 505). Substrate cleavage in the presence of the caspase-8 inhibitor or caspase-9 inhibitor was subtracted from the substrate cleavage in the absence of the caspase-8 or -9 inhibitor for each sample (in fluorescent units). Caspase-8 or -9 activity in cell extracts was determined by interpolation from the standard curve.

Loss of mitochondrial membrane potential

Quiescent alveolar macrophages cultured in chamber slides or BM-derived eosinophils were stained with a Mitoprobe kit (vide supra), and loss of JC-1 staining was quantified using Metamorph software (Molecular Devices, Sunnyvale, CA).

Efferocytosis of apoptotic target cells by macrophages in vitro and in vivo

Peritoneal macrophages isolated from WT and Adam8−/− mice (vide supra) were plated at a density of 2 × 10⁵ cells/ml in eight-well chamber-slides and cultured for 4 d to render them quiescent. Murine polymorphonuclear neutrophils (PMNs) were isolated from the BM of unchallenged BALB/c WT and BALB/c Adam8−/− mice by positive selection using Gr-1 Ab and immunomagnetic beads (39) and incubated in serum-free RPMI 1640 medium at 37°C in the presence of 5% CO₂ for 18 h to induce apoptosis. Apoptotic WT or Adam8−/− PMNs (2 × 10⁵) were added to chamber slides containing WT or Adam8−/− macrophages, and cells were cultured for 4 h at 37°C. PMNs that had not been ingested by macrophages were removed by washing macrophages twice with PBS, the chamber slides stained using Diff Quick staining, and macrophage uptake of apoptotic cells quantified by counting apoptotic bodies in 300 macrophages per condition (41). We also quantified apoptotic bodies ingested by 200–300 macrophages isolated from the airways of OVA-treated BALB/c WT and BALB/c Adam8−/− mice 24 h after the last OVA challenge in Diff Quick–stained cytospin preparations, as outlined above.

Staining human lung sections for ADAMS

Formalin-fixed paraffin-embedded lung tissue samples from discarded human diagnostic specimens were retrieved from the files of Brigham and Women’s Hospital Department of Pathology. All studies of human subjects were approved by the Partners Healthcare Institutional Review Board. Note that it is very rare for our Pathology Department to receive lung biopsy specimens from adult patients with asthma for an asthma indication
alone. Thus, specimens were selected from patients who had undergone surgical resection for an incidental mass (diagnoses included three hamartomas, one metastatic renal cell carcinoma, and five primary lung adenocarcinomas), and lung tissue away from the area of the mass was examined. By history, four patients were current smokers, two were non-smokers, and three were former smokers. Cases were designated by a pathologist (L.S.) as a histologic or a morphometric basis on clinical history and pathologic criteria. Lung sections were deparaffinized, Ag retrieval was performed using Dako target retrieval solution (DakoCytomation) and Triton in PBS, and endogenous peroxidase activity was blocked using a peroxidase blocker. Nonspecific binding of Ab was blocked by incubating slides with 10% rabbit serum at room temperature. Slides were subsequently incubated with goat anti-human ADAM8 Ab or nonimmune goat IgG (both at 1 µg/ml) in Cadenza buffer containing 10% normal rabbit serum for 1 h at 4°C. Slides were then incubated at 4°C with rabbit anti-goat IgG conjugated to biotin (7.5 µg/ml), and the slides were stained using the Vector ABC kit (Vector Laboratories) using 3,3'-diaminobenzidine as the chromogen and counterstained with 1% methyl green.

Double-immunofluorescence staining of human lung sections for ADAM8 and markers of eosinophils and macrophages

To identify the leukocyte subsets that express ADAM8 in the airways of patients with asthma, we performed two-color immunofluorescence staining of lung sections from three patients with asthma. Briefly, the lung sections were deparaffinized, and Ag retrieval was performed by heating the slides in a microwave in citrate buffer. The sections were incubated overnight at 4°C for 18 h with either a murine IgG to human MBP (diluted 1:50) as a marker of eosinophils or a rabbit anti-human CD68 IgG (diluted 1:100) as a marker of macrophages. After washing the lung sections with PBS, the sections were incubated at 37°C for 1 h with rhodamine-conjugated donkey anti-murine IgG (diluted 1:100) or rhodamine-conjugated donkey anti-rabbit IgG (diluted 1:100). Sections were then washed in PBS and incubated at 37°C for 2 h with goat anti-human ADAM8 IgG (diluted 1:100). After washing the lung sections in PBS, fluorescein-conjugated donkey anti-goat IgG (diluted 1:100) was applied, and slides were incubated for an additional 1 h at 37°C. Nuclei were counterstained with DAPI. Images of the stained lung sections were analyzed using a confocal microscope (Leica Microsystems, Exton, PA) fitted with air-cooled argon and krypton lasers. Fluorescent confocal micrographs were recorded under dual fluorescent imaging mode in which the cells were simultaneously exposed to 488 and 568 nm light attenuated by an acusto-tunable optical filter. A band pass (530 ± 30 nm) filter was used to select light emitted from the fluorescein-labeled ADAM8, and a long-pass 590 nm filter was used to detect the rhodamine-labeled CD68 or MBP. MetaMorph software was used (39) to measure cell-associated fluorescence (in arbitrary fluorescence units) on images captured under the green filter of cells that were positively stained for MBP (eosinophils) or CD68 (macrophages).

Statistics

Statistical analysis was performed using the Sigma Stat statistics program. The p values were calculated using paired t tests if data were normally distributed and using a Mann–Whitney rank-sum test if not normally distributed. All data are represented as mean ± SEM unless otherwise indicated. A p value ≤0.05 was considered significant.

Results

Adam8 reduces lung levels of some Th2 cytokines and other pro- and anti-inflammatory mediators

Lung levels of IL-4 and IL-5 were significantly higher in the lungs of OVA-treated Adam8−/− mice than OVA-treated WT mice 24 h after the last OVA challenge (Fig. 2A, 2B). However, lung levels of IL-13 (Fig. 2C) and TSLP (Supplemental Table I) did not differ between OVA-treated WT and OVA-treated Adam8−/− mice. We also assessed whether Adam8 limits AAI in mice by reducing lung levels of cytokines and chemokines that promote migration of eosinophils and mononuclear leukocytes into the airways. Lung levels of RANTES (CCL5), IP-10 (CXCL10), and TNF-α were higher in OVA-treated Adam8−/− than OVA-treated WT mice (Fig. 2D–F). However, OVA-treated WT versus Adam8−/− mice did not differ in lung levels of other key chemokines that drive recruitment of eosinophils and mononuclear leukocytes into allergen-exposed airways including eotaxin (CCL11), MIP-1α (CCL3), JE (CCL2), MDC (CCL22), or TARC (CCL17; Supplemental Table I). We also found no differences in lung levels of the anti-inflammatory mediator, TGF-β, in OVA-treated WT and Adam8−/− mice (Supplemental Table I). However, lung levels of anti-inflammatory IL-10 were higher in OVA-treated Adam8−/− mice compared with OVA-treated WT mice. Thus, OVA-treated Adam8−/− mice had greater AAI and AHR than OVA-treated WT mice despite having higher lung levels of a key anti-inflammatory mediator.

