Plasminogen activator inhibitor type 1 is protective during severe Gram-negative pneumonia

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Plasminogen activator inhibitor type-1 (PAI-1) levels are consistently elevated in patients with severe pneumonia and sepsis and highly predictive for an unfavorable outcome. In addition, pneumonia is associated with strongly elevated PAI-1 levels in the pulmonary compartment. However, whether PAI-1 causally affects antibacterial host defense in vivo remains unknown. We report here that pneumonia caused by the common respiratory pathogen *Klebsiella pneumoniae* is associated with local production of PAI-1 in the lungs of wild-type mice. PAI-1 deficiency impaired host defense as reflected by enhanced lethality and increased bacterial growth and dissemination in mice with a targeted deletion of the *PAI-1* gene. Conversely, transgenic overexpression of PAI-1 in the lung using a replication-defective adenoviral vector markedly improved host defense against *Klebsiella* pneumonia and sepsis. PAI-1 deficiency reduced accumulation of neutrophils in the lungs during pneumonia, whereas PAI-1 overexpression in healthy lungs resulted in neutrophil influx, suggesting that PAI-1 protects the host against *Klebsiella* pneumonia by promoting neutrophil recruitment to the pulmonary compartment. These data demonstrate for the first time that PAI-1 is essential for host defense against severe Gram-negative pneumonia. (Blood. 2007;109:1593-1601)

Introduction

Bacterial pneumonia remains associated with a high morbidity and mortality. Because of the high incidence of pneumonia and the increasing antimicrobial resistance,[1] further understanding of the nonspecific host defense is necessary to pave the way for new treatment options. During pneumonia several mediator systems become activated, culminating in a profound inflammatory response together with increased procoagulant activity and suppression of the fibrinolytic system.[2,3] Plasminogen activator inhibitor type-1 (PAI-1) is the main inhibitor of the fibrinolytic system. By inactivating both urokinase type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA), PAI-1 inhibits plasmin generation and subsequent fibrin degradation. Several studies in patients with pneumonia revealed elevated PAI-1 levels in bronchoalveolar lavage fluid,[2-5] and in patients with ventilator-associated pneumonia high PAI-1 concentrations correlated with a poor outcome.[4] In addition, elevated circulating levels of PAI-1 predicted lethality in patients with sepsis,[6-11] and the most common site of infection in such patients is the respiratory tract.[12,13] Hence, observational studies are highly suggestive of a role for PAI-1 in the pathogenesis of pneumonia and sepsis.

Besides its classic role as an inhibitor of fibrinolysis, PAI-1 has been implicated as a mediator in several other processes, including wound healing, atherosclerosis, tumor angiogenesis, rheumatoid arthritis, fibrosis, metabolic disturbances, and glomerulonephritis.[14-21] Of interest, different roles for PAI-1 in leukocyte migration have been described. For instance, PAI-1 can inhibit integrin-mediated cell migration by the binding to vitronectin, thereby competing with integrins and the uPA receptor (uPAR).[22-24] In contrast, PAI-1 can support interleukin-8 (IL-8)–mediated neutrophil transendothelial migration by inhibition of the constitutive shedding of endothelial IL-8/heparan sulfate/syndecan-1 complexes.[25] Furthermore, the absence of PAI-1 prevents cancer invasion in mice,[17] which provides more evidence for a stimulatory role of PAI-1 in cell migration. Moreover, bleomycin-induced lung inflammation was almost absent in PAI-1 gene–deficient (PAI-$1^{-/-}$) mice and strongly enhanced by transgenic PAI-1 overexpression.[15] The migration of leukocytes, especially neutrophils, to the lungs is an important part of the innate immune response to bacterial pneumonia.[26] Therefore, in theory, elevation of PAI-1 levels might influence the inflammatory response and host defense during severe pneumonia.

Recently, our laboratory showed that PAI-$1^{-/-}$ mice have an unremarkable host defense in a model of community-acquired pneumonia caused by the Gram-positive bacterium *Streptococcus pneumoniae*.[5] In light of the clear association between elevated PAI-1 concentrations and the outcome of severe pneumonia and sepsis,[4,6-11] we here wondered whether PAI-1 plays a functional role in the host response to severe Gram-negative pneumonia and the ensuing sepsis syndrome. Therefore, in the present study we studied the local and systemic consequences of PAI-1 deficiency and adenoviral-mediated PAI-1 overexpression in a murine model

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of pneumonia and sepsis caused by *Klebsiella pneumoniae,* a common Gram-negative respiratory pathogen.[1,27,28] We show for the first time an important protective role for PAI-1 in host defense against severe pneumonia.

Materials and methods

Animals

The Institutional Animal Care and Use Committee approved all experiments. Normal C57BL/6 wild-type (Wt) mice were obtained from Harlan Sprague-Dawley (Horst, The Netherlands). PAI-1$^{-/-}$ mice on a C57BL/6 genetic background were obtained from the Jackson Laboratory (Bar Harbor, ME). Female, 8- to 10-week-old mice were used in all experiments.

Klebsiella pneumoniae infection

Pneumonia was induced by intranasal inoculation of 1×10^4 colony-forming units (CFU) *K pneumoniae* serotype 2 (ATCC 43816; American Type Culture Collection, Manassas, VA) as described before.[29,30]

Assays

Lung homogenates were prepared as described.[29,30] The following enzyme-linked immunosorbent assays (ELISAs) were used: D-dimer (Asserachrom D-dimer; Roche, Woerden, The Netherlands); murine PAI-1 and human PAI-1 (both from Kordia, Leiden, The Netherlands); macrophage inflammatory protein 2 (MIP-2) and keratinocyte-derived chemokine (KC) (both from R&D Systems, Abingdon, United Kingdom); and myeloperoxidase (MPO; Hycult Biotechnology BV, Uden, The Netherlands). Tumor necrosis factor-α (TNF-α), IL-6, interferon-γ (IFN-γ), and IL-10 levels were determined using a cytometric beads array (CBA) multiplex assay (BD Biosciences, San Jose, CA). Aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT), urea, and creatinine were determined with kits from Sigma (St Louis, MO), using a Hittachi analyzer (Boehringer Mannheim, Mannheim, Germany).

