The fibrinolytic system in infection & inflammation

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The Fibrinolytic System in Infection & Inflammation

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Voor mijn oma

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Chapter 1

General introduction

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Introduction

The fibrinolytic system is activated during infection and inflammation. The primary function of the fibrinolytic system is to dissolve fibrin clots. Interestingly, recent evidence suggests that components of the fibrinolytic system have properties beyond fibrinolysis, mainly involving inflammatory responses. In this introduction we discuss the different components of the fibrinolytic system and what is known about their roles in inflammation and infection. We also introduce the infectious diseases studied in this thesis.

The fibrinolytic system

Figure 1 shows an overview of the fibrinolytic system. The different components that are relevant for this thesis are described in detail below.

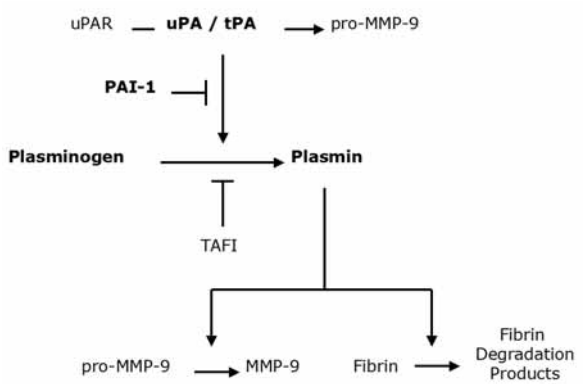

Figure 1. The fibrinolytic system and its relation with mediators studied in this thesis.

Plasmin(ogen)

Plasminogen is the pro-enzyme of plasmin. Plasminogen is primarily synthesized in the liver and circulates in plasma. The role of plasmin(ogen) became evident with the development of gene-targeted plasminogen deficient ($^{-/-}$) mice, which had a normal embryogenesis and survived to adulthood, but were predisposed to severe thrombosis (1). Young animals developed multiple spontaneous thrombotic lesions in liver, stomach, colon, rectum, lung, pancreas and other tissues. These results show that plasmin plays a pivotal role in the protection against thrombosis and sustaining the homeostatic balance. Beyond fibrinolysis, plasmin has also been implicated to play a role in inflammatory responses. Besides binding to fibrin, plasmin(ogen) can bind to many cell types, including neutrophilic granulocytes, monocytes, lymphocytes, platelets and endothelial cells. Cell-associated plasmin is considered to play a role in extracellular matrix degradation and, *in vitro,* plasmin can induce cytokine release, cell adhesion and migration. Furthermore, studies using plasminogen$^{-/-}$ mice have provided *in vivo* evidence for an essential role of the plasminogen system in cell migration towards inflammatory sites (2).

Plasminogen activators

Tissue-type plasminogen activator

Plasminogen needs to be activated to convert into plasmin. TPA is one of the two major endogenous plasminogen activators. TPA is synthesized and secreted mainly by endothelial cells. TPA-deficient mice exhibit normal fertility and embryonic development and have a normal spontaneous phenotype, but have less efficient lysis of artificially induced pulmonary thrombi, as well as enhanced thrombus formation in response to endotoxin (3). The circulating levels of tPA are markedly elevated in patients suffering from severe infections as peritonitis and sepsis. Recent studies using $tPA^{-/-}$ mice showed that tPA promotes the induction of matrix metalloproteinase (MMP)-9 (4), which plays a role in extracellular matrix degradation and thereby in cell migration. Thus, in theory, tPA might be able to affect inflammatory responses through MMP-9 induction. However, not much is known about the role of tPA in inflammation or host defense against bacterial infection.

Urokinase-type plasminogen activator and its receptor

uPA is the other important plasminogen activator in the fibrinolytic system. It is expressed and secreted by several cell-types, such as epithelial cells, endothelial cells, monocytes and neutrophils. The receptor of uPA (uPAR) is expressed on endothelial cells, monocytes, macrophages, fibroblasts and a variety of tumour cells. uPA binds to uPAR on cells, resulting in cell-surface associated plasmin activation which facilitates cellular migration by enhancing pericellular proteolysis and thereby extracellular matrix degradation. In addition, cell-surface associated plasmin also enhances migration by activation of matrix MMPs. Independent of catalytic activity, uPAR is involved in many other cellular processes like cell signaling, interactions with integrins, cell motility, adhesion, invasion and angiogenesis. In mice, uPAR deficiency does not compromise fertility, development or hemostasis which makes it possible to study the role of uPAR in murine models (5). Furthermore, the circulating levels of uPA and the expression of uPAR on monocytes and neutrophils become upregulated during bacterial infection (6). Moreover, $uPAR^{-/-}$ mice displayed a strongly reduced neutrophil recruitment to the pulmonary compartment after induction of *Pseudomonas* or pneumococcal pneumonia, which was associated with an impaired antibacterial host defense (7, 8).

Inhibitors of fibrinolysis

Plasminogen activator inhibitor type-1

PAI-1 is another major player in the fibrinolytic system. PAI-1 is the most important and most rapidly acting physiological inhibitor of both tPA and uPA, and thereby a strong inhibitor of plasmin generation. PAI-1 is released by endothelial cells, monocytes, macrophages, hepatocytes, adipocytes and platelets. Transgenic mice that overexpress PAI-1 exhibit thrombotic occlusion of tail veins and swelling of hind limbs within 2 weeks of birth (9). On the other hand, mice deficient in PAI-1 exhibit normal fertility, viability, tissue histology and development, and show no evidence of hemorrhage (10). Besides its classic role as an inhibitor of fibrinolysis, PAI-1 has been implicated to play a role in many other

processes like wound healing, atherosclerosis, tumor angiogenesis, rheumatoid arthritis, fibrosis, metabolic disturbances, glomerulonephritis, cell migration, bleomycin-induced lung injury, asthma and nasal allergy. Inflammatory mediators, like endotoxin and the cytokines interleukin (IL)-1 and tumor necrosis factor (TNF)-α, can induce the release of PAI-1 during an inflammatory response. In humans, PAI-1 is increased by trauma, surgery and burn injury (all associated with sterile tissue injury accompanied by an acute phase response), and during infectious diseases. In addition, PAI-1 is associated with a poor outcome in severe bacterial infections like sepsis. These observations suggest that PAI-1 might play a functional role during inflammation and infection; however, this role of PAI-1 has not yet been elucidated.

Thrombin-activatable fibrinolysis inhibitor

TAFI is another inhibitor of fibrinolysis, which also known as procarboxypeptidase B, procarboxypeptidase U (unstable) and procarboxypeptidase R (for Arg). TAFI is expressed by the liver and circulates in plasma as a zymogen. TAFI can be activated by thrombin, which is accelerated by the presence of thrombomodulin, and by plasmin (although only at very high concentrations). Activated TAFI binds to carboxy-terminal Arg and Lys-residues on fibrin, which are also the binding sites of plasminogen and tPA. Thus by competing with plasminogen and tPA for the same binding sites, TAFI can inhibit the activation of plasmin and thereby attenuate the fibrinolysis. TAFI$^{-/-}$ mice showed a normal development and no differences in models of thrombosis or endotoxin-induced diffuse intravascular coagulation compared to wild-type (WT) mice (11). Besides its role in fibrinolysis, TAFI is able to inactivate the complement factors C3a and C5a (12, 13), which play an important role in the attraction of neutrophils during inflammation. These new data suggest a possible role for TAFI in inflammation and infection *in vivo*.

The inflammatory response

Tissue injury and bacterial infection can both initiate a profound inflammatory response. The inflammatory response may include leukocyte activation, cytokine and chemokine release, acute phase protein release, inflammatory cell recruitment, MMP-9 and complement activation.

The acute phase response

The acute phase response is a non-specific systemic inflammatory host response which is induced by sterile tissue injury and therefore is often seen in patients after trauma, major surgery, burn injury, tissue infarction or during advanced cancer. The cytokines IL-1β and IL-6 play an essential role in the initiation of the acute phase response. In particular IL-6 triggers the liver to produce so-called acute phase proteins, like C-reactive protein (CRP) and serum amyloid A (SAA). The function of the strong rise of the plasma concentrations of acute phase proteins is still unknown. The subcutaneous injection of turpentine into the hindlimbs of mice is a well-established experimental model to study local inflammation and a classical systemic acute phase response in response to sterile tissue injury. Therefore, we used this model to investigate the role of PAI-1 in sterile inflammation (chapter 4).

Cytokine and chemokine response

Cytokines and chemokines are small proteins that play a major role in the initiation and regulation of the host immune reponse. They act mainly as messenger proteins to inhibit or activate other immune cells. Cytokines are grossly divided by there pro-inflammatory (IL-1, IL-12, TNF-α, IFN-γ) and anti-inflammatory (IL-10) properties. However, several cytokines have overlapping activities. Chemokines are very important for the recruitment of specific leukocyte subsets to the site of infection. In humans, the main neutrophil attracting chemokine is IL-8; in mice two major neutrophil attracting chemokines are macrophage inflammatory protien (MIP)-2 and keratinocyte-like cytokine (KC).

Neutrophil recruitment

Neutrophil recruitment to the site of infection is an important part of the local host defense against bacteria. Neutrophils protect against dissemination of infection by phagocytosing bacteria and killing them by releasing reactive oxygen and proteolytic enzymes. The migratory response of neutrophils is strongly regulated by cytokines, chemokines, MMPs and the complement system. Cellular migration is a multistep process including slowing down, rolling, adhesion to endothelial cells and finally diapedesis. Furthermore, MMPs degrade the extracellular matrix, thereby facilitating the movement of cells through the endothelial cell layer. Moreover, the complement system is an important part of the innate immune response, especially complement factors C3a and C5a play an important role in neutrophil activation and chemotaxis.

MMP-9

MMP-9 was first identified in neutrophils but can also be produced by monocytes, macrophages and lymphocytes. Upon activation of cells by cytokines MMP-9 is released as a pro-enzyme. MMP-9 is linked with the fibrinolytic system since the release of pro-MMP-9 can be induced by tPA (4) and pro-MMP-9 can be activated by various proteases including plasmin (14). The main function of MMP-9 is the regulation of the extracellular matrix composition. It cleaves denatured collagens (gelatins) and type 4 collagen, which is a major component of the basement membrane. This cleavage facilitates the migration of leukocytes out of the blood circulation through the extracellular matrix membrane towards inflammatory sites. In line, MMP-9 deficient mice were protected against mortality in an endotoxin-induced shock model (15).

Infectious diseases

Sepsis

Severe sepsis remains a major challenge in the care of critically ill patients. The outcome is poor and mortality rates remain up to 30-40%. Recent insights into the molecular mechanisms responsible for the pathogenesis of the severe sepsis syndrome shows that sepsis is associated with the activation of multiple inflammatory pathways, including the cytokine network, and the coagulation and fibrinolytic systems. Pro- and anti-inflammatory pathways are simultaneously activated. Subsequently, cellular activity involving leukocyte-endothelial cell interactions occur leading to expression of membrane surface molecules such as toll-like receptors, adhesion molecules and cytokine receptors.

Furthermore, sepsis leads to a hemostatic imbalance, which can result in disseminated intravascular dissemination (DIC). This pro-coagulant state is a consequence of coagulation activation together with inhibition of the fibrinolytic system. Impaired fibrin degradation is mainly due to high circulating levels of PAI-1, and contributes to enhanced intravascular fibrin deposition and subsequent organ failure. Studying the specific roles of the different components of the fibrinolytic system during sepsis might help us to further understand the pathogenesis of the sepsis syndrome.

Pneumonia

Pneumonia is the most frequent cause of sepsis accounting for approximately 50% of the cases in recent clinical trials (16). *Klebsiella (K.) pneumoniae* is a Gram-negative opportunistic bacterium, which is an important cause of hospital-acquired pneumonia and sepsis. We used this bacterium to induce severe Gram-negative pneumonia in mice to study the role of PAI-1 and tPA in local and systemic host defense against severe pneumonia (chapter 5 and 6).

Peritonitis

Acute bacterial peritonitis is a life-threatening disease with a mortality rate ranging between 30 and 50%. Furthermore, peritonitis is the second most common cause of sepsis and the mortality of peritonitis-induced sepsis can be as high as 80%. Therefore we also investigated the role of different components of the fibrinolytic system in the inflammatory responses to and outcome of this disease (chapter 7-10). The most common causative organisms are enteric Gram-negative bacteria. *Escherichia (E.) coli* is found in 60% of the cases (17), therefore we used *E. coli* to induce abdominal sepsis in mice.

Aim and outline of this thesis

The aim of this thesis is to obtain more insight into the specific roles of the components of the fibrinolytic system in the innate immune response during severe Gram-negative infection and sterile inflammation. In chapter 2 we investigated the role of plasmin activity during the acute systemic inflammatory response to endotoxin in human volunteers. In chapter 3 we studied the role of PAI-1 in the cytokine response during systemic inflammation induced by endotoxin or staphylococcal enterotoxin B (SEB). In chapter 4 we investigated the role of PAI-1 during a more chronic inflammatory response (with abscess formation) and the systemic acute phase response induced by subcutaneous turpentine injection. In Chapter 5 and 6, we subsequently studied the effect of PAI-1 and tPA deficiency and overexpression on host defense against *Klebsiella* pneumonia. In chapter 7-10 we investigated the role of different components related to the fibrinolytic system in the inflammatory responses and host defense against *E. coli* peritonitis. Respectively, we studied the role of tPA, MMP-9, TAFI and uPAR in abdominal sepsis. In addition, in chapter 10 we also investigated the effect of uPAR on sterile inflammation induced by endotoxin.

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Chapter 2

Inhibition of plasmin activity by tranexamic acid does not influence inflammatory pathways during human endotoxemia

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Abstract

Plasmin activates several pro-inflammatory pathways at the cellular level *in vitro*. LPS administration to healthy humans results in a rapid generation of plasmin activity, accompanied by activation of a number of inflammatory systems. To determine the role of early plasmin activity in LPS-induced inflammation *in vivo*, 16 healthy males received an intravenous bolus injection with LPS (from *Escherichia coli*, 4 ng/kg) directly preceded by a 30-min intravenous infusion of tranexamic acid (2 g, $n = 8$), a plasmin activation inhibitor, or placebo ($n = 8$). LPS injection induced marked increases in the plasma levels of D-dimer and plasmin-α2-antiplasmin complexes, indicative for plasmin activation and generation respectively, which were strongly attenuated by tranexamic acid (both $P < 0.01$ vs placebo). However, tranexamic acid did not influence LPS-induced coagulation activation, granulocytosis, neutrophil activation (expression of CD11b, CD66b and L-selectin) or degranulation (plasma concentrations of elastase-α1-antitrypsin and bactericidal permeability-increasing protein), endothelial cell activation (plasma levels of von Willebrand factor and soluble E-selectin), or cytokine release. These data argue against a role of early plasmin generation in the subsequent activation of other inflammatory pathways during human endotoxemia.

Introduction

LPS, present in the outer membrane of Gram-negative bacteria, plays a pivotal role in triggering inflammatory responses during Gram-negative sepsis. The human endotoxemia model, in which a bolus dose of LPS is administrated intravenously to healthy subjects, has been frequently used to study the mechanisms by which inflammatory systems are activated in man *in vivo* (1, 2). Intravenous injection of LPS into healthy humans is associated with activation of the fibrinolytic system, the coagulation cascade, neutrophilic granulocytes, endothelial cells and the cytokine network (3, 4). In particular activation of fibrinolysis is a very early phenomenon in the human response to LPS administration. Within two hours a marked increase in tissue-type plasminogen activator (tPA) can be detected, which leads to the generation of plasmin as reflected by an increase in plasmin-α2-antiplasmin (PAP) complexes. This activation is rapidly followed by an increase in plasminogen activator inhibitor-1 (PAI-1) levels, inhibiting the fibrinolytic system (3, 4). These fibrinolytic changes precede and occur independently from activation of the coagulation system in this model (5-7).

Recent evidence indicates that the fibrinolytic system likely has functions different from its classical fibrin dissolving properties. Binding of plasmin(ogen) to surfaces plays a pivotal role in regulating the function of this system (8). Besides binding to fibrin, plasmin(ogen) can bind to many cell types, including neutrophilic granulocytes, monocytes, lymphocytes, platelets and endothelial cells (9, 10). Upon binding to cells, conversion of plasminogen to plasmin is facilitated and cell-bound plasmin is protected from inactivation by α2-antiplasmin (11). Although the biological function of cell-bound plasmin has been regarded mainly in terms of fibrinolytic activity, in recent years, it has become clear that plasmin can affect various cell functions. Cell-associated plasmin is considered to play an important role in extracellular matrix degradation and tissue remodeling (12). Interestingly, plasmin can also induce pro-inflammatory responses independent of its proteolytic properties. *In vitro*, plasmin was demonstrated to stimulate the release of cytokines and other inflammatory mediators by different cell types (13-16). Furthermore, plasmin induced cell adhesion and migration *in vitro* (17-20), and studies using plasminogen-deficient mice have provided *in vivo* evidence for an essential role of the plasminogen system in cell migration towards inflammatory sites (21, 22). Moreover, plasmin can activate the p38 mitogen-activated protein kinase (MAPK) signaling pathway in monocytes (14, 23), and activation of this pathway was recently shown to be of key importance for the inflammatory response to LPS in humans (24, 25). Together, these findings implicate plasmin as a mediator of several cellular inflammatory responses. However, at present, the role of plasmin in systemic inflammation *in vivo* is unknown.

Tranexamic acid (Cyklokapron®) is a synthetic anti-fibrinolytic substance, which acts by competitively blocking the lysine binding sites of plasmin(ogen), thereby preventing binding to fibrin or cells (26). *In vitro*, tranexamic acid potently inhibited plasmin-induced proinflammatory responses (13, 19, 27).

The fact that the formation of plasmin is one of the earliest events after intravenous administration of LPS, together with the recent findings that plasmin is able to induce several cellular proinflammatory responses, including activation of p38 MAPK, led us to hypothesize that plasmin may play a role in the induction of LPS-induced inflammatory pathways. To test this hypothesis, we studied the effect of tranexamic acid infusion on activation of coagulation, granulocytes, endothelial cells and the cytokine network in healthy humans injected with a single dose of LPS.

Methods

Study design

Sixteen healthy men (19-34 years) were studied. The study was approved by the institutional scientific and ethics committees and written informed consent was obtained from all volunteers. Tranexamic acid (Cyklokapron®; Pharmacia & Upjohn, Woerden, the Netherlands) (2 gram in 100 mL sterile NaCl 0.9%; N = 8) or placebo (100 mL 0.9% sterile NaCl; N = 8) was administered intravenously over 30 minutes directly prior to LPS injection. All participants received a bolus intravenous injection of LPS (*Escherichia coli* lipopolysaccharide, lot G; US Pharmacopeia, Rockville, MD) at a dose of 4 ng/kg. Oral temperature, blood pressure, heart rate, and oxygen saturation were measured at half-hour intervals (Dinamap1846 SX; Critikon, Tampa, FL). Clinical symptoms such as headache, chills, nausea and myalgia were recorded throughout the study periods using a graded scale (0, absent; 1, weak; 2, moderate; 3, severe).

Blood collection

Blood was obtained before and at the end of the infusion of tranexamic acid or placebo (t = -0.5 h and t = 0 h), and at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8 and 23 h after LPS injection. All blood samples, except those for the determination of leukocyte counts and differentials, were centrifuged at 3000 rpm for 15 minutes at 4˚C, and plasma was stored at –20˚C until assays were performed. Blood for FACS analysis was obtained directly before tranexamic acid or placebo infusion (t = -0.5 h) and at 1, 4, 6, and 23 h after LPS administration and put on ice.

Assays

Coagulation and fibrinolysis assays were done in citrated plasma, all other assays in EDTA plasma. The following ELISA's were performed according to the instructions of the manufacturer and/or as described previously: D-dimer, F1+2 prothrombin fragment and thrombin-antithrombin (TAT) complexes (all Dade Behring, Marburg, Germany), tissue-type plasminogen activator (tPA) (Asserachrom tPA, Diagnostics Stago, Asnieres-sur-Seine, France), plasminogen activator inhibitor type 1 (PAI-1) (Monozyme, Charlottelund, Denmark), Elastase-α1-antitrypsin complex concentrations were measured with an ELISA modified from a previously described RIA procedure (28), bactericidal permeability-increasing protein (BPI)(29), von Willebrand factor (Dako, Glostrup, Denmark), soluble E-selectin (Diaclone, Fleming, France), tumor necrosis factor (TNF)-α, IL-6, IL-10 and IL-8

(all Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). Plasmin-α2-antiplasmin (PAP) complexes were measured by RIA as described previously.(30) Leukocyte counts and differentials were assessed by a STKR Coulter counter (Coulter, Bedfordshire, U.K.).

Flow Cytometry

Erythrocytes were lysed with ice-cold isotonic NH_4Cl solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA [pH 7.4]) for 10 minutes. The cells were centrifuged at 250 × *g* for 10 minutes at 4˚C. The remaining cells were washed twice with FACS buffer (phosphate-buffered saline supplemented with 0.5% bovine serum albumin [BSA], 0.01% NaN3, and 0.35 mM EDTA) and brought to a concentration of 4×10^6 cells/mL in FACS buffer. All procedures were performed at 4˚C. All FACS reagents were titrated to obtain optimal results, as recommended by the manufacturers. For each test at least 10^4 cells were analyzed using a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA). Cell surface staining was performed using the following anti-human mAbs: FITC-labeled mouse anti-human CD66b (clone 80H3; Immunotech, Marseille, France), PE-labeled mouse anti-human L-selectin (clone DREG-56), and APC-labeled mouse anti-human CD11b (clone ICRF44) (all BD Pharmingen, San Diego, CA). To correct for nonspecific staining, all analyses were also conducted with the appropriate isotype control Abs (FITC-, PE- and APC-labeled murine IgG1, BD Pharmingen). Granulocytes were identified by forward and side-angle light scatter gating. Data are presented as the difference between mean fluorescence intensities (MFI) of specifically and nonspecifically stained cells.

Statistical analysis

All values are given as means ± SEM. Differences in time and between treatment groups were analyzed by mixed models analysis using SPSS for Windows (SPSS 11.5, Chicago, IL). A value of $P < 0.05$ was considered to represent a statistically significant difference.

Results

Clinical features

Intravenous injection of LPS elicited a febrile response, peaking after 4 h (38.4 ± 0.3 ˚C), together with tachycardia and transient flu-like symptoms, including headache, chills, nausea and myalgia. Infusion of tranexamic acid did not influence LPS-induced signs and symptoms, and no adverse events attributable to tranexamic acid infusion were observed (data not shown).

Inhibition of plasmin activity by tranexamic acid

Tranexamic acid competitively binds to the high affinity lysine binding sites on plasmin(ogen), thereby preventing direct action of plasmin on fibrin and cells and the surface-facilitated conversion of plasminogen to plasmin. To obtain evidence for the *in vivo* plasmin inhibitory activity of tranexamic acid during endotoxemia, we determined the plasma concentrations of D-dimer, a split product cleaved off from cross-linked fibrin by a direct action of plasmin, as a measure for plasmin activity and the plasma levels of PAP

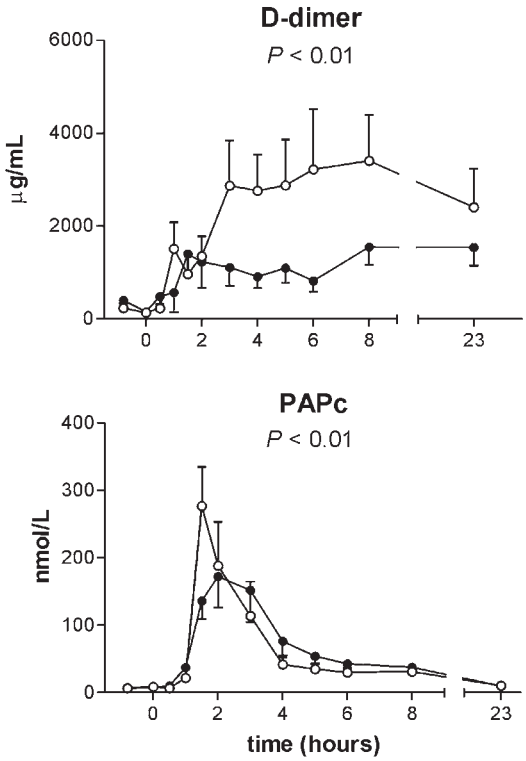

Figure 1. Tranexamic acid inhibits LPS-induced plasmin generation and activity. Plasma concentrations of D-dimer and PAP complexes after LPS administration (4 ng/kg IV, t = 0 h), preceded by a 30-minute infusion of placebo (open symbols) or tranexamic acid (2 g IV,black symbols). Data are means ± SEM. P value indicates the difference between treatment groups.

complexes, as a measure for plasmin generation **(Fig. 1)**. LPS administration resulted in a profound rise in D-dimer, which reached a plateau phase from 3 h and peaked at 8 h (3400 ± 1000 µg/L), and a transient rise in PAP complexes, peaking after 1.5 h (276 ± 57 nmol/L). Tranexamic acid essentially prevented the increase in D-dimer levels (8 h: 1554 ± 388 µg/L; $P < 0.01$ vs placebo) and blunted the rise in PAP complexes (peak at 2 h: 172 ± 46 nmol/L; $P < 0.01$ vs placebo). Hence, these data indicate that tranexamic acid effectively inhibited plasmin generation and activity.

Activation and inhibition of fibrinolysis

LPS injection resulted in an early stimulation of the fibrinolytic system **(Fig. 2)**, measured by a rise in tPA levels, peaking after 3 h ($P < 0.001$ vs baseline). This increase in tPA levels was followed by the secretion of its inhibitor PAI-1, peaking after 4 h ($P < 0.001$ vs baseline). Consistent with its mode of action (26), tranexamic acid did not influence the LPS-induced rises in tPA and PAI-1 concentrations.

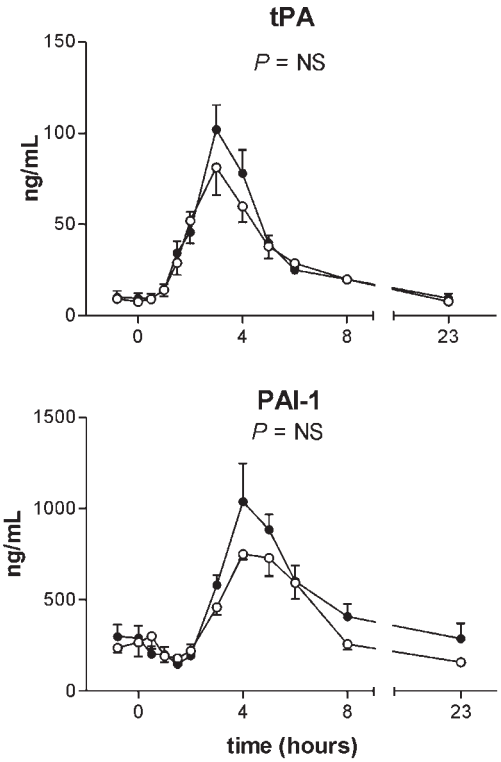

Figure 2. Activation and inhibition of fibrinolysis. Plasma levels of t-PA and PAI-1 after LPS administration (4 ng/kg IV, t = 0 h), preceded by a 30-minute infusion of placebo (open symbols) or tranexamic acid (2 g IV, black symbols). Data are means ± SEM. P values (NS, not significant) indicate the difference between treatment groups.

Activation of the coagulation system

LPS administration was associated with thrombin generation, as reflected by increases in the plasma levels of the prothrombin fragment F1 + 2 and TAT complexes (both $P < 0.001$ vs baseline). Tranexamic acid did not affect the LPS-induced thrombin generation **(Fig. 3)**.

Leukocyte activation

LPS injection induced activation of neutrophilic granulocytes, as reflected by a biphasic change in neutrophil counts involving an initial decrease with a nadir at 1 hour, followed by neutrophilia peaking at 8 h ($P < 0.001$ vs baseline; **Fig. 4**). Furthermore, LPS administration induced an up-regulation of the activation markers CD11b (Fig. 4) and CD66b (data not shown) at the surface of circulating granulocytes with a concurrent down-modulation of L-selectin **(Fig. 4)** (all $P < .01$ vs baseline). Moreover, LPS injection resulted in an increase in degranulation products as measured by BPI **(Fig. 4)** and elastase-α1-antitrypsin complexes (data not shown) (both $P < 0.001$ vs baseline). Tranexamic acid did not modify any of these LPS-induced changes.

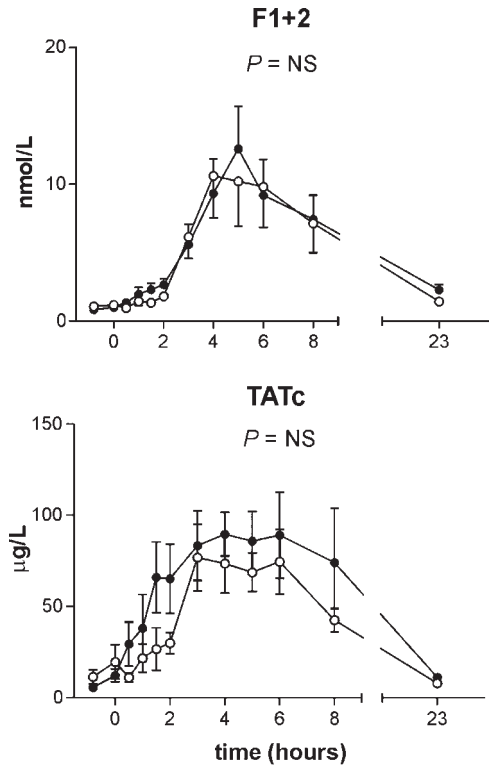

Figure 3. Activation of coagulation. Plasma concentrations of F1 + 2 and TAT complexes after LPS administration (4 ng/kg IV, t = 0 h), preceded by a 30-minute infusion of placebo (open symbols) or tranexamic acid (2 g IV, black symbols). Data are means ± SEM. P values (NS, not significant) indicate the difference between treatment groups.

