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ADAM9 Is a Novel Product of Polymorphonuclear Neutrophils: Regulation of Expression and Contributions to Extracellular Matrix Protein Degradation during Acute Lung Injury

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A disintegrin and metalloproteinase domain (ADAM) 9 is known to be expressed by monocytes and macrophages. In this study, we report that ADAM9 is also a product of human and murine polymorphonuclear neutrophils (PMNs). ADAM9 is not synthesized de novo by circulating PMNs. Rather, ADAM9 protein is stored in the gelatinase and specific granules and the secretory vesicles of human PMNs. Unstimulated PMNs express minimal quantities of surface ADAM9, but activation of PMNs with degranulating agonists rapidly (within 15 min) increases PMN surface ADAM9 levels. Human PMNs produce small quantities of soluble forms of ADAM9. Surprisingly, ADAM9 degrades several extracellular matrix (ECM) proteins, including fibronectin, entactin, laminin, and insoluble elastin, as potently as matrix metalloproteinase-9. However, ADAM9 does not degrade types I, III, or IV collagen or denatured collagens in vitro. To determine whether ADAM9 regulates PMN recruitment or ECM protein turnover during inflammatory responses, we compared wild-type and Adam9−/− mice in bacterial LPS- and bleomycin-mediated acute lung injury (ALI). Adam9 lung levels increase 10-fold during LPS-mediated ALI in wild-type mice (due to increases in leukocyte-derived Adam9), but Adam9 does not regulate lung PMN (or macrophage) counts during ALI. Adam9 increases mortality, promotes lung injury, reduces lung compliance, and increases degradation of lung elastin during LPS- and/or bleomycin-mediated ALI. Adam9 does not regulate collagen accumulation in the bleomycin-treated lung. Thus, ADAM9 is expressed in an inducible fashion on PMN surfaces where it degrades some ECM proteins, and it promotes alveolar–capillary barrier injury during ALI in mice. The Journal of Immunology, 2014, 193: 2469–2482.

Abbreviations used in this article: ADAM, a disintegrin and metalloproteinase domain; ADAM9, a disintegrin and metalloproteinase domain 9; BAL, bronchoalveolar lavage; BALF, BAL fluid; ECM, extracellular matrix; i.e., intratracheal; KRPG buffer, Krebs–Ringer phosphate glucose buffer; MMP, matrix metalloproteinase; MP, metalloproteinase; MPO, myeloperoxidase; NE, neutrophil elastase; PAF, platelet-activating factor; PMN, polymorphonuclear neutrophil; proMMP, pro–matrix metalloproteinase; s, soluble; WT, wild-type.

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also contributes to the formation of multineucleate giant cells from monocytes and macrophages by mechanisms that are not clear (3). Although there have been a small number of reports that a few ADAM proteases cleave a limited number of extracellular matrix (ECM) proteins (19, 20), it is not known whether ADAM9’s MP domain cleaves ECM proteins to promote tissue remodeling or injury.

Less is known about the function of ADAM9’s other domains. The disintegrin domain of ADAM9 binds to various integrins, including: 1) αβ integrin to promote adhesion of fibroblast cell lines (21); 2) α1, α5, α6, αs, and β1 integrins to regulate adhesion of human embryonic kidney-293 cells (6); and 3) β integrins on macrophages to promote macrophage fusion to form multineucleate giant cells in mycobacterial-induced granulomas (22). Both the disintegrin and cysteine-rich domains of ADAM9 interact with the β integrin subunit on keratinocytes to promote keratinocyte migration (23). The cytoplasmic domain of ADAM9 binds protein kinase C δ isoform to promote shedding of heparin-binding epidermal growth factor from cell surfaces (4).

Although ADAM9 is expressed by monocytes and macrophages, it is not known whether ADAM9 is expressed by polymorphonuclear neutrophils (PMNs) and, if so, whether it regulates PMN function or contributes to pericellular proteolysis and tissue injury. Neither ADAM9’s expression in the lung during acute lung injury (ALI) nor its activities in regulating ALI have been investigated previously. To begin to address these knowledge gaps, we assessed whether ADAM9 is expressed by PMNs, characterized the forms of ADAM9 that are produced by PMNs, and assessed the biology of ADAM9 in PMNs. We assessed whether PMN-derived ADAM9 contributes to PMN pericellular proteolysis of ECM proteins in vitro. We also compared wild-type (WT) and Adam9−/− mice in murine models of acute neutrophilic lung inflammation and injury to determine whether ADAM9 regulates PMN accumulation in the lung, injury to the lung, and/or ECM protein turnover during ALI syndromes.

Materials and Methods

Reagents, Abs, and Kits

Recombinant human ADAM9 ectodomain and the human ADAM9 ELISA kit were purchased from R&D Systems (Minneapolis, MN). Rabbit anti-ADAM9 IgG (Ab36176) and murine anti-pancytokeratin IgG and Abs to PMN granule markers were purchased from Abcam (Cambridge, MA). Rat anti-murine Ly6G and rat anti-murine Mac-3 IgGs were purchased from BD Pharmingen (San Jose, CA). Purified human pro–matrix MP (proMMP)-8 and proMMP-9 were purchased from Chemicon International (Temecula, CA). DQ-FITC–conjugated gelatin, DQ-FITC–conjugated type IV collagen, DQ-FITC–conjugated type I collagen, and goat anti-rabbit F(ab′)2 conjugated to Alexa Fluor 488 were purchased from Invitrogen (Carlsbad, CA). FITC-conjugated particulate elastin (particles having a diameter of 37–74 μm) was purchased from ICM Biomedicals (Aurora, OH). L-α-phosphatidylcholine-β-γ-α-alkyl platelet-activating factor (PAF) was purchased from Bachem (Torrance, CA). Optimized ADAM9 and 18S TaqMan primer and probe sets were purchased from Ambion (Austin, TX). Vectashield mounting medium with DAPI was purchased from Vector Laboratories (Burlingame, CA). The murine desmosine ELISA kit was purchased from Antibodies-Online (Atlanta, GA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Mice

The Harvard Medical School Animal Care and Use Committee approved all procedures performed on mice. C57BL/6 strain Adam9−/− mice were provided by Dr. Carl Blobel (Hospital for Special Surgery, New York, NY). Age- and gender-matched adult C57BL/6 WT mice (8–12 wk of age) were purchased from The Jackson Laboratory (Bar Harbor, ME) and studied as experimental controls. The genotype of the Adam9−/− mice was determined using PCR-based genotyping protocols conducted on genomic DNA extracted from murine tail biopsies.

Isolation of human PMNs

All human subject studies were approved by the Partners Healthcare Institutional Review Board. Human PMNs (>95% pure and >99% viable) were isolated from the peripheral blood of healthy human volunteers using the Ficoll-Hypaque method (24).

Activation of PMNs

Human PMNs (10^7/ml in PBS) were incubated for 30 min at 37°C with or without PMA (0.3 μM), calcium ionophore (A23187; 1 μM), IMLP (10^-6–10^-11 M), IL-8 (10^-7–10^-10 M), or TNF-α (10^-7–10^-10 M). Other aliquots of cells were incubated with or without IMLP (10^-7 M), TNF-α (10^-7 M), or IL-8 (10^-5 M) at 37°C for up to 120 min. To test whether agonists have synergistic or additive effects on regulating surface ADAM9 levels on PMNs, we incubated human PMNs at 37°C for 15 min with or without optimal concentrations of bacterial LPS from Escherichia coli 011B4 (100 ng/ml), PAF (10^-7 M), or TNF-α (10^-7 M), followed by the optimal concentration of IMLP (10^-10 M) for 30 min at 37°C. Cells were then fixed for 5 min at 4°C in PBS containing 3% (w/v) paraformaldehyde and 0.5% (v/v) glutaraldehyde (pH 7.4), washed in PBS, and then immunostained for surface ADAM9, as outlined below.

Isolation and activation of murine PMNs

PMNs (>85% pure and >99% viable) were isolated from the bone marrow of Adam9−/− or WT mice by positive selection for Ly6G using immunomagnetic beads (25). Murine PMNs were incubated without agonists for 45 min or incubated with 10^-6 M PAF or 10^-7 M IMLP for 30 min at 37°C, or incubated at 37°C for 15 min with 10^-6 M PAF followed by 10^-7 M IMLP for 30 min. PMNs were fixed and washed (as described above) and then immunostained for surface Adam9 or used in cell-based proteolysis assays as outlined below.

