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

Citation for published version (APA):

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POTENTIAL NOVEL TARGETS:
Protease-Activated Receptors In Idiopathic Pulmonary Fibrosis

By
Cong Lin
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on Tuesday
2nd September
at 14:00 in the Agnietenkapel
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Followed by a reception
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Potential novel targets:
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The research described in this thesis was performed at the Center for Experimental and Molecular Medicine (CEMM) at the Academic Medical Center in Amsterdam, the Netherlands.

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Cover design Vivi and Eelco Roos
Layout Eelco Roos
Print Ridderprint

The print of this thesis was financially supported by the Academic Medical Center, Amsterdam, the Netherlands.

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Faculteit der Geneeskunde
To my parents
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Chapter 1

General introduction and outline of the thesis
General Introduction

Idiopathic pulmonary fibrosis (IPF) is the most frequent diffuse fibrosing lung disease with unknown cause of pulmonary fibrogenesis\textsuperscript{1, 2}. It was first described as “chronic pneumonitis” in the late 19\textsuperscript{th} century based on a vague clinical characterization of inflammatory exudation and fibrosis of the lung. Although several similar cases were documented during the following decades, there was no clear and definite classification of this rare pulmonary disease for many years. In 1944, a more detailed description of the clinical and pathological features of a disease referred as “acute diffuse interstitial fibrosis” was provided, which helped greatly on identification of further cases of IPF. Due to the increasing recognition of IPF, the understanding of the pathogenesis of IPF has greatly improved in the past decades. However, it did not remarkably prolong the survival of IPF patients\textsuperscript{3}. Possible targets for novel therapeutic interventions for IPF are thus eagerly awaited for. In this thesis, pharmacological inhibition of blood coagulation factor receptors for the treatment of pulmonary fibrosis is evaluated and based on the obtained results the underlying mechanisms by which the receptors may influence pulmonary fibrosis were studied.

**Idiopathic pulmonary fibrosis**

The understanding of processes underlying lung repair after injury are important to appreciate the development of pulmonary disease. The architecture and physiology of the lung is complex as evident from the fact that more than forty cell types are involved in the development and repair of the lung\textsuperscript{4}. Of these cell types, lung epithelial cells are key components as they form the structural barrier between the capillaries and the alveoli. Type I alveolar epithelial cells (AECI) are the most abundant pulmonary epithelial cells with attenuated cytoplasm and minimal thickness, facilitating gas exchange such as oxygen and carbon dioxide. Type II AECs (AEC II), which take up around 10\% of the alveolar surface area, are normally responsible for the production and secretion of surfactant but can also act as facultative progenitors as they are capable of replacing themselves and differentiating into AECI after injury\textsuperscript{4}. Under pathologic conditions AEC II cells are activated and promote fibroblast accumulate in injured areas where they differentiate into myofibroblasts that secrete collagen and other extracellular matrix (ECM) proteins.
IPF is a lethal disease, characterized by (myo)fibroblast proliferation and excessive ECM formation leading to destruction of the lung architecture. The lungs of IPF patients become stiff and lose their ability for gas exchange and patients usually suffer from progressively worsening cough and dyspnea caused by decreasing lung compliance. The prognosis of IPF is devastating with a median survival of 3 years after diagnosis and a mortality rate that exceeds many types of cancer. IPF occurs in middle-aged and older adults with an age range from 55–75. The prevalence of IPF rises in the world population and deaths attributed to IPF still continues to increase in the 21st century. There are approximately 14,000 persons diagnosed with IPF each year in the U.S and >5000 new cases to be expected in the U.K. Although the etiology of IPF remains elusive, several genetic and environmental risk factors predispose patients to IPF. For example, mutations in the TERT or TERC genes are causal genetic defects in over 15% of pulmonary fibrosis families. Other than genetic mutations, smoking, viral or bacterial infections, exposure to therapeutic or environmental toxins are also risk factors for IPF.

Although considerable effort has been made to develop pharmacologic therapies for IPF in order to meet the urgent needs of patients, the progress in patient care is modest. IPF was originally considered a chronic inflammatory response caused by lung injury and IPF patients were consequently treated with anti-inflammatory agents like prednisone or with cytotoxic agents such as azathioprine. Although these agents may help to improve symptoms and give patients pain relief to some extent, they have shown little clinical efficacy in IPF. The failure of anti-inflammatory therapy in diminishing IPF progression or delaying death boosted research for alternative antifibrotic therapies. Currently, more than 20 agents have been tested in clinical trials for the treatment of IPF only a few have completed phase 3 clinical trials. Of the four completed phase 3 trials, the ones with Bosentan and Ambrisentan were negative as the primary endpoints (delaying worsening of respiratory function and/or death) were not met, whereas the trials analyzing pirfenidone and nintedanib showed effective antifibrotic effects of these drugs in IPF patients. Both of these latter drugs were officially approved in 2014 by the U.S. Food and Drug Administration (FDA) for the treatment of IPF. Indeed, both pirfenidone and nintedanib treatment slow down the decline of lung function and reduce disease progression in patients with IPF. However, these drugs are associated with side effects and do not
stop nor reverse disease progression. Therefore, lung transplantation is still the most effective treatment for IPF, not only prolonging patient survival but also enhancing the quality of life. Importantly however, not all patients can undergo lung transplantation due to strict requirements for patients’ health condition and limited supply of donor lungs. Novel therapeutic targets and strategies are thus still eagerly awaited for throughout the world and such targets will only become available due to a comprehensive and integrated understanding of the pathogenesis of IPF.

**General mechanisms underlying IPF**

As already touched upon, IPF was originally thought to be the result of chronic inflammation in response to lung injury with unknown cause. However, a growing body of evidence obtained in the past years shows that inflammation is not a vital event in the pathogenesis of IPF\textsuperscript{21}. As mentioned above, anti-inflammatory therapies generally present a disappointing outcome in patients with IPF, which is in line with the recent notion that inflammation is hardly observed in histopathological samples of advanced IPF\textsuperscript{22}. In addition, clinical analysis of inflammatory markers did not correlate with disease severity in IPF patients\textsuperscript{21}. Furthermore, animal studies show that epithelial injury caused by repeated irritations of the lung is sufficient to induce pulmonary fibrosis in the absence of inflammatory responses\textsuperscript{23}.

More recent hypotheses suggest that the pathogenesis of IPF is independent of inflammation. Instead, it is proposed that wound repair signaling pathways triggered by repeated alveolar epithelial injury in some way become dysregulated and aberrant, leading to progressive and irreversible fibrosis over time\textsuperscript{3,5,24}. In the early stages after lung tissue damage, the coagulation cascade is activated thereby triggering platelet activation and the subsequent local secretion of soluble mediators resulting in increased vascular permeability and further damage to the basement membrane\textsuperscript{25,26}. Concurrently, injured epithelial or endothelial cells produce and release excessive inflammatory mediators, inducing the subsequent entry of leucocytes through the disrupted basement membrane (Figure 1). Recruited leucocytes such as macrophages, secrete profibrotic cytokines (e.g. TGF-β, IL-13 and IL-1)\textsuperscript{27,28} and they contribute to a series of events leading to myofibroblast accumulation. Myofibroblasts are highlighted as the main effector cell in fibrosis as they are the major source of ECM components, like collagen and fibronectin.
CHAPTER 1

Profibrotic cytokines together with other growth factors such as platelet-derived growth factor (PDGF) induce resident lung fibroblasts to proliferate, migrate and differentiate into myofibroblasts. Myofibroblasts may also be derived from either fibrocytes from the bone marrow that are attracted by CXCL12 to the injured site\textsuperscript{2,5,29}, or epithelial cells via epithelial–mesenchymal transition (EMT)\textsuperscript{24,30}. Finally, in the tissue remodeling phase, provisional ECM formation promotes wound healing and restores tissue structure. Fibrosis develops when wound repair processes keeps going on because of repeated injury or when any part of the process fails to function normally, changing the provisional ECM formation at the site of tissue injury into a permanent scar. Thus, the initiation and maintenance of fibrosis is a very complex process that involves many effector cells and mediators and identifying the critical contributors may provide new insight into treatment of IPF.

Figure 1. A general mechanism of the pathogenesis of pulmonary fibrosis. 1: After injury, epithelial cells release inflammatory mediators and activates the coagulation cascade, followed by leucocyte influx from capillaries into the alveolus through disrupted basement membrane. 2: Recruited leukocytes secrete profibrotic mediators leading to myofibroblasts accumulation by inducing resident lung fibroblasts and fibrocytes to differentiate into myofibroblasts and/or triggering epithelial cells to undergo EMT process. 3: Fibrosis is formed when tissue repair pathways are dysregulated, which leads to fibroblasts/myofibroblasts foci formation and excessive ECM components release.
General introduction

Coagulation and protease-activated receptors (PARs)

The coagulation cascade is responsible for fibrin formation at sites of damaged blood vessels thereby preventing blood loss. Coagulation is initiated instantly upon tissue injury and the coagulation pathway is triggered by the exposure of tissue factor (TF) to plasma Factor VII (FVIIa), facilitating the formation of the TF-FVIIa complex. This complex, either directly or via the intrinsic FIX/FVIII pathway, catalyzes the activation of factor X (FX) to FXa, which together with factor V (FVa) converts pro-thrombin to thrombin. Minute amounts of thrombin subsequently amplify the pathway via activation of FVIII and IX, generating more FXa and thrombin. Finally, thrombin turns soluble fibrinogen into insoluble fibrin strands, leading to a blood clot. Once formed, thrombin also induces a negative feed-back loop to prevent excessive coagulation. After binding to thrombomodulin, thrombin activates protein C (PC), which in turn inactivates factors Va and VIIIa thereby preventing further thrombin formation (Figure 2).

Figure 2. Schematic representation of the coagulation cascade. TF-FVIIa complex formation initiates coagulation through intrinsic and extrinsic pathways, leading to thrombin production and fibrin formation. Coagulation can either be amplified by thrombin via an amplification loop (dashed black lines) or down regulated by active protein C (APC) through inactivation of FVa and FVIIIa (dashed red lines).
Interestingly, coagulation proteinases also contribute to a striking range of pathophysiological functions independent of fibrin formation. The cellular effects of coagulation factors are mediated by protease-activated receptors (PARs), which are seven-transmembrane G protein-coupled receptors. As opposed to classical GPCRs that are activated by ligand binding, PARs are irreversibly activated by proteolytic cleavage. Protease binding to PARs and subsequent cleavage of the extracellular N-terminus reveals a novel tethered ligand that folds back over the receptor to trigger their transmembrane signaling to intracellular G proteins. To date, there are four known members of the PAR family of which PAR-1 was the first to be discovered in the early 1990s. After intensive studies centered on revealing how thrombin influences cellular responses, PAR-1 was originally identified on human platelets and termed “the cloned thrombin receptor” by Coughlin and co-workers. The second member of PAR family (i.e. PAR-2) was identified in 1994 by Nystedt, who cloned a mouse genomic DNA sequence encoding a protein that shares the identical structure and activation mechanism with PAR-1 but has a different tethered ligand pharmacology from PAR-1. Later on, PAR-3 and PAR-4, the other two thrombin receptors, were also identified by the same group which discovered PAR-1. All four PARs can be activated by individual coagulation factors but they have their distinct N-terminal cleavage sites and tethered ligand sequences which trigger different functional responses. Thrombin is a major activator of PAR-1, PAR-3, and PAR-4, whereas PAR-1 can also be activated by FXa and APC. As for PAR-2, it is the target of both FXa and the TF-FVIIa complex (Figure 2). In addition to direct interactions with receptors, thrombin is also capable of trans-activating PAR-2 by generating the tethered ligand of PAR-1, which in turn binds to adjacent PAR-2. Apart from proteinases, synthetic agonist peptides designed according to the sequence of the cleaved N-terminus can activate PARs in the absence of proteases-induced cleavage (SFLLRN for PAR-1, SLIGKV for PAR-2 and GYPGKF for PAR-4). PAR-3, however, cannot be activated by synthetic peptides and it appears that PAR-3 may act as a cofactor to enhance the affinity of PAR-4 for thrombin-mediated activation or PAR-3 may form heterodimers with PAR-1 to signal together, rather than signaling by itself.

Although blood coagulation factors are the archetypal proteases activating PARs, it is now well established that multiple other proteases can activate individual PARs with different affinity and triggering specific responses via biased agonist signaling. Besides thrombin, PAR-1 can for instance be activated by matrix
metalloproteinases, kallikreins and Granzymes. PAR-2 may also be activated by trypsin, and trypsin-like peptidases such as mast cell tryptase or matriptase.

**Coagulation and PARs in the pathogenesis of IPF**

Fibrin deposition is a key histological feature of IPF and coagulation activation in response to tissue injury is increasingly recognized to be a critical contributor in the pathogenesis of fibrotic lung disorders. Indeed, patients with IPF are more likely to have a hypercoagulable state and prothrombotic mutations are overrepresented in IPF patients as compared to the general population. In line, increased thrombin activity is considered as one of the characteristic features of pulmonary fibrosis. Gene expression and protein levels of coagulation factors, such as TF, FVII, FXa and thrombin, are increased in patients with progressive IPF and individual coagulation factors exert pro-fibrotic cellular effects through PARs. Thrombin, as the best-known profibrotic coagulation factor, activates PAR-1 leading to myofibroblast accumulation through activating resident lung fibroblasts, promoting epithelial-mesenchymal transition of lung epithelial cells and/or by inducing differentiation of fibrocytes. Thrombin is also a potent inducer of several profibrotic cytokines, including TGF-β, connective tissue growth factor and PDGF. Moreover, FXa induces pro-fibrotic effects of fibroblasts via PAR-2, resulting in the secretion of cytokines like monocyte chemoattractant protein (MCP)-1, interleukin (IL)-6 and TGF-β. Additionally, FXa induces myofibroblast differentiation and TGF-β activation in a PAR-1 dependent manner. More recently, FVIIa was shown to stimulate proliferation and ECM production of human lung fibroblasts via PAR-2 and these proliferative effects of FVIIa were considerably potentiated in the presence of TF, suggesting that the PAR-2/TF/FVIIa axis may contribute to the progression of IPF. Furthermore, FXIIa, a coagulation factor from the intrinsic pathway, strongly stimulates migration of human lung fibroblasts (HLF) derived from IPF patients. More interestingly, HLF from IPF patients exhibit an enhanced FXIIa-binding capacity, suggesting FXIIa contributes to fibrogenesis. The potential importance of coagulation factors in IPF is further underscored by the fact that inhibiting coagulation limits pulmonary fibrosis in preclinical experimental animal models.

In contrast to the pro-coagulant coagulation factors, the endogenous anticoagulant activated protein C (APC) exhibits a protective effect in lung injury. Endogenous APC inhibits infection-induced coagulation activation and APC over-
expression modifies neutrophil recruitment during experimental pneumococcal pneumonia\textsuperscript{57}. In addition, APC administration attenuates the expression of inflammatory mediators in acute lung injury induced by hyperoxia and protects mice against sepsis\textsuperscript{58,59}. Interestingly, decreased protein C activation is actually associated with the severity of IPF at diagnosis\textsuperscript{60} and APC treatment is proposed to protect mice from bleomycin-induced lung fibrosis\textsuperscript{61}.

**Anticoagulant treatment in IPF**

Due to the fact that the coagulation cascade seems to be instrumental for the etiology in IPF and anticoagulants have proven efficacious in lung fibrosis models, inhibition of the coagulation cascade may be a good strategy for the treatment of IPF. This hypothesis was first addressed in a trial with 56 IPF patients\textsuperscript{62}. In this study, patients received prednisolone alone or prednisolone in combination with anticoagulant therapy\textsuperscript{62}. The anticoagulants included oral warfarin (an anticoagulant that inhibits the vitamin K–dependent synthesis of thrombin, FVII, FIX, FX and protein C\textsuperscript{63}) in an outpatient setting and low-molecular-weight heparin for rehospitalized patients with severely progressive respiratory failure. Anticoagulant therapy showed a beneficial effect on survival in patients with IPF. However, the IPF population in this study was criticized not to be representative, because all patients were nonsmokers while IPF is strongly associated with smoking. In addition, it may contain a misclassification bias, which may have resulted in an overestimated efficacy of the anticoagulant therapy\textsuperscript{64}. Later on, heparin inhalation was tested in 20 IPF patients for safety only in an open-label exploratory pilot study. The treatment appeared to be safe and well tolerated in IPF patients, however, whether it will improve any disease related symptoms still needs to be investigated\textsuperscript{65}. Recently, a placebo-controlled trial of warfarin was performed including 145 IPF patients\textsuperscript{66}. In contrast to the aforementioned study\textsuperscript{62}, warfarin did not show a benefit in patients with progressive IPF. Instead, it increased the risk of mortality and hospitalization, which was not caused by major and/or minor bleeding complications. This finding was later supported by a retrospective cohort study, showing that warfarin deteriorated respiratory status and decreased survival of IPF patients\textsuperscript{67}. Although coagulation factors seem to play center roles in the pathogenesis of IPF, general inhibition of the coagulation cascade may not be an ideal approach to combat IPF. It may be better to target individual coagulation factors and/or their specific receptors modifying fibrotic disease.
Targeting PARs in fibrotic diseases

PAR-1 and PAR-2 are recently highlighted as potential targets for therapeutic interventions in treating several fibrotic disorders. Activated PARs mediate a number of pathophysiological pathways involved in inflammatory and fibrotic diseases of different organs throughout the body, including brain, lung, heart, liver, kidney and gastrointestinal tract. For example, PAR-1 receptor gene polymorphisms significantly influence the progression of liver fibrosis and the expression of PAR-1 is elevated during acute and chronic human liver injury, whereas PAR-2 activation leads to increased TGF-β production and induces a profibrogenic phenotype in human hepatic stellate cells. Moreover, PAR-2 regulates PAR-1-driven hyperplasia in response to arterial injury and overexpression of PAR-2 in mice induces cardiac fibrosis, inflammation and heart failure. In the context of brain diseases, PAR-1 mediates neurotoxicity induced by Granzyme B released from T cells during neuro-inflammatory disorders, whereas upregulation of PAR-2 has been observed during neuro-inflammation in the brain tissue from patients with HIV-1-associated dementia. During renal fibrotic disorders, PAR-1 activation by plasmin(ogen) triggers the induction of EMT, while PAR-2 deficiency reduces unilateral ureteral obstruction-induced renal tubular injury and fibrosis. Furthermore, both PAR-1 and PAR-2 modulate radiation-induced intestinal fibrosis. Next to vital organs in the body, PAR-1 and PAR-2 also mediate pro-inflammatory and profibrotic cellular events in important tissues, such as the skin and the joint.

