Potential novel targets: Protease-activated receptors in idiopathic pulmonary fibrosis

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Chapter 5

Protease activated receptor-1 regulates macrophage-mediated pulmonary fibrosis

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ABSTRACT

Background
Pulmonary fibrosis is a devastating disease of unknown etiology. Protease-activated receptor-1 recently emerged as a critical component in the context of fibrotic lung diseases. In the present study, we aimed to address the potential importance of macrophages in PAR-1-driven pulmonary fibrosis.

Methods
Macrophage numbers in lungs of bleomycin-instilled mice treated or not with a specific PAR-1 antagonist (i.e. P1pal-12) were assessed by (immuno)histochemical analysis. Macrophage migration was assessed by trans-well migration assays while PAR-1 ligand production was addressed by qPCR. In vitro, fibroblasts were stimulated with macrophage-derived conditioned medium after which fibrotic marker expression and Smad-2 phosphorylation was analyzed by Western blot.

Main results
Macrophage numbers were significantly reduced in lungs of P1pal-12 treated animals after bleomycin instillation. In line with these data, PAR-1 stimulation increased monocyte/macrophage recruitment in response to epithelium injury in in vitro trans-well assays. Moreover, macrophages induced fibroblasts migration, differentiation and the secretion of collagen, which were inhibited in the presence of TGF-β receptor inhibitors. Interestingly, these profibrotic effects were partially inhibited by P1pal-12 treatment. Using shRNA mediated PAR-1 knock down in fibroblasts, we demonstrate that fibroblast PAR-1 contributes to TGF-β activation and production. Finally, we show that the macrophage-dependent induction of PAR-1 driven TGF-β activation was mediated by FXa.

Conclusions
Our data identify novel mechanisms by which PAR-1 stimulation on different cell types can contribute to pulmonary fibrosis and pinpoint macrophages as key players in PAR-1 dependent development of pulmonary fibrosis.
INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a devastating disease, characterized by (myo)fibroblast proliferation and excessive extracellular matrix (ECM) formation leading to destruction of the lung architecture\(^1\). The current paradigm postulates that pulmonary fibrosis results from a chronic epithelial lesions leading to an aberrant wound healing response.\(^2-3\) Although knowledge of the pathogenesis of pulmonary fibrosis continues to evolve, therapeutics that effectively improve the clinical outcome of IPF are limited\(^4\). To date, only pirfenidone and nintedanib slow the decline of lung function in patients with IPF. However, both drugs have side effects, have no benefit on quality of life, and do not stop nor reverse the disease\(^5-7\). Novel treatment options for IPF are thus eagerly awaited for and such options will only become available due to the comprehensive understanding of the underlying mechanisms.

Protease-activated receptor (PAR)-1 is a cell surface seven-transmembrane G protein-coupled receptor that is activated by proteolytic cleavage, inducing transmembrane signaling to intracellular G proteins leading to a broad range of pathophysiological pathways\(^8\). Importantly, PAR-1 recently emerged as a critical component in the context of fibrotic lung disease. Indeed, PAR-1 expression is increased within fibroproliferative and inflammatory foci in IPF patients\(^9\). Moreover, PAR-1 activation stimulates fibroblast differentiation and ECM production\(^10,11\), whereas PAR-1 seem to synergize with PAR-3 to mediate epithelial-mesenchymal transition of alveolar epithelial cells\(^12\). In line with these \textit{in vitro} data, PAR-1 deficiency in mice affords protection from bleomycin-induced pulmonary fibrosis whereas pharmacological inhibition of PAR-1 also limits bleomycin-induced pulmonary fibrosis\(^9,10\).

Interestingly, PAR-1 overexpression is found in alveolar macrophages from patients with chronic airway disease and PAR-1 expression in IPF patients is associated with macrophages\(^9,13\). This may be particularly important as macrophages are known to be key regulators in the progression of pulmonary fibrosis\(^14-16\). In this context, macrophage influx is an early event following lung injury and macrophages secrete large amounts of profibrotic cytokines like TGF-\(\beta\)\(^17\). TGF-\(\beta\) on its turn induces fibroblast proliferation and differentiation into myofibroblasts leading to extracellular matrix (ECM) deposition thereby promoting fibrosis\(^16\).