Endothelial cell response

LPS administration elicited endothelial cell activation, as indicated by profound increases in the plasma concentrations of vWF and soluble E-selectin (both $P < 0.001$ vs baseline). Tranexamic acid infusion did not alter these LPS-induced endothelial cell responses **(Fig. 5)**.

Cytokine response

LPS injection was associated with a transient rise in the plasma concentration of TNF, IL-6, IL-8 and IL-10, peaking after 2-3 h (all $P < 0.001$ vs baseline). Tranexamic acid infusion did not influence these LPS-induced cytokine responses **(Fig. 6)**.

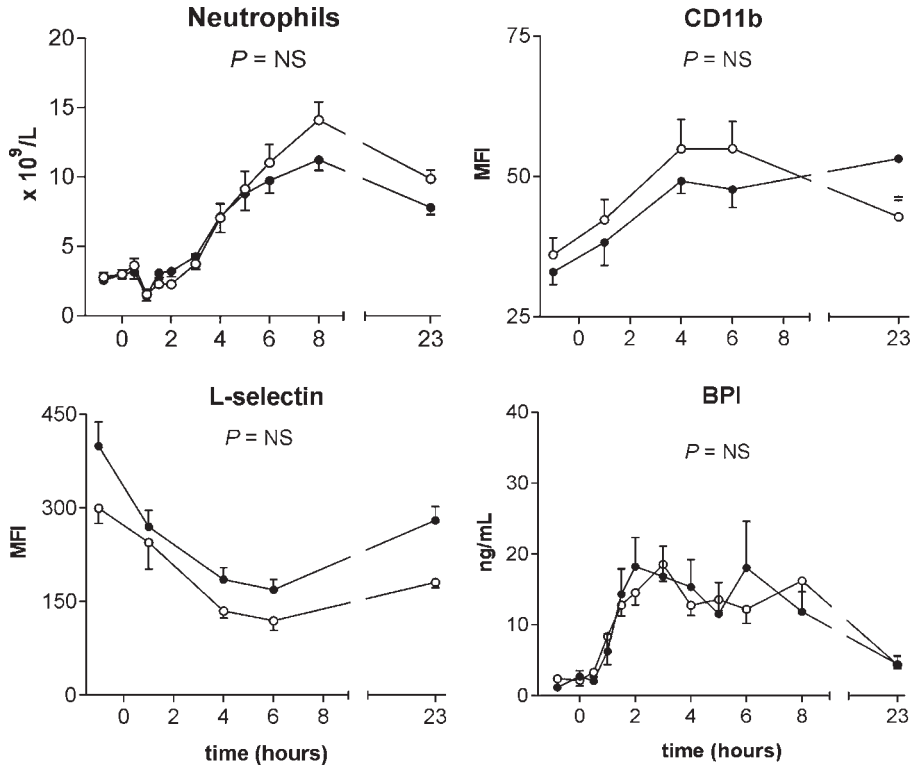

Figure 4. Activation of neutrophils. Circulating neutrophil counts, mean fluorescence intensity (MFI) of CD11b and L-selectin on granulocytes and the degranulation product BPI after LPS administration (4 ng/kg IV, t = 0 h), preceded by a 30-minute infusion of placebo (open symbols) or tranexamic acid (2 g IV, black symbols). Data are means ± SEM. FACS data (CD11b and L-selectin) are expressed as the difference between specific MFI and nonspecific MFI. P values (NS, not significant) indicate the difference between treatment groups.

Discussion

In recent years it has become clear that plasmin has functions beyond its classical proteolytic and fibrin degrading properties. *In vitro* and animal studies have provided evidence for a stimulatory effect of plasmin on cellular proinflammatory responses. The current investigation is the first to examine the role of plasmin during a systemic inflammatory response in humans *in vivo*. We here demonstrate that although pre-treatment with tranexamic acid strongly inhibited LPS-induced plasmin activation, it did not influence sensitive markers of the activation of proinflammatory pathways that accompany endotoxemia, including effects on the coagulation cascade, granulocytes, endothelial cells and the cytokine network. These findings suggest that plasmin is not involved in the induction of systemic inflammatory responses during human endotoxemia.

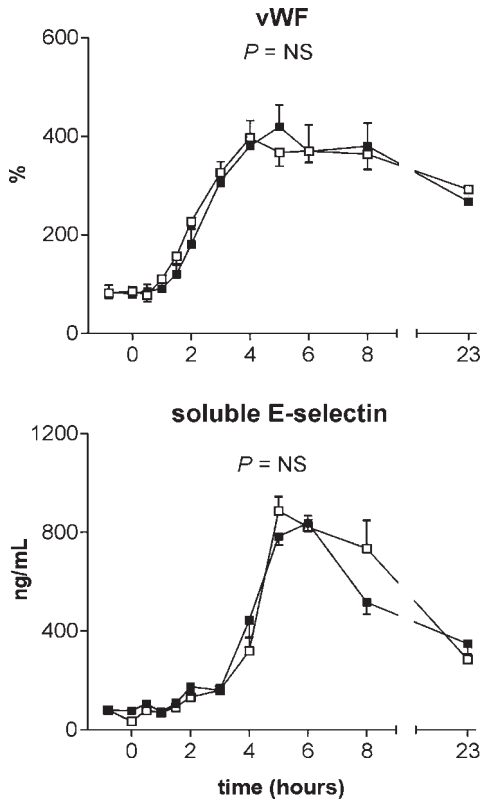

Figure 5. Endothelial cell activation. Plasma concentrations of vWF and soluble E-selectin after LPS administration (4 ng/kg IV, t = 0 h), preceded by a 30-minute infusion of placebo (open symbols) or tranexamic acid (2 g IV, black symbols). Data are means ± SEM. P values (NS, not significant) indicate the difference between treatment groups.

Binding of plasmin(ogen) to fibrin or cell surfaces is of crucial importance in regulating its function. Plasmin(ogen) binds to fibrin and cells via its lysine binding sites, which are associated with its kringle domains and recognize carboxy-terminal lysines of surface proteins (26, 31). Upon binding plasmin activity is increased and the conversion of plasminogen to plasmin is facilitated (8, 11). Tranexamic acid competitively binds to the lysine binding sites of plasmin(ogen), thereby blocking the binding to fibrin and cells (26). Although plasmin can still be formed under these circumstances, its activity and the surface-facilitated plasmin generation are inhibited. In line with this mode of action, we found that pre-treatment with tranexamic acid strongly reduced the LPS-induced rise in the plasma levels of D-dimer, a split product cleaved off from cross-linked fibrin by a direct action of plasmin, providing direct evidence for the virtually complete inhibition of plasmin activity on fibrin *in vivo*. Furthermore, pre-treatment with tranexamic acid decreased the levels of circulating PAP complexes, indicating that apart from the strong inhibition of plasmin activity, plasmin generation was also inhibited by tranexamic acid, albeit to a lesser extent. As expected, pre-treatment with tranexamic acid did not influence the

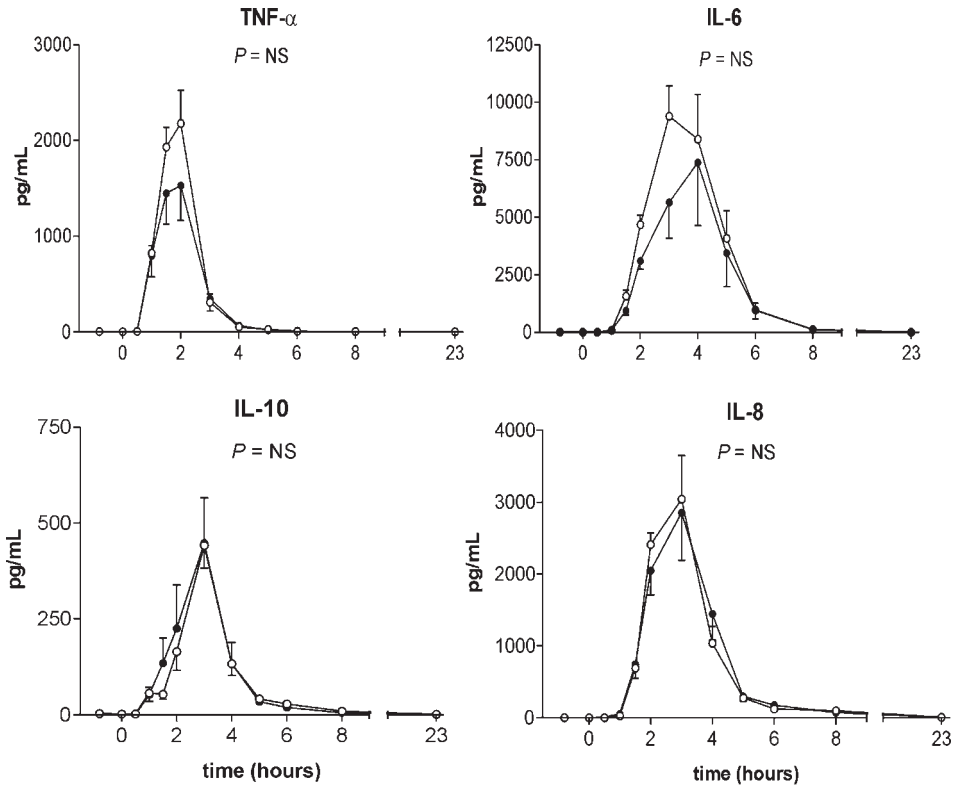

Figure 6. Cytokine response. Plasma levels of TNF-α, IL-6, IL-10 and IL-8 after LPS administration (4 ng/kg IV, t = 0 h), preceded by a 30-minute infusion of placebo (open symbols) or tranexamic acid (2 g IV, black symbols). Data are means ± SEM. P values (NS, not significant) indicate the difference between treatment groups.

LPS-induced increase in tPA levels, nor did it change the subsequent rise in PAI-1 concentration.

LPS injection induced thrombin generation, as shown by a rise in the plasma concentrations of F1+2 and TAT complexes, which indicates activation of the coagulation cascade. Tissue factor plays a pivotal role herein. Indeed, LPS administration to healthy humans resulted in a marked increase in monocytic tissue factor mRNA expression (32), and treatment with recombinant tissue factor pathway inhibitor strongly inhibited the associated coagulation activation (5). Interestingly, plasmin is able to induce tissue factor expression on monocytes *in vitro*, which was inhibited by tranexamic acid (13). This finding led us to investigate the effect of tranexamic acid on coagulation activation after LPS injection. However, we did not find any influence of inhibition of plasmin activity by tranexamic acid on activation of the coagulation cascade. Of note, earlier investigations have demonstrated that the fibrinolytic changes during endotoxemia are completely independent of coagulation activation (5-7). In the present study we show, in turn, that the activation of the coagulation system occurs independent of plasmin activation.

Neutrophilic granulocytes are activated upon infection or inflammation and have been implicated in the pathogenesis of tissue injury during severe sepsis (33). Plasmin induced neutrophil aggregation and increased neutrophil adhesion to endothelial cells *in vitro*, an effect that could be inhibited by tranexamic acid (19, 20, 27). The plasmin-induced neutrophil adherence was mediated through an upregulation of CD18 neutrophil cell surface glycoprotein, reflecting neutrophil activation. These data suggest that plasmin is able to activate neutrophils, which can be abrogated by tranexamic acid. In contrast, our findings show that infusion of tranexamic acid does not influence the neutrophil response to LPS administration in humans *in vivo* and has no effect on neutrophil activation, as reflected by unaltered upregulation of CD11b and CD66b, downmodulation of L-selectin and rise in circulating neutrophilic degranulation products.

Endothelial cells play a pivotal role in the inflammatory response to systemic infection (34, 35). Plasmin can influence endothelial cell behavior *in vitro*. Endothelial cells incubated with plasmin showed an enhanced release of arachidonate, the precursor of leukotriene B4 (LTB4) and other eicosanoids (16), which was inhibited by tranexamic acid. Furthermore, plasmin induced endothelial cell retraction evidenced by loss of cell-cell contacts and increased permeability (17), and stimulated endothelial cell migration *in vitro* (18). Together these data implicate plasmin as a mediator of endothelial cell activation. However, in the present study, inhibition of LPS-induced plasmin activity did not affect the endothelial cell activation, measured by plasma levels of von Willebrand factor and soluble E-selectin.

The release of cytokines into the circulation is a characteristic feature of endotoxemia, predominantly mediated by monocytes and macrophages. Stimulation of human peripheral monocytes with plasmin *in vitro* induced an up-regulation of several inflammatory mediators, including TNF-α, IL-1α, IL-1ß, monocyte chemoattractant protein (MCP)-1 and LTB4 (13-15). Tranexamic acid attenuated cytokine mRNA expression elicited by plasmin (13). Plasmin-induced expression of TNF-α, IL-1α and IL-1ß involved AP-1 and NF-κB activation (13), whereas plasmin-induced monocyte expression of MCP-1 and CD40 was triggered via activation of the p38 MAPK and Janus Kinase/STAT signaling pathways (14). Syrovets et al. demonstrated that ciglitazone inhibited cytokine release from plasmin-stimulated monocytes by inhibition of AP-1 and NF-κB activation via modulation of p38 MAPK activity (23). In accordance, a specific p38 MAPK inhibitor significantly diminished proinflammatory gene expression by plasmin-stimulated peripheral monocytes (23, 36). Together, these data indicate that plasmin induces monocytic cytokine production at least in part via p38 MAPK activation. Recently, our laboratory demonstrated that the p38 MAPK signaling pathway is important for induction of the inflammatory response to LPS in humans. Indeed, intravenous injection of LPS resulted in a transient activation of p38 MAPK (24), and more importantly, a specific p38 MAPK inhibitor strongly inhibited the LPS-induced cytokine production and other proinflammatory responses in humans *in vivo* (24, 25). In spite of this abundant *in vitro* evidence that plasmin can induce p38 MAPK activation and cytokine production, inhibition of plasmin activity by tranexamic acid did not affect the cytokine response in the present study. It should be noted that we did not measure p38 MAPK activation in blood cells in the current study, and thus we can only speculate on the effects of tranexamic acid on p38 MAPK activation in our subjects.

Indeed, in a more general way, it remains to be established whether plasmin can activate p38 MAPK *in vivo*.

In vitro and animal studies have indicated that plasmin can activate various inflammatory pathways implicated in the host response to endotoxemia. We here demonstrate that although active plasmin is generated early after intravenous injection of LPS into normal subjects, it does not contribute to a significant extent to activation of the coagulation system, granulocytes, the vascular endothelium or the cytokine network. By the nature of our experiment, performed in healthy human beings, we cannot exclude that plasmin does play a role in endotoxemia or infection models in which more severe challenges are given. Investigations in animals are warranted to determine the potential role of plasmin in a lethal systemic inflammatory response syndrome.

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Chapter 3

Plasminogen activator inhibitor type-1 deficient mice have an enhanced interferon-γ response to lipopolysaccharide and staphylococcal enterotoxin B

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Abstract

Plasminogen activator inhibitor type I (PAI-1) is a major inhibitor of fibrinolysis by virtue of its capacity to inhibit urokinase type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). Systemic inflammation is invariably associated with elevated circulating levels of PAI-1 and during human sepsis plasma PAI-1 concentrations predict an unfavorable outcome. Knowledge about the functional role of PAI-1 in a systemic inflammatory response syndrome is highly limited. We here determined the role of endogenous PAI-1 in cytokine release induced by administration of LPS or staphylococcal enterotoxin B (SEB). Both LPS and SEB elicited secretion of PAI-1 into the circulation of normal wild-type mice. Relative to wild-type mice, PAI-1 gene deficient (PAI-$1^{-/-}$) mice demonstrated strongly elevated plasma IFN-γ concentrations after injection of either LPS or SEB. In addition, PAI-$1^{-/-}$ splenocytes released more IFN-γ after incubation with LPS or SEB than wild-type splenocytes. Both PAI-$1^{-/-}$ CD4+ and CD8+ T cells produced more IFN-γ upon stimulation with SEB. LPS-induced IFN-γ release in mice deficient for uPA, the uPA receptor or tPA was not different from IFN-γ release in LPS treated wild-type mice. These results identify a novel function of PAI-1 during systemic inflammation, where endogenous PAI-1 serves to inhibit IFN-γ release by a mechanism that does not depend on its interaction with uPA/uPA receptor or tPA.

Introduction

Mediators of the fibrinolytic system have been associated with functions besides their classical fibrin dissolving properties (1, 2). Plasminogen activator inhibitor-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 activation of the key-enzyme plasmin and subsequent fibrin degradation. However, PAI-1 has also been implicated in several other processes and diseases by actions that are not or only partially related to its capacity to inhibit plasminogen activation, including wound healing, tumor angiogenesis, rheumatoid arthritis, fibrosis, metabolic disturbances, glomerulonephritis, cell migration, bleomycin-induced lung injury, asthma and nasal allergy.

During severe infection or sepsis, the systemic inflammatory response is almost invariably associated with a strong rise in circulating PAI-1 levels. In sepsis patients, PAI-1 concentrations predict lethality in a very sensitive manner (3, 4). Moreover, a (4G/5G) promoter deletion/insertion polymorphism in the PAI-1 gene has been found to influence the risk of the development of septic shock and to be associated with a poor outcome in patients with meningococcal sepsis (5, 6). Although these observations suggest that PAI-1 plays a functional role in the inflammatory response during severe infection, such a role has not been established thus far. One can hypothesize that PAI-1 influences the innate immune response by inhibition of mediators of the fibrinolytic system. For instance, plasmin can activate the p38 MAPK signaling pathway in monocytes (7, 8), and activation of this pathway was recently shown to be of key importance for the inflammatory response to endotoxin in humans (9, 10). Furthermore, *in vitro*, plasmin was demonstrated to stimulate the release of cytokines and other inflammatory mediators by different cell types (7, 11-13). Moreover, PAI-1 inhibits uPA, which can enhance LPS-induced cytokine expression *in vitro* and *in vivo* (14) and studies using genetically modified mice have implicated uPA as an important regulator of inflammatory responses to bacterial and other stimuli (15, 16).

LPS, present in the outer membrane of Gram-negative bacteria, plays a pivotal role in triggering inflammatory responses during Gram-negative sepsis. Staphylococcal enterotoxin B (SEB) is a product of *Staphylococcus aureus,* which stimulates both antigen presenting cells and T-cells *in vivo* (17, 18). We investigated the role of PAI-1 in cytokine responses to LPS and SEB-induced inflammation *in vivo,* using PAI-1 gene deficient (PAI-$1^{-/-}$) mice. We show that PAI-1 deficiency is associated with a strongly enhanced LPS and SEB-induced IFN-γ release *in vivo* and *in vitro* by a mechanism that does not depend on its interaction with tPA, uPA or its receptor uPAR.

Materials and Methods

Animals

PAI-1$^{-/-}$, uPA$^{-/-}$, uPAR$^{-/-}$ and tPA$^{-/-}$ mice, all backcrossed to a C57BL/6 genetic background, and C57BL/6 wild type (Wt) mice were purchased from the Jackson Laboratories (Bar Arbor, ME). Eight weeks old female mice were used for all experiments. All experiments were approved by the institutional animal care and use committee of the Academic Medical Center (Amsterdam, The Netherlands).

LPS and SEB-induced inflammation in vivo

200 µg LPS (*Escherichia coli* 055:B5, Sigma, St. Louis, MI) or 100 µg SEB (Sigma) was injected i.p. in 200 µl of sterile NaCl 0.9%.

Sample harvesting

At the time of sacrifice, mice were first anesthetized by i.p. injection of 0.07 ml/g FFM mixture (Fentanyl (0.315 mg/ml)-Fluanisone (10 mg/ml) (Janssen, Beersen, Belgium), Midazolam (5 mg/ml) (Roche, Mijdrecht, The Netherlands). Next, blood was drawn via direct heart puncture with a sterile syringe, and transferred to tubes containing heparin; plasma was prepared by centrifugation at 1400 x *g* for 10 min at 4 °C, after which aliquots were stored at -20 °C.

Assays

Murine PAI-1 was measured by ELISA (Korida, Leiden, the Netherlands). TNF-α, IL-6, MCP-1, IL-10, IL-12p70, IFN-γ, IL-5, IL-4 and IL-2 were measured by cytometric bead array (CBA) multiplex assay (Pharmingen, San Diego, CA) in accordance with the manufacturer's recommendations. The detection limit of all cytokines was 2.5-5.0 pg/ml.

Ex vivo splenocyte stimulation

Single-cell suspensions were obtained from Wt and PAI-1$^{-/-}$ mice by crushing spleens through a 40-µm cell strainer (Pharmingen). Erythrocytes were lysed with ice-cold isotonic NH_4Cl solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 100 mM EDTA [pH 7.4]), and the remaining cells were washed twice with RPMI 1640 (BioWhittaker Europe, Verviers, Belgium). Splenocytes were suspended in medium (RPMI 1640 with L-glutamine, 5% autologous serum, 5% antibiotic-antimycotic [GIBCO BRL; Life Technologies Inc., Rockville, ML]), seeded in 96-well round-bottomed culture plates at a cell density of 1 x 10^6 cells per well in triplicate, and stimulated with medium, 10 ng/ml LPS or 10 µg/ml SEB in an end-volume of 200 µl. Supernatants were harvested after a 48-hour incubation at 37°C in 5% CO_2, and cytokine levels were analyzed by CBA.

Ex vivo peritoneal macrophage stimulation

Peritoneal macrophages were harvested from Wt and PAI-1$^{-/-}$ mice by washing the peritoneal cavity with 5 ml sterile saline. Collected cells were allowed to adhere to 96-well tissue-culture plates (10^5 cells per well) for 2 hours at 37°C, after which nonadherent cells were removed by rinsing with medium. More than 95% of the cells were peritoneal macrophages, as identified by cytospin preparations stained with modified Giemsa stain.

Macrophage monolayers were stimulated with medium, 10 ng/ml LPS or 10 µg/ml SEB in an end-volume of 200 µl for 48 hours at 37°C; then supernatants were aspirated and cytokine levels were analyzed by CBA.

FACS analysis

For FACS analysis splenocytes suspensions obtained from Wt and PAI-$1^{-/-}$ mice were washed with FACS buffer (PBS supplemented with 0.5% BSA, 0.01% NaN_3 and 100 mM EDTA) and resuspended in 150 µl FACS buffer. Immunostaining for cell surface molecules was performed for 30 minutes at 4°C using directly labeled Ab's against CD3, CD4, CD8, NK1.1, CD11b, CD11c, F4/80 and GR1. All Ab's were used in concentrations recommended by the manufacturer (Pharmingen). To correct for nonspecific staining, an appropriate control Ab (rat IgG2; Pharmingen) was used. For the ex vivo stimulation experiments splenocytes were seeded in 24-well tissue-culture plates at a cell density of 1 x 10^6 cells per well in duplicate, and stimulated with medium or 10 µg/ml SEB in an end-volume of 2 ml. After 1 hour of incubation at 37°C in 5% CO_2, the protein transport inhibitor brefeldin A (2 µg/ml, Sigma) or medium was added to the wells. Cell cultures were incubated at 37°C in 5% CO_2 for 48 hours, after which cells were washed with FACS buffer and resuspended in FACS buffer. Cells were fixed with 4% formaldehyde and permeabilised using 100 µl cytofix/cytoperm™ (Pharmingen) for 20 minutes at 4°C. Next cells were stained for intracellular IFN-γ (Pharmingen) for 30 minutes at 4°C, after which they were washed and resuspended in FACS buffer for FACS analysis (using FACSCalibur , Beckton en Dickinson , San Jose, CA).

Statistical analysis

Data are expressed as means ± SEM, unless indicated otherwise. Comparison between time-curves were conducted using 2-way analysis of variance, with bonferroni post-hoc tests. Comparisons between groups were conducted using the Mann-Whitney *U* test. A *P* value of <0.05 was considered to represent a statistically significant difference.

Results

PAI-1 is upregulated by LPS and SEB

To confirm PAI-1 production in Wt mice in our models, we measured PAI-1 levels in plasma before and at 4 and 8 hours after i.p. injection of LPS (200 µg) or SEB (100 µg). Baseline plasma PAI-1 concentrations were 5 ± 0.3 ng/ml. At 4 and 8 hours after LPS administration PAI-1 levels were significantly increased compared to baseline values (512 ± 34 and 669 ± 54 ng/ml respectively, both $P< 0.05$). SEB injection also resulted in a elevation of plasma PAI-1 concentrations at 4 and 8 hours post-injection (18 ± 2.3 and 31 ± 2.3 ng/ml respectively, both $P<0.05$ versus baseline).

The LPS-induced cytokine response

To investigate whether PAI-1 deficiency influences LPS-induced cytokine production, we measured plasma levels of TNF-α, IL-6, MCP-1, IL-10, IL-12p70 and IFN-γ at various time points up to 48 hours after i.p. injection of LPS. At 48 hours all cytokine levels were back

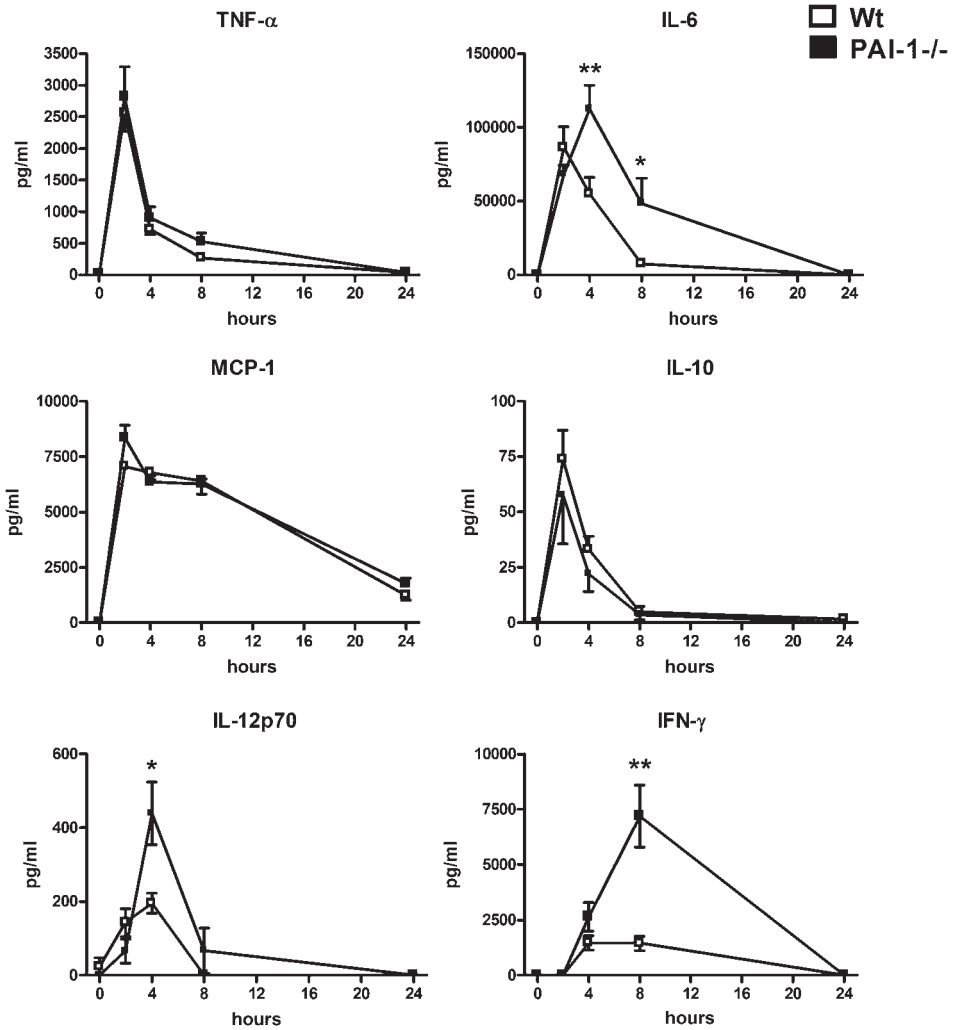

Figure 1. Effect of PAI-1 deficiency on LPS-induced cytokine release. TNF-α, IL-6, MCP-1, IL-10, IL-12p70 and IFN-γ were measured in plasma at several time points after intraperitoneal injection of LPS (200 µg). Results are expressed as means ± SE of 8 mice per strain at each time point. * P <0.05, ** P <0.001 versus Wt mice at the same time-point.

to baseline in both groups of mice, therefore only results obtained during the first 24 hours are shown. The plasma concentrations of all cytokines measured showed a profound rise after LPS injection **(Figure 1)**. Plasma TNF-α, MCP-1 and IL-10 levels were not different between Wt and PAI-$1^{-/-}$ mice. In Wt mice plasma IL-6 levels showed a peak at 2 hours post injection; in contrast, in PAI-$1^{-/-}$ mice IL-6 levels peaked after 4 hours and were still significantly higher than in Wt mice at 8 hours after injection. IL-12p70 levels peaked at 4 hours after LPS in both groups of mice, but were much higher in PAI-$1^{-/-}$ mice compared to

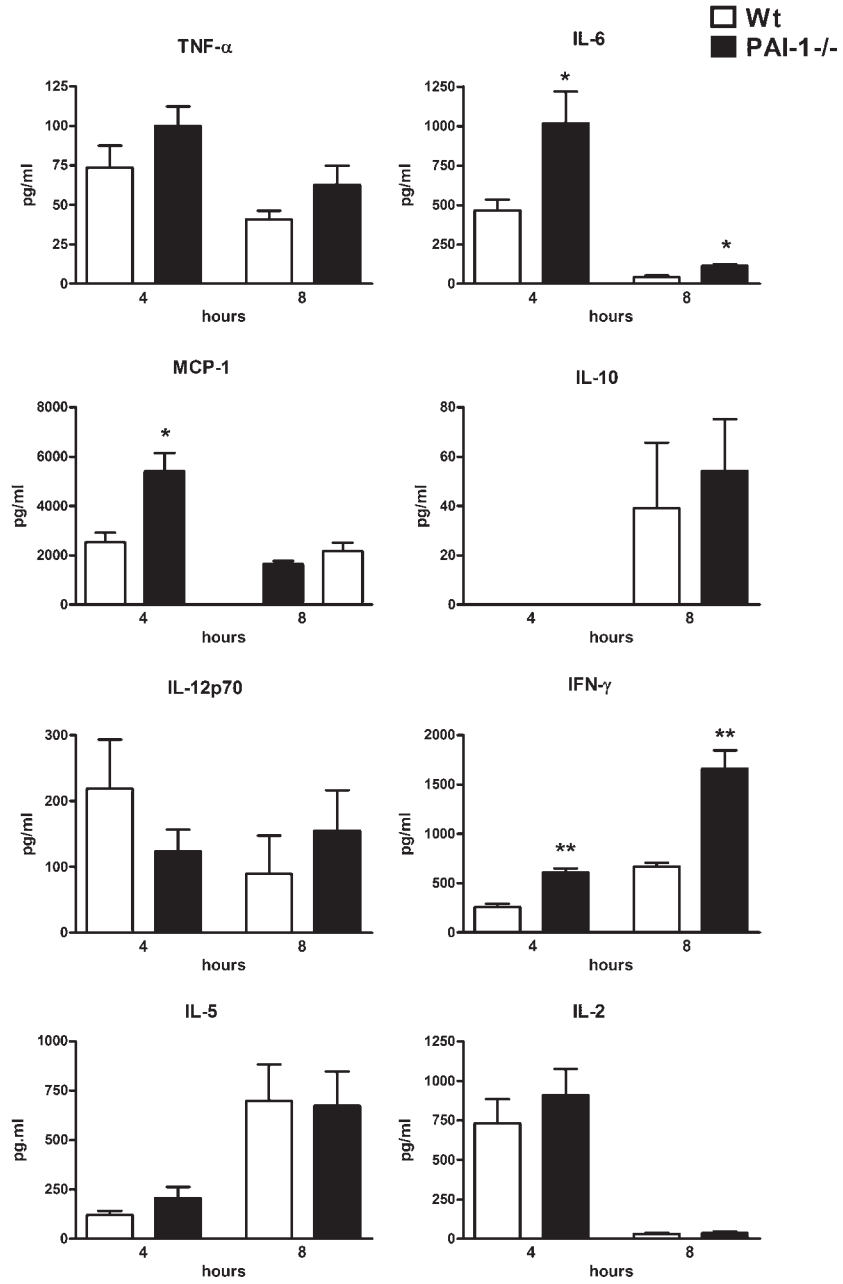

Figure 2. Effect of PAI-1 deficiency on SEB-induced cytokine release. TNF-α, IL-6, MCP-1, IL-10, IL-12p70, IFN-γ, IL-5 and IL-2 were measured in plasma at 4 and 8 hours after intraperitoneal injection of SEB (100 μg). Results are expressed as means ± SE of 8 mice per strain at each time point. * P <0.05, ** P <0.001 versus Wt mice at the same time-point.