With respect to lung injury and pulmonary fibrosis, accumulating evidence suggests that both PAR-1 and PAR-2 induce pro-inflammatory and profibrotic processes that aggravate disease progression. PAR-1 and PAR-2 are both widely expressed on many different pulmonary cell types like fibroblasts, macrophages, epithelial and endothelial cells. PAR-1 activation enhances inflammation in the pulmonary epithelium, by mediating macrophage/microcyte recruitment, excessive cytokine release and endothelial barrier disruption. PAR-1 also promotes profibrotic effects by facilitating TGF-β activation, inducing the differentiation of fibroblasts into myofibroblasts and stimulating ECM synthesis. In addition, PAR-1 seems to synergize with PAR-3 to mediate epithelial-mesenchymal transition of alveolar epithelial cells. The importance of these in vitro findings is emphasized by the fact that genetic ablation of PAR-1 limits bleomycin-induced acute lung inflammation and fibrosis, as evident from...
reduced total collagen levels in the lung in combination with decreased levels of proinflammatory and profibrotic mediators, such as TGF-β, IL-6 and MCP-1. More clinically relevant, PAR-1 expression is increased within fibroproliferative and inflammatory foci in IPF patients\textsuperscript{85}. Similar to PAR-1, PAR-2 also plays a crucial role in promoting pulmonary inflammatory and fibrotic responses. PAR-2 activation leads to endothelial barrier dysfunction and vascular permeability during acute lung injury\textsuperscript{86}. Activated PAR-2 also enhances inflammatory signaling in airway epithelial cells by increasing cytokine production, such as IL-8, and PAR-2 deficiency reduces allergic lung inflammation in mice\textsuperscript{87,88}. Moreover, PAR-2 triggers fibroproliferative responses such as proliferation, migration, differentiation into myofibroblasts and release of profibrogenic cytokines (e.g. TGF-β) in human and murine fibroblasts (Figure 3). The absence of PAR-2 affords protection from bleomycin-induced pulmonary fibrosis, as evident from a reduction in the extent and

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure3}
\caption{Schematic representation of pro-fibrotic effects of PARs in fibroblasts. After activation by proteases, PARs induce profibrotic cytokines (e.g. TGF-β) release and activation. PARs also contribute to fibroblast migration, proliferation and ECM production.}
\end{figure}
### Table 1. Application of antagonists of PARs in different fibrotic or inflammatory disorders.

<table>
<thead>
<tr>
<th>PARs</th>
<th>Antagonists</th>
<th>Organs/Tissue</th>
<th>Models</th>
<th>Key results</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAR-1</td>
<td>SCH79797</td>
<td>Heart&lt;sup&gt;90&lt;/sup&gt;</td>
<td>Ischemia-reperfusion injury</td>
<td>Limited left ventricular (LV) dilatation and improved LV systolic function of the reperfused myocardium</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kidney&lt;sup&gt;92&lt;/sup&gt;</td>
<td>Ischemia-reperfusion injury</td>
<td>Significantly attenuated kidney damage by improving serum creatinine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intestine&lt;sup&gt;94&lt;/sup&gt;</td>
<td>Ischemia-reperfusion injury</td>
<td>Intestinal myeloperoxidase and adhesion molecule expression were significantly decreased</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Brain&lt;sup&gt;95&lt;/sup&gt;</td>
<td>Surgically induced brain injury (SBI)</td>
<td>Reduced secondary brain injury by decreasing both brain edema and apoptosis</td>
</tr>
<tr>
<td></td>
<td>SCH602539</td>
<td>Intestine&lt;sup&gt;93&lt;/sup&gt;</td>
<td>Chronic intestinal radiation fibrosis</td>
<td>Markedly reduced early intestinal radiation injury, but had no effect on the level of delayed intestinal radiation fibrosis</td>
</tr>
<tr>
<td></td>
<td>P1pal-12S</td>
<td>Lung&lt;sup&gt;96&lt;/sup&gt;</td>
<td>CLP-induced sepsis</td>
<td>Early administration significantly increased survival rate</td>
</tr>
<tr>
<td></td>
<td>RWJ-56110</td>
<td>Liver&lt;sup&gt;96&lt;/sup&gt;</td>
<td>Bile duct ligation-induced fibrosis</td>
<td>Reduced liver type I collagen mRNA and protein expression, as well as hepatic and urinary excretion of hydroxyproline</td>
</tr>
<tr>
<td>PAR-2</td>
<td>NDGA</td>
<td>Skin&lt;sup&gt;101&lt;/sup&gt;</td>
<td>Oxazolone-induced atopic dermatitis</td>
<td>Rebuilt skin barrier and increased transepidermal water loss recovery</td>
</tr>
<tr>
<td></td>
<td>GB88</td>
<td>Joint&lt;sup&gt;97&lt;/sup&gt;</td>
<td>Collagen-induced arthritis</td>
<td>Reduced pathological and histopathological changes (i.e. edema, macrophage invasion, mast cell degranulation, etc.)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Colon&lt;sup&gt;98&lt;/sup&gt;</td>
<td>PAR2 agonist or 2,4,6-trinitrobenzenesulfonic acid-induced Colitis</td>
<td>Reduced mortality and pathology (including colon obstruction, ulceration, wall thickness, and myeloperoxidase release)</td>
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<tr>
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<td>ENMD-1068</td>
<td>Joint&lt;sup&gt;99&lt;/sup&gt;</td>
<td>Intra-articular carrageenan/kaolin injection induced joint swelling</td>
<td>Dose dependently attenuated joint inflammation</td>
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<tr>
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<td>FSLLRY-amide</td>
<td>Lung&lt;sup&gt;102&lt;/sup&gt;</td>
<td>Monocrotaline induced pulmonary hypertension (PH)</td>
<td>Reversed established PH in hypoxia–exposed mice</td>
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<tr>
<td></td>
<td>P2pal-18S</td>
<td>Pancreas&lt;sup&gt;100&lt;/sup&gt;</td>
<td>Retrograde intraductal bile acid infusion induced biliary pancreatitis</td>
<td>Reduces the severity of biliary pancreatitis</td>
</tr>
</tbody>
</table>
severity of fibrotic lesions and diminished collagen expression\textsuperscript{89}. Further, PAR-2 mRNA and protein levels are elevated in lungs of IPF patients as compared to healthy donor lungs and the increased PAR-2 expression is associated with hyperplastic alveolar type II cells and fibroblasts/myofibroblasts\textsuperscript{46}. Noteworthy, unlike PAR-1, which expression is already relatively high in normal lung fibroblasts, PAR-2 expression is low in quiescent lung fibroblasts but may considerably increase under inflammatory and fibrotic conditions\textsuperscript{90}.

It is thus tempting to speculate that inhibition of PARs may be a promising therapeutic option in the treatment of IPF. In recent years, pharmacological inhibition of PAR-1 or PAR-2 has proven efficacious in different inflammatory and fibrotic preclinical models (Table 1). PAR-1 inhibition with SCH79797 protects mice from ischemia-reperfusion induced heart, kidney and intestine injury\textsuperscript{91-94}, as well as surgically induced brain injury\textsuperscript{95}. Blocking PAR-1 with pepducin-based inhibitors at early time points of cecal ligation and puncture (CLP)-induced sepsis significantly prolonged survival\textsuperscript{38}. Furthermore, PAR-1 inhibition with RWJ-56110 limits experimental liver fibrosis, as evident from reduced collagen production in the liver\textsuperscript{96}. Compared to PAR-1, pharmacological inhibition of PAR-2 is more widely applied in inflammatory models. Targeting PAR-2 ameliorated pathological changes in inflammatory colon and joint diseases\textsuperscript{97-99}. Moreover, PAR-2 inhibition attenuated the severity of experimental biliary pancreatitis and blocked PAR2-mediated inflammatory signaling pathways in atopic dermatitis\textsuperscript{100-101}. Additionally, PAR-2 inhibition by FSLRRY-NH\textsubscript{2} reduced the number of fully muscularized vessels and reversed established pulmonary hypertension in a hypoxia mouse model\textsuperscript{102}.

Overall, given the critical role of PARs in mediating pro-inflammatory and pro-fibrotic responses on different cell types, pharmacological PAR-1 and PAR-2 inhibition may benefit a broad range of (PAR-dependent) disorders, like among others IPF.
Aim and outline of this thesis

This thesis aims to explore the efficacy of pharmacologically targeting PAR-1 and PAR-2 in experimental lung fibrosis and intends to obtain more insight on the pathogenesis of pulmonary fibrosis. We show that specific pepducin-based inhibitors of PAR-1 (Chapter 2) and PAR-2 (Chapter 3) are able to limit lung fibrosis when administered before and after induction of fibrosis by bleomycin instillation. After showing that both PAR-1 and PAR-2 inhibition limits pulmonary fibrosis, we hypothesized that the simultaneous inhibition of PAR-1 and PAR-2 would be superior to targeting either receptor alone in pulmonary fibrosis. However, the experiments described in Chapter 4 refute this hypothesis and instead show that the pro-fibrotic effects induced by PAR-1 require the presence of PAR-2. Next, we sought to provide mechanistic explanations for observations obtained in chapter 2 and we identified novel mechanisms by which PAR-1 stimulation on different cell types can contribute to pulmonary fibrosis (Chapter 5). Finally, in Chapter 6 we study the role of the endogenous anticoagulant factor APC in bleomycin-induced pulmonary fibrosis. We end the thesis with Chapter 7, a summary and general discussion chapter providing the overall conclusions and profound implications derived from this thesis.
Reference


CHAPTER 1


CHAPTER 1


Chapter 2

Targeting protease activated receptor (PAR)-1 with P1pal-12 limits bleomycin-induced pulmonary fibrosis

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ABSTRACT

Background

Idiopathic pulmonary fibrosis is the most devastating fibrotic diffuse parenchymal lung disease which remains refractory to pharmacological therapies. Therefore, novel treatments are urgently required. Protease-activated receptor (PAR)-1 is a G protein-coupled receptor that mediates critical signaling pathways in pathology and physiology. Bleomycin-induced lung fibrosis has been shown to be diminished in PAR-1 deficient mice. The purpose of this study is to investigate whether pharmacological PAR-1 inhibition is a potential therapeutic option to combat pulmonary fibrosis.

Methods

Pulmonary fibrosis was induced by intranasal instillation of bleomycin into wild-type mice with or without a specific PAR-1 antagonist (i.e. P1pal-12, a pepducin that blocks the PAR-1/G-protein interaction). Fibrosis was assessed by hydroxyproline analysis, immunohistochemistry, q-PCR and western blot for fibrotic markers expression.

Results

We first show that P1pal-12 effectively inhibits PAR-1 induced pro-fibrotic responses in fibroblasts. Next, we show that once daily treatment with 0.5, 2.5 or 10 mg/kg P1pal-12 reduced the severity and extent of fibrotic lesions in a dose-dependent manner. These findings correlated with significant decreases in fibronectin, collagen and α-SMA expression on both the mRNA and protein level in treated mice. To further establish the potential clinical applicability of PAR-1 inhibition, we analyzed fibrosis in mice treated with P1pal-12 1 or 7 days after bleomycin instillation. Interestingly, when administered 7 days after the induction of fibrosis, P1pal-12 was as effective in limiting the development of pulmonary fibrosis as when administration was started before bleomycin instillation.

Conclusions

Overall, targeting PAR-1 may be a promising treatment for pulmonary fibrosis.
INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is the most frequent diffuse fibrosing lung disease of unknown etiology.\cite{1,2} The prognosis of IPF is devastating with a 20-30% survival rate at 3-5 years after diagnosis and a mortality rate that exceeds many types of cancer. Since treatment approaches for IPF are limited, considerable effort has been made to reveal the underlying mechanisms allowing novel treatments to prolong survival.\cite{2} The molecular mechanisms underlying the pathogenesis of IPF are far from understood. However, current paradigms postulate that the development of fibrosis involves repeated epithelial cell injury leading to aberrant wound healing responses. Consistently, a hallmark of IPF is the presence of fibroblastic foci with differentiated fibroblasts which show myofibroblast phenotypes and secrete extracellular matrix (ECM) proteins that form depositions which subsequently establish fibrotic lesions.\cite{1-3}

Protease activated receptors (PARs) are seven transmembrane domain receptors that belong to the family of G-protein-coupled receptors (GPCRs).\cite{4} In contrast to other GPCRs, PAR-1 activation involves proteolytic cleavage of the receptor by serine proteases. PAR-1 was originally identified on human platelets and its best-known agonist is thrombin,\cite{5} although other ligands like FXa and certain matrix metalloproteinases have been described.\cite{6,7} Proteolytic activation of PAR-1 reveals a tethered ligand with a new N-terminus. Once irreversibly activated, PAR-1 initiates the recruitment of G\(\alpha\)-subunits, Gq, Gi and G\(_{12/13}\). Together with G\(_{\beta\gamma}\) they trigger several downstream signaling events, which contribute to a striking range of pathophysiological functions.\cite{5,8-9} In addition to proteinases, PAR-1 can be activated by synthetic agonist peptides, which are designed according to the sequence of the cleaved N-terminus.

Accumulating evidence indicates that PAR-1 induces multiple processes that may promote pulmonary fibrosis. PAR-1 modulates mitogenesis and angiogenesis, alters lung vascular permeability, stimulates fibroblasts migration, proliferation, ECM synthesis, and enhances inflammation in the pulmonary epithelium.\cite{10-13} In line, PAR-1 deficient mice are protected from bleomycin-induced acute lung inflammation and pulmonary fibrosis.\cite{14} The attenuated fibrotic response in PAR-1 deficient mice was associated with a reduction in the total collagen content in the lung and with decreased levels of proinflammatory and profibrotic mediators like interleukin (IL)-6 and monocyte chemoattractant protein-1 (MCP-1). Furthermore, PAR-1 expression is highly increased within fibroproliferative and inflammatory foci in fibrotic human lung. Overall, PAR-1 may thus have a critical contribution in promoting pulmonary fibrosis and PAR-1 may be a promising target to combat the development and progression of IPF.
In recent years, several PAR-1 antagonists have been designed; among which pepducins seem to be most promising. P1pal-12 is a cell-penetrating pepducin derived from the third intracellular loop of PAR-1. Once inserted into the plasma membrane it is delivered to the PAR-1 intracellular surface, thereby interfering with the receptor/G-proteins interaction.\(^1\^) P1pal-12 was initially described to inhibit PAR-1-driven calcium fluxes and platelet aggregation.\(^1\^,\(^2\^) In addition, P1pal-12 has been shown to block PAR-1-mediated ERK activation \textit{in vitro}.\(^1\^) More importantly, PAR-1 specific pepducins also showed promising \textit{in vivo} effects. Indeed, treatment with a PAR-1 specific pepducin significantly attenuated the growth of mice xenograft breast tumors,\(^1\^) whereas the same pepducin provided remarkable inhibition of lung tumor growth in nude mice.\(^2\^) P1pal-12 thus seems a promising PAR-1 inhibitor for preclinical experiments.

Here, we hypothesized that targeting PAR-1 with P1pal-12 may limit the development and progression of pulmonary fibrosis and tested this hypothesis using a well-established murine model of bleomycin-induced pulmonary fibrosis.

**MATERIALS AND METHODS**

**Cells and reagents**

Mouse embryonic NIH3T3 fibroblasts (ATCC, Manassas, VA; CRL-1658) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS). Cells were grown at 37°C in an atmosphere of 5% CO\(_2\). Unless indicated otherwise, cells were washed twice with PBS and serum-starved for 4 hours before stimulation. Thrombin was from Sigma (St-Louis, MO), whereas PAR-1 agonist peptide (PAR-1-AP; H-SFLLRN-NH\(_2\)) and P1pal-12 (palmitate-RCLSSSAVANRSP-NH\(_2\)) were from GL Biochem Ltd (Shanghai, China).

**Western Blot**

Western blots were performed essentially as described before.\(^2\) For details, see the supplementary method section.

**Wound Scratch assay**

Scratch assays were performed essentially as described before.\(^2\) For details, see the supplementary method section.
Animal Model of Pulmonary Fibrosis

Ten-week-old wild-type C57Bl/6 mice were purchased from Charles River (Someren, Netherlands). All procedures were performed in accordance with the Institutional Standards for Humane Care and Use of Laboratory Animals. Experiments were approved by the Animal Care and Use Committee of the Academic Medical Center Amsterdam.

Bleomycin (Sigma, St-Louis) was administered intranasally (1 mg/kg) under anesthesia. In the dose finding experiment, animals were instilled with 0.5, 2.5 or 10 mg/kg P1pal-12 30 minutes before bleomycin administration and subsequently once daily until the end of the experiment. In the delayed treatment experiment, mice were treated once daily with 2.5 mg/kg P1pal-12 starting one or seven-days after bleomycin instillation. In both experiments, 6% DMSO in saline was administered as solvent control. Mice were sacrificed 14 days after bleomycin instillation, after which one lung was taken for histology and one was homogenized.

Cytokine/Chemokine Assays

TGF-β1 was measured with the Mouse TGF-beta 1 DuoSet kit (R&D systems, UK). IL-6 and MCP-1 were measured using the BD Cytometric Bead Array Mouse Inflammation Kit (BD, NJ, USA) following the manufacturer’s instructions. Detection limits were 2.5 pg/ml for IL-6 and 20 pg/ml for MCP-1.

Hydroxyproline Assay

Right lungs were homogenized after which samples were processed for hydroxyproline content analysis using the hydroxyproline assay kit (Sigma, Netherlands) as per the manufacturer’s instructions. For details, see the supplementary method section.

(Immun{o})Histological Analysis

Histological examination and Ashcroft score were performed as described before.22 Smooth muscle actin (α-SMA) staining was graded in a blinded fashion on a scale from 0 to 3 as described before.23 Pictures of collagen staining 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 before.24,25 For details, see the supplementary method section.

Quantitative PCR

mRNA expression levels were quantified by real-time PCR as indicated in the supplementary method section.
Statistics
Statistical analyses were conducted using GraphPad Prism (GraphPad software, San Diego). Comparisons between conditions were analyzed using two tailed unpaired t-tests for normally distributed data, otherwise Mann-Whitney analysis was performed. \( P<0.05 \) was considered significant.