Immunofluorescence staining for surface Adam9 protein levels

Unstimulated or activated human or murine PMNs were incubated at 4°C for 2 h in PBS containing 1% albumin and 50 μg/ml goat IgG to block binding of Abs to PMN Fc receptors. Cells were then incubated for 2 h at 4°C with rabbit anti-ADAM9 IgG or nonimmune rabbit IgG (both at 1 μg/10^6 cells). The cells were washed twice in PBS and incubated for 2 h at 4°C with 4 μg/ml goat anti-rabbit F(ab′)2 conjugated to Alexa Fluor 488. The cells were then washed twice in PBS, and cytospin preparations were examined using either a confocal microscope or bright field and epifluorescence microscopy (Eclipse E-800; Nikon, Tokyo, Japan). For quantitative analysis of surface ADAM9 staining, images of the immunostained cells were captured using a Leica DFC480 camera (Leica Microsystems, Buffalo Grove, IL), and ADAM9 staining was measuring using MetaMorph software (Universal Imaging, West Chester, PA) exactly as described previously for other proteases expressed on human PMNs (26).

Double immunostaining of permeabilized PMNs for ADAM9 and markers of PMN granules

Unstimulated human PMNs were permeabilized by incubating them in 100% methanol for 30 min at 4°C and then incubated overnight at 4°C in PBS containing 1% albumin, 10% normal goat serum, and 50 μg/ml murine IgG (for cells incubated with primary Abs raised in mice) or 1% albumin, 10% normal mouse serum, and 50 μg/ml murine IgG (for cells incubated with primary Abs raised in goats) to block nonspecific binding of Abs. Cells were then incubated with rabbit anti-ADAM9 IgG (or nonimmune rabbit IgG) followed by goat anti-rabbit F(ab′)2 conjugated to Alexa Fluor 488 as outlined above. ADAM9-labeled cells were then washed and incubated with either murine anti-myeloperoxidase (MPO) IgG, murine anti-lactoferrin IgG, or goat anti–MMP-9 IgG (or nonimmune murine or goat IgG as negative controls) cells were then washed in PBS and incubated with goat anti-murine F(ab′)2 conjugated to Alexa Fluor 546 or rabbit anti-goat F(ab′)2 conjugated to Alexa Fluor 546. Nuclei were counterstained with DAPI and cells were then examined using a confocal microscope.

Subcellular fractionation of PMNs

Fresh buffy coats were obtained from the central blood bank at Rigshospitalet, Denmark. Dextran 500 solution (Sigma-Aldrich; 500 ml) was added to induce sedimentation of the red cells. The leukocytes with supernatant was layered on Lymphoprep (Axis-Shield, Oslo, Norway) and centrifuged. Contaminating red cells were lysed by hypotonic shock as described previously (27). The PMNs were resuspended at 3 × 10^7 cells/ml in Krebs–Ringer phosphate glucose (KRPB buffer; pH 7.4) and split in two equal aliquots. One aliquot was incubated at 4°C (unstimulated), and the other was incubated at 37°C for 5 min and then PMA (2–5 μg/ml) was added. After 15 min, equal volumes of ice-cold KRPB buffer were added to both samples. The cells were centrifuged and the supernatant fluids were frozen to −80°C for analysis. The cells were resuspended at 3 × 10^7 cells/ml in KRPB buffer containing disopropyl fluorophosphatase (Calbiochem,
Darmstadt, Germany), centrifuged and resuspended in 12.5 ml relaxation buffer (28), and subjected to nitrogen cavitation (27).

The postnuclear supernatant was subjected to fractionation on a three-layer Percoll density gradient, which efficiently separates the organelles into fractions enriched in azurophil granules (a band, identified by MPO); specific granules (β1 band, identified by neutrophil gelatinase–associated lipocalin); gelatinase granules (β2 band, identified by MPP-9); and a fraction rich in both the secretory vesicles (identified by albumin) and the plasma membrane (enriched by HLA) (29). Equal volume (1 ml total of each) were aspirated and those with peak MPO concentrations (fractions 1–8), peak neutrophil gelatinase–associated lipocalin concentrations (fractions 9–14), peak MMP-9 concentrations (fractions 15–19), and peak albumin and HLA concentrations (fractions 20–28) were pooled. Percoll was removed by ultracentrifugation and the biological material was collected and re-suspended in 12.5 ml of radioimmunoprecipitation assay containing 100 mM PMSF and 100 mM 1,10-phenanthroline. Proteins in the fractions along with the cytosol were separated on 12% SDS-PAGE and then immunoblotted for ADAM9 using rabbit anti-ADAM9 IgG (Abcam), goat anti-rabbit IgG conjugated to HRP, and an ECL Plus detection system (Pierce, Rockford, IL). ADAM9 was quantified in solubilized granules and vesicles, as well as in the cytosolic and cell-free supernatant samples, using a commercial ELISA, and ADAM9 levels were normalized to total protein levels using a commercial kit (Bio-Rad Laboratories, Hercules, CA).

**Quantitative real-time RT-PCR to measure ADAM9 steady-state mRNA levels in PMNs**

Human PMNs (24 × 10⁶ cells/assay) were activated at 37°C for 15 min with 10⁻⁷ M TNF-α followed by 10⁻⁷ M IL-8 for up to 4 h. RNA was isolated from the cells using a RNaseasy kit (Qiagen, Valencia, CA), and RNA concentration and purity were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA). RNA samples (500 ng) were reverse transcribed using Moloney murine leukemia virus reverse transcriptase, reverse transcriptase buffer, deoxynucleobase triphosphate mixture, and RNAse-1–γ inhibitor. We used TaqMan gene expression probes and primer sets for human ADAM9 and 18S as the expression probes and primer sets for human ADAM9 and 18S as the reference gene.

**Analysis of ADAM9 levels and forms in PMNs**

Human PMNs (20 × 10⁶/ml) were incubated in PBS with or without 10⁻⁷ M HMLP for up to 2 h at 37°C and PMNs and cell-free supernatant samples were separated by centrifugation (500 × g for 5 min). PMNs were washed once in PBS, and PMN extracts were prepared at 10⁵ cells/ml in radioimmunoprecipitation assay containing 100 mM PMSF and 100 mM 1,10-phenanthroline. ADAM9 was quantified in cell extracts (from 0.5 million cells per experimental condition) and cell-free supernatant samples using a commercial ELISA kit. ADAM9 forms in these samples were assessed by Western blot analysis as outlined above.

**Effects of proteinase inhibitors on PMN production of soluble ADAM9**

To determine whether soluble (s)ADAM9 forms produced by PMNs are generated by proteolytic shedding from the PMN surface, we incubated human PMNs (20 × 10⁶/ml) with or without 10⁻⁷ M HMLP or PMA (200 nM) in the presence and absence of the following inhibitors: 1) 4-(2-aminoethyl)-benzenesulfonyl fluoride (100 μM), a serine proteinase inhibitor; 2) GM6001 (50 μM), a general inhibitor of MMPs; 3) pepstatin A (50 μM), an inhibitor of aspartic acid proteinases; 4) leupeptin (50 μM), an inhibitor of cysteine proteinases; and 5) E64 (50 μM), an inhibitor of cysteine proteinases. After 2 h, cell-free supernatant fluids were harvested, and sADAM9 levels were measured using an ELISA. Results of sADAM9 levels produced by PMNs incubated with proteinase inhibitors were expressed as a percentage of the sADAM9 levels produced by PMNs in the absence of inhibitors.

**Binding of exogenous ADAM9 ectodomain to the PMN surface**

To determine whether ADAM9's ectodomain can bind to the plasma membrane of PMNs, we incubated PMNs (2 × 10⁶/assay in 100 μl PBS) with or without 1 or 2 μM recombinant ADAM9 ectodomain for 2 h at 4°C. Unbound ADAM9 was removed by washing the cells twice with PBS. PMNs were then fixed and immunostained for ADAM9 as outlined above.

**Active-site titration of human ADAM9, MMP-9, MMP-8, and neutrophil elastase**

Purified human proMMP-9 and proMMP-8 were activated by incubation for 3 h at 37°C with 1 mM 4-amino-phenylmercuric acetate and active-site titrated using human tissue inhibitor of MP-2 as described previously (25, 30, 31).

