Role of Urokinase Plasminogen Activator Receptor–Associated Protein in Mouse Lung

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Urokinase plasminogen activator receptor–associated protein (uPARAP, or Endo180) is a transmembrane endocytic receptor that mediates collagen internalization and degradation. uPARAP may be a novel pathway for collagen turnover and matrix remodeling in the lung. The function of uPARAP in lung injury has not been described. We analyzed the pulmonary mechanics of uPARAP$^{-/-}$ and wild-type mice at baseline and examined their response after bleomycin instillation. We compared collagen internalization in primary mouse lung fibroblasts (MLFs) from wild-type and uPARAP$^{-/-}$ mice using flow cytometry and fluorescent microscopy, and we examined the role of cytokines in regulating uPARAP expression and collagen internalization. We show that uPARAP is highly expressed in the lung, and that uPARAP$^{-/-}$ mice have increased lung elastance at baseline and after injury. uPARAP$^{-/-}$ mice are protected from changes in lung permeability after acute lung injury and have increased collagen content after bleomycin injury. uPARAP is the primary pathway for internalization of collagens in MLFs. Furthermore, collagen internalization through uPARAP does not require matrix metalloproteinase digestion and is independent of integrins. Mediators of lung injury, including transforming growth factor-β, TNF-α, and IL-1, down-regulate both uPARAP expression and collagen internalization. uPARAP is highly expressed in the murine lung, and loss of uPARAP leads to differences in lung mechanics, lung permeability, and collagen content after injury. uPARAP is required for collagen internalization by MLFs. Thus, uPARAP is a novel pathway that regulates matrix remodeling in the lung after injury.

Keywords: urokinase plasminogen activator receptor–associated protein; Endo180; collagen internalization; lung fibroblasts; matrix remodeling

Urokinase plasminogen activator receptor–associated protein (uPARAP, Endo180, or mannose receptor, C type 2) is a 180-kD transmembrane receptor that can bind and internalize both fibrillar and nonfibrillar collagens (1, 2). After internalization, uPARAP targets collagen to the lysosome for degradation and then uPARAP recycles to the plasma membrane (3). uPARAP is expressed in mesenchymal cells, predominantly fibroblasts. In addition, dermal macrophage and human placental endothelial cells express low levels of uPARAP (1, 4, 5).

We previously demonstrated high expression of uPARAP in the mesenchyme throughout lung development (6). Despite the high expression of uPARAP in the developing lung, development proceeds normally in uPARAP$^{-/-}$ mice, and is not associated with any differences in matrix metalloproteinase (MMP), tissue inhibitor of metalloproteinases, or collagen expression in the lung (6). uPARAP$^{-/-}$ mice appear phenotypically normal in the unchallenged state, and have a normal lifespan. One possible explanation for the lack of lung phenotype is use of an alternative pathway for collagen internalization. Although previous work demonstrated that uPARAP was the primary receptor for collagen internalization in dermal fibroblasts and mouse embryonic fibroblasts (3, 7–9), other studies have implicated integrins (specifically, α2β1) and MMPs for collagen internalization in different cells (10, 11). Therefore, we asked whether lung fibroblasts required uPARAP for collagen internalization. Given the potential role of uPARAP in extracellular matrix remodeling during injury and repair, we also examined the response of uPARAP$^{-/-}$ mice to lung injury induced by bleomycin instillation.

CLINICAL RELEVANCE

Matrix remodeling is important for lung injury and repair. In this study, we demonstrate that the newly described endocytic receptor, urokinase plasminogen activator receptor–associated protein (uPARAP), plays a role in regulating lung mechanics, lung permeability, and collagen content after injury. Thus, uPARAP is a novel pathway that regulates matrix remodeling in the lung after injury.

MATERIALS AND METHODS

Bleomycin-Induced Lung Injury

uPARAP$^{-/-}$ mice (8) or wild-type (WT) littermates underwent intratracheal instillation with 2 U/kg bleomycin (SICOR Pharmaceuticals, Inc., Irvine, CA) or saline. At the time mice were killed, the right main stem bronchus was tied off and the left lung was lavaged and processed as previously described (13). The left lung was snap frozen and used for hydroxyproline measurement (13). Hydroxyproline concentration was extrapolated from a standard curve. For measurements of alveolar permeability, FITC-Dextran 70 kD (Invitrogen, Carlsbad, CA) was injected retro-orbitally 3 hours before death. After necropsy, bronchoalveolar lavage (BAL) fluorescence–to–plasma fluorescence ratio was measured with a standard plate reader.