RESULTS
P1pal-12 inhibits PAR-1-mediated signaling pathways in fibroblasts
Several studies have shown that PAR-1 activation leads, among others, to extracellular regulated kinase (ERK) phosphorylation, which is an important signaling pathway regulating fibroblast proliferation and migration.26 Pepducins have been shown to efficiently inhibit PAR-1-driven ERK phosphorylation in cancer cells.20 Importantly however, pepducins may act in a cell type dependent manner.27 Therefore, we assessed whether P1pal-12 also inhibits PAR-1-driven ERK activation in fibroblasts. As shown in Figure 1A, treatment of fibroblasts with 100 μM PAR-1-AP resulted in ERK1/2 phosphorylation. This PAR-1-AP-induced ERK phosphorylation was completely inhibited in 10 μM P1pal-12-pretreated cells. P1pal-12 thus effectively antagonizes PAR-1-mediated signaling in murine fibroblasts.

P1pal-12 inhibits PAR-1-dependent fibroblast differentiation, proliferation and migration
PAR-1 activation on fibroblasts has been demonstrated to induce several profibrotic processes like fibroblast migration, proliferation and differentiation.6,28 Consequently, we assessed whether P1pal-12 also antagonizes these PAR-1-driven fibroproliferative responses. As shown in Figure 1B, both PAR-1-AP (100 μM) and thrombin (1 U/ml) induced fibroblast differentiation and ECM synthesis as evident from increased \( \alpha \)-SMA (hallmark of fibroblast differentiation) and collagen expression. This increased \( \alpha \)-SMA and collagen expression was clearly down-regulated by the treatment with 10 μM P1pal-12. Importantly, TGFβ-induced collagen production is independent from P1pal-12 treatment (Supplementary Figure 1). Furthermore, we observed that PAR-1 stimulation induced fibroblast migration/proliferation in wound scratch assays, as evident from significantly smaller wound sizes (70-80%). Again, P1pal-12 treatment inhibited PAR-1-AP-induced wound closure (Figure 1C-D). Overall these in vitro experiments show that P1pal-12 effectively blocks PAR-1-induced profibrotic effects of fibroblasts.
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Figure 1. P1pal-12 inhibits PAR-1 induced pro-fibrotic responses in fibroblasts. (A) Western blot analysis of ERK phosphorylation in NIH3T3 cells after stimulation with 100 μM PAR-1-AP in the absence or presence of P1pal-12 (10 μM). P1pal-12 was added 30 minutes before PAR-1-AP stimulation. Total ERK served as loading control. (B) Western blot analysis of α-SMA and collagen expression in NIH3T3 cells 24 hours after stimulation with thrombin (1 U/ml) or 100 μM PAR-1-AP in the absence or presence of P1pal-12 (10 μM). P1pal-12 was added 30 minutes before thrombin or PAR-1-AP stimulation. β-actin served as a loading control. (C) Wound size of NIH3T3 fibroblast monolayers after treatment with either DMSO (control) or PAR-1-AP (100 μM) for 18 hours in the presence or absence of P1pal-12. Cells were pre-incubated with 10 μM P1pal-12 for 30 min as indicated. Shown are photographs of representative microscopic fields. (D) Quantification of the results depicted in (C) as described in the Materials and Methods section. Data are expressed as mean±SEM (n=6). *** P<0.001.
P1pal-12 dose-dependently limits the development of pulmonary fibrosis in a murine bleomycin model

We next examined whether P1pal-12 limits fibrosis in a murine model of bleomycin-induced pulmonary fibrosis. To this end, mice were intranasally instilled with different concentrations of P1pal-12, after which the extent and severity of fibrosis was determined. As shown in Figure 2A-B, bleomycin instillation induced extensive patchy fibrotic foci accompanied by a marked accumulation of inflammatory cells and increased deposition of ECM. Low dose P1pal-12 treatment (0.5 mg/kg, Figure 2C) marginally reduced bleomycin-induced fibrosis (about 10%) whereas intermediate (2.5 mg/kg) and high (10 mg/kg) P1pal-12 doses significantly reduced the severity of regional interstitial fibrosis (approximately 25% and 20%) and diminished the destruction of alveolar units (compare figures 2D-E with 2B). Quantification of the bleomycin-induced histological changes using the Ashcroft score shows that P1pal-12 treatment results in less severe fibrotic lesions (Figure 2F).

It is well recognized that accumulation of α-SMA and ECM proteins (like collagen and fibronectin) can lead to organ-destructive remodeling, which also occur in fibrotic foci and are considered as hallmarks of IPF. To substantiate that P1pal-12 limits experimental fibrosis, we next analyzed α-SMA expression immunohistochemically. Dramatic α-SMA expression was seen in focal fibrotic lesions after bleomycin instillation (compare Figure 2G and H). Low dose P1pal-12 treatment did marginally reduce α-SMA expression, whereas intermediate P1pal-12 doses significantly reduced (about 40%) α-SMA levels (Figure 2I-J). Noteworthy, although the 10 mg/kg P1pal-12 dose also strongly reduced α-SMA levels; it did not reach statistical significance (Figure 2K-L).

To confirm the inhibitory effect of P1pal-12 on bleomycin-induced fibrosis, we analyzed collagen accumulation in the lung. Collagen levels in lung homogenates largely increased after bleomycin instillation. As shown in Figure 3A-B, 2.5 and 10 mg/kg P1pal-12 administration considerably reduced collagen deposition in the lungs. In contrast, mice treated with 0.5 mg/kg P1pal-12 showed similar collagen deposition as mice not treated with P1pal-12. In line, Masson-trichrome and collagen stainings showed high collagen levels in bleomycin instilled mice, which were significantly reduced in mice treated with 2.5 or 10 mg/kg P1pal-12 (Figure 3C-D). Finally, we confirmed these observations by analyzing hydroxyproline content in right lung homogenates. As shown in Figure 3E, collagen level (calculated according to the hydroxyproline content) increased almost 3 fold in bleomycin treated mice compared with solvent controls. Importantly, collagen
Figure 2. P1pal-12 treatment affords protection against bleomycin-induced pulmonary fibrosis. (A-E, ×100) Representative H&E stained lung tissue sections obtained 14 days after saline (A) or bleomycin instillation in mice treated with 0 mg/kg (B), 0.5 mg/kg (C), 2.5 mg/kg (D), or 10 mg/kg P1pal-12 (E). (F) Quantification of pulmonary fibrosis using the Ashcroft score in control mice and mice treated with P1pal-12. Data are expressed as mean±SEM (n=8 per group). (G-K, ×100) Representative pictures of α-SMA deposition in lung tissue sections obtained 14 days after saline (G) or bleomycin instillation of untreated (H) mice and mice treated with 0.5 mg/kg (I), 2.5 mg/kg (J) or 10 mg/kg (K) P1pal-12. (L) Quantification of α-SMA deposition as depicted in panels G-K. Data are expressed as mean±SEM (n=8 per group). * P<0.05. Dashed lines in the quantification figure represent solvent-treated controls.
levels decreased 24.9±7.8%, 36.4±4.5% and 29.7±3.5% after treatment with 0.5, 2.5 and 10 mg/kg P1pal-12 respectively.

Next, we analyzed mRNA expression levels of α-SMA, collagen and fibronectin in the lungs. All these genes were highly expressed in response to bleomycin treatment and high as well as intermediate doses of P1pal-12 treatment strongly reduced their expression (Figure 4A-C). Low dose P1pal-12 treatment did not inhibit bleomycin-induced profibrotic gene expression.
Finally, we assessed TGF-β1 levels in lung homogenates as TGF-β1 is one of the most typical profibrotic mediators and is frequently over-expressed in fibrotic diseases. TGF-β1 levels increased in bleomycin-instilled untreated mice (two-fold increase, Figure 4D) compared with saline treated mice. The 2.5 mg/kg dose of P1pal-12 attenuated the TGF-β1 increase by about 50%. IL-6 and MCP-1 levels were also increased in bleomycin-instilled mice and again treatment with 2.5 mg/kg P1pal-12 significantly attenuated these increases by 65±3% and 36±3% respectively. Treatment with 10 mg/kg P1pal-12 also led to a decrease in these cytokine levels, although it failed to reach statistical significance (Figure 4E-F).

Overall, P1pal-12 limits bleomycin-induced pulmonary fibrosis, and the most significant effects were reached at the dose of 2.5 mg/kg. Therefore, we selected this intermediate dose for our subsequent studies.

**Figure 4. P1pal-12 treatment reduces bleomycin-induced pro-fibrotic gene expression and cytokine increases.** Expression of α-SMA (A), collagen (B) and fibronectin (C) mRNA levels in lung homogenates obtained 14 days after saline or bleomycin instillation in mice treated with 0-10 mg/kg P1pal-12 was assessed by real-time RT-PCR. Data are expressed relative to the amount of input RNA. TGF-β1 (D), IL-6 (E) and MCP-1 (F) levels in lung homogenates obtained 14 days after saline or bleomycin instillation in mice treated with 0-10 mg/kg P1pal-12. Data are expressed as mean±SEM (n=8 per group). * P<0.05, ** P<0.01. Dashed lines represent solvent-treated controls.
CHAPTER 2

Delayed treatment with P1pal-12 effectively limits pulmonary fibrosis progression in a murine bleomycin model of pulmonary fibrosis

After having established that long-term treatment with P1pal-12 effectively limits pulmonary fibrosis, we next investigated whether delayed treatment starting after the initiation of fibrosis would still limit pulmonary fibrosis. Daily P1pal-12 (2.5mg/kg) administration was started either 1 (inflammatory phase) or 7 (fibrotic phase) days after bleomycin instillation. As shown in Figure 5A-C, lungs from mice not treated with P1pal-12 again showed severe fibrotic lesions induced by bleomycin treatment (more than fourfold increase compared with saline control). Interestingly, delayed P1pal-12 treatment reduced bleomycin-induced lung damage as evident from lower Ashcroft scores. However, scores of the one-day delayed-treatment group did not reach

Figure 5. Delayed P1pal-12 treatment effectively attenuates bleomycin-induced pulmonary fibrosis. (A-C) Representative H&E stained lung tissue sections obtained 14 days after bleomycin instillation in untreated mice (A) and mice treated with 2.5 mg/kg P1pal-12 from day 1 (B) or day 7 (C) after bleomycin instillation. (D) Quantification of pulmonary fibrosis using the Ashcroft score in untreated mice and mice treated with P1pal-12. Data are expressed as mean±SEM (n=8 per group). (E-G) Representative pictures of α-SMA deposition in lung tissue sections obtained 14 days after bleomycin instillation of untreated (E) mice and mice treated with 2.5 mg/kg P1pal-12 from day 1 (F) or day 7 (G) after bleomycin instillation. (H) Quantification of α-SMA deposition as depicted in panels E-G. Data are expressed as mean±SEM (n=8 per group). * P<0.05. Dashed line in the quantification figure represents solvent-treated controls.
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Figure 6. Delayed P1pal-12 treatment attenuates bleomycin-induced collagen deposition and reduces gene expression of fibrotic markers. (A) Western blot analysis of collagen expression in lung homogenates obtained 14 days after bleomycin instillation in untreated mice and mice treated with 2.5 mg/kg P1pal-12 from day 1 or day 7 after bleomycin instillation. GAPDH served as a loading control. Shown are three representative samples per condition from a group of 8. (B) Quantification of the results depicted in (A). (C) Representative pictures of Masson-trichrome (×100) and collagen stained lung tissue sections (×200) obtained 14 days after bleomycin instillation in untreated mice and mice treated with 2.5 mg/kg P1pal-12 from day 1 or day 7 after bleomycin instillation. (D) Quantification of collagen immunostaining (semi-quantitative image analysis). (E) Collagen content in lung homogenates obtained 14 days after saline or bleomycin instillation in mice treated with different doses of P1pal-12. Expression of α-SMA (F), collagen (G) and fibronectin (H) mRNA levels in lung homogenates obtained 14 days after bleomycin instillation in untreated mice and mice treated with 2.5 mg/kg P1pal-12 from day 1 or day 7 post-bleomycin instillation as assessed by real-time RT-PCR. Data are expressed relative to the amount of input RNA. Data are expressed as mean±SEM (n=8 per group; * P<0.05, ** P<0.01, *** P<0.001). Dashed lines in the quantification figures represent solvent-treated controls.
statistical significance (Figure 5D). Consistently, $\alpha$-SMA expression levels were increased in bleomycin instilled lungs and these levels were significantly reduced in the 1- and 7-day delayed treatment groups by 27±3.8% and 32±8.0% respectively (Figure 5E-H).

Next, we determined pulmonary collagen levels and both 1- and 7-days delayed P1pal-12 treatment clearly reduced the expression of collagen (Figure 6A-B). However, again, the effect of 1-day delayed treatment did not reach statistical significance. The decrease in collagen deposition by delayed P1pal-12 treatment was confirmed using collagen immunohistochemistry analysis and Masson-trichrome staining (Figure 6C-D). In line, collagen content of bleomycin instilled lungs increased by more than 4 fold compared to saline control and this increase was attenuated by 34.5±3.8% and 40.7±3.7% in the 1- and 7-day delayed treatment groups (Figure 6E). Moreover, the decreases of $\alpha$-SMA and collagen expression after delayed-treatment were confirmed on the mRNA level (Figure 6F-H), whereas fibronectin mRNA levels were also reduced after delayed treatment as compared to P1pal-12 untreated controls (Figure 6H).

Overall, delayed treatment with P1pal-12 effectively limits the progression of bleomycin-induced pulmonary fibrosis. It is noteworthy however, that P1pal-12 treatment starting 7 days after bleomycin instillation seems to be more effective compared to one-day delayed-treatment.

**DISCUSSION**

IPF is the end result of a heterogeneous group of disorders with a devastating prognosis and very few therapeutic options.\(^1\) Interestingly, PAR-1 has been proven to play an important role in mediating pro-inflammatory and pro-fibrotic effects *in vitro*, and preclinical experimental animal data showed that PAR-1 drives the progression of pulmonary fibrosis as mice that lack PAR-1 were protected against bleomycin-induced pulmonary fibrosis.\(^14\) In this study, we assessed whether treatment with a PAR-1 inhibitor limits experimental pulmonary fibrosis, and we show that pharmacological inhibition of PAR-1 with P1pal-12 indeed effectively limits pulmonary fibrosis in the murine bleomycin model. More specifically, we first show that P1pal-12 effectively blocks PAR-1 pro-fibrotic effects in fibroblasts. Pre-incubation with P1pal-12 before PAR-1-AP or thrombin stimulation blocked ERK activation, diminished $\alpha$-SMA expression and reduced proliferation/migration. Importantly, we were able to confirm these data in *in vivo* experiments by showing that bleomycin-induced fibrosis was significantly
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In mice treated with both 2.5 and 10 mg/kg P1pal-12 administered once daily. Both histological hallmarks of fibrosis and pro-fibrotic protein and gene expression were significantly reduced after P1pal-12 treatment. Interestingly, finally we show that P1pal-12 still effectively limits pulmonary fibrosis when administration started 7 days after the induction of fibrosis by bleomycin instillation.

Interestingly, the observed effects of P1pal-12 are comparable to those observed in PAR-1 deficient animals, which confirms the efficacy of P1pal-12. In both PAR-1 deficient and P1pal-12 treated mice, the severity of the fibrotic lesions was reduced by around 1 point in the Ashcroft score as compared to untreated control mice. Moreover, collagen accumulation in the lung was reduced by around 55% in PAR-1 deficient mice, whereas P1pal-12 treatment reduced collagen deposition by approximately 40%. Finally, MCP-1 and TGF-β1 levels were increased in response to bleomycin instillation and these increases were diminished by 35% and 50% in PAR-1 deficient mice and by 36% and 50% in P1pal-12 treated animals for MCP-1 and TGF-β1 respectively. Overall, P1pal-12 treatment thus efficiently blocks PAR-1 driven pulmonary fibrosis.

Obviously, the fact that P1pal12 limits pulmonary fibrosis as efficiently as PAR-1 deficiency is scientifically interesting. However, more clinically relevant is the observation that when P1pal-12 treatment was started 7 days after the induction of fibrosis, it still significantly reduced fibrosis. The reductions in Ashcroft score, collagen deposition and profibrotic gene expression were similar to those observed in mice in which treatment was started before the induction of fibrosis. Inhibition of PAR-1 may thus be a promising therapeutic strategy for treating fibrosis, although future clinical studies are required to confirm this notion.

Two interesting findings of our study are that 2.5 mg/kg P1pal-12 seemed more effective in reducing fibrosis than the 10 mg/kg dose, and that initiation of treatment 7 days after bleomycin instillation was more effective than when treatment was started one day after the induction of fibrosis. Although we do not have a conclusive explanation for these surprising findings, it is tempting to speculate that the different dose- and time-dependent effects are related to the dual role of PAR-1 in inflammation and fibrosis. In pulmonary fibrosis, PAR-1 is mainly considered to trigger inflammatory responses; however recent data suggest that PAR-1 also exerts anti-inflammatory functions. Therefore, the extent and/or timing of PAR-1 inhibition may influence the balance of its anti-inflammatory and pro-inflammatory properties. Alternatively, the slight differ-
ences observed may merely reflect the variability/inaccuracy associated with the analysis rather than any definable biological phenomenon.

When interpreting our data, several issues should be taken into consideration. First, although the bleomycin model is the best available model to study pulmonary fibrosis, it does not completely mimic the progression of fibrosis in IPF patients. For example, the spontaneous resolution of fibrosis in this model fails to represent the irreversibility seen in IPF patients. In addition, fibrosis develops fast in the bleomycin model, whereas it actually takes years to progress in patients.32 Second, we expressed our mRNA data as relative to the amount of input RNA as opposed to the more frequently used normalization of gene expression against housekeeping genes. We opted for this approach because it is well-known that expression of commonly used housekeeping genes is highly dependent on the experimental conditions33-34 and the use of total cellular RNA has been proposed as the best alternative for data normalization.35 In agreement, we analyzed several different “housekeeping genes” (Supplementary Figure 2 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Hypoxanthine–Guanine Phosphoribosyltransferase (HPRT) and 18S rRNA) and observed considerable variability between the different “house-keeping” genes. Importantly however, mRNA levels as expressed relative to the amount of input RNA do correspond with the histological scores and hydroxyproline analysis. Finally, we did observe a significant difference in IL-6 levels between untreated mice and mice receiving 2.5 mg/kg once daily. Such a difference in IL-6 was not shown in PAR-1 deficient animals. Most likely this is explained by the different time point at which IL-6 was measured (6 and 14 days after bleomycin instillation for PAR-1 deficient and P1pal-12 treated mice respectively), although we cannot exclude small differences due to genetic PAR-1 deficiency versus pharmacological PAR-1 inhibition.