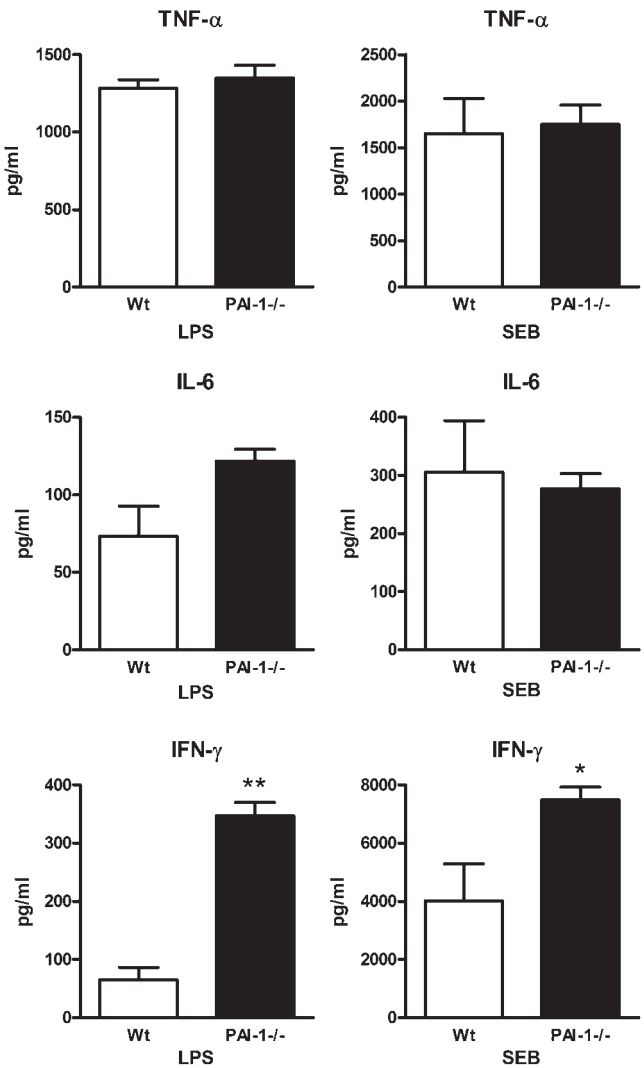

Figure 3. PAI-1$^{-/-}$ splenocytes produce more IFN-γ *in vitro*. TNF-α, IL-6 and IFN-γ were measured in the supernatants of splenocyte cultures after 48 hours of incubation with either LPS (10 ng/ml) or SEB (10 μg/ml). Results are expressed as means ± SE of 6 mice. * P <0.05, ** P <0.01 versus Wt mice.

Wt mice. The plasma concentrations of IFN-γ peaked at 8 hours after LPS injection in both groups. However, IFN-γ levels were strongly increased in PAI-1$^{-/-}$ mice compared to Wt mice.

The SEB-induced cytokine response

Having found that PAI-1 deficiency is associated with strongly enhanced LPS-induced IFN-γ, IL-6 and IL12p70 release, we decided to investigate the cytokine response to a

Table 1. Cellular composition of spleens from Wt and PAI-1$^{-/-}$ mice.

Cell type (%)	Wt	PAI-1$^{-/-}$
CD4+ T-cells	11.5 ± 0.7	12 ± 0.6
CD8+ T-cells	8.6 ± 0.5	8.1 ± 0.7
NK cells	3.6 ± 0.1	3. 8 ± 0.2
Dendritic cells	0.7 ± 0.1	0.8 ± 0.1
Macrophages	4.4 ± 0.3	3.5 ± 0.4
Granulocytes	2.7 ± 0.3	2.1 ± 0.3

Percentages of total splenocyte cell counts. Data are means ± SEM.
N= 6 mice per genotype.

polyclonal T cell activator. Therefore, we measured plasma levels of TNF-α, IL-6, MCP-1, IL-10, IL-12p70, IFN-γ, IL-5, IL-4 and IL-2 at 4 and 8 hours after i.p. injection of SEB in Wt and PAI-1$^{-/-}$ mice **(Figure 2)**. We chose these two time points since they have been found representative for superantigen induced cytokine release in vivo; in particular IFN-γ reaches peak levels at 8 hours after SEB administration (17, 19). The SEB-induced cytokine response was less strong than the LPS-induced cytokine response, although all cytokines with the exception of IL-4 displayed elevated plasma concentrations after SEB administration. TNF-α levels were similar in the PAI-1$^{-/-}$ mice and Wt mice. In line with the LPS-induced IL-6 response, SEB-induced IL-6 levels were higher in PAI-1$^{-/-}$ mice than in Wt mice at both time points. MCP-1 concentrations were higher in PAI-1$^{-/-}$ mice at 4 hours after SEB administration. IL-10, IL-12p70, IL-5 and IL-2 concentrations were similar between both genotypes. In line with the results obtained after LPS injection, IFN-γ levels were markedly elevated in PAI-1$^{-/-}$ mice when compared to Wt mice at both 4 and 8 hours after SEB injection **(Figure 2)**.

PAI-1$^{-/-}$ splenocytes release more IFN-γ during ex vivo stimulation

In an attempt to determine which cell populations in PAI-1$^{-/-}$ mice show a changed cytokine response and to determine which cytokine changes are influenced directly by PAI-1 deficiency, we stimulated splenocytes and peritoneal macrophages harvested from Wt and PAI-1$^{-/-}$ mice that had not received LPS or SEB *in vivo* with LPS, SEB or culture medium for 48 hours *ex vivo* and measured TNF-α, IL-6, IL-12p70, IFN-γ, IL-5, IL-4 and IL-2 levels in the supernatants. The cellular distribution of spleens obtained from PAI-1$^{-/-}$ and wild-type mice did not differ **(table 1)**. None of these cytokines were detectable in supernatants of cell cultures incubated with medium only. Stimulation with either LPS or SEB resulted in TNF-α, IL-6 and IFN-γ release by splenocytes **(Figure 3)**, whereas IL-12p70, IL-5, IL-4 and IL-2 remained undetectable. LPS and SEB-induced TNF-α and IL-6 levels were similar in supernatants of Wt and PAI-1$^{-/-}$ splenocytes. However, in line with the *in vivo* findings, IFN-γ release by PAI-1$^{-/-}$ splenocytes stimulated with either LPS or SEB was significantly increased. Peritoneal macrophages only released detectable levels of TNF-α and IL-6 upon

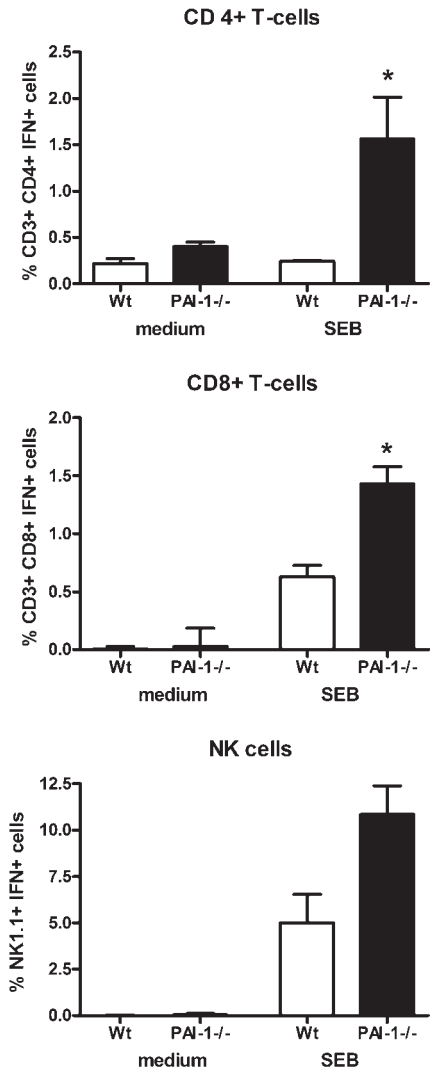

Figure 4. PAI-1$^{-/-}$ CD4+ and CD8+ T-cells produce more IFN-γ *in vitro*. Splenocytes were stimulated with SEB (10 μg/ml) for 48 hours. The percentage of cells that positively stained for intracellular IFN-γ was measured by FACS analysis. Results are expressed as means ± SE of 6 mice. * P <0.01 versus Wt mice.

stimulation with LPS or SEB; these concentrations did not differ between Wt or PAI-1$^{-/-}$ macrophages (data not shown).

CD4+ and CD8+ T cells are involved in the stronger IFN-γ response in PAI-1$^{-/-}$ mice

To determine which cell types are involved in the production of IFN-γ in response to SEB and to examine whether they were influenced by PAI-1 deficiency, we measured the percentage of cells positive for intracellular (IC) IFN-γ in splenocyte cultures after 48 hours

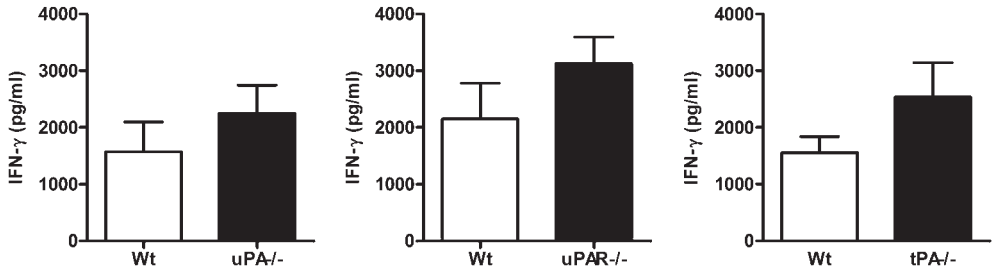

Figure 5. uPA, uPAR or tPA deficiency does not influence LPS-induced IFN-γ release. Plasma IFN-γ levels were measured 8 hours after intraperitoneal injection of LPS (200 μg) in uPA$^{-/-}$, uPAR$^{-/-}$, tPA$^{-/-}$ or Wt mice. Results are expressed as means ± SE of 8 mice per group. Differences between groups were not significant.

of SEB stimulation and differentiated between CD3+CD4+, CD3+CD8+ and NK1.1+ cells by FACS analysis **(Figure 4)**. In these studies we focused on SEB-induced IFN-γ production since this stimulus had proved to be a far more potent IFN-γ inducer than LPS (see Figure 3). The percentage IFN-γ positive T-cells was low, and there was no increase in IC IFN-γ after SEB stimulation in Wt CD4+ T-cells; in contrast, in PAI-1$^{-/-}$ cultures we did find an increase in the percentage IFN-γ positive CD4+ T-cells that was significantly different from Wt CD4+ T cells. CD8+ T-cells showed a rise in IC IFN-γ in both Wt and PAI-1-/- cells; however, the percentage was significantly higher in PAI-1$^{-/-}$ cultures. Finally, NK-cells also showed a rise in the percentage IC IFN-γ positive cells after SEB stimulation; although PAI-1$^{-/-}$ NK cells showed a higher percentage with positive IC IFN-γ staining, the difference with Wt cells did not reach statistical significance. These data show that both CD4+ and CD8+ T-cells from PAI-1$^{-/-}$ mice produced more IFN-γ upon SEB stimulation *in vitro*.

uPA, uPAR or tPA-deficiency does not influence LPS-induced IFN-γ release

A major function of PAI-1 in the fibrinolytic system is inhibition of the plasminogen activators uPA and tPA. We therefore were interested to determine the role of uPA and the uPA receptor and tPA in IFN-γ release induced by LPS. To address this issue, uPA$^{-/-}$, uPAR$^{-/-}$ and tPA$^{-/-}$ mice were i.p. injected with LPS and after 8 hours plasma IFN-γ concentrations were measured, i.e. the time point at which plasma IFN-γ reached peak levels in this model. None of the mice displayed an altered IFN-γ response when compared with Wt mice **(Figure 5)**.

Discussion

Patients with sepsis consistently display elevated plasma concentrations of PAI-1 and such elevated circulating PAI-1 levels are highly predictive for an unfavorable outcome (3, 4). However, these clinical studies failed to provide insight into a possible functional role of PAI-1 in sepsis, *i.e.* the elevated PAI-1 levels could merely be indicative of a strong inflammatory response of the host, rather than bearing any pathophysiological significance. We here attempted to provide a first insight into the role of endogenous PAI-1 in the systemic inflammatory response to two different challenges: LPS, the most

important proinflammatory component of Gram-negative bacteria that mainly activates monocytes and macrophages (20), and SEB, a superantigen derived from the Gram-positive pathogen *S. aureus*, that triggers polyclonal T cell activation (17, 18). The main finding of our study was that PAI-1$^{-/-}$ mice demonstrate markedly elevated plasma IFN-γ levels upon administration of either LPS or SEB. Further investigations identified CD4+ and CD8+ T cells, and to a lesser extent NK cells, as likely cellular sources for the enhanced IFN-γ release in PAI-1$^{-/-}$ mice, and in addition showed that the effect of PAI-1 on IFN-γ secretion occurred independent from tPA, uPA or uPAR.

LPS has been used extensively to obtain insight in the mechanisms contributing to systemic inflammation during sepsis. Bolus injection of LPS into humans results in a strong rise in plasma PAI-1 concentrations peaking after 4 hours (10, 21). We here used the LPS challenge model to obtain a first insight in the role of PAI-1 in systemic release of cytokines. We first confirmed PAI-1 release after LPS administration to Wt mice. When we subsequently found elevated IFN-γ concentrations in LPS challenged PAI-1$^{-/-}$ mice, we decided to investigate whether this observation could be reproduced after administration of a polyclonal T cell activator. For this we used SEB, a superantigen produced by *S. aureus* implicated in nonmenstrual toxic shock syndrome (18). Indeed, SEB injection elicited PAI-1 secretion into the circulation and higher IFN-γ levels were detected in PAI-1$^{-/-}$ mice. Of note, not all cytokine responses were similarly affected by PAI-1 deficiency in the LPS and SEB models. These differences might be explained by the fact that LPS and SEB activate different cell types (20). LPS primarily activates mononuclear cells (20). SEB can bind directly to regions of the class II MHC molecule that are outside the physiological MHC haplotype-restricted antigen-binding groove, which eventually results in activation of both antigen-presenting cells and SEB-reactive Vβ8+ T cells (17). Such differences in cell activation likely contributed to the differential effects of PAI-1 deficiency on IL-12p70 and MCP-1 release. In particular the different impact of PAI-1 deficiency on IL-12p70 release is important. IL-12p70 is a strong inducer of IFN-γ production (22); the fact that IFN-γ release was enhanced in PAI-1$^{-/-}$ mice after both LPS and SEB injection, whereas only in the former model IL-12p70 released was increased in these animals, strongly suggests that the effect of endogenous PAI-1 on IFN-γ release does not rely on IL-12p70. This notion is further supported by the fact that PAI-1$^{-/-}$ splenocytes released more IFN-γ upon stimulation with either LPS or SEB under conditions where IL-12p70 release remained undetectable.

Mice with a targeted deletion of the gene encoding uPA have been found to mount a reduced type 1 response upon pulmonary infection with the opportunistic yeast *Cryptococcus neoformans*, as reflected by lower IFN-γ levels in bronchoalveolar lavage fluid (16). In addition, lung mononuclear cells and regional lymph node cells obtained from infected uPA$^{-/-}$ mice released less IFN-γ upon antigen-specific stimulation in this model (16). These findings led us to hypothesize that PAI-1 may facilitate a type 2 response through inhibition of uPA. However, this appeared not to be the case: not only uPA$^{-/-}$ mice, but also uPAR$^{-/-}$ and tPA$^{-/-}$ mice demonstrated an unremarkable IFN-γ response upon injection of LPS. These data suggest that the effect of endogenous PAI-1 is not mediated by an effect on uPA/uPAR or tPA.

While our study was in progress, Sejima et al. reported on the consequences of PAI-1 deficiency in a model of nasal allergy (23). In this latter investigation, OVA-sensitized mice demonstrated increased PAI-1 levels in nasal washings upon intranasal OVA challenge; this locally produced PAI-1 contributed to a T helper 2 response as reflected by T helper 1 biased responses in sensitized PAI-1$^{-/-}$ mice characterized by reduced OVA-specific circulating IgE and elevated plasma IgG2a levels, as well as lower IL-4 and IL-5 and higher IFN-γ concentrations in nasal lavage fluid (23). Our present findings extend and are partly in line with these results: naive PAI-1$^{-/-}$ mice displayed enhanced systemic IFN-γ release after i.p. injection of either LPS or SEB, and these *in vivo* observations could be reproduced using PAI-1$^{-/-}$ splenocytes *in vitro*. However, in contrast to the results obtained by Sejima et al. (23), we did not find a diminished release of the type 2 cytokine IL-5 in the SEB model, whereas IL-4 could not be studied since even in Wt mice plasma IL-4 remained undetectable after SEB administration. Nonetheless, these data together suggest that PAI-1 inhibits IFN-γ release under markedly different conditions, thereby identifying a novel biological activity of this protein. Further studies are warranted to unravel the mechanisms by which PAI-1 influences IFN-γ production.

Inhibition or elimination of IFN-γ has been reported to reduce LPS-induced lethality in mice (24-26). On the other hand pretreatment with recombinant IFN-γ increased LPS-induced lethality (24). Thus, it is possible that PAI-1 deficiency influences survival during endotoxemia. We considered survival studies beyond the scope of the current investigations, since our study focused on the regulation of IFN-γ production by PAI-1 and since many other factors besides IFN-γ have been implicated as mediators of LPS toxicity and lethality. In addition, in this respect should be noted that one study reported that IFN-γ receptor deficient mice sensitized with D-galactosamine were protected against LPS-induced lethality, whereas the toxicity evoked by high dose LPS in the absence of D-galactosamine sensitization was similar in IFN-γ receptor deficient and wild-type mice (26) . Of note, the influence of endogenous PAI-1 on IFN-γ production could be of relevance to different conditions and diseases besides sepsis in which IFN-γ has been found to play a role, including allergy (see above), viral and parasitic infections and intracellular infections such as tuberculosis.

Enhanced PAI-1 release is a consistent part of the systemic inflammatory response syndrome induced by sepsis or administration of bacterial products. We here demonstrate for the first time that elevated PAI-1 concentrations are functionally important for attenuating IFN-γ release after injection of LPS or SEB. These results further exemplify the complex role of PAI-1 in the host response to severe infection, which reaches far beyond its classical role as an inhibitor of fibrinolysis.

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Chapter 4

The role of plasminogen activator inhibitor type 1 in the inflammatory response to local tissue injury

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Abstract

The plasma levels of the plasminogen activator-inhibitor type 1 (PAI-1) are consistently elevated in patients with sterile tissue injury, often accompanied by a systemic acute phase protein response. It remains, however, unknown whether and to what extent PAI-1 affects the host response to trauma. By using the well-established murine model of turpentine-induced tissue injury we compared local and systemic inflammatory responses in PAI-1 gene deficient (PAI-$1^{-/-}$) and normal wild type (Wt) mice. Subcutaneous turpentine injection elicited strong increases in PAI-1 protein concentration in plasma and at the site of injury, but not in liver. PAI-1 mRNA was locally increased and mainly expressed by macrophages and endothelial cells. PAI-1 deficiency greatly enhanced the early influx of neutrophils to the site of inflammation, which was associated with increased edema and necrosis at 8 hours after injection. Furthermore, PAI-$1^{-/-}$ mice showed a reduced early interleukin (IL)-6 induction with subsequently lower acute phase protein levels and a much slower recovery of body weight loss. These findings suggest that PAI-1 is not merely a marker of tissue injury but plays a functional role in the local and systemic host response to trauma.

Introduction

Local tissue injury is often associated with a systemic inflammatory response, the so-called acute phase response (1). This non-specific host response to tissue damage is often seen in patients after trauma, major surgery, burn, tissue infarction or during advanced cancer. The subcutaneous injection of turpentine is a well-established murine model to study the acute phase response induced by local tissue damage (2-6). The inflammatory reaction to turpentine is characterized by local inflammation and abscess formation, fever, cytokine production, changes in acute phase protein levels, loss of body weight and anorexia. The cytokines interleukin (IL)-1β and IL-6 play an essential role in initiation of the acute phase response (2-6). IL-1β deficient mice as well as mice treated with anti-IL-1 receptor antibodies were not able to mount a normal inflammatory reaction to turpentine-induced local tissue damage as reflected by undetectable IL-6 levels and the absence of weight loss and the acute phase protein response (2, 6-8). Administration of anti-IL-6 antibodies and IL-6 deficiency also caused a dramatic reduction of turpentine-induced acute phase protein expression and weight loss in mice (3, 4, 9). Together these data indicate that the acute phase response to sterile tissue injury is initiated by IL-1, and that the subsequently induced IL-6 is the essential mediator of the systemic acute phase response.

Plasminogen activator inhibitor type 1 (PAI-1) is the main physiological inhibitor of both tissue-type and urokinase-type plasminogen activator and thereby plays an important role in regulation of the fibrinolytic system. PAI-1 has also been reported to act like an acute phase protein (10, 11) and plasma PAI-1 levels rise markedly during disease states often associated with a sterile acute phase response including trauma, surgery and burn injury (11-15). In line, turpentine injection elicits an increase in plasma PAI-1 levels in mice, a response that is absent in IL-1β deficient mice (16). Recently, it has become clear that PAI-1 likely has other properties besides its inhibitory role in the fibrinolytic system. In particular, PAI-1 has been suggested to be involved in extracellular matrix proteolysis, cellular adhesion and migration (17, 18). However, the overall knowledge about the role of PAI-1 in inflammatory processes is very limited. We here used the turpentine induced abscess model to obtain insight into the role of endogenous PAI-1 in inflammation considering that this model allows us to determine the function of PAI-1 in both the (systemic) acute phase protein response as well as in the local inflammatory response and the recruitment of neutrophils during the formation of an abscess.

Methods

Animals

All experiments were approved by the institutional animal care and use committee of the Academic Medical Center (Amsterdam, The Netherlands). C57BL/6 mice were purchased from the Jackson Laboratories (Bar Arbor, ME). PAI-1-deficient (PAI $^{-/-}$) mice (19) and wild-type (Wt) mice were on a mixed Ola129/C57BL/6 background. Eight weeks old female mice were used for all experiments (eight mice per genotype per time-point). The animals were maintained with a 12 h light/12 h dark cycle and had free access to food and water.

Experimental design

Mice were subcutaneously (s.c.) injected with 100 µl of turpentine oil (Sigma, St Louis, MO) or saline into both hind limbs. Mice were weighed 24 hours before and at 24 hour intervals after turpentine injection for 2 weeks. At different time points after turpentine or saline injection groups of eight mice were anesthetized by intraperitoneal injection of Hypnorm (Janssen Pharmaceutica, Beerse, Belgium) and midazolam (Roche, Meidrecht, The Netherlands), and blood was taken from the inferior vena cava. Blood was collected in EDTA-coated tubes; spun at 3400 rpm at 4 °C for 15 min and the supernatants were aliquoted and frozen at – 20 °C until assayed.

Preparation of tissue homogenates

The subcutis and muscle tissue were removed at the injection site of each hind limb, weight and snap frozen in liquid nitrogen. These frozen specimens were crushed to a powder like suspension, suspended in 4 volumes of sterile isotonic saline and subsequently lysed in 1 volume of lysis buffer (300 mM NaCl, 15 mM Tris [tris(hydroxymethyl)aminomethane], 2 mM $MgClH_2O$, 2 mM Triton X-100, pepstatin A, leupeptin, and aprotinine [20 ng/mL], pH 7.4) on ice for 30 minutes and spun at 3400 rpm at 4 °C for 15 min. The supernatant was frozen at –20°C. Livers were harvested and homogenized at 4°C in 4 volumes sterile isotonic saline with a tissue homogenizer (Biospect Products, Bartlesville, OK), lysed in 1 volume of lysis buffer and spun at 3400 rpm at 4°C for 15 minutes; the supernatant was frozen at –20°C until assayed.

PAI-1 in situ hybridization

Visualization of PAI-1 mRNA was performed as described (20). First, 5-µm paraffin sections were mounted on SuperFrost Plus glass slides (Menzel-Gläser, Braunschweig, Germany) and subjected to in situ hybridization with a PAI-1 antisense riboprobe. In vitro transcription of linearized plasmid DNA was performed using (^{35}S)-uridine triphosphate (UTP) (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) to obtain the radiolabeled antisense PAI-1 riboprobe. In situ hybridization was executed by standard procedures. In situ sections were covered with nuclear research emulsion (ILFORD Imaging UK, Cheshire, United Kingdom), exposed for 3 weeks, and then developed and counterstained with hematoxylin and eosin.

Histology

Injected hind limb tissues were removed, post-fixed in 10% formaldehyde in PBS for 24 hours, dehydrated in increasing concentrations of ethanol followed by xylene, and embedded in paraffin. Sections (4 um) were cut and stained with hematoxylin and eosin. Semi-quantitative evaluation of tissue histology was performed independently by a pathologist without knowledge of the type of mice and treatment. The histology scores were evaluated by the size of the infiltrate, necrosis and edema. Each was scored separately from 0-6 in which 0= absent and 1 to 6 ranges from very mild to extremely severe respectively. Slides were scored in random order. The 3 scores per section were summarized to the total histology score. To evaluate the amount of collagen fibers formed around the infiltrates, picrosirius red staining of hind limb sections was performed at 7 days after turpentine injection. The percentage of area stained was quantified in ten random non-overlapping fields of the area around the edge of the abscess (magnification, x 20) from each animal

using a computer assisted image analysis system (Image-pro plus, MediaCybernetics, Silver Spring, MD).