Collagen Internalization by Fluorescence Microscopy

Collagen internalization studies were performed using a modified protocol from Curino and colleagues (12). uPARAP$^{-/-}$ and WT mouse lung fibroblasts (MLFs) were plated onto poly-L-lysine coated coverslips and serum starved overnight. MLFs were then preincubated with 20 μM (25,35)trans-epoxysuccinyl-L-leucylamindo-3-methylbutane ethyl ester, a lysosomal cathepsin inhibitor (Calbiochem, San Diego, CA), at 37°C for 1 hour to prevent degradation of internalized collagen. We then added Oregon green–labeled type IV collagen (Invitrogen) or Oregon green–labeled gelatin (Invitrogen) at 25 μg/ml for 24 hours at 37°C. To confirm that internalized collagens were targeted to the lysosomal compartment, 0.5 μM LysoTracker Red (Invitrogen) was added 1 hour before the end

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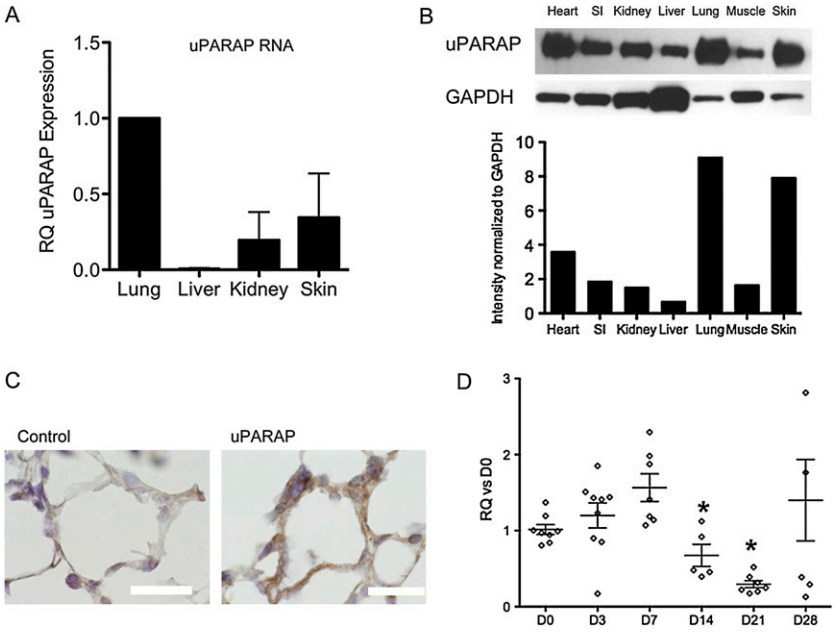

Figure 1. Expression of urokinase plasminogen activator receptor–associated protein (uPARAP) is highest in the mouse lung. (*A*) Real time PCR analysis of uPARAP mRNA expression. Data were normalized to hypoxanthine phosphoribosyltransferase expression. *Y axis* represents fold increase compared with lung. (*B*) Western blot analysis of uPARAP expression from whole-organ lysates. Graph demonstrates densitometry analysis of uPARAP expression normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) loading control. S.I., small intestine. (*C*) uPARAP immunoreactivity in adult mouse lung. *Left panel*, isotype control antibody; *right panel*, uPARAP immunoreactivity (*brown*), counterstained with hematoxylin. *Bar*, 20 μm. (*D*) Real-time PCR analysis of uPARAP mRNA expression after bleomycin administration. Data were normalized to HPRT expression. *Y axis* represents fold increase compared with Day 0. Each *point* represents an individual mouse. Mean value is indicated. $*P < 0.05$ compared with Day 0. RQ, relative quantity.

of collagen incubation. The cells were then washed, fixed in 4% paraformaldehyde, and nuclei were counterstained with 4',6-diamidino-2-phenylindole (1 μg/ml). Coverslips were mounted with Vectashield Hardset (Vector, Burlingame, CA). Images were obtained using a Nikon Eclipse 80i microscope with a DS Camera Head for fluorescent microscopy and a Nikon Eclipse TE200 inverted fluorescent microscope (Nikon, Melville, NY) with a Bio-Rad Confocal Laser Scanning System Radiance 2,000 (Bio-Rad Laboratories, Hercules, CA) equipped with krypton–argon and red diode lasers, using LaserSharp 2,000 software (Bio-Rad). Images were superimposed and processed with Adobe Photoshop version 7.0 (Adobe, San Jose, CA).

Collagen Internalization by Flow Cytometry

MLFs were serum starved overnight, preincubated with 20 μM EST (Calbiochem) for 1 hour, followed by 1-hour incubation with 10 μg/ml integrin subunit β1 blocking antibody (Ha2/5; BD Biosciences, San Diego, CA), 10 μg/ml of integrin subunit α2 blocking antibody (Ha1/29; BD Biosciences), isotype control (Hamster IgM; BioLegend, San Diego, CA), or 25 μg/ml MMP inhibitor (GM6001; Calbiochem).