Evaluation of PAI-1 mRNA

Total RNA was isolated using the RNeasy Mini Kit system (Qiagen, Venlo, The Netherlands) and treated with RQ1 RNase-Free DNase (Promega, Leiden, The Netherlands) and reverse transcribed using oligo (dT) primer and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Breda, The Netherlands). Reverse-transcription–polymerase chain reactions (RT-PCRs) were performed in a LightCycler (Roche) apparatus using the following conditions: 5-minute 95°C hot-start, followed by 40 cycles of amplification (95°C for 15 seconds, 60°C for 5 seconds, 72°C for 20 seconds). For quantification, standard curves were constructed by PCR on serial dilutions of a concentrated cDNA, and data were analyzed using LightCycler software. Gene expression is presented as a ratio of the expression of the housekeeping gene β2-microglobulin.[31] Primers used for murine PAI-1 were as follows: mPAI-1 S1019, ATCCTGCCTAAGTTCTCTCTG; mPAI-1 AS1164, ACCTCGATCCTGACCTTTTG. Primers for the housekeeping gene were as follows: mB2M S74, TGGTCTTTCTGGTGCTTGTCT; mB2M AS231, ATTTTTTTCCCGTTCTTCAGC. Oligonucleotides were derived from Eurogentec (Seraing, Belgium). Visualization of PAI-1 mRNA by in situ hybridization was performed as described previously using radiolabeled [^{35}S]-UTP (Amersham, Arlington Heights, IL) human (NM_000602, bp 76-1109) and mouse (NM_008871) PAI-1–specific riboprobes.[32]

Determination of survival and bacterial outgrowth

For survival studies, mice (n = 12-16 per group) were monitored every 12 hours for 10 days after infection. Bacterial loads were determined as described (n = 8 per group).[29,30]

Histologic examination

Hematoxylin and eosin–stained lung and liver slides were scored according to the following parameters: (1) the number of thrombi counted in 5 fields at a magnification of × 200 (lungs) or × 100 (liver); (2) the presence and degree of inflammation, which included the presence of interstitial influx of leukocytes, endothelialitis, edema, pleuritis, and bronchitis; and (3) for liver only, the presence and degree of necrosis. All parameters were rated from 0 to 3, wherein 0 = absent, 1 = occasionally, 2 = regularly, and 3 = massively.

Slides were mounted with glycergel (DAKO, Glostrup, Denmark) and stained with hematoxylin (inset images in Figures 3, 5, and 7 were stained with Ly6G). Images were visualized using an Olympus BX51 microscope, and were acquired using an Olympus DP70 camera as well as DPController software version 1.2 and DP Manager software version 1.2 (Olympus Optical, Zoeterwoude, The Netherlands). Objectives used were as follows: 10×/0.30 numerical aperture (NA) UPlanF1 (Figures 1, 3, 4), 20×/0.50 NA UPlan F1 (Figures 3 insets, 5, 5 insets, 6, and 7), or 40×/0.85 NA UPlanApo (Figure 7 insets).

Adenovirus-mediated transfer of PAI-1 gene

The replication-defective adenoviral vector expressing human PAI-1 cDNA (Ad.PAI-1) was generated as described.[33,34] After transfection, recombinant viral plaques were harvested and amplified as described.[35,36] To overexpress PAI-1 in the lung, we administered 5×10^8 plaque-forming units (PFU) Ad.PAI-1 diluted in sterile NaCl 0.9% to a final volume of 50 μL intranasally. As a control adenovirus, Ad.RR5 was used at the same dose. The dose of 5×10^8 PFU was based on previous studies using adenoviral gene transfer to murine lungs.[37-40]

Statistical analysis

All data are expressed as means ± SEM. Comparisons between groups were conducted using the Mann-Whitney *U* test. Survival was compared by Kaplan-Meier analysis followed by a log-rank test. Significance was set at *P* values less than .05.

Results

Endogenous PAI-1 is up-regulated during *Klebsiella* pneumonia

To obtain insight into local PAI-1 concentrations in the lungs, we measured PAI-1 protein levels in lung homogenates of uninfected Wt mice and Wt mice with a respiratory tract infection with *K pneumoniae* (Figure 1A). PAI-1 was detected at levels of 2.5 to 4.1 ng/mL in lung homogenates of uninfected mice. *Klebsiella* pneumonia was associated with a mean 9-fold increase in lung PAI-1 levels at 24 hours and a 7-fold increase at 48 hours after infection (both $P < .05$ versus controls). This rise in PAI-1 protein was a result of an increased expression of PAI-1 mRNA in the lungs during infection, as shown by RT-PCR (Figure 1B) and in situ hybridization (Figure 1C-D). Pulmonary PAI-1 expression was mostly colocalized with vessel walls and inflammatory infiltrates.

Fibrinolysis is enhanced in PAI-1$^{-/-}$ mice during *K pneumoniae* pneumonia

Since PAI-1 is the main inhibitor of the fibrinolytic system, we wanted to investigate whether PAI-1 deficiency influenced the fibrinolytic activity in the lungs during *K pneumoniae* pneumonia. Therefore, we measured the levels of D-dimer, an end-product derived from plasmin-mediated degradation of cross-linked fibrin clots, in lung homogenates of Wt and PAI-1$^{-/-}$ mice at 0, 24, and

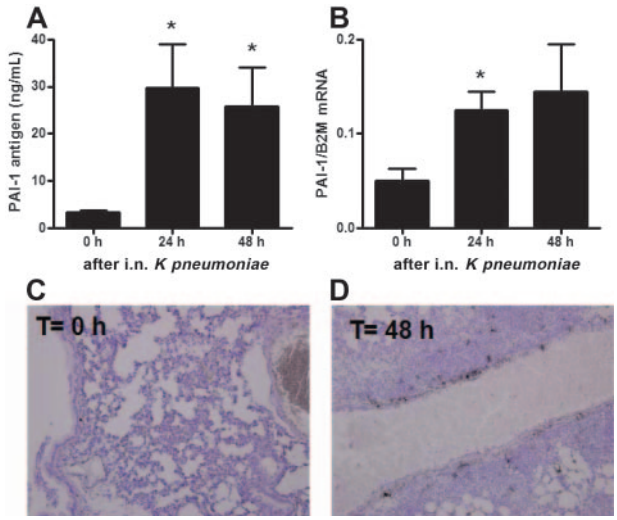

Figure 1. Endogenous PAI-1 levels increase during *Klebsiella* pneumonia. PAI-1 protein concentrations in lung homogenates (A) and mRNA levels in lung tissues (B) were determined before and at 24 and 48 hours after intranasal (i.n.) administration of *K pneumoniae*. Data are means ± SE. n = 6 per group. $^*P < .05$ versus 0 hours (uninfected mice). Murine PAI-1 in situ hybridization was performed on lung tissues before (C) and 48 hours after (D) infection. Positive signal is indicated in black. Magnification, × 100.