Assays

Murine PAI-1 antigen levels were measured in plasma and tissue homogenates by ELISA as described (20, 21). Levels of myeloperoxidase (MPO) were measured as described elsewhere (22, 23). Macrophage inflammatory protein (MIP)-2, keratinocyte derived chemokine (KC) and IL-1β levels were measured using commercially available ELISA kits (R&D systems, Abingdon, UK). IL-6 levels were measured using a commercially available cytometric beads array (CBA) multiplex assay (BDBiosciences, San Jose, CA) in accordance with the manufacturer's recommendations. Serum amyloid P (SAP) was measured by a sandwich ELISA as described previously (24, 25). In short, we used a sheep anti-mouse SAP as a coating antibody, and a rabbit anti-mouse SAP as detecting antibody (both Calbiochem-Novabiochem International, San Diego, CA), after which an anti-rabbit antibody alkaline phosphatase-conjugated (Sigma Chemical Co., Saint Louis, MO) was added. The assay was developed using *p*-nitrophenylphosphate; absorption was measured at 405 nm. Serum C3 was also detected by sandwich ELISA using goat anti-mouse C3 (ICN, Costa Mesa, CA) as coating antibody, and goat anti-mouse C3c (Nordic, Tilburg, the Netherlands) as detecting antibody. The assay was developed using tetramethyl benzidine and measured at 450 nm. In both ELISA's a standard curve was made by serial dilutions of acute phase mouse serum (Calbiochem-Novabiochem International) with known concentrations of SAP and C3. Haptoglobin was measured using a commercially available colorimetric assay (Tridelta Development Ltd, Wicklow, Ireland).

Statistical analysis

All values are given as means ± SE. Comparisons between groups were analyzed by Mann-Whitney U test or two-way analysis of variance (ANOVA, using GraphPad Software, Prism version 4.0, San Diego, CA) followed, when statistically significant, by a post hoc Bonferroni test. A P value of <0.05 was considered as a significant difference between groups.

Results

PAI-1 production upon turpentine-induced tissue injury

To evaluate the role of PAI-1 in the sterile acute phase response, we used a well-established murine model of turpentine induced tissue injury and compared inflammatory responses in PAI-$1^{-/-}$ and Wt mice. First, to confirm PAI-1 production in this model, we measured PAI-1 protein levels in C57BL/6 Wt mice at the site of inflammation (hind limb tissue homogenates), in plasma and in liver homogenates at different time points after turpentine or saline injection. Turpentine injection increased PAI-1 concentrations locally in hind limb tissue homogenates ($P < 0.05$ vs saline; **Figure 1A**), as well as systemically in plasma ($P<0.05$ vs saline; **Figure 1B**), but not in liver homogenates **(Figure 1C)**.
To obtain insight into the cellular expression of locally produced PAI-1, we performed in situ hybridization on turpentine injected hind limb tissues. At 1, 3, 5 and 7 days after

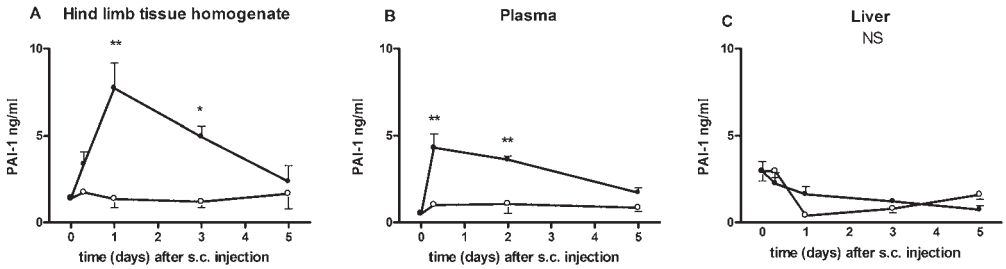

Figure 1. PAI-1 protein levels. PAI-1 protein concentrations in (A) hind limb homogenates, (B) plasma and (C) liver homogenates measured at different time points after turpentine (black circles) or saline (open circles) injection. Data are mean ± SEM. N = 8 per group per time point. * P<0.05, ** P<0.001 vs. saline. NS, non significant.

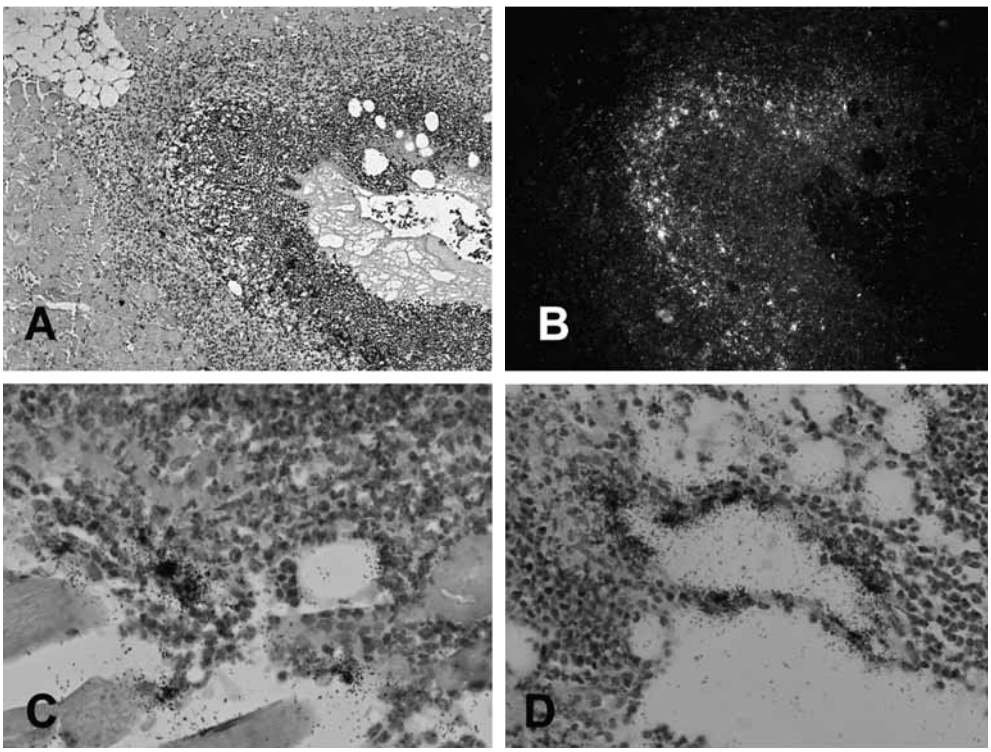

Figure 2. Local PAI-1 mRNA expression. PAI-1 mRNA expression in hind limb tissue by in situ hybridization 1 day after subcutaneous injection of turpentine. (A) Black and white picture showing an overview of a turpentine-induced abscess. (B) Dark field image of the same microscopic field as in (A) showing PAI-1 mRNA expression as white points surrounding the abscess correlating with the encapsulating macrophages. Magnification, x 100. (C) Detail of PAI-1 mRNA expression co-localized with macrophages and (D) endothelial cells. Magnification, x 400 and x 200 respectively.

turpentine injection strong expression of PAI-1 mRNA was observed. PAI-1 expression was predominantly co-localized with macrophages encapsulating the inflammatory infiltrates, and was also present in local endothelial cells **(Figure 2 A-D)**. This local PAI-1 mRNA expression was most pronounced at 1 day after turpentine injection. Neutrophilic granulocytes did not show any PAI-1 expression. Together these data suggest that the site of local tissue injury is a major source of circulating PAI-1 after turpentine administration.

Increased local inflammation in PAI-1$^{-/-}$ mice early after turpentine injection

To obtain insight into the composition of the turpentine induced infiltrate, histological evaluation of the inflamed hind limb tissue from Wt and PAI-1$^{-/-}$ mice was performed before and at 8 hours and 1, 3, 5 and 7 days after turpentine injection. In both mouse strains turpentine injection elicited a strong inflammatory response resulting in local abscess formation characterized by a neutrophilic granulocyte infiltrate encapsulated by macrophages and fibroblasts. After 8 hours PAI-1$^{-/-}$ mice displayed significantly more local tissue injury than Wt mice, as judged by the total histology scores of the size of the granulocytic inflammatory infiltrate and the amount of necrosis and edema (mean score 4.0 $\pm$ 0.5 and 2.0 $\pm$ 0.3 respectively, $P<0.01$, **Figure 3A and B**). Furthermore, at this time point, MPO activity in hind limb homogenates was higher in the PAI-1$^{-/-}$ mice compared to Wt mice (13.1 $\pm$ 1.3 versus 9.1 $\pm$ 0.7 units/gram/minute, $P<0.05$), also indicating a stronger neutrophilic migratory response.

Thereafter, the extent and the severity of the inflammation increased in both mouse strains, with maximal histology scores reached after 7 days. At the later time points, the local inflammatory response did not differ histologically between PAI-1$^{-/-}$ and Wt mice (shown for days 1 and 5 in **Figure 3 C-F**). Also, MPO activtiy was similar at 7 days after turpentine injection (data not shown).

Since PAI-1 has been implicated to play a role in the formation of fibrosis (26-29), we measured the amount of fibrotic tissue in Wt and PAI-1$^{-/-}$ mice at 7 days after turpentine injection. However, there was no difference between Wt and PAI-1$^{-/-}$ mice in fibrosis formation around the inflammatory infiltrate at 7 days after turpentine injection, as investigated by image analysis of picrosirius red staining (2.8 $\pm$ 0.5 versus 3.6 $\pm$ 0.9 respectively, not significant).

Local chemokine response

The mouse CXC chemokines KC and MIP-2 have been implicated in the attraction of neutrophils to the site of inflammation (30). Therefore, the concentrations of these mediators were measured in hind limb homogenates. In both genotypes KC and MIP-2 levels increased significantly after turpentine injection and peaked at 1 and 3 days, respectively. There were no differences between Wt and PAI-1$^{-/-}$ mice in local MIP-2 or KC levels at any time-point after turpentine injection (data not shown).

Reduced local and systemic IL-6 response in PAI-1$^{-/-}$ mice

IL-1β and IL-6 play a pivotal role in the systemic acute phase response upon local tissue injury (2-6). Therefore we measured the concentrations of these cytokines in hind limb homogenates derived from the site of inflammation and in plasma at different time points after turpentine injection. Local IL-1β levels showed a quick rise and peaked after 8 hours

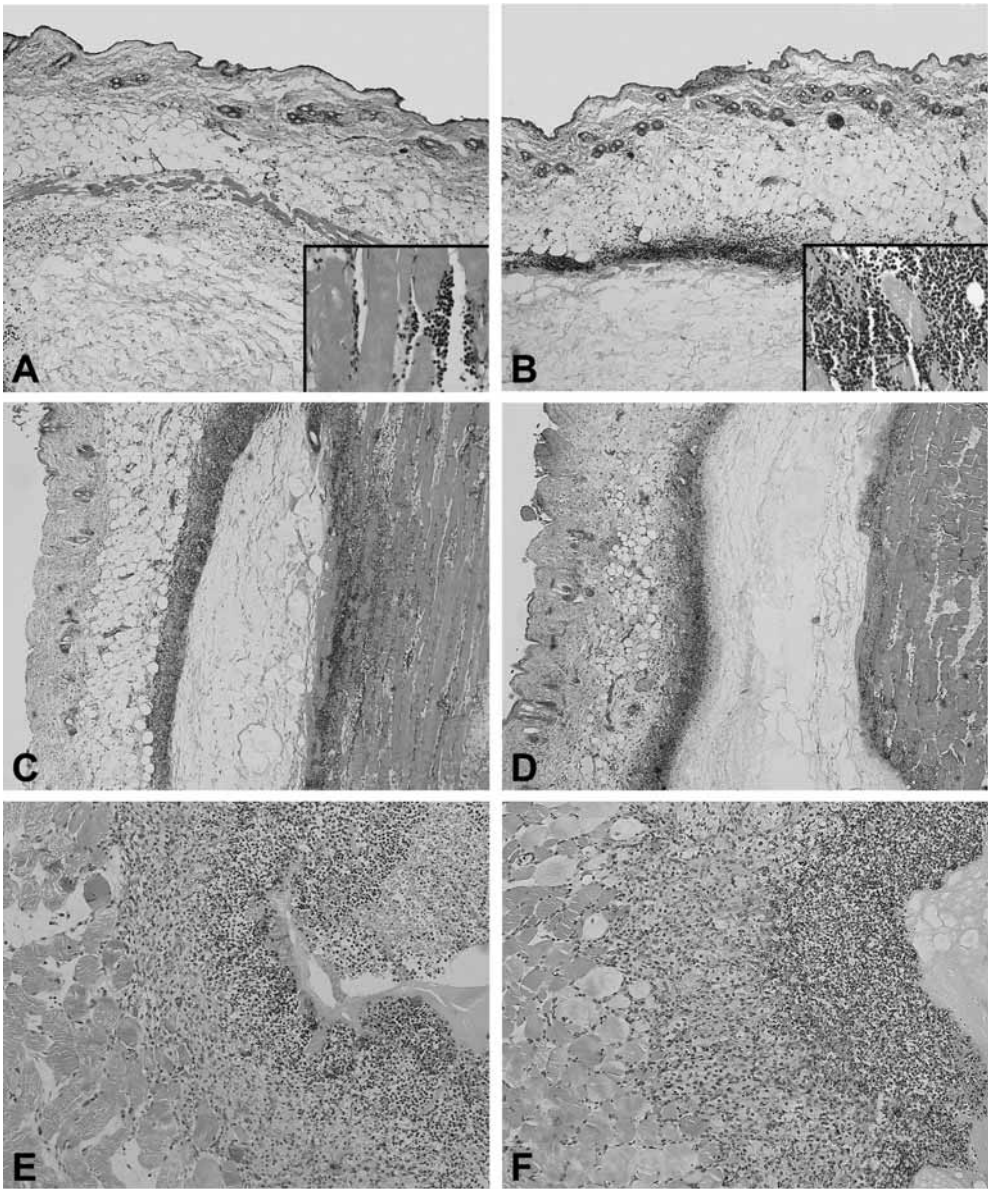

Figure 3. Histopathology of local inflammation. Wt (A, C, E) and PAI-1$^{-/-}$ (B, D, F) mice were injected subcutaneously with 100 µl of turpentine. Hind limb tissues were removed after 8 hours (A, B), 1 (C, D) and 5 (E, F) days. At 8 h PAI-1$^{-/-}$ mice displayed significantly more tissue injury with larger inflammatory infiltrates and more necrosis and edema. The inserts show destruction of skeletal muscle tissue by neutrophils, which is clearly more extensive in PAI-1$^{-/-}$ mice compared to Wt mice. At 1 and 5 days the extent and severity of tissue injury and inflammation did not differ between PAI-1$^{-/-}$ and Wt mice. Slides are representative for 8 mice per group per time point. Magification, A-B x 40, inserts x 400, C-D x 100, E-F x 200.

Table 1. Plasma acute phase protein levels after turpentine injection

	Serum amyloid P		Complement 3		Haptoglobin	
time	µg/ml		µg/ml		mg/ml	
	Wt	PAI-1 $^{-/-}$	Wt	PAI-1 $^{-/-}$	Wt	PAI-1 $^{-/-}$
0 h	39 ± 0.9	40 ± 0.2	340 ± 70	375 ± 47	ND	ND
8 h	50 ± 5.8	45 ± 3.7	530 ± 49	396 ± 39	0.1 ± 0.1	0.4 ± 0.4
1 day	506 ± 44	343 ± 24*	703 ± 71	396 ± 73*	7.3 ± 0.5	9.8 ± 2.7
3 days	540 ± 35	615 ± 71	744 ± 69	1008 ± 60*	16.3 ± 1.7	14.1 ± 2.1
5 days	209 ± 44	269 ± 46	342 ± 41	371 ± 29	4.5 ± 0.5	9.1 ± 1.9
7 days	153 ± 35	116 ± 31	539 ± 45	533 ± 30	2.5 ± 0.9	5.9 ± 2.4

Data are means ± SEM (N=8 mice per group at each time point) before and at 8 hours and 1, 3, 5 and 7 days after turpentine injection. * P < 0.05 versus Wt mice. ND, not detectable.

with no differences between PAI-1$^{-/-}$ and Wt mice (**Figure 4**). IL-1β remained undetectable in plasma at all time points in both mouse strains. Local IL-6 levels also peaked at 8 hours; at this time point IL-6 levels were much lower in PAI-1$^{-/-}$ mice than in Wt mice, although the difference did not reach statistical significance due to a relatively large interindividual variation **(Figure 4)**. In addition, plasma IL-6 levels were also markedly reduced in PAI-1$^{-/-}$ mice at 8 hours after turpentine injection compared to Wt mice (P<0.05, **Figure 4**).

Acute phase protein response

Since IL-6 is the essential mediator of the systemic acute phase protein response in this model (3, 4, 9), we next determined the magnitude of the acute phase protein response in PAI-1$^{-/-}$ and Wt mice (**Table 1**). Turpentine injection caused a significant increase in all acute phase proteins measured in both mouse strains. In Wt mice SAP and C3 reached a plateau phase after 24 hours and started to decline after 3 days, whereas haptoglobin

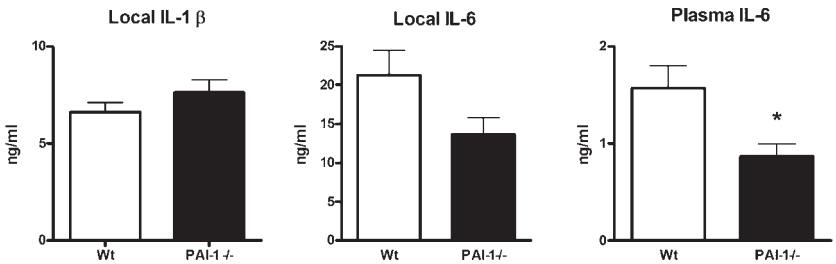

Figure 4. Reduced IL-6 levels in PAI-1$^{-/-}$ mice. IL-1β and IL-6 concentrations in hind limb homogenates and IL-6 levels in plasma at t= 0 and 8 hours after turpentine injection in Wt and PAI-1e$^{-/-}$ mice. Data are means ± SEM. N=8 per group per time point. * P<0.05 vs. Wt.

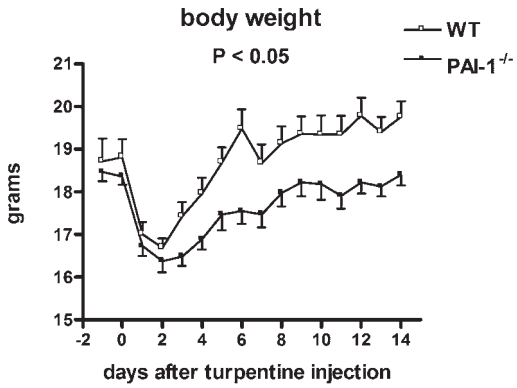

Figure 5. Increased turpentine-induced weight loss in PAI-1$^{-/-}$ mice. Wt and PAI-1$^{-/-}$ mice were injected with 100 µl of turpentine in both hind limbs. Body weight was measured daily. Data are expressed as means ± SEM. N=8 per group. P value represents difference between Wt and PAI-1$^{-/-}$ mice. Table 1. Plasma acute phase protein levels after turpentine injection

levels peaked after 3 days. In PAI-1$^{-/-}$ mice SAP and C3 levels were significantly lower than in Wt mice at 24 hours (both P<0.05 vs Wt) and showed a delayed peak at 3 days after injection. However, at day 3 PAI-1$^{-/-}$ mice demonstrated higher C3 peak levels than Wt mice. Haptoglobin levels did not differ between the two mouse strains at any time point after turpentine injection.

Prolonged turpentine-induced weight loss in PAI-1$^{-/-}$ mice

Weight loss is a clinically important feature of the acute phase response (1-6, 8, 9). To study the role of PAI-1 in turpentine-induced weight loss, we measured the body weight of PAI-1$^{-/-}$ and Wt mice daily for two weeks after turpentine injection. Both groups of mice demonstrated considerable weight loss in the first 2 days after turpentine injection. Thereafter Wt mice started to regain weight, recovering to their initial body weight after 5 days. In contrast, PAI-1$^{-/-}$ mice did not recover and were still below their initial body weight 2 weeks after turpentine injection (P < 0.05 vs Wt; **Figure 5**).

Discussion

PAI-1 is an acute phase protein, which is markedly induced upon sterile tissue injury in humans and mice. In this study, we investigated the role of increased PAI-1 levels during the turpentine-induced sterile acute phase response in mice. We here show that PAI-1 is upregulated after turpentine injection and is produced at the site of tissue injury, mainly by macrophages and endothelial cells. Endogenously produced PAI-1 influenced the inflammatory reaction to turpentine at various levels. Indeed, PAI-1$^{-/-}$ mice had a more pronounced local inflammatory response at 8 hours after turpentine injection, characterized by increased neutrophil influx, edema and necrosis. Furthermore, PAI-1$^{-/-}$ mice showed a reduced early IL-6 induction and subsequently lower acute phase protein levels after

turpentine injection. Clinically, the altered inflammatory response in PAI-1$^{-/-}$ mice was associated with a much slower recovery of their weight loss.

In an earlier investigation Seki et al. found elevated plasma PAI-1 levels after turpentine injection; these authors also reported increased PAI-1 mRNA in the liver (16). In contrast, we could not detect an increase in PAI-1 protein levels in liver homogenates. Instead, at the site of injury we found high protein levels of PAI-1 together with clear PAI-1 mRNA upregulation by local macrophages and endothelial cells. Hence, our data suggest that elevated PAI-1 plasma concentrations that accompany a sterile trauma at least in part are the result of increased production at the site of tissue injury.

PAI-1 can play a role in the regulation of several physiological and pathological processes implicated in inflammation. First, PAI-1 is the main inhibitor of urokinase plasminogen activator (uPA) and thereby a strong inhibitor of uPA mediated pericellular plasmin generation (31, 32). Cell-associated plasmin is able to degrade various extra-cellular matrix components, which facilitates cellular migration. Therefore, PAI-1 might be a negative regulator of plasmin-mediated cell migration. Secondly, PAI-1 can influence cellular migration by inhibition of uPA receptor and vitronectin mediated cell adhesion (31, 32). The uPA system regulates cell migration by an interaction of uPA with its receptor uPAR (CD87); in addition, uPAR can bind vitronectin, a component of the extra cellular matrix and a ligand for integrins. PAI-1 can inhibit cell adhesion and migration by inhibiting the activity of uPAR-bound uPA, and by preventing integrin association to vitronectin. Studies with uPAR$^{-/-}$ mice have emphasized the eminent role of this receptor in leukocyte trafficking. Indeed, uPAR$^{-/-}$ mice displayed a profoundly reduced neutrophil recruitment to the peritoneal cavity after intraperitoneal administration of thioglycollate (33). However, the in vivo relevance of PAI-1 in cell migration has not been directly addressed thus far. We showed that PAI-1 deficiency facilitated the initial influx of neutrophils in response to turpentine injection. These data provide the first in vivo evidence for a negative role for PAI-1 in neutrophil recruitment to the site of inflammation. However this difference in local cellular accumulation disappeared after 1 day. Thus, PAI-1 seems to act as a negative regulator of neutrophil recruitment only during the early phase of turpentine-induced inflammation. Conceivably, other mediators, like the CXC chemokines, overcome the inhibitory effects of PAI-1 later on. This theory is strengthened by the fact that local PAI-1 concentrations decrease substantially during the later phase of turpentine-induced inflammation and that, on the other hand, local MIP-2 levels stay strongly elevated up to 7 days after turpentine injection.

Besides its influence on cellular migration PAI-1 also plays a role in extracellular matrix remodelling and the development of fibrosis (26). PAI-1 can promote the formation of fibrosis by inhibiting plasmin activation and thereby inhibiting proteolysis of various extracellular matrix components, like type IV collagen, fibronectin, laminin, proteoglycan and fibrin (26). Indeed, previous studies with PAI -1$^{-/-}$ mice showed reduced fibrosis formation in lungs after bleomycin-induced lung damage (27) as well as in kidneys after ureteral obstruction (28). Furthermore, PAI-1$^{-/-}$ mice had less fibrotic tissue accumulation after subcutaneous polyvinyl alcohol sponge implantation (29). During turpentine-induced

chronic tissue inflammation an abscess is formed which finally becomes encapsulated with fibrotic tissue. Therefore, we investigated whether there was a difference in the accumulation of collagen fibers around the turpentine-induced inflammatory infiltrate between Wt and PAI-$1^{-/-}$ mice at 7 days after s.c. injection. In contrast to previous studies we could not detect any difference in the formation of fibrotic tissue. This might be explained by the fact that this is a chronic inflammation model and that by the time fibrosis starts to develop PAI-1 levels are already back to baseline.

The induction of IL-1β and IL-6 plays a very important role in this model of sterile inflammation. IL-$1\beta^{-/-}$ and IL-$6^{-/-}$ mice do not develop an acute phase protein response and do not suffer from weight loss after turpentine injection (4, 6). In the present study, PAI-$1^{-/-}$ mice showed lower local and systemic IL-6 levels early after turpentine injection, whereas the induction of IL-1β, which at least in part is responsible for the IL-6 response (2, 6-8), was not different between the genotypes. These data suggest that PAI-1 deficiency reduces the early IL-6 response to turpentine by an IL-1β independent mechanism. Whether PAI-1 can directly influence IL-6 production, remains to be established. In any case, since IL-6 is responsible for the liver acute phase protein response in this model (3, 4), the lower SAP and C3 levels at 1 day after turpentine injection were probably at least in part caused by the reduced IL-6 levels in PAI-$1^{-/-}$ mice at 8 hours. In addition, the attenuated IL-6 response might have played a role in the delayed and reduced recovery of body weight in PAI-$1^{-/-}$ mice. Indeed, although IL-$6^{-/-}$ mice did not mount an acute phase response after turpentine injection, they started losing weight at the time that Wt mice were recovering, and IL-$6^{-/-}$ mice even died later on (4). These findings indicate that the IL-6 induced acute phase response is protective and necessary to repair and recover from the localized tissue inflammation. Of note, we did not follow the mice beyond 14 days since at that time point all animals had already in part recovered from their injury. In addition, due to the lack of sufficient plasma obtained from mice with a turpentine induced abscess, we were not able to study the metabolic response to injury here.

Notably, several hormones and drugs are known to decrease PAI-1 levels, including estrogen, angiotensine converting enzyme inhibitors and metformin [34]. Of these, the effect of estrogen on the inflammatory response to subcutaneous turpentine has been investigated earlier [35]. In that study, published in 1949, oestradiol dipropionate administration to rats of which the adrenals and gonads were removed, was associated with the formation of poorly demarcated abscesses with thin walls which were almost completely void of granulation tissue and a fibroblastic response, consisting almost entirely of neutrophils. Although this study is difficult to compare with ours, the data combined suggest that part of the effect of estrogens in this model could be due to diminished PAI-1 levels. The effects of angiotensine converting enzyme inhibitors and metformin in this model are unknown at present and further investigations are warranted to determine these.

While our study investigated the role of endogenously produced PAI-1 in the host inflammatory response to a turpentine induced abscess, the effect of high circulating levels of PAI-1 in this model remains to be established. In order to investigate this appropriately sustained overexpression of PAI-1 has to be achieved either by using transgenic mice or

by administration of a PAI-1 expressing viral vector. Such experiments are of considerable interest and our laboratory is currently examining this important issue.

The current study reveals several novel findings on the role of PAI-1 in inflammation. We show that PAI-1 is produced at the site of inflammation after turpentine injection, where it plays a role in local inflammation by inhibition of early neutrophil influx. Furthermore, PAI-1 deficiency influences the induction of IL-6 and the subsequent acute phase protein response and delays weight loss recovery. Although our data indicate that endogenously produced PAI-1 influences the local and systemic inflammatory host response to tissue injury, the effect of PAI-1 in human inflammation remains to be established.

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Chapter 5

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

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Abstract

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.

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 non-specific 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). Interestingly, 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 (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 of pneumonia and sepsis caused by *Klebsiella (K.) 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.

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 weeks old mice were used in all experiments.

Klebsiella pneumoniae infection

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

Assays

Lung homogenates were prepared as described (29, 30). The following ELISA´s were used: D-dimer (Asserachrom D-dimer, Roche, Woerden, the Netherlands), murine PAI-1 and human PAI-1 (both Kordia, Leiden, The Netherlands), macrophage inflammatory protein (MIP)-2, keratinocyte derived chemokine (KC) (both R&D systems, Abingdon, UK), and myeolperoxidase (MPO) (Hycult biotechnology BV, Uden, The Netherlands). Tumor necrosis factor (TNF)-α, IL-6, interferon (IFN)-γ and IL-10 levels were determined using a cytometric beads array (CBA) multiplex assay (BD Biosciences, San Jose, CA). Aspartate aminotranspherase (ASAT) and alanine aminotranspherase (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). RT-PCR reactions were performed in a LightCycler (Roche) apparatus using the following conditions: 5 min 95 ^{o}C hot-start, followed by 40 cycles of amplification (95^{o}C for 15 s, 60^{o}C for 5 s, 72^{o}C for 20 s). For quantification standard curves were constructed by PCR on serial dilutions of a concentrated cDNA and data were analyzed using the LightCycler software. Gene expression is presented as a ratio of the expression of the house keeping gene β2-microglobulin (31). Primers used for murine PAI-1 were mPAI-1 S1019: ATCCTGCCTAAGTTCTCTCTG; mPAI-1 AS1164: ACCTCGATCCTGACCTTTTG. Primers for the house-keeping gene were mB2M S74 TGGTCTTTCTGGTGCTTGTCT and 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) human (NM_000602, bp 76-1109) and mouse PAI-1 (NM_008871) specific riboprobes (32).

Determination of survival and bacterial outgrowth

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

Histological 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 x200 (lungs) or x100 (liver), (2) the presence and degree of inflammation, which included the presence of interstitial influx of leukocytes, endothelialitis, oedema, pleuritis and bronchitis, and (3) for liver only: the presence and degree of necrosis. All parameters were rated from 0-3, wherein 0= absent, 1= occasionally, 2= regularly, 3= massively.

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 $P < 0.05$.