To determine the role of cytokines in collagen internalization, serum-starved MLFs were incubated for 24 hours with: 10 ng/ml transforming growth factor (TGF)-β1 (R&D Systems, Minneapolis, MN), 10 ng/ml TNF-α (R&D Systems), 10 ng/ml IFN-γ (Chemicon, Temecula, CA), 10 ng/ml platelet-derived growth factor-BB (R&D Systems), or 1 ng/ml IL-1α (R&D Systems). After incubation with cytokines, EST 20 μM was added for 1 hour.

Oregon green–labeled type IV collagen or gelatin was added for 8–24 hours. Fibroblasts were washed and incubated with 0.4% trypan blue (Hyclone; Thermo Scientific, Logan, UT) to quench extracellular, uninternalized fluorescent collagens. Fibroblasts were then trypsinized, resuspended in dye-free Dulbecco's modified Eagle's medium/10% FBS, fixed in 2% paraformaldehyde, and analyzed using the Guava PCA System (Guava Technologies, Hayward, CA) with the Guava Express-Plus program, and analyzed using CellQuest 2.0 (BD Biosciences).

RESULTS

uPARAP Is Highly Expressed in Mouse Lung

We compared the protein and RNA expression of uPARAP in different murine whole-organ lysates. We found that uPARAP is expressed highest in the lung compared with other whole-organ homogenates (Figures 1A and 1B). uPARAP immunoreactivity was visualized throughout the lung parenchyma, especially at the junctures of alveolar septae (Figure 1C). We then examined expression levels of uPARAP after bleomycin lung injury (Figure 1D). We found that uPARAP expression was maintained for the first 7 days after injury, then decreased significantly from

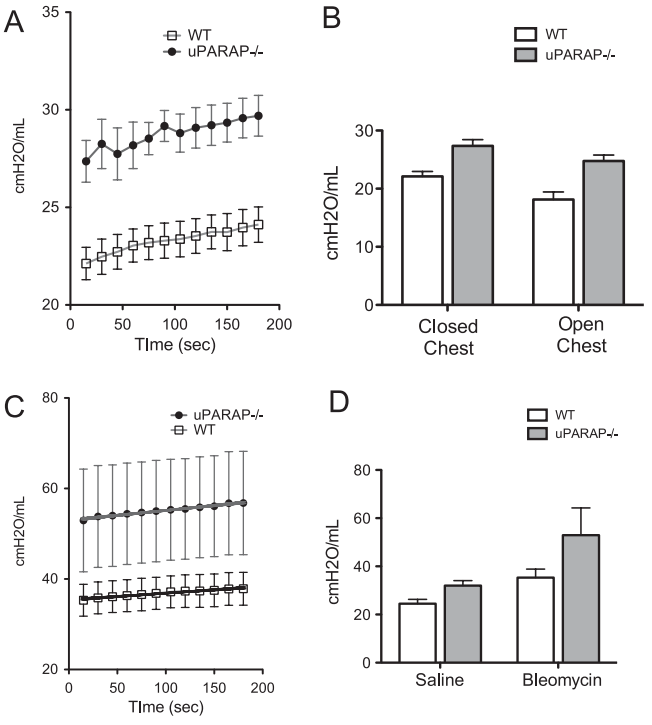

Figure 2. uPARAP$^{-/-}$ mice have increased elastance compared with wild-type (WT) mice at baseline and after bleomycin lung injury. (*A*) Repeated measurements of thoracic elastance after total lung capacity (TLC) maneuver at baseline. (*B*) Initial elastance after TLC in open and closed chest at baseline. (*C*) Repeated measurements of thoracic elastance after TLC maneuver at Day 7 after bleomycin instillation. (*D*) Initial elastance after TLC at Day 7 after bleomycin instillation. $n = 7$–8/group. $P < 0.001$ WT versus uPARAP$^{-/-}$ in all conditions.

baseline by 14 days, which persisted through Day 21. uPARAP expression increased in a subset of mice by Day 28.

uPARAP$^{-/-}$ Mice Have Increased Elastance Compared with WT Mice

Despite no obvious differences upon gross histological evaluation in uPARAP$^{-/-}$ versus WT, we did found that uPARAP$^{-/-}$ mice have higher elastance compared with WT mice in the uninjured state (Figure 2A). This difference persisted after thoracotomy, indicating that the mechanical difference observed is due to lung structure rather than the chest wall (Figure 2B). The differences in elastance persisted after lung injury (Figures 2C and 2D). Despite the difference in elastance, uPARAP$^{-/-}$ mice had similar total lung hydroxyproline content to WT mice (Figure 3F).