In the present study, we aimed to address the potential importance of macrophages in PAR-1-dependent pulmonary fibrosis. We show that PAR-1 modifies macrophage
recruitment to the lung during pulmonary fibrosis, and we identify a potential mechanism by which PAR-1 mediates macrophage induced profibrotic responses.

**MATERIALS AND METHODS**

**Reagents**

Thrombin and bleomycin were from Sigma (St-Louis, MO), the recombinant TGF-β was from Tebu-bio (Heerhugowaard, Netherlands), the specific FXa inhibitor antistasin core peptide was purchased from Bachem (Bubendorf, Switzerland), TGF-β receptor (TGFBR) 1 and 2 were inhibited by a combination of inhibitors SB-431542 and LY-2157299, which were from Axon medchem (Groningen, Netherlands) whereas PAR-1 inhibitor P1pal-12 (palmitate-RCLSSAVANRS-NH2) was from GL Biochem Ltd (Shanghai, China).

**Cell Lines and Conditioned Medium Preparation**

Murine NIH3T3 fibroblasts and RAW264.7 macrophages were cultured in DMEM and IMMDM, respectively, supplemented with 10% fetal calf serum (FCS). Murine lung epithelial cells (MLE-15) were cultured in HITES medium (RPMI supplemented with 5 μg/ml insulin, 10 μg/ml transferrin, 10 nM estradiol and 10 nM hydrocortisone). Cells were grown at 37°C in an atmosphere of 5% CO₂. Unless indicated otherwise, cells were washed twice with PBS and serum-starved for 4 hours before stimulation. For conditioned medium preparations, cells were seeded and grown overnight under normal growth conditions to reach subconfluency. Next, the cells were washed with PBS and incubated for 24 hours in FCS free medium. Finally, the collected media were centrifuged, put through a 0.2 μm filter and stored at -20 °C.

**Lentiviral Knockdown of PAR-1**

PAR-1 knock down cells were established as described before. Briefly, PAR-1 (clone TRCN0000026806) and control (clone SHC004) shRNA in the pLKO.1-puro backbone were purchased from Sigma-Aldrich (St. Louis, MO; MISSION shRNA library). Lentiviral production and subsequent cell transduction was performed using standard protocols. shRNA transduced NIH3T3 cells were selected in the presence of 5 μg/ml puromycin for 72 h.

**Cell Viability Assays**

Cells were seeded in 96-well plates at a concentration of 5000 cells/well, after which cell
viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay at 12 and 24 hours according to routine procedures.

**Calcium Assay** - Calcium signaling responses were analyzed using the Fluo-4 Direct™ Calcium Assay Kit (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. Cells were challenged with thrombin. Ca^{2+} flux was monitored for the indicated time points on a Bio-Tek HT Multi-Detection Microplate Reader (Winooski, United States).

**Wound Scratch Assay**
Scratch assays were performed essentially as described before. Briefly, fibroblasts were seeded in six-well plates in DMEM supplemented with 10% FCS. After the cells formed a confluent monolayer, a scratch was created in the center of the monolayer by a sterile p200 pipette tip. Next, medium was removed and cells were washed with serum-free medium to remove floating debris. The cells were subsequently incubated for 18 hours with serum-free medium (negative control), RAW264.7 conditioned medium or RAW264.7 conditioned medium containing 10 μM of each TGF-β receptor inhibitors or 10 μM P1pal-12. When indicated, cells were pre-incubated with 10 μM P1pal-12 for 30 minutes before scratching. The ability of cells to close the wound was assessed by comparing the 0- and 18-hour phase-contrast micrographs of 6 marked points along the wounded area. The percentage of non-recovered wound area was calculated by dividing the non-recovered area after 18 hours by the initial area at 0 hour as previously described.