Results

Endogenous PAI-1 is upregulated 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 2.5–4.1 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 $P < 0.05$ 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 and D)**. Pulmonary PAI-1 expression was mostly co-localized 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 crosslinked fibrin clots, in lung

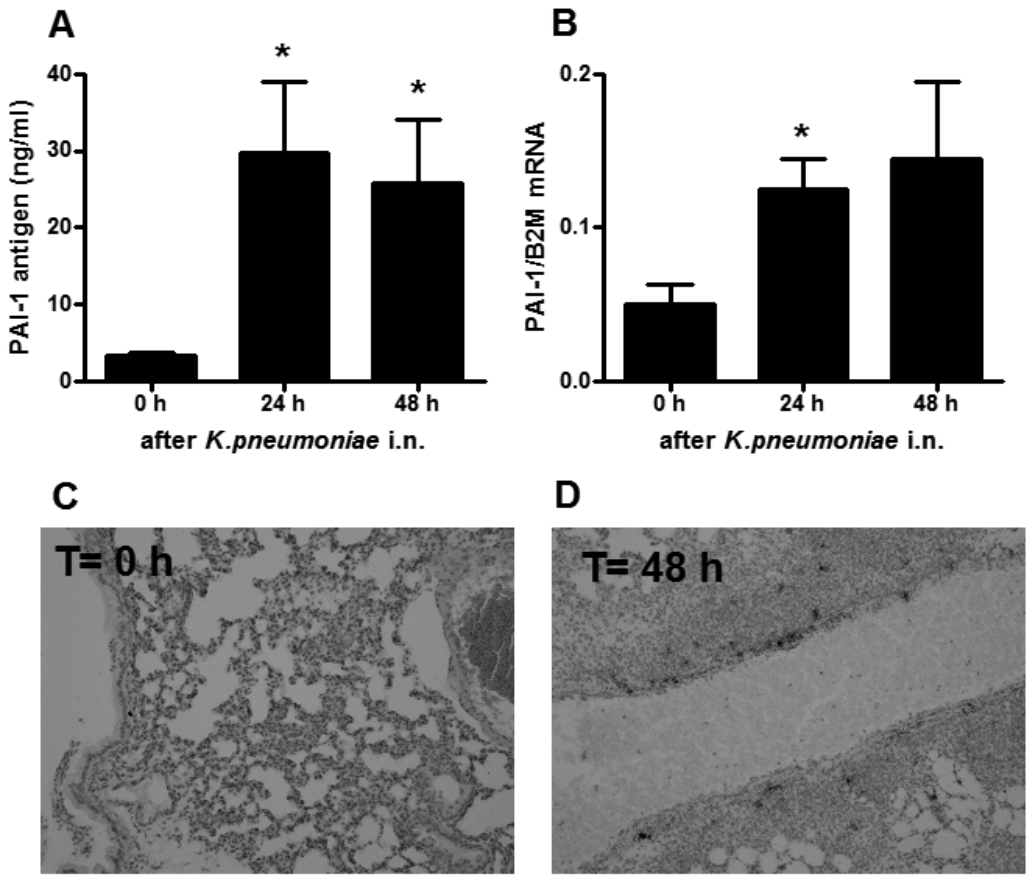

Figure 1. Endogenous PAI-1 levels increase during *Klebsiella* pneumonia. PAI-1 protein concentrations in lung homogenates (A) and mRNA levels in lung tissues (B) were determined before and at 24 and 48 hours (h) after intranasal (i.n.) administration of *K.pneumoniae*. Data are means ± SE. N= 6 per group. *P < 0.05 versus 0 h (uninfected mice). Murine PAI-1 in situ hybridization was performed on lung tissues before (C) and 48 hours after infection (D). Positive signal in black. Magnification x100.

homogenates of Wt and PAI-1$^{-/-}$ mice at 0, 24 and 48 hours after i.n. 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 i.n. 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 ($P < 0.05$). 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 and after 6 days 91 % of PAI-1$^{-/-}$ versus 55 % of Wt mice. Thus, the lack of PAI-1 rendered mice more susceptible to *K.pneumoniae* induced death.

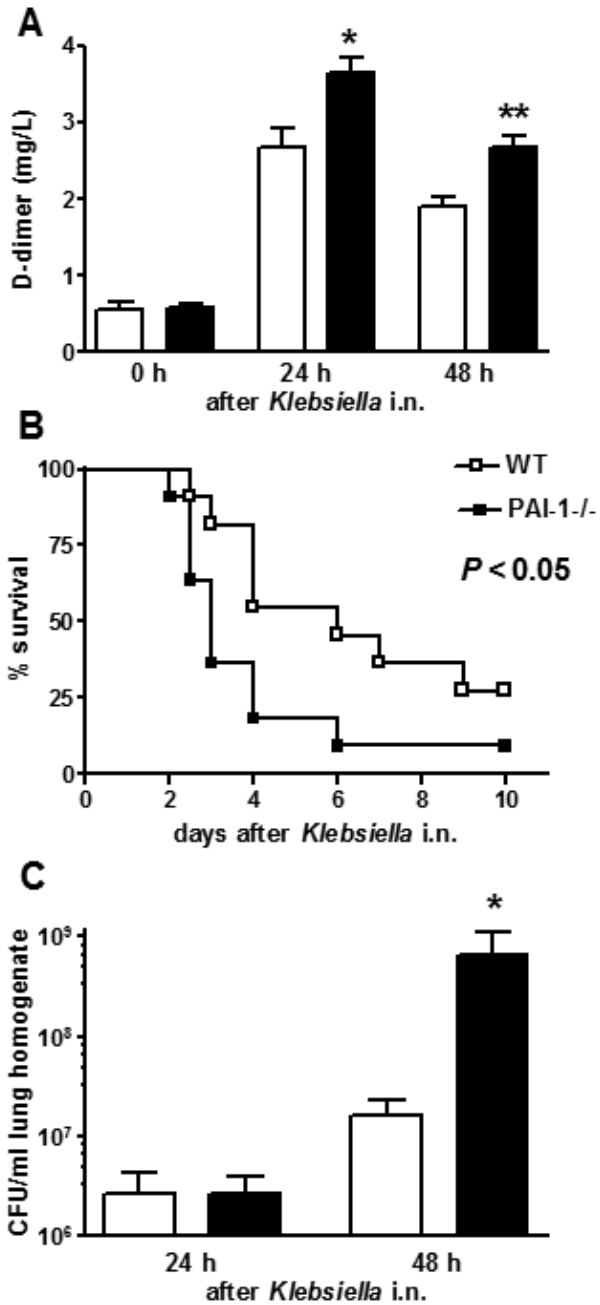

Figure 2. PAI-1 deficiency impairs host defense against *Klebsiella* pneumonia. (A-C) Wt and PAI-1-/- mice were inoculated intranasally with 10^4 CFU *K.pneumoniae*. D-dimer was measured in lung homogenates at 0, 24 and 48 hours after infection (A). Survival was monitored (B) and bacterial load was determined at 24 and 48 hours postinfection (C). Data are means ± SE of 8 mice per genotype at each time point (B + C). Survival was evaluated using 12 mice per genotype. * $P < 0.05$ versus Wt mice at the same time-point.

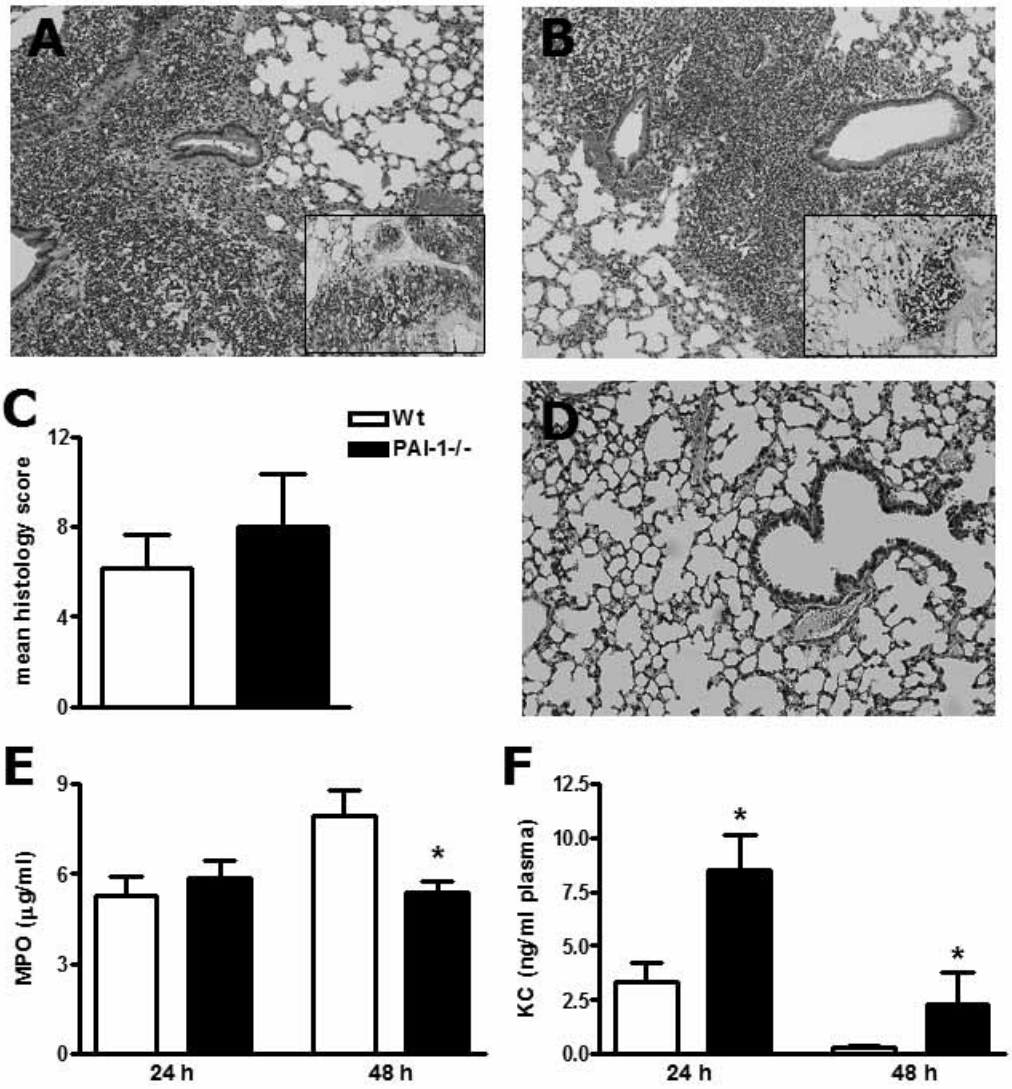

Figure 3. Influence of PAI-1 deficiency on lung inflammation during *Klebsiella* pneumonia. Representative HE stainings of lung tissue of Wt (A) and PAI-$1^{-/-}$ (B) mice at 48 hours after i.n. inoculation of 10^4 CFU *K.pneumoniae*. Original magnification x100. Insets show representative neutrophil stainings at the same time-point, original magnification x200. (C) Graphical representation of the degree of lung inflammation, determined according to the scoring system described in the Methods. 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 postinfection. Data are means ± SE. N= 8 per group at each time point. * $P < 0.05$ versus Wt mice at the same time-point.

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 (*i.e.* 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 to Wt mice ($P < 0.01$; **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 to 40 % of the Wt mice; at 48 hours, all PAI-1$^{-/-}$ mice were bacteremic compared to 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 h: 1.45 $\pm$ 0.89 x 10^3 CFU/ml versus 12.46 $\pm$ 3.55 x 10^3 CFU/ml, respectively, P <0.05; 48h: 2.14 $\pm$ 2.12 x 10^7 CFU/mL versus 3.48 $\pm$ 2.46 x 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 semi-quantitative analyses of lung histological 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 and 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 less infiltrating neutrophils as visualized by Ly-6 staining **(Figure 3a and 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 postinfection 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 ($P < 0.05$, **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 postinfection. 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 ($P < 0.05$). 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.

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 lead to distant organ injury. Therefore, we performed histopathological 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 for 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.

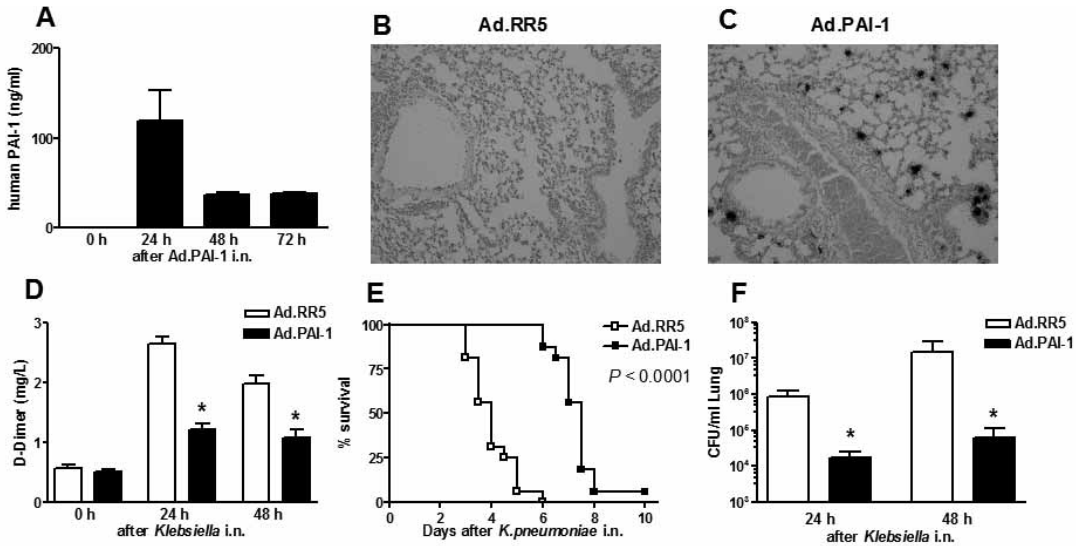

Figure 4. Transgenic PAI-1 expression improves host defense against *Klebsiella* pneumonia. (A) Wt mice were intranasally inoculated with 5x10^8 PFU Ad.PAI-1 and human PAI-1 levels were measured in lung homogenates after 0 and 24, 48 and 72 hours. Human PAI-1 in situ hybridization (B-C) showed a strong expression of PAI-1 mRNA in murine lung tissues 24 hours after Ad.PAI-1 i.n. (C) but not after Ad.RR5 (B). Positive signal in black. Magnification x100. (D-F) *Klebsiella pneumonia* was induced in Wt mice at 24 hours after i.n. inoculation with 5x10^8 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 postinfection (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 < 0.05 versus Ad.RR5 treated mice at the same time-point.

Transgenic over-expression 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 an adenoviral vector expressing human PAI-1 cDNA (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 i.n. administration of Ad.PAI-1 lead 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 hybridisation was performed on lung tissues obtained 24 hours after i.n. 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 i.n. administration of Ad.PAI-1 or Ad.RR5 24 hours prior to i.n. infection with *K. pneumoniae*. Mice treated with Ad.PAI-1 showed strongly reduced D-dimer levels during pneumonia compared to 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 ($P < 0.0001$ versus mice treated with Ad.RR5; **Figure 4E**). Whereas control mice started dying after 3 days and all had died by day 6, mice that had received Ad.PAI-1 did not show clear signs of illness up to 6 days postinfection with the first deaths occurring shortly thereafter. Furthermore, anti-bacterial defense was clearly improved in Ad.PAI-1 treated mice, as shown by 100-fold lower bacterial loads as compared to Ad.RR5 treated mice at both 24 and 48 hours after induction of pneumonia ($P < 0.001$ 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 to 75% of Ad.RR5 treated control mice.

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

Upon histopathologic examination at 48 h 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 to Ad.RR5 treated mice **(Figure 5B)**. In accordance, the total histopathology score of the lungs (quantified according to the scoring system described in the Methods section) was significantly reduced in the Ad.PAI-1 treated mice ($P < 0.01$; **Figure 5C**). Ly-6 stainings of lung tissue slides revealed a strongly reduced

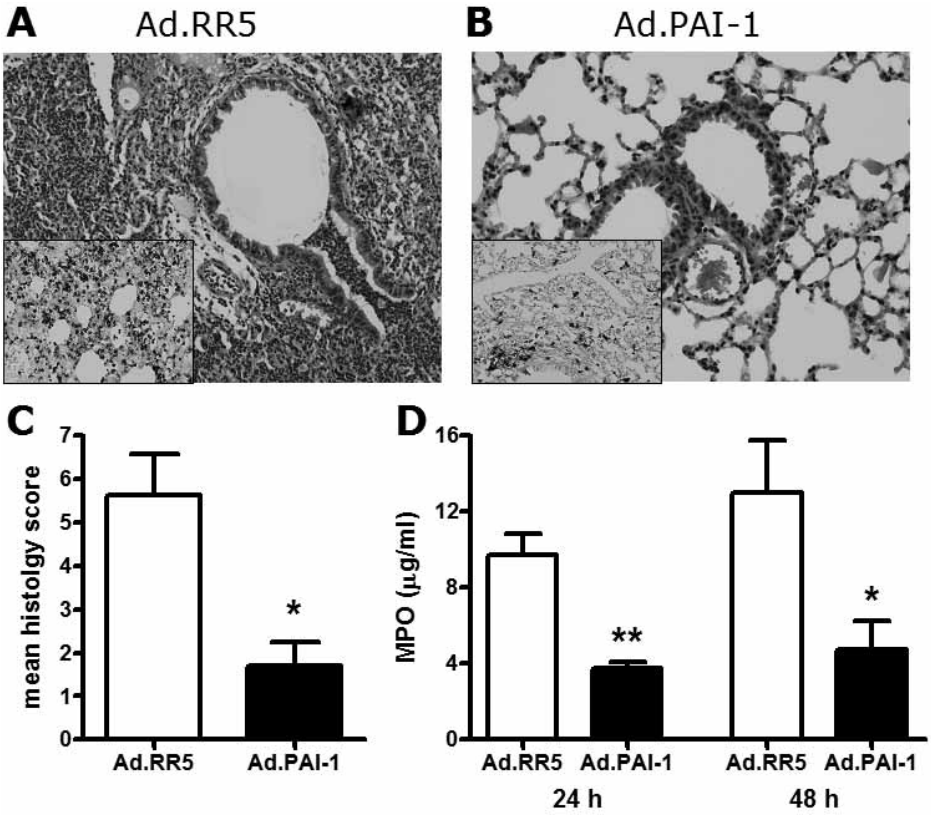

Figure 5. Transgenic PAI-1 expression reduces lung inflammation during *Klebsiella* pneumonia. Representative HE stainings of lung (A-B) tissue at 48 hours after i.n. inoculation of 10^4 CFU *K.pneumoniae*. Mice were pretreated with $5x10^8$ PFU Ad.PAI-1 or Ad.RR5 intranasally at 24 hours before infection. Magnification x200. Insets show representative neutrophil stainings of the same time-point, magnification x 200. (C) Graphical representation of the degree of lung inflammation, determined according to the scoring system described in the Methods. (D) MPO levels were determined at 24 and 48 hours postinfection. Data are means ± SE. N= 8 per group at each time point. * $P < 0.01$, **$P < 0.001$ versus Ad.RR5 treated mice at the same time-point.

influx of neutrophils in Ad.PAI-1 treated mice when compared with Ad.RR5 administered control mice (**Figure 5A and B**, insets). In line, mice that had received Ad.PAI-1 showed strongly reduced MPO levels in lung homogenates at 24 and 48 hours after infection with *Klebsiella* ($P < 0.05$ 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).**

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 histological examination at 48 hours after bacterial infection **(Figure 6A and B).** 75% of Ad.RR5 treated mice showed profound liver injury, as characterized by inflammation

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 h after intranasal inoculation of 10^4 CFU K.*pneumoniae*. Mice were inoculated with $5x10^8$ PFU of Ad.RR5 or Ad.PAI-1 at 24 hours before infection. Eight mice per group were used at each time point. *P <0.05, **P <0.001 versus Ad.RR5 mice at the same time-point.

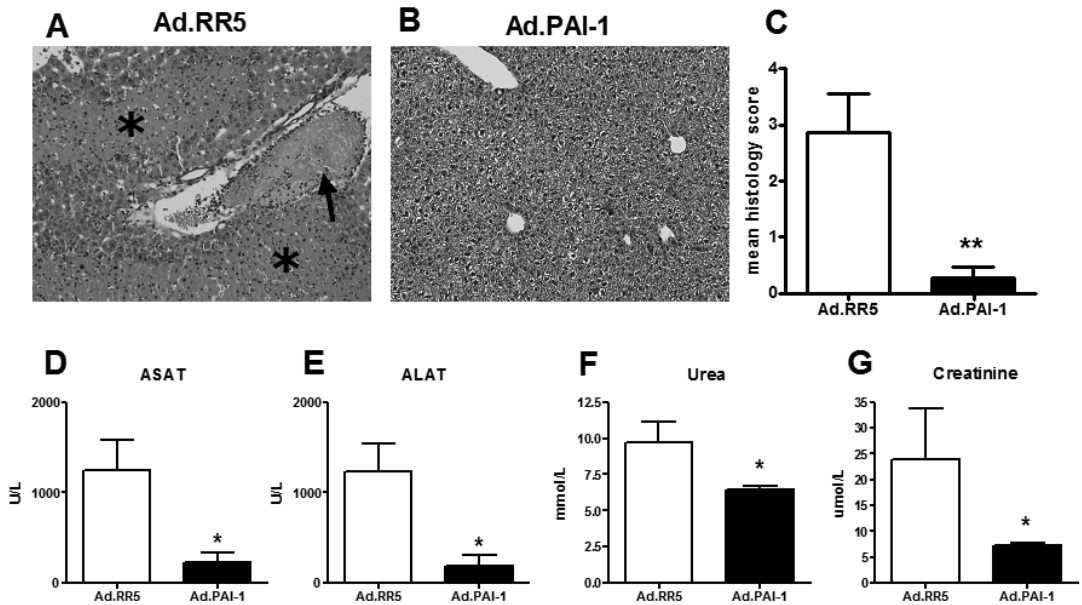

Figure 6. Transgenic PAI-1 expression reduces distant organ injury during *Klebsiella* pneumonia. Representative HE stainings of liver (A-B) tissue at 48 hours after i.n. inoculation of 10^4 CFU *K.pneumoniae*. Mice were pretreated with $5x10^8$ PFU Ad.PAI-1 or Ad.RR5 intranasally at 24 hours before infection. Magnification x200. Livers of Ad.RR5 injected mice (A) showed inflammation, necrosis (*) and thrombi (arrow). (C) Graphical representation of the degree of liver damage at 48 hours postinfection, determined according to the scoring system described in the Methods. ASAT (D), ALAT (E), urea (F) and creatinine (G) were measured in plasma at the same time-point. Data are means ± SE of eight mice per genotype. *P <0.05, **P < 0.01 versus Ad.RR5 treated mice.

Table 2. PAI-1 induces pro-inflammatory 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 h after intranasal inoculation of 5x108 PFU of Ad.RR5 or Ad.PAI-1. Eight mice per group were used. *P<0.001 versus Ad.RR5 mice.

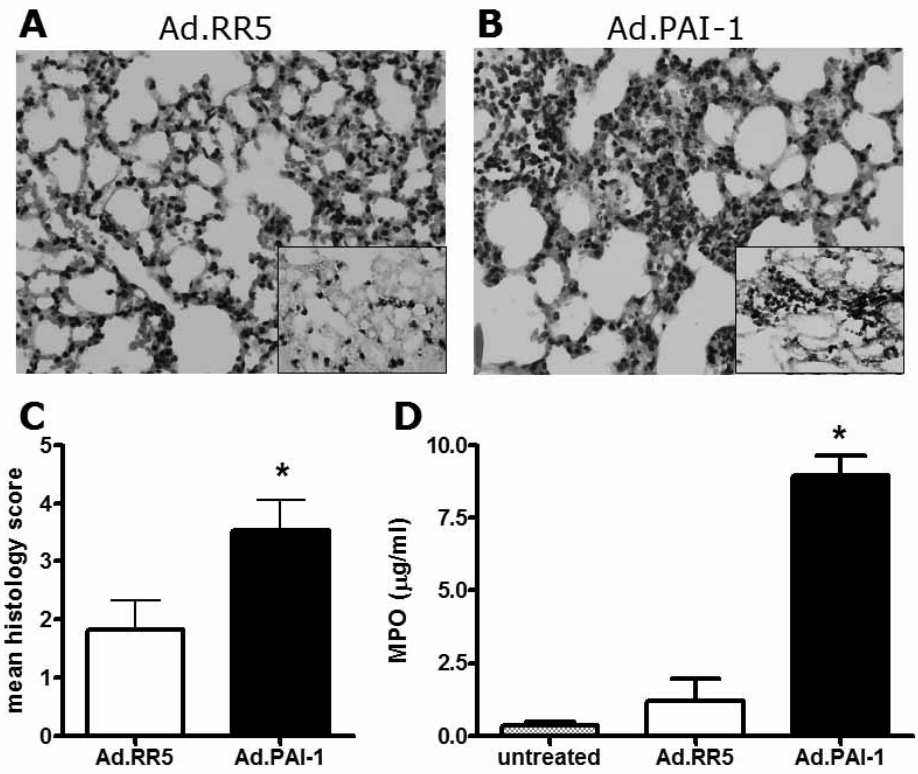

Figure 7. Transgenic PAI-1 expression induces inflammation in healthy lungs. Representative HE stainings of lung tissue at 24 hours after i.n. inoculation of healthy mice with $5x10^8$ PFU Ad.RR5 (A) or Ad.PAI-1 (B). Original magnification x200. Insets show representative neutrophil stainings of the same time-point, original magnification x 400 (C) Graphical representation of the degree of lung inflammation, determined according to the scoring system described in the Methods. (D) MPO levels. Data are means ± SE. N= 8 per group. * P < 0.01 versus Ad.RR5 treated mice.

(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, the total liver histopathology score of Ad.PAI-1 treated mice was significantly lower than that in Ad.RR5 treated mice ($P < 0.01$; **Figure 6C**). These differences in liver injury found upon histopathological examination were confirmed by the plasma concentrations of ALAT and ASAT, which were much lower in transgenic PAI-1 expressing mice (both $P < 0.05$; **Figure 6D and 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 postinfection. Both urea and creatinine levels were lower in Ad.PAI-1 treated mice (both $P < 0.05$; **Figure 6F and 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 similar effects as 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, $P < 0.01$). 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 i.n. administration of Ad.RR5 or Ad.PAI-1, at which time-point mice were infected with *K.pneumoniae* in the experiments described above. 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 and B)** and confirmed by histopathologic scores ($P < 0.05$ 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 and B, insets)** and lung MPO levels ($P < 0.001$ 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 to Ad.RR5 administered control mice (all $P < 0.001$, **Table 2**). IL-10 and MIP-2 levels were not different. Together, these data show that elevated PAI-1 levels exert a pro-inflammatory 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 upregulated 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 upregulation 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 to survivors (4). Furthermore, in sepsis patients increased PAI-1 activity predicts lethality in a very sensitive manner (6-11). In line, 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 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, 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 was most likely the result of the impaired inflammatory response discussed above. 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** and **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 = 0h) are transient and have disappeared at 24/48 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 Figure 2B and 4E are somewhat different. Several issues deserve attention in this respect. The control groups used in these studies differed in that they either received 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 mouse than in man. Therefore, with respect to transgene delivery of human PAI-1 our data should be interpreted with caution. In addition, the effect 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 upregulated 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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Chapter 6

Transgenic tissue-type plasminogen activator expression protects during *Klebsiella* pneumonia

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Abstract

Severe pneumonia is associated with a local inhibition of fibrinolysis in the lung as reflected by strongly reduced pulmonary plasminogen activator activity. We studied the effect of elevation of local plasminogen activator activity during pneumonia caused by the common respiratory pathogen *Klebsiella pneumoniae*. Female C56Bl/6 mice were inoculated intranasally with a replication-defective adenoviral vector expressing human tissue-type plasminogen activator or a control vector 24 hours before intranasal infection with *Klebsiella pneumoniae*. Mice infected with *Klebsiella* via the airways developed overt pneumonia, which was accompanied by a downregulation of pulmonary tissue-type plasminogen activator levels at protein and mRNA levels. Pulmonary overexpression of human tissue-type plasminogen activator resulted in increased fibrinolytic activity in the lungs during pneumonia, as indicated by higher D-dimer levels and reduced fibrin deposition. Interestingly, overexpression of tissue-type plasminogen activator markedly improved host defense against pneumonia: mice treated with the tissue-type plasminogen activator vector displayed a reduced mortality, less bacterial growth and dissemination and attenuated distant organ injury. Furthermore, although chemokine levels were similar in both groups, tissue-type plasminogen activator overexpression was associated with an earlier inflammatory response in the lungs. In conclusion, these data demonstrate for the first time that local elevation of plasminogen activator activity in the lungs improves host defense against severe Gram-negative pneumonia and sepsis.

Introduction

Gram-negative pneumonia is a common and serious illness that is a major cause of morbidity and mortality in humans. *Klebsiella pneumoniae* is a frequently isolated causative pathogen in lower respiratory tract infection (1-3). The increasing microbial resistance to antibiotics resulting in therapy failure and higher mortality rates is an issue of major concern (1). Therefore it is important to gain more insight into the pathogenesis of pneumonia.

Tissue-type plasminogen activator (tPA) is one of the main activators of the fibrinolytic system. tPA exerts its pro-fibrinolytic effect by the conversion of plasminogen into the active protease plasmin, the key enzyme of the fibrinolytic system. Patients with severe pneumonia display profound changes in local fibrinolytic activity, characterized by a reduced PA activity in bronchoalveolar lavage fluid (BALF) primarily due to elevated PA inhibitor type I (PAI-1) levels (4-6). In line, intrapulmonary delivery of lipopolysaccharide (LPS), which is part of the outer membrane of Gram-negative bacteria, also reduced PA activity and increased PAI-1 concentrations in BALF of healthy volunteers (7-9). Hence, ample evidence indicates that the presence of bacteria or bacterial products in the alveolar space results in suppression of local PA activity. The functional consequences hereof during pneumonia are unknown. In theory, alterations in fibrinolytic activity in the lung could impact on multiple processes important for a balanced response to respiratory pathogens. In particular, mediators of the fibrinolytic system have biological functions that go beyond the lysis of fibrin per se, including degradation of extracellular matrix components, tissue remodeling and cell migration (10-12).