We confirmed that our rADAM9 preparation was pure by demonstrating a single band corresponding to the size of the ectodomain (~60 kDa) when we analyzed rADAM9 on silver-stained 12% SDS-PAGE gels. We also detected no contaminating MMP-8 or MMP-9 when we performed Western blotting for MMP-8 or MMP-9 on rADAM9 that had been subjected to electrophoresis on 12% SDS-PAGE gels. We used RS113456, a general hydroxamate inhibitor of MPs, to active-site titrate ADAM9. Human PMNs (24 × 10⁶ cells/ml) were incubated at 100°C for 30 min with 0.5–250 nM RS113456, and then 10 μM Mca-Lys cleaved–Lys(P) (a quenched fluorogenic peptide substrate containing the sequence in pro-TNF-α that is cleaved by ADAM17 and ADAM9; see Ref. 15) was added and samples were incubated at 37°C. Cleavage of this substrate was measured over 18 h using fluorimetry (Hitachi F2500 fluorescence spectrophotometer; Hitachi, Tokyo, Japan) using an excitation wavelength of 320 nm and an emission wavelength of 405 nm. Substrate cleavage was plotted against the concentration of RS113456 tested, and the concentration of active ADAM9 was determined by regression analysis using SigmaPlot (Jandel Scientific Software, San Jose, CA). Human neutrophil elastase (NE) was active-site titrated as described previously (32).

**Cleavage of ECM proteins by soluble rADAM9 versus sMMPs**

We incubated equimolar amounts (2 μM) of active-site–treated ADAM9 (or MMP-9 or MMP-8 or buffer alone as controls) for 18 h at 37°C with 2 μg/ml collagen, laminin, fibronectin, or entactin, or 20 μg/ml type III collagen in 20–50 μl Tris assay buffer (50 mM Tris containing 150 mM NaCl and 20 mM CaCl₂; pH 7.4) containing 0.05% Triton X-100. The reaction was terminated by adding 10 μl 3× SDS sample buffer (50 mM Tris HCl containing 100 mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol; pH 6.8) and heating the reaction mixtures at 100°C for 30 min. ADAM9 (25 μM) or buffer alone was also incubated with fibronectin (2 μg) for 2, 4, 8, and 18 h at pH 7.4. The reaction mixtures were separated on 4–20% Tris-glycine SDS gradient electrophoresis gels (Bio-Rad Laboratories), and proteins were stained with Coomassie blue R-250. We used Scion Image software (Scion, Frederick, MD) to quantify the intensities of the bands of residual intact substrate and calculate the percentage of the substrate that was cleaved by each proteinase in three to four separate experiments.

For ECM proteins that are commercially available in quenched FITC-conjugated forms, we incubated 25 nM active MMP-9, MMP-8, or ADAM9 or buffer alone in 200 μl Tris assay buffer (at pH 7.4) with 50 μg/ml quenched DQ-FITC–conjugated gelatin, quenched DQ-FITC–conjugated type IV collagen, or quenched DQ-FITC–conjugated type I collagen at 37°C for 18 h. Cleavage of the substrates was quantified using fluoroimetry (excitation wavelength of 495 nm and emission wavelength of 520 nm). To quantify cleavage of elastin, equimolar amounts (25 nM) of active-site–treated ADAM9, MMP-8, MMP-9, or human NE, or buffer alone were incubated at 37°C in 200 μl Tris assay buffer with 20 μg/ml elastin-FITC, and elastin cleavage was quantified at intervals in aliquots of eluates using fluoroimetry (25).

**Cleavage of ECM by membrane-bound Adaman on PMNs**

We used a loss-of-function strategy to determine whether membrane-bound Adam9 was expressed on the surface of activated murine PMNs has the same spectrum of catalytic activity as soluble ADAM9 ectodomain by comparing the surface MP-mediated proteolytic activity associated with equal numbers of activated PMNs from WT versus Adaman9−/− mice or Mmp-8−/− or Mmp-9−/− mice as controls. We and other investigators have previously validated similar cell-based assay systems to quantify the contributions of MMPs and other ADAM proteinases to proteolytic events occurring on cell surfaces (25, 31, 33, 34).

WT, Adam9−/−, Mmp-8−/−, and Mmp-9−/− PMNs were optimally activated with 10⁻⁶ M PAF for 15 min followed by 10⁻⁶ M IL-8 for 30 min to induce surface expression of Adam9, Mmp-8 (31), and Mmp-9 (25) on WT cells. Cells were then fixed as described above to prevent release of soluble proteinases during the assays (25, 31) and resuspended in Tris assay buffer (0.05 M Tris containing 0.15 M NaCl and 0.02 M CaCl₂; pH 7.4). Equal numbers of cells from each genotype were incubated in triplicate for 37°C for 30 min with 1 mM PMSF to completely inactivate cell surface serine proteinases on PMNs that contribute to cleavage of the substrates studied (25, 31, 32, 35, 36). Samples were divided into two equal aliquots, and one
ALADIN was quantified using the SD9 Adam17, forward, 5'AGT GGC AGG ACT TCT TCA GTG G-3', numbers of WT PMNs in four to six separate experiments. The MP activity associated with the surface of proteinase-deficient PMNs was expressed as a percentage of the activity that was associated with equal numbers of WT PMNs in four to six separate experiments.

**LPS-mediated ADAM9 in mice**

WT and Adam9<sup>−/−</sup> mice were anesthetized with i.p. injections of ketamine (100 mg/kg), xylazine (10 mg/kg), and acepromazine (3 mg/kg). LPS from *E. coli* 0111:B4 (10 µg in 30 µl endotoxin-free PBS) or 30 µl endotoxin-free PBS alone was delivered to WT and Adam9<sup>−/−</sup> mice by the intratracheal (i.t.) route to induce robust PMN accumulation in the lungs of C57BL/6 WT mice (31, 38).

Adam-9, -10, and -17 steady-state mRNA levels in the lungs of mice during LPS-mediated ALI

Lungs were removed from mice 4 and 24 h after delivering LPS or PBS by the i.t. route. RNA was extracted from the lungs using TRIzol reagent and a RNasey kit (39), and RNA samples were reverse transcribed as described above. We used a SYBR Green protocol to amplify the samples using the following primers: Adam9, forward, 5'-GGC CTG TGT GGA AAG CTT-3', reverse, 5'-AAA CAC CGG CAT GTC CTG TAC-3'; GAPDH, forward, 5'-CCAGGAAT GAGCTTGAGAAGT-3', reverse, 5'-CCC-ACTCTCCAC C TTG TAC-3'; Adam10, forward, 5'-TTG CCG CCT CTT AAA CCA C-3', reverse, 5'-TGG CCG TCT CTT T-3'; Adam17, forward, 5'-AGT GGC AGG AGT ACT TCT TCA GTG G-3', reverse, 5'-CCC TAG AFT CAG GCT CAC CAA C-3'. Fold change in Adam gene expression was quantified using the 2<sup>ΔΔCt</sup> method and GAPDH as the housekeeping control gene.

**Immunofluorescence staining for Adam9 in lung sections**

We performed immunoperoxidase staining on inflated and formalin-fixed lung sections obtained from WT mice 24 h after LPS was instilled by the i.t. route (38) or lung sections from unchallenged WT mice using rabbit anti-Adam9 IgG (Ab36176) or nonimmune rabbit IgG as a control. To identify the cell type expressing Adam9, we used a SYBR Green protocol to amplify the samples using the following primers: Adam9, forward, 5'-GGC CTG TGT GGA AAG CTT-3', reverse, 5'-AAA CAC CGG CAT GTC CTG TAC-3', GAPDH, forward, 5'-CCAGGAAT GAGCTTGAGAAGT-3', reverse, 5'-CCC-ACTCTCCAC C TTG TAC-3'; Mmp-8, forward, 5'-GTC CCG CCT CTT AAA CCA C-3', reverse, 5'-TGG CCG TCT CTT T-3'; Adam17, forward, 5'-AGT GGC AGG AGT ACT TCT TCA GTG G-3', reverse, 5'-CCC TAG AFT CAG GCT CAC CAA C-3'. For immunofluorescence staining for Adam9 in lung sections, we double immunostained lung sections obtained from unchallenged WT mice or WT mice 24 h after LPS was instilled for Adam9 and markers of leukocyte subsets or lung epithelial cells. Sections from formalin-fixed lungs were subjected to Ag retrieval by heating them at 100°C in 0.01 M sodium citrate and 2 mM citric acid buffer for 10 min. Lungs were then incubated with rabbit anti-Adam9 IgG or nonimmune rabbit IgG as a control. To identify the cell type expressing Adam9, we used a SYBR Green protocol to amplify the samples using the following primers: Adam9, forward, 5'-GGC CTG TGT GGA AAG CTT-3', reverse, 5'-AAA CAC CGG CAT GTC CTG TAC-3', GAPDH, forward, 5'-CCAGGAAT GAGCTTGAGAAGT-3', reverse, 5'-CCC-ACTCTCCAC C TTG TAC-3'; Mmp-8, forward, 5'-GTC CCG CCT CTT AAA CCA C-3', reverse, 5'-TGG CCG TCT CTT T-3'; Adam17, forward, 5'-AGT GGC AGG AGT ACT TCT TCA GTG G-3', reverse, 5'-CCC TAG AFT CAG GCT CAC CAA C-3'. Fold change in Adam gene expression was quantified using the 2<sup>ΔΔCt</sup> method and GAPDH as the housekeeping control gene.