In conclusion, P1pal-12 significantly blocks PAR-1-induced pro-fibrotic effects in vitro and inhibits bleomycin-induced pulmonary fibrosis in mice. Thus, targeting PAR-1 may be clinically relevant and may meet the urgent medical need for treating patients with pulmonary fibrosis.
REFERENCES


Supplementary figures

Supplementary figure 1. TGFbeta induced collagen production is independent from P1pal-12 treatment. Western blot analysis of collagen expression by NIH3T3 cells stimulated with TGFbeta in the presence and absence of P1pal-12. Tubulin was used as loading control.

Supplementary figure 2. Expression of GAPDH, HPRT and 18S rRNA mRNA levels in lung homogenates obtained 14 days after bleomycin instillation in untreated mice and mice treated with 2.5 mg/kg P1pal-12 from day 1 or day 7 post-bleomycin instillation as assessed by real-time RT-PCR.

Supplementary methods

Western Blot In brief, cells or lung homogenates were lysed in Laemmli lysis buffer and the lysates were incubated for 5 minutes at 95°C. Afterwards, 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 (α-SMA), β-actin, GAPDH, collagen (all Santa Cruz, CA), phospho-ERK1/2 or total ERK1/2 (both Cell Signaling, Leiden) 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). For quantification, densitometry was performed...
with ImageJ (NIH, Maryland, U.S) using the histogram function in a selected area of mean gray value for each band. Values for the protein of interest were corrected for those of β-actin or GAPDH.

**Wound scratch assays** Cells 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), serum-free medium supplemented with 100 μM PAR-1-AP, or serum-free medium containing 100 μM PAR-1-AP and 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.

**Hydroxyproline Assay** Right lungs were homogenized in saline (100 mg lung in 900 μl saline) and stored at -20°C. Next, hydroxyproline content was analysed using the hydroxyproline assay kit (Sigma, Netherlands) as per the manufacturer’s instructions. In detail, 40 μl homogenate was added to 60 μl water after which 100 μl 12N HCL was added, Samples were hydrolyzed at 120°C for 3 hours after which 20 μl of the hydrolyzed samples were transferred to a 96 well plate. Subsequently, the plate (also containing a hydroxyproline standard curve) was incubated at 60°C till all fluid was evaporated (approximately 2 hours) after which the chloramine T/Oxidation buffer mixture was added. After 5 minute incubation on a shaker at room temperature, 100 μL of the Diluted DMAB Reagent was added and the samples were incubated for 90 minutes at 60 °C. Finally, the absorbance was measured at 560 nm and hydroproline content was calculated according to the standard curve. Right lung collagen content was calculated by multiplying the hydroxyproline values with a factor 7.4 (because collagen contains on average 13.5% hydroxyproline). Finally, right lung collagen levels were multiplied by a factor 2 to obtain total lung collagen content.

**Histological Analysis** Briefly, the excised lung was fixed in formalin, embedded in paraffin and 4-μm-thick slides were subsequently deparaffinized, rehydrated and washed in deionized water. Slides were stained with hematoxylin and eosin (H&E) and Masson’s
trichrome according to routine procedures. In H&E staining, severity of fibrosis was assessed according to the Ashcroft scoring system using a 200× magnification. Two independent observers were blinded to the treatment group and an average of 10 fields of each lung section were selected and scored. The average Ashcroft score was calculated by averaging the individual field scores.

**Immunohistochemistry** Four-μm sections were first deparaffinized and rehydrated. Endogenous peroxidase activity was quenched with 0.3% H₂O₂ in methanol. Smooth muscle actin (α-SMA) and collagen staining were performed with an anti-α-smooth muscle actin antibody (1:1000, 24 hour at 4°C, Santa Cruz, CA) or anti-collagen-I antibodies (1:800, overnight at 4°C, GeneTex, CA). 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).

**qPCR** Total RNA was isolated from lung homogenates with TriPure (Roche, Almere, Netherlands) following the manufacturer’s recommendations. q-PCR was performed with SYBR Green PCR master Kit (Roche) using the following primers: α-SMA: forward 5’-TCCCTGGAGAAGAGCTACGAACT-3’ and reverse 5’-GATGCCCGCTGACTCCAT-3’; collagen 1A1: forward 5’-ACCTAAGGGTACCAGCTGGA-3’ and reverse 5’-TCCAGCTTCTCCATCTTTGC-3’; Fibronectin forward 5’-CCATGTAGGAGAACAGTGGCA-3’ and reverse 5’-GAAGCACTCAATGGGGCA-3’. The results were calculated as Efficiency**Cp**.

**Reference**
Chapter 3

Pharmacological targeting of protease activated receptor-2 affords protection from bleomycin-induced pulmonary fibrosis

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Accepted by Molecular Medicine.
ABSTRACT

Background
Idiopathic pulmonary fibrosis is the most devastating diffuse fibrosing lung disease which remains refractory to therapy. Protease activated receptor (PAR)-2 is an important mediator of fibrosis and PAR-2 deficiency limits bleomycin-induced pulmonary fibrosis. Here, we addressed whether PAR-2 deficiency persistently reduces bleomycin-induced pulmonary fibrosis or merely delays disease progression and whether pharmacological PAR-2 inhibition limits experimental pulmonary fibrosis.

Methods
Bleomycin was instilled intranasally into wild-type or PAR-2 deficient mice in the presence/absence of a specific PAR-2 antagonist. Fibrosis was assessed by hydroxyproline analysis, immunohistochemistry and western blot for fibrotic markers expression.

Results
Pulmonary fibrosis was consistently reduced in PAR-2 deficient mice throughout the fibrotic phase as evident from reduced Ashcroft scores (29%) and hydroxyproline levels (26%) at day 28. Moreover, P2pal-18S inhibited PAR-2-induced pro-fibrotic responses in both murine and primary human pulmonary fibroblasts (p<0.05). Treatment with P2pal-18S reduced the severity and extent of fibrotic lesions in lungs of bleomycin-treated wild-type but did not further reduce fibrosis in PAR-2 deficient mice. Importantly, P2pal-18S treatment starting even 7 days after the onset of fibrosis still as effectively limits pulmonary fibrosis as when treatment was started together with bleomycin instillation (p<0.05).

Conclusions
Overall, PAR-2 contributes to the progression of pulmonary fibrosis and targeting PAR-2 may be a promising therapeutic strategy for treating pulmonary fibrosis.
INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is lethal diffuse fibrosing lung disease with a 5-year mortality rate greater than 50%, which exceeds many types of cancers. IPF comprises a group of conditions characterized by interstitial fibroblast proliferation, alveolar destruction and excessive extracellular matrix (ECM) synthesis and accumulation. Studies exploring the mechanisms that are crucially involved in the development of IPF identified several possible targets for therapeutic interventions. Among those, protease-activated receptors (PARs) are key candidates, as these receptors mediate the cellular effects of coagulation factors and play central roles in influencing inflammatory and fibrotic responses.

PARs are seven-transmembrane G protein-coupled receptors which are activated by proteolytic cleavage by serine proteases. PAR-2 is one of four members of the PAR family that is widely expressed in many different cell types like fibroblasts and epithelial cells. Proteases such as trypsin, factor V/F/VIIa, FXa, mast cell tryptase or matriptase cleave the N-terminal extracellular domain of PAR-2, thereby revealing a novel tethered ligand that binds to PAR-2 and activates its transmembrane signaling to intracellular G proteins. Importantly, activated PAR-2 mediates a number of pathophysiological pathways involved in acute/chronic inflammatory and fibrotic diseases of the joints, skin, brain, lung and gastrointestinal tract.

With respect to lung injury and pulmonary fibrosis, there is increasing evidence that PAR-2 is a critical contributor in the pathogenesis of IPF. Increased PAR-2 expression has been detected in the lungs of patients with IPF and a recent study proposed that the PAR-2/F/VIIa axis may contribute to the development and/or progression of IPF. This study indeed provided in vitro evidence that F/VIIa exerts pro-fibrotic effects in human fibroblasts by specifically activating PAR-2. In line, the prototypical PAR-2 agonist tryptase stimulates the growth of human lung fibroblasts and potentiate extracellular matrix production in a PAR-2-dependent manner. Moreover, PAR-2 expression significantly correlates with the extent of honeycombing and PAR-2 activation has been shown to be highly relevant to the progression of pulmonary fibrosis in an experimental animal model of bleomycin-induced injury. Indeed, genetic ablation of PAR-2 in mice affords protection from pulmonary fibrosis, as evident from a reduction in the extent and severity of fibrotic lesions and diminished collagen expression. In addition, treatment of pulmonary fibrosis bearing rats with diallylsulfide results in amelioration of collagen production and alveolar epithelial cell apoptosis through the involvement of PAR-2.
Notwithstanding the overwhelming amount of data supporting an important role of PAR-2 in pulmonary fibrosis, some controversy has also emerged over the topic. Indeed, using a similar model of bleomycin-induced pulmonary fibrosis as in the study mentioned above, edema and hydroxyproline levels were not different between wild type and PAR-2 deficient mice in a study by Su and colleagues. Also, a recent study showed that protein and mRNA expression levels of PAR-2 in IPF patients were not different from controls, and it has been claimed that it is doubtful whether blocking PAR-2 would serve as an effective treatment strategy for IPF. It is thus fair to state that, despite intriguing data supporting a role for PAR-2 in pulmonary fibrosis, its potential clinical relevance remains controversial.

In the present study, we addressed the controversy by first assessing whether PAR-2 deficiency limits bleomycin-induced pulmonary fibrosis or merely delays disease progression. We reaffirmed the importance of PAR-2 and subsequently evaluated the efficacy of pharmacological PAR-2 inhibition in pulmonary fibrosis.

MATERIALS AND METHODS

Cells and Reagents
Mouse embryonic NIH3T3 fibroblasts (American Type Culture Collection, Manassas, VA; CRL-1658) and primary human lung fibroblast (derived from pulmonary control and IPF patient explants as described in; provided by INSERM U1152) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS). 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. Human platelets were donated by a healthy volunteer and isolated as described before. Thrombin and trypsin were from Sigma (St-Louis, MO), whereas P2pal-18S (PAR-2 inhibitor; palmitate-RSSAMDENSEKRRKSAIK-NH2) was from GL Biochem Ltd (Shanghai, China).

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 pre-incubated with or without 10 μM P2pal-18S and then challenged with PAR-1 agonist. Ca²⁺ flux was monitored for the indicated time points on a Bio-Tek HT Multi-Detection Microplate Reader (Winooski, United States).
Western Blot
Western blots were performed essentially as described before\textsuperscript{19}. In brief, cells were lysed in Laemmli lysis buffer and the lysates were incubated for 5 minutes at 95°C. Afterwards, 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 or collagen (all Santa Cruz, CA) 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). For quantification, densitometry was performed using ImageJ.

Animal Model of Pulmonary Fibrosis
Wild type C57Bl/6 mice were purchased from Charles River (Someren, Netherlands). PAR-2 deficient (PAR-2\textsuperscript{-/-}) C57Bl/6 mice were originally provided by Jackson Laboratories (Maine) and bred at the animal care facility of the Academic Medical Center. All procedures were performed on ten-week-old mice in accordance with the Institutional Standards for Humane Care and Use of Laboratory Animals. Experiments were approved by the Animal Care and Use Committee of the Academic Medical Center (Amsterdam, Netherlands).

Bleomycin (Sigma, St-Louis, MO) was administered by intranasal instillation (1 mg/kg body weight in 45 μl saline) under anesthesia. In the PAR-2 pepducin dose finding experiment, animals were intranasally instilled with 2.5 or 10 mg/kg P2pal-18S half an hour before bleomycin administration and subsequently once daily until the end of the experiment. In the delayed treatment experiment, mice were treated once daily with 2.5 mg/kg pepducin starting one, three or seven-day after bleomycin instillation. In both experiments, 6% DMSO in saline was administered as a solvent control. Unless stated otherwise, mice were sacrificed 14 days after bleomycin instillation after which one lung was taken for histological analysis and one was homogenized.

Bronchoalveolar Lavage (BALF)
At 14 days after bleomycin instillation, the lungs were lavaged three times with 0.3 ml of saline by a 22-gauge Abbocath-Tcatheter into the trachea via a midline incision before animals were sacrificed. The BALF was centrifuged, and the supernatant was frozen at \(-70°C\) until use.
CHAPTER 3

**TGF-β ELISA**

TGF-β1 was measured with the Mouse TGF-beta 1 DuoSet kit (R&D systems, UK) as per the manufacturer’s instructions.

**Hydroxyproline Assay**

Hydroxyproline analysis was performed by the hydroxyproline assay kit as per the manufacturer’s instructions (Sigma, Netherlands) and as described before.

**Histological Analysis**

Histological examination was performed essentially as described before. Briefly, the excised lung was fixed in formalin, embedded in paraffin and 4-μm-thick slides were subsequently deparaffinized, rehydrated and washed in deionized water. Slides were stained with hematoxylin and eosin (H&E) according to routine procedures. In H&E staining, the severity of fibrosis was assessed according to the Ashcroft scoring system using a 100× magnification. Two independent observers were blinded to the treatment group and an average of 10 fields of each lung section was selected and scored. The average Ashcroft score was calculated by averaging the individual field scores.

**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-2 deficiency attenuates bleomycin-induced pulmonary fibrosis**

Although PAR-2 deficiency has been shown to afford protection from bleomycin-induced lung injury before, those studies used day 14 as the endpoint which may just point to a delay of the onset of fibrosis. To determine whether PAR-2 deficiency indeed limits bleomycin-induced pulmonary fibrosis or just delays progression, we compared lungs from wild type and PAR-2 deficient mice at day 28 and 48 after the instillation of bleomycin. As shown in Figure 1A, bleomycin-induced inflammatory and fibrotic processes culminate in severe pulmonary fibrosis at day 28 in wild type mice. In contrast, lung tissue sections from PAR-2 deficient mice at day 28 showed significantly
Targeting PAR-2 in pulmonary fibrosis

Three-dimensional culture of primary human lung fibroblasts pointed to reduced interstitial fibrosis, with diminished ECM deposition and less destruction of alveolar units (Figure 1B). Both wild type and PAR-2 deficient mice showed some degree of resolution by day 48 (Figure 1C and D). Quantification of the bleomycin-induced histological changes in wild type and PAR-2 deficient mice using the Ashcroft score shows that PAR-2 deficient mice develop less severe fibrotic lesions at day 28 as compared to wild type mice (Figure 1E and F). These observations are also supported by hydroxyproline levels, depicting collagen deposition is significantly reduced in PAR-2 deficient lungs at day 28 (Figure 1G and H). Overall, these results show that PAR-2 deficiency indeed provides protection against bleomycin-induced pulmonary fibrosis throughout the fibrotic stage and does not merely delay disease onset.

P2pal-18S effectively inhibits PAR-2 mediated pro-fibrotic responses in fibroblasts

Next, we determined the specificity of the observed effects in PAR-2 deficient mice by assessing whether pharmacological PAR-2 inhibition by P2pal-18S would reduce lung fibrosis as well. Before assessing the in vivo effect of P2pal-18S, we first determined its specificity for blocking PAR-2 dependent fibrotic responses in vitro. Although the specificity of P2pal-18S has been tested previously, we here use human platelets (not expressing PAR-2) to confirm that P2pal-18S only targets PAR-2 and does not interact with other PARs. As shown in Figure 2A, PAR-1 activation by thrombin induced Ca\(^{2+}\) influxes that peaked within 1 minute after stimulation, whereas pretreatment with 10 μM P2pal-18S did not interfere with this reaction. We next assessed whether P2pal-18S inhibits PAR-2 driven pro-fibrotic responses in fibroblasts. We show that trypsin induced fibroblast differentiation, as evident from increased α-SMA expression and collagen production compared to vehicle treated cells. Trypsin-induced α-SMA and collagen expression were clearly down-regulated by the treatment with 10 μM P2pal-18S (Figure 2B).

Bleomycin administration is known to increase PAR-2 agonist expression in BALF which may lead to fibroblast differentiation or ECM synthesis. We next thus assessed the inhibitory effect of P2pal-18S against BALF induced pro-fibrotic effects on murine fibroblasts. NIH3T3 cells seeded in BALF of wild type mice treated with bleomycin showed increased expression of α-SMA and collagen as compared to cells seeded in BALF derived from saline-treated mice, while preincubation with P2pal-18S partially decreased this effect (Figure 2C). Finally, we determined whether P2pal-18S also limits PAR-2 induced fibrotic responses on primary human lung fibroblasts. We stimulated...
fibroblasts isolated from nonfibrotic controls and IPF patients with trypsin in the absence or presence of P2pal-18S. IPF-derived fibroblasts exhibited high α-SMA and collagen expression persistently (Figure 2D and E showing three independent fibroblast isolations) while control fibroblasts only showed increased α-SMA and collagen expression after treated with trypsin. The expression of α-SMA and collagen were reduced by P2pal-18S in both control and IPF fibroblasts (Figure 2F and G). Overall these in vitro data show that P2pal-18S effectively antagonizes PAR-2-mediated profibrotic effects in both murine and human fibroblasts.

**P2pal-18S limits the development of pulmonary fibrosis in the bleomycin model**

After having shown that P2pal-18S inhibits PAR-2-induced fibroproliferative responses in fibroblasts, we set out to examine whether P2pal-18S would effectively limit fibrosis in the bleomycin model. To this end, mice were intranasally instilled with different concentrations of P2pal-18S, after which the extent and severity of fibrosis was determined at day 14. As shown in Figure 3A, bleomycin instillation resulted in extensive fibrotic foci accompanied by increased deposition of ECM. Both 2.5 mg/kg and 10
mg/kg P2pal-18S treatment significantly reduced the severity of regional interstitial fibrosis (compare Figures 3B and C with 3A). Quantification of the bleomycin-induced histopathological changes in the different groups using the Ashcroft score shows that P2pal-18S treatment results in less severe fibrotic lesions (Figure 3D). We also analyzed total collagen accumulation in the lung and, as shown in figure 3E, bleomycin-induced
collagen levels were attenuated by P2pal-18S treatment, although the 10 mg/kg dose did not reach statistical significance.