48 hours after intranasal inoculation of *K pneumoniae*. As expected, PAI-1$^{-/-}$ mice showed a stronger increase in D-dimer levels during infection than Wt mice (Figure 2A).

PAI-1 deficiency accelerates lethality due to *K pneumoniae* pneumonia

To study the contribution of endogenous PAI-1 to the outcome of *Klebsiella* pneumonia, Wt and PAI-1$^{-/-}$ mice were intranasally inoculated with *K pneumoniae* and monitored for 10 days (Figure 2B). Although the first deaths occurred after 2 days in both strains, lethality was significantly accelerated among PAI-1$^{-/-}$ mice thereafter ($P < .05$). Indeed, after 3 days 64% of PAI-1$^{-/-}$ versus 18% of Wt mice had died, after 4 days 82% of PAI-1$^{-/-}$ versus 45% of Wt mice had died, and after 6 days 91% of PAI-1$^{-/-}$ versus 55% of Wt mice had died. Thus, the lack of PAI-1 rendered mice more susceptible to *K pneumoniae*–induced death.

PAI-1 deficiency facilitates bacterial outgrowth and dissemination

To investigate whether the enhanced lethality of PAI-1$^{-/-}$ mice was associated with changes in bacterial outgrowth, we examined the bacterial loads in the lungs of PAI-1$^{-/-}$ and Wt mice at 24 and 48 hours after induction of pneumonia (ie, directly before the first mice started dying). At 24 hours, bacterial counts in the lungs were similar in PAI-1$^{-/-}$ and Wt mice. However, after 48 hours the number of *Klebsiella* CFU was almost 50-fold higher in the lungs of PAI-1$^{-/-}$ mice when compared with Wt mice ($P < .01$; Figure 2C). To obtain insight into the dissemination of the infection, we examined the number of positive blood cultures in PAI-1$^{-/-}$ and Wt mice. At 24 hours after infection, 60% of the PAI-1$^{-/-}$ mice showed positive blood cultures compared with 40% of the Wt mice; at 48 hours, all PAI-1$^{-/-}$ mice were bacteremic compared with 62.5% of the Wt mice. Moreover, PAI-1$^{-/-}$ mice with positive blood cultures had a higher bacterial load in their circulation than WT mice with positive cultures (24 hours: $1.45 \pm 0.89 \times 10^3$ CFU/mL versus $12.46 \pm 3.55 \times 10^3$ CFU/mL, respectively [$P < .05$]; 48 hours: $2.14 \pm 2.12 \times 10^7$ CFU/mL versus $3.48 \pm 2.46 \times 10^7$ CFU/mL, respectively [not significant]). Thus, endogenous PAI-1 serves to limit the outgrowth of *K pneumoniae* in the lung and the ensuing dissemination to the blood stream.

Influence of PAI-1 deficiency on pulmonary neutrophil recruitment during pneumonia

Pneumonia results in inflammatory-cell recruitment and local inflammation, which is an integral part of the host immune response.[26,41] Therefore, we performed semiquantitative analyses of lung histologic slides prepared from PAI-1$^{-/-}$ and Wt mice 48 hours after infection. At this time point, all mice showed inflammatory infiltrates, characterized by interstitial inflammation together with endothelialitis, bronchitis, pleuritis, and edema (Figure 3A-B). Although there was no difference in total lung histopathology scores between PAI-1$^{-/-}$ and Wt mice (Figure 3C), the lungs of PAI-1$^{-/-}$ mice clearly contained fewer infiltrating neutrophils as visualized by Ly-6 staining (Figure 3A-B insets). The reduced neutrophil influx in PAI-1$^{-/-}$ mice was confirmed by measurements of MPO levels in whole lung homogenates. Indeed, whereas at 24 hours after infection MPO concentrations were similar in the lungs of PAI-1$^{-/-}$ and Wt mice, at 48 hours lung MPO levels were lower in the former mouse strain ($P < .05$, Figure 3E). Since cytokines and chemokines are important regulators of the inflammatory response to bacterial pneumonia,[26,41] we also measured the levels of KC, MIP-2, TNF-α, IL-6, IFN-γ, and IL-10 in lung homogenates obtained at 24 and 48 hours after infection. No differences were found between PAI-1$^{-/-}$ and Wt mice with the exception of IL-6 levels after 48 hours, which were higher in PAI-1$^{-/-}$ mice (data not shown). In light of recent findings described by Arndt et al,[42] revealing reduced neutrophil influx in BALF of PAI-1$^{-/-}$ mice after inhalation of LPS aerosols in the presence of elevated KC plasma levels, we measured the concentrations of this mouse CXC chemokine in plasma samples obtained 24 and 48 hours after infection with *Klebsiella* (Figure 3F). Indeed, PAI-1$^{-/-}$ mice displayed higher KC plasma levels than Wt mice at both time points ($P < .05$). Together, these data indicate that the major differences between PAI-1$^{-/-}$ and Wt mice with respect to the inflammatory response to *Klebsiella* pneumonia were a diminished recruitment of neutrophils into lung tissue combined with increased intravascular KC.

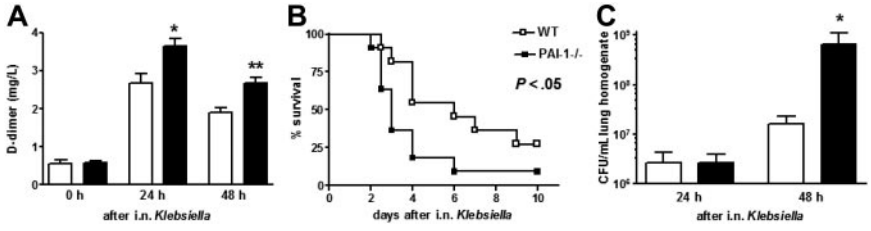

Figure 2. PAI-1 deficiency impairs host defense against *Klebsiella* pneumonia. (A-C) Wt and PAI-1$^{-/-}$ mice were inoculated intranasally with 10^4 CFU *K pneumoniae*. D-dimer was measured in lung homogenates at 0, 24, and 48 hours after infection (A). Survival was monitored (B) and bacterial load was determined at 24 and 48 hours after infection (C). Data are means ± SE of 8 mice per genotype at each time point. Survival was evaluated using 12 mice per genotype. $^*P < .05$ versus Wt mice at the same time point. $^{**}P < .01$.

