PAI-1 is an essential component of the pulmonary host response during *Pseudomonas aeruginosa* pneumonia in mice

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ABSTRACT

Rationale Elevated plasma and bronchoalveolar lavage fluid plasminogen activator inhibitor 1 (PAI-1) levels are associated with adverse clinical outcome in patients with pneumonia caused by *Pseudomonas aeruginosa*. However, whether PAI-1 plays a pathogenic role in the breakdown of the alveolar—capillary barrier caused by *P aeruginosa* is unknown.

Objectives The role of PAI-1 in pulmonary host defence and survival during *P aeruginosa* pneumonia in mice was tested. The in vitro mechanisms by which *P aeruginosa* causes PAI-1 gene and protein expression in lung endothelial and epithelial cells were also examined.

Methods and results PAI-1 null and wild-type mice that were pretreated with the PAI-1 inhibitor Tiplaxtinin had a significantly lower increase in lung vascular permeability than wild-type littermates after the airspace instillation of 1×10^7 colony-forming units (CFU) of *P aeruginosa* bacteria. Furthermore, *P aeruginosa* in vitro induced the expression of the PAI-1 gene and protein in a TLR4/p38/RhoA/NF-κB (Toll-like receptor 4/p38/RhoA/nuclear factor-κB) manner in lung endothelial and alveolar epithelial cells. However, in vivo disruption of PAI-1 signalling was associated with higher mortality at 24 h (p<0.03) and higher bacterial burden in the lungs secondary to decreased neutrophil migration into the distal airspace in response to *P aeruginosa*.

Conclusions The results indicate that PAI-1 is a critical mediator that controls the development of the early lung inflammation that is required for the activation of the later innate immune response necessary for the eradication of *P aeruginosa* from the distal airspaces of the lung.

Key messages

What is the key question?
► The role of PAI-1 in pulmonary host defence and survival during *P aeruginosa* pneumonia in mice is not known.

What is the bottom line?
► PAI-1 is a critical mediator that controls the development of the early lung inflammation that is required for the activation of the later innate immune response necessary for the eradication of *P aeruginosa* from the distal airspaces of the lung.

Why read on?
► Although associated with an early attenuation of the severity of pulmonary oedema formation, in vivo disruption of PAI-1 signalling is associated with higher mortality at 24 h secondary to decreased neutrophil migration into the distal airspace in response to *P aeruginosa*.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen that causes lethal pneumonia in immunocompromised individuals and in critically ill patients.[1 2] *P aeruginosa* pneumonia is characterised by a shift in the balance between procoagulant and anticoagulant/fibrinolytic activity in the alveoli followed by intra-alveolar fibrin deposition. Early procoagulant activity in the alveolar space may exert beneficial effects by decreasing the protein leakage through the alveolar—capillary barrier caused by *P aeruginosa*. However, excessive fibrin deposition causes deleterious effects by increasing the lung inflammatory response and vascular permeability. When fibrin is present in the alveolar space, it is usually degraded by plasmin, a proteolytic enzyme that is present in the lung tissue as an inactive zymogen. However, decreased fibrinolytic activity has been reported in the airspaces of the lungs of patients with *P aeruginosa* pneumonia.[3]

The plasminogen activator inhibitor 1 (PAI-1), a member of the serine protease inhibitors superfamily,[4] which is expressed by endothelial cells, epithelial cells, monocytes and macrophages, is the principal inhibitor of tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) and thus inhibits fibrinolysis. PAI-1 levels have been shown to be elevated in the airspace and plasma of patients with ventilator-associated pneumonia (VAP), aspiration pneumonia and acute lung injury (ALI),[5—7] and to correlate with severe disease and adverse clinical outcome.[8] Furthermore, bronchoalveolar lavage (BAL) fluid levels of PAI-1 are higher in patients with *P aeruginosa*-induced VAP than those found in other infections[9] and increased BAL levels of PAI-1 were associated with increased mortality in a cohort of intensive care patients with *P aeruginosa* VAP,[10] suggesting a role for PAI-1 in the development of severe *P aeruginosa* VAP. However, these clinical associations do not establish a direct causal link between PAI-1 and the development of more severe *P aeruginosa* pneumonia, particularly because

contrasting results have been reported with PAI-1 gene deletion in several experimental models of bacterial pneumonia in mice.[11][12] PAI-1 gene deletion has also been shown to be protective in non-infectious experimental models of lung injury induced by ischaemia—reperfusion or hyperoxia.[13][14] Thus, it is not clear whether PAI-1 itself is beneficial or deleterious for the host during *P aeruginosa* pneumonia, and answering this question constitutes the goal of the present study.

MATERIAL AND METHODS
More details are available in the online supplement.

Reagents
All cell culture media were prepared by the University of California San Francisco Cell Culture Facility using deionised water and analytical grade reagents.

Cell culture
Bovine pulmonary arterial endothelial cells (BPAECs; ATCC, CCL-209; passages all <8) were cultured, as previously described.[15]

Human lung microvascular endothelial cells (HMVECs; Lonza; CC-064) were cultured either on transwells (Costar 3495, 2×10^5 cells per filter) for 4 days or in 6-well plates until they formed confluent monolayers.

Primary rat alveolar epithelial type II (ATII) cells were isolated and cultured as previously described.[16]

Preparation of *P aeruginosa*
The wild-type, PAK strain of *P aeruginosa* was a kind gift from Dr Stephen Lory at Harvard University, Massachusetts. The PA103 strain of *P aeruginosa* was generously provided by Dr Dara Frank at the Medical College of Wisconsin. The preparation of *P aeruginosa* was performed as previously described.[15]

Measurement of transendothelial albumin flux
Transendothelial albumin flux across BPAECs and HMVECs was measured as previously described.[15]

Western blot analysis
Western blot analysis was performed as described previously.[17]

Primers and probes
Real-time reverse transcription—PCR primers and probes were designed using Primer Express software (PE-Applied Biosystems, Warrington, UK) and purchased from Biosearch Technologies Novato, CA, USA.

Quantitative real-time RT—PCR
Quantitative real-time RT—PCR analyses were performed as described previously.[17]

RhoA and Rac-1 activity assay in cells
Rac-1 and RhoA activities were determined using a luminescence-based G-LISA Rac-1 and RhoA activation assay (Cytoskeleton, Denver, Colorado, USA).

Cell treatment with exogenous PAI-1
This was performed as described in our previous study.[18]

Cell viability assay
Cell viability was measured by the Alamar Blue assay after exposure to the various experimental conditions.

Mice
Wild-type C57BL/6 (C57BL/6J #000664) and PAI-1 null (strain: C57BL/6J #000257) mice were purchased from Jackson Laboratories Bar Harbor, ME, USA.

Pneumonia model
This model was produced and used as described in our previous study.[19]

Lung vascular permeability measurement
Lung excess lung water (ELW, μl) and extravascular plasma equivalent (EVPE, μl) were measured as previously described.[20]

BAL, cell count and keratinocyte-derived chemokine (KC) measurements
BAL fluid was collected by infusing 1 ml of sterile phosphate-buffered saline (containing 5 mM EDTA) into the lungs of the mice after tracheal cannulation, as previously described.[21] Cytospin and KC measurements were performed after centrifugation.

Bacterial cultures from the lung homogenates
The lungs were homogenised in sterile containers using a tissue homogeniser (Tissue Tearor model 985-370, Biospec products, Racine, Wisconsin, USA). The homogenates were serially diluted and plated in triplicate on sheep-blood agar plates.

Lung myeloperoxidase measurement
After homogenisation, the lungs were clarified by centrifugation. The measurement of myeloperoxidase was performed according to the manufacturer's protocol.

Survival protocol
Twenty wild-type mice were randomly assigned to two groups (untreated mice n=10; PAI-039-treated mice n=10). A third group was composed of 10 PAI-1 null mice. Mice were exposed to *P aeruginosa* as described previously.[19]

Statistical analysis
All data are summarised as mean ± SD. For the statistical analysis we used Statview Version 5.0 and MedCalc Version 7.2.0.2. The normal distribution was verified using the Kolmogorov—Smirnov test. For normally distributed series, comparison of three or more experimental groups was performed by one-way analysis of variance (ANOVA). For non-normally distributed series or series of three measurements, the Mann—Whitney test was used to compare two experimental conditions. A Kaplan—Meier analysis followed by a logrank (Mantel—Cox) test was used to compare the survival between the three experimental groups of mice at 48 h. A p value of <0.05 was considered statistically significant.