Epithelial cell–derived Adam8 does not regulate mucin gene expression in airway epithelial cells

IL-13 is a key cytokine that induces mucus metaplasia during AAI in mice. However, although OVA-treated Adam8−/− mice had greater mucus metaplasia (Fig. 1B), they had similar levels of IL-13 when compared with OVA-treated WT mice (Fig. 2C). Adam8 is expressed by activated airway epithelium as well as leukocytes in mice (19), and other ADAM proteinases expressed by airway epithelial cells regulate mucin production by airway epithelial cells (43). Thus, we assessed whether epithelial-derived Adam8 regulates airway epithelial cell mucus production directly by measuring mucin gene expression in airway epithelial cells isolated from naive WT versus Adam8−/− mice and cultured under basal or IL-13–stimulated conditions. Muc5ac transcripts were not detected in epithelial cell cultures from either genotype under either basal or IL-13–stimulated conditions (data not shown). Muc5b gene expression was ~6-fold higher in unstimulated Adam8−/− epithelial cells when compared with unstimulated WT epithelial cells (Supplemental Fig. 2A). When WT and Adam8−/− epithelial cells were both incubated with IL-13, Muc5b gene expression increased ~11-fold in WT cells when compared with unstimulated
WT cells, but IL-13 did not increase Muc5b gene expression in Adam8−/− cultures when compared with expression levels in unstimulated Adam8−/− cells (Supplemental Fig. 2B). When the results for Muc5b gene expression in IL-13–activated epithelial cells were normalized to Muc5b expression in unstimulated WT epithelial cells, Muc5b gene expression increased to a similar extent in WT and Adam8−/− cells. Thus, epithelial cell–derived Adam8 restrains basal Muc5b gene expression in airway epithelial cells. However, under conditions in which IL-13 levels in the airways of mice are high (e.g., during AAI), our in vitro results indicate that Adam8 that is expressed by activated epithelial cells is unlikely to contribute to the Adam8-mediated reduction in mucus metaplasia that we observed in mice with OVA-induced AAI (Fig. 1B).

**Adam8 limits HDM protein–induced acute AAI and AHR in mice**

We also compared AAI and AHR in BALB/c WT versus Adam8−/− mice sensitized and challenged with a second Ag that is a common trigger for human asthma (HDM). HDM-treated Adam8−/− mice had higher BAL leukocyte counts (Fig. 3A) and greater peribronchial inflammation than HDM-treated BALB/c WT mice (Fig. 3B). In addition, HDM-treated Adam8−/− mice had greater AHR to aerosolized methacholine challenges than HDM-treated WT mice (Fig. 3C). Together, these data indicate that Adam8 potently limits AAI and AHR in response to two different allergens in mice sensitized by delivering allergen by two different routes (i.p. for OVA and the respiratory mucosal route for HDM), using either high or low doses of allergen (with or without an adjuvant) for sensitization. In all subsequent experiments, we studied WT and Adam8−/− mice in the BALB/c background in the more commonly used OVA model of AAI and AHR (using a low dose of OVA 10 μg) along with alum delivered by the i.p. route for the allergen sensitization step.

**Leukocytes are the crucial source of anti-inflammatory Adam8 in the airways of mice with AAI**

Adam8 is expressed both by leukocytes and activated airway epithelium in mice (19). Thus, we performed BM transplant experiments to generate Adam8 BM chimeric mice to determine whether leukocyte-derived or lung parenchymal cell–derived Adam8 mediates its anti-inflammatory activities in the airways of mice with AAI. OVA-treated WT recipients transplanted with Adam8−/− BM had greater peribronchial inflammation (Fig. 4A), higher BAL total leukocyte counts (Fig. 4B), greater BAL eosinophil counts (Fig. 4C), and higher BAL macrophage counts (data not shown) than WT recipients transplanted with WT BM. In addition, Adam8−/− recipients transplanted with WT BM had less peribronchial inflammation (Fig. 4A), lower BAL total leukocyte counts (Fig. 4B), lower BAL eosinophil counts (Fig. 4C), and lower BAL macrophage counts (data not shown) than Adam8−/− mice transplanted with Adam8−/− BM. Thus, BM-derived leukocytes are crucial sources of anti-inflammatory Adam8 in mice with AAI. To provide insights into which leukocytes are important

**FIGURE 1.** Adam8−/− mice have greater OVA-induced AAI than WT mice. In (A)–(D), BALB/c WT and BALB/c Adam8−/− mice were sham sensitized with PBS or sensitized with a low dose (10 μg) of OVA along with alum via the i.p. route and then challenged with aerosolized PBS or OVA. In (A) and (B), lungs were inflated and fixed in formalin 24 h after the last PBS or OVA challenge and stained with H&E (Fig. 1A, original magnification ×100) or PAS (Fig. 1B, original magnification ×400). The lung sections shown are representative of six mice per group. Arrows indicate peribronchial and perivascular inflammation in (A) and mucus metaplasia in (B). In (C), BAL was performed 24 h after the last PBS or OVA challenge, and absolute numbers of all BAL leukocytes (All WBCs), eosinophils (Eos), macrophages (Macs), lymphocytes (Lymphs), and PMNs were counted. Data are mean ± SEM; n = 4 mice in the PBS-treated groups and 14–19 mice in the OVA-treated groups. *p ≤ 0.001, **p ≤ 0.017 compared with PBS-treated mice belonging to the same genotype. In (D), Rn to aerosolized methacholine was measured 24 h after the last PBS or OVA challenge. Data are mean ± SEM; n = 5 to 6 mice/group for PBS-treated mice and n = 14–20 mice/group for OVA-treated mice. *p ≤ 0.014 versus OVA-treated WT mice. **p ≤ 0.021 when compared with PBS-treated mice belonging to the same genotype as the OVA-treated mice. Responses for OVA-treated WT mice ranged from 0.5–1.6 cm H2O/s/ml for 10 mg/ml of methacholine; 0.6–1.9 cm H2O/s/ml for 30 mg/ml of methacholine; and 1.0–1.8 cm H2O/s/ml for 45 mg/ml of methacholine. Responses for OVA-treated Adam8−/− mice ranged from 0.7–1.7 cm H2O/s/ml (for 10 mg/ml of methacholine); 1.1–3.4 cm H2O/s/ml (for 30 mg/ml of methacholine); and 1.5–3.4 cm H2O/s/ml (for 45 mg/ml of methacholine).
sources of Adam8 in the airways of mice with AAI, we isolated eosinophils and macrophages from the airways of OVA-sensitized and -challenged WT mice and measured Adam8 protein levels in extracts of the cells using an ELISA. Airway eosinophils and macrophages both robustly expressed Adam8 protein and contained similar amounts of Adam8 protein (Table I; p = 0.802). When we measured Adam8 protein levels in extracts of BM-derived eosinophils versus BM-derived monocytes, we detected Adam8 protein in both cell types. However, BM-derived eosinophils contained modestly (1.7-fold) but statistically significantly more Adam8 protein than BM-derived monocytes (1042.3 ± 189.5 vs. 605.2 ± 81.5 pg of Adam8/ng solubilized protein; n = 6 and 7 preparations, respectively; p = 0.047). These results suggest that eosinophils, monocytes, and macrophages are all important sources of this anti-inflammatory proteinase in the airways of WT mice with AAI.

