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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

Robin Roychaudhuri,*1 Anja H. Hergrueter,*1 Francesca Polverino,*†‡§ Maria E. Laucho-Contreras,* Kushagra Gupta,* Niels Borregaard,‡ and Caroline A. Owen*†

A disintegrin and metalloproteinase (MP) domain (ADAM) proteins are a family of ~30 type I transmembrane proteins belonging to the zinc-dependent MP superfamily. They are characterized by a multidomain structure that includes: 1) a pro-domain that maintains the MP domain in a latent form, 2) an MP domain, 3) a disintegrin domain that binds integrins to regulate cell–cell or cell–matrix adhesion and/or migration, 4) a cysteine-rich and an epidermal growth factor-like domain that may promote cell–cell fusion and/or regulate cell adhesion, 5) a transmembrane domain that anchors ADAMs to cell surfaces, and 6) a cytoplasmic tail that can participate in intracellular signaling (1, 2).

ADAM9 (MDC-9/Meltrin-γ) is expressed by monocytes (3), activated macrophages (4), fibroblasts (5), epithelial cells (6), activated vascular smooth muscle cells (7), and keratinocytes (8). It is also upregulated in these cells during pathologic processes and is expressed by some tumor cells (9–12). ADAM9 is first synthesized as a precursor proenzyme (M₉ of ~110 kDa). It is then processed to an active form (M₉ of ~84 kDa) in the medial Golgi apparatus by a furin-like pro-protein convertase that cleaves proADAM9 at a consensus cleavage sequence between the pro and MP domains (13). Human and murine ADAM9 share 82% sequence homology at the amino acid level (14).

Little is known about the activities of ADAM9 in regulating physiologic or pathologic processes. Most is known about the function of ADAM9’s MP domain. ADAM9 is an active MP and has the characteristic consensus sequence (HEXXHXXGXHX) of MPs with three histidine residues binding the catalytically essential zinc atom in the catalytic domain. ADAM9’s MP domain is not inhibited by tissue inhibitors of MPs (15), and its physiologic inhibitors have not yet been identified. ADAM9’s MP domain cleaves a limited number of extracellular proteins, including 1) the insulin B chain (13), 2) insulin-like growth factor binding proteins (16, 13) amyloid precursor protein at the α-secretase cleavage site in the non–amyloidogenic pathway (17, 14) ligands for the epidermal growth factor receptor from cell surfaces (18, and 5) fibroblast growth factor receptor 2 (19) from tumor cell surfaces to contribute to tumor cell growth and metastasis (20). ADAM9’s MP domain...
ADAM9 IS A PMN PRODUCT THAT DEGRADES ECM PROTEINS

also contributes to the formation of multinucleate 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) αβ2 integrin to promote adhesion of fibroblast cell lines (21); 2) α1, α3, α5, αv, and β1 integrins to regulate adhesion of human embryonic kidney-293 cells (6); and 3) β1 integrins on macrophages to promote macrophage fusion to form multinucleated giant cells in mycobacterial-induced granulomas (22). Both the disintegrin and cysteine-rich domains of ADAM9 interact with activated giant cells in mycobacterial-induced granulomas (22). Both ADAM9 and 18S were purchased from Bachem (Torrance, CA). Optimized ADAM9 and 18S extracted from murine tail biopsies.

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-pancytokerin 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 from cell surfaces (4).

Isolation of human 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 10−6 M iMLP for 30 min at 37˚C, or incubated at 37˚C for 15 min with 10−6 M PAF followed by 10−6 M iMLP for 30 min. PMNs were fixed and washed (as described above) and then immunoassisted 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/106 cells).

PMNs activated with LPS or TNF-α were incubated for 30 min at 37˚C (27). 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 and goat anti-murine F(ab)2 conjugated to Alexa Fluor 488. The 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 immunoassisted for surface ADAM9, as outlined below.