**Activities of Adam9 in LPS-mediated acute lung inflammation and lung injury in mice**

At intervals after inducing ALI, mice were euthanized, bronchialveolar lavage (BAL) was performed, and total and differential leukocytes were counted in BAL samples (38). To assess whether Adam9 regulates lung injury in mice, we measured total protein and hemoglobin levels in BAL samples and quantitated wet-to-dry lung weight ratios, as described previously (38).

**Activities of Adam9 in regulating bleomycin-mediated ALI and collagen accumulation in the lungs of mice**

WT and Adam9<sup>−/−</sup> mice were anesthetized with i.p. injections of ketamine (100 mg/kg), xylazine (10 mg/kg), and acepromazine (3 mg/kg) and 30–100 µl bleomycin in 30 µl endotoxin-free saline or 30 µl saline alone was delivered to WT versus Adam9<sup>−/−</sup> mice by the i.t. route. In one cohort of mice, weight loss and mortality were monitored during 21 d. Other cohorts of mice were euthanized at intervals during 10 d and BAL was performed. Absolute numbers of PMNs, macrophages, monocytes, and lymphocytes were counted in BAL samples 3, 7, and 10 d after instilling bleomycin.

In other cohorts of mice, respiratory mechanics (lung elastance, lung resistance, and quasi-static compliance) were measured on anesthetized and tracheostomized mice using a digitally controlled mechanical ventilator (FlexiVent device; Scireq, Montreal, QC, Canada) 7 d after instilling 100 µl bleomycin or saline, exactly as described previously (38). Wet-to-dry lung weight ratios were also measured after euthanizing the animals. Collagen deposition in the lung was measured 21 d after delivering bleomycin or saline by measuring hydroxyproline levels in hydrolysates of left lungs and staining formalin-fixed right lung sections with Masson’s trichrome stain, as described previously (39).

**Statistical analysis**

Data are expressed as means ± SEM or means ± SD. The results for paired and unpaired data were compared using the Mann–Whitney rank sum test for nonparametric data using SigmaStat. A p value <0.05 was considered significant.

**Results**

**ADAM9 is expressed in an inducible manner on the surface of human and murine PMNs**

To determine whether ADAM9 is expressed by PMNs, we immunostained nonpermeabilized unstimulated and fMLP-activated human PMNs with a green fluorophore for surface ADAM9 and examined the cells using a confocal microscope. Unstimulated PMNs expressed minimal quantities of ADAM9 on their surface. However, fMLP induced robust expression of ADAM9 on the surface of PMNs (Fig. 1A).

To trigger the effects of agonists on surface ADAM9 levels on PMNs, we incubated human PMNs for varying times with varying concentrations of pharmacologic and biologically relevant agonists and quantitated surface ADAM9 levels. Pharmacologic agonists that robustly induce PMN degranulation (PMA and A23187) induced striking increases in surface ADAM9 levels on PMNs within 30 min (Fig. 1B). Proinflammatory agonists that also promote PMN degranulation (fMLP, IL-8, and TNF-α) induced concentration-dependent increases in surface expression of ADAM9 on PMNs (Fig. 1C, 1D, and 1E, respectively). The effect of these mediators was also rapid. Significant increases in surface ADAM9 levels were detected within 5–15 min of adding iMLP (Fig. 1F), TNF-α (Supplemental Fig. 1), or IL-8 (Supplemental Fig. 2) to PMNs. Surface ADAM9 levels returned to or below baseline levels after incubating PMNs with agonists for 120 min. Proinflammatory mediators induced additive increases in surface ADAM9 levels on PMNs, as surface ADAM9 levels were higher on PMNs activated with LPS, PAF, or TNF-α followed by fMLP when compared with cells incubated with optimal concentrations of each agonist alone (Fig. 1G). Proinflammatory mediators also rapidly increased surface Adam9 levels on PMNs isolated from unchallenged WT mice (Fig. 1H).

**ADAM9 is not synthesized de novo by PMNs**

We used real-time RT-PCR to determine whether ADAM9 expression is regulated at the steady-state mRNA level in activated human PMNs. Although we detected transcripts for a housekeeping gene (18β) in PMNs (mean Ct of 23.95 ± 0.45 [SD] for unstimulated PMNs versus Ct of 25.56 ± 0.47 for PMNs activated for 4 h with 10⁻⁷ M fMLP), we did not detect ADAM9 transcripts in either unstimulated or fMLP-activated PMNs (no cycle detected for any experimental condition using the ADAM9 primer and probe sets).

When we measured total ADAM9 protein levels in extracts of unstimulated human PMNs using an ELISA, we detected ~66 pg ADAM9 per million cells, but ADAM9 protein levels did not change significantly when cells were activated with proinflammatory agonists.
FIGURE 1. Proinflammatory mediators upregulate surface ADAM9 levels on human and murine PMNs. In (A), PMNs were isolated from healthy human volunteers, incubated with or without 10⁻⁷ M fMLP at 37˚C for 30 min, and fixed. PMNs were then immunostained with Alexa Fluor 488 for surface ADAM9 or incubated with an isotype-matched nonimmune primary Ab and examined using confocal microscopy. Images shown are representative of six separate experiments. Original magnification ×400. In (B)–(D), human PMNs were incubated at 37˚C for 30 min with or without 10⁻⁷ M PMA or 10⁻⁶ M A23187 (B), 10⁻⁵–10⁻² M fMLP (C), 10⁻⁶–10⁻⁴ M IL-8 (D), or 10⁻⁵–10⁻³ M TNF-α (E). In (F), PMNs were incubated for up to 120 min at 37˚C with or without 10⁻⁷ M fMLP. In (G), PMNs were incubated for 30 min at 37˚C with or without 100 ng/ml LPS, 10⁻⁷ M PAF, 100 U/ml TNF-α, or 10⁻⁷ M fMLP alone (open bars), or PMNs were incubated at 37˚C for 15 min with 100 ng/ml LPS, 10⁻⁷ M PAF, or 100 U/ml TNF-α and then activated for 30 min at 37˚C with 10⁻⁷ M fMLP (filled bars). In (B)–(G), cells were fixed, immunostained for surface ADAM9, and surface ADAM9 levels were quantified as described in Materials and Methods. In (B) and (F), *p < 0.001 versus unstimulated cells. In (C)–(E), *p < 0.04, **p = 0.004, ***p < 0.0001 versus unstimulated PMNs. In (G), *p < 0.001 compared with unstimulated cells and **p < 0.001 versus each agonist when tested alone. In (H), PMNs were isolated from unchallenged C57BL/6 WT mice and incubated at 37˚C without agonists for 45 min (unstim), with 10⁻⁶ M fMLP or 10⁻⁶ M PAF for 30 min, or with 10⁻⁶ M PAF for 15 min and then 10⁻⁶ M fMLP for 30 min. Cells were immunostained for surface ADAM9 as described in Materials and Methods. Data are expressed as means ± SEM as a percentage of surface staining associated with unstimulated cells (B–E, G, H) or in arbitrary fluorescence units (F); n = 150–300 cells/group. Results are representative of three to four separate experiments.

for 30 min (Table I), 1–4 h (Table II), or 6 h (data not shown). Thus, PMNs do not synthesize ADAM9 de novo.