To address the mechanism by which Adam8 reduced AAI and AHR in mice, we tested whether Adam8: 1) inhibited sensitization of mice to allergen; 2) reduced circulating leukocyte counts; 3) inhibited leukocyte adhesion or migration; 4) cleaved and thereby inactivated proinflammatory mediators that promote leukocyte recruitment into the airways; 5) decreased airway leukocyte survival; and/or 6) increased leukocyte clearance from the airways of mice with AAI.

Adam8 does not regulate sensitization of mice to OVA

OVA-treated WT and Adam8−/− mice did not differ in serum levels of OVA-specific IgE (Fig. 5A), indicating that Adam8 did not limit AAI in mice by reducing the sensitization of mice to OVA. Adam8 is expressed by dendritic cells (44), which regulate adaptive immune responses to allergens. Thus, we also quantified dendritic cell numbers in enzymatic lung digests from OVA-sensitized and -challenged WT and Adam8−/− mice. WT and Adam8−/− mice did not differ in their numbers of lung dendritic cells when treated with PBS or OVA (Fig. 5B). Additionally, Adam8 likely did not reduce OVA-induced AAI and AHR via Adam8’s MP domain degrading OVA because we detected no cleavage or degradation of OVA protein when we incubated OVA with 200 nM recombinant active human ADAM8 in vitro (data not shown).

Adam8 does not regulate circulating leukocyte counts or leukocyte adhesion or migration

Unchallenged WT and Adam8−/− mice do not differ in their peripheral total and differential WBC counts (20). OVA-treated WT and Adam8−/− mice also had similar total and differential leukocyte counts in blood samples (data not shown), indicating that Adam8 did not influence circulating leukocyte counts in mice with AAI.

Next, we tested whether Adam8 restrained AAI and AHR in mice by shedding leukocyte cell adhesion molecules to reduce leukocyte transendothelial migration, as ADAM8 sheds L-selectin from the surface of human PMNs activated in vitro (24). We measured circulating leukocyte surface L-selectin levels and levels of sL-selectin in serum samples from PBS- or OVA-treated WT versus Adam8−/− mice. Neither serum sL-selectin levels (data not shown) nor surface L-selectin levels on blood leukocytes (data not shown) differed between PBS- or OVA-challenged WT and Adam8−/− mice. Adam8 also sheds VCAM-1 in vitro (27), and VCAM-1 is an important vascular endothelial cell molecule regulating leukocyte migration into the airways during AAI (27). However, WT and Adam8−/− mice with AAI did not differ in serum levels of sVCAM-1 (PBS-treated WT mice, 106 ± 29 pg/ml; OVA-treated Adam8−/− mice, 271 ± 75 pg/ml; OVA-treated WT mice, 1581 ± 127 pg/ml; OVA-treated Adam8−/− mice, 1894 ± 112 pg/ml; n = 4 PBS-treated mice/group and n = 8 OVA-treated mice/group). Thus, it is unlikely that Adam8 inhibited leukocyte migration into the airways of mice with AAI by shedding leukocyte or endothelial cell adhesion molecules.

Adam8 has a disintegrin domain that binds α9β3 integrin expressed on the surface of osteoclasts (18), and binding of the disintegrin domain of other ADAM family members to integrins regulates cell adhesion and migration (16). Thus, we compared the capacity of WT versus Adam8−/− leukocytes isolated from unchallenged mice to: 1) adhere to murine LMVECs and a representative extracellular matrix protein (fibronectin); and 2) migrate in response to chemoattractants in vitro. WT and Adam8−/− eosinophils and monocytes did not differ in their capacity to adhere to murine LMVECs (Fig. 6A, 6B) or fibronectin (data not shown) either under basal or stimulated conditions. WT and Adam8−/− eosinophils and monocytes also did not differ in their ability to migrate in response to buffer or chemoattractants in either Boyden microchemotaxis chambers (Fig. 6C, 6D) or Matrigel Invasion Chambers.

FIGURE 2. OVA-treated Adam8−/− mice have higher lung levels of Th2 cytokines, RANTES, IP-10, and TNF-α compared with OVA-treated WT mice. In (A)–(F), BALB/c WT and BALB/c Adam8−/− mice were treated with PBS or sensitized with a low dose (10 μg) of OVA along with alum by the i.p. route and then challenged with aerosolized PBS or OVA. Levels of IL-4 (A), IL-5 (B), IL-13 (C), RANTES (D), IP-10 (E), and TNF-α (F) protein were measured in homogenates of lungs isolated from PBS- and OVA-treated BALB/c WT and BALB/c Adam8−/− mice 24 h after the last PBS or OVA challenge using ELISAs. Data are mean ± SEM; n = 4–6 for PBS-treated mice and n = 6–10 for OVA-treated mice. In (A)–(C), *p ≤ 0.046 (and p ≤ 0.003 for OVA- versus PBS-treated mice belonging to the same genotype) (data not shown). In (D)–(F), *p = 0.041; **p ≤ 0.022 compared with PBS-treated mice belonging to the same genotype as the OVA-treated mice.
Adam8 IS AN ANTI-INFLAMMATORY PROTEIN DURING AAI

We found higher levels of some Th2 cytokines and mediators of inflammation in the lungs of OVA-treated Adam8−/− mice versus OVA-treated WT mice, and Adam8 has an active MP domain. Thus, we tested whether Adam8 reduced lung levels of RANTES, IP-10, TNF-α, IL-4, and/or IL-5 (Fig. 2) by its MP domain degrading these mediators. We studied the activity of recombinant human Adam8 (rhAdam8) because purified murine Adam8 is not commercially available. First, we confirmed that rhAdam8 ectodomain (which contains the MP domain) is catalytically active by showing that it progressively degraded a quenched fluorogenic peptide substrate that is sensitive to cleavage by ADAM8 (22 and data not shown). However, active rhAdam8 did not degrade recombinant purified human IL-4, RANTES, TNF-α, or IP-10 (Supplemental Fig. 3 or data not shown) even when high concentrations (200 nM) of rhAdam8 were tested in vitro. Although a lower Mγ cleavage product of IL-5 was detected when IL-5 was incubated with 200 nM rhAdam8, most of the IL-5 tested was not degraded (Supplemental Fig. 3). Thus, given the high concentration of Adam8 tested in vitro and the low amount of the IL-5 cleavage product generated, it is unlikely that Adam8 reduced lung levels of IL-5 during AAI in mice via its MP domain degrading IL-5. Likely, the higher lung levels of some proinflammatory mediators (Fig. 2) and anti-inflammatory IL-10 (Supplemental Table I) in Adam8−/− mice with AAI compared with WT mice with AAI were a consequence of the higher leukocyte counts in the airsaws of Adam8−/− mice with AAI as leukocytes are important cellular sources of these mediators.