Isolation and activation of murine 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 liquid 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 × 106 cells/ml in Krebs–Ringer phosphate solution (KRPG 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 KRPG 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 × 106 cells/ml in KRPG buffer containing disopropyl fluorophosphate (Calbiochem,
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 × 10^6/ml) with or without 10^-7 M ILMP 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 hydroxamate inhibitor of MPs, to active-site titrate ADAM9. We confirmed that our rADAM9 preparation was pure by demonstrating only a single band corresponding to the size of the ectodomain (∼60 kDa) when we analyzed rADAM9 on silver-stained 12% SDS-PAGE gels. We 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 p3113456, a general hydroxamate inhibitor of MPs, to active-site titrate ADAM9. We used p3113456, a general hydroxamate inhibitor of MPs, to active-site titrate ADAM9. We used p3113456, a general hydroxamate inhibitor of MPs, to active-site titrate ADAM9.
expression was quantified using the DD Adam17, forward, 5'-9'- and backward, 5'-9'- probes, and WT PMNs, as the 1,10-o-phthalaldehyde–inhibitable cleavage of each substrate. The MP association 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 ALI in mice**

WT and Adam9+/− 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+/− mice by the intracheal (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 an RNeasy 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'-GCC CGG TCT CCT CCT CTT T-3'; reverse, 5’-AAA CAC CGG CAT GTC CTG TAC-3'; GAPDH, forward, 5'-CAGAGGAAAT GAGCTTGAGAAAGT-3'; reverse, 5’-CCCCACTCTCCCACTTTGAC-3'; Adam10, forward, 5'-TTG CCT CCG CCT AAA CCA C-3'; reverse, 5’-TGG CCT TCT CCT T-3'; Adam17, forward, 5’-AGT GGC AGG ACG TCT TCA GTG G-3'; reverse, 5’-CCCTAGT CTG CCT GAC CAA C-3'; and GAPDH primers (18S) in PMNs (mean ± SEM).t of 25.56 ± 0.47 for PMNs activated for 4 h

**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 iMLP-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, iMLP induced robust expression of ADAM9 on the surface of PMNs (Fig. 1A).

To quantify 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 quantified 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 (iMLP, IL-8, and TNF-α) induced concentration-dependent increases in surface ADAM9 levels 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 iMLP 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 (18S) in PMNs (mean Ct of 23.95 ± 0.45) [SD] for unstimulated PMNs versus Cts of 25.56 ± 0.47 for PMNs activated for 4 h with 10−7 M iMLP], we did not detect ADAM9 transcripts in either unstimulated or iMLP-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 $37^\circ$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 $\times 400$. In (B)-(D), human PMNs were incubated at $37^\circ$C for 30 min with or without $10^{-7}$ M PMA or $10^{-6}$ M A23187 (B), $10^{-5}$–$10^{-7}$ M fMLP (C), $10^{-6}$–$10^{-10}$ M IL-8 (D), or $10^{-7}$–$10^{-10}$ M TNF-$\alpha$ (E). In (F), PMNs were incubated for up to 120 min at $37^\circ$C with or without $10^{-7}$ M fMLP. In (G), PMNs were incubated for 30 min at $37^\circ$C with or without 100 ng/ml LPS, $10^{-7}$ M PAF, 100 U/ml TNF-$\alpha$, or $10^{-7}$ M fMLP alone (open bars), or PMNs were incubated at $37^\circ$C for 15 min with 100 ng/ml LPS, $10^{-7}$ M PAF, or 100 U/ml TNF-$\alpha$ and then activated for 30 min at $37^\circ$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 $37^\circ$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. *$p < 0.001$. Data are expressed as means $\pm$ 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 ADAM9**

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). iMLP-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 iMLP, and with or without inhibitors of serine proteinases (AEBSF), MPs (GM6001), aspartic acid proteinases (pepstatin 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 MPs, 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 assessed 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 II

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### Table I. ADAM9 protein levels in PMN extracts and release of sADAM9 from unstimulated and activated PMNs

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>ADAM9 Protein Levels in PMN Cell Extracts (pg/10⁶ PMNs)ᵃ</th>
<th>sADAM9 Released by PMNs (% of Amount Contained within Unstimulated PMNs)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
<td>65.6 ± 10.3</td>
<td>4.7 ± 2.5</td>
</tr>
<tr>
<td>TNF-α</td>
<td>59.0 ± 11.5</td>
<td>4.6 ± 1.8</td>
</tr>
<tr>
<td>PAF</td>
<td>64.8 ± 12.2</td>
<td>4.1 ± 2.4</td>
</tr>
<tr>
<td>iMLP</td>
<td>49.4 ± 8.9</td>
<td>4.2 ± 2.4</td>
</tr>
<tr>
<td>TNF-α + iMLP</td>
<td>59.1 ± 6.5</td>
<td>4.3 ± 2.0</td>
</tr>
<tr>
<td>PAF + iMLP</td>
<td>60.9 ± 9.8</td>
<td>5.3 ± 2.3</td>
</tr>
</tbody>
</table>