**ADAM9 is stored in the tertiary and specific granules and also in the secretory vesicles of human PMNs**

Because serine proteinases and MMPs are stored within different PMN granules (2) and translocate to the PMN surface when cells undergo degranulation (25, 26, 31, 36), we tested whether this is the case for ADAM9. We double immunostained methanol-permeabilized, unstimulated human PMNs for intracellular ADAM9 and markers of the three types of granules present in PMNs: the azurophil (using MPO as a marker), specific (using lactoferrin as a marker), and the tertiary or gelatinase granules (using MMP-9 as a marker). These studies localized ADAM9 mainly to the tertiary granules of PMNs, as intense colocalization of ADAM9 and MMP-9 was detected using confocal microscopy (Fig. 2A). Colocalization of ADAM9 and the specific granule marker lactoferrin was also detected (Fig. 2A). However, ADAM9 was not detected in the MPO-containing PMN azurophil granules. Cells stained with isotype-matched nonimmune primary Abs showed minimal or no staining (Fig. 2A).

We also subjected unstimulated and PMA-activated human PMNs to subcellular fractionation as described in Materials and Methods. When the PMN granule fractions and the fraction containing both the plasma membrane and secretory vesicles were detergent-solubilized and subjected to immunoblotting for ADAM9, a single band of this proteinase was detected in the tertiary (or gelatinase) granules and to a lesser extent in specific granules in unstimulated PMNs, but no signal was present in the azurophil granule fraction (Fig. 2B, top panel). The intensity of the ADAM9 band present in the tertiary and specific granules of PMA-stimulated cells (Fig. 2B, lower panel) was lower than that in the same granule fractions in unstimulated PMNs. The latter result is consistent with PMA-triggered degranulation and translocation of these granules (and their complement of ADAM9) to the plasma membrane. However, the most intense ADAM9 signal in unstimulated PMNs was detected in the fraction containing both the plasma membranes and secretory vesicles of PMNs (the MV fraction). Secretory vesicles are present in segmented PMNs and undergo exocytosis. Secretory vesicles contain plasma proteins, suggesting that they are formed (in part) by endocytosis (40, 41). This MV fraction contained a second higher molecular mass form of ADAM9. Both ADAM9 signals in the MV fraction decreased in intensity (albeit more modestly than did the ADAM9 signals in the gelatinase and specific granules) when PMNs...
were activated with PMA. Several faint ADAM9 bands were detected in the cytosol fraction of unstimulated PMNs. The form having the same Mr as that detected in the granule and MV fractions was not present in the cytosol fraction of PMA-activated PMNs. We confirmed these findings when we quantified ADAM9 protein levels in the solubilized granule and MV fractions using an ELISA (Fig. 2C).

Taken together, these results indicate that mature PMNs store ADAM9 protein in their tertiary and specific granules, and that substantial quantities of ADAM9 are also present in the combined plasma membrane and secretory vesicle fraction of PMNs. Moreover, activation of PMNs results in a redistribution of ADAM9 with translocation of ADAM9 from the tertiary and specific granules and the secretory vesicles to the PMN surface.

**PMNs produce several soluble forms of ADAM**

Western analysis of PMN supernatant samples revealed that human PMNs produce several forms of sADAM9. Two main sADAM9 forms (Mr of ~87 and ~78 kDa) were detected in cell-free supernatant fluids from PMNs incubated without agonists for 60 min (data not shown). fMLP-activated PMNs produced additional sADAM9 forms, including a major form having an Mr of ~75 kDa and a less abundant form having an Mr of ~56 kDa (data not shown). When we quantified sADAM9 levels in cell-free supernatant samples from PMNs using an ELISA, the total amount of sADAM9 released by the cells did not change significantly when cells were activated (Tables I, II).

To assess whether sADAM9 forms are generated by proteolytic shedding of ADAM9 ectodomain from PMN surfaces, we incubated PMNs with or without fMLP and with or without inhibitors of serine proteinases (AEBSF), MP3 (GM6001), aspartic acid proteinases (peptatin A), and cysteine proteinases (leupeptin or E64). None of these inhibitors, either when tested separately or together, blocked the generation of sADAM9 by PMNs under either basal or stimulated conditions (data not shown). Thus, sADAM9 forms are not generated by proteolytic shedding of ADAM9’s ectodomain from the PMN surface.

**Soluble ADAM9 can bind to the PMN surface**

Other soluble proteinases expressed by PMNs, including serine proteinases and MMPs, can bind to the external surface of the plasma membrane of PMNs following their release by PMNs (25, 26, 31, 36). To test whether this is also the case for sADAM9, we incubated PMNs with or without exogenous sADAM9 ectodomain at 4°C, removed unbound protein by washing the cells, and then measured surface ADAM9 levels by immunostaining. The results show that sADAM9 can bind to the PMN plasma membrane but only when relatively high concentrations (2 μM) are present in extracellular fluids (Supplemental Fig. 3).

**sADAM9 ectodomain degrades ECM protein**

Activated PMNs are associated with potent pericellular proteolytic activity when they adhere to and migrate on ECM proteins (42, 43). To determine whether ADAM9 contributes to PMN pericellular proteolysis, we tested the activity of purified human rADAM9 ectodomain against basement membrane proteins and structural ECM proteins (including insoluble elastin and interstitial collagens) at physiologic pH (7.4). To estimate the efficiency of ADAM9 as an ECM protein-degrading proteinase, we also compared its activities to those of equimolar amounts of soluble MMP-8 and MMP-9. We chose these MMPs as controls because, similar to ADAM9, they are produced by PMNs and expressed on the surface of activated PMNs where they contribute to PMN-mediated pericellular proteolysis (25, 31). MMP-8 potently degrades interstitial collagens and some basement membrane proteins (31), whereas MMP-9 degrades denatured collagen (gelatin), elastin, and many basement membrane proteins (25). Preliminary experiments confirmed that neither MMP-8 nor MMP-9 were present (as contaminants) in our rADAM9 preparation as assayed by Western blot analysis (data not shown).

**Basement membrane proteins.** Soluble human rADAM9 degrades fibronectin, entactin, and laminin (Fig. 3). ADAM9 was modestly but significantly more efficient at degrading fibronectin than MMP-9 but less potent than MMP-8 (Fig. 3A). When tested against entactin, ADAM9 was ~3-fold more efficient than MMP-9 and had similar potency as MMP-8 (Fig. 3B). ADAM9 was only slightly less efficient than MMP-9 at degrading laminin (Fig. 3C). However, unlike MMP-9 or NE, a serine proteinase that is also expressed by PMNs, ADAM9 was not a type IV collagenase (Fig. 3D), even when a high concentration (up to 2 μM) of ADAM9 was tested. When we assessed the time course for ADAM9-mediated degradation of one basement membrane protein (fibronectin), ADAM9 progressively degraded this substrate during 18 h, and significant degradation of this protein occurred as early as 2 h after adding ADAM9 (Fig. 3E).

**Structural components of the ECM.** Soluble human ADAM9 had no detectable activity against type I collagen (Fig. 3F) or type III collagen.
collagen (data not shown). Unlike MMP-9, ADAM9 also had no activity against denatured type I collagen (gelatin; Fig. 3G). Surprisingly, ADAM9 degraded particulate insoluble elastin and was as potent at degrading insoluble elastin as MMP-9 (Fig. 3H). Both MMP-9 and ADAM9 were only one third as potent as NE at degrading insoluble elastin (Fig. 3H). When we assessed the time course for ADAM9-mediated degradation of elastin, ADAM9 progressively degraded this substrate during 18 h. Significant degradation of this protein was detected as early as 2 h after adding ADAM9 (Supplemental Fig. 4).

Taken together, these data indicate that ADAM9 is an ECM protein-degrading protease. Moreover, ADAM9 has a unique spectrum of catalytic activity degrading some basement membrane components and elastin (similar to MMP-9). However, ADAM9 has no activity against gelatin or basement membrane collagen (unlike MMP-9) and no activity against type I or III interstitial collagens (unlike MMP-8).

Membrane-bound ADAM9 degrades a similar spectrum of ECM proteins as does sADAM9

ADAM9 is a transmembrane protease, and most (~95%) of the ADAM9 that is expressed by PMNs is cell associated (Tables I, II). Thus, we tested whether membrane-bound ADAM9 has a similar spectrum of activity as does sADAM9 ectodomain against ECM proteins. To accomplish this, we used a published loss-of-function strategy that we and others have used to assess the contributions of MMPs and other ADAM proteases expressed on cell surfaces to pericellular proteolysis that is associated with intact cells (25, 31, 33, 34). We compared the surface MP activities associated with equal numbers of activated PMNs isolated from WT mice versus mice deficient in individual proteases against ECM substrates found to be susceptible (elastin) or not susceptible (type I collagen and gelatin) to cleavage by sADAM9 (see Fig. 3). Unlike membrane-bound Mmp-8 on activated PMNs, membrane-bound Adam9 had no detectable type I collagenase activity (Fig. 4A). Unlike membrane-bound Mmp-9 on activated PMNs, membrane-bound Adam9 had no gelatinase activity (Fig. 4B). However, membrane-bound Adam9 degraded particulate elastin as potently as did membrane-bound Mmp-9 on activated PMNs (Fig. 4C). Thus, both soluble and membrane-bound ADAM9 have significant elastase but not interstitial collagenase or gelatinase activities.