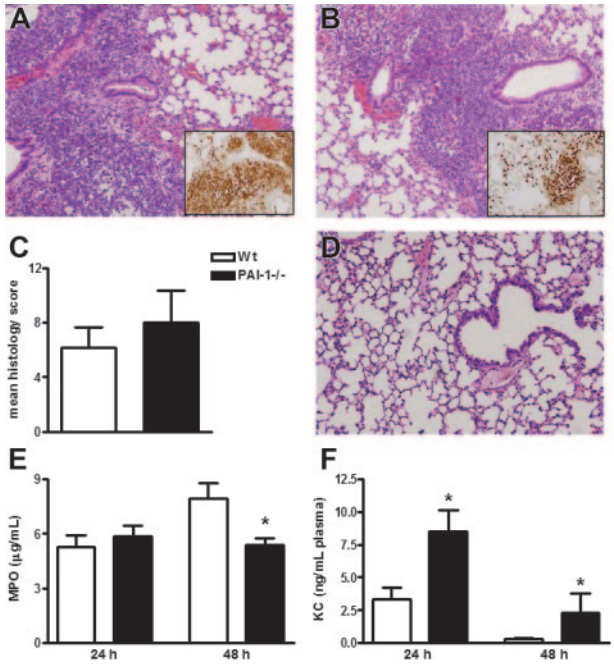

Figure 3. Influence of PAI-1 deficiency on lung inflammation during *Klebsiella* pneumonia. Representative HE stainings of lung tissue of Wt (A) and PAI-$1^{-/-}$ (B) mice at 48 hours after intranasal inoculation of 10^4 CFU *K pneumoniae.* Original magnification, × 100. Insets show representative neutrophil stainings at the same time point; original magnification, × 200. (C) Graphic representation of the degree of lung inflammation, determined according to the scoring system described in "Histologic examination." As a reference, normal mouse lung tissue is shown in panel D. (E) MPO levels and (F) plasma KC levels were determined at 24 and 48 hours after infection. (C,E-F) Data are means ± SE. n = 8 per group at each time point. *$P < .05$ versus Wt mice at the same time point.

Effect of PAI-1 deficiency on distant organ injury

Considering that PAI-$1^{-/-}$ mice displayed an accelerated lethality together with more frequent bacteremia, we determined whether the enhanced dissemination of the infection had led to distant organ injury. Therefore, we performed histopathologic analyses of liver tissue and evaluated liver injury and kidney function by clinical chemistry at 48 hours after infection in both PAI-$1^{-/-}$ and Wt mice. At this time point, all mice showed evidence of hepatic injury, as characterized by inflammation of liver tissue (the influx of leukocytes into the hepatic parenchyma), thrombi formation, and foci of liver necrosis; the mean liver histology scores were similar in PAI-$1^{-/-}$ and Wt mice (2.7 ± 0.7 versus 3.4 ± 0.6, respectively). In accordance, the plasma levels of ASAT and ALAT, indicative of hepatocellular injury, were indistinguishable in both mouse strains (data not shown). Moreover, plasma creatinine and urea levels were similar in PAI-$1^{-/-}$ and Wt mice (data not shown). Hence, these data suggest that PAI-1 deficiency does not have a significant impact on distant organ injury during *K pneumoniae* pneumonia and sepsis, at least not during the first 48 hours after infection.

Transgenic overexpression of PAI-1 reduces lethality and limits bacterial outgrowth during *Klebsiella* pneumonia

Having established that endogenous PAI-1 contributes to an effective host defense against *Klebsiella* pneumonia, we next determined whether enhanced expression of PAI-1 would be of benefit to the host in the same model. For this, we used Ad.PAI-1, which previously has been used successfully to enhance systemic PAI-1 expression after intravenous administration.[14] Considering that intrapulmonary delivery of adenoviral vectors has been demonstrated to result in increased expression of various transgenes in the respiratory tract,[37-40] we here examined whether intranasal administration of Ad.PAI-1 led to expression of human PAI-1 in the lungs. Indeed, human PAI-1 protein was present in the lungs (but not in plasma); human PAI-1 lung concentrations peaked at 24 hours after inoculation and remained elevated for at least 2 more days (Figure 4A). In addition, in situ hybridization was performed on lung tissues obtained 24 hours after intranasal administration of a control adenovirus Ad.RR5 or Ad.PAI-1 (Figure 4B and C, respectively). Transgenic PAI-1 expression was absent in the Ad.RR5-inoculated mice. In the Ad.PAI-1–treated mice, human PAI-1 mRNA expression was found diffuse throughout the lungs. As expected, human PAI-1 mRNA was not detected in livers (data not shown), suggesting that human PAI-1 expression remained confined to the pulmonary compartment. Based on these experiments, we decided to determine the effect of enhanced pulmonary PAI-1 expression on host defense during pneumonia by intranasal administration of Ad.PAI-1 or Ad.RR5 24 hours prior to intranasal infection with *K pneumoniae*. Mice treated with Ad.PAI-1 showed strongly reduced D-dimer levels during pneumonia compared with Ad.RR5-treated mice, indicating that the overexpression of human PAI-1 in murine lungs resulted in local inhibition of the fibrinolytic system (Figure 4D). Furthermore, mice treated with Ad.PAI-1 showed a significantly delayed mortality ($P < .001$ versus mice treated with Ad.RR5; Figure 4E). Whereas control mice started dying after 3 days and all had died by