RESULTS
PAI-1 is an essential component of the pulmonary host response during the early phase of *P aeruginosa* pneumonia in mice
We first found that there was a time-dependent increase in plasma and BAL fluid levels of PAI-1 in C57Bl6 mice instilled with *P aeruginosa* (figure 1). We also found that the increase in lung vascular permeability caused by the airspace instillation of two different strains of *P aeruginosa* (PA103 that produces exoU and PAK that does not produce exoU) was significantly attenuated in PAI-1 null mice (figure 2A—D). These results were confirmed by treating the mice with Tiplaxtinin (also called PAI-039), a newly developed pharmacological inhibitor of PAI-1 (figure 2E,F). We next showed that PAI-1 inhibition using PAI-039 (11 μM)
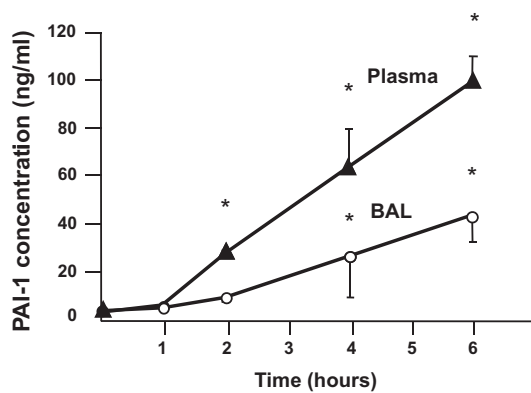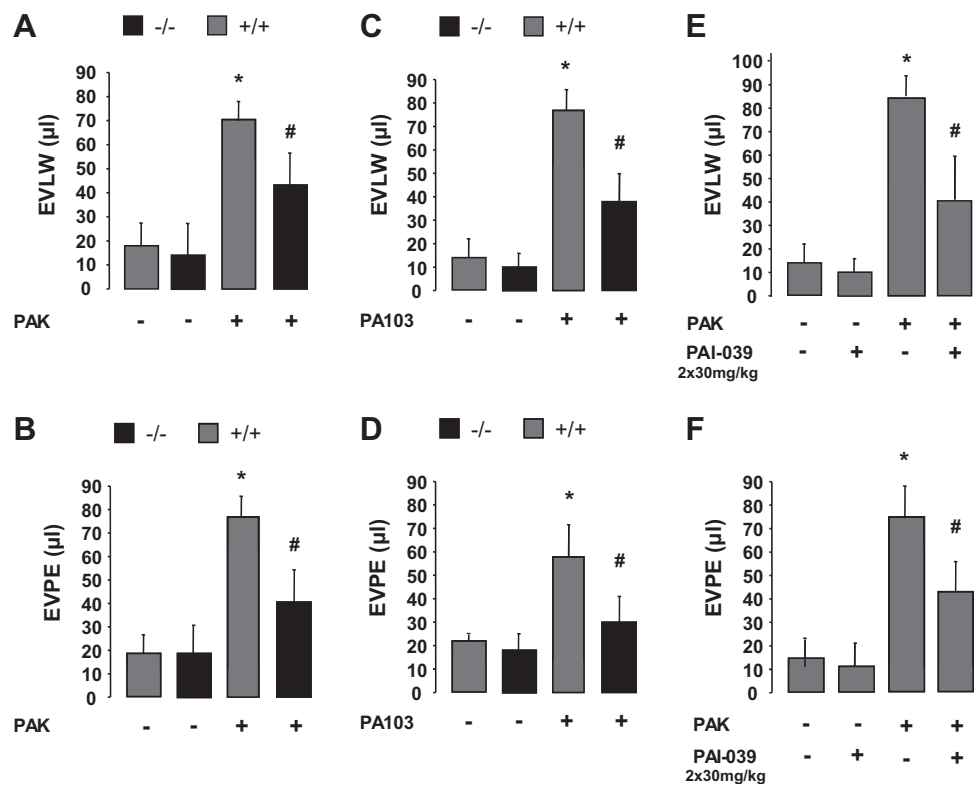
Figure 1 Airspace instillation of *P aeruginosa* causes a time-dependent increase in plasma and bronchoalveolar lavage (BAL) fluid levels of plasminogen activator inhibitor-1 (PAI-1) in mice. C57BL/6 mice were instilled with *P aeruginosa* (PAK strain, 1×10^7 colony-forming units) or its vehicle. Data are mean $\pm$ SD (n=8 mice in each experimental group); *p<0.05 from time 0 (control mice).

significantly decreased PAK-induced paracellular permeability in both human and bovine pulmonary endothelial cells (figure 3A,B). Figure 3C,D also shows that PAI-1 inhibition significantly attenuated PAK-induced RhoA activation in both BPAEC and HMVEC monolayers. Finally, the Alamar Blue assay showed that exposure to PAK for 3 h did not decrease the viability of lung endothelial or alveolar epithelial cells (data not shown).

P aeruginosa (PAK) induces PAI-1 expression in pulmonary endothelial and alveolar epithelial cells via a RhoA-dependent mechanism

Exposure of endothelial cells to PAK or transforming growth factor β1 (TGFβ1) resulted in the release of PAI-1 into the cell supernatant (figure 4A). Exposure to PAK also resulted in an

increase in PAI-1 gene and protein expression (figure 4B,C). Furthermore, pretreatment of BPAEC monolayers with a ROCK inhibitor (Y-27632, 10 μM) or with a nuclear factor-κB (NF-κB)-dependent kinase (IκB) inhibitor (BMS-345541, 10 μM) significantly decreased PAI-1 protein expression in BPAEC monolayers (figure 4C,D). We also found that inhibition of p38 mitogen-activated protein (MAP) kinase activity with SB98450 (10 μg/ml) significantly decreased PAK-mediated induction of PAI-1 gene and protein expression (figure 5A,B) and protein permeability across BPAEC monolayers induced by *P aeruginosa* (figure 5C). Interestingly, inhibition of the p38 MAP kinase decreased PAK-mediated RhoA activation whereas the inhibition of the RhoA-dependent kinase by its inhibitor Y-27632 did not prevent *P aeruginosa*-induced p38 MAP kinase activation (figure 5D,E). Next, we found that PAK-induced p38 MAP kinase activation was largely dependent on activation of Toll-like receptor 4 (TLR4) by this bacterium (figure 5F). Finally, we found that exposure of BPAEC monolayers to human recombinant PAI-1 caused a reversal of the RhoA/Rac-1 activity ratio that resulted in the activation of RhoA and a corresponding inhibition of Rac-1 which could further increase the direct effect of *P aeruginosa* on lung vascular permeability (figure 6A,B).

As figure 1 shows, there was a time-dependent increase in PAI-1 BAL fluid levels after airspace instillation of PAK in mice. Therefore, we also examined whether there was an increase in PAI-1 protein expression in alveolar epithelial cell monolayers exposed to *P aeruginosa*. Figure 7 shows that PAI-1 protein was significantly increased after PAK exposure (4 h) and that PAK-mediated induction of PAI-1 expression was RhoA dependent (figure 7).

PAI-1 deletion and inhibition inhibits the lung innate immune response and increases mortality during *P aeruginosa* pneumonia in mice

We next examined the effect of PAI-1 deletion or inhibition on bacterial clearance, mortality and lung innate immune response

Figure 2 Plasminogen activator inhibitor-1 (PAI-1) gene deletion or pharmacological inhibition attenuates the increase in lung vascular protein permeability caused by the airspace instillation of *P aeruginosa* in mice. (A−D) Wild-type or PAI-1 (−/−) (C57BL/6 strain) mice were instilled with *P. aeruginosa* (PAK strain, 1×10^7 colony-forming units (CFU)) or its vehicle. Four hours later, mice were euthanised and excess lung water (ELW) and lung vascular permeability (extravascular plasma equivalent (EVPE)) to protein were measured as described in the Materials and methods section. For all experiments, results are shown as mean $\pm$ SD (n=8 mice in each experimental group); *p $\leq$0.05 from controls; #p $\leq$0.05 from wild-type mice instilled with *P aeruginosa*. (E−F) C57BL/6 mice were instilled with *P aeruginosa* (PAK strain, 1×10^7 CFU or PA103 strain 5×10^5 CFU) or their vehicle. Mice were treated with a specific PAI-1 inhibitor Tiplaxtinin (PAI-039) (30 mg/kg) or its vehicle given intraperitoneally twice, 1 h before and at the time of airspace instillation of *P aeruginosa*. For all experiments, results are shown as mean