Activities of Adam9 in regulating PMN accumulation and lung ECM protein degradation during ALI in mice

During ALI or its more severe form, the acute respiratory distress syndrome (ARDS), there is robust recruitment of PMNs to the lung, and PMN-derived proteases contribute to lung injury (44). Degradation of lung ECM proteins by PMN-derived proteases contributes significantly to alveolar capillary barrier injury, lung edema, and mortality during ALI in human subjects and experimental animals (45–50). Levels of an elastin degradation product (desmosine) are increased in urine and/or lung samples from human ALI/ARDS patients and animals with ALI, and levels correlate positively with lung injury and mortality (50, 51). It is not known whether the MP or disintegrin domains of PMN-derived Adam9 regulate PMN transendothelial migration and PMN recruitment into inflamed tissues. It is also not known whether the MP domain of PMN-derived Adam9 contributes to PMN proteolysis of ECM proteins and tissue injury during inflammatory responses in the lung. To begin to address these knowledge gaps, we compared WT and Adam9−/− mice in murine models of neutrophilic lung inflammation (i.e. instillation of LPS or bleomycin) and quantified Adam9 expression, PMN accumulation in...
the lungs, lung injury, and turnover of two ECM proteins, one that is sensitive (elastin) and one that is resistant (type I collagen) to ADAM9 degradation in vitro (see Figs. 3, 4).

**Adam9 is upregulated in the lungs of mice with ALI**

After i.t. instillation of LPS in C57BL/6 WT mice, we measured steady-state Adam9 mRNA levels in whole-lung samples using quantitative real-time RT-PCR. Adam9 steady-state mRNA lung levels increased 10-fold 4 h after delivering i.t. LPS to WT mice, and Adam9 levels returned to baseline after 24 h (Fig. 5A). In contrast, no significant changes in Adam10 or Adam17 steady-state mRNA levels were detected in lung samples 4 or 24 h after instillation of LPS.

Immunoperoxidase staining of lung sections from unchallenged versus LPS-treated WT mice showed minimal staining for Adam9 in the lungs of unchallenged mice, but robust staining for Adam9 in the lungs 24 h after delivering LPS mainly in leukocytes recruited to the lungs (Fig. 5B). To identify the cells in which Adam9 expression is increased in the lung, we double immunostained lung sections for Adam9 and markers of leukocyte subsets (Ly6G for PMNs and Mac-3 for macrophages) and lung epithelial cells (pancytokeratin). There was minimal staining for Adam9 in resident lung macrophages or lung epithelial cells in unchallenged WT mice (Fig. 5C). In contrast, intense staining for Adam9 was detected in lung macrophages and PMNs recruited to the lungs of LPS-treated WT mice (Fig. 5D). Positive staining for Adam9 was also detected in bronchial epithelial cells in LPS-treated WT mice (Fig. 5D), but there was minimal staining for Adam9 in alveolar epithelial cells (data not shown).

**Adam9 is not required for PMN accumulation in the lung during LPS- or bleomycin-mediated ALI**

To assess whether Adam9 regulates the accumulation of PMNs (or macrophages) following lung injury, we compared PMN and macrophage counts in BAL samples from LPS-treated WT and Adam9−/− mice. WT and Adam9−/− mice did not differ in BAL total leukocyte counts (Fig. 6A), PMN counts (Fig. 6B), or macrophage counts (data not shown) at any time point from 4 h to 7 d.
after i.t. LPS. BAL PMN and macrophage counts did not differ 3 or 7 d after i.t. instillation of 30 mU bleomycin (data not shown).

Adam9 promotes lung injury and proteolysis of lung elastin in mice with LPS-mediated ALI

When compared with WT mice, Adam9−/− mice were significantly protected from lung injury following instillation of LPS as assessed by measuring 1) wet-to-dry lung weight ratios (Fig. 6C), 2) BAL fluid (BALF) total protein levels (Fig. 6D), and 3) BAL hemoglobin levels (a marker of leakage of erythrocytes across the alveolar–capillary barrier; Fig. 6E). To assess whether Adam9 degrades lung elastin during ALI to contribute to alveolar capillary barrier injury, we measured BALF desmosine levels in WT and Adam9−/− mice with LPS-mediated ALI. LPS increased BALF desmosine levels in WT mice, but LPS-treated Adam9−/− mice had significantly lower BALF desmosine levels than did LPS-treated WT mice (Fig. 6F).

Adam9 promotes lung injury but does not regulate lung fibroproliferative responses during bleomycin-induced ALI

Saline-treated WT and Adam9−/− mice did not lose weight, and all of the saline-treated mice survived as expected (Fig. 7A, 7B). However, compared with bleomycin-treated WT mice, bleomycin-treated Adam9−/− mice lost less body weight (Fig. 7A) and had higher survival rates (~80% survival versus ~50% survival; Fig. 7B). When compared with WT mice, Adam9−/− mice were protected from bleomycin-mediated ALI as assessed by measuring wet-to-dry lung weight ratios 7 d after delivering bleomycin or saline (Fig. 7C). To further assess ALI, we measured respiratory mechanics on WT and Adam9−/− mice 7 d after they were treated with bleomycin or saline. Bleomycin-treated Adam9−/− mice had significantly lower increases in lung elastance (a measure of lung stiffness; Fig 7D) and lower reductions in quasi-static lung compliance (Fig. 7E), indicating that Adam9−/− mice have less severe bleomycin-mediated ALI than do WT mice. The lower weight loss and higher survival rates in the bleomycin-treated Adam9−/− mice likely reflect the less severe ALI in these animals when compared with bleomycin-treated WT mice.

Because Adam9 does not degrade interstitial collagens or denatured collagens in vitro (see Figs. 3, 4), we next assessed whether Adam9 regulates collagen accumulation in the lung following i.t. instillation of bleomycin. WT and Adam9−/− mice had similar lung fibroproliferative responses to bleomycin as assessed by H&E staining (data not shown) and Masson’s trichrome staining of lung sections from the mice (Fig. 7F). WT and Adam9−/− mice also had similar lung collagen levels 21 d after instilling bleomycin as assessed by hydroxyproline assays performed on lung hydrolysates (Fig. 7G). Thus, Adam9 does not regulate collagen accumulation in the lung after instillation of bleomycin.

**Discussion**

To our knowledge, this is the first study to report that ADAM9 is expressed by both human and murine PMNs. PMNs produce both transmembrane and sADAM9 forms, but transmembrane-bound ADAM9 is the most abundant (>90%) form produced by PMNs. ADAM9 is not synthesized de novo by circulating PMNs. Rather, ADAM9 protein is stored in PMN tertiary and specific granules. Substantial quantities are also detected in the combined secretory vesicle and plasma membrane fraction of PMNs. Moreover, ADAM9 translocates to the PMN surface from these granules and vesicles when PMNs degranulate. Surprisingly, ADAM9 degrades some basement membrane proteins and insoluble elastin, but has no type IV or interstitial collagenase activity. We also provide novel insights into the expression and activities of Adam9 during ALI by showing that Adam9 levels are robustly upregulated in lung PMNs (and lung macrophages) during ALI in mice, but Adam9 is not required for PMN (or macrophage) accumulation in the lungs of mice with ALI. Adam9 promotes lung injury, mortality, and lung elastin degradation during ALI. However, Adam9 does not regulate lung collagen deposition following i.t. instillation of bleomycin. Thus, ADAM9 is a novel PMN product that contributes to PMN extracellular proteolysis. Adam9 promotes ALI in mice and this may be mediated, in part, by Adam9 degrading lung elastin (and possibly basement membrane proteins) in the lung.
Biology of ADAM9 in PMNs

ADAM9 has not been studied previously in leukocytes other than monocytes and macrophages (3, 22). Although ADAM9 is regulated at the transcriptional level in monocytes and macrophages (3, 10, 52), ADAM9 is not regulated at the mRNA level in mature PMNs. ADAM9 is likely transcribed by PMN precursors in the bone marrow and stored as a preformed proteinase within PMN granules and vesicles. ADAM9 is redistributed to the PMN surface when PMNs are activated by proinflammatory mediators as is the case for PMN-derived serine proteinases and MMPs (2). ADAM8 and ADAM17 are the only other members of the ADAM family known to be expressed by primary PMNs (53–55), but ADAM10 is expressed by PMN-like HL60 cells (56). However, little is known about the biology of ADAM10 or ADAM17 in PMNs.