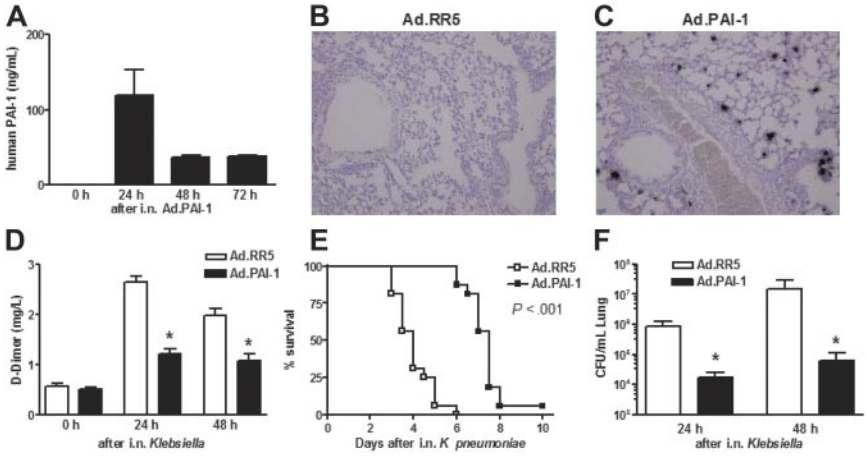

Figure 4. Transgenic PAI-1 expression improves host defense against *Klebsiella* pneumonia. (A) Wt mice were intranasally inoculated with 5 × 10^8 PFU Ad.PAI-1, and human PAI-1 levels were measured in lung homogenates after 0 and 24, 48, and 72 hours. Human PAI-1 in situ hybridization (B-C) showed a strong expression of PAI-1 mRNA in murine lung tissues 24 hours after Ad.PAI-1 intranasal (C) but not after Ad.RR5 (B) inoculation. Positive signal is indicated in black. Magnification, × 100. (D-F) *Klebsiella* pneumonia was induced in Wt mice at 24 hours after intranasal inoculation with 5 × 10^8 PFU Ad.PAI-1 or Ad.RR5; D-dimer was measured in lung homogenates at 0, 24, and 48 hours after infection (D), survival was monitored (E), and bacterial load was determined at 24 and 48 hours after infection (F). Data are means ± SE of 8 mice per genotype at each time point (A,D,F). Survival was evaluated using 16 mice per genotype. *$P < .05$ versus Ad.RR5-treated mice at the same time point.

day 6, mice that had received Ad.PAI-1 did not show clear signs of illness up to 6 days after infection, with the first deaths occurring shortly thereafter. Furthermore, antibacterial defense was clearly improved in Ad.PAI-1–treated mice, as shown by 100-fold lower bacterial loads compared with Ad.RR5-treated mice at both 24 and 48 hours after induction of pneumonia ($P < .001$ at both time points; Figure 4F). Moreover, dissemination of the infection to the circulation, as measured by positive blood cultures for *K pneumoniae* after 48 hours, was present in only 12.5% of Ad.PAI-1–treated mice compared with 75% of Ad.RR5-treated control mice.

PAI-1 overexpression attenuates neutrophil recruitment and lung inflammation during *Klebsiella* pneumonia

Upon histopathologic examination at 48 hours after *K pneumoniae* infection, the lungs of all Ad.RR5-treated mice showed inflammatory infiltrates, characterized by interstitial inflammation together with pleuritis, bronchitis, and edema (Figure 5A). In contrast, only 12% of the mice that had received Ad.PAI-1 showed any infiltrated areas in their lungs, which were also of a lesser extent compared with Ad.RR5-treated mice (Figure 5B). In accordance, the total histopathology score of the lungs (quantified according to the scoring system described in "Materials and methods") was significantly reduced in the Ad.PAI-1–treated mice ($P < .01$; Figure 5C). Ly-6 stainings of lung tissue slides revealed a strongly reduced influx of neutrophils in Ad.PAI-1–treated mice when compared with Ad.RR5-administered control mice (Figure 5A-B insets). In line with these findings, mice that had received Ad.PAI-1 showed strongly reduced MPO levels in lung homogenates at 24 and 48 hours after infection with *Klebsiella* ($P < .05$ versus Ad.RR5-treated mice; Figure 5D). In addition, the lung concentrations of TNF-α, IL-6, IFN-γ, IL-10, KC, and MIP-2 were markedly diminished in Ad.PAI-1–treated mice at 24 and 48 hours after induction of *Klebsiella* pneumonia (Table 1).

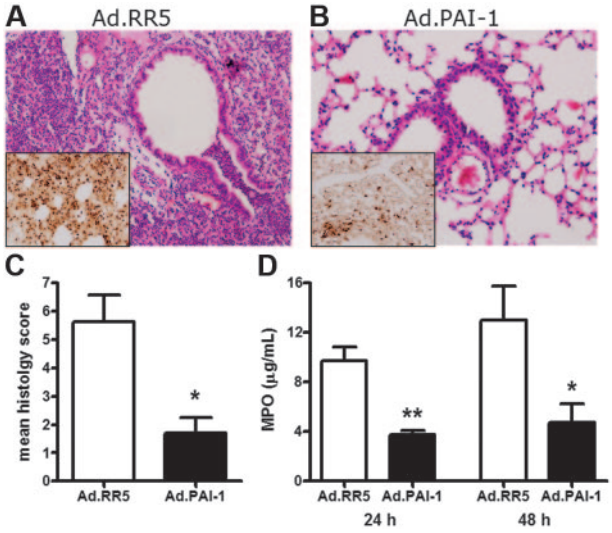

Figure 5. Transgenic PAI-1 expression reduces lung inflammation during *Klebsiella* pneumonia. Representative HE stainings of lung (A-B) tissue at 48 hours after intranasal inoculation of 10^4 CFU *K pneumoniae.* Mice were pretreated with 5×10^8 PFU Ad.PAI-1 or Ad.RR5 intranasally at 24 hours before infection. Magnification, × 200. Insets show representative neutrophil stainings of the same time point; magnification, × 200. (C) Graphic representation of the degree of lung inflammation, determined according to the scoring system described in "Histologic examination." (D) MPO levels were determined at 24 and 48 hours after infection. Data are means ± SE. n = 8 per group at each time point. $*P < .01$, $**P < .001$ versus Ad.RR5-treated mice at the same time point.

Table 1. Effect of PAI-1 on cytokine levels during *Klebsiella* pneumonia

	24 h		48 h	
pg/mL	Ad.RR5	Ad.PAI-1	Ad.RR5	Ad.PAI-1
TNF-α	1152 ± 324	162 ± 14*	829 ± 609	56 ± 14
IL-6	1413 ± 362	140 ± 27†	1564 ± 507	119 ± 69‡
IFN-γ	45 ± 24	9 ± 1*	243 ± 128	48 ± 43
IL-10	763 ± 706	51 ± 11	2576 ± 916	659 ± 620†
KC	695 ± 244	123 ± 8‡	797 ± 270	323 ± 240†
MIP-2	1496 ± 137	983 ± 31†	1986 ± 507	991 ± 40†

Data are means ± SEM at 24 and 48 hours after intranasal inoculation of 10^4 CFU *K pneumoniae.* Mice were inoculated with 5×10^8 PFU Ad.RR5 or Ad.PAI-1 at 24 hours before infection. Eight mice per group were used at each time point.

$*P < .05$, $†P < .01$, $‡P < .001$ versus Ad.RR5 mice at the same time point.