Response of uPARAP$^{-/-}$ Mice to Injury

To determine whether absence of uPARAP impacted response to lung injury and fibrosis, we analyzed the uPARAP$^{-/-}$ and WT mice after bleomycin instillation. There was a trend toward improved survival in uPARAP$^{-/-}$ mice compared with WT mice (Figure 3A), although this did not achieve statistical significance. We also noted less weight loss in uPARAP$^{-/-}$ mice (Figure 3B). We found significant differences in lung permeability at Day 3 after bleomycin, as measured by FITC-dextran extravasation into the lung and wet lung weight (Figures 3C and 3D). At baseline, no FITC-dextran extravasation was detected in BAL of WT or uPARAP$^{-/-}$ mice. There was a small, but statistically significant, decrease in BAL total protein in saline-instilled uPARAP$^{-/-}$ mice (Figure 3E). At Day 3 after bleomycin, BAL total protein and IgM were also decreased in uPARAP$^{-/-}$ mice, although this did not achieve statistical significance (Figure 3E and data not

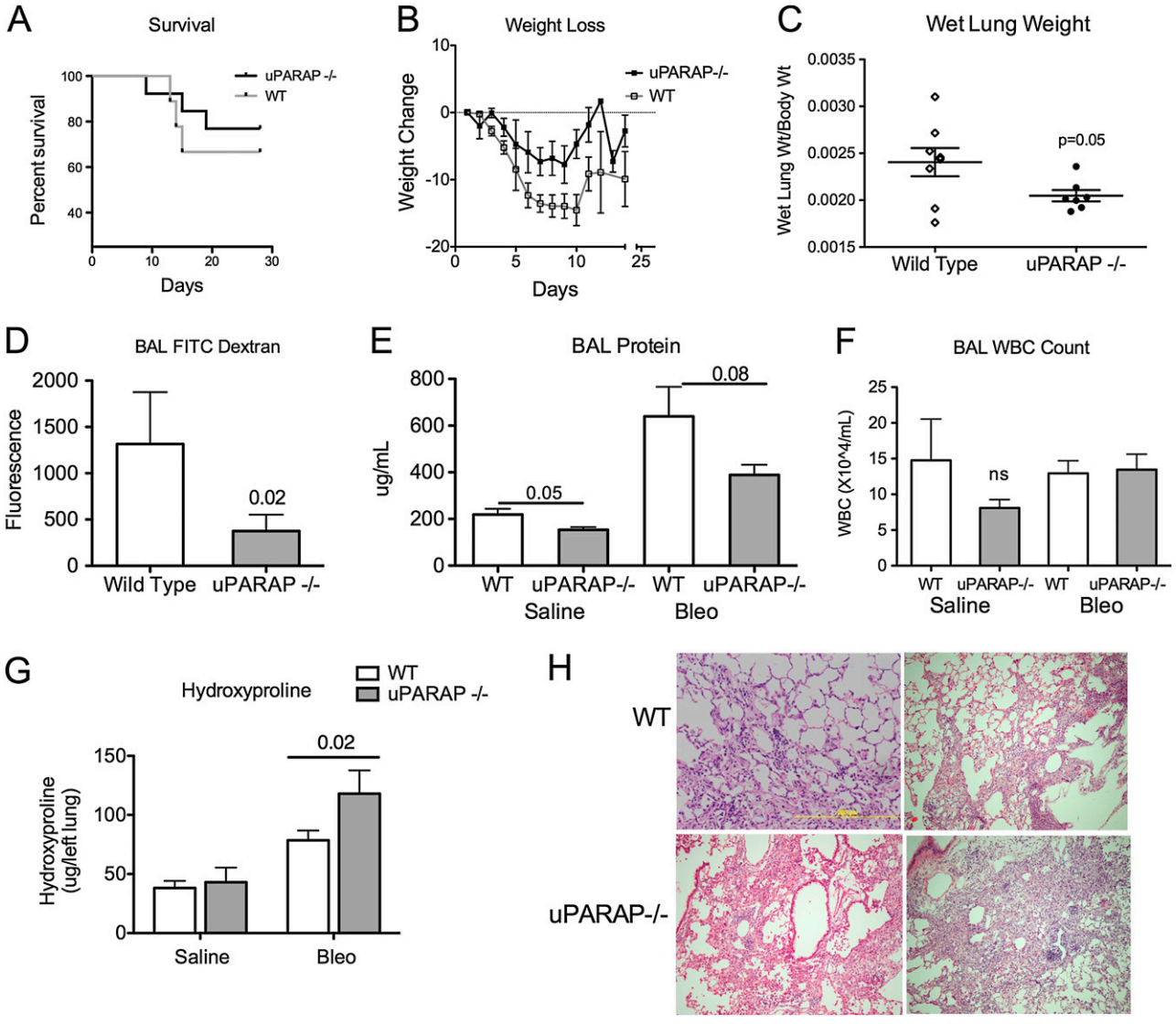

Figure 3. Response of uPARAP$^{-/-}$ mice after bleomycin instillation. (*A*) Kaplan-Meier survival curve after bleomycin instillation (2 U/kg; $n = 10$–13/group). (*B*) Percent weight loss after bleomycin instillation. (*C*) Wet lung weight at Day 3 after bleomycin or saline instillation. (*D*) Bronchoalveolar lavage (BAL) FITC dextran at Day 3 after bleomycin or saline instillation. (*E*) BAL total protein at Day 3 after bleomycin or saline instillation. (*F*) BAL total cell count at Day 3 after bleomycin or saline instillation ($n = 7$–8/group). (*G*) Hydroxyproline content at Day 28 after bleomycin instillation or saline instillation ($n = 9$/WT, 13/uPARAP$^{-/-}$). Mean (±SEM) is shown for all assays. (*H*) Histology of WT (*top*) and uPARAP$^{-/-}$ mice (*bottom*) at Day 28 after bleomycin instillation. Two mice per group are shown. Hematoxylin and eosin stain.

shown). There were no differences in total BAL cell count or differential at any time point examined (Figure 3F and Figure E1 in the online supplement). We analyzed hydroxyproline content as a measure of lung fibrosis at Day 28 after bleomycin instillation (Figures 3G and 3H). We found increased hydroxyproline in uPARAP$^{-/-}$ mice compared with WT mice after injury, consistent with the role of uPARAP in collagen clearance. To look for compensatory changes in other proteins involved in matrix remodeling and injury response, we examined levels of collagen type I, MMPs, tissue inhibitor of metalloproteinases, and several cytokines in WT and uPARAP$^{-/-}$ mice at baseline and after injury. We did not find significant differences at any time point examined (Figure 4).

uPARAP Is the Primary Pathway of Internalization of Type IV Collagen and Gelatin in Primary Lung Fibroblasts

We compared collagen internalization by uPARAP$^{-/-}$ MLFs and WT MLFs. WT lung fibroblasts internalized type IV collagen and gelatin beginning at 1 hour (Figure 5 and data not shown). In contrast, there was minimal internalization of collagen IV or gelatin by uPARAP$^{-/-}$ MLFs at any time point up to 24 hours (Figure 5 and Figure E2). Internalized collagen IV and