$\pm$ SD (n=8 mice in each experimental group); *p $\leq$0.05 from controls; #p $\leq$0.05 from mice instilled with *P aeruginosa* and treated with Tiplaxtinin vehicle.
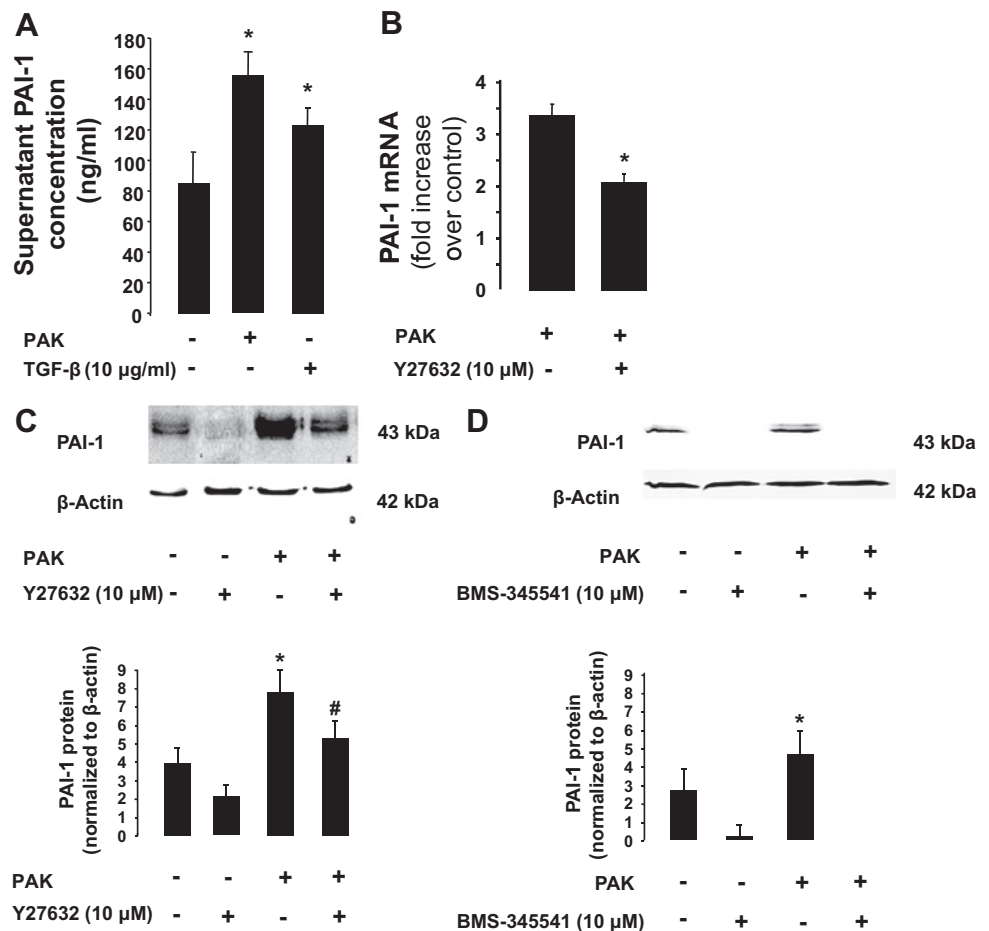
Figure 3 Specific plasminogen activator inhibitor-1 (PAI-1) inhibition attenuates *P aeruginosa*-induced increase in protein permeability and RhoA activity in bovine pulmonary artery and human microvascular lung endothelial cell monolayers. (A–D) Bovine pulmonary artery (BPAEC) and human microvascular (HMVEC) lung endothelial cell monolayers were treated with the wild-type *P aeruginosa* strain PAK (bacterial to bovine cell ratio: 1:2) or its vehicle for 30 min (RhoA activity) or 4 h (permeability measurement). Cell monolayers were pretreated with Tiplaxtinin (PAI-039) (11 μM) or its vehicle for 1 h prior to stimulation with *P aeruginosa* (PAK). All experiments were performed at least in triplicate and repeated three times. Results are shown as mean ± SD; *$p \leq 0.05$ from controls; #$p \leq 0.05$ from cell monolayers treated with PAK and PAI-039 vehicle.
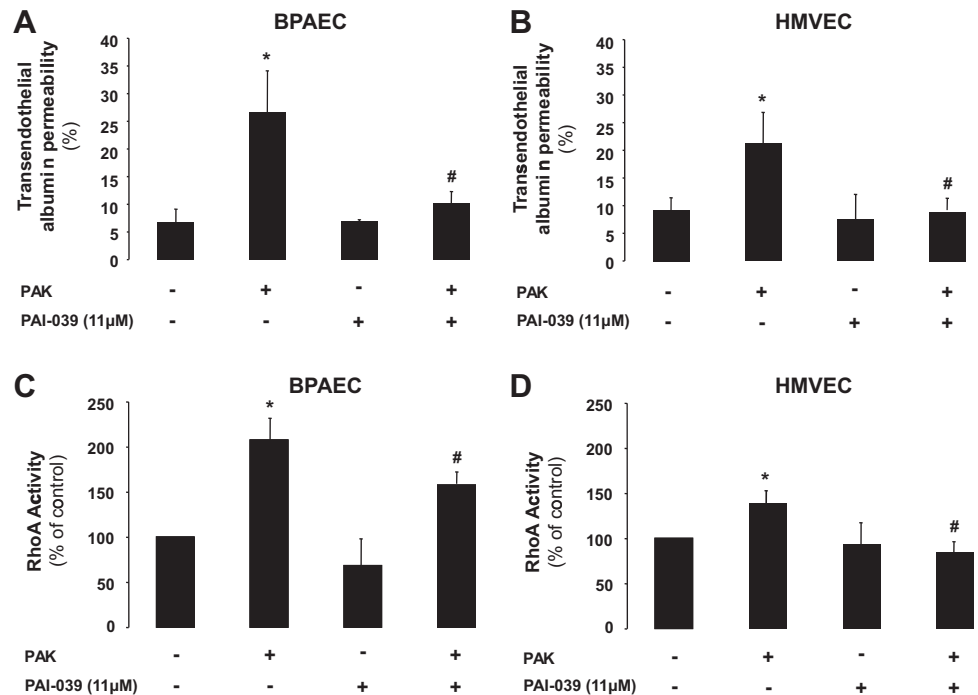
using our murine model of *P aeruginosa* pneumonia. PAI-1 deletion or inhibition was associated with a significant decrease in lung bacterial clearance (figure 8A,B) and higher mortality

(figure 8C). In the next series of experiments, we examined whether the decreased bacterial clearance observed in mice with disrupted PAI-1 signalling was caused by a defect in neutrophil

Figure 4 *P aeruginosa* causes plasminogen activator inhibitor-1 (PAI-1) gene and protein expression via a RhoA- and nuclear factor-κB (NF-κB)-dependent mechanism in bovine pulmonary artery epithelial cell (BPAEC) monolayers. (A) *P aeruginosa* causes the extracellular release of PAI-1 protein by BPAEC monolayers. BPAEC monolayers were treated with the wild-type *P aeruginosa* strain PAK or its vehicle for 24 h (bacterial to bovine cell ratio: 1:5). Transforming growth factor β1 (TGFβ1) (10 ng/ml) served as a positive control. (B and C) *P aeruginosa* causes PAI-1 gene and protein expression via a RhoA-dependent mechanism in BPAEC monolayers. BPAEC monolayers were treated with the wild-type *P aeruginosa* strain PAK or its vehicle for 4 h (bacterial to bovine cell ratio: 1:2). Cell monolayers were treated with the ROCK inhibitor Y27632 (10 μM) or its vehicle for 1 h prior to stimulation with *P aeruginosa* (PAK). (D) *P aeruginosa* causes PAI-1 protein expression via an NF-κB-dependent mechanism in BPAEC monolayers. BPAEC monolayers were treated with the wild-type *P aeruginosa* strain PAK or its vehicle for 4 h (bacterial to bovine cell ratio: 1:2). Cell monolayers were treated with the NF-κB-dependent kinase (IκB) inhibitor BMS-345541 (10 μM) or its vehicle for 1 h prior to