PAI-1 overexpression reduces distant organ injury

To obtain insight into the effect of intrapulmonary transgenic PAI-1 expression on the development of distant organ damage during *Klebsiella* pneumonia, livers were harvested for histologic examination at 48 hours after bacterial infection (Figure 6A-B). Seventy-five percent of Ad.RR5-treated mice showed profound liver injury, as characterized by inflammation (influx of leukocytes into the hepatic parenchyma) and foci of necrosis; in 50% of these mice vascular thrombi were observed. In contrast, no liver necrosis or thrombi were seen in mice treated with Ad.PAI-1, and only 25% of these mice showed any sign of hepatic inflammation. In line with these findings, the total liver histopathology score of Ad.PAI-1–treated mice was significantly lower than that in Ad.RR5-treated mice ($P < .01$; Figure 6C). These differences in liver injury found upon histopathologic examination were confirmed by the plasma concentrations of ALAT and ASAT, which were much lower in transgenic PAI-1–expressing mice (both $P < .05$; Figure 6D-E). Furthermore, to evaluate the effect of Ad.PAI-1 on kidney function during *Klebsiella* pneumonia, we measured urea and creatinine in plasma of both groups of mice at 48 hours after infection. Both urea and creatinine levels were lower in Ad.PAI-1–treated mice (both $P < .05$; Figure 6F-G).

PAI-1 overexpression also reduces bacterial loads and neutrophil recruitment in PAI-$1^{-/-}$ mice

We next wished to establish whether transgenic expression of PAI-1 in the lungs of PAI-$1^{-/-}$ mice has effects similar to those in Wt mice. Therefore, PAI-$1^{-/-}$ mice were intranasally administered with either Ad.PAI-1 or Ad.RR5 24 hours prior to infection with *Klebsiella.* In accordance with the effects observed in Wt mice, at 48 hours after induction of pneumonia, Ad.PAI-1–treated PAI-$1^{-/-}$ mice displayed much lower bacterial loads in their lungs (Ad.PAI-1: $7.53 \pm 5.81 \times 10^4$ CFU/mL; Ad.RR5: $1.04 \pm 0.54 \times 10^7$ CFU/mL, $P < .01$). In addition, Ad.PAI-1 treatment was associated with a reduced recruitment of neutrophils to the lungs, as reflected by neutrophil stainings of lung tissue slides (data not shown).

Transgenic expression of PAI-1 induces lung inflammation in healthy mice

Having established that enhanced pulmonary expression of PAI-1 confers resistance to *Klebsiella* pneumonia, we speculated about possible underlying mechanisms and hypothesized that elevated PAI-1 levels might influence the inflammatory milieu in the respiratory tract in a way that facilitates an immediate immune response to *K pneumoniae.* To test this hypothesis, we examined lung tissues obtained from healthy mice 24 hours after intranasal

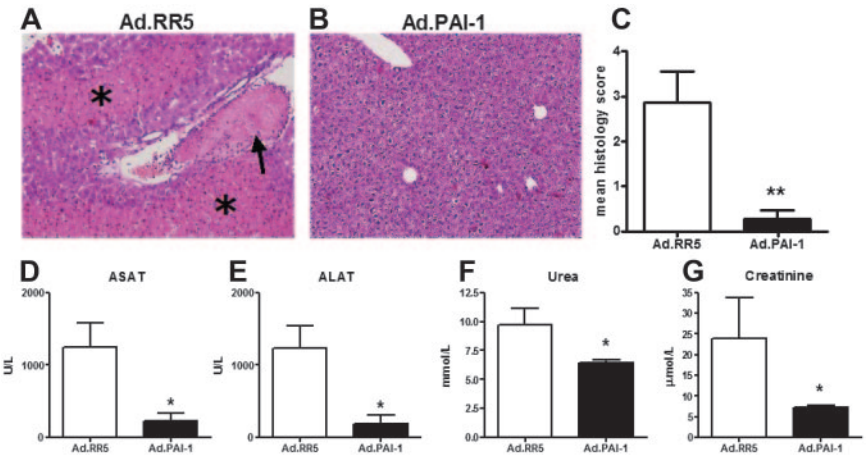

Figure 6. Transgenic PAI-1 expression reduces distant organ injury during *Klebsiella pneumonia.* Representative HE stainings of liver (A-B) tissue at 48 hours after intranasal inoculation of 10^4 CFU *K pneumoniae.* Mice were pretreated with 5×10^8 PFU Ad.PAI-1 or Ad.RR5 intranasally at 24 hours before infection. Magnification, × 200. Livers of Ad.RR5-injected mice (A) showed inflammation, necrosis (*), and thrombi (arrow). (C) Graphic representation of the degree of liver damage at 48 hours after infection, determined according to the scoring system described in "Histologic examination." ASAT (D), ALAT (E), urea (F), and creatinine (G) were measured in plasma at the same time point. Data are means ± SE of 8 mice per genotype. *$P < .05$, **$P < .01$ versus Ad.RR5-treated mice.

administration of Ad.RR5 or Ad.PAI-1, at which time point mice were infected with *K pneumoniae*. Indeed, whereas mice treated with Ad.RR5 showed little if any evidence of lung inflammation, mice administered with Ad.PAI-1 displayed a clear inflammatory reaction in their lungs as assessed by histopathology (Figure 7A-B) and confirmed by histopathologic scores ($P < .05$ versus Ad.RR5 control mice; Figure 7C). In addition, PAI-1 gene transfer was associated with influx of neutrophils into lung tissue as indicated by Ly-6 stainings (Figure 7A-B insets) and lung MPO levels ($P < .001$ versus Ad.RR5 control mice; Figure 7D). Moreover, the levels of TNF-α, IL-6, IFN-γ, and the chemokine KC were all increased in lungs of Ad.PAI-1–treated mice compared with Ad.RR5-administered control mice (all $P < .001$, Table 2). IL-10 and MIP-2 levels were not different. Together, these data show that elevated PAI-1 levels exert a proinflammatory effect in the pulmonary compartment.