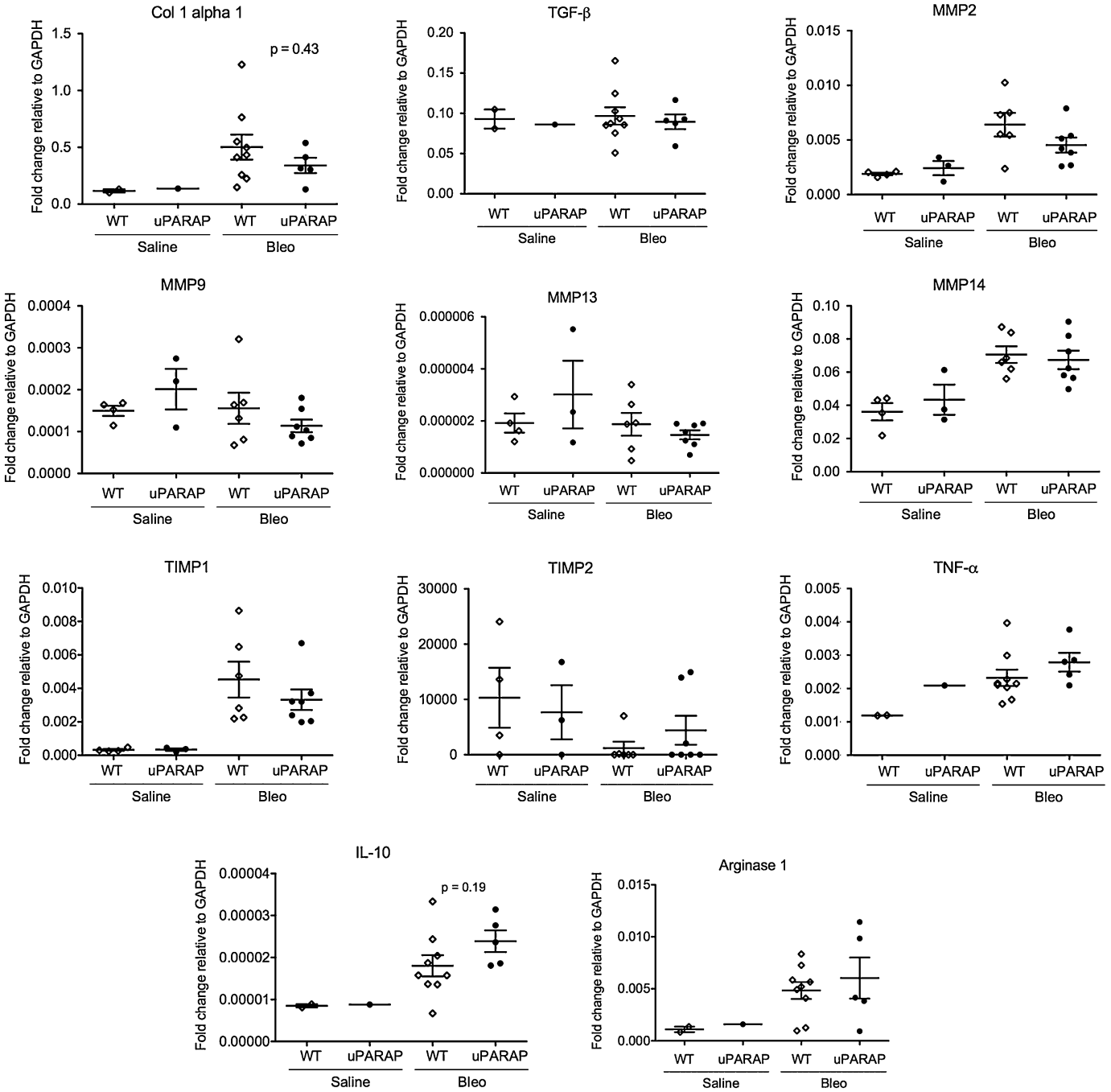

Figure 4. Real-time PCR analysis of select cytokines, matrix metalloproteinases (MMPs), and tissue inhibitor of metalloproteinases, in mouse lungs after saline or bleomycin instillation (Day 7). Data were normalized to GAPDH expression. Each *point* represents an individual mouse. Mean value (±SEM) is indicated. Col, collagen.

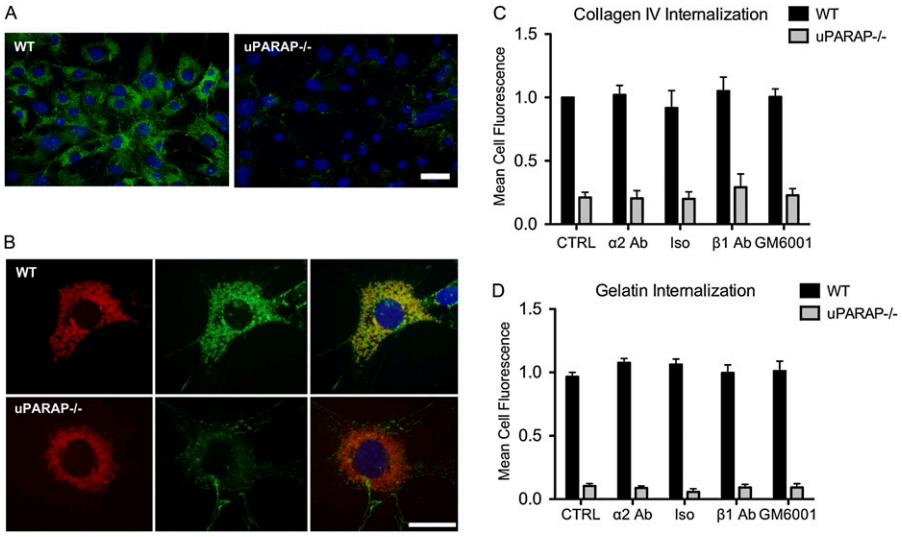

Figure 5. uPARAP mediates collagen IV and gelatin internalization in mouse lung fibroblasts (MLFs) and targets it to the lysosome. (*A*) WT lung fibroblasts, but not uPARAP$^{-/-}$ fibroblasts, internalize collagen type IV (*green*) at 24 hours. *Blue*, nuclei; *bar*, 40 mm. (*B*) WT MLFs (*top*) colocalize internalized collagen IV (*middle panel*, *green*) and lysosomes (*left panel*, *lysotracker red*). Merge channels on *right panel*. uPARAP$^{-/-}$ (*bottom*) MLFs do not internalize collagen or target collagen to lysosomes at 24 hours. The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (*blue*). *Bar*, 20 μm. (*C* and *D*). Quantification of internalized collagen IV (*C*) and gelatin (*D*) by flow cytometry. Blockade of integrin α2, β1, or MMPs have no effect on collagen or gelatin internalization by WT MLFs (3,000 cells/condition analyzed).

gelatin localized to the lysosomes in WT fibroblasts by confocal microscopy (Figure 5B and gelatin not shown). Collagen internalization findings were confirmed and quantified by flow cytometry (Figures 5C and 5D). Integrin α2 or β1 antibodies or MMP inhibitor (GM6001) did not block internalization of type IV collagen or gelatin in WT fibroblasts (Figures 5C and 5D). Similar results were found in lung fibroblasts derived from both FVB and C57Bl/6 mice.