PMN products other than ADAM9 are also localized in more than one granule in PMNs. For example, ADAM8 is localized mainly in the tertiary and specific granules and to a lesser extent in azurophil granules (55), CD11b/CD18 is also stored in all three PMN granules, the fMLP receptor and SNAP23–25 are stored in the specific and gelatinase granules, and lysozyme is detected in the azurophil and gelatinase granules (57). ADAM9 may be distributed to different PMN compartments during granule biogenesis during the maturation of myeloid precursor cells in the bone marrow. PMN granules are formed sequentially during the differentiation of myeloid cells in the bone marrow. The MPO-rich azurophil granules form first during the early promyelocyte stage, and this is followed by the appearance of the lactoferrin-rich specific granules at the myelocyte and metamyelocyte stages. The MMP-9–rich tertiary granules form next during the band cell and segmented PMN stages, and lastly the secretory vesicles (which also undergo exocytosis) appear during the segmented PMN stage (57). These granules and vesicles are packaged with proteins that are synthesized at the same time during granulopoiesis. The known heterogeneity in PMN granule protein content is thus due to differences in the biosynthetic windows of individual granule proteins that, in turn, reflects different patterns of transcription factors present at distinct stages of myeloid cell development (58).

Although little is know about the transcription factors that regulate the expression of ADAM9 in PMNs, likely the transcription factors involved are mostly activated during the later myelocyte to segmented PMN stages, which explains the partitioning of ADAM9 into the gelatinase granules and secretory vesicles and, to a lesser extent, the specific granules. It is noteworthy that we detected large quantities of ADAM9 in the combined plasma membrane and secretory vesicle fraction in unstimulated PMNs, and the ADAM9 signal in this fraction decreased when cells were activated with PMA. The latter result supports our conclusion that ADAM9 is also stored within the secretory vesicles, as our immunostaining results show that 1) minimal amounts of ADAM9 are associated with the plasma membrane of unstimulated PMNs, and 2) the amount of ADAM9 in the plasma membrane strikingly increases when cells are acti-

FIGURE 5. Adam9 is upregulated in the lung during ALI in mice. In (A), we delivered 10 μg LPS or PBS to C57BL/6 WT mice by the i.t. route, and 4 and 24 h later we measured steady-state Adam9, Adam10, and Adam17 mRNA levels in whole-lung samples using quantitative real-time RT-PCR. Data are means ± SEM; n = 6 mice/group. *p < 0.001. In (B), we immunostained lungs sections from unchallenged C57BL/6 WT mice (Unchal) or C57BL/6 WT mice harvested 24 h after 10 μg LPS was delivered by the i.t. route using rabbit anti-Adam9 IgG or non-immune rabbit IgG and the immunoperoxidase method. Images shown are representative of four mice per group (original magnification ×400 and ×1000 as indicated). Black arrows indicate PMNs staining positively for Adam9, and yellow arrows indicate macrophage staining positively for Adam9. In (C) and (D), sections of lungs from unchallenged mice (C) or lungs harvested 24 h after LPS was instilled by the i.t. route (D) were immunostained with Alexa Fluor 488 for Adam9 (second columns) and Alexa Fluor 546 for markers of epithelial cells (pancytokeratin [Pck]), PMNs (Ly6G), or macrophages (Mac-3). Sections were then counterstained with DAPI (first columns). Lung sections were examined using confocal microscopy, and merged images are shown in the fourth columns (original magnification ×200 for Pck-stained sections and ×500 for Ly6G and Mac3-stained sections). No staining was detected in lung sections stained with isotype-matched control primary Abs (data not shown).
FIGURE 6.  Adam9 is not required for PMN recruitment into the lung but promotes LPS-mediated ALI in mice. In (A)–(F), we delivered 10 μg LPS or PBS by the i.t. route to WT versus Adam9−/− mice. Four h to 1 wk later, we performed BAL or removed lungs from the mice. We counted total leukocytes (A) and PMNs (B) in BAL samples. In (A) and (B), data are means ± SEM; n = 5–8 PBS-treated mice and n = 15–18 LPS-treated mice. *p ≤ 0.05 compared with PBS-treated mice belonging to the same genotype at the same time point in (A) and (B). In (C), wet-to-dry lung weight ratios were measured 24–72 h after LPS or PBS was delivered by the i.t. route to WT and Adam9−/− mice. Data are means ± SEM; n = 4–9 PBS-treated mice, n = 9–25 LPS-treated WT mice, and n = 7–16 LPS-treated Adam9−/− mice. *p = 0.016, **p ≤ 0.002. In (D) and (E), total protein (D) and hemoglobin levels [in (E) as a marker of leakage of erythrocytes across the alveolar–capillary barrier (38)] were measured in BALF samples 24 h after instilling PBS or LPS in mice. In (D) and (E), data are means ± SEM; n = 4–9 PBS-treated mice and n = 5–12 LPS-treated mice. In (D), *p < 0.05, **p = 0.018. In (E), *p < 0.035 and **p < 0.035 versus PBS-treated mice belonging to the same genotype. In (F), desmosine levels were measured in BALF samples 24 h after PBS or LPS were instilled by the i.t. route. Data are means ± SEM; n = 4–9 PBS-treated mice and n = 8–16 LPS-treated mice. *p = 0.016 and **p = 0.021 versus PBS-treated WT mice.

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vated with PMA. Also, the secretory vesicles contain plasma proteins (such as albumin), which suggests that they are formed by endocytosis as well as granulopoiesis. Thus, ADAM9 that translocates to the surface of PMNs via fusion of the tertiary and specific granules with the plasma membrane may subsequently be endocytosed from the plasma membrane into the secretory vesicles. This possibility might explain (in part) the large quantities of ADAM9 that we detected in the combined plasma membrane and secretory vesicle fraction both in unstimulated and PMA-activated PMNs. The localization of ADAM9 in PMN tertiary granules and secretory vesicles is consistent with the very rapid (within 5 min) increases in surface ADAM9 levels on PMNs after adding agonists, as both storage compartments are known to translocate to the PMN plasma membrane more rapidly than the more dense azurophil granules (42).

PMNs produce small amounts of sADAM9 forms, but these are not generated via proteolytic shedding of transmembrane-bound ADAM9 from the PMN surface, as we could not block their generation by incubating PMNs with synthetic inhibitors of all four classes of proteinases. It is possible that ADAM9 forms lacking the transmembrane domain are also stored within PMN granules and freely released by the cells during PMNs degranulation. Some tumor cells produce sADAM9 forms having biologic activities (12). However, to our knowledge, PMNs are the only nonmalignant cell type that produces sADAM9. Whether PMN-derived sADAM9 forms contribute to ADAM9’s activities in vivo is not clear. However, it is noteworthy that the amount of sADAM9 generated by PMNs is small relative to the total amount that is expressed by the cells (~5%). Likely, transmembrane-bound ADAM9 is the main form contributing to ADAM9’s activities in vivo. Although our results showed that the sADAM9 ectodomain can bind to the external surface of the PMN plasma membrane, this occurs only when PMNs are incubated with high concentrations of sADAM9 (2 μM). Because we detected only relatively low concentrations of sADAM9 in cell-free supernatants fluids from both unstimulated and activated PMNs (Fig. 2C, Tables I, II), it is unlikely that binding of sADAM9 contributes significantly to the pericellular proteolytic activity that is associated with activated PMNs unless PMNs are present in tissues in very high numbers such as in abscesses. Rather, our results indicate that most of the surface-bound ADAM9 that we detected on the surface of activated PMNs is the result of translocation of ADAM9 from intracellular storage sites rather than sADAM9 that is released from the cells binding to the plasma membrane.

ECM protein-degrading activities of ADAM9

ADAM proteins are closely related to snake venom proteinases, which can degrade basement membrane (type IV) collagen. However, until now, there has been little evidence that ADAMs are significant ECM-degrading proteinases (2). Whereas ADAM10 has type IV collagenase activity, this activity is very modest in magnitude (60). ADAM9’s ectodomain and PMN-derived transmembrane Adam9 both potently degrade several ECM proteins. Surprisingly, ADAM9 was as potent as MMP-9 in degrading insoluble elastin, and as potent (or more potent) than MMP-9 in degrading several basement membrane proteins.