Discussion

Severe pneumonia is associated with the concurrent local activation of various mediator systems, in particular the inflammatory, procoagulant, and fibrinolytic responses. PAI-1 is an important inhibitor of the fibrinolytic system of which the production is up-regulated during pneumonia and sepsis in humans.[2-11] Although numerous clinical studies have documented a strong correlation between elevated PAI-1 concentrations and a poor outcome of either sepsis or pneumonia,[4,6-11] it remains unknown whether PAI-1 causally affects antibacterial host defense in vivo. We here used a model of Gram-negative pneumonia and sepsis to determine the role of PAI-1 in the host response to severe infection. In line with clinical studies, we found an up-regulation of endogenous PAI-1 mRNA and protein in the lungs of mice during *Klebsiella* pneumonia. We then demonstrated that endogenous PAI-1 has a protective function during *Klebsiella* pneumonia, as reflected by an enhanced bacterial outgrowth and dissemination and a reduced survival in PAI-$1^{-/-}$ mice. Furthermore, we show that the opposite approach, accomplished by transgenic overexpression of PAI-1 in the pulmonary compartment, conferred a significant protective effect in the same model, not only limiting the outgrowth and dissemination of the infection, but also reducing distant organ failure and delaying mortality. Together our data provide the first evidence for a protective role of PAI-1 during severe infection in vivo.

The respiratory tract is the most common primary site of infection in sepsis.[12,13] *Klebsiella* species is the second most commonly isolated Gram-negative organism in sepsis[13,43] and a frequent causative pathogen in pneumonia.[1,27,28] In severe Gram-negative pneumonia, nonsurvivors showed significantly higher PAI-1 levels in bronchoalveolar lavage fluid compared with survivors.[4] Furthermore, in sepsis patients increased PAI-1 activity predicts lethality in a very sensitive manner.[6-11] In line with these findings, a (4G/5G) promoter deletion/insertion polymorphism in the PAI-1 gene (which has been linked to higher circulating levels of PAI-1) was found to influence the risk of the development of septic shock and to be associated with a poor outcome in patients

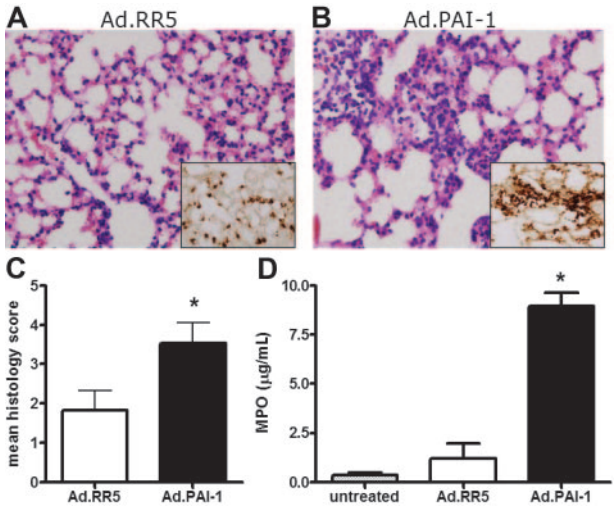

Figure 7. Transgenic PAI-1 expression induces inflammation in healthy lungs. Representative HE stainings of lung tissue at 24 hours after intranasal inoculation of healthy mice with 5×10^8 PFU Ad.RR5 (A) or Ad.PAI-1 (B). Original magnification, × 200. Insets show representative neutrophil stainings of the same time point; original magnification, × 400. (C) Graphic representation of the degree of lung inflammation, determined according to the scoring system described in "Histologic examination." (D) MPO levels. Data are means ± SE. n = 8 per group. *$P < .01$ versus Ad.RR5-treated mice.

Table 2. PAI-1 induces proinflammatory cytokines in healthy lungs

pg/mL	Ad.RR5	Ad.PAI-1
TNF-α	36 ± 12	806 ± 102*
IL-6	73 ± 17	564 ± 33*
IFN-γ	4 ± 0.5	25 ± 5*
IL-10	7 ± 1	8 ± 2
KC	1059 ± 52	1689 ± 93*
MIP-2	2348 ± 1077	2444 ± 471

Data are means ± SEM at 24 hours after intranasal inoculation of 5×10^8 PFU Ad.RR5 or Ad.PAI-1. Eight mice per group were used.

*$P < .001$ versus Ad.RR5 mice.

with sepsis caused by *Neisseria meningitides.*[44,45] These observational studies have suggested a harmful role for enhanced PAI-1 expression in the course of severe pneumonia and sepsis. However, the current data clearly indicate that the production of PAI-1 contributes to an effective host response to *Klebsiella* pneumonia and sepsis.

PAI-1 may play a role in the regulation of several processes implicated in the pathogenesis of sepsis. First, PAI-1 is a strong inhibitor of intravascular fibrinolysis. In line with these findings, PAI-1$^{-/-}$ mice have an accelerated spontaneous whole blood clot lysis.[46] We here add to this that endogenous PAI-1 plays a role in the regulation of the fibrinolytic activity in the lung during pneumonia. Indeed, pneumonia was associated with a rise in D-dimer concentrations in lungs, and this increase was stronger in PAI-1$^{-/-}$ mice, providing evidence for an in vivo role for PAI-1 in inhibiting pulmonary plasmin generation. Second, PAI-1 can prevent the association between vitronectin with integrins and uPAR, which inhibits cell adhesion and migration. uPAR has been implicated as an important positive regulator of neutrophil migration during Gram-negative infection, as indicated by diminished neutrophil recruitment to the lungs of uPAR$^{-/-}$ mice with *Pseudomonas* pneumonia.[47] Our present data suggest that PAI-1 does not inhibit this uPAR function. On the contrary, PAI-1 deficiency was associated with a reduced neutrophil influx into the pulmonary compartment during *Klebsiella* pneumonia. In addition, transgenic overexpression of PAI-1 in healthy mouse lungs resulted in neutrophil recruitment. While our studies were in progress, Arndt et al provided further support for a role of PAI-1 as a positive regulator of neutrophil recruitment during Gram-negative inflammation in the lungs.[42] Indeed, these authors found a diminished influx of neutrophils into BALF of PAI-1$^{-/-}$ mice after aerosol LPS exposure, which was associated with elevated intravascular KC levels.[42] We here confirm and extend these data by showing that PAI-1 deficiency also results in elevated plasma KC levels in the setting of Gram-negative pneumonia. Intratracheal administration of plasmin also caused a rise in plasma KC concentrations in mice with LPS-induced lung inflammation[42]; together with our present finding, these data strongly suggest that PAI-1 deficiency negatively regulates neutrophil influx into the alveolar space at least in part by augmenting intravascular KC levels (thereby "trapping" neutrophils in the intravascular compartment) by a plasmin-dependent mechanism. Our findings are also in line with the results obtained in a model of bleomycin-induced lung injury, in which PAI-1 deficiency protected against inflammation-induced lung damage and overexpression of PAI-1 enhanced the accumulation of leukocytes in the lung.[15] Furthermore, in an antigen-induced arthritis model, PAI-1$^{-/-}$ mice showed significantly reduced joint inflammation.[18] Also, during glomerulonephritis PAI-1 deficiency reduced the number of infiltrating leukocytes in the glomeruli and mice overexpressing PAI-1 showed a profound increase in leukocyte infiltration.[21] Together, these data strongly suggest that the role of PAI-1 in inflammatory-cell migration is often stimulatory rather than inhibitory. Of note, our laboratory recently did not find a role for endogenous PAI-1 during Gram-positive pneumonia caused by *S pneumoniae*.[5] The discrepancy with our current findings might be due to the fact that the mechanisms of cell recruitment to the lung in response to Gram-positive and Gram-negative bacteria appear to be different. In particular, neutrophil migration toward Gram-negative bacterial stimuli present in the lung occurs via a β2 integrin–dependent mechanism, while Gram-positive stimuli elicit β2 integrin–independent leukocyte migration.[48-50] The absence of an effect on neutrophil recruitment during *S pneumoniae* pneumonia[5] may also explain why PAI-1$^{-/-}$ mice had an unremarkable host defense against this infection.