We next assessed TGF-β1 levels in lung homogenates as TGF-β1 is a key profibrotic cytokine and its gene and protein expression levels are known to be increased by bleomycin. As shown in Figure 3F, TGF-β1 levels increased in bleomycin-instilled mice, while the 2.5 mg/kg dose of P2pal-18S significantly attenuated the TGF-β1 increase by about 50%. The 10 mg/kg dose of P2pal-18S also reduced TGF-β1 level but this reduction again did not reach statistical significance.

**Delayed treatment with P2pal-18S effectively limits pulmonary fibrosis progression in the bleomycin model**

After having established that long-term treatment with P2pal-18S effectively limits bleomycin-induced pulmonary fibrosis, we assessed whether therapeutic modes of

![Figure 3](image)

**Figure 3. P2pal-18S treatment attenuates bleomycin-induced pulmonary fibrosis.** (A-C) Representative H&E stained lung tissue sections obtained 14 days after bleomycin instillation in saline treated mice (A) and mice treated with 2.5 mg/kg (B) or 10 mg/kg P2pal-18S (C). (D) Quantification of pulmonary fibrosis in control and P2pal-18S treated mice using the Ashcroft score. Data are expressed as mean ± SEM (n=8 per group). Total collagen content (E) and TGF-β1 levels (F) in lung homogenates of the different groups of mice obtained 14 days after saline or bleomycin instillation. Data are expressed as mean ± SEM (n=8 per group; * P<0.05; **P<0.01).
administration would still be effective. P2pal18 treatment (2.5 mg/kg) was started either 1, 3 (preventive treatment; concurrent with the inflammatory phase) or 7 (therapeutic treatment; upon the establishment of lung fibrosis) days after bleomycin instillation and continued once daily till the end of the experiment. As shown in Figure 4A-C, histological analysis of lung slides from bleomycin-instilled mice not treated with P2pal-18S again showed severe fibrotic lesions (about 5 fold increase compared to lungs of non-bleomycin-instilled controls). Treatment with P2pal-18S in the preventive mode strongly inhibited bleomycin-induced pulmonary fibrosis, as evident from lower Ashcroft scores (Figure 4G) and reduced collagen accumulation (Figure 4H).

Figure 4. Delayed P2pal-18S treatment effectively attenuates bleomycin-induced pulmonary fibrosis. (A-F) Representative H&E stained lung tissue sections obtained 14 days after bleomycin instillation in wild type control treated mice (A), wild type mice treated with 2.5 mg/kg P2pal-18S from day 1 (B), day 3 (C) or day 7 (D) after bleomycin instillation, PAR-2-/- control treated mice (E) and PAR-2-/- mice treated with 2.5 mg/kg P2pal-18S (F). (G) Quantification of pulmonary fibrosis in delayed P2pal-18S treated wild type mice using the Ashcroft score. (H) Total collagen content in lung homogenates from the different groups of WT mice obtained 14 days after saline or bleomycin instillation. (I) Quantification of pulmonary fibrosis in PAR-2-/- mice treated with or without P2pal-18S using the Ashcroft score (Note that the wild type untreated controls are the same as depicted in panel G, as the experiments were performed at the same time.) (J) Total collagen content in lung homogenates from PAR-2-/- mice and PAR-2-/- mice treated with P2pal-18S obtained 14 days after bleomycin instillation. The wild type controls are the same as depicted in panel H. Data are expressed as mean ± SEM (n=8 per group; * P<0.05**P<0.01).
Interestingly, similar findings were obtained when P2pal-18S was administered in the therapeutic mode (compare Figure 4A and D). Indeed, P2pal-18S treatment starting on day 7 reduced the bleomycin-induced increase in Ashcroft score (although not statistically significant) and collagen deposition (Figure 4G and H).

To corroborate the specificity of P2pal-18S in an *in vivo* setting, we analyzed the effect of P2pal-18S treatment (2.5 mg/kg) in bleomycin-treated PAR-2 deficient mice. As shown in figure 4E and F, PAR-2 deficient mice developed less fibrosis as compared to wild type mice. More importantly, P2pal-18S treatment did not further reduce fibrosis and collagen deposition in the PAR-2 deficient mice (Figure 4I and J), suggesting that P2pal-18S limits pulmonary fibrosis by specifically targeting PAR-2.

**DISCUSSION**

IPF is a progressive lung disorders for which very few therapeutic options are available. It is often suggested as an uncontrolled wound healing response, in which multiple effectors are tightly involved. PAR-2, as a critical receptor that orchestrates a diverse range of signaling pathways, plays an important role in mediating pro-fibrotic effects in fibroblasts and in preclinical experimental animal models, although there is still some controversy regarding the role of PAR-2 in pulmonary fibrosis. In the present study, we aimed to address the controversy and provide several lines of evidence to support the crucial role of PAR-2 in the progression of pulmonary fibrosis.

Bleomycin-induced pulmonary fibrosis is stably formed at around day 14, which is consequently frequently used as endpoint in literature. However, the fibrotic stage induced by bleomycin peaks at around 3–4 weeks and the reduction in fibrosis observed in PAR-2 deficient mice at day 14 may thus only indicate a delay in the onset of fibrosis instead of reduced progression. To address this concern, we increased the follow-up time, and show that the severity of fibrosis as well as collagen accumulation are still significantly reduced in PAR-2 deficient mice compared with wild type mice 28 days after bleomycin instillation. Furthermore, both wild type and PAR-2 deficient mice enter the resolution phase of fibrosis by day 48, indicating the peak of fibrosis has passed. By using different endpoints of the murine bleomycin model, we provide evidence that PAR-2 deficiency provides persistent protection along the development of pulmonary fibrosis, suggesting PAR-2 is indeed an interesting target for therapeutic intervention.
P2pal-18S is a cell-penetrating lipopeptide “pepducin” antagonist of PAR-2 that efficiently blocks PAR-2-dependent inflammatory responses in mouse models. We confirm and extend these findings by showing that P2pal-18S effectively blocks PAR-2 driven pro-fibrotic responses *in vitro*. Indeed, pre-incubation of murine fibroblasts with P2pal-18S blocks trypsin-induced ERK phosphorylation, reduces proliferation and migration and diminishes α-SMA and collagen expression (Figure 2). Moreover, P2pal-18S significantly inhibits collagen expression not only in control fibroblasts but also in IPF-derived human fibroblasts. IPF-derived human fibroblasts actually exhibit a more differentiated and fibrotic myofibroblast phenotype, as evident from elevated basal expression levels of both α-SMA and collagen, which may explain the reduced responsive to the trypsin stimulation as compared to control fibroblasts. As opposed to complete inhibition of trypsin-induced signaling, P2pal-18S did only partly block ECM deposition induced by BALF from bleomycin-treated wild type mice. This suggests that BALF partially exerts pro-fibrotic effects through PAR-2 activation but that BALF also contains additional pro-fibrotic components. Overall, these data show that P2pal-18S efficiently block PAR-2-dependent fibrotic responses in vitro.

In line with a potential therapeutic role of PAR-2 in pulmonary fibrosis, we show that P2pal-18S limits bleomycin-induced pulmonary fibrosis even when administered at the fibrotic phase. Treatments during the first seven days after bleomycin administration are generally considered “preventive” whereas treatments starting at day 7 are considered “therapeutic”. After showing that once daily P2pal-18S treatment (2.5mg/kg) during the 14 days of the experiment significantly limited bleomycin-induced pulmonary fibrosis, we next showed that administration of P2pal-18S also effectively decreases the histological grade of pulmonary fibrosis and collagen contents in both a preventive (1 and 3 days delayed treatment) and therapeutic (7 days delayed treatment) mode. Importantly, the protective effect afforded by PAR-2 deficiency against bleomycin-induced pulmonary fibrosis was nearly identical to that observed in wild type mice treated with P2pal-18S. In PAR-2 deficient mice as well as in P2pal-18S treated mice, the severity of the fibrotic lesions assessed by the Ashcroft score was decreased by around 1 point. In line, quantitative measurement of collagen deposition in the lungs reduced by about 34% in PAR-2 deficient mice and 29% in P2pal-18S treated mice. Interestingly, the anti-fibrotic effect of P2pal-18S was lost in PAR-2/-/- mice, demonstrating that P2pal-18S is highly specific for PAR-2 *in vivo*. This is in accordance with a previous study showing that P2pal-18S significantly reduces mouse paw edema and inflammation in wild type but not PAR-2 deficient mice. Moreover, our *in vitro* data show that P2pal-
18S does not interfere with thrombin-induced calcium fluxes in human platelets. As platelets respond to thrombin through PAR-1 and/or PAR-4, these data indicate that P2pal-18S does not have cross-reactivity with other PARs. Together, our data indicate that P2pal-18S provides effective and specific pharmacologic inhibition of PAR-2 in an animal model of pulmonary fibrosis and that inhibition of PAR-2 may thus be a promising therapeutic strategy for treating pulmonary fibrosis, although future clinical studies are needed to confirm this notion.

It is noteworthy that, similarly as observed for targeting PAR-1 in pulmonary fibrosis, P2pal-18S at a dose of 2.5 mg/kg seemed to be more effective in reducing fibrosis than at a high dose of 10 mg/kg. Although we do not have a definite explanation for this finding, it is tempting to speculate that the phenomenon may be due to the fact that PAR-2, again similarly to PAR-1, acts both as a pro- and anti-inflammatory receptor in the respiratory system. In cultured human epithelial cells, activation of PAR-2 causes release of pro-inflammatory cytokines such as interleukin (IL)-8. PAR-2 stimulation is also required for serralysin to activate the critical transcription factors for host inflammatory responses. On the other hand, PAR-2 activation enhances LPS-induced expression of the anti-inflammatory cytokines while suppressing gene expression of pro-inflammatory cytokines and PAR2-TLR signaling integration drives “customized” inflammatory responses. Therefore, the extent of PAR-2 inhibition may affect the balance of its pro- and anti-inflammatory properties. Alternatively, the slight differences observed between the 2.5 and 10 mg doses may simply be attributed to the variability within the analysis rather than any definable biological phenomenon.

Pharmacological PAR-2 inhibition may not just benefit pulmonary fibrosis due to the fact that PAR-2 influences a large range of pathophysiological pathways and indeed PAR-2 deficiency affords protection to, amongst others, renal fibrosis, heart failure and pulmonary hypertension. In line, PAR-2 inhibition reduced, again amongst others, pulmonary hypertension, acute biliary pancreatitis, and osteoarthritis. It is thus tempting to speculate that P2pal-18S may have an impact on a large plethora of other (PAR-2 dependent) disorders although this tantalizing hypothesis needs to be addressed experimentally.

In summary, our data endorse the importance of PAR-2 in mediating pro-fibrotic effects and identify the PAR-2 antagonist P2pal-18S as an effective inhibitor of bleomycin-induced pulmonary fibrosis. Thus, targeting PAR-2 signaling with P2pal-18S may offer a novel therapeutic option for patients with pulmonary fibrosis.
REFERENCES


Chapter 4

Protease-activated Receptor (PAR)-2 Is Required For PAR-1 Signaling In Pulmonary Fibrosis

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ABSTRACT

Background
Idiopathic pulmonary fibrosis is the most devastating diffuse fibrosing lung disease of unknown etiology. Compelling evidence suggests that both protease-activated receptor (PAR)-1 and PAR-2 participate in the development of pulmonary fibrosis. Previous studies have shown that bleomycin-induced lung fibrosis is diminished in both PAR-1 and PAR-2 deficient mice. We thus hypothesized that combined inactivation of PAR-1 and PAR-2 would be more effective in blocking pulmonary fibrosis.

Methods
Human and murine fibroblasts were stimulated with PAR-1 and PAR-2 agonists in the absence or presence of specific PAR-1 or PAR-2 antagonists after which fibrotic markers like collagen and smooth muscle actin were analyzed by Western blot. Pulmonary fibrosis was induced by intranasal instillation of bleomycin into wild type and PAR-2 deficient mice with or without a specific PAR-1 antagonist (P1pal-12). Fibrosis was assessed by hydroxyproline quantification and (immuno)histochemical analysis.

Results
We show that specific PAR-1 and/or PAR-2 activating proteases induce fibroblast migration, differentiation and extracellular matrix production. Interestingly however, combined activation of PAR-1 and PAR-2 did not show any additive effects on these profibrotic responses. Strikingly, PAR-2 deficiency as well as pharmacological PAR-1 inhibition reduced bleomycin-induced pulmonary fibrosis to a similar extent. PAR-1 inhibition in PAR-2 deficient mice did not further diminish bleomycin-induced pulmonary fibrosis. Finally, we show that the PAR-1-dependent profibrotic responses are inhibited by the PAR-2 specific antagonist.

Conclusions
Targeting PAR-1 and PAR-2 simultaneously is not superior to targeting either receptor alone in bleomycin-induced pulmonary fibrosis. We postulate that the profibrotic effects of PAR-1 require the presence of PAR-2.
INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a characteristic form of fibrosing idiopathic interstitial pneumonia which has a devastating prognosis\(^1\,^2\). The therapeutic options are limited and, to date, only pirfenidone has been granted orphan drug status in the EU for the treatment of mild to moderate IPF\(^3\). Although the understanding of IPF continues to evolve, the molecular mechanisms underlying the pathogenesis of IPF are still far from completely understood. The current paradigm postulates that the abnormal wound healing response to lung epithelial injury subsequently leads to pulmonary fibrosis\(^4\). IPF lesions are distinctively characterized by the formation and proliferation of fibroblast foci in the background of excessive extracellular matrix (ECM) deposition\(^1\,^2\,^4\). Therefore, unraveling the mechanisms by which fibroblasts replicate and secrete ECM proteins could be beneficial for conceiving effective therapeutic strategies\(^5\).

Protease activated receptors (PARs) belong to the superfamily of G-protein-coupled receptors (GPCRs)\(^6\). Unlike other GPCRs, which are activated by ligand binding, PARs are irreversibly activated by proteolytic cleavage\(^7\). After proteolytic activation of PARs, a novel tethered ligand is exposed that folds back over the receptor to trigger several downstream signaling pathways, contributing to a broad range of pathophysiological functions\(^6\,^8\,^9\). Although blood coagulation factors are the archetypal activating proteases of PARs, it is now well established that multiple proteases, such as thrombin, matrix metalloproteinase-1, factor (F)VII, FXa, trypsin, and tryptase, can activate individual PARs with different affinity and trigger specific responses via biased agonist signaling\(^6\,^10\).

In the context of lung injury and pulmonary fibrosis, accumulating evidence suggests that both PAR-1 and PAR-2 induce pro-inflammatory and pro-fibrotic processes that aggravate disease progression. PAR-1 activation enhances inflammation in the pulmonary epithelium, it induces the differentiation of fibroblasts into myofibroblasts and stimulates ECM synthesis\(^12\,^13\). Moreover, genetic ablation of PAR-1\(^15\), as well as pharmacological PAR-1 inhibition\(^16\), limit bleomycin-induced acute lung inflammation and fibrosis, as evident from reduced total collagen level in the lung in combination with decreased levels of proinflammatory and profibrotic mediators, such as transforming growth factor (TGF)-\(\beta\), interleukin (IL)-6 and monocyte chemoattractant protein-1 (MCP-1). Furthermore, PAR-1 expression is increased within fibroproliferative and inflammatory foci in IPF patients\(^14\). PAR-2 activation induces acute lung inflammation and also triggers fibroproliferative responses in fibroblasts, such as proliferation, migration and differentiation into myofibroblasts\(^17\,^19\). In line, the absence of PAR-2 affords
protection from bleomycin-induced pulmonary fibrosis, as evident from a reduction in the extent and severity of fibrotic lesions and diminished collagen expression\textsuperscript{20}. PAR-2 expression is also increased in lungs of IPF patients and its expression highly correlates with the extent of honeycombing\textsuperscript{20-22}.

Overall, these studies highlight PAR-1 and PAR-2 as critical contributors in promoting pulmonary fibrosis. Importantly, in the experimental bleomycin model, pulmonary fibrosis is not completely abolished in mice that harbor deficiency for either PAR-1 or PAR-2. Therefore, in the present study, we hypothesized that the simultaneous inhibition of PAR-1 and PAR-2 would be superior to targeting either receptor alone in pulmonary fibrosis.

**MATERIALS AND METHODS**

**Cells and Reagents**

Mouse embryonic NIH3T3 fibroblasts (American Type Culture Collection, Manassas, VA; CRL-1658) and human lung fibroblast (HLFs from control lungs, isolated as described before\textsuperscript{23}) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS). Cells were grown at 37°C in an atmosphere of 5% CO\textsubscript{2}. Unless indicated otherwise, cells were washed twice with PBS and serum-starved for 4 hours before stimulation. Thrombin (T7009; ≥ 1000 NIH Units/mg) and trypsin (T0303; 13,000-20,000 BAEE Units/mg) were from Sigma (St-Louis, MO), whereas P1pal-12 (palmitate-RCLSSAVANRS-NH\textsubscript{2})\textsuperscript{24} and P2pal-18s (palmitate-RSSAMDENSEKRRKSAIK-NH\textsubscript{2})\textsuperscript{25} were from GL Biochem Ltd (Shanghai, China). Both pepducins, which are insoluble in water, were dissolved in DMSO followed by dilutions in PBS or saline leading to final DMSO concentrations of 6% for the in vivo experiment and 0.1% for in vitro experiments.

**Western Blot**

Western blots were performed essentially as described before\textsuperscript{19}. In brief, cells were lysed in Laemmli lysis buffer and the lysates were incubated for 5 minutes at 95°C. Afterwards, 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 (α-SMA), tubulin, collagen, (all Santa Cruz, CA), phospho-ERK1/2 or total ERK1/2 (both Cell Signaling, Leiden) at 4°C. All secondary antibodies were horse-
radish 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).

**Wound Scratch Assay**

Scratch assays were performed essentially as described before. Cells 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), serum-free medium supplemented with 10 nM thrombin/trypsin or serum-free medium containing 10 nM thrombin/trypsin and 10 μM PAR-1 or PAR-2 antagonist (P1pal-12/P2pal-18s). When indicated, cells were pre-incubated with 10 μM pepducin 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.