stimulation with *P aeruginosa* (PAK). All experiments were performed at least in triplicate and repeated at least three times. For western blots, a representative blot is shown. Three additional experiments gave comparable results. Results are shown as mean ± SD; *$p \leq 0.05$ from negative or positive controls; #$p \leq 0.05$ from cell monolayers treated with PAK and Y27632 or BMS-345541 vehicle.
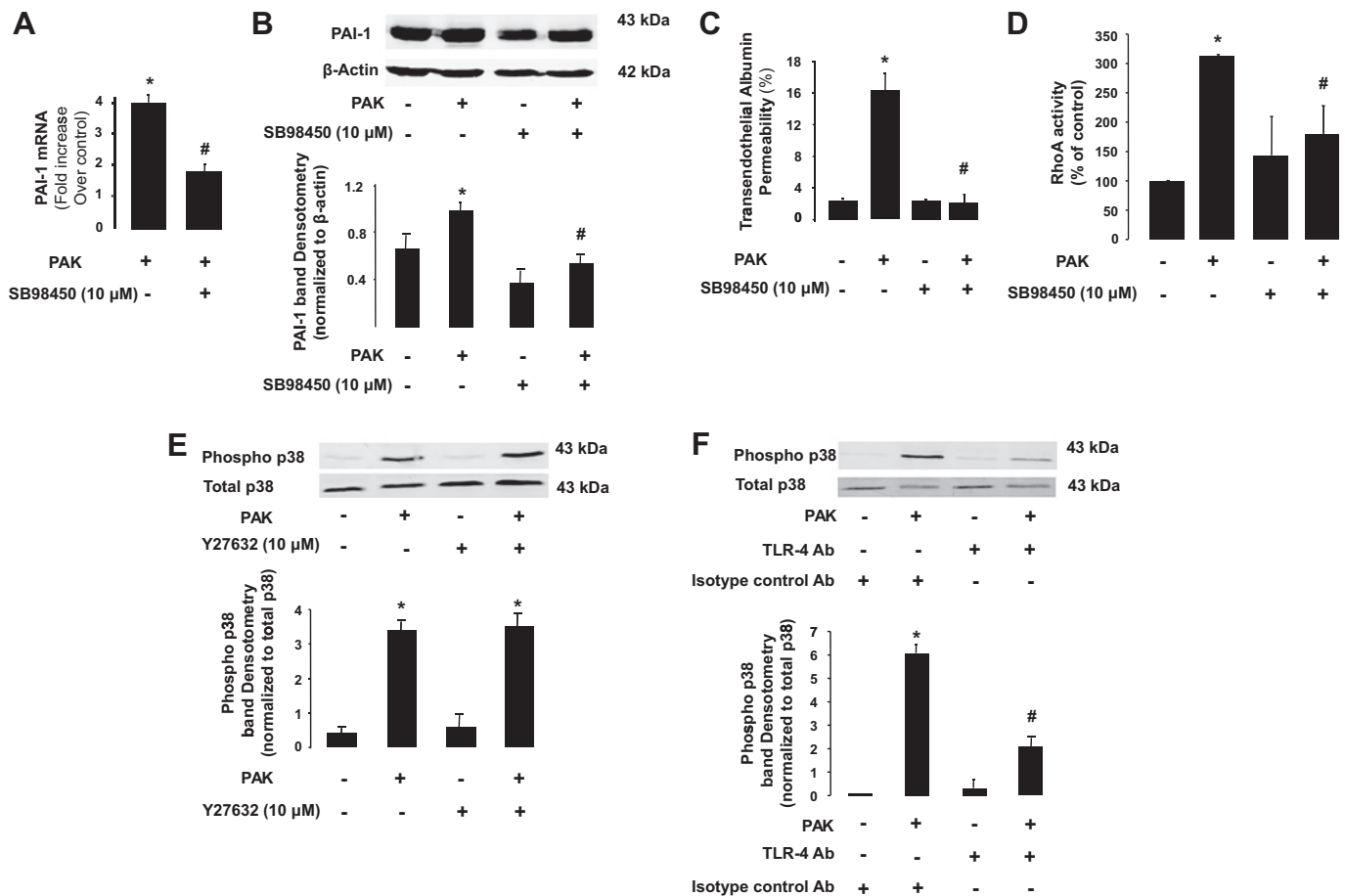
Figure 5 *P aeruginosa* causes plasminogen activator inhibitor-1 (PAI-1) gene and protein expression via a p38 mitogen-activated protein (MAP) kinase- and Toll-like receptor 4 (TLR4)-dependent mechanism in lung endothelial cell monolayers. (A and B) *P aeruginosa* causes PAI-1 gene and protein expression via a p38 MAP kinase-dependent mechanism in bovine pulmonary artery epithelial cell (BPAEC) monolayers. BPAEC monolayers were treated with the wild-type *P aeruginosa* strain PAK or its vehicle for 4 h (bacterial to bovine cell ratio: 1:2). Cell monolayers were treated with the p38 MAP kinase inhibitor SB88450 (10 µg/ml) or its vehicle for 1 h prior to stimulation with *P aeruginosa* (PAK). (C) Specific p38 MAP kinase inhibition attenuates *P aeruginosa*-induced increase in protein permeability in BPAEC monolayers. BPAEC monolayers were treated with the wild-type *P aeruginosa* strain PAK or its vehicle for 4 h (bacterial to bovine cell ratio: 1:2). Cell monolayers were treated with the p38 MAP kinase inhibitor SB88450 (10 µg/ml) or its vehicle for 1 h prior to stimulation with *P aeruginosa* (PAK). (D) Pretreatment with the p38 MAP kinase inhibitor SB88450 attenuates the *P aeruginosa*-mediated RhoA activation in BPAEC monolayers. BPAEC monolayers were treated with the wild-type *P aeruginosa* strain PAK or its vehicle for 30 min (bacterial to bovine cell ratio: 1:2). Cell monolayers were treated with the p38 MAP kinase inhibitor SB88450 (10 µg/ml) or its vehicle for 1 h prior to stimulation with *P aeruginosa* (PAK). (E) Pretreatment with the ROCK inhibitor Y27632 does not attenuate the *P aeruginosa*-mediated p38 MAP kinase phosphorylation in BPAEC monolayers. BPAEC monolayers were treated with the wild-type *P aeruginosa* strain PAK or its vehicle for 4 h (bacterial to bovine cell ratio: 1:2). Cell monolayers were treated with the ROCK inhibitor Y27632 (10 µM) or its vehicle for 1 h prior to stimulation with *P aeruginosa* (PAK). (F) Pretreatment with a specific blocking antibody (Ab) against TLR4 attenuates the *P aeruginosa*-mediated p38 MAP kinase phosphorylation in human lung microvascular endothelial cell (HMVEC) monolayers. HMVEC monolayers were treated with the wild-type *P aeruginosa* strain PAK or its vehicle for 4 h (bacterial to bovine cell ratio: 1:2). Cell monolayers were treated with a specific blocking Ab against TLR4 or its isotype control Ab for 1 h prior to stimulation with *P aeruginosa* (PAK). All experiments were performed at least in triplicate and repeated at least three times. For western blots, a representative blot is shown. Three additional experiments gave comparable results. Results are shown as mean ± SD; *$p \leq 0.05$ from negative or positive controls; #$p \leq 0.05$ from cell monolayers treated with PAK and SB98450 vehicle or a control isotype Ab.

migration into the lung in response to the airspace instillation of *P aeruginosa*. We found that PAI-1 null mice or wild-type mice treated with PAI-039 had a decreased neutrophil recruitment into the lung in response to the airspace instillation of *P aeruginosa* (figure 9A,B). Disruption of PAI-1 signalling was also associated with a lower myeloperoxidase activity in the lungs (figure 9C) and lower BAL fluid levels of KC, a major murine neutrophil chemokine (figure 9D).

DISCUSSION
A growing body of literature has highlighted clinical correlations between elevated plasma, BAL or pulmonary oedema fluid levels of PAI-1 and severe disease, as well as adverse clinical outcome in both patients with ALI and with pneumonia caused by *P aeruginosa*.[8–10 22] Decreased airspace fibrinolysis in patients with pneumonia has mainly been attributed to an elevation in PAI-1 activity[11] and precedes the clinical recognition of VAP by several days in critically ill patients.[5] However, it was still unknown whether there is a role for PAI-1 in lung injury induced by bacteria, such as *P aeruginosa*. Using the combined approach of PAI-1 gene deletion and pharmacological inhibition, we found that the release of PAI-1 in response to the exposure to *P aeruginosa* is an important contributor to the early alterations in the lung fluid balance caused by *P aeruginosa*. Moreover, PAI-1
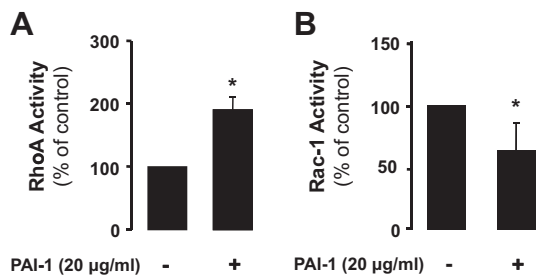
Figure 6 Recombinant plasminogen activator inhibitor-1 (PAI-1) increases RhoA and inhibits Rac-1 activity in bovine pulmonary artery epithelial cell (BPAEC) monolayers. (A and B) BPAEC monolayers were treated with human recombinant PAI-1 (20 μg/ml) or its vehicle for 30 min. All experiments were performed at least in triplicate and repeated at least three times. Results are shown as mean ± SD; *p≤0.05 from controls.

inhibition also protected against *P aeruginosa*-mediated lung cell endothelial leak in vitro, indicating a direct effect of PAI-1 released by *P aeruginosa* on the barrier function of lung endothelial cells via a RhoA-dependent mechanism.

The importance of RhoA in mediating the inflammatory response elicited by *P aeruginosa* is supported by our previous in vivo and in vitro studies that reported a central role for this small GTPase in the regulation of the endothelial barrier permeability after *P aeruginosa* challenge.[15][19] There are several pathways that could lead to RhoA activation by *P aeruginosa* in pulmonary endothelial cells that include the activation of TLR4 and TLR5 by the bacterial endotoxin and flagellin.[23][24] Our results indicate that RhoA activation and PAI-1 expression induced by *P aeruginosa* depend in part on a TLR4-mediated phosphorylation of the p38 MAP kinase and activation of RhoA. However, *P aeruginosa* may also activate RhoA signalling via the
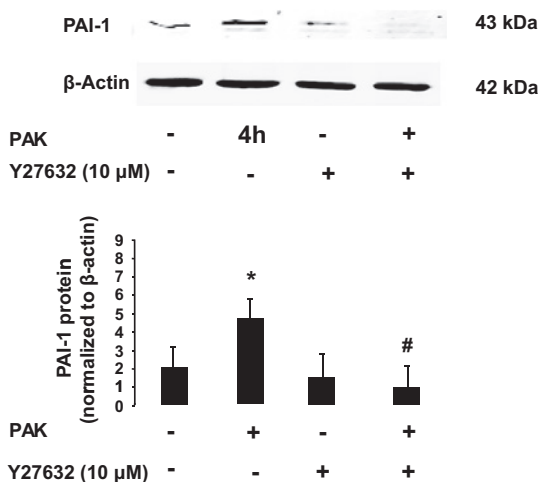

Figure 7 *P aeruginosa* causes plasminogen activator inhibitor-1 (PAI-1) protein expression via a RhoA-dependent mechanism in primary cultures of alveolar epithelial type II cell monolayers. Rat alveolar epithelial type II cell monolayers cultured on transwells with an air–liquid interface were treated with the wild-type *P aeruginosa* strain PAK or its vehicle for 3 h (bacterial to rat cell ratio: 1:2). Cell monolayers were treated with the ROCK inhibitor Y27632 (10 μM) or its vehicle for 1 h prior to stimulation with *P aeruginosa* (PAK). A representative western blot is shown. Three additional experiments gave comparable results. Results are shown as mean ± SD; *p≤0.05 from controls; #p≤0.05 from cell monolayers treated with PAK and Y27632 vehicle.

exotoxins S and T from the type III secretion system.[25] Interestingly, a recent study from Dr Lynch's laboratory has demonstrated cooperation between the endotoxin O-antigen and the type III secretion system in both laboratory and clinical isolates of *P aeruginosa*.[26] These investigators found that the loss of O-antigen facilitates secretion of the toxins of the type III secretion system, documenting an association between endotoxin O-antigen structural composition and type III cytotoxin secretion in a mouse model of *P aeruginosa* pneumonia.[26] These results indicate that the activation of RhoA signalling by *P aeruginosa* is a critical step both for the induction of the PAI-1 gene and protein and for the modulation of lung vascular permeability by this bacterium.