Since the recruitment of neutrophils is an important part of host defense against pneumonia,[26,41] the increased local bacterial load, higher occurrence of bacteremia, and the increased mortality in the PAI-1$^{-/-}$ mice were most likely the result of the impaired inflammatory response. Conversely, transgenic overexpression of PAI-1 was associated with a proinflammatory response in the normal lung. The fact that such a response was not seen after intranasal administration of the empty adenoviral vector strongly suggests that locally induced PAI-1 stimulates inflammation, in particular neutrophil influx and cytokine release. Multiple studies have shown that an early local inflammatory response (influx of neutrophils and/or production of proinflammatory cytokines) is of utmost importance for host defense against bacterial pneumonia[26,41]; hence, this proinflammatory milieu likely was at least in part responsible for the improved host defense in mice treated with Ad.PAI-1. Our current observation that PAI-1 can induce proinflammatory effects (and thereby protect the host against infection) is supported by a very recent report by Kwak et al[51] showing that PAI-1 potentiates LPS-induced neutrophil activation in vitro through a c-Jun N-terminal kinase–mediated pathway.[51] The fact that Ad PAI-1–treated animals displayed a reduced inflammatory response at 24 and 48 hours after the induction of pneumonia, as reflected by histopathology, a reduced neutrophil influx into the lungs, and strongly reduced cytokine levels in lung homogenates likely was the consequence of the strongly reduced outgrowth of *Klebsiella,* resulting in a diminished proinflammatory stimulus provided by bacteria in the lung. Thus, it is our hypothesis that overexpression of PAI-1 elicits an inflammatory response in the lungs at the time of infection (Table 2; Figure 7), which limits the subsequent growth of bacteria; and that as a result of the reduced growth of *Klebsiella,* the bacterial loads in lungs were much lower in Ad.PAI-1–treated mice at later phases during the infection (24 and 48 hours), which can explain the reduced inflammation in these animals at these time points. These results also suggest that the proinflammatory effects of Ad.PAI-1 (as measured at $t = 0$ h) are transient and have disappeared at 24 or 48 hours after infection. In this respect, it is worth mentioning that the differences in bacterial loads between PAI-1$^{-/-}$ mice and Wt mice were much lower than the differences in bacterial numbers between Ad.PAI-1– and Ad.RR5-treated mice; whereas the lung inflammation observed in PAI-1$^{-/-}$ mice overall was similar to that in Wt mice, with the exception of neutrophil influx, Ad.PAI-1–treated mice displayed a strong reduction in all lung inflammatory parameters measured when compared with Ad.RR5-treated mice. These data suggest that extent of lung inflammation is at least in part dependent on the bacterial load, an assumption supported by several investigations on experimental pneumonia.[52,53] Notably, whereas mice treated with Ad.RR5 all died between 3 and 6 days, Ad.PAI-1–treated mice did not get sick initially and survived the first 6 days of infection. However, at day 6 they also became clinically ill and they all died between 6 and 10 days. These data are in line with the kinetics of transgenic expression after adenoviral gene transfer to the lungs, which shows a peak at 24 hours with a strong decline thereafter and disappearing after 7 to 10 days.[54] Thus, the strong decrease in PAI-1 expression at 7 days after adenoviral administration (which coincides with 6 days after infection) might have abolished the protection against mortality in these mice.

It should be noted that the survival curves of the respective control groups shown in Figures 2B and 4E are somewhat different. Several issues deserve attention in this respect. The control groups used in these studies differed in that they received either no treatment (Figure 2B) or Ad.RR5 (Figure 4E). Possibly, Ad.RR5 has a modest impact on the outcome of *Klebsiella* pneumonia; in fact, this is exactly why Ad.RR5 was given in the experiments shown in Figure 4: it is the empty control vector for Ad.PAI-1.

Notably, we induced high lung levels of human PAI-1 in mice. Although human PAI-1 clearly was active in mice (as reflected, for example, by reduced D-dimer levels in Ad.PAI-1–treated mice), it is not excluded that human PAI-1 acts differently in mice than in humans. Therefore, with respect to transgene delivery of human PAI-1, our data should be interpreted with caution. In addition, the effects of exogenous PAI-1 (the experiments with Ad.PAI-1) were stronger than the effects of endogenous PAI-1 (the studies with PAI-$1^{-/-}$ mice), which may have been caused by the fact that Ad.PAI-1 treatment produced much higher PAI-1 levels than endogenous PAI-1 concentrations in Wt mice.

PAI-1 production and release are strongly up-regulated during severe pneumonia and sepsis. In spite of a strong positive association between PAI-1 concentrations and an adverse outcome, the functional role of this enhanced PAI-1 production during severe infection had not been investigated thus far. To our knowledge, this study is the first to show that the local rise in PAI-1 levels plays an important protective role during Gram-negative pneumonia and the ensuing sepsis syndrome induced by *K pneumoniae*.

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Authorship

Contribution: R.R. designed and performed the research, analyzed the data, and wrote the paper; J.J.T.H.R. and S.F. analyzed the pathology slides and took part in writing the corresponding sections of the paper; P.I.B. and C.J.M.V. performed the in situ hybridization of murine and human PAI-1; M.L., P.C., and C.V.V. contributed vital reagents and analytical tools; T.V.D.P. designed and supervised the research and took part in writing the paper.

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