**Animal Model of Pulmonary Fibrosis**

Wild-type C57Bl/6 mice were purchased from Charles River (Someren, Netherlands). PAR-2 deficient (PAR-2-/-) C57Bl/6 mice were originally provided by Jackson Laboratories (Maine) and bred at the animal care facility of the Academic Medical Center. All procedures were performed on ten-week-old mice in accordance with the Institutional Standards for Humane Care and Use of Laboratory Animals. Experiments were approved by the Animal Care and Use Committee of the Academic Medical Center (Amsterdam, Netherlands).

Bleomycin (Sigma, St-Louis) was administered by intranasal instillation (1 mg/kg body weight) under anesthesia. We specifically opted for intranasal instillation instead of intratracheal instillation as the former administration route, which is also a well-recognized manner to induce pulmonary fibrosis, causes less discomfort to the mice and is therefore the preferred model of the Animal Welfare Committee of our institute. Bleomycin was instilled in 16 wild type and 16 PAR-2 deficient mice. Per genotype, 8 mice were subsequently treated with P1pal-12 (dissolved in 6% DMSO) whereas the other 8
mice were treated with DMSO alone. The latter mice are indicated as solvent controls throughout the manuscript. Eight wild type mice were instilled with saline instead of bleomycin were used as non-fibrotic controls and are indicated as saline controls. P1pal-12 (PAR-1 antagonist) was administered 30 minutes before bleomycin administration and subsequently once daily until the end of the experiment at a dose of 2.5 mg/kg (based on previous dose finding experiments). Since the most suitable time point for assessing lung fibrosis is day 14 after bleomycin challenge, mice were sacrificed at this time point, after which the left lung was taken for histology and the right one was homogenized.

**TGF-β ELISA**

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

**Hydroxyproline Assay**

Hydroxyproline analysis was performed by the hydroxyproline assay kit as per the manufacturer’s instructions (Sigma, Netherlands) and as described before.

**Histological Analysis**

The excised lung was fixed in formalin, embedded in paraffin and 4-μm-thick slides were subsequently deparaffinized, rehydrated and washed in deionized water. Slides were stained with hematoxylin and eosin (H&E) and Masson’s trichrome according to routine procedures. As for the immunohistochemistry, four-μm sections were first deparaffinized and rehydrated. Endogenous peroxidase activity was quenched with 0.3% H2O2 in methanol. Smooth muscle actin (α-SMA) and collagen staining were performed with an anti-α-smooth muscle actin antibody (1:1000, 24 hour at 4°C, Santa Cruz, CA) or an anti-collagen-I antibody (1:800, overnight at 4°C, GeneTex, CA). 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).

Histological examination and Ashcroft score were performed as described before. Smooth muscle actin (α-SMA) staining was graded in a blinded fashion on a scale from 0 to 3 as described before. Pictures of collagen staining 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 before.
Statistics
Statistical analyses were conducted using GraphPad Prism (GraphPad software, San Diego). Comparisons between conditions were analyzed using two tailed unpaired t-tests when the data were normally distributed; otherwise Mann-Whitney analysis was performed. Results are expressed as mean±SEM, P values < 0.05 are considered significant.

RESULTS
PAR-1 and PAR-2 activating proteases induce profibrotic responses
PAR-1 is prototypically activated by thrombin whereas trypsin is the best characterized PAR-2 agonist. Compelling evidence shows that PAR stimulation of fibroblasts leads to the phosphorylation of extracellular signal-regulated kinase (ERK)1/2 (a surrogate marker for PAR-1 and PAR-2 activation), cell migration, differentiation into myofibroblasts and ECM synthesis. We previously showed that NIH3T3 cells express functional PAR-1 and PAR-2 and here we first validated the efficacy of thrombin and trypsin to induce these cellular responses. As shown in Figure 1A, both thrombin (10 nM) and trypsin (10 nM) induced ERK1/2 activation in murine NIH3T3 fibroblasts. In wound scratch assays, thrombin treatment led to wound closure in a dose dependent manner (Figure 1B-C), whereas only the highest concentration of trypsin strongly induced wound closure by about 60% compared to solvent treated cells (Figure 1B and D). Furthermore, both thrombin and trypsin induced fibroblast differentiation (reflected by increased α-SMA expression) and collagen synthesis (Figure 1E). These data thus indicate that thrombin and trypsin both can induce pro-fibrotic responses in NIH3T3 fibroblasts. Based on these data, we opted to use 10 nM of thrombin and trypsin in our subsequent experiments.

Simultaneous stimulation of both PAR-1 and PAR-2 does not show additive pro-fibrotic effects
After having established that both PAR-1 and PAR-2 promote pro-fibrotic responses in fibroblasts, we next assessed whether simultaneous activation of PAR-1 and PAR-2 induces a more robust pro-fibrotic response by stimulating cells with thrombin and trypsin at the same time. Interestingly, as shown in Figure 2A-B, no additive effect could be observed on wound closure. Wound sizes were decreased by approximately 50% in cells treated with thrombin, trypsin or a combination of thrombin and trypsin. Likewise, combined thrombin and trypsin treatment did not induce higher α-SMA and collagen expression than that observed after single PAR agonist treatment (Figure 2C).
Interestingly, delayed trypsin treatment (either 2, 4, 8 or 12 hours after thrombin stimulation) still did not show any additive effect on thrombin-induced wound-healing and/or fibrotic marker expression (Fig. S1A and C).

PAR-1 inhibition in PAR-2 deficient mice does not further limit pulmonary fibrosis in vivo

In previous experiments, we showed that blocking PAR-1 by P1pal-12 limits bleomycin-induced pulmonary fibrosis in a dose-dependent manner. Here, we applied the optimal P1pal-12 dose (2.5 mg/kg once daily) to treat both wild type and PAR-2 deficient mice, and compared bleomycin-induced fibrosis with solvent control treated wild type and PAR-2 deficient mice. As shown in Figure 3, extensive patchy areas of fibrosis were formed 14 days after bleomycin instillation in solvent treated wild type
PAR-2 is required for PAR-1 signalling in pulmonary fibrosis mice, accompanied by a marked accumulation of inflammatory cells and significant ECM deposition (Figure 3A). Both P1pal-12 treatment and PAR-2 deficiency significantly reduced the severity of regional interstitial fibrosis as assessed by the Ashcroft score (reduction of approximately 22% and 27% respectively, Figure 3B-C, E). Surprisingly, PAR-2 deficient mice treated with the PAR-1 antagonist P1pal-12 did not show a further reduction in fibrosis as that observed in solvent treated PAR-2 deficient mice (about 26% reduction; Figure 3D-E).
To substantiate our findings that PAR-1 inhibition does not further decrease fibrosis in PAR-2 deficient mice, we next analyzed α-SMA expression immunohistochemically. A considerable increase in α-SMA expression was seen in focal fibrotic lesions of solvent treated wild type mice (Figure 3F). Both pharmacological PAR-1 inhibition and genetic PAR-2 ablation significantly attenuated bleomycin-induced α-SMA expression (Figure 3G-H and J). Again, PAR-1 inhibition in PAR-2 deficient mice was not superior to either PAR-1 inhibition or PAR-2 deficiency alone (Figure 3I-J).

We next analyzed collagen deposition in the lungs. As shown in Figure 4A-C, Masson-trichrome and collagen I analysis showed similar reductions of collagen deposition in
PAR-2 is required for PAR-1 signalling in pulmonary fibrosis

P1pal-12 (PAR-1 antagonist) treated wild type mice, solvent control treated PAR-2 deficient mice or P1pal-12 treated PAR-2 deficient mice. In line, compared with bleomycin instilled solvent treated wild type mice, hydroxyproline levels decreased by 41±7%, 49±5% and 46±5% in P1pal-12 treated wild type mice, solvent control treated PAR-2 deficient mice and P1pal-12 treated PAR-2 deficient mice respectively (Figure 4D).

TGF-β1 is one of the most important profibrotic mediators and its expression is frequently associated with PAR regulation in fibrotic diseases. We therefore assessed TGF-β1 levels in lung homogenates of saline or bleomycin-instilled mice. As shown in Figure 4E, TGF-β1 levels increased around 2-fold in solvent treated bleomycin-instilled wild type mice compared with saline treated controls. Again, the increase in TGF-β1
was attenuated in PAR-2 deficient and P1pal-12 treated wild type or PAR-2 deficient mice. Altogether, these data show that the combined inhibition of PAR-1 and PAR-2 also has no additive effect in vivo.

**PAR-2 is required for PAR-1-induced pro-fibrotic responses in fibroblasts**

Our data so far show that combined activation of PAR-1 and PAR-2 is just as effective as single PAR activation on promoting fibrotic responses in fibroblasts. Moreover, simul-
PAR-2 is required for PAR-1 signalling in pulmonary fibrosis

Inhbitory inhibition of PAR-1 and PAR-2 was not superior to targeting either receptor alone in vivo, suggesting that PAR-1 and PAR-2 may actually act in concert to promote fibrosis. Consequently, we analyzed PAR agonist-induced pro-fibrotic responses in fibroblasts in the absence or presence of specific PAR-1 (P1pal-12) or PAR-2 (P2pal-18s) inhibitors. As shown in Figure 5A, thrombin-induced ERK1/2 phosphorylation was largely inhibited in the presence of P1pal-12. Surprisingly however, thrombin-induced ERK1/2 phosphorylation is also inhibited by the PAR-2 inhibitor P2pal-18s. In contrast, trypsin-induced ERK1/2 activation is only inhibited by P2pal-18s but not by P1pal-12 treatment (Figure 5A). In wound scratch assays, P1pal-12 pre-treatment blocked thrombin induced wound closure but only slightly reduced trypsin-induced closure, whereas P2pal-18s pre-treatment completely inhibited both trypsin and thrombin induced wound closure (Figure 5B-C). Consistent with these results, thrombin induced α-SMA and collagen expression was significantly down-regulated in P2pal-18s-pretreated cells (Figure 5D). In addition, delayed P2-pal-18s treatment was less efficient as compared to pretreatment, as evident from a gradual decrease in preventing wound healing and fibrotic marker expression over time (Fig. S1B and D). These data suggest that once the signaling pathways are activated additional PAR-2 activation is irrelevant. Overall, PAR-1-induced responses in fibroblasts are blocked by a PAR-2 specific antagonist, suggesting that the presence of PAR-2 is required for PAR-1 dependent pro-fibrotic signaling.

PAR-2 is also pivotal for PAR-1 to induce pro-fibrotic effects in HLFs

Finally, we aimed to confirm our in vitro findings using primary human lung fibroblasts (HLFs) derived from (non-fibrotic) patients. As shown in Figure 6A, stimula-
tion of HLFs with thrombin induced ERK1/2 phosphorylation, which was blocked by pretreatment with the PAR-1 antagonist P1pal-12 but also by pretreatment with the PAR-2 antagonist P2pal-18s. Furthermore, thrombin-induced differentiation of HLFs into myofibroblasts (as assessed by a-SMA expression) and collagen production were also inhibited by P2pal-18s (Figure 6B), indicating that pro-fibrotic effects of PAR-1 in HLFs also require the presence of PAR-2.

DISCUSSION

Compelling evidence suggests that aberrant wound healing caused by acute lung injury may play a pathophysiological role in IPF. It has been documented that many proteases exert pro-inflammatory and pro-fibrotic effects by proteolytically activating PAR-1 and/or PAR-2. Even more importantly, preclinical experimental data show that mice lacking either receptor are protected against bleomycin-induced pulmonary fibrosis. However, bleomycin-induced pulmonary fibrosis was not completely diminished by pharmacological inhibition of PAR-1 or genetic ablation of either PAR-1 or PAR-2. In the current study, we aimed to assess whether PAR-1 and PAR-2 synergically promote fibrosis progression and thus whether the simultaneous inhibition of PAR-1 and PAR-2 would more efficiently limit pulmonary fibrosis as compared to single receptor inhibition. Strikingly, we show that, both in vitro and in vivo, the simultaneous stimulation or inhibition of PAR-1 and PAR-2 does not lead to additive effects. In fact, we show that the pro-fibrotic effects induced by PAR-1 stimulation require the presence of PAR-2.

The most interesting finding of our current study is the fact that PAR-2 is pivotal for PAR-1-induced fibrotic processes. We show that pharmacological inhibition of PAR-1 does inhibit bleomycin-induced fibrosis in wild type mice but does not further diminish bleomycin-induced fibrosis in PAR-2 deficient mice, as evident from similar reductions in Ashcroft score, α-SMA expression and hydroxyproline content in the lungs. We unraveled the molecular basis for these findings in vitro. We show that PAR-1 dependent fibroblast migration, differentiation and ECM production is abolished in the presence of the specific PAR-2 inhibitor P2pal-18s (Figure 5) and we further confirmed these findings in HLFs. Overall, these results indicate that PAR-2 modulates the activity of PAR-1 thereby inducing profibrotic responses.

In recent years, several studies showed that PAR-1 and PAR-2 might facilitate each other’s activity in different pathophysiological processes. For instance, protective effects of PAR-1 during sepsis require transactivation of PAR-2 signaling pathways.
PAR-2 is required for PAR-1 signalling in pulmonary fibrosis while PAR-2 regulates the PAR-1 hyperplastic response to arterial injury leading to stenosis. Moreover, in tumor biology it is shown that thrombin-induced melanoma cell migration and metastasis are dependent on both PAR-1 and PAR-2 activation. Finally, mammary adenocarcinoma cells lacking PAR-2 failed to express PAI-1 in response to thrombin activation, and a very recent study shows that PAR-1 and PAR-2 act as a functional unit in breast cancer development. Here, we extend these observations by showing cooperative signaling between PAR-1 and PAR-2 in the setting of pulmonary fibrosis (Figure 7).

The mechanism by which PAR-1 interacts with PAR-2 signaling in fibrosis remains elusive. Interestingly, several potential mechanisms have been suggested (excellently reviewed in ). First, it has been described that the thrombin-generated tethered ligand of PAR-1 may transactivate PAR-2. However, P1pal-12 (PAR-1 antagonist) does not prevent thrombin-induced PAR-1 cleavage. Indeed, it is a cell-penetrating pepducin derived from the third intracellular loop of PAR-1 that - once

![Figure 7. Schematic overview of potential mechanisms by which PAR-1 and PAR-2 act in concert to contribute to pulmonary fibrosis.](image-url)

- Fibroblast migration
- Cytokine release
- Fibroblast differentiation
- Extracellular matrix deposition

Bleomycin administration leads to the release of a PAR-1 agonist that subsequently activates PAR-1 on fibroblasts. This activation may subsequently lead to transactivation of PAR-2 or to the production of a PAR-2 agonist thereby inducing profibrotic processes like migration, differentiation and extracellular matrix deposition. As elaborated in the discussion section however, most likely the PAR-1 agonist activates PAR-1/PAR-2 heterodimers thereby inducing the profibrotic responses.
inserted into the plasma membrane interferes with interaction between the receptor and its G-proteins thereby blocking PAR-1 dependent signaling. Consequently, PAR-2 transactivation by the PAR-1 tethered ligand seems not to be the main mechanism in the present setting. An alternative explanation could be that PAR-1 activation induces the expression of a PAR-2 ligand that would subsequently induce fibrosis in a PAR-2 dependent manner. However, this explanation is not very likely because PAR-1 dependent ERK1/2 phosphorylation, which is also partially blocked by PAR-2 inhibition, occurs within minutes. It is difficult to envision that PAR-2 ligands are synthesized during this short time frame. Moreover, conditioned medium of thrombin-treated fibroblasts did not induce PAR-2 dependent fibrotic effects (data not shown). Finally, PAR-1 and PAR-2 may directly interact and form heterodimers that induce different signaling pathways compared to those induced by monomers. In line with such a mechanism, PAR-2 expression is low in quiescent lung fibroblasts but may considerably increase under inflammatory and fibrotic conditions thereby favoring the formation of PAR-1/PAR-2 complexes. Indeed, while PAR-1 expression remains constant on normal and IPF-derived fibroblasts, PAR-2 expression is low in normal fibroblasts but undergoes a dramatic up-regulation in IPF-derived fibroblasts. In line, bleomycin instillation induced PAR-2, and also PAR-1, mRNA expression levels increase in our experimental animals (Fig. S2). In addition, TGF-β stimulations increase PAR-2 levels both on the mRNA and protein level and treatment with thrombin results in an upregulation of PAR-2 mRNA level (data not shown). It is tempting to speculate that this latter notion also explains our observation that PAR-2 inhibition by P2pal-18 only partially blocked thrombin-induced ERK1/2 phosphorylation. The rapid phosphorylation of ERK (within minutes) may still largely be induced by PAR-1 monomers as the PAR-1/PAR-2 complexes have not yet been formed in large quantities. Irrespective the actual mechanism, our data strongly suggest that PAR-1-induced fibrosis is dependent on PAR-2 signaling.

Several issues should be kept in mind when interpreting our data. First, we used a single dose bleomycin model to induce pulmonary fibrosis. Although this model is sometimes criticized not to completely mimic the progression of fibrosis in IPF patients, this model show typical histological patterns, like patchy parenchymal inflammation and interstitial fibrosis, as observed in IPF patients. A recent paper actually shows that bleomycin induces clinically meaningful molecular responses in the lungs of mice mimicking those occurring in the lungs of IPF patients (even in a quantitative manner). Interestingly, the single dose bleomycin model was shown to be as effective
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in terms of producing a more substantial or progressive fibrotic response in the lung as compared to a model of repetitive bleomycin exposures\textsuperscript{42}, which has been argued to be superior of the single dose model\textsuperscript{43}. Despite the fact that there was no significant advantage in using the repetitive bleomycin model instead of the single challenge model, future studies using alternative fibrosis models should obviously validate our findings. Second, as thrombin also activates PAR-4, one may suggest that PAR-4 could also be involved in thrombin-induced fibrosis both \textit{in vitro} and \textit{in vivo}. However, PAR-4 is not expressed by human lung fibroblasts\textsuperscript{39} and several studies show that PAR-4 does not show pro-fibrotic effects after its activation\textsuperscript{44}. In addition, we previously showed that PAR-4 does not modify bleomycin-induced pulmonary fibrosis\textsuperscript{45}. The observed effects can consequently not depend on PAR-4. Moreover, PAR-1 agonist peptide showed similar responses as thrombin (Fig. S3) and the thrombin-induced responses are (almost) completely inhibited by a specific PAR-1 antagonist all suggesting thrombin induces fibrosis in a PAR-1 dependent and PAR-4 independent manner. Third, P1pal-12 (PAR-1 antagonist) treatment was started before bleomycin instillation and one could argue that delayed PAR-1 inhibition may alter our results. However, we previously showed that administration of P1pal-12 at different time points after bleomycin instillation (i.e. either after 1 or 7 days) had similar effects in limiting the development of pulmonary fibrosis as compared to when administration was started before bleomycin instillation\textsuperscript{16}. Fourth, pharmacological inhibition of PAR-1 signaling and genetic ablation of PAR-2 either alone or in combination did significantly reduce pulmonary fibrosis but did not completely prevent fibrosis. Although reducing fibrosis or slowing down its progression may be clinically relevant, future studies need to establish whether PARs are prime candidates for the treatment of pulmonary fibrosis. Irrespective the potential clinical relevance, we highlight a cooperative contribution of PAR-1 and PAR-2 to pulmonary fibrosis.