Since there are no known intracellular functions reported for PAI-1, we next hypothesised that PAI-1 could exert its effects from the extracellular milieu as the extracellular release of PAI-1 by lung endothelial cells was also increased after exposure to *P aeruginosa*. We observed that human recombinant PAI-1 increased RhoA, but decreased Rac-1 activity in these cell monolayers. Interestingly, we reported a similar shift in the balance between RhoA and Rac-1 activity in a previous study [15] where lung endothelial cell monolayers were simply exposed to *P aeruginosa*, indicating that the effect of *P aeruginosa* on the endothelial barrier permeability may depend in part on PAI-1-mediated RhoA activation. Although the mechanism(s) of small GTPase regulation by extracellular PAI-1 remain largely unknown, PAI-1 interaction with the extracellular matrix may explain in part the activation of RhoA by this coagulation protein. Indeed, PAI-1 prevents the binding of αv-integrins to the RGD domain of vitronectin, their extracellular ligand.[27] Under steady-state conditions, some members of the αv-integrin family, such as the αvβ3 integrin, are anchored to the cortical actin and are associated with other cell adhesion protein complexes within the basal membrane called focal adhesion complexes. The spatial association of these proteins is under the control of local membrane activity of various kinases, such as the focal adhesion kinase and src. Among these kinases, src has been reported to cause a sustained activation of Rac-1 through Cdc-42, allowing the maintenance of cortical actin.[28] It is therefore likely that the destabilisation of this adhesion complex by PAI-1 competition with αvβ3 integrin for vitronectin may affect the basal Rac-1 activity in these cells and promote the formation of actin stress fibres through RhoA activation. Taken together, these results indicate that PAI-1 amplifies *P aeruginosa*-mediated RhoA activation and may play an important role in the increase of lung vascular permeability associated with *P aeruginosa* pneumonia.

We next sought to determine if *P aeruginosa* increases PAI-1 release by the alveolar epithelium. Our initial in vivo results indicate that there is a time-dependent increase in PAI-1 protein released in the distal airspace in mice with *P aeruginosa* pneumonia. Furthermore, clinical studies have reported that BAL fluid levels of PAI-1 are higher in patients with *P aeruginosa*-induced VAP than those found in other infections,[9] and increased BAL levels of PAI-1 were associated with increased mortality in a cohort of intensive care patients with *P aeruginosa* VAP.[10] Here, we found that exposure of rat alveolar type II epithelial cells to *P aeruginosa* also resulted in an increased PAI-1 protein expression by these cells. As observed for lung endothelial cells, the induction of PAI-1 expression by *P aeruginosa* in rat alveolar type II cells was also RhoA dependent, underscoring the central role of the small GTPase RhoA in the control of PAI-1 expression in the lung after exposure to this bacterium. These results are also significant because we have previously

Figure 8 Plasminogen activator inhibitor-1 (PAI-1) gene deletion or pharmacological inhibition decreases lung bacterial clearance and increases mortality in a mouse model of *P aeruginosa* pneumonia. (A) Wild-type or PAI-1 (−/−) (C57BL/6 strain) mice were instilled with *P aeruginosa* (PAK strain, 1×10^7 colony-forming units (CFU)) or its vehicle. Wild-type mice were treated with a specific PAI-1 inhibitor Tiplaxtinin (PAI-039) (30 mg/kg) or its vehicle given intraperitoneally twice, 1 h before and at the time of airspace instillation of *P aeruginosa*. Mice were studied for 8 h. For all experiments, results are shown as mean ± SD (n=6 mice in each experimental group); *p≤0.05 from wild-type mice treated with Tiplaxtinin vehicle. (B) Low-power photomicrographs of the cytospin of bronchoalveolar lavage fluids from wild-type or PAI-1 (−/−) (C57BL/6 strain) mice stained with H&E. Wild-type mice were treated with a specific PAI-1 inhibitor Tiplaxtinin (PAI-039) (30 mg/kg) or its vehicle given intraperitoneally twice, 1 h before and at the time of airspace instillation of *P aeruginosa*. Mice were studied for 8 h. One representative cytospin for each condition is shown. Three additional experiments gave comparable results. (C) Wild-type or PAI-1 (−/−) (C57BL/6 strain) mice were instilled with *P aeruginosa* (PAK strain, 1×10^7 CFU) or its vehicle. Wild-type mice were treated with a specific PAI-1 inhibitor Tiplaxtinin (PAI-039) (30 mg/kg) or its vehicle given intraperitoneally twice,

1 h before and at the time of airspace instillation of *P aeruginosa*. Kaplan—Meier survival analysis was performed (n=10 mice in each experimental group). Mice with a gene deletion or pharmacological inhibition of PAI-1 died significantly earlier than the wild-type mice after airspace instillation of *P aeruginosa* and treatment with Tiplaxtinin vehicle.
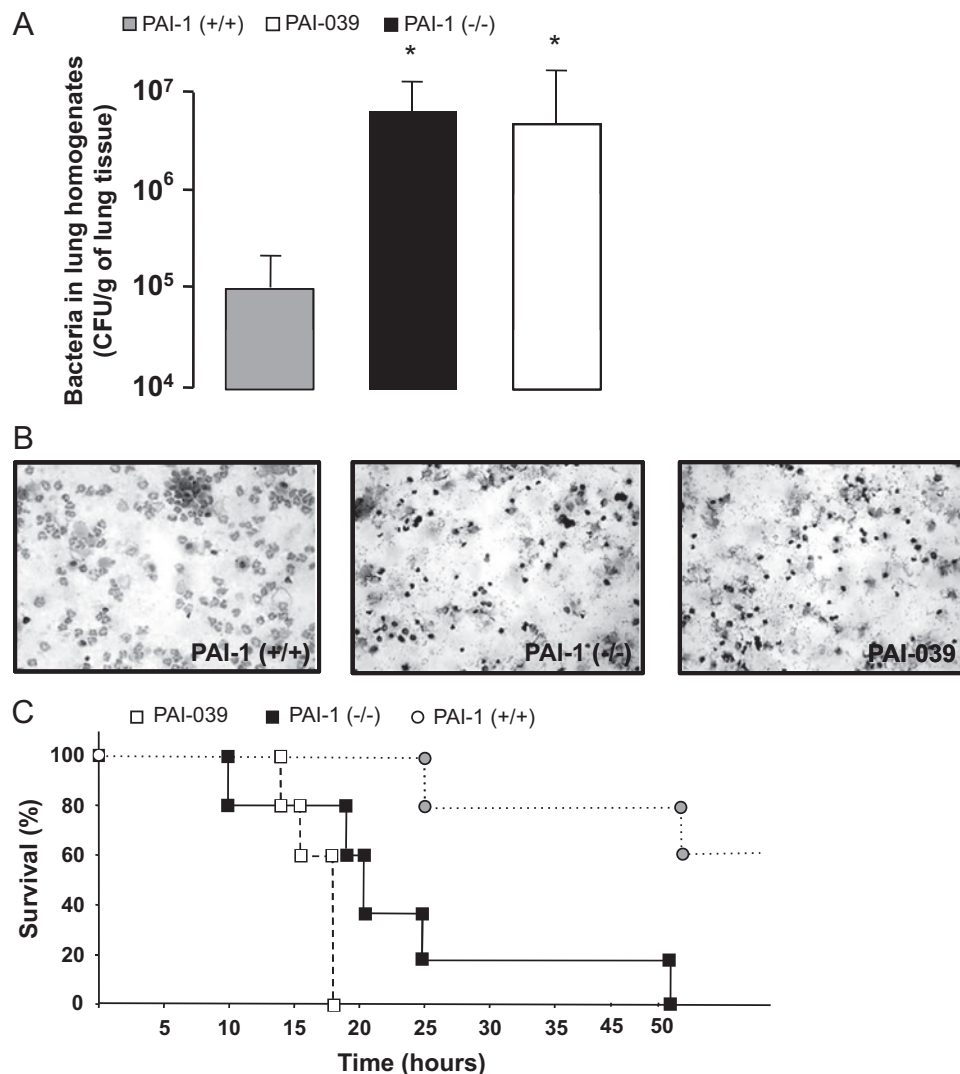
reported an important role for TGFβ1, a major inducer of PAI-1,[29] in mediating the breakdown of the alveolar epithelial barrier associated with ALI.[20] TGFβ1 also mediates the increase in protein permeability and inhibition of baseline and cAMP-dependent vectorial fluid transport across the alveolar epithelial barrier in experimental rodent models of ALI.[20 30] In addition, *P aeruginosa* increases protein permeability across rat alveolar epithelial type II cell monolayers, an effect that was inhibited by a specific inhibitor of RhoA or a soluble type II receptor for TGFβ1.[19] Interestingly, one of the major mechanisms mediating TGFβ1 activation in the distal lung epithelium is the activation of RhoA by interleukin 1β or thrombin agonists that results in an αvβ6-integrin-mediated activation of TGFβ1 on alveolar epithelial cells,[20] suggesting that PAI-1 could activate TGFβ1 signalling via a RhoA-dependent mechanism. These results indicate that *P aeruginosa* causes the expression of PAI-1 protein by the distal lung epithelium and that the release of PAI-1 in the distal airspace may contribute to the breakdown of the alveolar epithelial barrier associated with *P aeruginosa* pneumonia.