Among MP family members, ADAM9’s spectrum of catalytic activity most resembles that of MMP-9 (25, 42), as elastin, fibronectin, laminin, and entactin were susceptible to cleavage by both proteinases. However, ADAM9 had no activity against basement membrane (type IV) collagen or gelatin (denatured collagen), both of which are degraded by MMP-9. Adam9 is a significant elastase in vivo, as LPS-treated Adam9−/− mice had substantially reduced BALF levels of desmosine (an elastin degradation product), which were associated with less severe ALI.
when compared with LPS-treated WT mice. Adam9 did not increase lung elastin degradation or promote lung injury by increasing inflammatory cell burdens in the lung, as LPS- and bleomycin-treated WT and Adam9−/− mice had similar lung PMN and macrophage counts. Thus, whereas NE is a more potent elastase than ADAM9 on a molar basis in vitro, Adam9 may have more potent elastin-degrading activities in the murine lung than either NE or MMP-9, as neither neutrophil elastase-deficient nor Mmp9-deficient mice are protected from LPS-mediated ALI (61, 62). As expected, ADAM9 had no activity in vitro against interstitial collagens present in the lung (types I and III), and Adam9 did not regulate type I collagen accumulation in the lungs following bleomycin instillation in mice, suggesting that it is not an interstitial collagenase in the lung.

A prior study reported that ADAM9 has ECM protein-degrading activities, as sADAM9 released by tumor cells degrades laminin (59). Our study adds to the literature by more fully characterizing the ECM protein-degrading profile of sADAM9 and comparing its efficiency to that of equimolar amounts of other key ECM protein-degrading proteases that are expressed by PMNs. We also assessed the ECM-degrading activities of transmembrane-bound Adam9 by murine PMNs.

**FIGURE 7.** Adam9 promotes weight loss, mortality, and ALI but does not regulate lung collagen accumulation in bleomycin-treated mice. In (A) and (B), we delivered 30 mU bleomycin versus saline by the i.t. route to WT versus Adam9−/− mice and measured changes in body weight relative to baseline body weight (A) and recorded survival of the mice (B) during 21 d. In (A), n = 4–5 saline-treated mice and n = 22 bleomycin-treated mice were studied. *p = 0.044 for bleomycin-treated WT versus bleomycin-treated Adam9−/− mice. In (B), n = 5–8 saline-treated mice and n = 9–15 bleomycin-treated mice were studied. In (C)–(E), we delivered 100 mU bleomycin versus saline by the i.t. route to WT versus Adam9−/− mice and measured wet-to-dry lung weight ratios and respiratory mechanics using a FlexiVent device. In (C), 7–17 saline-treated mice and 8–10 bleomycin-treated mice were studied per group. *p < 0.001 when compared with saline-treated mice belonging to the same genotype; **p < 0.001. In (D) and (E), 4–6 saline-treated mice and 5–9 bleomycin-treated mice were studied per group. *p < 0.001 for saline-treated mice belonging to the same genotype; **p < 0.001. In (F) and (G), 30 mU bleomycin or saline was instilled by the i.t. route to WT versus Adam9−/− mice and 21 d later, the right lungs were inflated, removed, fixed, and stained with Masson’s trichrome stain. Representative images of lung sections from saline- and bleomycin-treated WT and Adam9−/− mice are shown in (F) (original magnification ×100; insets, ×400). In (G), lung collagen levels were assessed using hydroxyproline assays performed on hydrolysates of left lungs removed after 21 d. In (G), data are means ± SEM; n = 5 saline-treated mice and n = 9–15 bleomycin-treated mice. *p = 0.043 when compared with saline-treated mice belonging to the same genotype.
lungs during LPS- or bleomycin-mediated ALI in mice, suggesting that neither the MP nor the disintegrin domain of PMN or monocytic/macrophage-derived ADAM9 regulates inflammatory cell recruitment into the lung. In contrast, the MP domains of PMN-derived Adam-8 and Adam-17 regulate inflammatory responses in tissues by shedding L-selectin, TNF-α, and TNF receptors from PMN surfaces (53, 63, 64). During LPS- and bleomycin-mediated ALI, Adam9 promoted lung injury and reduced lung compliance without altering lung inflammatory cell counts in mice. Adam9 also increased weight loss and mortality in bleomycin-treated mice, which likely reflected the more severe ALI in mice expressing Adam9.

Strengths and limitations of this study
Strengths of our present study include our comprehensive analysis of the biology of ADAM9 in PMNs. We identified an unexpected function for ADAM9 ectodomain (ECM protein degradation) and compared the relative activities of ADAM9 in this respect to those of other key proteinases expressed by PMNs. We confirmed that 1) transmembrane-bound Adam9 on intact PMNs has elastin-degrading activities, 2) Adam9 has significant elastin-degrading (but not collagenase) activity in the lungs of mice with ALI, and 3) Adam9 promotes lung injury in two models of neutrophilic lung inflammation in mice. We also showed that PMNs recruited to the lungs of mice are a significant source of Adam9 in the lung using immunostaining methods. A limitation of our study is that we focused our in vivo studies on only one ECM protein that is sensitive (elastin) or resistant (type I collagen) to ADAM9-mediated cleavage in vitro, as we currently lack reliable methods for assessing proteolysis of basement membrane proteins that are susceptible to cleavage by ADAM9 in lung injury model systems. Additionally, we did not determine whether ADAM9 promotes ALI in mice by cleaving proteins other than ECM proteins in the lung. These topics are beyond the scope of the present study, but will be investigated in our future studies as appropriate tools become available.

Clinical relevance
Our results have clinical relevance, as proteolysis of ECM proteins occurs in the lung during ALI. Levels of protein fragments of elastin and other ECM proteins are increased in BALF, plasma, and urine samples from ALI/ARDS patients, and levels correlate positively with lung injury and mortality (44, 66, 67). Our results suggest that Adam9 contributes significantly to this process. Strategies that inhibit the activity of ADAM9’s MP domain or reduce its expression or catalytic activity of serine proteinases.

References
Supplemental Fig. 1: TNF-α rapidly increases surface ADAM9 levels on human PMNs. PMNs were isolated from healthy human volunteers, incubated with or without $10^{-7}$M TNF-α at 37°C for up to 120 min. At intervals, PMNs were removed, fixed, and immunostained with Alexa-488 for surface ADAM9 or incubated with an isotype-matched non-immune primary antibody. Surface ADAM9 levels on PMNs were quantified as described in Methods. Data are expressed as mean ± SEM arbitrary fluorescence units; $n = 150 - 300$ cells per group. Asterisks indicated $p < 0.031$ and **$p < 0.001$ versus unstimulated PMNs.
**Supplemental Fig. 2**: IL-8 rapidly increases surface ADAM9 levels on human PMNs. PMNs were isolated from healthy human volunteers, incubated with or without 10^{-7} M IL-8 at 37°C for up to 60 min. At intervals, PMNs were removed, fixed, and immunostained with Alexa-488 for surface ADAM9 or incubated with an isotype-matched non-immune primary antibody. Surface ADAM9 levels on PMNs were quantified as described in Methods. Data are expressed as mean ± SEM arbitrary fluorescence units; n = 150 - 300 cells per group. Asterisks indicate p < 0.001 versus unstimulated PMNs.
Supplemental Fig. 3: Exogenous soluble ADAM9 ectodomain binds to the external surface of human PMNs. PMNs were isolated from healthy human volunteers, incubated at 4°C with or without 1 μM or 2 μM soluble human ADAM9 ectodomain for 2 h. Unbound ADAM9 was removed by washing the cells twice with PBS. The cells were fixed and then immunostained for surface ADAM9 which was quantified as described in Methods. Data are expressed as % surface ADAM9 present on PMNs incubated without exogenous ADAM9 ectodomain (mean ± SEM). Asterisk indicates p < 0.001.
Supplemental Fig. 4: ADAM9 progressively degrades particulate elastin over 20 h.

Human soluble active ADAM9 (25 nM) versus buffer alone were incubated in duplicate with 20 mg/ml particulate elastin-FITC at 37°C for up to 20 h in Tris assay buffer (pH 7.4). Cleavage of the substrate was quantified in fluorescence units using fluorimetry. Results are mean ± SD. The data shown are representative of those obtained from 7 separate experiments.