uPARAP Is Down-Regulated by Cytokine Mediators of Lung Injury and Inflammation

Given the role of uPARAP in matrix turnover, we asked whether cytokine mediators of extracellular matrix remodeling in lung injury regulate uPARAP expression or function. TGF-β1 has been previously shown to up-regulate uPARAP RNA expression in human gingival fibroblasts (14). In contrast, we found decreased uPARAP RNA expression in lung fibroblasts incubated with TGF-β1, TNF-α, IL-1α, but not PDGF-BB (Figure 6). Consistent with decreased uPARAP expression, we found decreased collagen internalization in lung fibroblasts incubated with TGF-β1, TNF-α, IFN-γ, IL-1α, but not PDGF-BB (Figure 6C). Myeloid differentiation primary response gene 88 (Myd88) is an adapter protein necessary for IL-1 signaling (15). We demonstrated that MyD88$^{-/-}$ MLFs did not down-regulate uPARAP expression or collagen internalization in response to IL-1 (Figures 6D and 6E). However, uPARAP expression and collagen internalization were still decreased by TGF-β1. These results demonstrate that Myd88 regulates IL-1, but not TGF-β–mediated down-regulation of uPARAP expression and function.

DISCUSSION

We now demonstrate that although uPARAP$^{-/-}$ mice appear phenotypically normal, are fertile, and have a normal lifespan,

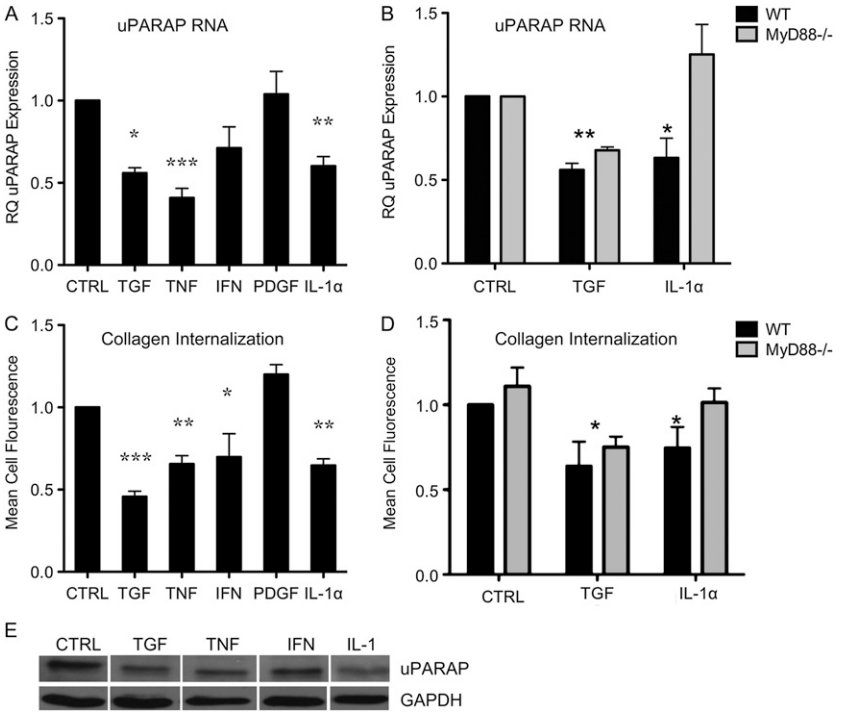

Figure 6. uPARAP expression and function is decreased by cytokines. WT MLFs were incubated in serum-free media, transforming growth factor (TGF)-β1 (10 ng/ml), TNF-α (10 ng/ml), IFN-γ (10 ng/ml), platelet-derived growth factor-BB (10 ng/ml), or IL-1α (1 ng/ml) for 24 hours. (*A*) Real-time PCR analysis of uPARAP mRNA expression. Data were normalized to HPRT expression. *Y axis* represents fold increase compared with serum free media. (*B*) After 24-hour incubation with cytokines, MLFs were incubated with labeled type IV collagen for 8 hours. Collagen internalization was determined by flow cytometry and reported as mean cell fluorescence. (*C*) uPARAP protein expression in MLFs after incubation with indicated cytokines. (*D* and *E*) WT or myeloid differentiation primary response gene 88$^{-/-}$ MLFs were incubated with indicated cytokines for 24 hours. (*D*) Real-time PCR analysis of uPARAP mRNA expression. Data were normalized to HPRT expression. *Y axis* represents fold increase compared with serum-free media. (*E*) After 24-hour incubation with cytokines, MLFs were incubated with labeled gelatin for 8 hours. Gelatin internalization was determined by flow cytometry and reported as mean cell fluorescence (n = 2–6 mice per condition). *$P < 0.05$, **$P <$ 0.01, ***$P < 0.001$ compared with serum-free media alone.