Adam8 increases apoptosis of eosinophils and macrophages in the airsaws of mice with AAI, but has no effect on regulating macrophage uptake of apoptotic leukocytes

To assess whether Adam8 limited AAI by reducing the survival of airway macrophages and eosinophils, we compared rates of apoptosis of macrophages and eosinophils recruited to the airsaws of WT versus Adam8−/− mice with AAI. Intracellular levels of active caspase-3 (Fig. 7A) and loss of mitochondrial membrane potential (which is a marker of activation of the intrinsic apoptosis pathway; Fig. 7B) were significantly lower in all Adam8−/− airway leukocytes and also in Adam8−/− airway eosinophils and airway macrophages (but not lymphocytes) than airway leukocytes from WT mice with AAI. This result indicates that Adam8 reduced the survival of airway leukocytes in mice with AAI. Airway macrophages isolated from Adam8−/− mice with AAI also contained fewer apoptotic bodies than airway macrophages from WT mice with AAI. We assessed whether the latter finding was due to impaired macrophage uptake of apoptotic cells (efferocytosis) in the absence of Adam8. When WT and Adam8−/− macrophages isolated from unchallenged mice were incubated with equal numbers of granulocytes that had been induced to undergo apoptosis in vitro, the mean number of apoptotic target cells ingested per WT and Adam8−/− macrophage was similar (Fig. 7D). These data indicate that the reduced number of apoptotic bodies in airway macrophages from Adam8−/− mice with...
AAI in Fig. 7C was likely to be due to reduced rates of airway leukocyte apoptosis in mice lacking Adam8 rather than to defects in efferocytosis of airway macrophages lacking Adam8. Adam8 does not regulate surface levels of apoptosis ligands or receptors on airway leukocytes during AAI in mice. Adam8 has an active MP domain that has the potential to shed and activate apoptosis ligands from cell surfaces. However, surface levels of TNF-α, Fas-ligand, and TRAIL, and their receptors did not differ on airway eosinophils or macrophages isolated from OVA-treated WT and Adam8<sup>−/−</sup> mice (data not shown). Thus, it is unlikely that Adam8 reduced survival of airway leukocytes by activating the extrinsic apoptosis pathway in vivo.

Adam8 promotes activation of the intrinsic but not extrinsic apoptosis pathway in myeloid leukocytes in vitro

To determine which apoptosis pathway in myeloid leukocytes is regulated by Adam8, we isolated macrophages or eosinophils from naive WT and Adam8<sup>−/−</sup> mice and compared their rates of apoptosis.

Table I. Airway eosinophils and macrophages isolated from WT mice with AAI express Adam8 protein

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<th>BAL macrophages&lt;sup&gt;a&lt;/sup&gt;</th>
<th>BAL eosinophils&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>236.0 ± 65.7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>174.5 ± 24.2</td>
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</tbody>
</table>

Data are mean ± SEM.

<sup>a</sup>Adam8 protein and total protein levels were measured in extracts of BAL macrophages versus BAL eosinophils using an ELISA and a Bradford dye-binding assay kit, respectively. The results are expressed as picograms of Adam8 per microgram of total protein.

<sup>b</sup>BALB/c WT mice were sensitized with 10 μg of OVA by the i.p. route and then challenged with aerosolized OVA. Twenty-four hours after the last OVA challenge, airway eosinophils and macrophages were isolated, and cell extracts were prepared as described in Materials and Methods.

<sup>c</sup>n = 16 different paired BAL macrophage and eosinophil preparations (p = 0.802 for the comparison of airway macrophages and eosinophils).

FIGURE 4. Leukocyte-derived Adam8 predominantly mediates Adam8’s anti-inflammatory activities in the airways during AAI. We generated BALB/c strain Adam8 BM chimeric mice, as described in Materials and Methods. Adam8 BM chimeric mice were sham-sensitized with PBS or sensitized with a low dose (10 μg) of OVA along with alum by the i.p. route and then challenged with aerosolized PBS or OVA. Lungs were inflated and fixed in formalin, or BAL was performed. (A) shows images of H&E-stained sections of lungs from mice 24 h after the last OVA challenge (images are representative of six mice per group; original magnification ×100). PBS-treated mice had no airway inflammation (data not shown). Twenty-four hours after the last OVA or PBS challenge, total leukocytes (B) and granulocytes (>80% eosinophils in [C]) were counted in BAL samples. Data are mean ± SEM n = 3 to 4 mice/group for PBS-treated mice and n = 8–22 mice/group for OVA-treated mice. *p ≤ 0.01, **p ≤ 0.003 in (B) and (C).

FIGURE 5. Adam8 is not required for sensitization of mice to OVA. BALB/c WT and BALB/c Adam8<sup>−/−</sup> mice were sham-sensitized with PBS or sensitized with a low dose (10 μg) of OVA along with alum delivered by the i.p. route and then challenged with aerosolized PBS or OVA. In (A), levels of OVA-specific IgE were measured in serum samples from mice treated with either with PBS (n = 3 mice/group) or OVA (n = 8 to 9 mice/group). Data are mean ± SEM. *p < 0.002 when compared with PBS-treated mice of the same genotype. In (B), lungs were harvested from the mice 24 h after the last OVA or PBS challenge, and lungs were enzymatically digested, as described in Materials and Methods. Dendritic cells were enumerated in single-cell suspensions of lung digests by immunostaining and flow cytometry, as described in Materials and Methods. Data are mean ± SEM (n = 3 mice/group for PBS-treated mice and n = 5 mice/group for OVA-treated mice).
Adam8 is not required for murine eosinophils or monocytes to adhere to LMVEC monolayers or migrate in vitro. In (A), equal numbers (10⁶ cells) of BM-derived monocytes isolated from unchallenged BALB/c WT or BALB/c Adam8−/− mice were incubated at 37°C for 1 h in duplicate with or without 1 μg/ml LPS for 2 h on LMVEC monolayers that had been preincubated for 4 h at 37°C with or without 10 ng/ml TNF-α. In (B), equal numbers (10⁶ cells) of BM-derived eosinophils isolated from unchallenged BALB/c WT and BALB/c Adam8−/− mice were incubated in duplicate at 37°C with or without 10−⁷ M LTB₄ on LMVEC monolayers that had been preincubated for 18 h with or without 10−⁷ M IL-4 and 10−⁷ M IL-13. In (A) and (B), the percentage of leukocytes adhering to the LMVEC monolayers was determined. Data are mean ± SEM; n = 3 to 4 experiments. In (C) and (D), we used Boyden microchemotaxis assay chambers to compare the migration of BM-derived WT versus Adam8−/− monocytes to buffer alone, 10−⁷ M IL-6, or 10−⁷ M MIP-1α in the lower chambers [in (C)] or the migration of WT versus Adam8−/− BM-derived eosinophils in response to buffer alone, 10−⁷ M LTB₄, or 10−⁷ M murine eotaxin in the lower chambers [in (D)]. Data are expressed as fold increase in cellular migration when compared with cellular migration in response to buffer alone. Data are mean ± SEM from three to six wells per experimental condition and three to six separate experiments. *p ≤ 0.031 in (C) and p ≤ 0.01 in (D) when compared with rates of migration of cells of the same genotype in response to buffer.

after triggering activation of either: 1) the extrinsic apoptosis pathway in eosinophils by ligating FAS; or 2) the intrinsic apoptosis pathway by serum starving the cells. When we activated the extrinsic apoptosis pathway in WT and Adam8−/− macrophages and eosinophils in vitro, we found no differences in their rates of apoptosis, as assessed by measuring intracellular levels of active caspases-3 and -8 (Table II) and binding of Annexin V–Alexa 488 and propidium iodide to the cells (Table III). WT and Adam8−/− eosinophils and macrophages had similar cell-surface levels of apoptosis ligands and their receptors including FAS-L, TRAIL, TNF-R1, and FAS (data not shown), with the exception of surface TNF-α levels, which were significantly higher on Adam8−/− macrophages compared with WT cells (data not shown). The relevance of the latter in vitro finding to events occurring in the airways of mice with AAI is not clear given that: 1) we found similar levels of TNF-α on the surface of macrophages from the airways of WT and Adam8−/− mice with AAI (data not shown); and 2) studies of conditional Adam17−/− mice lacking Adam17 only in myeloid leukocytes have confirmed that Adam17 is the main TNF-α sheddase expressed by murine macrophages in vitro and in vivo (45).