The primary objective of the present study was to determine the effect of elevation of local PA activity in the pulmonary compartment of mice suffering from severe pneumonia caused by *K. pneumoniae*. For this we induced transiently enhanced expression of tissue type PA (tPA) in the lungs of mice by intranasal administration of a replication-defective adenoviral vector expressing human tPA. We here report that enhanced pulmonary expression of tPA strongly improved host defense against severe *Klebsiella* pneumonia.

Materials and Methods

Animals

Female 8 to 10 weeks old C57BL/6 WT mice were obtained from Harlan Sprague-Dawley (Horst, The Netherlands). $tPA^{-/-}$ mice on a C57BL/6 genetic background were obtained from the Jackson Laboratory (Bar Harbor, ME). The Institutional Animal Care and Use Committee approved all experiments.

Induction of pneumonia and sample harvesting

Pneumonia was induced as described before (13-15). *K. pneumoniae* serotype 2 (ATCC 43816; American Type Culture Collection, Manassas, VA) was cultured for 16 h at 37°C in 5% CO_2 in tryptic soy broth (TSH, Difco, Detroit, MI). This suspension was diluted 1:100 in fresh medium and grown for 3 h to midlogarithmic phase. Bacteria were harvested

by centrifugation at 1,500 x *g* for 15 min, washed twice in sterile 0.9% saline and resuspended in saline. The number of colony forming units (CFU) was determined by plating 10-fold dilutions of the suspensions on blood agar plates. After preparation of the bacterial inoculum, mice were lightly anesthetized by inhalation of isoflurane (Upjohn, Ede, The Netherlands) and 50 µl (1 x 10^4 CFU) of the bacterial suspension was inoculated intranasally. Uninfected mice (controls) and mice infected with *K. pneumoniae* 24 or 48 hours (h) earlier were anesthetized by the intraperitoneal administration of FFM (fentanyl citrate [0.079 mg/ml], fluanisone [2.5 mg/ml], midazolam [1.25 mg/ml in H_2O]) (7.0 ml/kg of body weight) and sacrificed by bleeding out the vena cava inferior. Blood was collected in heparin-containing tubes and whole lungs and livers were harvested. Lung homogenates were diluted 1:2 in lysis buffer (150 mM NaCl, 15 mM Tris, 1 mM $MgCl.H_2O$, 1 mM $CaCl_2$, 1% Triton X-100, 100 µg of pepstatin A/ml, leupeptin, aprotinin, pH 7.4) and incubated at 4°C for 30 min. Homogenates were centrifuged at 1,500 x *g* for 15 min, after which the supernatants were stored at -20°C until further use.

Assays

The following ELISA´s were used: mouse tPA (Molecular Innovations, Inc. Southfield, MI), human tPA (TintElize, Biopool, Sweden), thrombin-antithrombin complexes (TATc) (Dade Behring, Marburg, Germany), D-dimer (Asserachrom D-dimer, Roche, Woerden, the Netherlands), macrophage inflammatory protein (MIP)-2 and keratinocyte derived chemokine (KC) (both R&D Systems, Abingdon, UK). Human tPA activity and PA activity were measured by amidolytic assays (16). Tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-10 levels were determined using a cytometric beads array (CBA) multiplex assay (BDBiosciences, San Jose, CA) in accordance with the manufacturer's recommendations. Aspartate aminotranspherase (ASAT), alanine aminotranspherase (ALAT), urea and creatinine were determined with commercially available kits (Sigma, St. Louis, MO), using a Hittachi analyzer (Boehringer Mannheim, Mannheim, Germany).

Evaluation of lung tPA mRNA levels by quantitative RT-PCR.

Total RNA was isolated using the RNeasy Mini Kit system (Qiagen Venlo, the Netherlands) and RT-PCR was performed as described previously (17).

Adenovirus-mediated tPA gene transfer

The replication-defective adenoviral vector expressing human tPA (Ad.tPA) was generated by homologous recombination in 293 cells. After transfection, recombinant viral plaques were harvested and amplified, and large-scale production of recombinant adenovirus was performed. Mice were lightly anesthesized by inhalation of isoflurane (Upjohn), and 5×10^8 plaque-forming units (PFU) of either Ad.tPA or Ad.RR5 (control adenovirus) diluted in sterile saline to a final volume of 50 µL was inoculated intranasally. This dose was based on previous studies using adenoviral vector gene transfer to the lungs of mice. For kinetic studies of tPA after gene transfer, mice were sacrificed at 4, 24 and 72 hours after intranasal inoculation of Ad.tPA. For BAL the trachea was exposed by a midline incision and cannulated by use of a sterile 22-gauge Abbocath-T catheter (Abbott, Sligo, Ireland). BAL was performed by instilling two 0.5-ml aliquots of sterile saline. The supernatant was frozen at –20°C until tPA measurements.

Determination of survival and bacterial outgrowth

For survival studies mice (N = 12 per group) were monitored every 12 h up to 10 days after infection. In separate mice, lungs and blood were collected as described above at 24 or 48h after infection. Bacterial outgrowth was determined as described before (15).

Histologic examination

Lungs for histologic examination were harvested 24 h after infection, fixed in 4% formaldehyde and embedded in paraffin. Sections (4 µm thick) were stained with hematoxylin-eosin and analyzed by a pathologist who was blinded for group identity. To score lung inflammation and damage, the lung samples were screened for the following parameters: interstitial inflammation, intra-alveolar inflammation, edema, endothelialitis, bronchitis, pleuritis, and thrombi formation. Each parameter was graded on a scale of 0 to 3 (0, absent; 1, mild; 2, moderate; and 3, severe). Livers were scored according to the following parameters: number of thrombi, presence and degree of inflammation and presence and degree of necrosis. Each parameter was graded on a scale of 0 to 3 (0, absent; 1, mild; 2, moderate; and 3, severe). The total histology score was expressed as the sum of the score for all parameters. Granulocyte (18, 19) and fibrin (13) stainings were performed as described previously.

Effect of rtPA on the growth and killing of *K. pneumoniae*

The direct effect of recombinant human (rhu) tPA on the growth of *K. pneumoniae* was investigated by adding rhu-tPA to the growth medium. 100 µl of 1 x 105 CFU/ml *K.pneumoniae* was added to 9,4 ml TSH medium. 500 µl of tPA (Molecular Innovations, the Netherlands; final concentration of 100 µg/ml) in NaCl 0,9% or 500 ul NaCl 0,9% alone were also added to the medium and the medium was incubated at at 37°C in 5% CO_2 for 2 and 4 hours. The bacteria were washed and plated out as described above. Bacterial killing was determined according to a protocol published recently (20).

Statistical analysis

Data are expressed as mean ± SE. Statistics were performed as described before (15). Differences were compared using the Mann-Whitney *U* test or one-way analysis of variance where appropriate. Survival was compared by Kaplan-Meier analysis followed by a log rank test. $P < .05$ was considered to be significant.

Results

Endogenous tPA during *K. pneumoniae* pneumonia

To obtain insight into local tPA concentrations in the lungs, we measured tPA protein levels in lung homogenates of uninfected WT mice and WT mice after infection with *K. pneumoniae*. tPA was detected at levels of 30 - 41 ng/ml in lung homogenates of uninfected mice. *K. pneumoniae* pneumonia was associated with a significant decrease in lung tPA levels at 24 and 48 hours after infection ($P < 0.05$ and $P < 0.01$ versus control respectively; **figure 1A**). This reduction of pulmonary tPA protein levels during *K. pneumoniae* pneumonia was associated with a downregulation of tPA mRNA expression in lung tissue, as measured by RT-PCR **(Figure 1B)**.

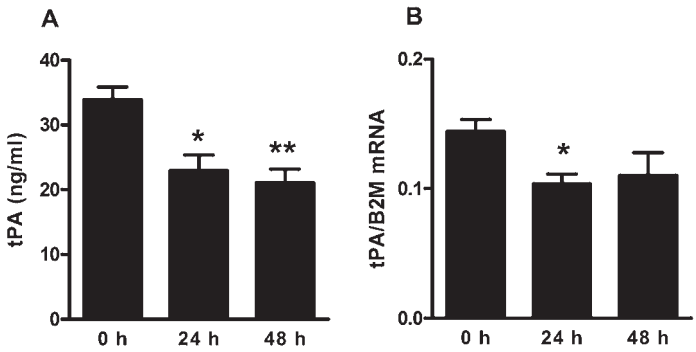

Figure 1. Endogenous tPA during *K. pneumoniae* pneumonia. (A-B) Mice were sacrificed before and at 24 and 48 hours (h) after intranasal administration of *K.pneumoniae*. tPA protein concentrations were measured in lung homogenates by ELISA and tPA mRNA expression was determined in lung tissues by quantitative RT-PCR. Data are means ± SE. N= 5 per group. **P* < 0.05, ***P* < 0.01 versus 0 h (uninfected mice). Statistics by one-way analysis of variance.

Adenoviral-mediated tPA gene transfer to the lungs

In order to increase local PA activity in the lung we used a replication-defective adenoviral vector expressing human tPA cDNA (Ad.tPA), which previously has been used successfully to enhance systemic tPA expression after intravenous or intraperitoneal administration (21). Considering that intrapulmonary delivery of adenoviral vectors has been demonstrated to result in increased expression of various transgenes in the respiratory tract (22-25), we here examined whether intranasal administration of Ad.tPA lead to expression of human tPA in the lungs. Thus, we intranasally inoculated mice with 5×10^8 PFU Ad.tPA and measured tPA protein levels in lung homogenates, BALF and plasma before and at 4, 24 and 72 hours thereafter. We found a strong rise of tPA protein levels in both lung homogenates and BALF with peak levels after 24 hours **(figure 2A-B)**. In line, tPA and PA activity in BALF were significantly increased at 24 hours after Ad.tPA administration **(Figure 2C-D)**. Therefore, we used this time-point to infect mice with *K. pneumoniae* in all subsequent experiments. In plasma human tPA levels were undetectable at all time-points.

Transgenic expression of tPA protects against *K. pneumoniae*-induced pneumonia

To investigate the effect of local tPA overexpression on *K. pneumoniae* pneumonia induced mortality mice received Ad.tPA or Ad.RR5 (control) intranasally 24 hours before infection with *K. pneumoniae* and were followed for 30 days. The first deaths among ad.RR5 treated control mice occurred 4 days after infection and overall mortality was 75% **(figure 3A)**. In contrast, mortality among mice treated with Ad.tPA did not occur until 8 days postinfection and overall mortality was only 33% (*P* < 0.05 versus control mice). Hence, enhanced expression of tPA in the pulmonary compartment protected mice from death due to *Klebsiella* pneumonia. To investigate whether the reduced mortality of Ad.tPA treated mice was associated with changes in bacterial outgrowth, we compared the bacterial loads in lungs and blood of Ad.tPA and Ad.RR5 treated mice at 24 and 48 hours after infection with *K. pneumoniae*. Mice that had received Ad.tPA had a reduced bacterial load in their lungs at

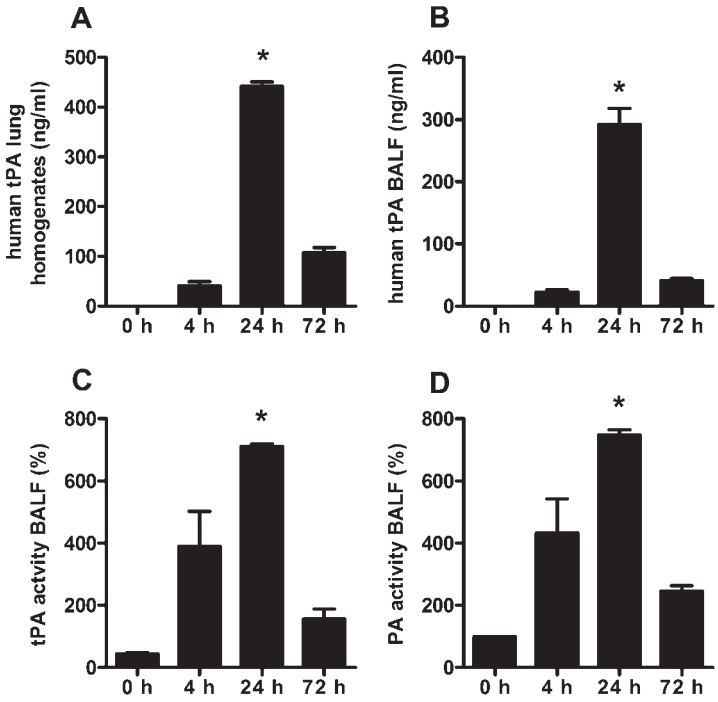

Figure 2. tPA gene transfer to the lungs. WT mice were sacrificed before and at 4, 24 and 72 hours after intranasal inoculation with 5×10^8 PFU of Ad.tPA. Human tPA protein levels were measured in lung tissue homogenates (A) and bronchoalveolar lavage fluid (BALF) (B). Human tPA (C) and PA (D) activity was measured in BALF. N= 4 per group. * $P < 0.05$ versus 0 h. Statistics by one-way analysis of variance.

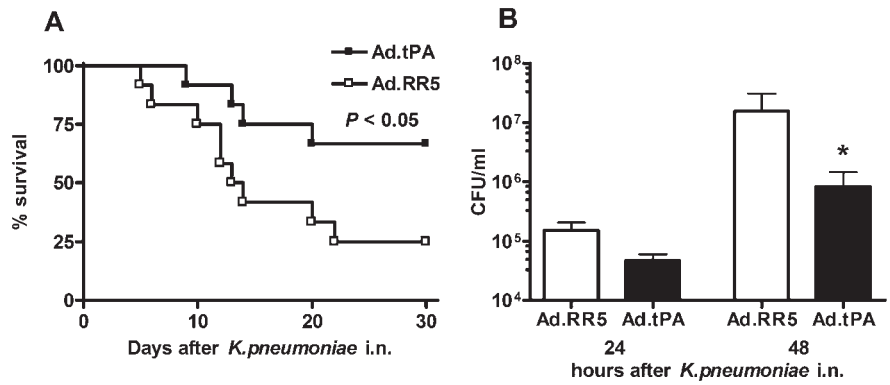

Figure 3. tPA gene transfer protects against *K. pneumoniae* pneumonia. Mice were inoculated with 10^4 CFU *K. pneumoniae* at 24 hours after administration of Ad.tPA or Ad.RR5 and survival was monitored of 12 mice per group (B). *K. pneumoniae* CFU were determined in lungs at 24 and 48 hours postinfection (C). N = 8-10 mice per group per time-point. Data are means ± SE. * $P< 0.05$ compared to Ad.RR5 at the same time-point.

24 and 48 hours postinfection compared to the control treated mice (P<0.05 at T= 48 hours; **figure 3B**). Furthermore, in control mice bacteremia occurred in 25% at 24 hours and 75% at 48 hours postinfection. In contrast, none of the mice overexpressing tPA showed positive blood cultures after 24 hours and only 25% was bacteremic at 48 hours postinfection. Thus, enhanced pulmonary expression of tPA reduced the bacterial outgrowth in the lungs and limited dissemination of the infection.

Transgenic tPA expression increases local fibrinolysis during pneumonia

To investigate the effect of tPA overexpression on local coagulation and fibrinolytic activity we measured TATc **(Figure 4A)**, as a marker for coagulation activation, and D-dimer **(Figure 4B)**, reflecting fibrinolytic activity, in lung homogenates of Ad.tPA and Ad.RR5 treated mice before and at 24 and 48 hours after pulmonary infection with *K. pneumoniae*. In both groups TATc and D-dimer were significantly increased during pneumonia compared to baseline values. As expected, Ad.tPA administration did not affect coagulation activation (TATc). However, tPA overexpression did strongly increase local D-dimer levels at 24 and 48 hours postinfection, showing that the higher pulmonary tPA levels resulted in a higher fibrinolytic activity in the lungs during pneumonia. These results were confirmed by fibrin stainings of lung tissues obtained at 48 hours after infection from Ad.tPA and Ad.RR5

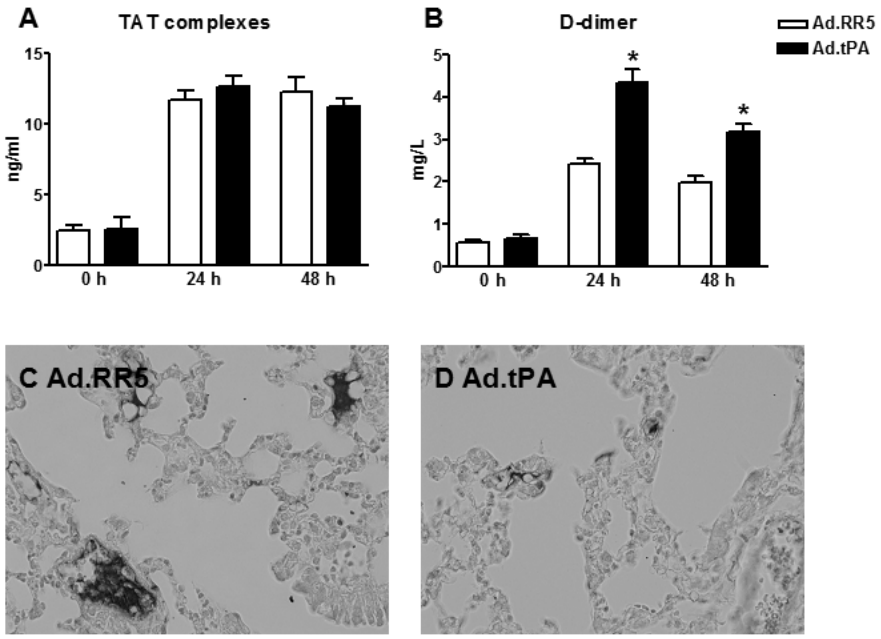

Figure 4. tPA gene transfer increases pulmonary fibrinolytic activity during pneumonia. Mice were inoculated with 10^4 CFU *K. pneumoniae* at 24 hours after administration of Ad.tPA or Ad.RR5. TATc (A) and D-dimer (B) were measured in lung homogenates before and at 24 and 48 hours (h) after infection with *K. pneumoniae* in both groups. N = 8 mice per group per time-point. Data are means ± SE. * P< 0.001 compared to Ad.RR5 at the same time-point. Fibrin stainings of lung tissues were performed at 48 after infection with *K. pneumoniae* of Ad.RR5 (C) and Ad.tPA (D) treated mice. Magnification x 400.

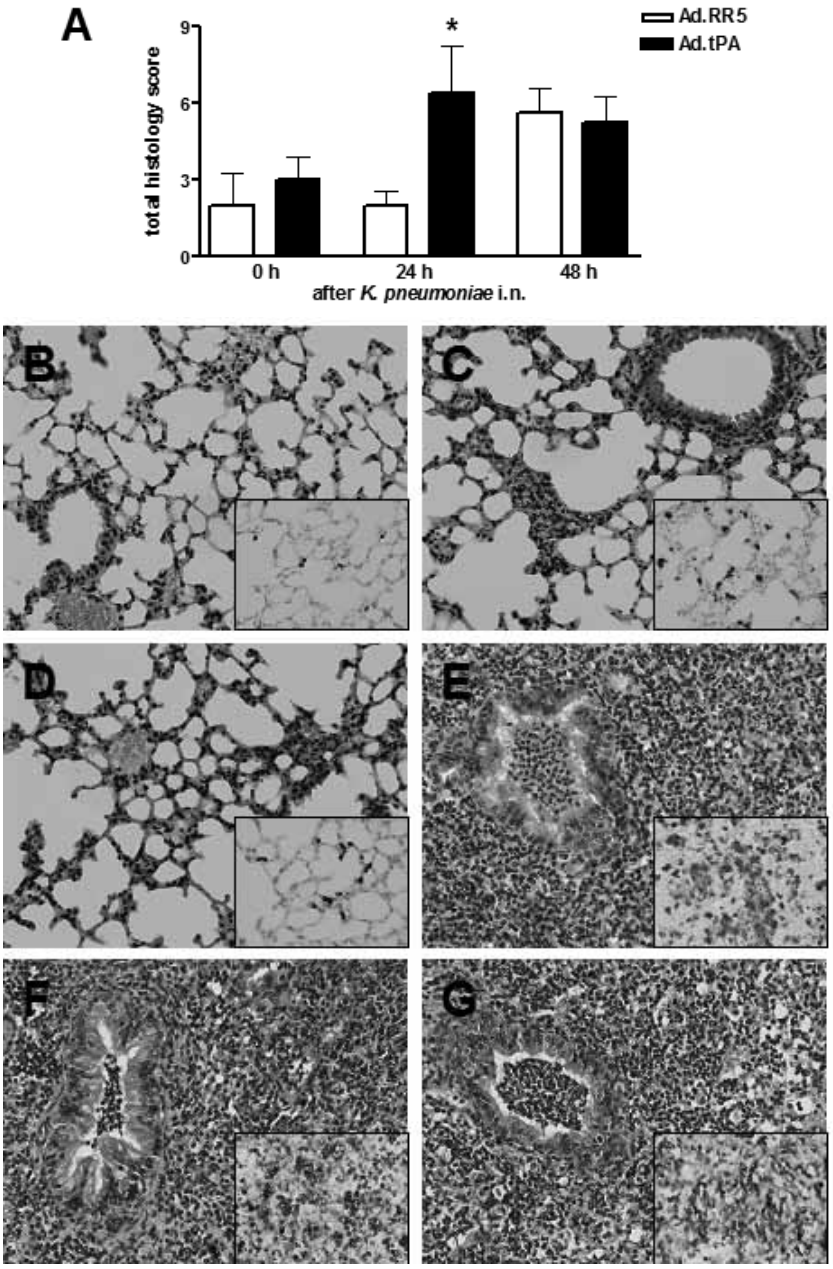

Figure 5. Lung histopathology. (A) Total histology scores of the lungs were determined in Ad.RR5 and Ad.tPA treated mice before and at 24 and 48 hours after infection with *K. pneumoniae*, as described in the Methods section. Representative HE stainings of lung tissue before (B-C), at 24 (D-E) and at 48 (F-G) hours after intranasal inoculation of *K.pneumoniae* in mice previously (–24 hours) infected with Ad.RR5 (B,D,F) or Ad.tPA (C,E,G). Data are means ± SE. N= 8-10 per group. Microscopic magnification: x200, insets x400.

administered mice **(Figure 4 C and D)**, which showed less positive staining for fibrin in the tPA overexpressing mice compared to the Ad.RR5 injected control mice.

Transgenic tPA expression results in a more rapid inflammatory response in the lungs during pneumonia

Lung inflammation, characterized by recruitment of leukocytes to the site of infection and local production of cytokines and chemokines, is an important part of the immune response during pneumonia (26-28). We therefore semi-quantitatively scored the inflammatory response in lung tissue slides obtained from mice at the time of infection with *Klebsiella* (24 hours after the intranasal administration of Ad.tPA or Ad.RR5) and 24 and 48 hours after induction of bacterial pneumonia **(Figure 5)**. At t = 0 (*i.e.* 24 hours after Ad.RR05 or Ad.tPA administration) Ad.tPA showed slightly more lung inflammation, but the difference with Ad.RR5 treated mice did not reach statistical significance **(figure 5A-C)**. However, Ad.tPA inoculation did result in increased neutrophil influx into lung tissue, as reflected by neutrophil stainings **(figure 5B and C, insets)**. The subsequent induction of *Klebsiella pneumonia* resulted in a brisk inflammatory response in the lungs of Ad.tPA treated mice, but not in Ad.RR5 treated animals **(figures 5A, D and E)**: whereas at 24 hours after infection with *K. pneumoniae*, the lungs of the Ad.RR5 treated mice hardly showed inflammation, the lungs of mice overexpressing tPA showed profound areas of pneumonia, interstitial inflammation, endothelialitis, pleuritis and edema resulting in a significantly higher total histology score **(Figure 5A, D and E)**. In line, neutrophil stainings showed more neutrophils in the lungs of the Ad.tPA treated group **(Figure 5D and E, insets)**.

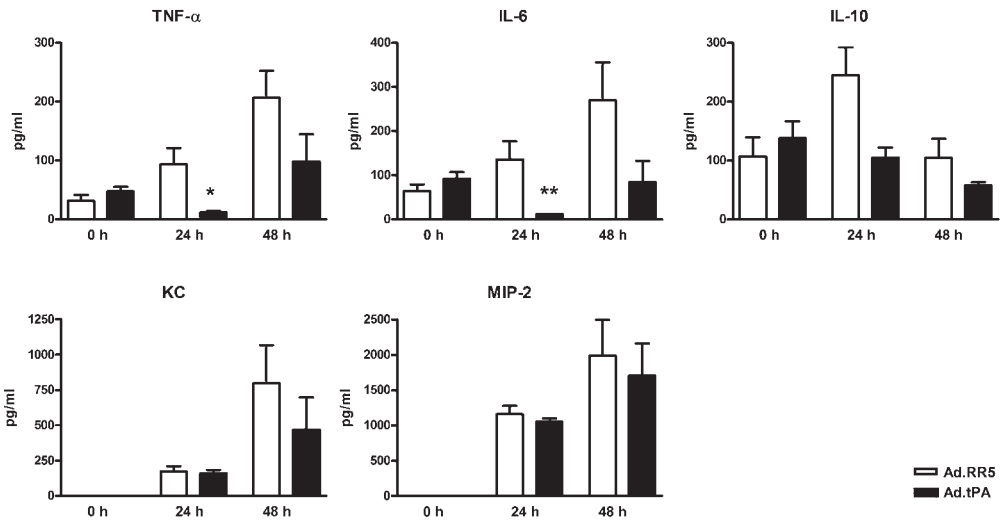

Figure 6. Effect of tPA gene transfer on cytokine and chemokine levels. TNF-α, IL-6, IL-10, KC and MIP-2 levels were measured in lung homogenates at 0, 24 and 48 hours after inoculation of *K.pneumoniae* in mice previously (– 24 hours) infected with Ad.RR5 or Ad.tPA. Data are means ± SE. N= 8 per group. **P*<0.05, ***P*<0.001 versus Ad.RR5.

At 48 hours postinfection, the Ad.RR5 group also displayed areas of pneumonia and the difference in total histological inflammation scores between both strains had disappeared **(Figure 5A and figure 6 E and F)**. In addition, neutrophil stainings showed a similar accumulation of neutrophils in the lungs of both groups at 48 hours after inoculation with *K. pneumoniae* **(Figure 6 E and F, insets)**. Hence, Ad.tPA treatment resulted in a faster inflammatory reaction in the lungs in the first 24 hours after infection with *Klebsiella*. Next, we measured cytokine and chemokine levels in lung tissues harvested before and at 24 and 48 hours after infection with *K. pneumoniae* **(Figure 6)**. Directly before infection with *Klebsiella* (i.e. 24 hours after administration of Ad.RR5 or Ad.tPA) cytokine and chemokine levels did not differ between groups. In Ad.RR5 treated mice, all cytokines increased during *K. pneumoniae* infection. In contrast, TNF-α, IL-6 and IL-10 levels were not increased in tPA overexpressing mice at 24 hours after bacterial infection and TNF-α and IL-6 levels were significantly lower than in infected Ad.RR5 treated mice. At 48 hours after infection there were no significant differences between both groups anymore. The murine neutrophil-attracting chemokines MIP-2 and KC were also elevated during *K. pneumoniae* pneumonia; the concentrations of these chemokines were similar in both groups. Hence, tPA gene transfer resulted in a reduced pulmonary cytokine, but not chemokine, response during *Klebsiella* pneumonia.

Transgenic tPA expression reduces distant organ damage

Having established that Ad.tPA treated mice were protected against lethality together with a reduced local bacterial outgrowth and dissemination of the infection, we determined whether the pulmonary tPA gene transfer also protected against distant organ injury. We previously established that *K. pneumoniae* pneumonia is associated with clear evidence of liver and kidney dysfunction (15). Therefore, we performed histopathological analyses of liver tissue and evaluated liver injury and kidney function by clinical chemistry at 48 hours after infection with *K. pneumoniae* in both Ad.tPA and Ad.RR5 treated mice. Upon histopatologic examination livers showed clear signs of inflammation, as reflected by accumulation of leukocytes in the interstitium, in 75% of Ad.RR5 treated mice and 37.5% of Ad.tPA treated mice **(figure 7A and B)**. tPA overexpression was also associated with reduced thrombi formation in the livers (12.5% showed thrombi in 5 random microscopic fields compared to 50% of the control mice), together with less liver necrosis (25% of Ad.tPA treated mice showed liver necrosis versus 75% of control mice; **figure 7A and B**). The total liver histology score was lower in the Ad.tPA treated group compared to the Ad.RR5 group, but the difference was not significant ($P = 0.1$; **figure 7C**). In line with the histopathological findings, clinical chemistry showed much lower plasma levels of the liver enzymes ASAT and ALAT in mice which expressed tPA compared to controls (both $P < 0.05$; **Figure 7D**), indicating reduced hepatocellular injury. Furthermore, to evaluate the effect of tPA expression on kidney function during *Klebsiella* pneumonia we measured urea and creatinine in plasma of both groups of mice at 48 hours postinfection. Both urea and creatinine levels were significantly lower in Ad.tPA treated mice **(Figure 7E and F)**.