In conclusion, the simultaneous inhibition of PAR-1 and PAR-2 is not superior to targeting either receptor alone in limiting pulmonary fibrosis. In fact, both \textit{in vitro} and \textit{in vivo}, we show that the pro-fibrotic effects induced by PAR-1 require the presence of PAR-2.
REFERENCES

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Supplementary figures

Supplementary figure 1: (A-B) Wound size of NIH3T3 fibroblast monolayers after treatment with PBS (control), thrombin (10 nM) or thrombin in combination with trypsin (10 nM; (A)) or P2pal-18s (10 µM; (B)) for 18 hours. Trypsin or P2pal-18s treatment was started simultaneously with thrombin treatment (indicated as t=0, or at different time points after thrombin stimulation (either 2, 4, 8 or 12 hours)). Data are expressed as mean±SEM (n=6). ** P<0.01, *** P<0.001. (C-D) Western blot analysis of α-SMA and collagen expression in NIH3T3 cells 24 hours after stimulation with thrombin or thrombin in combination with trypsin (C) or P2pal-18s (D). Tubulin served as a loading control. The timing of trypsin or P2pal-18s treatment is indicated in the figure.
Supplementary figure 2: mRNA expression levels of PAR-1 and PAR-2 in lung homogenates of wildtype mice obtained 14 days after bleomycin or saline instillation. Data are expressed as mean±SEM (n=8).

Supplementary figure 3: (A) Quantification of wound closure of NIH3T3 fibroblast monolayers induced by PAR-1 agonist peptide or thrombin as described in Materials and Methods. Data are expressed as mean±SEM (n=6). ** P<0.01, *** P<0.001. (B) Western blot analysis of collagen and α-SMA expression in NIH3T3 cells 24 hours after stimulation with the indicated concentrations of thrombin or PAR-1 agonist peptide. Tubulin served as a loading control.
Protease activated receptor-1 regulates macrophage-mediated pulmonary fibrosis

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Manuscript submitted for publication.
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 \textit{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. The current paradigm postulates that pulmonary fibrosis results from a chronic epithelial lesions leading to an aberrant wound healing response. Although knowledge of the pathogenesis of pulmonary fibrosis continues to evolve, therapeutics that effectively improve the clinical outcome of IPF are limited. 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. 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. 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. Moreover, PAR-1 activation stimulates fibroblast differentiation and ECM production, whereas PAR-1 seem to synergize with PAR-3 to mediate epithelial-mesenchymal transition of alveolar epithelial cells. In line with these 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.

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. This may be particularly important as macrophages are known to be key regulators in the progression of pulmonary fibrosis. In this context, macrophage influx is an early event following lung injury and macrophages secrete large amounts of profibrotic cytokines like TGF-β. TGF-β on its turn induces fibroblast proliferation and differentiation into myofibroblasts leading to extracellular matrix (ECM) deposition thereby promoting fibrosis.

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 mecha-
nism 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-RCLSSAVANS-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 IMDM, 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.  

*Magnesium 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 before10. 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 before17. 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 (α-SMA), GAPDH (all Santa Cruz, CA), collagen (SouthernBiotech, AL) or p-SMAD2 (Cell Signaling Technology, Boston, MA) at 4°C. All secondary antibodies were horse-radish 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 (1×10^5 CellTrace CFSE labeled for real-time analysis or 2×10^4 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 before. 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% H_2O_2 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 before.

**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’-GGCAACCTAGACGCAGTGAGT-3’ and reverse 5’-TAGCACA-GCGACCTTCCAGA-3’; FX: forward 5’-GACCCATATAAAAGACGGGAC-3’ and reverse 5’-TCCGAAACAAAGAGCTCAGT-3’; Granzyme K: forward 5’-TGAGCC-CATGAAGCACAGCAT-3’ and reverse 5’-TGGCATTTGTTCCCATCTCTCT-3’; Matrix metalloproteinases(MMP)-1: forward 5’-TTACGGCTCATGAACTGGGT-3’ and reverse 5’-GTTGGCTTGGATGGGATTCTG-3’; MMP-13: forward 5’-AACATCATCCCGTGACCTT-3’ and reverse 5’-TTCTCAAGTGAACCGGACG-3’; Kallikrein (KLK)-1: forward 5’-CCCACAACCTGAGACTCT-3’ and reverse 5’-GCTTGAGGTTTCACACACTG-3’; KLK-4: forward 5’-ATCTCTCAGTGCGTGAGCTGAG-3’ and reverse 5’-CTGCCACACTTCTTCTGGTC-3’; KLK-6: forward 5’-GCTTGAGGTTTCACACACTG-3’ and reverse 5’-CATGCCACACTTCTTCTGGTC-3’; GAPDH: forward 5’-CTCATGACCACAGTCCATGC-3’ and reverse 5’-CACATGGGGTGGAGGAGTC-3’; TBP: forward 5’-GGAGAATCATGAGACCAGA-3’ and reverse 5’-GATGGGAATTCCAGGAGTC-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. As macrophage recruitment in response to chemoattractant production by injured epithelial cells is a key process in fibrosis, 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 analysed 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
PAR-1 regulates macrophage-mediated pulmonary fibrosis

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. 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 TGBR1 and TGBR1I. 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.
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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REFERENCES


Chapter 6

High endogenous activated protein C levels afford protection against bleomycin-induced pulmonary fibrosis

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Manuscript submitted for publication
ABSTRACT

Background
Coagulation activation accompanied by reduced anticoagulant activity is a key characteristic of patients with idiopathic pulmonary fibrosis (IPF) and the consequent hypercoagulability may play an important role in the pathogenesis of IPF. Although the importance of coagulation activation in IPF has been well studied, the potential relevance of diminished endogenous anticoagulant activity in IPF progression remains elusive.

Aims
To assess the importance of the endogenous anticoagulant protein C pathway on disease progression during bleomycin-induced pulmonary fibrosis and to assess the underlying mechanism by which APC may modify fibrosis.

Methods
Wild-type mice and mice with high endogenous APC levels (APC\text{high}) were intranasally instilled with bleomycin. After 14 and 28 days, pulmonary fibrosis was assessed by hydroxyproline and histochemical analysis. Macrophage recruitment to lungs of bleomycin-instilled mice was assessed immunohistochemically. In vitro, macrophage migration was analyzed by trans-well migration assays after stimulation with thrombin and/or recombinant APC.

Results
14 days after bleomycin instillation, APC\text{high} mice developed pulmonary fibrosis to a similar degree as wild type mice as evident from similar Ashcroft scores and hydroxyproline levels. Interestingly however, Ashcroft scores as well as lung hydroxyproline levels were significantly lower in APC\text{high} mice than in wild type mice on day 28. The reduction in fibrosis in APC\text{high} mice was accompanied by reduced macrophage numbers in their lungs and subsequent in vitro experiments show that APC inhibits thrombin-dependent macrophage migration.

Conclusion
High endogenous APC levels inhibit the progression of bleomycin-induced pulmonary fibrosis. We suggest that APC modifies pulmonary fibrosis by limiting macrophage recruitment.
INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a specific form of fibrosing idiopathic interstitial pneumonia, characterized by progressive and irreversible pathological changes, with a median survival of 3 years.1-2 IPF comprises a group of conditions characterized by the formation and proliferation of (myo)fibroblast foci and exaggerated extracellular matrix (ECM) accumulation.3 The current pathogenesis paradigm suggests that pulmonary fibrogenesis results from an uncontrolled wound healing response that is initiated after repeated epithelium injury.4

Beyond its primary role in hemostasis, coagulation activation in response to tissue injury seems to be a critical contributor in the pathogenesis of fibrotic lung disorders.5 A hypercoagulable state is commonly observed in IPF patients 6,7 and coagulation factors, such as tissue factor (TF), factor (F)VII, FXa and thrombin, are increased in these patients. All these individual coagulation factors exert pro-fibrotic cellular effects through activation of the cell surface protease-activated receptors (PARs). Indeed, FVIIa may contribute to the development and/or progression of IPF by activating PAR-2, whereas FXa induces pro-fibrotic effects via either PAR-1 or PAR-2.8-11 Thrombin, as the best-described profibrotic coagulation factor, activates PAR-1 leading to myofibroblast accumulation and subsequent fibrotic responses of lung (myo)fibroblasts, such as proliferation, migration and ECM synthesis (e.g. collagen).12-14 The potential importance of coagulation factors in IPF is underscored by the fact that inhibiting coagulation limits pulmonary fibrosis in preclinical experimental animal models.10,15-17

The hypercoagulable state observed in IPF patients may not only be due to increased coagulation factor expression but may at least in part result from reduced anticoagulant activity. Indeed, the balance between pro- and anticoagulant pathways is compromised in patients with IPF, and especially the anticoagulant protein C pathway seems down regulated.18 Protein C, once activated by the thrombin-thrombomodulin complex, prevents excessive coagulation via inactivation of factors Va and VIIIa.19-20 Next to inhibiting coagulation, activated protein C (APC) exhibits anti-inflammatory and vascular protective effects through PAR-1, the same receptor activated by thrombin.21 In the context of lung injury, endogenous APC inhibits infection-induced coagulation activation22 and APC overexpression modifies neutrophil recruitment during experimental pneu-
mococcal pneumonia. Moreover, exogenous APC instillation limits bleomycin-induced pulmonary fibrosis probably through its anti-inflammatory activity.

Despite the clear potential importance of endogenous anticoagulant activity in IPF progression, previous studies mainly focussed on the importance of coagulation activation whereas the relevance of disturbed anticoagulant pathways in IPF has not been addressed. In the present study, we consequently aimed to assess the significance of the endogenous anticoagulant protein C pathway in IPF. To this end, we subjected mice with different endogenous APC levels to the preclinical bleomycin induced pulmonary fibrosis model.

**MATERIALS AND METHODS**

**Animal Model of Pulmonary Fibrosis**

Wild type (WT) C57Bl/6 mice were purchased from Charles River (Someren, Netherlands). APC\textsuperscript{high} mice, with plasma APC levels almost forty times higher than in WT mice, were generated and backcrossed to a C57BL/6 genetic background as described\textsuperscript{25} and bred at the animal care facility of the Academic Medical Center. All procedures were performed on eight to ten-week-old mice, and in accordance with the Institutional Standards for Humane Care and Use of Laboratory Animals. Experiments were approved by the Animal Care and Use Committee of the Academic Medical Center (Amsterdam, Netherlands). Bleomycin (Sigma, St-Louis, MO) was administered by intranasal instillation (1 mg/kg body weight) under anesthesia. Mice were sacrificed 14 or 28 days after bleomycin instillation, following which the left lungs were excised for histological analysis whereas the right lungs were homogenized for hydroxyproline and cytokine assays.

**Cells and Reagents**

Murine NIH3T3 fibroblasts and RAW264.7 macrophages were cultured in DMEM and IMDM, respectively, supplemented with 10% fetal calf serum (FCS). 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. Thrombin was from Sigma (St-Louis, MO), recombinant human activated protein C (rhAPC; Xigris) was obtained from Eli Lilly (Houten, The Netherlands) and recombinant mouse monocyte chemotactic protein (MCP-1) was from R&D systems.
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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 24 hours according to routine procedures.26

Wound Scratch Assay
Scratch assays were performed essentially as described before.27 In detail, 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 with 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 without (negative control) or with 10 μM thrombin or APC. 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.

Western Blot
Fibroblasts were seeded in 12-well plates in DMEM supplemented with 10% FCS. After serum starvation for 4 hours, the cells were incubated with serum-free medium (negative control) with or without 10 μM thrombin or APC. Twenty four hours later, cells were lysed in Laemmli lysis buffer and Western blots were performed as described before.27 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 (both Santa Cruz, CA) or collagen (SouthernBiotech, AL) 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 2×10⁴ RAW264.7 cells 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/APC, and the inserts were incubated at 37°C for 10 hours in serum-free medium with MCP-1 as chemoattractant. 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. 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.

(Immunoh)histological Analysis
Four-μm sections were deparaffinized and rehydrated. Slides were stained with hematoxylin and eosin (H&E) according to routine procedures. In H&E stainings, the severity of fibrosis was assessed according to the Ashcroft scoring system using a 100× magnification as described before. Two independent observers, blinded to the treatment group, scored the average Ashcroft score of 10 fields of each lung section as calculated by averaging the individual field scores. For F4/80 staining, endogenous peroxidase activity was quenched with 0.3% H2O2 in methanol and the F4/80 antibody was incubated for 24 hours at 4°C (1:500, 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 staining 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.

ELISA
Active transforming growth factor-beta 1 (TGF-β1) was measured using a Mouse DuoSet kit (R&D systems, UK) as per the manufacturer’s instructions.

Hydroxyproline Assay
Hydroxyproline analysis was performed by the hydroxyproline assay kit as per the manufacturer’s instructions (Sigma, Netherlands) and as described before.

Statistics
Statistical analyses were conducted using GraphPad Prism version 5.00 (GraphPad software, San Diego, CA, USA). Data were expressed as means ± SEM. Compar
isons 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

**APC**\textsuperscript{high} mice are protected from bleomycin-induced pulmonary fibrosis

To study the effect of high endogenous APC levels during the progression of pulmonary fibrosis, APC\textsuperscript{high} and wild type mice were subjected to bleomycin-induced fibrosis for either 14 or 28 days. As shown in Figure 1, bleomycin-induced extensive patchy areas of fibrosis were present to a similar extent in both wild type and APC\textsuperscript{high} mice on day 14. During disease progression, the inflam-

![Figure 1. High endogenous APC levels limit the progression of pulmonary fibrosis.](image-url)

(A) Representative pictures of lungs of mice 14 and 28 days after saline bleomycin instillation (100× magnification). (B) Quantification of pulmonary fibrosis 14 and 28 days after bleomycin instillation using the Ashcroft score. (C) Collagen content in lung homogenates obtained 14 or 28 days after bleomycin instillation. (D) Transforming growth factor (TGF)-β1 levels in lung homogenates obtained 14 or 28 days after bleomycin instillation. Data are expressed as mean ± SEM (n=8 per group, *p<0.05 and **P<0.01).
matory and fibrotic effects culminated in severe pulmonary fibrosis at day 28 in wild type mice (Figure 1A). Interestingly however, the increase in pulmonary fibrosis over time was not observed in APC\textsuperscript{high} mice, and indeed Ashcroft scores are similar in APC\textsuperscript{high} mice at day 14 and day 28 (Figure 1B). In line with the Ashcroft scores, lung hydroxyproline levels did not show differences between APC\textsuperscript{high} and wild type mice on day 14, whereas these levels were significantly higher in wild type mice at 28 days after bleomycin instillation (Figure 1C). Similar to Ashcroft scores and hydroxyproline levels, TGF-\(\beta\)\textsubscript{1} concentrations are relatively low in wild type and APC\textsuperscript{high} mice at day 14. During disease progression, TGF-\(\beta\)\textsubscript{1} levels increase in both wild type and APC\textsuperscript{high} mice although the increase is clearly reduced in APC\textsuperscript{high} mice at day 28 (Figure 1D). Overall, these results show that high endogenous APC levels provide protection against bleomycin-induced pulmonary fibrosis.

**APC inhibits thrombin-induced monocyte/macrophage recruitment during pulmonary fibrosis**

Macrophage recruitment in response to inflammatory mediators produced by injured epithelial cells is a key process in fibrosis.\textsuperscript{30} Moreover, we recently showed that thrombin-dependent PAR-1 signaling potentiates macrophage migration towards bleomycin-treated epithelial cells, thereby driving pulmonary fibrosis (manuscript submitted). Consequently, we determined macrophage numbers in fibrotic lungs of both wild type and APC\textsuperscript{high} mice. As shown in Figure 2A, F4/80 positive macrophages were diffusely present in lungs of wild type mice 28 days after bleomycin instillation. In APC\textsuperscript{high} mice, macrophage numbers are reduced by around 50\% as compared to wild type mice (Figure 2A-B). Interestingly, the reduced macrophage numbers in APC\textsuperscript{high} mice at day 28 after bleomycin-instillation are not due to a direct effect of APC on macrophage migration. As shown in Figure 2C, RAW264.7 macrophage migration towards MCP-1 in vitro was not modified by APC treatment. APC did however (almost) completely prevent thrombin-induced RAW264.7 cell migration towards MCP-1 (Figure 2D). Together, the results suggest that APC inhibits thrombin-induced macrophage migration during pulmonary fibrosis.

**APC does not interfere with thrombin-induced profibrotic effects on fibroblasts**

Thrombin induces several profibrotic processes on fibroblasts, such as fibroblast proliferation, migration, differentiation and ECM production.\textsuperscript{13,16} Therefore we
assessed whether APC may, next to reducing thrombin-dependent macrophage recruitment, also modify pulmonary fibrosis by inhibiting thrombin-dependent profibrotic responses in fibroblasts. As shown in Figure 3A-B, as opposed to APC treatment, thrombin stimulation increased fibroblast proliferation and migration. Interestingly, APC did not modify these thrombin-induced profibrotic responses. In line, APC also did not modify thrombin-induced fibroblast
differentiation and ECM synthesis (Figure 3C). Hence, APC neither directly affects fibrotic responses of fibroblasts nor does it limit thrombin-induced profibrotic effects of fibroblasts.

**DISCUSSION**

Coagulation activation is a frequent phenomenon in IPF and IPF patients are more than four times more likely to have a hypercoagulable state than general population controls. The presence of a hypercoagulable state is not only associated with disease severity at diagnosis but also adversely impacts on survival of IPF patients. At least in part, hypercoagulability in IPF patients may be due to
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reduced anticoagulant activity, and here we consequently addressed the importance of the endogenous protein C pathway. We show that endogenous APC modifies disease progression and affords protection against bleomycin-induced pulmonary fibrosis.