**Western Blot**
Fibroblasts were seeded in 12-well plates in DMEM supplemented with 10% FCS. Next, medium was removed and cells were washed with serum-free medium. After serum starvation for 4 hours, the cells were incubated with serum-free medium (negative control) or RAW264.7 conditioned medium with or without 10 μM of each TGF-β receptor inhibitors or 10 μM P1pal-12. When indicated, cells were pre-incubated with 10 μM P1pal-12 for 30 minutes. Twenty four hours later, cells were lysed in Laemmli lysis buffer and Western blots were performed as described before. In brief, protein samples were separated by 10% SDS gel electrophoresis and transferred to a PVDF membrane (Millipore, Billerica, MA). Membranes were blocked for 1 hour in 4% milk in TBST and incubated overnight with monoclonal antibodies against α-smooth muscle actin (a-SMA), GAPDH (all Santa Cruz, CA), collagen (SouthernBiotech, AL) or p-SMAD2 (Cell Signaling Technology, Boston, MA) at 4°C. All secondary antibodies were horseradish peroxidase (HRP)-conjugated from DakoCytomation (Glostrup, Denmark) and diluted according to the manufacturer’s instructions. Blots were imaged using Lumilight.
plus ECL substrate from Roche (Almere, The Netherlands) on an ImageQuant LAS 4000 biomolecular imager from GE Healthcare (Buckinghamshire, U.K).

**Trans-well Migration Assays**

Serum starved Raw264.7 cells (1×10⁵ CellTrace CFSE labeled for real-time analysis or 2×10⁴ unlabeled for analysis by microscopy) were transferred to 8 μm pore-size Cell Culture inserts coated with 0.1% (w/v) collagen. The cells were incubated in serum-free medium with or without thrombin, and the inserts were incubated at 37°C for 10 hours in serum-free medium or MLE-15 conditioned medium as chemoattractant. For real time analysis, fluorescence values representing the number of cells on the bottom side of the insert were read on a BioTek plate reader at 485/528 nm (BioTek®, Bad Friedrichshall, Germany). For microscopic analysis, cells on the upper side of the transwell membrane were removed with a cotton swab after which the inserts were fixed and stained in a crystal violet solution as described before¹⁹. The membranes were subsequently mounted on a glass slide, and migrated cells were counted by light microscopy. Cells were counted in five different fields using a 200× magnification.

**Immunohistological Analysis**

Four-μm sections were deparaffinized and rehydrated. Endogenous peroxidase activity was quenched with 0.3% H₂O₂ in methanol. F4/80 staining was performed using an anti-F4/80 antibody (1:500, 24 hour at 4°C, AbD Serotec, Kidlington, UK). A horseradish peroxidase-conjugated polymer detection system (ImmunoLogic, Duiven, the Netherlands) was applied for visualization, using an appropriate secondary antibody and diaminobenzidine (DAB) staining. Slides were photographed with a microscope equipped with a digital camera (Leica CTR500). Pictures of F4/80 stainings were taken to cover the entirety of all sections. Color intensity of stained areas was analyzed semi-quantitatively with ImageJ and expressed as percentage of the surface area essentially as described before²⁰.

**TGF-β ELISA**

TGF-β1 was measured with the Mouse TGF-beta 1 DuoSet kit (R&D systems, UK) as suggested by the manufacturer.

**Quantitative real-time PCR**

Total RNA was isolated from cells with TriPure (Roche, Almere, Netherlands) following the manufacturer’s recommendations. q-PCR was performed with SYBR Green PCR
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master Kit (Roche) using the following primers:
Factor(F)II: forward 5'-GGCAACCTAGACGTGAGT-3' and reverse 5'-TAGCACA-GCGACCTTCCAGA-3'; FX: forward 5'-GACCAATATAAGACGGGCAC-3' and reverse 5'-TCCGAACAAAGAGCTCACAGT-3'; Granzyme K: forward 5'-TGAGCC-CATGAAGCAGACAT-3' and reverse 5'-TGGCATTGCTCCCATCTCTCTCT-3'; Matrix metalloproteinases (MMP)-1: forward 5'-TTACGCGCTCATGAACTGGGT-3' and reverse 5'-GTTGGCTGGATGGGATTTGG-3'; MMP-13: forward 5'-AACATCCATCCCCGTGACCTT-3' and reverse 5'-TTCTCAAAGTGACCCCGACG-3'; Kallikrein (KLK)-1: forward 5'-CCCACAACCTGAGGATGAC-3' and reverse 5'-GCTTGAGGTTCACACACTTG-3'; KLK-4: forward 5'-ATCTCTCAGTG-GGTCAGAG-3' and reverse 5'-CTGCCCACACTTTCCTGTC-3'; KLK-6: forward 5'-GCTTGAGGTTCACACACTTG-3' and reverse 5'-GCTGCCACACTTTCCTGTC-3'; GAPDH: forward 5'-TCTGACAGACTTCCCTGAAC-3' and reverse 5'-CCATGGAACCAC-CTTCTCCTGTG-3'; TBP: forward 5'-GGAGAATCATG-GACCAGAACA-3' and reverse 5'-GATGGGAATTCCAGGAGTCA-3'.