they demonstrate differences in lung mechanics, as illustrated by increased lung elastance in uPARAP$^{-/-}$ mice at baseline, which persists after injury. We also demonstrate that uPARAP$^{-/-}$ mice are partially protected from increases in lung permeability after acute lung injury and, furthermore, that uPARAP$^{-/-}$ mice have increased collagen content after bleomycin-induced lung injury. Consistent with these findings, we demonstrate that uPARAP is the primary pathway for collagen internalization in MLFs, and that internalization was not dependent on the integrins, α1 and β2, or MMP-mediated proteolysis. These results demonstrate an unsuspected role of uPARAP in maintaining lung integrity, in addition to a role in matrix remodeling during injury.

Although uPARAP internalizes collagen I and collagen IV, we previously demonstrated that uPARAP colocalized most closely with collagen IV, not collagen I, during lung development (6). Collagen type IV is a major component of the alveolar basement membrane basal lamina. In the lung, the basal lamina of the alveoli and capillary are fused to facilitate gas transfer. The importance of type IV collagen in regulating basement membrane integrity is well illustrated in Goodpasture's syndrome, an autoimmune disease in which antibodies against type IV collagen result in alveolar and renal hemorrhage (16). We speculate that loss of uPARAP-mediated collagen IV internalization decreases basement membrane turnover. Absence of uPARAP-mediated collagen IV internalization and degradation may subtly alter the basement membrane conformation such that it is more resistant to breakdown after injury. For example, decreased collagen IV turnover may lead to increased crosslinking of collagen and increased functional integrity that is only apparent with stress. Despite differences in elastance, we did not find differences in total hydroxyproline content in the lung at baseline. Because turnover in the lung of extracellular crosslinked collagen is relatively slow (20–22), the assay may not detect small changes in collagen content that may be physiologically relevant. In addition, differences in the basement membrane, such as increased collagen cross-linking, would not be reflected by hydroxyproline measurements. In concert, our data suggest that collagen IV may be the physiologically relevant substrate for uPARAP in the lung during normal homeostasis.

We also found that uPARAP$^{-/-}$ mice had increased collagen content after bleomcyin-induced injury. This finding is consistent with the known function of uPARAP in collagen internalization and degradation, which would lead to increased accumulation of collagen. Much focus has been paid to extracellular pathways of collagen degradation, including MMPs and cathepsin-mediated degradation (17). In contrast to these other pathways of degradation, uPARAP-mediated collagen internalization and degradation allows cells to clear matrix components after protease cleavage and recycle collagen components, key features of cell housekeeping. This makes uPARAP an attractive candidate for collagen turnover during active matrix remodeling such as with lung injury and repair. Collagen that has been precleaved by a mammalian collagenase, such as MMP-14, is internalized much more efficiently than intact collagen, suggesting that fibroblast-mediated collagen degradation proceeds as a sequential mechanism in which extracellular collagen degradation is followed by uPARAP-mediated endocytosis of collagen fragments (10, 23).

Due to the profibrotic role of TGF-β1 in extracellular matrix remodeling in lung injury, we asked whether TGF-β1 regulated collagen clearance through uPARAP. Honardoust and colleagues (14) showed that uPARAP is up-regulated by TGF-β1 in human gingival fibroblasts. In contrast, we found that uPARAP expression and collagen internalization were both decreased in MLFs treated with TGF-β1. This suggests that uPARAP is differentially regulated in fibroblasts derived from different tissues. Furthermore, our finding is consistent with the general role of TGF-β in extracellular matrix accumulation (18, 19). Differences in results may be related to the different baseline expression of uPARAP in MLFs compared with gingival fibroblasts. Interestingly, additional mediators of injury (TNF-α, IFN-γ, IL-1) also decreased uPARAP expression. We also find that *in vivo* expression of uPARAP is decreased by Day 14 after bleomycin injury, when collagen accumulation occurs. These data suggest that cytokine mediators of lung injury may promote development of fibrosis by decreased uPARAP-mediated clearance of collagen.

In summary, we demonstrate that uPARAP$^{-/-}$ mice have increased lung elastance, decreased lung permeability, and increased collagen deposition after bleomycin-induced lung injury. We demonstrate that uPARAP, but not other receptors, is required for collagen internalization in lung fibroblasts. Furthermore, we demonstrate that uPARAP and collagen internalization are down-regulated by several cytokine mediators of lung injury and fibrosis, in contrast to studies using fibroblasts of different origins. Thus, uPARAP-mediated collagen internalization contributes to both structural integrity and matrix remodeling after lung injury.

Author disclosures are available with the text of this article at www.atsjournals.org.

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