Conflicting results have been reported concerning the overall role of PAI-1 in various experimental models of pneumonia.[11 12] For example, Renckens *et al* have reported that PAI-1 is

protective during severe Gram-negative pneumonia caused by *Klebsiella pneumoniae*, but did not influence the outcome of Gram-positive pneumonia caused by *Streptococcus pneumoniae* in mice.[12] We found that PAI-1 plays an important role in bacterial clearance from the lung and survival in our experimental model of *P aeruginosa* pneumonia because deletion or inhibition of this protein caused a decreased neutrophil recruitment into the lung in response to the airspace instillation of *P aeruginosa*. Our results are in accordance with previous studies that showed that PAI-1 regulates neutrophil recruitment into several organs, such as the lung and kidney.[11 31] In addition to its role in recruiting neutrophils into the lung, PAI-1 expression on the non-apoptotic neutrophil plasma membrane protects these immune cells against efferocytosis (phagocytosis of viable cells) by alveolar macrophages.[32] These results indicate that PAI-1 is required for the activation of the innate immune response necessary for the eradication of *P aeruginosa* from the distal airspaces of the lung.

In summary, we have demonstrated a dual role for PAI-1 during the course of *P aeruginosa* pneumonia in mice. Despite an early protective effect of the function of the alveolar capillary barrier, the lack of PAI-1 activity was later associated with the inability to clear the bacteria from the airspace and increased
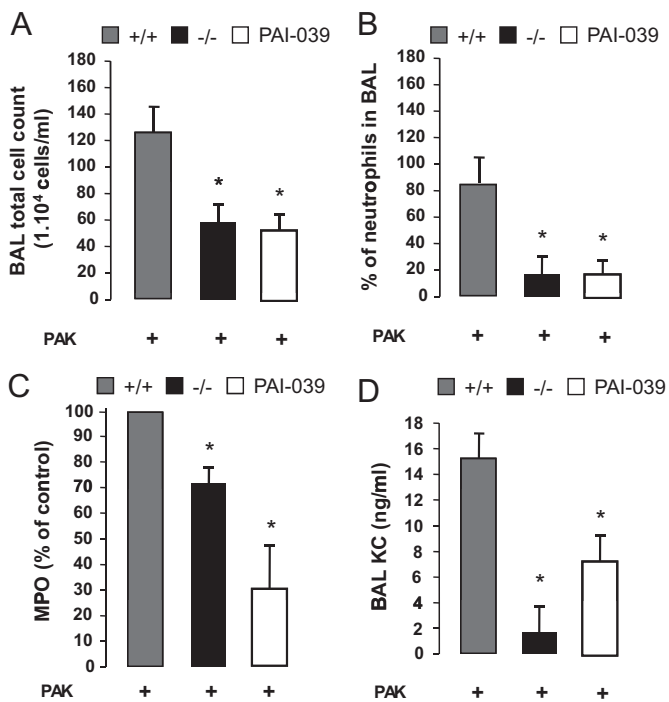
Figure 9 Plasminogen activator inhibitor-1 (PAI-1) gene deletion or pharmacological inhibition attenuates neutrophil recruitment into the airspace and bronchoalveolar lavage (BAL) fluid levels of keratinocyte-derived chemokine (KC) in a mouse model of *P aeruginosa* pneumonia. (A–D) Wild-type or PAI-1 (−/−) (C57BL/6 strain) mice were instilled with *P aeruginosa* (PAK strain, 1×10^7 colony-forming units (CFU)) or its vehicle. Wild-type mice were treated with a specific PAI-1 inhibitor Tiplaxtinin (PAI-039) (30 mg/kg) or its vehicle given intraperitoneally twice, 1 h before and at the time of airspace instillation of *P aeruginosa*. Mice were studied for 8 h. BAL was performed, and cells were counted. and KC levels measured as described in the Materials and methods sections. For all experiments, results are shown as mean ± SD (n=6 mice in each experimental group); *p≤0.05 from wild-type mice treated with Tiplaxtinin vehicle. MPO, myeloperoxidase.

mortality. This apparent dichotomy may in fact reflect a synergistic host response to bacterial lung infection that has to be tightly controlled. Indeed, an exaggerated release of PAI-1 in the extracellular space may lead to the development of severe pulmonary oedema that would overwhelm PAI-1's beneficial effects on the lung innate immune response and explain the clinical correlation between high BAL fluid and plasma levels of PAI-1 and poor outcome in patients with *P aeruginosa* pneumonia.[10] Additional studies that will include multiple time points and several strains of *P aeruginosa* will be needed to answer this critical question. Despite this limitation, the present study demonstrates that PAI-1 controls the development of the early lung inflammation that is required for the activation of the innate immune response necessary for the eradication of *P aeruginosa* from the distal airspaces of the lung, suggesting that therapeutic approaches that would inhibit the early inflammation may be deleterious for the host. Indeed, patients with high *P aeruginosa* burden in their lungs, but with a weak inflammatory response, have an increased mortality from *P aeruginosa* pneumonia.[33]

Competing interests None.

Provenance and peer review Not commissioned; externally peer reviewed.

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Journal club

Combined use of transoesophageal and transbronchial ultrasonography may lead to fewer unnecessary thoracotomies while staging NSCLC

This study investigated the use of endosonography versus surgical staging in non-small cell lung cancer. Patients in the endosonography arm underwent transbronchial and transoesophageal ultrasonography to detect mediastinal involvement followed by surgical staging if no nodal involvement was found.

Two hundred and forty-one patients were randomised for surgical staging (118) or endosonography (123). Sixty-five patients in the endosonography group also underwent surgical staging. Nodal metastasis was found in 41 patients (35%) by surgical staging compared with 56 patients (46%) by endosonography alone and 62 patients (50%) by endosonography followed by surgical staging. Thoracotomy was unnecessary in 21 patients (18%) in the mediastinoscopy group compared with 9 patients (7%) in the endosonography group.

The use of combined endosonography and surgical staging resulted in an improvement in the detection of nodal metastasis and a reduction in unnecessary thoracotomies compared with surgical staging alone in patients with suspected non-small cell lung cancer. However, several nodes were out of reach of endosonography, and the technique is not available in all centres. Further research is needed, and the question remains as to whether all routinely negative endosonography patients should have mediastinoscopy or whether this approach is meant for a particular subgroup.

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Online methods supplement

Reagents. All cell culture media were prepared by the University of California San Francisco Cell Culture Facility using deionized water and analytical grade reagents. HMVEC cell culture media EGM-2MV was purchased from Lonza (CC-064). The protein concentration of cell lysates was determined using Bio-Rad protein assay kit (Biorad, CA). G-LISA^{TM} RhoA and Rac-1 activity assay were measured using commercial kits from Cytoskeleton Inc. (Denver, CO). Collagen-coated PFTE membrane Costar Transwells were obtained from Fisher Scientific (Santa Clara, CA). DuoSet$^{®}$ ELISA kits for mouse keratinocyte-derived chemokine (KC) were purchased from R&D (Minneapolis, MN). Myeloperoxidase activity was measured using a mouse MPO kit HK210 from Hycult biotechnology (Uden, Netherlands). Tiplaxtinin (PAI-039) was purchased from Axon Medchem BV (Groningen, Netherlands). Human Plasminogen Activator inhibitor-1 recombinant protein was purchased from Chemicon international INC (Billerica, MA). ^{125}I-labeled human serum albumin (Jeanatope ISO-TEX Diagnostics, Friendswood, TX) was used as radioactive tracer. All other reagents were purchased from Sigma (St-Louis, MI).

Cell culture. *Bovine pulmonary arterial endothelial cells* (BPAEC; ATCC, CCL-209; passages all < 8) were cultured, as previously described **(1)**. Bovine macrovascular lung pulmonary arterial endothelial cells were cultured either on transwells (Costar 3495, 1x10^5 cells per filter) for 4 days or in 6 wells plastic plates until they formed confluent monolayers. Cells were kept in Dulbecco's modified Eagle's medium/H21 medium containing 10% low endotoxin FBS and 1% penicillin/streptomycin/amphotericin in a humidified 95% air and 5% CO_2 environment at 37 °C. Confluent monolayers were exposed to *P. aeruginosa* or vehicle as described in the specific protocols.

Human lung microvascular endothelial cells (HMVEC; Lonza; CC-064) were cultured either on transwells (Costar 3495, 2x10^5 cells per filter) for 4 days or in 6 wells plastic plates until they formed confluent monolayers. Cells were kept in EGM-2MV (Bulletkit$^{®}$; Lonza; Walkersville, MD) medium containing 1% penicillin/streptomycin. Confluent monolayers were exposed to *P. aeruginosa* or vehicle as described in the specific protocols.