The qPCR data were normalized to the average of the housekeeping genes GAPDH and TBP.

Statistics
Statistical analyses were conducted using GraphPad Prism version 5.00, (GraphPad software, San Diego, CA, USA). Data were expressed as means ± SEM. Comparisons between two conditions were analyzed using two tailed unpaired t-tests when the data where normally distributed, otherwise Mann-Whitney analysis was performed. P values of less than 0.05 were considered significant.

RESULTS
PAR-1 regulates monocyte/macrophage recruitment during pulmonary fibrosis
We previously showed that pharmacological inhibition of PAR-1 by P1pal-12 reduced bleomycin-induced pulmonary fibrosis\(^9\). As macrophage recruitment in response to chemoattractant production by injured epithelial cells is a key process in fibrosis\(^16\), we set out to determine whether PAR-1 would modify macrophage recruitment into fibrotic lungs. As shown in Figure 1A, macrophages were omnipresent in lungs of wild type mice instilled with bleomycin as evident from large amounts of F4/80 positive cells. Interestingly, macrophage numbers were reduced by approximately 50% in fibrotic mice treated with P1pal-12 (Figure 1B, C).
To assess whether the reduced macrophage numbers in P1pal-12 treated mice are due to a direct effect of PAR-1 on macrophage migration towards injured epithelium, we next analyzed migration of RAW264.7 macrophages in vitro. To mimic the in vivo setting, MLE-15 epithelial cells were exposed to bleomycin (10 μg/ml) for 48 or 72 hours after which the medium was used as chemoattractant for RAW264.7 cells. As shown in Figure 2A and D, medium of bleomycin-exposed MLE-15 cells served as a chemoattractant for RAW264.7 cells. Interestingly, stimulation of RAW264.7 cells with thrombin did not have an effect on migration towards control medium, but potentiated migration towards bleomycin-treated MLE-15 conditioned medium (Figure 2B-D). These results thus indicate that macrophage recruitment into injured lungs seems (at least in part) PAR-1 dependent.

**Macrophages induce fibrotic responses in fibroblasts via TGF-β in a PAR-1 dependent manner**

To assess whether the decreased number of macrophages in lungs of P1pal-12 treated mice correlate with the observed reduction in fibrosis, we subsequently analyzed macrophage-induced profibrotic responses in fibroblasts. RAW264.7 conditioned medium induced

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Figure 1. PAR-1 inhibition reduces macrophage numbers in the lung of bleomycin treated mice. Representative macrophage marker F4/80 stained sections obtained 14 days after bleomycin instillation in wild type mice (A) and wild type mice treated with the PAR-1 inhibitor P1pal-12 (2.5 mg/kg) (B). The arrows indicate an example of F4/80 positive macrophages. (C) Quantification of macrophage numbers in fibrotic mice treated or not with P1pal-12 (mean±SEM, n=8 per group). * P<0.05.
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Figure 2. PAR-1 regulates macrophages migration in trans-well assays. (A) Real-time migration of RAW264.7 cells towards MLE-15 epithelial cell conditioned medium collected after exposure to 10 μg/ml bleomycin 48 or 72 hours. RAW264.7 cell migration towards plain medium was used as control. (B) Real-time migration of RAW264.7 cells towards control or MLE-15 conditioned medium (10 μg/ml bleomycin for 72 hours) in the absence or presence of thrombin (1 U/ml). Shown is the mean ± SEM, n=3. (C) Representative pictures of RAW264.7 cells migrated through the trans-well toward plain control or MLE-15 epithelial cells conditioned medium (CM) stimulated with or without thrombin (1 U/ml). (D) Quantification of the data presented in (C) (mean ± SEM of an experiment performed three times, *P<0.05 and **P<0.01).
fibroblast migration as evident from efficient wound closure as compared to control medium that did not induce wound closure (Figure 3A-B). In line, RAW264.7 conditioned medium also induced fibroblast differentiation and extracellular matrix production as evident from increased α-SMA and collagen expression levels (Figure 3C). To determine whether the macrophage-induced profibrotic responses of fibroblasts rely upon PAR-1 activation on fibroblasts, we next pre-incubated fibroblasts with P1pal-12 before assessing the macrophage-dependent fibrotic responses. As shown in Figure 3,
P1pal-12 treatment significantly inhibited macrophage-induced wound closure, fibroblast differentiation and collagen deposition suggesting macrophages potentiate fibroblast-driven fibrosis in a PAR-1 dependent manner.