When we triggered the intrinsic apoptosis pathway in myeloid leukocytes isolated from unchallenged WT and Adam8−/− mice,
and Adam8<sup>−/−</sup> mice with AAI. As mentioned above, lung levels of IL-5 (which is an important survival factor for eosinophils) were increased in Adam8<sup>−/−</sup> mice with AAI compared with WT mice with AAI (Fig. 2B). However, WT and Adam8<sup>−/−</sup> mice with AAI did not differ in lung levels of M-CSF (which is a key survival factor for macrophages) or GM-CSF (which is an important survival signal for both eosinophils and macrophages; Supplemental Table I). Thus, it is unlikely that Adam8 promotes apoptosis of macrophages by reducing lung levels of macrophage survival factors.

**ADAM8 is robustly expressed in peribronchial granulocytes and airway epithelium, but not in macrophages, in lung sections from human patients with asthma**

To gain insights into the cell types that express ADAM8 in asthmatic airways, we immunostained lung sections from asthma patients and control subjects without asthma for ADAM8. ADAM8 was expressed to a similar extent in bronchial epithelium and type II pneumocytes in the lungs of patients with and without asthma (Fig. 9A). The bronchial epithelium had membranous and cytoplasmic staining patterns, with an apical accentuation in both asthmatic and healthy human subjects expressed minimal quantities of ADAM8 staining (Fig. 10). Peripheral blood eosinophils or monocytes from healthy human subjects, whereas macrophages had relatively low-level ADAM8 staining in asthmatic subjects, whereas macrophages had relatively low-level ADAM8 staining (Fig. 10). Peripheral blood eosinophils or monocytes from healthy human subjects expressed minimal quantities of ADAM8 on their surface under basal conditions, but incubating the cells with a proinflammatory mediator (10<sup>−7</sup> M LTB<sub>4</sub>) for 30 min at 37°C induced 2- to 3-fold increases in surface ADAM8 protein levels.

### Table II. Intracellular levels of active caspase-3 and -8 do not differ in eosinophils undergoing apoptosis induced by activating the extrinsic apoptosis pathway in vitro

<table>
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<tr>
<th>Time Point (h)</th>
<th>WT Eosinophils&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Adam8&lt;sup&gt;−/−&lt;/sup&gt; Eosinophils</th>
<th>WT Eosinophils</th>
<th>Adam8&lt;sup&gt;−/−&lt;/sup&gt; Eosinophils</th>
<th>WT Eosinophils</th>
<th>Adam8&lt;sup&gt;−/−&lt;/sup&gt; Eosinophils</th>
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Data are mean (SEM), n = 3 to 4 mice studied per group. We were not able to induce significant activation caspase-3 or -8 in either WT or Adam8<sup>−/−</sup> macrophages by treating cells with the FAS cross-linking Ab for up to 48 h (data not shown).<sup>a</sup>Eosinophils were isolated from the BM of unchallenged BALB/c WT and BALB/c Adam8<sup>−/−</sup> mice, as described in Materials and Methods. Macrophages were isolated from the peritoneal cavities of BALB/c WT and BALB/c Adam8<sup>−/−</sup> mice 4 d after thioglycollate was delivered by the i.p. route, and cells were cultured for 5 d in tissue-culture plates to render them quiescent. Equal numbers of eosinophils or macrophages were incubated with a cross-linking Ab to CD95 (FAS), which activates this death domain-containing receptor, or a nonimmune isotype control Ab for up to 20 h for eosinophils or 48 h for macrophages. At intervals, cell extracts were prepared at 5 × 10<sup>6</sup> cells/ml, and intracellular levels of active caspase-3 and -8 were measured using specific fluorogenic substrates, as described in Materials and Methods.

### Table III. Binding of Annexin V and propidium iodide to WT versus Adam8<sup>−/−</sup> eosinophils does not differ when the extrinsic apoptosis pathway is triggered in vitro

<table>
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<th>Time Point (h)</th>
<th>WT Eosinophils&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Adam8&lt;sup&gt;−/−&lt;/sup&gt; Eosinophils</th>
<th>WT Eosinophils</th>
<th>Adam8&lt;sup&gt;−/−&lt;/sup&gt; Eosinophils</th>
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Data are mean (SEM), n is number of independent experiments. We were not able to induce significant positive staining for either Annexin V or propidium iodide in either WT or Adam8<sup>−/−</sup> macrophages by treating cells with the FAS cross-linking Ab for up to 48 h (data not shown).<sup>a</sup>Eosinophils or macrophages were incubated with a cross-linking Ab to CD95 (FAS), which activates this death domain-containing receptor, or a nonimmune isotype control Ab for up to 20 h for eosinophils or 48 h for macrophages. At intervals, cells were stained with Annexin V conjugated to Alexa 488 and propidium iodide, and the percentage of cells staining positively with Annexin V and negatively with propidium iodide, late apoptotic cells (cells staining positively with both Annexin V and propidium iodide), and nonviable cells (cells stained with either Annexin V and/or propidium iodide).
on monocytes and eosinophils (data not shown). Thus, surprisingly, ADAM8 (which has anti-inflammatory actions in the airways of mice with AAI) is expressed at only low levels in airway macrophages in human asthma patients.

Discussion
We report consistent and robust anti-inflammatory actions for leukocyte-derived Adam8 in murine AAI by studying WT versus Adam8−/− mice in two genetic backgrounds exposed to different allergens and different sensitization protocols. Our results showed that airway eosinophils and macrophages robustly expressed Adam8 in WT mice with AAI, and both cell types are likely to be crucial sources of anti-inflammatory Adam8 in mice with AAI.

We also report that an Adam family member promotes activation of the intrinsic apoptosis pathway in both macrophages and eosinophils, thereby restraining allergen-induced AAI and AHR in mice.