Recombinant tPA does not influence the growth or killing of *K. pneumoniae*

To examine whether tPA has a direct effect on the growth of *K. pneumoniae* we added rhu-tPA or vehicle to TSH medium containing *K. pneumoniae*. Rhu-tPAdid not influence

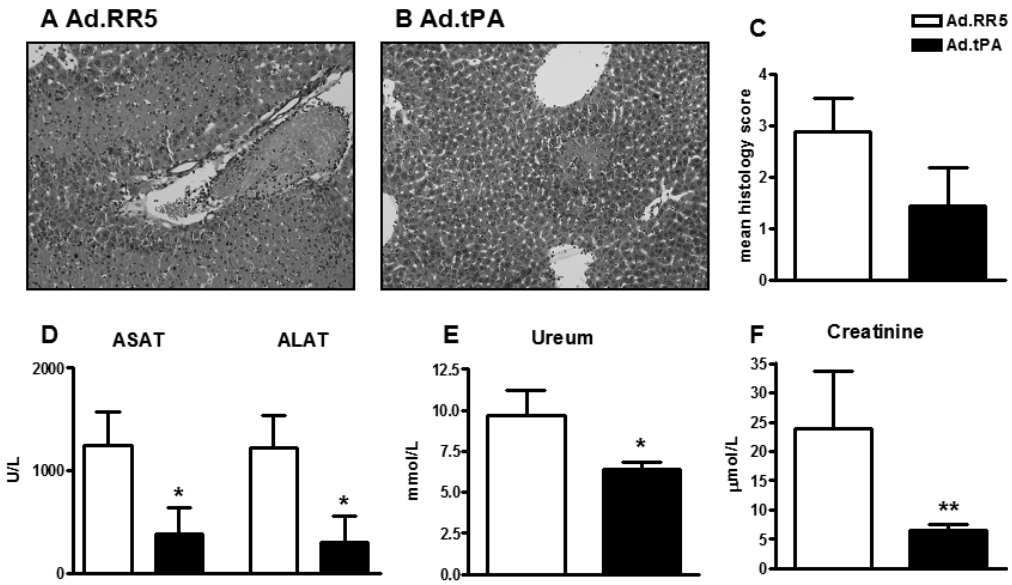

Figure 7. tPA reduces distant organ damage. Representative HE stainings of liver (A-B) tissue at 48 hours after i.n. inoculation *K.pneumoniae*. Mice were pretreated with Ad.tPA or Ad.RR5 intranasally at 24 hours before infection. Original magnification x100. (C) Graphical representation of the degree of liver damage at 48 hours postinfection, determined according to the scoring system described in the methods. ASAT and ALAT (D), urea (E) and creatinine (F) were measured in plasma at the same time-point. Data are means ± SE of eight mice per genotype. **P* <0.05, ***P* < 0.01 versus Ad.RR5 treated mice.

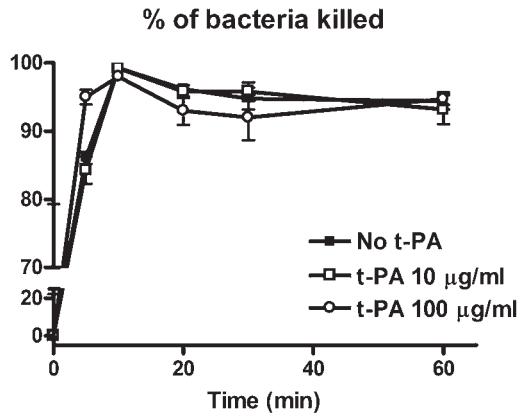

Figure 8. tPA does not influence *K. pneumoniae* killing. The effect of rhu-tPA on bacterial killing by MH-S cells was determined as described in the Methods section. Data are % of bacteria killed. N=3 per concentration per time-point.

the growth of *Klebsiella* after 2 and 4 hours of incubation (data not shown). To investigate whether tPA has an effect on the killing of bacteria by alveolar macrophages, we examined the effect of rhu-tPA on killing of *K. pneumoniae* by MH-S cells *in vitro*, determining the percentage of bacteria killed at different time-points. As shown in **figure 8**, rhu-tPA did not affect bacterial killing by MH-S alveolar macrophages.

Host defense in tPA$^{-/-}$ mice

The experiments described above established that elevation of pulmonary tPA levels enhanced host defense against *Klebsiella* pneumonia. Next, we wished to investigate whether a total lack of tPA might influence host defense against *K. pneumoniae* pneumonia. Therefore, we inoculated tPA$^{-/-}$ and Wt mice with *K. pneumoniae* and studied survival and bacterial outgrowth. Mortality did not differ between tPA$^{-/-}$ and Wt mice (91% versus 88% respectively). Moreover, the growth and dissemination of *Klebsiella* occurred equally in both mouse strains (data not shown).

Discussion

Pneumonia is associated with a reduction in the fibrinolytic activity in the bronchoalveolar space (4-6). The primary objective of the present investigation was to examine the effect of overexpression of transgenic tPA in the lung on the outcome of severe Gram-negative pneumonia. We here show that elevation of tPA levels at the primary site of the infection, accomplished by intranasal administration of a replication-defective adenoviral vector expressing human tPA, improved survival, decreased the growth of bacteria in the lungs and the subsequent dissemination of the infection, and reduced distant organ damage. Moreover, transgenic tPA induced a rapid inflammatory response to *K.pneumoniae* infection in the lungs, which probably contributed to the improved host defense. These data indicate that enhanced expression of tPA in the pulmonary compartment improves host defense against *Klebsiella* pneumonia.

Bacterial infection of the lower airways results in an inhibition of constitutively present fibrinolytic activity in the lung primarily due to enhanced local release of PAI-1 (4-6). Similar changes in pulmonary fibrinolysis were detected after inhalation or bronchial instillation of LPS in healthy humans (7-9). Our laboratory recently reported that experimental pneumonia induced in mice by either *Streptococcus pneumoniae* (29) or *Klebsiella pneumoniae* (15) elicits local production of PAI-1 at the site of the infection. We here show that healthy lungs constitutively express tPA, confirming previous reports (17, 30), and that *Klebsiella* pneumonia is accompanied by a reduction in this basal tPA production, as reflected by reduced tPA mRNA and protein levels in lung tissue of infected mice relative to uninfected mice. Together these data convincingly show that respiratory tract infection results in a reduced activity of the fibrinolytic system in the lung, which at least in the *Klebsiella* model used here is characterized by enhanced production of the major inhibitor PAI-1 (15) and a diminished production of the major agonist tPA (the present study).

Mediators of the fibrinolytic system have been implicated to play a role in various processes relevant for the pathophysiology of lung inflammation that reach beyond fibrinolysis (10). Mice deficient for the urokinase-type PA receptor (uPAR$^{-/-}$ mice) demonstrated a diminished neutrophil accumulation in their lungs when compared with WT mice, which was associated with enhanced bacterial growth during *Pseudomonas aeruginosa* (31) and *S. pneumoniae* pneumonia (18). In contrast, uPA deficiency did not impact on host defense during *Pseudomonas* pneumonia (31), whereas it improved resistance against pneumococcal pneumonia (18). Our laboratory recently investigated the role of PAI-1 in gram-positive and Gram-negative pneumonia (15, 29). Whereas PAI-1 deficiency did not influence the outcome of *S. pneumoniae* pneumonia (29), PAI-1$^{-/-}$ mice were clearly more susceptible to *Klebsiella* pneumonia, as reflected by an enhanced growth and dissemination of bacteria and a reduced survival (15). The mechanism by which PAI-1 protected mice against *Klebsiella* pneumonia likely involved a stimulating effect on neutrophil recruitment to the site of the infection and neutrophil activation (15, 32, 33). Neither the effect of exogenous tPA nor the role of endogenous tPA have, to our knowledge, been studied before in the setting of pneumonia. The main finding of our study is that sustained elevation of tPA levels in the lung induces a more rapid cellular inflammatory response and limits the bacterial load during *Klebsiella* pneumonia. Transgenic overexpression of tPA already resulted in a modest proinflammatory response in the lungs of healthy mice, as reflected by histopathology scores and neutrophil stainings at 24 hours after intranasal administration of Ad.tPA. At 24 hours after bacterial infection this difference in inflammatory response was much more pronounced and the mice overexpressing tPA showed significantly higher histology scores compared to mice treated with the empty vector Ad.RR5. Hence, in the beginning of the infection with *K. pneumoniae* more neutrophils were present in lung tissue of mice treated with Ad.tPA, which considering that neutrophils are an important part of host defense against pneumonia (27, 28), may at least in part explain the protection provided by transgenic tPA expression. The fact that neutrophil influx was not seen after intranasal administration of the empty adenoviral vector strongly suggests that locally induced tPA stimulates the recruitment of neutrophils into the lungs. Our group made two earlier observations supporting the notion that tPA may stimulate neutrophil migration: in models of renal ischemia reperfusion injury and thioglycolate-induced peritonitis tPA deficiency resulted in a diminished neutrophil influx into post-ischemic renal tissue and peritoneal lavage fluid respectively (17, 34). The stimulatory effect of tPA on the cellular inflammatory response to the lungs was not mediated by chemokines since the concentrations of the murine chemokines MIP-2 and KC were similar in both groups.

An alternative mechanism by which transgenic tPA overexpression could have conferred protection against pneumonia may be that tPA either directly or indirectly influences the killing of *Klebsiella*. In this context it is interesting to note that a recent study has suggested that uPA can kill *Staphylococcus aureus* (35). However, such an uPA effect was not found on *Pseudomonas* (36) and we were unable to detect any effect of tPA on the growth or killing of *Kebsiella in vitro*.

Recently we reported that endogenous and transgenic PAI-1 also induces neutrophil influx in the same model of *Klebsiella* pneumonia (15). The fact that PAI-1 and tPA both show similar effects on neutrophil recruitment in this model is surprising since they play opposite roles in the fibrinolytic network. Hence, these findings suggest that the mechanism by which at least one of these proteins influences neutrophil migration is independent of its fibrinolytic function. PAI-1 deficiency negatively regulates neutrophil influx into the alveolar space (15, 32), which at least in part could be explained by an increase in intravascular KC levels (thereby "trapping" neutrophils in the intravascular compartment) (15, 32). Since intratracheal plasmin administration also increased intravascular KC levels, this effect of PAI-1 seems to be plasmin dependent (32). However, tPA is able to directly activate MMP-9 (37), which has been implicated as an important enzyme during inflammation because of its ability to assist leukocyte trafficking through the extracellular matrix (38-40). Therefore, in theory, tPA could exert its effect via MMP-9, independent of plasmin. These mechanisms should be further investigated in future studies.

The humoral inflammatory response to *K. pneumoniae* was reduced by tPA gene transfer, as shown by lower pro-inflammatory cytokine levels at 24 hours postinfection. Although the difference in CFU's at 24 hours was not significantly different yet, the mice overexpressing tPA already showed less bacterial outgrowth, which may have provided a less potent proinflammatory stimulus to the immune cells, thereby delaying the initial proinflammatory cytokine response. tPA did not influence proinflammatory cytokine production by alveolar macrophages stimulated with *Klebsiella* LPS *in vitro* (data not shown). Of note, the improved host defense conferred by Ad.tPA occurred in spite of the lower TNF-α levels. Indeed, TNF-α serves a protective role in the host response to *Klebsiella* pneumonia (14, 41).

$tPA^{-/-}$ mice did not show any differences in mortality or bacterial outgrowth during *Klebsiella* pneumonia. We recently reported that $tPA^{-/-}$ mice are more susceptible to *Escherichia coli* peritonitis, as reflected by a reduced survival and an increased growth and dissemination of bacteria (17). These data suggest that the role of tPA in severe infection may vary depending on the infectious source and pathogen. The observation that endogenous tPA does not play a significant role in *Klebsiella* pneumonia may be related to the fact that tPA levels already are suppressed in the lungs of WT mice as a consequence of the infection.

Local tPA activity is downregulated during severe pneumonia. We here artificially increased lung tPA concentrations during experimentally induced lower respiratory tract infection resulting in sepsis. Using gene transfer of tPA to the lungs of mice we show that local tPA overexpression protects against Gram-negative pneumonia induced by *K. pneumoniae* and subsequent systemic dissemination, organ failure and death. Although in our studies enhanced tPA expression in the lungs did not result in bleeding complications, caution is warranted to extrapolate these data to humans. Nonetheless, these results provide the first evidence that elevated tPA levels within the pulmonary compartment provides protection against severe pneumonia.

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Chapter 7

Endogenous tissue-type plasminogen activator is protective during *Escherichia coli*-induced abdominal sepsis in mice

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Abstract

Sepsis is associated with enhanced production of tissue-type plasminogen activator (tPA). We investigated the function of endogenous tPA in the immune responses to *Escherichia coli*-induced abdominal sepsis using tPA gene-deficient ($tPA^{-/-}$) and normal wild-type (WT) mice. $tPA^{-/-}$ mice demonstrated an impaired defense against *E. coli* peritonitis as indicated by higher bacterial loads at the primary site of the infection, enhanced dissemination and reduced survival. The protective function of tPA was independent of plasmin since plasminogen gene-deficient ($Plg^{-/-}$) mice were indistinguishable from WT mice. Relative to WT mice, $tPA^{-/-}$ mice demonstrated similar neutrophil counts in the peritoneal cavity in spite of much higher bacterial loads and higher local concentrations of neutrophil attracting chemokines, suggesting a reduced migratory response. In line, $tPA^{-/-}$ mice demonstrated a reduced thioglycollate-induced neutrophil influx into the peritoneal cavity and i.p. injection of WT mice with a replication-defective adenoviral vector expressing tPA caused an enhanced cell migration to the peritoneal cavity during *E. coli* peritonitis. These findings identify a novel protective function of tPA in abdominal sepsis caused by *E. coli* that seems independent of its role in the generation of plasmin.

Introduction

Sepsis is the leading cause of death in critically ill patients in the developed world (1). Whereas the overall mortality rate of sepsis is 25-30%, mortality in patients with abdominal sepsis can be as high as 60% (1, 2). Although different bacteria have been identified as causative organisms in peritonitis, *E. coli* remains one of the most common pathogens in intraperitoneal infections (2, 3).

Sepsis results in the activation of various host mediator systems including the cytokine network and the coagulation and fibrinolytic systems. tPA is a serine protease which main function is to activate the fibrinolytic system by the conversion of plasminogen into the active protease plasmin (4). Evidence exists that systemic and abdominal infection are associated with increased production of tPA. Patients with sepsis demonstrated elevated tPA levels, increasing further during severe sepsis and septic shock (5). In patients with bacterial peritonitis tPA levels in peritoneal fluid were found to increase 65-fold (6). Furthermore, plasma tPA levels strongly increased in healthy humans i.v. injected with *E. coli* LPS (7, 8). Some attempts have been made to treat patients with severe sepsis, in particular meningococcal purpura fulminans, with recombinant tPA, but the results have been variable and concern has been raised about hemorrhagic complications (9).

In the last decade, evidence has accumulated showing that mediators of the fibrinolytic system have more functions besides their fibrin degrading properties. Plasmin plays an important role in degradation of extracellular matrix components, tissue remodeling and cellular migration (10). Furthermore, recent studies using $tPA^{-/-}$ mice showed that tPA promotes the induction of matrix metalloproteinase-9 during focal cerebral ischemia (11, 12) and renal interstitial fibrosis (13) thereby affecting various inflammatory responses. In spite of its enhanced production during bacterial peritonitis and sepsis and its potential impact on the host response, the function of endogenous tPA in the pathogenesis of sepsis has not been investigated thus far. By using $tPA^{-/-}$ mice we here studied the role of tPA in *E. coli*-induced abdominal sepsis *in vivo*. Our results show for the first time that endogenous tPA is part of the protective immune response to abdominal sepsis.

Methods

Animals

The Institutional Animal Care and Use Committee approved all experiments. C57BL/6 (WT), tPA-/- and Plg-/- mice, both on a C57BL/6 genetic background, were obtained from the Jackson Laboratory (Bar Harbor, ME). Female 8 to 10 weeks old mice were used in all experiments.

Induction of peritonitis

Peritonitis was induced as described previously (14-16). In brief, *E. coli* O18:K1 was cultured in Luria-Bertani medium (Difco, Detroit, Mich.) at 37°C, harvested at mid-log phase, and washed twice with sterile saline before injection. Mice were injected i.p. with $1x10^4$ CFU of *E. coli* O18:K1 in 200 µl of sterile saline.

Sample harvesting

At the time of sacrifice, mice were first anesthetized by inhalation of isoflurane (Abbott Laboratories Ltd., Kent, UK). A peritoneal lavage was then performed with 5 ml of sterile isotonic saline using an 18-gauge needle, and peritoneal lavage fluid was collected in sterile tubes (Plastipack [BD Biosciences, San Jose, CA]). After collection of peritoneal fluid, deeper anesthesia was induced by i.p. injection of 0.07 ml of FFM mixture (fentanyl [0.315 mg/ml]-fluanisone [10 mg/ml] [Janssen, Beersen, Belgium], midazolam [5 mg/ml] [R°Che, Woerden, the Netherlands]) per gram body weight. The abdomen was opened, and blood was drawn from the vena cava inferior into a sterile syringe, transferred to tubes containing heparin, and immediately placed on ice. Thereafter, livers and lungs were harvested and pr°Cessed for histology and measurements of CFU as described before (14-16) and (for RT-PCR) below.

Evaluation of mRNA levels by quantitative RT-PCR

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) according to recommendations of the suppliers. RT-PCR reactions were performed on cDNA samples that were 4 fold diluted in H_20 using FastStart DNA Master SYBR Green I (Roche) with 2.5 mM $MgCl_2$ in a LightCycler (Roche) apparatus. PCR conditions were: 5 min 95 °C hot-start, followed by 40 cycles of amplification (95°C for 15 s, 60°C for 5 s, 72°C for 20 s). For quantification standard curves were constructed by PCR on serial dilutions of a concentrated cDNA and data were analyzed using the LightCycler software as described by the manufacturer. Gene expression is presented as a ratio of the expression of the house keeping gene β_2m (17). All PCR reactions generated a single DNA product of the expected length as judged by evaluation on ethidiumbromide stained 1.2% agarose gel electrophoresis. Primers used for murine tPA were mtPA-S1454 CTCCATTCTTCTCTGACCGG and mtPA-AS1630 TTGATCATGCACACCAGAGG. Primers for the house keeping gene were m β_2m S74 TGGTCTTTCTGGTGCTTGTCT and m β_2m AS231 ATTTTTTTCCCGTTCTTCAGC.

In situ hybridization

tPA-specific digoxigenin-labeled riboprobes were prepared by T7 RNA polymerase driven in vitro transcription from clone specific PCR products as template. Primers used in the PCR reaction were: 5'-ATTTAGGTGACACTATAGGGCCCTGTATTTCTCTGACTT T -3' and 5'-TAATACGACTCACTATAGGGGTCC TCC ACG CTG TGT AAC TCT-3', yielding a 466 bp product. The underlined primer regions encode the T7-promoter element. Using these probes, in situ hybridization was performed as described previously (18), using the DIG-labeled riboprobes at a concentration of 300 ng/ml. After hybridization, slides were washed and bound alkaline phosphatase activity was visualized with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate, toluidine salt (NBT/BCIP) (Roche).

Determination of bacterial outgrowth

Liver and lungs were homogenized as described earlier (14-16). In short, organs were weight and to correct for the differences in weight of the organs we added 4 times the weight of the organ (in mg) in µl of sterile saline, in which it was homogenized. Next, eight

serial 10-fold dilutions were made of each sample of the homogenates, peritoneal lavage fluid and blood, in sterile saline, and 50 µl of each dilution was plated onto blood agar plates. The plates were incubated at 37°C under 5% CO_2, and after 16 h CFU were counted and corrected for the dilution factor.

Monitoring of mortality

Our laboratory previously established that in this model mortality occurs predominantly between 24 and 72 h after the *E. coli* challenge (14). Therefore, mortality was assessed every hour during this period and at 6 h intervals thereafter. In this model, mice surviving for more than 3 days appeared healthy and remained alive for at least 4 weeks (after which they were killed).

Phagocytosis assay

The uptake of *E. coli* by peritoneal macrophages was analyzed as described previously (19, 20). In short, macrophages were isolated from the peritoneal cavities of untreated WT and $tPA^{-/-}$ mice, and were cultured overnight at 37°C to allow adherence. FITC-labeled heat-killed (HK)-*E. coli* O18:K1 (equivalent to 5 x 10^7 CFU) were added to the cells (bacterium/cell ratio of 50:1) and incubated for 2 hours at 37 °C. Phagocytosis was stopped and the cells were treated with vital blue stain (Orpegen, Heidelberg, Germany) to quench extracellular fluorescence, washed and analyzed using a FACScalibur flowcytometer (BD Biosciences). Results are expressed as phagocytosis index, defined as the percentage of cells with internalized *E. coli* times the mean fluorescence intensity.

Cell counts and differentials

Cell counts were determined in peritoneal lavage fluid using a hemacytometer (Beckman Coulter, Fullerton, CA). Subsequently, differential cell counts were performed on cytospin preparations stained with a modified Giemsa stain (Diff-Quick; Dade Behring AG, Düdingen, Switzerland).

Histology

Lungs and livers for histology were harvested 6 and 20 h after infection, fixed in 4% formalin, and embedded in paraffin. Four-micrometer sections were stained with hematoxylin and eosin and analyzed by a pathologist who was blinded for groups. To score liver injury, the following parameters were analyzed (16): interstitial inflammation, formation of thrombi, hepatocellular necrosis and portal inflammation. To score lung inflammation and damage, each entire left lung was screened for the following parameters: interstitial inflammation, edema, pleuritis, and thrombus formation. Each parameter was graded on a scale from 0 to 4, as follows: 0, absent; 1, mild; 2, moderate; 3, severe; 4, very severe. The total injury score was expressed as the sum of the scores for all parameters; the maximum values were 16.

Assays

Murine tPA (Kordia, Leiden, the Netherlands), keratinocyte-derived chemokine (KC) and MIP-2 (both R&D systems, Abingdon, UK) were measured by ELISA. TNF-α, IL-6 and IL-10 were measured by cytometric bead array multiplex assay (BD Biosciences). Aspartate

aminotranspherase and alanine aminotranspherase were determined with commercially available kits (Sigma-Aldrich, St. Louis, MO), using a Hittachi analyzer (Boehringer Mannheim, Mannheim, Germany). Human tPA was measured by ELISA (Kordia).

Intracellular TNF-α staining

For intracellular TNF-α staining peritoneal macrophages from untreated $tPA^{-/-}$ and WT mice were washed and resuspended in medium (RPMI 1640 containing 1 mM pyruvate, 2 mM L-glutamine, penicillin, streptomycin, and 10% tPA-deficient serum (Molecular Innovations, Inc., Southfield, MI)). Cells were then incubated in 96-well flat-bottom microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) (1×10^5/well) for 2 h at 37 °C, 5% CO_2 and than washed with medium to remove nonadherent cells. Next, the adherent monolayer cells were stimulated for 4 hours in 200 µl of medium alone or with LPS from *E. coli* (100 µg/ml; Sigma-Aldrich) or HK-*E. coli* O18:K1 (1×10^7 CFU/ml) at 37 °C, 5% CO_2. After 1 hour, brefeldin A (10 µg/ml) was added to the wells. The cells were harvested, fixed and permeabilized using the BD cytofix/cytoperm kit (BD biosciences) according to the manufacturers instructions. The cells were stained with APC-conjugated anti-TNF-α antibodies (BD biosciences) for 30 min at room temperature. Stained cells were analyzed on the FACScalibur flowcytometer with CellQuest software.

Sterile peritoneal inflammation model

A thioglycollate-induced inflammation model was used as described previously (21, 22). WT and $tPA^{-/-}$ mice (N = 8 per group) were injected i.p. with 0.5 ml of a 4% Brewer thioglycollate medium (Difco Laboratories, Detroit, Michigan). At 6 hours, the mice were sacrificed, peritoneal lavage and cell counts were performed as described above

Adenoviral tPA gene transfer

The recombinant replication-defective adenoviral vector expressing human tPA (Ad.tPA) was generated by homologous recombination in 293 cells (23-25). After transfection, recombinant viral plaques were harvested and amplified (26-28) and large-scale production of recombinant adenovirus was performed as described (26). The kinetics and organ distribution of tPA expression after adenoviral transfer by i.v. bolus injection have been reported in detail elsewhere (29). In a first series of experiments we injected WT mice i.p. with $2x10^9$ PFU of Ad.tPA and determined human tPA levels in plasma obtained 1, 2 and 4 days later. In a second experiment WT mice received an i.p. injection of $2x10^9$ PFU of a control replication-defective adenoviral vector (Ad.RR5) or Ad.tPA in 200 µl of sterile isotonic saline 4 days before i.p. injection with *E. coli* and were sacrificed 20 hours later as described above.

Statistical analysis

Differences between groups were calculated by using the Mann-Whitney U test. For survival analysis a Kaplan-Meier analysis followed by a log rank test was performed. Values were expressed as means ± SE. A P value of <0.05 was considered statistically significant.

Results

tPA is upregulated during *E. coli*-induced abdominal sepsis in mice

To evaluate the role of tPA during Gram-negative abdominal sepsis, we used a murine *E. coli* peritonitis model. To confirm tPA production in this model, we measured tPA mRNA levels in liver and lung tissue, and tPA protein concentrations in plasma of WT mice at various time points after the induction of peritonitis. I.p. injection of *E. coli* significantly increased mRNA and protein levels of tPA (all $P < 0.05$ vs baseline; **Figure 1A-C**). To obtain insight into the cellular source of tPA during abdominal sepsis, we performed in situ hybridization on liver and lung tissue of WT mice at 20 hours after *E. coli* injection. tPA mRNA expression co-localized mainly with the endothelium in both lung and liver tissues **(Figure 1D-E)**.

tPA$^{-/-}$ mice are more susceptible to *E. coli*-induced abdominal sepsis

In a first attempt to determine the role of tPA in *E. coli* abdominal sepsis, tPA$^{-/-}$ and WT mice were injected i.p. with 10^4 CFU of *E. coli* and either observed for 4 days to monitor survival or sacrificed after 6 or 20 hours. When compared with WT mice, tPA$^{-/-}$ mice

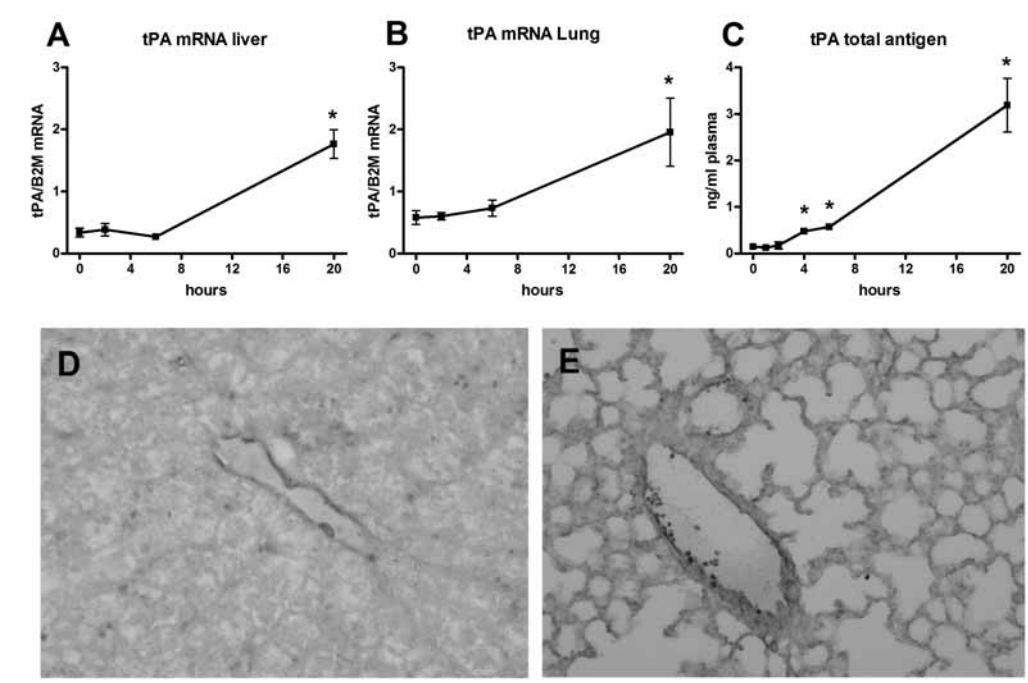

Figure 1. Enhanced tPA production during septic peritonitis. WT mice were injected i.p. with 10^4 CFU *E. coli* (0 h) and sacrificed just before and at various h after infection. Liver (A) and lung (B) tPA mRNA expression was determined by RT-PCR. Plasma tPA protein levels (C) were measured by ELISA. Results are expressed as means ± SE. N= 4 mice per time point. * P <0.05 vs 0h. Localization of tPA mRNA in liver (D) and lung (E) tissue as determined by in situ hybridization at 20 h postinfection. Slides are representatives of N= 8 mice. Magnification × 200.

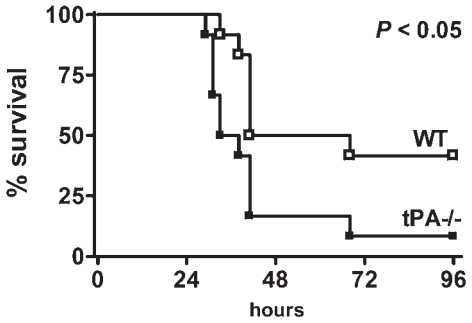

Figure 2. tPA protects against *E. coli*-induced mortality. Survival was monitored in tPA$^{-/-}$ (closed squares) and WT (open squares) mice after i.p. infection with 10^4 CFU of *E. coli*. N= 12 mice per group.