PMNs isolated from healthy human donors were incubated at 37°C for 30 min with or without 100 U/ml TNF-α, 10⁻⁷ M PAF, or 10⁻⁷ M iMLP. PMNs were also primed for 15 min with 100 U/ml TNF-α or 10⁻⁷ M PAF, and then activated for 30 min with 10⁻⁷ M iMLP (TNF-α + iMLP and PAF + iMLP). Cells and cell-free supernatant fluids were separated by centrifugation, and ADAM9 protein levels were measured in cell extracts and cell-free supernatant fluids using an ELISA. There are no significant differences in the amounts of ADAM9 quantified in extracts of unstimulated versus activated PMNs.

ᵃData are means ± SEM; n = 4 donors.

ᵇResults for amounts of sADAM9 released by PMNs are expressed as a percentage of the cellular content of ADAM9 in extracts of unstimulated PMNs.

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### Table II. Activation of PMNs with iMLP for up to 4 h does not alter ADAM9 protein levels in cell extracts or cell-free supernatant fluids

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>ADAM9 Protein Levels in PMN Cell Extracts (pg/10⁶ PMNs)ᵃ</th>
<th>sADAM9 Released by PMNs (% of Amount Contained within Unstimulated PMNs)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
<td>65.48 ± 7.84</td>
<td>3.2 ± 1.5</td>
</tr>
<tr>
<td>iMLP 1 h</td>
<td>73.67 ± 6.56</td>
<td>4.8 ± 2.7</td>
</tr>
<tr>
<td>iMLP 2 h</td>
<td>49.77 ± 12.10</td>
<td>3.9 ± 1.2</td>
</tr>
<tr>
<td>iMLP 4 h</td>
<td>59.18 ± 13.70</td>
<td>5.2 ± 1.6</td>
</tr>
</tbody>
</table>

PMNs were isolated from healthy human donors and an extract of freshly isolated cells was prepared (unstimulated). Other aliquots of PMNs were incubated at 37°C for 1–4 h with 10⁻⁷ M iMLP. Cell-free supernatant samples were removed and cell extracts prepared. ADAM9 protein levels were quantified in PMN extracts and cell-free supernatant fluids using an ELISA. Data for ADAM9 amounts released by PMNs are expressed as a percentage of the amount of ADAM9 protein measured in extracts of unstimulated cells from the same donor. There are no significant differences in the amounts of ADAM9 quantified in extracts of unstimulated versus iMLP-activated PMNs, or in the amount of sADAM9 released by unstimulated versus iMLP-activated PMNs.

ᵃData are means ± SEM; n = 4 donors.
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 proteinase, 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 proteinases 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 proteinases 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 proteinases contribute to lung injury (44). Degradation of lung ECM proteins by PMN-derived proteinases 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 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.