Until now, ADAM family members have been thought to regulate cellular apoptosis via their MP domains shedding apoptosis ligands and/or receptors (14). However, we found no evidence that Adam8 sheds apoptosis ligands or their receptors from the surface of myeloid leukocytes or activates the extrinsic apoptosis pathway in airway myeloid leukocytes in mice with AAI. Apoptosis promotes resolution of inflammatory responses, and nonphlogistic removal of apoptotic cells is associated with the release of anti-inflammatory mediators by macrophages (52). Asthma has been strongly linked to reduced rates of apoptosis of leukocytes in peripheral blood and airways (53–58), including two leukocyte subsets that express Adam8 [eosinophils and macrophages (54, 56, 59–61)]. There is evidence that prolonged survival of airway macrophages in asthma patients versus control subjects. (A) Lung sections from human patients without (top panels) or with (bottom panels) asthma were stained with either H&E (left panels), an isotype control Ab (middle panels), or an anti-Adam8 Ab (right panels; original magnification ×100). (B) High-magnification images (original magnification ×400) of lung sections from patients with and without asthma showing intense staining for Adam8 in peribronchial granulocytes (eosinophils; black arrows) in the asthma patients, minimal staining for Adam8 in macrophages in either control subjects or patients with asthma (black arrowheads), and a lack of staining for Adam8 in the predominantly lymphocytic peribronchial infiltration on a patient with chronic bronchitis (top right panel, red arrows). The images shown in (A) and (B) are representative of lung sections from five asthmatic subjects and four control subjects without asthma.

**FIGURE 8.** Adam8 promotes activation of the intrinsic apoptosis in eosinophils and macrophages in vitro. The intrinsic apoptosis pathway was activated in quiescent peritoneal macrophages (Macs in A, B, F), quiescent alveolar macrophages (D), or quiescent BM-derived eosinophils (Eos in C and E) isolated from unchallenged BALB/c WT and BALB/c Adam8−/− by incubating cells in the absence of serum, agonists, and survival factors for up to 48 h at 37°C. At intervals, cells were stained with Annexin V conjugated to Alexa 488 and propidium iodide (A), and the percentage of cells staining positively for both Annexin V and propidium iodide was quantified by flow cytometry. Data are mean ± SEM positive cells; n = 3 experiments. *p ≤ 0.027. Intracellular levels of active caspase-3 (B, C) or active caspase-9 (F) were quantified in macrophage and/or eosinophil cell extracts using quenched fluorogenic substrates that are specific for caspase-3 or caspase-9, respectively, and fluorimetry. Data are mean ± SEM positive cells; n = 3–8 experiments. In (B), *p ≤ 0.013; in (C), *p ≤ 0.003 versus Adam8−/− cells studied at the same time point. In (F), *p < 0.029 compared with WT cells studied at the same time point. Loss of mitochondrial membrane potential was measured in serum-starved alveolar macrophages (D) or eosinophils (E) isolated from unchallenged BALB/c WT or BALB/c Adam8−/− mice, as described in Materials and Methods. Data are mean ± SEM; n = 4 to 5 experiments. In (D) and (E), *p < 0.048, **p = 0.007.
FIGURE 10. ADAM8 is expressed at higher levels in eosinophils than macrophages in the lungs of patients with asthma. Lung sections from an asthmatic subject were double immunostained with FITC for ADAM8 (left panels in A, B), with rhodamine for a marker of eosinophils (MBP; middle panels in A), or with rhodamine for a marker of macrophages (CD68; middle panels in B) and examined using a confocal microscope (original magnification ×600). Merged images are shown in the right panels for (A) and (B). The images shown are representative of immunostained lung sections from three asthmatic subjects. Lung sections stained with non-immune control primary Abs showed no staining (data not shown). In (C), ADAM8 staining in cells identified as eosinophils (Eos) versus macrophages (Macs; by their positive staining with rhodamine using the markers listed above) was quantified using MetaMorph image analysis software. The lines in the boxes in the box plots show the 25th percentiles, medians, and 75th percentiles, and the error bars represent the 10th and 90th percentiles. Data are mean ± SEM (n = 18 eosinophils and 9 macrophages). *p = 0.025.

eosinophils and macrophages contributes to airway inflammation in asthma (47, 54, 62, 63). However, little is known about the pathways that regulate leukocyte apoptosis in asthma. Our study has identified Adam8 as a key molecule that promotes leukocyte apoptosis in the airways of mice with AAI. We speculate that the cytoplasmic tail of Adam8 binds to intracellular signaling molecules to trigger activation of the intrinsic pathway in myeloid leukocytes, and our future studies will investigate this possibility. Alternatively, Adam8 reduced the levels or activity of prosurvival factors in the airways of mice with AAI. Consistent with this, we found higher lung levels of the eosinophil prosurvival signal, IL-5 (47), in Adam8−/− mice with AAI compared with WT mice with AAI (Fig. 2B), which was unlikely to be due to the MP domain of Adam8 degrading IL-5 (Supplemental Fig. 3). Likely, the higher lung levels of IL-5 reflected the greater number of IL-5–producing cells in the airways of Adam8−/− mice with AAI when compared with WT mice with AAI. Although we found no evidence (Supplemental Table I) that Adam8 regulated lung levels of M-CSF or GM-CSF (key survival factors for myeloid leukocytes), it is possible that the MP domain of Adam8 cleaved and thereby regulated lung levels of other yet-to-be identified eosinophil or macrophage prosurvival factors in mice with AAI.

Although lung levels of some Th2 cytokines and proinflammatory mediators were increased in Adam8−/− mice compared with WT mice with AAI, the MP domain of rADAM8 did not degrade these mediators in vitro (Supplemental Fig. 3 or not shown). Adam8−/− mice had greater allergen-induced AAI and AHR than WT mice despite having higher levels of the anti-inflammatory cytokine IL-10 (Supplemental Table I). Likely, the higher lung levels of proinflammatory mediators (and IL-10) reflected the increased numbers of leukocytes resistant to apoptosis in the airways of Adam8−/− mice with AAI because leukocytes are key sources of these mediators. Adam8 may have reduced AAI in mice by polarizing airway macrophages from an alternatively activated (M2) phenotype [which other studies have reported to be increased in the airways of mice with AAI and linked to disease severity (64)] toward a classically activated (M1) phenotype. However, this is unlikely, because when compared with WT mice with AAI, Adam8−/− mice with AAI had higher lung levels of some markers of M1 macrophages (IL-12, TNF-α, and CCL-5) along with higher levels of a classic M2 marker (IL-10), but similar lung levels of other M1 (CCL2) and M2 (TGF-β) markers (Fig. 2, Supplemental Table I).

An interesting finding in our paper was that although lung levels of IL-4 and IL-5 levels were higher in OV A-treated Adam8−/− mice than OVA-treated WT mice, lung levels of IL-13 were similar in OV A-treated WT and Adam8−/− mice. Likely, CD4+ Th2 T cell counts were increased in OVA-treated Adam8−/− mice compared with OVA-treated WT mice, leading to the higher lung levels of IL-4 and IL-5 in the Adam8−/− mice. One explanation for the similar lung levels of IL-13 in OVA-treated WT and Adam8−/− mice is that IL-13 is produced by cells other than CD4+ Th2, including smooth muscle cells (65) and mucocytes, which are innate immune cells that produce early bursts of IL-13 in tissues with allergic inflammation (66). It is possible that in our study, Adam8 reduced Th2 T cell counts but increased the activation or numbers of these other IL-13–producing cells during AAI in mice, leading to overall similar lung levels of IL-13 in OVA-treated WT and Adam8−/− mice. These possibilities will be the focus of future studies in our laboratory.