Primary rat alveolar epithelial type II (ATII) cells were isolated as previously described **(2)**. Briefly, cells were isolated by elastase digestion followed by negative selection using four monoclonal antibodies (mAbs) against cell surface molecules expressed on rat macrophages (CD4/CD32/CD45/RMA). These mAbs were pre-incubated with Dynabeads M-450 (magnetic beads with sheep anti-mouse IgG, Dynal ASA, Oslo, Norway) in 0.1% BSA/PBS. After removing unbound mAbs, rat ATII cells were mixed with the bead suspension and rocked gently for 30 min at 4°C. Unbound cells were isolated and plated on polycarbonate Transwells with a 0.4 μm pore size (Costar 3401). Cells were seeded at a concentration of 1.5 x 10^6 cells/cm^2 in DMEM-H21 medium containing 10% low endotoxin fetal bovine serum, 1% penicillin and streptomycin and kept at 37°C in a humidified 95% air-5% CO_2 environment. Twenty-four hours later, nonadherent epithelial cells were removed by washing with PBS and fresh medium added to the lower compartments of the Transwells, thus maintaining the ATII cell monolayers with an air-liquid interface on their apical side. After 72-96 hours, cells that formed confluent monolayers reaching a transepithelial electrical resistance greater than 1500 ohms.cm^2 were used for experiments. Confluent monolayers were exposed to *P. aeruginosa* or vehicle as described in the specific protocols.

Preparation of P. aeruginosa. The wild-type, PAK strain of *P. aeruginosa* was a kind gift from Dr. Stephen Lory at Harvard University, MA. PA103 strain of *P. aeruginosa* was generously provided by Dr. Dara Frank at the Medical College of Wisconsin. For each experiment, frozen bacteria were inoculated into Luria-Bertani (LB) broth (Invitrogen, Carlsbad, CA), incubated for 6 h at 37°C on a rotating platform, and then diluted 1:100 in fresh LB broth. After 16–18 h of incubation at 37°C, the stationary phase bacteria were pelleted, washed three times in PBS, and suspended in PBS to a concentration adjusted by optical density at 600 nm, as 1 x 10^9 cfu/ml for *in vitro* experiments or 2 x 10^8 cfu/ml for instillation in mice (50μl preparation per mouse). Counts were confirmed by serial dilution and plating on LB agar.

Measurement of transendothelial albumin flux. Transendothelial albumin flux across BPAEC cells was measured, as previously described **(1)**.

Briefly, BPAEC cells were seeded onto 6.5-mm collagen-coated PFTE membrane Costar Transwells at 1 x 10^5 cells per well and cultured for 3 days. Cells were exposed to *P. aeruginosa* for 3 hours (PAK to bovine cell ratio 1:2). In some experiments, cells were pretreated with Y-27632 (10 µM) or SB98450 (10 µM) for 1 hour. Some cell monolayers were pretreated with the PAI-1 inhibitor Tiplaxtinin (PAI-039; 11 µM) just before exposure to *P. aeruginosa*. In all experiments, controls were treated with the respective drug vehicles. During the last hour of incubation with *P. aeruginosa* (PAK), ^{125}I-albumin (0.05 µCi) was applied to each upper compartment at 37°C. After 1 hour, the media from the lower compartment were collected and counted in a Wallac Wizard - counter (Perkin Elmer, Shelton, CT). Only monolayers retaining more than 95% of tracer at baseline were studied.

Transendothelial albumin flux across HMVEC cells was measured as described above. HMVEC cells were seeded onto 6.5-mm collagen-coated PFTE membrane Costar Transwells at 2 x 10^5 cells per well and cultured for 3 days. Cells were exposed to *P. aeruginosa* for 3 hours (PAK to human cell ratio 1:2). Cell monolayers were pretreated with the PAI-1 inhibitor Tiplaxtinin (PAI-039; 11 µM) or its vehicle just before exposure to *P. aeruginosa*.

Western blot analysis. Western blot analysis was performed as described previously **(3)**. After equal amounts of protein were loaded in each lane and separated by 10% SDS-PAGE, proteins were transferred using the iBlot™ Dry Blotting system from Invitrogen Corporation (Carlsbad, CA, USA). Membranes were blocked with the blocking buffer from Li-Cor Bioscience (cat# 927-40010; Lincoln, Nebraska USA) and incubated with the primary antibody (β-actin #4967, Phospho p38 MAP kinase #9216S; Total p38 MAP kinase #9212 all from Cell Signaling, Danvers, MA, PAI-1 #sc-5297 from Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. Primary antibodies were used at a dilution of 1:1000 for all proteins and 1:500 for p38 MAP kinase. IRDye® Secondary Antibodies from Li-Cor Bioscience (IRDye 680 Goat Anti-Rabbit IgG, 0.5mg, cat# 926-32221, IRDye 800CW Goat Anti-Mouse IgG, 0.5mg, cat# 926-32210; Lincoln, Nebraska USA) were used and Proteins were visualized using the Odyssey infrared imaging system from Li-

Cor Bioscience (Lincoln, Nebraska USA). Quantification was done using the digital image analysis system provided by Odyssey.

Primers and Probes. Real-time reverse transcription (RT)-PCR primers and probe were designed using Primer Express software (PE-Applied Biosystems, Warrington, United Kingdom). The TaqMan probes were labeled with a fluorophore reporter dye (6-carboxyfluorescein) at the 5′-end and a Black Hole Quencher dye (Biosearch Technologies, Inc.) at the 3′-end. The primers for PAI-1 (Bos Taurus, NM 174137) were the following: PAI-1 forward GCCTCTCCTTTCCTCGATTTTC ; PAI-1 reverse GTGAGCCGAAGTTGGATGGT ; Probe ACCCGATGGAGCCGCGTCC. GAPDH (Bos Taurus, glyceraldehyde-3-phosphate dehydrogenase) was chosen as the house keeping gene. The following primers were used: GAPDH forward GCATCGTGGAGGGACTTATGA ; GAPDH reverse GGGCCATCCACAGTCTTCTG ; Probe CACTGTCCACGCCATCACTGCCA.

Quantitative Real-time RT-PCR. After 4 days in culture, total RNA was extracted from BPAEC cells using the RNeasy mini kit (Qiagen Inc., Valencia, CA). One microgram of total RNA was reverse-transcribed using the Superscript first-strand synthesis system (Invitrogen). RT-PCRs were performed, and the results were analyzed using the ABI PRISM 7700 sequence detection system (PE-Biosystems, Foster City, CA). Briefly, RT-PCR was carried out in a 25-µl reaction mixture containing 1x TaqMan Universal PCR Master Mix (PE-Biosystems, Foster City, CA), 10 pmol of primers, 5 pmol of TaqMan probe, and an equivalent of 100 ng of total RNA for 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The number of cycles to threshold of fluorescence detection was normalized to the number of cycles to threshold of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for each sample tested. Results are expressed as fold induction of cDNA abundance compared with the control. The percentages were used for all statistical comparisons.

RhoA and Rac-1 activity assay in cells. Rac1 and RhoA activity of lung endothelial or alveolar epithelial cells were determined using a luminescence-based G-LISA Rac1 and RhoA activation assay biochemistry kit according to the manufacturer's instructions (Cytoskeleton Inc., Denver, CO). Briefly, the

endothelial cells (BPAEC and HMVEC) or rat alveolar epithelial type II cells were grown on 35-mm cell culture dishes to 50% confluence. After serum starvation for 6 hours, the *P. aeruginosa* strain PAK (bacterial to bovine cell ratio: 1:2) or its vehicle was placed on the cells for 30 minutes before harvesting the cell lysates. The cell lysis was performed using the lysis buffer and protease inhibitors cocktail provided within the kit. Lysates were centrifuged at 4°C (14,000 x *g*, 2 min), the protein concentration determined, and final protein concentrations adjusted to 1.0 mg/ml. The lysates were added to plates coated with a Rac- or Rho-GTP binding protein before incubation for 30 minutes at 4°C. Next a primary antibody specific for Rac-1 or RhoA was added and incubated for 45 minutes at room temperature. Finally, an HRP-conjugated secondary antibody is added and incubated for 45 minutes at room temperature. Luminescence was determined using the Wallac Victor 1420 (Perkin Elmer). In some experiments, BPAEC and HMVEC cells were treated with the PAI-1 inhibitor Tiplaxtinin (PAI-039; 11µM) or its vehicle just before exposure to PAK. In other experiments, BPAEC cells were pretreated with the p38 MAP kinase inhibitor SB98450 (11µM) for 1 hour.

Cells treatment with exogenous PAI-1. We proceeded as described in a previous study **(4)**. In brief, we cultured HMVEC cells in 6 wells plate until they reached confluence (4 days). The cells were rinsed 3 times with ice cold PBS and 1 time with EGM-2MV containing 0.02% BSA. The cells were then incubated in 0.84 ml of acid wash buffer (50mM glycine-HCl, 100mM NaCl, pH4) for 3 minutes at 4°C, in order to remove any endogenous PAI-1 present at the cell surface. After removal of the acid wash buffer, the cells were incubated with 1.70 ml of neutralizing buffer (100mM Tris-HCl, 100mM NaCl, pH 7.4) for 10 minutes. After replacement of the neutralizing buffer by fresh EGM-2MV (containing 0.02%), cells were exposed to 20µg/ml of human PAI-1 recombinant protein for 30 minutes. The medium containing recombinant PAI-1 was replaced by fresh EGM-2MV and the cells were transferred to a 37°C incubator for 10 min. Cells were then lysed for G-LISA as explained above.