Mice expressing high endogenous APC levels (i.e. APC\textsuperscript{high} mice) are protected from bleomycin-induced pulmonary fibrosis as evident from reduced Ashcroft scores, hydroxyproline concentrations and TGF-\(\beta\)1 levels at day 28 after bleomycin instillation. The reduction in fibrosis in APC\textsuperscript{high} mice was accompanied by significantly decreased macrophage numbers in their lungs. This may be particularly important as macrophage recruitment in response to lung epithelial cell injury is a key process in pulmonary fibrosis. Recruited macrophages produce profibrotic cytokines like TGF-\(\beta\) that activate fibroblasts, thereby potentiating their profibrotic responses.\textsuperscript{4, 30}

It is tempting to speculate that the reduction in pulmonary fibrosis observed at day 28 is explained by a direct inhibitory effect of endogenous APC on macrophage recruitment. Indeed, APC has previously been shown to inhibit migration of lymphocytes toward IL-8, RANTES and MCP-1\textsuperscript{31} and to limit migration and activation of rheumatoid arthritis monocytes via EPCR.\textsuperscript{32} Here, we show that APC does not directly inhibit migration of RAW264.7 macrophages towards MCP-1 by itself but instead blocks thrombin-induced macrophage migration. Most likely, APC competes for PAR-1 cleavage, thereby limiting thrombin-dependent PAR-1 signaling and subsequent macrophage migration. Such competition between APC and thrombin is well-known and APC- or thrombin-induced PAR-1 cleavage leads to distinct or even opposite downstream signaling events. For example, APC switches thrombin-induced PAR-1 signaling from a disruptive to a protective effect in human umbilical vein endothelial cells.\textsuperscript{21, 33}

As opposed to modifying thrombin-induced macrophage migration, APC does not affect thrombin-induced pro-fibrotic responses of fibroblasts, like fibroblast proliferation, migration, differentiation and collagen deposition. This may be surprising at a first glance, but this is most likely explained by the fact that fibroblasts do not express EPCR, which is actually essential for APC-dependent PAR-1 cleavage.\textsuperscript{34, 35}
In line with our data showing that endogenous APC limits bleomycin-induced pulmonary fibrosis, intratracheal administration of exogenous human APC reduced the progression of pulmonary fibrosis as well. Interestingly however, exogenous APC seemed more effective in reducing fibrosis at an earlier time point as hydroxyproline levels were already reduced 14 days after bleomycin infusion in case of exogenous APC administration. Although we do not have a definitive explanation for the increased efficacy of exogenous APC, it may well be due to higher initial concentrations of exogenous versus endogenous APC. Irrespective of the precise underlying molecular mechanisms, both studies emphasize the importance of the anticoagulant protein C pathway in disease progression of IPF and the availability of endogenous APC may thus be an important clinical and pharmacological parameter in patients with IPF. Consequently, preservation and/or restoration of endogenous APC generation might be an interesting target for limiting IPF progression.

In conclusion, the present study reveals that endogenous APC inhibits the progression of bleomycin-induced pulmonary fibrosis. We suggest that APC limits pulmonary fibrosis due to the inhibitory effect of APC on thrombin-induced macrophage recruitment rather than any direct antifibrotic effect of APC on fibroblasts.
High APC levels limit bleomycin-induced pulmonary fibrosis

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33. Bae JS, Rezaie AR. Protease activated receptor 1 (PAR-1) activation by thrombin is protective in human pulmonary artery endothelial cells if endothelial protein C receptor is occupied by its natural ligand. Thromb Haemost. 2008;100:101-9.
Chapter 7

Summary and general discussion
Nederlandse Samenvatting
Acknowledgements
PhD portfolio
List of publications
SUMMARY

Despite the high standards of modern medical care and the fast emerging anti-fibrotic agents, mortality rates for idiopathic pulmonary fibrosis (IPF) are still increasing over the years. At a conservative estimate, there are approximate 0.2 million patients suffering from IPF living in the Europe.

In this thesis, Chapter 1 gives an overview of IPF and introduces coagulation factors and their receptors, PARs, as potential contributors to fibrotic processes. Anticoagulant treatment in IPF is also discussed and to the overall conclusion of chapter 1 is that inhibition of PARs may be specific and effective therapeutic interventions for fibrotic disorders. We therefore set out to address this hypothesis and we show that pharmacological targeting of PARs with pepducin P1pal-12 (PAR-1 antagonist in Chapter 2) or P2pal-18S (PAR-2 antagonist in Chapter 3) effectively blocks PAR-1 or PAR-2 induced pro-fibrotic responses in fibroblasts and limits bleomycin-induced pulmonary fibrosis, even when administered 7 days after the induction of fibrosis. Importantly, in the experimental bleomycin model, pulmonary fibrosis is not completely abolished in mice that harbor deficiencies for either PAR-1 or PAR-2. Therefore, in Chapter 4, we studied the simultaneous inhibition of PAR-1 and PAR-2 in pulmonary fibrosis and we show that treatment of PAR-2 deficient mice with the PAR-1 antagonist P1pal-12 did not further reduce bleomycin-induced lung fibrosis as compared to wild type mice treated with P1pal-12 or PAR-2 deficient mice. Interestingly, PAR-1-induced pro-fibrotic effects in vitro are abolished in the presence of the specific PAR-2 inhibitor. We thus postulate that the pro-fibrotic effects induced by PAR-1 require the presence of PAR-2. Chapter 5 shows that PAR-1 on macrophages potentiate their recruitment towards injured lung epithelial cells. Once recruited, macrophages secrete a PAR-1 agonist, most likely FXa that acts upon fibroblasts leading to the production and activation of latent TGF-β which subsequently drives fibroblast migration, differentiation into myofibroblasts and ECM deposition. Finally, in Chapter 6 we addressed the importance of the endogenous anticoagulant system, especially APC, in the development of pulmonary fibrosis. We show that high endogenous APC levels reduce bleomycin-induced pulmonary fibrosis and this reduction in pulmonary fibrosis may be explained by the inhibitory effect of endogenous APC on macrophage recruitment during the fibrotic phase.
GENERAL DISCUSSION

With the research performed in this thesis, we sought to assess the potential clinical relevance of targeting PARs in pulmonary fibrosis and to identify potential effectors and mechanisms which contribute to the pathogenesis of IPF.

Most importantly, we show that pharmacological inhibition of PARs, even starting after the onset of fibrosis, affords protection against bleomycin-induced pulmonary fibrosis. As current treatment options for IPF are limited\(^2\), these findings may be particularly interesting for future medical interventions in IPF patients. Importantly, PAR-1 inhibition may be feasible on a short notice, as the PAR-1 inhibitor Zontivity just obtained FDA approval for antiplatelet therapy of patients with a previous myocardial infarction or leg ischemia\(^3\). Its application along with standard therapy effectively limits thrombotic cardiovascular events, yet Zontivity increases the rate of moderate to severe bleeding in a selection of patients\(^4\). Although PAR-1 inhibition thus seems an attractive strategy to pursue in IPF, potential bleeding complications should be taken into consideration and patients should be properly monitored.

Based on the notion that PAR-1 seems to drive pulmonary fibrosis in a PAR-2 dependent manner, it is tempting to speculate that blocking PAR-2 may be a better treatment strategy for IPF than using Zontivity. In addition, PAR-2 inhibition does not interfere with platelet activation as does PAR-1 inhibition. Therefore, targeting PAR-2 will not cause bleeding complications and may be the preferred treatment option. Unfortunately, there are no PAR-2 inhibitors currently available for clinical intervention.

PAR-1 is activated by coagulation factor Xa and thrombin, whereas PAR-2 is activated by the tissue factor-FVIIa complex and by factor Xa\(^5\). Consequently, anticoagulant treatment would presumably be effective in IPF as it blocks PAR-1 and PAR-2 simultaneously. Importantly however, anticoagulant therapy in the setting of IPF remains a matter of debate mainly due to the lack of effect of warfarin in recent clinical trials\(^6-7\). Compared to anticoagulant therapy, targeting PARs may be a more efficient (and safe) approach for limiting fibrosis, as PARs are not only activated by coagulation factors. Activation of PARs by several other proteases can also induce profibrotic effects, at least \textit{in vitro}. For example, Granzyme K is found to induce pro-inflammatory cytokine secretion and lung
fibroblast proliferation through PAR-1 whereas trypsin is well known to trigger fibroproliferative effects via PAR-28-9.

Our data that PAR inhibition limits pulmonary fibrosis may not only benefit IPF patients but may also have clinical impact in other disorders which involve PARs signaling. Indeed, PARs are suggested to contribute to (among others) heart failure, renal, liver and skin fibrosis10-13. Whether the application of PAR inhibitors will benefit patients suffering from these PAR-related human disorders needs however to be further studied.

The functional relevance of coagulation activation in the pathogenesis of IPF has received much attention, both in preclinical as well as in clinical studies. Surprisingly however, the endogenous anticoagulant pathways, such as APC pathway, received relatively little attention in IPF. In the current thesis, we highlight the potential importance of the endogenous anticoagulant protein C pathway by showing that high APC levels inhibit the progression of bleomycin-induced pulmonary fibrosis. In line, recombinant APC administration also limits bleomycin-induced pulmonary fibrosis, and PC activation is suppressed in IPF patients14-15. Moreover, infusion of APC was recently shown to reduce pulmonary coagulopathy and to decrease lung injury caused by acute respiratory distress syndrome16, suggesting APC may also attenuate lung injury in patients with pulmonary fibrosis. Increasing APC levels, either by the administration of APC or zymogen PC, may thus be an alternative strategy for the treatment of IPF. Importantly, recombinant human APC (Xigris) is well known for the treatment of sepsis and recombinant APC would thus be easily available for a clinical trial in IPF. However, the benefit-to-risk ratio of Xigris in patients with sepsis remained controversial for many years. Indeed, the most recent clinical trial on Xigris showed no evidence of benefit for treating patients with severe sepsis or septic shock. Consequently, Xigris was withdrawn from the worldwide market and is not available anymore17-18.

In addition to pinpointing the APC pathway as a potential therapeutic strategy in IPF, our data showing that endogenous APC limits pulmonary fibrosis may also explain (at least in part) the lack of efficacy of warfarin in improving IPF related symptoms. Indeed, warfarin inhibits the activity of all vitamin K dependent coagulation factors including APC. Warfarin would thus limit fibrosis by targeting the pro-coagulant factors but could potentiate fibrosis by inhibiting APC. Therefore,
alternative anticoagulants targeting the pro-coagulant factors without affecting endogenous anticoagulant pathways may have clinical potential.

Overall, we provided several lines of evidence to support the importance of PARs in the pathogenesis of IPF and suggest that targeting PARs, its endogenous agonists or the APC pathway may be novel therapeutic approaches for treating this disease.

REFERENCES

1. http://www.pulmonary-fibrosis.net/


**NEDERLANDSE SAMENVATTING**

Ondanks de hoge standaard van de moderne medische zorg en de snelle opkomst van anti-fibrotische medicijnen, neemt het sterftecijfer voor mensen met idiopathische longfibrose (IPF) nog steeds toe. Met een algemene schatting zijn er ongeveer 0,2 miljoen patiënten in Europa die lijden aan IPF. In dit proefschrift, geeft Hoofdstuk 1 een overzicht over IPF en introduceert het stollingsfactoren en hun receptoren, protease geactiveerde receptoren (PAR’s), als potentiële mediatoren van fibrotische processen. Antistollingsbehandeling in IPF wordt ook besproken en de algemene conclusie van hoofdstuk 1 is dat de remming van de PAR’s een specifieke en effectieve therapeutische interventie zou kunnen zijn voor fibrotische aandoeningen. Wij hebben er in dit proefschrift dan ook voor gekozen om ons te richten op deze hypothese en we laten zien dat de farmacologische remming van PARs met P1pal-12 (PAR-1 antagonist in **hoofdstuk 2**) of P2pal-18S (PAR-2 antagonist in **hoofdstuk 3**) effectief PAR-1 of PAR-2 geïnduceerde pro-fibrotische reacties blokkeert in fibroblasten en verder bleomycine-geïnduceerde longfibrose beperkt. Dit laatste is zelfs het geval indien de PAR remmers worden toegediend 7 dagen na de inductie van fibrose. Belangrijk echter, in het experimentele bleomycine model wordt longfibrose niet volledig voorkomen in muizen die geen PAR-1 of PAR-2 tot expressie brengen. Daarom hebben we in **hoofdstuk 4** de gelijktijdige remming van PAR-1 en PAR-2 in longfibrose bestudeert en laten we zien dat de behandeling van de PAR-2 deficiënte muizen met de PAR-1 antagonist P1pal-12 de bleomycine-geïnduceerde long fibrose niet verder verminderd in vergelijking met wild type muizen die zijn behandeld met P1pal-12 of PAR-2 deficiënte muizen. Interessant genoeg worden de PAR-1-geïnduceerde pro-fibrotische effecten in vitro geremd door de specifieke PAR-2 inhibitor P2pal-18. We concluderen in hoofdstuk 4 daarom dat de pro-fibrotische effecten geïnduceerd door PAR-1 de aanwezigheid van PAR-2 vereisen. **Hoofdstuk 5** laat zien dat PAR-1 op macrofagen hun migratie richting gewonde longepitheelcellen versterkt. Eenmaal in de long aangekomen, scheiden de macrofagen een PAR-1 agonist, waarschijnlijk factor Xa, uit die fibroblasten activeert wat leidt tot de productie en activering van latent TGF-β, wat vervolgens fibroblasten aanzet tot tot differentiatie in myofibroblasten en tot de afzetting van extracellulaire matrix productie. Tenslotte wordt er in **hoofdstuk 6** ingegaan op de betekenis van het endogene anticoagulante systeem, voornamelijk geactiveerd proteïne C (APC), in de ontwikkeling van longfibrose. We laten zien dat hoge endogene APC niveaus bleomycine-geïnduceerde longfibrose verlagen en deze vermindering in longfibrose kan worden verklaard door het remmende effect van endogeen APC op macrofaag migratietijds de fibrotische fase.
Acknowledgements / 致谢辞

At the very end of this adventurous journey, I would like to thank all the people who contributed to this achievement and accompanied me throughout this experience.

My first big thank you goes to my promoters, Tom, Arnold and Keren, for accepting me to this project and guiding me through to the end. Arnold, Thank you for always being there and for giving your helpful support. I really abused your “open door” policy during the starting period. Whenever I would receive a rejection from a journal or continuously get unexpected results from experiments (e.g. MTTs.) the first thing you would say to me is “tja, that’s life.” In the beginning I felt confused about this quote of yours, however as time passed by I got more and more used to it and it would somehow amuse me and cheer me up. I consider myself lucky to find you as my supervisor because you can always straighten things out for me with your great mind in combination with your kind personality. Keren, although you have had to face a lot of difficult issues in terms of health and the distance between Paris and Amsterdam, somehow you still always found a way to put maximum effort in supervising me. So what also inspires me is your resilience and perseverance and I am glad that we also shared our lives with each other through tons of emails, so that I have been able to get to know you as a close friend. As it does with every person, sometimes the motivation and passion fades away at times. However, the love you hold towards your profession is infectious and inspires those around you as it has with me. Importantly, it doesn’t matter how big your group is growing into, it has been an honor for me that I am always your first PhD student. I hope to see more and more of us joining you on this path.

I want to thank all the co-authors who contributed to my work. I could not have gotten so far without your professional help. Jan, thank you very much for always handling the histological scores of my endless slides and being so efficient
and specific towards my questions. JanWillem, thank you for your patience and that you are always ready to offer help. Joost and Marieke, you guys have the magic touch for handling animal, thank you for always taking good care of my mice and for the excellent performance of every experiment of mine. Bruno, thank you for your generous help and encouragement.

I would also like to thank the committee members for putting your time and effort in evaluating my thesis.

Eelco and Vivi, thank you for the nice formatting and the cover design of my thesis, the book could not have been made without your help.

Monique, thank you for your efficient ordering and tracking skills, I can always find solutions from you, especially when I bother you with my repeated question: when will my order arrive? Regina, thank you for all the slides staining that you have done for me. Heleen and Monique, thank you for always having a keen eye for the smallest details and for supporting me and making everything easier.

People from the Stolling group, thank you for the pleasant environment at work. Maaike and Kun thank you for all the coffee breaks and lunch times, it was fun. Hella, besides all the time we spent together, I want to also thank you for listening and sharing all the gossips, stories and experiences. Lab life is better with your company and your help with keeping the lab in order.

Teletubbies: Kaushal and Luigi, you guys totally complete each other. You are hilarious and thank you for making the whole scientific journey full of laughter and joy. I wish the Italian lab will be there one day, so the two of you can chill and fish and grow old together there, and live happily ever after. Akueni: “If you put your chair a bit that way, then people will pass by more easily” I remember that is the first thing you said to me when I placed my chair in F0 office. This was a lame starting line but then everything after that became much better. I appreciate your support when things were tough and I wish the same to you that
the future holds greater things for you no matter which path you choose to pave.

Dita: I doubted whether I should pick you as my paranimfen, since I was afraid that I will be overwhelmed with your passion and enthusiasm. It turns out that these are the exact things that I want and need. I appreciate you as a good friend because you always show your kindness and caring towards me. I also thank you for putting in effort and time in helping me to prepare all the stuff. As a noodle buddy, thank you for always backing me up with your healthy vegetarian noodle with extra spicy flavor. :D Finally, for all the teletubbies “You have to learn the rules of the game. And then you have to play better than anyone else.” (Albert Einstein)

Yet I save the biggest thank you for my family, while there is no word that can describe my appreciation enough towards them. I把最后的感谢留给我的家人，短短几句话并不能说清楚你们对我的重要。尤其是我的爸爸妈妈，你们教我做人，伴我成长，以身作则教会我什么是勤劳，正直和责任感，总是不遗余力地支持我的梦想，无条件地满足我的所有需要。我的成就无论大小总让你们觉得自豪和骄傲，但对于我来说，我只是踩着你们的足迹，一路追随着你们的背影。小呆，谢谢你给我的幸福，你满足了我对人生最大的期望。
PHD portfolio

Name PhD student: Cong Lin
PhD period: September 2010 - September 2014
Name PhD supervisor: Prof.dr.T.van der Poll

1. PhD training

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Protease-activated receptor (PAR)-2 is required for PAR-1 signalling in pulmonary fibrosis (oral).

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**3. Parameters of Esteem**

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List of publications