Another interesting finding in our paper was that OVA-treated Adam8−/− mice had greater mucus metaplasia in their airways than OVA-treated WT mice despite having similar lung levels of IL-13, an important cytokine driving mucus metaplasia in the airways of allergen-treated mice (67). Although epithelial cell–derived Adam10 and -17 regulate mucin gene expression in epithelial cells by activating the EGF receptor (43), we found no evidence that epithelial-derived Adam8 reduced mucus production by IL-13–activated airway epithelium (Supplemental Fig. 2). One possible explanation for the increased mucus metaplasia in the airways of OVA-treated Adam8−/− versus WT mice in the presence of similar lung levels of IL-13 is that OVA-treated Adam8−/− mice have higher airway macrophages and eosinophil counts (Fig.
Adam8 is an Anti-Inflammatory Protein during AAI

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et al. (28), we did not find any defects in the capacity of Adam8 to evaluate airway leukocyte apoptosis in sAdam8-transgenic mice. Divergent results in other studies of mice with AAI (69–71) have produced strain differences (reviewed elegantly in Ref. 64) that have produced divergent results in other studies of mice with AAI (69–71).

Adam8 was essential for the migration of T cells, macrophages, and monocytes (28, 29). Paulissen et al. (29) reported that Adam8 promoted AAI and AHR in C57BL/6 mice but by different mechanisms (28, 29). Paulissen et al. (29) reported that Adam8 increased AAI in mice by increasing lung dendritic cell counts and levels of eotaxin in vitro. Unlike Paulissen et al. (29), we did not find any differences in lung dendritic cell counts or lung levels of CCL22 or eotaxin in WT versus Adam8−/− mice with AAI. Unlike Naus et al. (28), we did not find any defects in the capacity of Adam8−/− eosinophils or mononuclear phagocytes to migrate in vitro using both Boyden chamber (Fig. 6) and Matrigel invasion assays (data not shown). Naus et al. (28) and our study both tested eotaxin as a chemotactant for eosinophils. However, we tested FMLF and MIP-1α as monocyte chemotactants, whereas Naus et al. (28) used stromal cell–derived factor-1 to stimulate dendritic cell migration and LPS and TNF-α to stimulate macrophage migration in vitro. It is possible that differences in the stimuli used for mononuclear phagocyte migration assays may explain the differences in the results obtained. It is interesting to note that although Naus et al. (28) reported that T lymphocytes from mice with AAI expressed Adam8 and T cell–derived Adam8 was essential for Adam8’s proinflammatory actions in marine airways, another group found that T lymphocytes do not express Adam8 (44). Overall, our results are more consistent with those of Matsuno et al. (27) on sAdam8-transgenic mice with OVA-induced AAI.

Possible explanations for the conflicting results of our study versus those of Naus et al. (28) and Paulissen et al. (29) on WT versus Adam8−/− mice with AAI include the strain of mouse studied and the sensitization dose and route used to deliver the allergen to mice. Most of our studies were conducted on Adam8−/− mice in the BALB/c strain, whereas Naus et al. (28) and Paulissen et al. (29) studied Adam8−/− C57BL/6 strain mice. These strain differences (reviewed elegantly in Ref. 64) have produced divergent results in other studies of mice with AAI (69–71). However, it is unlikely that strain differences explain the divergent results for Adam8−/− mice with AAI because we found similar anti-inflammatory activities for Adam8 when we studied Adam8−/− and WT mice in a mixed SvEv129 × C57BL/6 strain and the BALB/c strain. The sensitization dose of OVA used by Naus et al. (28) was significantly higher than the dose that we used in most of our studies (2 mg/ml versus 50 μg/ml or 10 μg/mouse, respectively). Sensitization of C57BL/6 strain mice with high rather than low doses of OVA leads to a Th1-type airway immune response (72) and a reduction in allergic phenotype due to the recruitment of anti-inflammatory effector CD8+ T cells into the airways (73). However, it is unlikely that the allergen sensitization dose explains the divergent results obtained for Adam8−/− mice with AAI in our study versus the studies of Naus et al. (28) and Paulissen and coworkers (29) because: 1) Paulissen et al. (29) used the same low dose of OVA in C57BL/6 mice that we used in BALB/c and mixed SvEv129 × C57BL/6 strain mice; and 2) when we sensitized Adam8−/− versus WT mice with a low versus high sensitization dose of OVA, Adam8−/− mice had greater AAI than WT mice with AAI using both protocols (Supplemental Fig. 1). Both Naus et al. (28) and Paulissen et al. (29) used lower OVA concentrations for challenging mice than we used (0.2, 1, and 6, respectively). Thus, it is possible that Adam8 has different activities in regulating mild versus severe AAI in mice. Future studies will assess the effects of varying concentrations of aerosolized OVA on the airway responses of OVA-sensitized WT versus Adam8−/− mice.

Our studies of Adam8 expression in lung sections from asthmatic patients showed that Adam8 was robustly expressed in airway epithelium and subepithelial granulocytes, which two pathologists (L.S. and L.K.) identified as being mainly eosinophils. These results are consistent with those from a previous study of Adam8 expression in bronchial biopsies from patients with asthma (26). In contrast to previous reports, we found that Adam8 was expressed by lung epithelial cells of human subjects without pathological features of asthma including epithelial cells in the small airways and type II pneumocytes (Fig. 9A) and that Adam8 expression in airway epithelial cells was similar in subjects with and without asthma. Overall, Adam8 expression was increased in the airways of asthmatic patients due to robust expression of Adam8 in granulocytes (but staining for Adam8 in macrophages was weak). Double immunofluorescence using markers of eosinophils versus macrophages confirmed robust expression of Adam8 in airway eosinophils but relatively low-level expression of Adam8 in airway macrophages. Adam8 was first cloned from macrophages and is expressed at high levels in activated macrophages (49–51), including macrophages present in other inflamed tissues (51, 74, 75). We detected robust levels of Adam8 protein in airway macrophages, which were similar to levels detected in airway eosinophils from WT mice with AAI (Table I). Surface Adam8 levels also increased on both monocytes and eosinophils from healthy human subjects that were activated in vitro with a proinflammatory mediator (LTB4). Thus, it was surprising that we detected only low-level staining for Adam8 in peribronchial macrophages in lung sections from all five asthmatic subjects studied because we expected peribronchial macrophages to be activated due to the inflammation present in the asthmatic airways. It is noteworthy that in our OVA model of AAI in mice, Adam8 was robustly expressed by airway macrophages, and airway inflammation resolved when we stopped challenging the mice with allergen. However, asthma tends to be a chronic disease in human subjects. Given the potent anti-inflammatory activity of Adam8 in mice with AAI, our results suggest that the lack of robust upregulation of Adam8 in airway macrophages in human asthmatic subjects could contribute to the severity or persistence of airway inflammation in asthma patients. This possibility will be the focus of our future studies.
The mechanism underlying the lack of robust upregulation of ADAM8 in peribronchial macrophages in asthmatic subjects is not clear, but could be due to reduced synthesis of ADAM8 or increased removal of ADAM8 from the surface of airway macrophages in patients with asthma. In this respect, it is noteworthy that single nucleotide polymorphisms (SNPs) in the ADAM8 locus are associated with increased plasma levels of a soluble form of ADAM8 and increased risk of myocardial infarction (76). However, SNPs in the ADAM8 locus were not found to be significantly associated with asthma or atopy in one cohort of subjects (77). Additional longitudinal studies are warranted in larger cohorts of patients with asthma to determine whether SNPs in the ADAM8 gene are associated with asthma risk or severity. However, if sADAM8 is found to have anti-inflammatory activities in human subjects as well as mice, reduced plasma or lung levels of sADAM8 in patients with severe asthma could contribute to their more severe airway inflammation.