Macrophages are a major source of TGF-β, a crucial pro-fibrotic cytokine which induces fibroblast migration, differentiation and ECM synthesis\textsuperscript{15-17}. Hence, we determined whether TGF-β plays a dominant role in the pro-fibrotic effects of RAW264.7 conditioned medium. We assessed macrophage-dependent fibroblast migration in the presence of TGF-β receptor inhibitors. As shown in Figure 3A-B, inhibition of TGF-β signaling inhibited RAW264.7 conditioned medium-induced wound closure. Consistently, TGF-β receptor inhibition also prevented macrophage-induced fibroblast differentiation (i.e. α-SMA expression) and ECM deposition (i.e. collagen production) (Figure 3).

To corroborate these findings, we next assessed macrophage-induced SMAD2 phosphorylation, a direct downstream target of TGFBR activation in fibroblasts. As shown in Figure 4A, RAW264.7 conditioned medium clearly caused a time-dependent increase in SMAD2 phosphorylation in NIH3T3 cells. Notably, SMAD2 phosphorylation was completely blocked by TGF-β receptor inhibitors, while it was also partly blocked by the PAR-1 inhibitor P1pal-12, suggesting that TGF-β signaling on fibroblasts is in part mediated by PAR-1.

To confirm the P1pal-12 data and to elucidate the mechanism by which PAR-1 influences TGF-β signaling, we generated a stable PAR-1 knockdown fibroblast cell line. As shown in Figure 4B, NIH3T3 cells lentivirally transduced with PAR-1 shRNA (indicated as PAR-1-/- cells) proliferated to a similar extent as NIH3T3 cells transduced with control shRNA (indicated as WT cells). The PAR-1-/- cells did however not respond to thrombin stimulation in calcium assays as opposed to WT cells confirming efficient knock-down (Figure 4C). As expected, WT fibroblasts showed increased SMAD2 phosphorylation after stimulation with RAW264.7 conditioned medium (Figure 4D). In line with p1pal-12 treatment, PAR-1 knock-down significantly inhibited macrophage-induced SMAD2 phosphorylation (Figure 4D). Interestingly, however, PAR-1-/- fibroblasts still responded to direct TGF-β stimulation and showed similar SMAD2 levels as WT fibroblasts (Figure 4D). Overall these data show that PAR-1 on fibroblasts modifies TGF-β signaling most likely by regulating the activation of latent TGF-β.
Finally, we assessed whether fibroblast PAR-1 would also modify TGF-β production induced by macrophage-conditioned medium. As shown in Figure 4E, baseline latent TGF-β levels were already reduced in PAR-1-/- fibroblasts as compared to WT fibroblasts. Moreover, RAW264.7 conditioned medium induced TGF-β expression by WT cells but not by PAR-1-/- cells (TGF-β level in PAR1-/- cells stimulated with RAW264.7 conditioned is similar to the levels in conditioned medium alone). It thus seems that PAR-1 expression on fibroblasts also potentiates TGF-β production.

**Factor Xa as PAR-1 agonist secreted by RAW264.7 cells**

The data presented above suggest a crosstalk between fibroblasts and macrophages, where macrophages are a source of one or several PAR-1 agonist(s) that subsequently