The reaction products were separated on 4–20% Tris-HCl SDS-PAGE gels, which were stained with Coomassie blue dye. Densitometry was used to quantify intact ECM proteins incubated without versus with proteinases. The results are expressed as percentage of ECM protein that was degraded by each proteinase. Data are means ± SEM; n = 3–6 experiments. In (A), *p = 0.031 and **p < 0.001 versus the percentage of fibronectin cleaved by MMP-9. In (B), *p < 0.001 versus the percentage of entactin degraded by MMP-9. In (C), *p < 0.001 versus the percentage of laminin that was degraded by MMP-9 and ADAM9. In (E), 25 nM ADAM9 or buffer alone were incubated with 2 μg fibronectin for up to 18 h at 37˚C and degradation of fibronectin was measured at intervals as outlined above. Data are means ± SD (n = 4 experiments), *p < 0.05 versus fibronectin incubated without ADAM9. In (D) and (F)–(H), equimolar (25 nM) concentrations of human soluble active Adam9, MMP-9, ADAM9, and MMP-8 versus buffer alone were incubated with 0.031 molar (25 nM) concentrations of human soluble active NE, MMP-8, or MMP-9 versus buffer alone were incubated with 50 μg/ml DQ-FITC–conjugated type IV collagen (D), 50 μg/ml DQ-FITC–conjugated type I collagen (F), 50 μg/ml DQ-FITC–conjugated gelatin (G), or 20 mg/ml particulate elastin-FITC (H) at 37˚C for 18 h at pH 7.4 (in [D] and [H], human soluble active NE was used as an additional control). Cleavage of each substrate was quantified in fluorescence units using fluorimetry. Results are means ± SEM; n = 6 experiments. In (A), *p = 0.001 versus the amount of type IV collagen degraded by ADAM9. In (F), *p = 0.001 versus the amount of type I collagen degraded by ADAM9 or MMP-9. In (G), *p < 0.001 versus the amount of gelatin degraded by ADAM9. In (H), *p = 0.033 versus the amounts of elastin degraded by MMP-8 and **p < 0.001 versus the amount of elastin degraded by all other proteinases.
FIGURE 4. Surface Adam9 on activated PMNs has similar ECM protein-degrading activity as does sADAM9. We incubated equal numbers of PAF- and fMLP-activated and fixed WT, Mmp-8−/−, Mmp-9−/−, or Adam9−/− PMNs versus buffer alone at 37˚C for 18 h at pH 7.4 with 50 μg/ml DQ-FITC–conjugated type I collagen (A), 50 μg/ml quenched DQ-FITC–conjugated gelatin (B), or 20 mg/ml FITC–conjugated particulate elastin (C) and quantified MP-mediated cleavage of the substrates by surface proteinases, as described in Materials and Methods. The results for proteinase-deficient PMNs are expressed as a percentage of the MP activity associated with the surface of WT PMNs. Data are means ± SEM; n = 3–4 experiments. In (A), *p = 0.008; in (B), *p < 0.001; and in (C), *p = 0.006, **p < 0.001.

After i.t. LPS, BAL PMN and macrophage counts did not differ 3 or 7 d after i.t. instillation of 30 μU 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 in vitro. 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 cells 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 known 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 plasma membrane of 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).

10 ADAM9 IS A PMN PRODUCT THAT DEGRADES ECM PROTEINS
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 (E) in BAL samples. In (D), *p < 0.05, **p = 0.016. 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 instilling PBS or LPS in mice. Data are means ± SEM; n = 4 PBS-treated mice and n = 5–12 LPS-treated mice. In (D), *p < 0.05, **p = 0.016. 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 instilling PBS or LPS in mice. 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.

immersed 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 PMN 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 supernatant 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, fibrinectin, 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 proteinases that are expressed by PMNs. We also assessed the ECM-degrading activities of transmembrane-bound Adam9 by murine PMNs.

**Adam9 expression and activities during lung inflammation**

Adam9 has not been studied previously in animal models of ALI. Adam9 gene expression increased in the lung during LPS-mediated ALI. Immunostaining studies showed that increases in lung Adam9 levels during ALI were due to the recruitment of PMNs to the lungs, as well as increased expression of Adam9 by lung macrophages and to a lesser extent by bronchial epithelial cells. Likely, the increased steady-state mRNA levels detected in whole-lung samples reflected increased Adam9 gene expression in lung macrophages and epithelial cells. We showed that Adam9 was not essential for PMNs (or macrophages) to accumulate in the

**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 monocytes/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 proteases 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 proteases 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 (45–50, 65). Proteolytic degradation of lung ECM proteins contributes to ALI pathogenesis by causing loss of epithelial cell–ECM attachments, alveolar epithelial cell anoxia, and alveolar edema (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 t12, in the lung could reduce alveolar capillary barrier injury and thereby reduce the high morbidity and mortality that are associated with ALI/ARDS.

In conclusion, Adam9 is a novel product of PMNs that is rapidly mobilized to the extracellular space when PMNs are activated. Adam9 contributes significantly to PMN pericyte proteolysis and elastin degradation in vitro. Adam9 expression is upregulated in the lung during neutrophilic ALI in mice and contributes to lung elastin degradation and alveolar capillary barrier injury in mice, but it does not regulate inflammatory cell recruitment in the lung. Thus, Adam9 is a novel PMN product that promotes ALI in mice.

**Disclosures**

The authors have no financial conflicts of interest.

**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.