In summary, we report that leukocyte-derived Adam8 has potent anti-inflammatory activity during in AAI in mice, and this is due to leukocyte-derived Adam8 increasing eosinophil and macrophage apoptosis in the airways of mice with AAI. We now show that an ADAM family member limits inflammatory responses in vivo by activating the intrinsic apoptosis pathway. Furthermore, human asthma patients appear to have defective upregulation of ADAM8 in their airway macrophages, which could contribute to the persistent inflammation in their airways by increasing the survival of these cells. Together, our results suggest that strategies that increase ADAM8 levels on leukocyte cell surfaces in human patients with asthma (or sADAM8 levels in extracellular fluids) may represent novel therapeutic approaches to limit the severity of airway inflammation, and thereby reduce the morbidity and mortality associated with asthma.

Acknowledgments

We thank Carl Blobel (Hospital for Special Surgery, New York, NY) and Andrew Docherty (UCB Cell Tech, Slough, U.K.) for providing us with Adam8−/− mice.

Disclosures

The authors have no financial conflicts of interest.

References

Supplemental Table: Chemokine and survival factor protein levels in lung homogenates from WT and Adam8−/− mice

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<th>Adam8−/− Mice A</th>
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^A^ BALB/c WT and BALB/c Adam8−/− mice were sham-sensitized with PBS or sensitized with a low dose of OVA (10 μg) along with alum in PBS by the i.p. route, and then challenged daily on days 14-17 with aerosolized PBS or OVA solution as described in Methods. Chemokines and survival factors were quantified in homogenates of lungs harvested 24 h after the last PBS or OVA challenge.

^B^ The results are expressed as pg of mediator/ml of lung homogenate. Data are mean ± SEM; n = number of mice studied per group.

^C^ N.D. indicates not detected.

^D^ There were no statistically significant differences between OVA-treated WT and OVA-treated Adam8−/− mice in lung levels of any of the mediators listed in Supplement Table 1, except for IL-12 and IL-10 which were
both significantly higher in homogenates of lungs from OVA-treated $\textit{Adam8}^+/-$ mice compared with samples from OVA-treated WT mice ($p = 0.045$ and $p = 0.002$, respectively).
Supplemental Figure 1: SvEv129 X C57BL/6 strain Adam8<sup>−/−</sup> mice have greater OVA-induced AAI and AHR when sensitized with either a low or a high dose of OVA. In A-C, SvEv129 X C57BL/6 Adam8<sup>−/−</sup> and WT littermate control mice were sham-sensitized with PBS or sensitized with a high dose of OVA (200 μg of OVA along with 1 mg of alum in 200 μl PBS) by the i.p. route on days 0 and 7, and then challenged with nebulized PBS alone or a 6% solution of OVA in PBS daily on days 14-17. In A, all leukocytes (WBCs), macrophages (Macs), granulocytes (Granulo), and lymphocytes (Lymphs) were counted in BAL samples 24 h after the last OVA or PBS challenge. Data are mean ± SEM; n = 4-5 and 9-10 animals/group for the PBS and OVA experimental conditions, respectively. Asterisk indicates p ≤ 0.045 and ** p ≤ 0.008 compared with PBS-treated mice belonging to the same genotype as the OVA-treated mice; NS indicates no significant differences between the groups indicated. B shows hematoxylin and eosin-stained lung sections (original magnification X 400) from PBS- and OVA-treated mice representative of n = 4 and 10 mice/group, respectively. In C, AHR to
aerosolized methacholine challenges was assessed 24 h after the last OVA challenge. Data are expressed as % increase in central airway resistance (Rn) over baseline (0 mg/ml methacholine). Data are mean ± SEM; n = 4-6 mice per group. Asterisk indicates p < 0.04 when compared with WT mice challenged with the same concentration of methacholine. In D, SvEv129 X C57BL/6 Adam8−/− and SvEv129 X C57BL/6 parental littermate WT mice were sham-sensitized with PBS or sensitized with a low dose of OVA (10 μg of OVA and 1 mg of alum in 200 μl PBS) by the i.p. route on days 0 and 7, and then challenged with PBS or 6% OVA in PBS daily on days 14-17. All WBCs and leukocyte subsets were counted in BAL samples 24 h after the last aerosolized PBS or OVA challenge. Data are mean ± SEM; n = 5 animals/group for PBS-treated mice and n = 18 mice/group for OVA-treated mice. Asterisk indicates p ≤ 0.03; ** p < 0.003, and *** p < 0.012 vs. PBS-treated mice belonging to the same genotype as the OVA-treated mice.
Supplemental Figure 2:  **ADAM8 regulates basal and stimulated Muc5b gene expression.**  Murine tracheal epithelial cells (MTECs) were isolated from naïve BALB/c WT vs. *Adam8<sup>−/−</sup>* mice and 100,000 cells were seeded onto 12-well tissue culture plates and grown to ~50% confluence on rat type I collagen-coated tissue culture dishes as previously described (1). In **A**, cells were incubated without agonists for 4 days. In **B**, WT vs. *Adam8<sup>−/−</sup>* epithelial cells were incubated with or without 10 ng/ml of interleukin-13 (IL-13) for 4 days at 37°C. In both **A** and **B**, RNA was isolated from the cells using Trizol (Life Technologies, Grand Island, NY) as described by the manufacturer, and qRT-RT PCR was performed to measure steady state mRNA levels of *Muc5b* using Taqman assays (Applied Biosystems, Life Technologies), cyclin-dependent kinase inhibitor-1 as the housekeeping gene and the ΔΔCT method ensuring that only mRNA amplification within the linear range was compared. In **A**, the results for *Muc5b* expression in unstimulated *Adam8<sup>−/−</sup>* epithelial cells were expressed as fold change relative to those in unstimulated WT cells. In **B**, the results for IL-13-stimulated cells were expressed as fold change from basal levels in cells of the same genotype. In **C**, results for IL-13 stimulated *Muc5b* expression in WT and *Adam8<sup>−/−</sup>* cells were expressed as fold change relative to *Muc5b* expression in unstimulated WT cells. In **A-C**, results are mean ± SEM fold change in *Muc5b* expression (n = 3-6 per experimental condition) Asterisk indicates p ≤ 0.017 when compared with unstimulated WT cells.
Supplemental Figure 3: ADAM8 does not degrade pro-inflammatory mediators in vitro. We incubated 200 nM active recombinant human ADAM8 ectodomain (which contains the MP domain) at 37°C for 18 h with and without 5 μM recombinant human (rh) IL-4 (left), 0.8 μM rh IL-5 (middle), and 5 μM rh RANTES (right). Reaction products were analyzed on silver-stained Tris-Tricine gels. Arrows indicate active ADAM8 and arrowheads indicate intact mediator. Note the lack of reduction in the intensity of the bands for each intact mediator and lack of generation of significant amounts of degradation products when the mediators were incubated with active ADAM8. These results indicate that ADAM8 has little or no capacity to degrade the mediators studied.