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**Figure 4. PAR-1 mediates TGF-β activation and production.** (A) Western blot analysis of SMAD2 phosphorylation in NIH3T3 cells stimulated with RAW264.7 CM in the absence or presence of TGFBRI inhibitors (10 μM of SB-431542 and 10 μM of LY-2157299) or P1pal-12 (10 μM). GAPDH served as loading control. (B) Cell viability of NIH3T3s lentivirally transduced with a control shRNA construct (WT fibroblasts, down-pointing triangle) or a PAR-1 shRNA construct (PAR-1-/- fibroblasts, up-pointing triangle) as evaluated by MTT assays (Mean+/SEM of an experiment performed two times in octoplo). (C) Intracellular Ca²⁺ fluxes in WT fibroblasts (circle) and PAR-1-/- fibroblasts (square) after stimulation with 1 U/ml thrombin. Ca²⁺ fluxes are expressed as arbitrary units of fluorescent intensity after background correction. (D) Western blot analysis of SMAD2 phosphorylation in WT fibroblasts or PAR-1-/- fibroblasts stimulated with RAW264.7 CM or recombinant TGF-β (1 ng). GAPDH served as loading control. (E) Total TGF-β production of RAW264.7 cells and WT or PAR-1-/- fibroblasts stimulated without or with RAW264.7 conditioned medium (CM) (mean ± SEM, n=6; *** P<0.001).
stimulate fibroblasts leading to TGF-β production and activation. Hence, to identify the potential PAR-1 agonist, we first analyzed mRNA expression levels of known PAR-1 agonists in RAW264.7 cells. As shown in Figure 5A, thrombin and MMP-13 seem not to be expressed by RAW264.7 cells, whereas MMP-1, KLK-1, KLK-4 and KLK-6 were expressed at relatively low levels. Granzyme K and particularly FX are expressed in significant amounts, and are thus likely candidates as macrophage-secreted PAR-1 agonist. To prove or refute that FXa is the PAR-1 agonist secreted by RAW264.7 cells, we next determined RAW264.7 cell-induced TGF-β signaling by assessing SMAD2 phosphorylation in fibroblasts in the absence or presence of antistasin, a direct FXa inhibitor. As shown in Figure 5B, RAW264.7 conditioned medium-induced SMAD2 phosphorylation was almost completely blocked by antistasin pinpointing FX as endogenous PAR-1 ligand secreted by Raw264.7 cells.

Figure 5. PAR-1-induced TGF-β activation on fibroblasts is mediated by FX. (A) Thrombin (FII), FX, Granzyme K (GZMK), MMP1, MMP13, KLK1, KLK4 and KLK6 mRNA levels in RAW264.7 cells as assessed by real-time reverse transcriptase PCR. Data are expressed relative to two housekeeping genes, GAPDH and TBP. Shown is the mean ± SEM, n=3. (B) Western blot analysis of SMAD2 phosphorylation in NIH3T3 cells stimulated with RAW264.7 CM in the absence or presence of the FX inhibitor antistasin. GAPDH served as loading control. (C) Proposed mechanism by which macrophages promote lung fibrosis in a PAR-1 dependent manner. During lung injury, epithelial cells release mediators that potentiate PAR-1 dependent macrophage migration towards the injured site (1). The recruited macrophages subsequently secrete TGF-β and FX. The PAR-1 agonist (FX) than activates PAR-1 on fibroblasts (2) leading to TGF-β production and activation. Finally, TGF-β induces TGFBR signaling (3) on fibroblast thereby inducing their migration, differentiation and ECM deposition.
DISCUSSION

There is compelling evidence that PAR-1 plays an important role in mediating profibrotic effects and pharmacological inhibition of PAR-1 affords protection from bleomycin-induced pulmonary fibrosis. The underlying mechanism by which PAR-1 modulates pulmonary fibrosis is however not yet fully understood. In the current manuscript, we identify macrophages as key players in PAR-1 driven pulmonary fibrosis. We show that PAR-1 on macrophage potentiates recruitment of macrophages towards injured lung epithelial cells. Once recruited, macrophages secrete the PAR-1 agonists FXa that act upon fibroblasts leading to the production and activation of latent TGF-β that subsequently drives fibroblast migration, differentiation into myofibroblasts and ECM deposition (Figure 5C).

A key finding of our study is the fact that macrophage numbers are significantly reduced in fibrotic lungs as a consequence of pharmacological PAR-1 inhibition. To prove or refute that PAR-1 directly modifies macrophage migration, we mimicked the in vivo setting by analyzing macrophage migration towards conditioned medium obtained from bleomycin-treated lung epithelial cells in trans-well assays. Interestingly, PAR-1 activation by thrombin did not affect macrophage migration towards control medium, but did potentiate directed migration towards conditioned medium of injured epithelial cells. This suggests that PAR-1 specifically modifies chemotaxis of macrophages in the setting of pulmonary fibrosis. The underlying mechanism by which PAR-1 modifies chemotaxis remains elusive although a recent study elegantly shows that PAR-1 activation by thrombin on THP-1 cells leads to cytoskeletal remodeling and migration in a Gα12/Pyk2/RAC1/RhoA/Pak2-dependent signaling pathway.