Cell viability assay. Cell viability was measured by the Alamar Blue assay after exposure to the various experimental conditions. Cell media were replaced with media containing 10% Alamar Blue and placed at 37 °C in a cell

incubator for 2h. The media were collected and read on a spectrophotometric plate reader at 530 nm.

Mice. Wild type C57BL/6 (C57BL/6J #000664) and PAI-1 null (strain: C57BL/6J #000257) mice were purchased from Jackson Laboratories. Mice were maintained in an air-filtered, temperature-controlled (24°C), pathogen-free barrier with free access to food and water. Room humidity was controlled between 35 and 40%. Mice were 8–10 wk of age at the time of experiment. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of California (San Francisco, CA).

Pneumonia model. The model was described in our previous study **(5)**. Mice were anesthetized with tribromoethanol (250 mg/kg, i.p.). The mouse was laid on a board with its head elevated at 45°. Then, 50 µl of PBS (containing 1 x 10^7 CFU of PAK) was instilled into both lungs through the trachea via the mouth by using a 27G gavage needle. The mouse was allowed to recover for 15 min prior replacement into the cage. Mice were active and appeared normal after 30 min. Four to eight hours after the bacterial instillation, mice were euthanized with a larger dose of tribromoethanol (500 mg/kg, i.p.). Blood samples were collected in a sterile fashion through puncture of the inferior vena cava after laparatomy and bilateral thoracotomies had been done. The mouse lungs were removed, weighed, and homogenized for lung vascular permeability measurements. Bronchoalveolar lavage (BAL) fluid was obtained, as described below. Bacterial concentration was determined by quantitative culture of homogenized lungs.

Lung vascular permeability measurement. Lung excess lung water (ELW, µl) and extravascular plasma equivalent (EVPE, µl) were measured, as previously described **(6)**. Briefly, 0.5 µCi of ^{125}I-albumin was injected i.p. 4 h before sacrificing the animals to ensure adequate tracer distribution. The blood was collected through puncture of the inferior vena cava. Then, the lungs were removed, counted in a Wizard γ-counter (Perkin-Elmer, Waltham, MA), weighed, and homogenized. The homogenate was weighed and a fraction centrifuged (12,000 x *g*, 8 min) for determination of the hemoglobin concentration in the supernatant. Another fraction of homogenate,

supernatant and blood were weighed and then dried in an oven (60°C for 48h) for gravimetric determination of the extravascular lung water. The lung wet-to-dry weight ratio (lung W/D ratio) was determined by standard formula as previously reported **(6)**.

Bronchoalveolar lavage (BAL), cell count and KC measurements. BAL fluid was collected by infusing 1 ml of sterile PBS (containing 5mM EDTA) into the lungs of the mice after tracheal cannulation, as previously described **(7)**. Gentle suction was applied and approximately 85% of the fluid was withdrawn from the lungs. The collected fluid was centrifuged at 6000 rpm for 5 min. 100 µl of the supernatant was immediately used for cytospin preparation (see below) and the remaining was stored immediately at -80°C for protein concentration and KC measurements.

Cytospin preparations were made on glass slides, and differential cell counts were performed by two independent operators using Diff-Quik-stained slides. Mean counts from duplicate slides were obtained and expressed as the number of cells per ml of BAL fluid recovered (x 10^4/ml BALF).

After BAL fluid was collected, BAL fluid samples were diluted 5 times for concentration measurements. DuoSet$^®$ ELISA kits (R&D) for mouse keratinocyte-derived chemokine (KC) were carried out according to the manufacturers' protocol.

Bacterial cultures from the lung homogenates. Mouse lungs were collected in a sterile fashion. The lungs were homogenized in sterile containers using a tissue homogenizer (Tissue tearor model 985-370, Biospec products Inc, Racine, WI). The homogenates were serially diluted and plated in triplicate on sheep-blood agar plates. Counts were performed by two independent operators.

Lung myeloperoxidase measurement. Lungs were isolated and quickly frozen into liquid nitrogen. Lungs were kept at -80°C until used. Lung homogenization was performed using a tissue homogenizer (Tissue tearor model 985-370, Biospec products Inc., Racine, WI) with the lysis buffer and the protease inhibitor provided by the company (mouse MPO kit HK210 from Cell Sciences, Canton MA). The lungs homogenates were then centrifuged

and the supernatants were transferred to clean tubes. All of the supernatant samples were adjusted to the same protein concentration for better accuracy. The measurement was then performed according to the manufacturers' protocol.

Survival protocol. 20 wild type mice were randomly assigned to two groups (untreated mice n=10; PAI-039 treated mice n=10). A third group was composed of 10 PAI-1 null mice. Mice were exposed to *P. aeruginosa* as described above. All mice received 1ml of subcutaneous saline to prevent dehydration. The mortality in the three groups was monitored over 48h. The mortality rates of the three groups were compared by a Kaplan-Meier analysis. Survival time was defined as the time between instillation and death.

Statistical analysis. All data are summarized as mean ± SD. For the statistical analysis we used Statview 5.0® (SAS Inc.) and MedCalc® 7.2.0.2 (MedCalc Software Inc.). The normal distribution was verified using the Kolmogorov-Smirnov test. Since all series of data were normally distributed, one-way ANOVA and the Fisher's exact *t* test were used to compare experimental with control groups. A *p* value of < 0.05 was considered statistically significant. A Kaplan-Meier analysis was used to compare the survival between the three experimental groups of mice at 48h.

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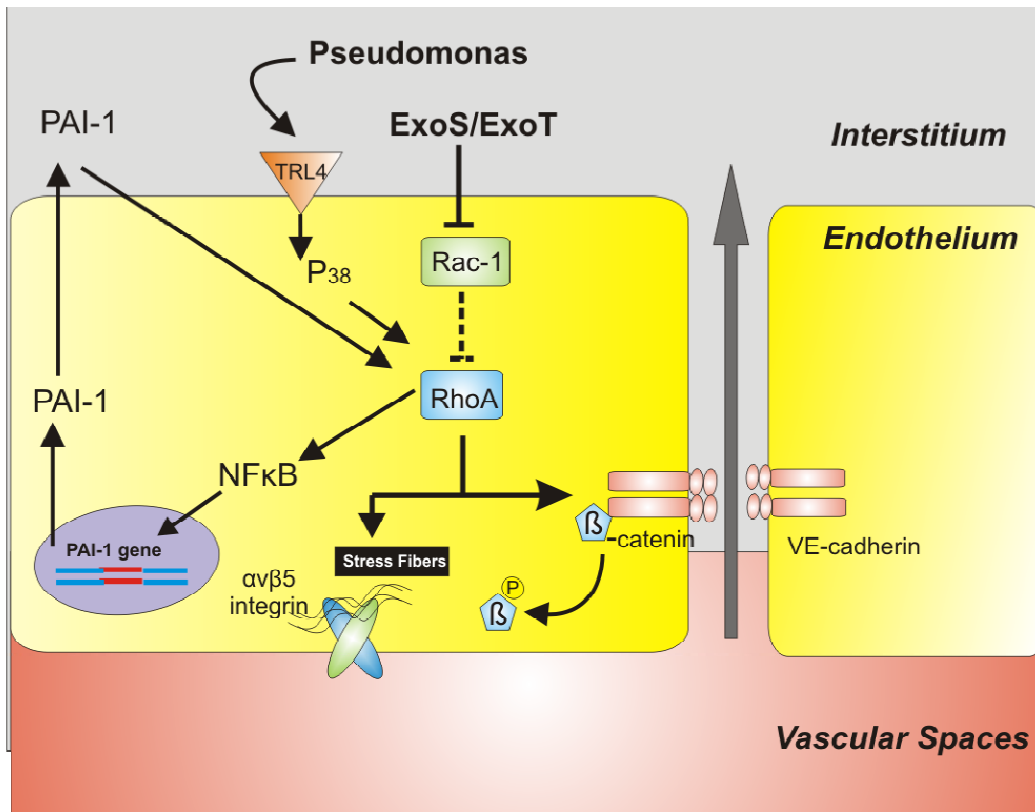
Figure 1. Schematic of the mechanisms by which *P. aeruginosa* increases PAI-1 expression via a RhoA-NFkappaB-dependent mechanism and by which PAI-1 released in the extracellular space further increases RhoA activation in lung endothelial cells. *P. aeruginosa* via the intracellular injection of ExoS/ExoT from the type III secretion system or via the activation of the Toll-like receptor 4 causes (a) an increase in RhoA activity resulting in (b) the expression of PAI-1 (c) its release in to the extracellular space and (d) the disassembly of the VE-cadherin/β-catenin adherens junction protein complex and increase in protein permeability in lung endothelial cells. Extracellular PAI-1 increases the activation of RhoA and further contributes to the *P. aeruginosa*-mediated disruption of the lung endothelial barrier function*.*