Macrophages recruited to injured lung tissue contribute to the development of fibrosis by secreting the profibrotic cytokine TGF-β that, once activated, targets fibroblasts. Here we show that macrophage TGF-β induces fibroblast migration, differentiation and ECM deposition. Of note, macrophages-induced pro-fibrotic responses were inhibited by a TGF-β receptor blocking cocktail, resulting in inactivation of both TGFBR1 and TGFBR2. Interestingly however, all macrophage conditioned medium-induced pro-fibrotic responses were also partially inhibited by the specific PAR-1 inhibitor P1pal-12 which may suggest that PAR-1 directly regulates TGF-β receptor signaling. However, the fact that TGF-β still efficiently triggered TGF-β receptor dependent Smad2 phosphorylation in PAR-1 knock down cells suggests that PAR-1 is not required once TGF-β is activated. Most likely, PAR-1 contributes to TGF-β activation on fibroblasts and...
indeed thrombin-dependent PAR-1 cleavage leads to TGF-β activation on respiratory epithelial cells\textsuperscript{22}. Mechanistically, PAR-1 activation seems to induce RhoA-dependent actin polymerization with subsequent conformational changes in the αvβ6 integrin/latent TGF-β complex that allows the interaction between active TGF-β and its adjacent receptors\textsuperscript{23,24}. Additionally, blocking αvβ6 integrin signaling inhibited TGF-β activation during acute lung injury, confirming the importance of this PAR-1-dependent TGF-β activation pathway \textit{in vivo}\textsuperscript{22}.

Our data show that macrophage conditioned medium contains a PAR-1 ligand leading to PAR-1 dependent activation of latent TGF-β. Although thrombin is the best-known PAR-1 agonist, several other PAR-1 agonists, like MMP-1, MMP-13, KLK-1, KLK-4, KLK-6, FX and Granzyme K have been described\textsuperscript{25-31}. Of these potential PAR-1 agonists, we identified FXa as the most likely endogenous agonist secreted by macrophages (in line with a previous study\textsuperscript{32}) based on the notion that a specific FX inhibitor blocked macrophage conditioned medium-induced TGF-β signaling. This is particularly interesting as FX has already been implicated in the pathogenesis of pulmonary fibrosis by inducing TGF-β activation in a PAR-1 and integrin-dependent manner and FXa inhibition limited bleomycin-induced pulmonary fibrosis\textsuperscript{31}. In addition to FX, other PAR-1 agonists may also be able to contribute to TGF-β activation. Our data show that macrophages also express relatively high mRNA levels of Granzyme K, which may be also interesting as Granzyme K is known to induce pro-inflammatory cytokine secretion and lung fibroblast proliferation through PAR-1\textsuperscript{25}. Although all other potential PAR-1 agonists only showed low expression levels in RAW264.7 cells in our experiments, these proteases may obviously not be ruled out as key players in PAR-1 driven pulmonary fibrosis.

Another interesting finding of our study is that PAR-1 seems also to be important for TGF-β secretion by fibroblasts. Although fibroblasts produce relatively low levels of latent TGF-β as compared to macrophages, PAR-1 knock down fibroblasts produced significantly less latent TGF-β than PAR-1 expressing fibroblasts in unstimulated conditions, suggesting PAR-1 may actually be essential for fibroblasts to secrete latent TGF-β. Moreover, fibroblast stimulation by macrophages conditioned medium (containing PAR-1 agonists as discussed above) induced secretion of latent TGF-β in wild type but not PAR-1 knock down fibroblasts. Our data thus confirm and extent previous findings that activation of PARs may indeed lead to cytokine release and TGF-β production\textsuperscript{33-34}. The underlying mechanism by which PAR-1 induces TGF-β expression by fibroblasts and the potential significance of fibroblast TGF-β in pulmonary fibrosis remains to be elucidated.
CHAPTER 5

In conclusion, the present study demonstrate novel mechanisms by which PAR-1 stimulation on different cell types can contribute to the lung fibrotic process and therefore provided a more integrated understanding of PAR-1-mediated pathogenesis of pulmonary fibrosis (Figure 5C). We pinpoint macrophages as key players in PAR-1 dependent lung fibrosis development and suggest that macrophages secrete FXa that subsequently targets fibroblasts to enhance TGF-β driven fibrotic effects.
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