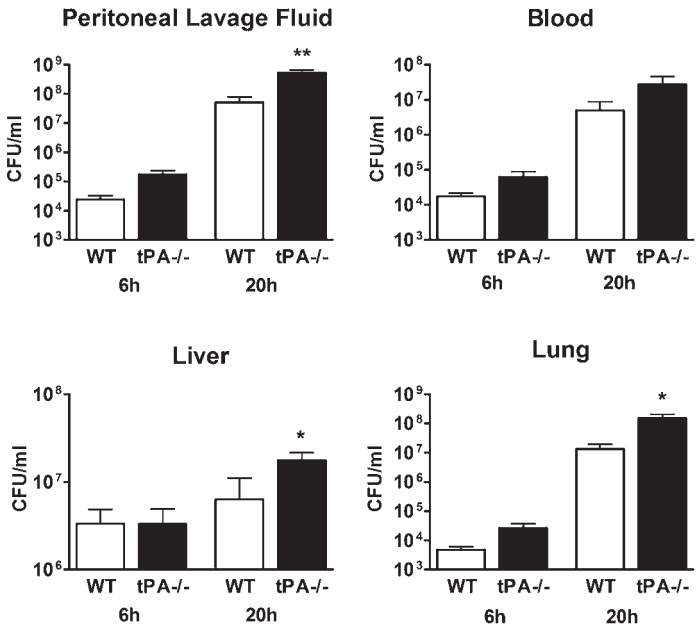

Figure 3. tPA$^{-/-}$ mice demonstrate an enhanced bacterial outgrowth and dissemination. Numbers of *E. coli* CFU in peritoneal lavage fluid, blood, liver and lungs at 6 and 20 h after i.p. injection of *E. coli* in tPA$^{-/-}$ (closed bars) and WT (open bars) mice. Data are expressed as means ± SE. N= 8 mice per group per time point. $^{**}P$ <0.001 and $^{*}P$ <0.05 vs WT at the same time-point.

showed a significantly reduced survival **(Figure 2)**. The mice that survived the infection stayed alive for at least 4 weeks, after which they were killed. Furthermore, at the time points indicated we counted the number of *E. coli* CFU's in the peritoneal lavage fluid to assess the bacterial load at the site of the infection, in the blood to evaluate to which extent the bacteria escaped to the circulation and the infection became systemic, and in the liver and lungs to evaluate whether the infection had spread to distant organs **(Figure 3)**. Whereas at 6 hours post infection tPA$^{-/-}$ mice tended to have higher bacterial loads, significantly more *E. coli* CFU´s were recovered from their peritoneal fluid, liver and lungs at 20 hours. These data indicate that endogenous tPA limits the outgrowth of bacteria at the primary site of infection, contributes to containment of the infection in the peritoneal cavity and thereby plays a protective role against lethality during *E. coli*-induced abdominal sepsis.

Unchanged phagocytosis of *E. coli* by tPA$^{-/-}$ peritoneal macrophages

To investigate whether the increased bacterial outgrowth in tPA$^{-/-}$ mice could be the result of an intrinsic defect in the ability of tPA$^{-/-}$ macrophages to phagocytose *E. coli*, we harvested macrophages from uninfected tPA$^{-/-}$ and WT mice and compared their capacity to phagocytose HK-*E. coli*. Peritoneal macrophages from the tPA$^{-/-}$ mice displayed a normal ability to phagocytose *E. coli* (phagocytosis index of 1131 ± 121 versus 1007 ± 70 in WT mice, non significant).

Inflammatory cell influx

The recruitment of leukocytes to the site of an infection is an essential part of the host defense to invading bacteria. The mouse CXC chemokines KC and MIP-2 have been implicated to play an important role in the attraction of neutrophils during inflammation (30, 31). Therefore, we determined chemokine levels and leukocyte counts and differentials in peritoneal fluid at 6 and 20 hours after *E. coli* or saline injection in tPA$^{-/-}$ and WT mice. KC and MIP-2 levels were strongly elevated at 6 and 20 hours postinfection. tPA$^{-/-}$ mice had significantly increased levels of both chemokines compared to WT mice at 20 hours postinfection **(Table 1)**. Saline injection did not result in a change in leukocyte counts or differentials (data not shown). However, *E. coli* injection resulted in a profound increase in total leukocyte numbers in the peritoneal fluid, which was mainly due to neutrophil influx **(Table 1)**. In spite of the much higher local chemokine levels and the higher local bacterial load (providing a more potent proinflammatory stimulus), tPA$^{-/-}$ mice showed similar neutrophil numbers in their peritoneal lavage fluid compared to WT mice at both time points.

Higher cytokine levels in tPA$^{-/-}$ mice

To determine whether tPA influenced the production of cytokines during septic peritonitis, local and systemic levels of pro- and anti-inflammatory cytokines were measured in WT and tPA$^{-/-}$ mice **(Figure 4)**. At 6 hours after infection peritoneal lavage fluid and plasma TNF-α, IL-6 and IL-10 levels were similar in both mouse strains. However, at 20 hours both peritoneal and plasma levels of these cytokines were significantly higher in tPA$^{-/-}$ mice. Hence, tPA$^{-/-}$ deficiency was associated with an exaggerated release of cytokines.

Table 1. Chemokine levels and inflammatory cell counts in peritoneal fluid

	6 h		20 h	
	WT	tPA$^{-/-}$	WT	tPA$^{-/-}$
Chemokines (ng/ml)				
KC	0.5 ± 0.1	1.2 ± 0.34	2.2 ± 0.5	4.9 ± 0.8*
MIP-2	0.5 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	1.7 ± 0.3*
Cells (x 10^4/ml)				
Total cells	21.1 ± 5.3	17.3 ± 2.3	194 ± 28	179 ± 33
Neutrophils	16.3 ± 4.7	9.3 ± 2.3	150 ± 19	148 ± 27
Macrophages	3. 6 ± 1.1	6.7 ± 1.0	40.1 ± 10.2	29.5 ± 7.5
Lymphocytes	0.9 ± 0.4	1.3 ± 0.6	2.9 ± 1.2	1.3 ± 0.3

Data are means ± SEM at 6 and 20 h after i.p. injection of 10^4 CFU *E.coli*. N= 8 mice per time-point. * $P < 0.05$ vs. WT mice at the same time-point.

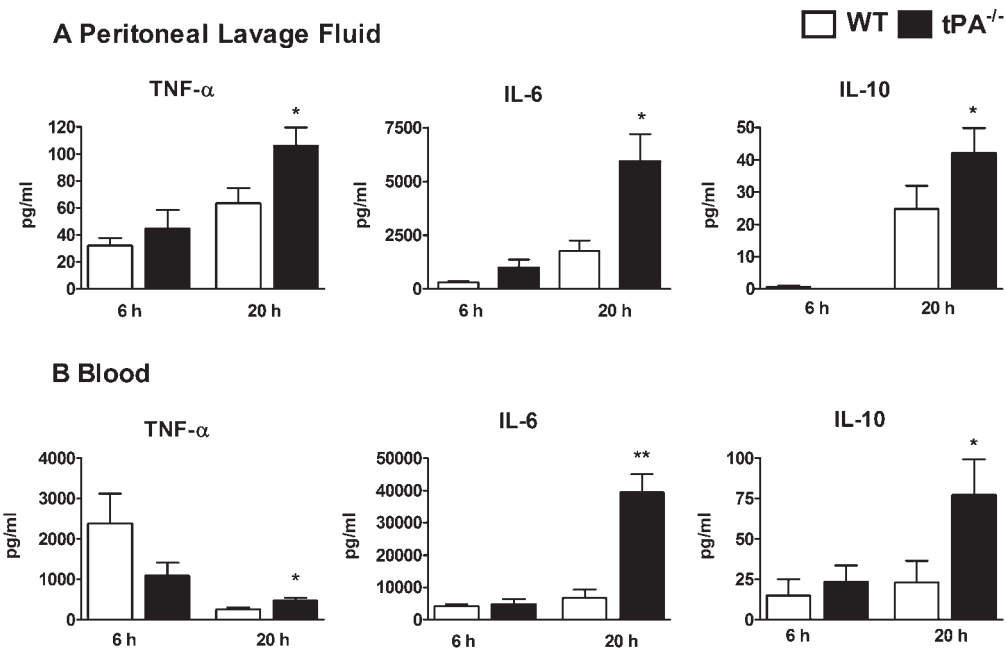

Figure 4. Higher cytokine levels in tPA$^{-/-}$ mice. TNF-α, IL-6 and IL-10 levels in peritoneal fluid and plasma of tPA$^{-/-}$ and WT mice at 6 and 20 h after i.p. infection with *E. coli*. Data are expressed as means ± SE. N= 8 per group per time point. **P <0.001 and *P <0.05 vs WT at the same time-point.

Normal TNF-α production by tPA-/- peritoneal macrophages ex vivo

Having established that tPA$^{-/-}$ mice display altered cytokine levels during abdominal sepsis, we next investigated whether tPA deficiency directly influenced TNF-α production by peritoneal macrophages ex vivo. Therefore we stimulated peritoneal macrophages from WT and tPA$^{-/-}$ mice for 16 hours with either LPS, HK-*E. coli* or medium alone. LPS and HK-*E. coli* significantly increased the percentage of TNF-α-positive cells in both groups (all $P < 0.05$ versus medium alone, data not shown). WT and tPA$^{-/-}$ peritoneal macrophages showed no significant differences in percentages of TNF-α-positive cells after stimulation with LPS (71 ± 5 versus 63 ± 8 %, respectively) or HK-*E. coli* (63 ± 3 versus 51 ± 8 %, respectively).

Organ injury

Our model of *E. coli* peritonitis is associated with profound liver injury (14-16). To evaluate the role of endogenous tPA in liver injury during abdominal sepsis, we determined liver damage in tPA$^{-/-}$ and WT mice 20 h after infection **(Figure 5)**. Upon histopathological examination by a pathologist who was blinded for groups, both tPA$^{-/-}$ and WT mice showed inflammation of the hepatic parenchyma, areas of liver necrosis **(Figure 5A and B)** and presence of intravascular thrombi **(Figure 5A and B, inserts)**. There was no difference in

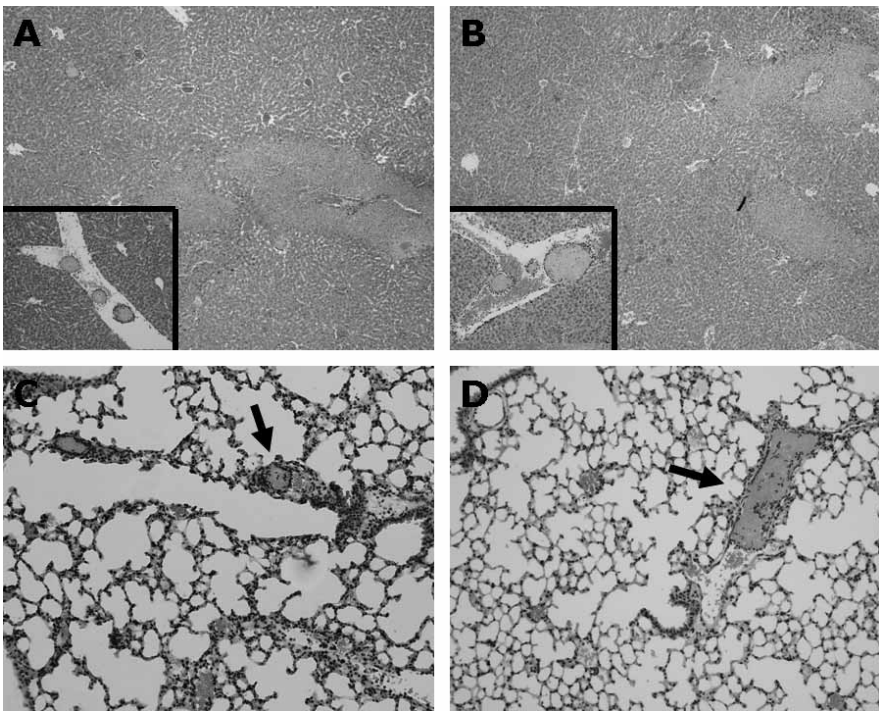

Figure 5. Organ damage. Representative HE stainings of liver (A-B) and lung (C-D) tissue at 20 hours after i.p. injection of 10^4 CFU *E. coli* in WT (A, C) and tPA $^{-/-}$ (B, D) mice. Microscopic magnification: liver ×40, inset in A ×200, inset in B ×125, lungs ×100.

Table 2. Cell influx in Plg$^{-/-}$ mice

Cells (x 10^4/ml PLF)	WT	Plg$^{-/-}$
Total cells	226 ± 22	255 ± 26
Neutrophils	192 ± 18	207 ± 24
Macrophages	32 ± 5.4	44 ± 6.4
Lymphocytes	2.3 ± 0.8	4.3 ± 1.9

Data are means ± SE at 20 h after i.p. injection of 10^4 CFU *E. coli.*
PLF, peritoneal lavage fluid.

the mean total histology scores of the liver tissues (quantified according to the scoring system described in the Methods section) between WT and tPA$^{-/-}$ mice (score of 7.56 ± 1.25 versus 8.19 ± 0.89, respectively). This was confirmed by clinical chemistry, i.e. tPA $^{-/-}$ mice showed similar plasma levels of aspartate aminotranspherase (3127 ± 612 versus 3204 ± 808 U/L; $P = 0.9$) and alanine aminotranspherase (976 ± 240 versus 1335 ± 333 U/L; $P = 0.4$) compared to WT mice. To obtain insight into the role of tPA in the development of inflammation in a more distant organ, lungs were harvested at 20 hours after the induction of *E. coli* infection. Lungs showed clear signs of inflammation in both WT and tPA$^{-/-}$ mice, as reflected by accumulation of leukocytes in the interstitium **(Figure 5C and D)**. The total histological scores were significantly higher in tPA$^{-/-}$ mice (score of 5.94 ± 0.47 versus 3.83 ± 0.50 in WT mice; $P < 0.05$), which was mainly due to higher scores for the amount and size of thrombi in the lungs **(Figure 5C and D, arrows)**.

Impaired host defense of tPA$^{-/-}$ mice is independent of plasmin

These studies with tPA$^{-/-}$ mice showed an important role for tPA during *E. coli* peritonitis. Since the major function of tPA is to convert plasminogen into the active protease plasmin, the key enzyme of the fibrinolytic system, we wanted to investigate whether the changes in host defense in tPA$^{-/-}$ mice could be the result of diminished plasmin generation. Therefore, we injected Plg$^{-/-}$-deficient and WT mice with $1x10^4$ CFU *E. coli* and sacrificed them at 20 hours postinfection. In contrast to the tPA$^{-/-}$ mice, Plg$^{-/-}$ mice showed bacterial loads in peritoneal lavage fluid, blood, liver and lungs that were similar to those recovered from WT mice **(Figure 6)**. In addition, leukocyte counts and differentials **(Table 2)**, local KC and MIP-2 levels and local and circulating cytokine concentrations were similar in Plg$^{-/-}$ and WT mice (data not shown). Liver and lung histology scores were indistinguishable between the two genotypes (data not shown). These data show that plasminogen deficiency does not affect the anti-bacterial host defense or inflammatory responses in this model, indicating that the role of tPA during *E. coli* peritonitis is independent of its function as an activator of plasmin.

Role of tPA in neutrophil migration during peritonitis

We hypothesized that tPA$^{-/-}$ mice had a relatively impaired neutrophil recruitment into the peritoneal cavity after *E.coli* injection, which was masked by the differences in neutrophil

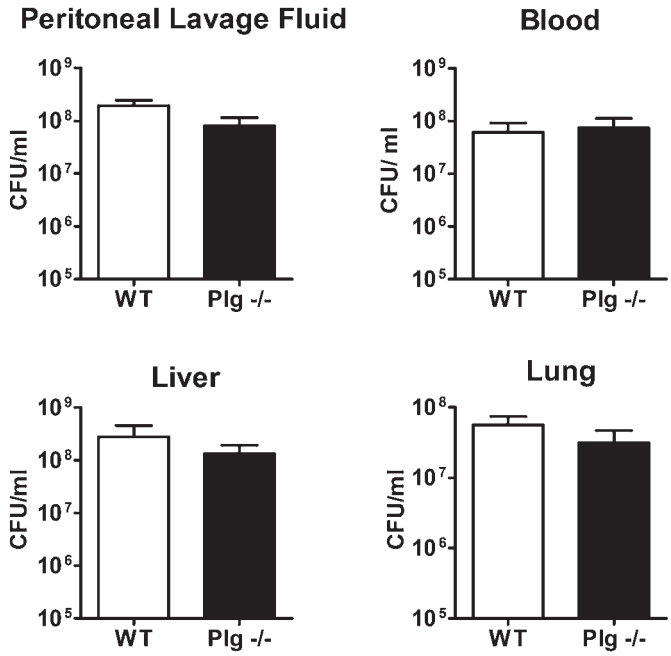

Figure 6. Bacterial outgrowth in plasminogen$^{-/-}$ mice. Numbers of *E. coli* CFU in peritoneal lavage fluid, blood, liver and lungs at 20 h after i.p. injection of *E. coli* in plasminogen$^{-/-}$ (closed bars) and WT (open bars) mice. Data are expressed as means ± SE. N= 6 per group.

attracting stimuli (higher bacterial load and CXC chemokines) between WT and tPA$^{-/-}$ mice in this model. Therefore, we wanted to examine whether tPA$^{-/-}$ mice have an impaired neutrophil recruitment to the peritoneal cavity during acute sterile inflammation. For this we used a well-known model of sterile peritonitis induced by thioglycollate. Indeed, at 6 hours after i.p. injection of 1 ml of a 4% thioglycolate solution, the tPA$^{-/-}$ mice had lower numbers of neutrophils in their peritoneal lavage fluid compared to WT mice **(Figure 7A)**. To obtain further evidence for a role of tPA in neutrophil recruitment, we administered $2x10^9$ PFU Ad.tPA i.p. to WT mice. By using this approach we sought to examine the effect of high levels of tPA on the host response, i.e. the "reverse experiment" with regard to the experiments with tPA$^{-/-}$ mice. Administration of this vector produced high levels of tPA in plasma peaking after 4 days **(Figure 7B)**. Transgenic tPA was undetectable in peritoneal fluid. To evaluate the effect of tPA overexpression on neutrophil migration during *E. coli* peritonitis, we injected WT mice with Ad.tPA or Ad.RR5 4 days prior to infection. At the time of *E. coli* injection there were no differences in leukocyte counts or differentials between Ad.tPA or Ad.RR5 injected mice (data not shown). At 20 hours postinfection, mice overexpressing tPA had significantly higher numbers of neutrophils and macrophages in their peritoneal lavage fluid compared to the controls **(figure 7C)**. There was no effect of tPA overexpression on the bacterial outgrowth (data not shown).

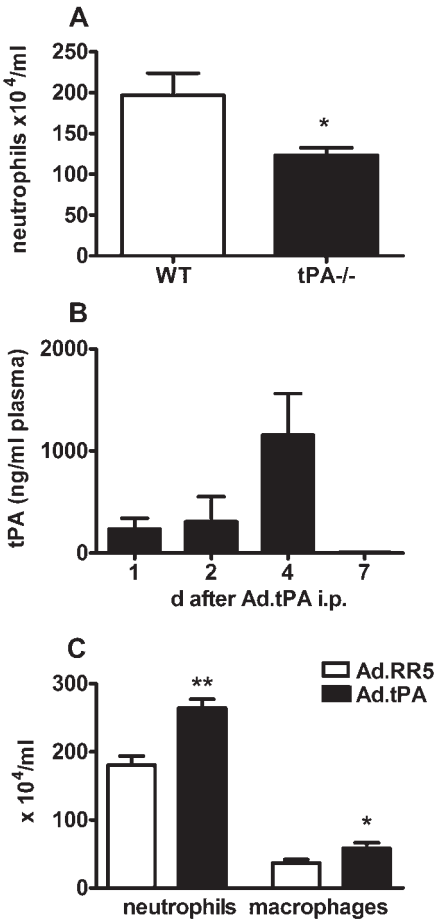

Figure 7. Role of tPA in neutrophil migration. (A) Mice were injected i.p. with 1 ml 4% thioglycollate solution and neutrophil numbers were counted in peritoneal lavage fluid harvested after 6 hours. Data are means ± SE. N=8 mice per genotype. * P<0.05 vs WT. (B) tPA levels were in measured in plasma at different time-points after i.p. injection of $2x10^9$ PFU Ad.tPA. (C) $1x10^4$ CFU *E. coli* was injected i.p. 4 days after injection of $2x10^9$ PFU Ad.tPA or AdRR5. Neutrophil and macrophage numbers were counted in peritoneal lavage fluid harvested after 20 hours. Data are means ± SE. N=8 mice per group. **P<0.01, *P<0.05 vs WT.

Discussion

Peritonitis is the second most common cause of sepsis and especially abdominal sepsis bears a grim prognosis (32, 33). Severe sepsis is associated with an early fibrinolytic response, which is primarily mediated by enhanced release of tPA. Recent studies have indicated that tPA may influence several inflammatory pathways by actions that at least in part are unrelated to its classical function in the fibrinolytic system (12, 13, 34-37). We here demonstrate for the first time that endogenous tPA serves a protective role during *E. coli* peritonitis that is independent of plasmin.

We investigated the changes in tPA levels during *E. coli* peritonitis in mice. tPA is expressed constitutively in most organs in the mouse, including lungs and liver (38). We found that *E. coli* peritonitis caused an upregulation of tPA mRNA in liver and lung tissue. These data are in line with a previous study showing enhanced tPA mRNA expression in various murine tissues after *E. coli* LPS injection (38). In our model, plasma tPA levels started to rise from 4 hours after infection onward and peak plasma concentrations were detected at the end of the 20-hour observation period (i.e. shortly before the first animals died). This prolonged time course of tPA release, corresponding with the gradually growing bacterial load, is in line with studies in patients with sepsis in whom plasma tPA concentrations were proportional to the severity of disease (5). Furthermore, in experimental models of self-limiting acute systemic inflammation, such as induced by i.v. injection of low dose LPS into healthy humans, tPA release into the circulation is transient (7, 8). Previous studies also documented elevated tPA levels in peritoneal fluid of patients and rats with bacterial peritonitis (6, 39, 40). In our study tPA levels remained undetectable in peritoneal lavage fluid, which was probably due to the high dilution factor since we performed the peritoneal lavages with 5 ml saline.

This is the first study investigating the role of tPA in host defense to abdominal sepsis in mice. The most striking finding of our study was that endogenous tPA played an important role in reducing the outgrowth of *E. coli*, which was associated with an improved survival of WT mice relative to $tPA^{-/-}$ mice. $TPA^{-/-}$ mice also showed higher chemokine and cytokine levels in their peritoneal lavage fluid and plasma at this time point, which most likely was the consequence of the increased bacterial load, providing a more potent proinflammatory stimulus. Indeed, the ex vivo production of TNF-α by peritoneal macrophages was not influences by the absence of tPA. Theoretically, one would expect that the locally higher bacterial load and more elevated chemokine levels would result in an increased neutrophil influx to the peritoneal cavity in $tPA^{-/-}$ mice. However, we did not find any difference in inflammatory cell recruitment between $tPA^{-/-}$ and WT mice. This led us to the possibility of a relatively impaired migratory response in $tPA^{-/-}$ mice. To our knowledge a direct role of tPA in inflammatory cell migration has not been described to date. However, Roelofs et al. (41) recently reported that tPA deficiency was associated with an impaired neutrophil influx into the kidneys in a model of ischemia reperfusion injury, which is associated with a profound inflammatory response (41). Furthermore, they found no differences in cytokine levels between WT and $tPA^{-/-}$ mice (41), which is in agreement with our ex vivo experiments. To investigate whether the neutrophil migratory response to the peritoneal cavity was indeed impaired in $tPA^{-/-}$ mice, we used the well-known model of thioglycollate-induced sterile peritonitis. Although it should be noted that thioglycollate induced cell migration cannot be directly compared with *E.coli* induced cell recruitment, we indeed found that the absence of tPA was associated with a reduced neutrophil influx in this model of sterile inflammation. Furthermore, enhanced expression of tPA by the administration of Ad.tPA increased neutrophil influx into the peritoneal cavity during peritonitis. This transgenic overexpression of tPA was done in order to determine the effects of artificially elevated levels of tPA on host defense in this model, i.e. the approach opposite to the total absence of tPA such as in $tPA^{-/-}$ mice. It should be emphasized that human tPA is produced by this vector (not mouse tPA) and that the plasma levels of human tPA after

Ad.tPA administration were > 1000 ng/mL whereas endogenous tPA levels increased to approximately 3 ng/mL in WT mice. Furthermore, this approach cannot reproduce the kinetics of endogenous tPA production and release such as observed in WT mice during abdominal sepsis. Thus, we chose to administer Ad.tPA to WT mice, not to $tPA^{-/-}$ mice, since Ad.tPA would not make $tPA^{-/-}$ mice phenotypically comparable to WT mice for the reasons described above.

Together these data suggest that there might be a role for tPA in inflammatory cell recruitment to the peritoneal cavity, which may have contributed to the reduced antibacterial defense in $tPA^{-/-}$ mice. Finally, we examined organ damage in both genotypes at 20 hours postinfection. We found more and bigger thrombi in the lungs of the tPA-/- mice compared to WT mice. The higher susceptibility of $tPA^{-/-}$ mice to thrombi formation might have been due to the stimulatory role of tPA in plasmin activation and subsequent fibrin degradation. However, previous studies using anti-IL-6 antibodies showed that IL-6 plays a very important role in both systemic and pulmonary coagulation activation after i.v. *E. coli* LPS injection in chimpanzees, which was measured by prothrombin activation fragment F1+2 and thrombin-antithrombin complexes in plasma and bronchoalveolar lavage fluids (42, 43). Thus, it is possible that the strongly enhanced circulating IL-6 levels in the $tPA^{-/-}$ mice might also have contributed to the enhanced thrombi formation in the lungs of these mice in this model.

The main physiological function of tPA is to convert plasminogen into the active protease plasmin. Besides binding to fibrin, plasmin(ogen) can bind to many cell types, including neutrophils (44, 45). Plasmin has the ability to directly degrade extracellular matrix proteins and can also activate MMP's. Thereby cell-associated plasmin might promote cellular migration (10). Indeed, plasmin induced neutrophil aggregation and adhesion *in vitro* (46, 47). Moreover, studies using $Plg^{-/-}$ mice have provided *in vivo* evidence for an essential role of the plasminogen system in thioglycollate-induced macrophage migration (21). To investigate whether plasmin might play a role in the impaired host defense of $tPA^{-/-}$ mice during *E. coli* peritonitis, we infected $Plg^{-/-}$ mice and studied the number of *E. coli* CFU´s in several body compartments and the inflammatory response (cell influx, chemokine or cytokine levels, histopathology) at 20 hours postinfection. We found no differences between $Plg^{-/-}$ and WT mice, strongly suggesting that the role of tPA in host defense against *E. coli* is independent of its fibrinolytic function.

Peritonitis is a common cause of sepsis in humans. I.p. administration of live *E. coli* results in a paradigm that resembles a clinical condition commonly associated with septic peritonitis, with diaphragmatic lymphatic clearance, and systemic bacteremia and endotoxemia (48). We here used this model to investigate the function of tPA in host defense against septic peritonitis. Our results identify for the first time a protective role for tPA in the immune response to abdominal sepsis. The mechanism by which tPA mediates this effect seems independent of its plasminogen activating function. Our data extend rapidly increasing knowledge of the biology of fibrinolytic mediators involved in plasminogen activation, such as tPA, urokinase-type plasminogen activator and its receptor

and plasminogen activator inhibitor-1, which clearly exert activities that go beyond their ability to stimulate or inhibit fibrinolysis.

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Chapter 8

Matrix metalloproteinase 9 deficiency impairs host defense against abdominal sepsis

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Abstract

Matrix metalloproteinase (MMP)-9 is involved in extracellular matrix degradation and leukocyte migration. To determine the role of MMP-9 in the innate immune response to peritonitis, MMP-9 gene-deficient (MMP-$9^{-/-}$) and normal wild-type (Wt) mice were intraperitoneally infected with *Escherichia coli (E. coli)*. MMP-9 mRNA and pro-MMP-9 protein levels increased rapidly upon induction of peritonitis. Although MMP-$9^{-/-}$ neutrophils showed a normal phagocytosis of *E.coli* in vitro, MMP-$9^{-/-}$ mice displayed a reduced resistance against *E. coli* peritonitis, as indicated by an enhanced bacterial outgrowth in the peritoneal cavity and increased dissemination of the infection. Furthermore, the cytokine response to LPS was not influenced by MMP-9 deficiency. However, during *E.coli* peritonitis, MMP-$9^{-/-}$ mice showed much higher peritoneal chemokine and cytokine levels compared to Wt mice. Despite the increased local chemokine concentrations, MMP-$9^{-/-}$ mice displayed a diminished recruitment of leukocytes to the site of infection, indicating that cellular migration was impaired. Moreover, MMP-$9^{-/-}$ mice developed more severe distant organ damage during infection. These data suggest that MMP-9 is an essential component of an effective host response to *E. coli* peritonitis.

Introduction

Sepsis is the most common cause of death in noncoronary critical care units in the U.S. with more than 750,000 cases per year (1). Peritonitis is the second most common cause of sepsis (2), with *E. coli* as one of the major pathogens involved (3). Since *E. coli* peritonitis is a life threatening disease, an immediate and adequate host defense is necessary to contain and kill the pathogen.

Proteases released by activated leukocytes and degradation of connective tissue structures have been implicated to play an important role in inflammatory host responses. Matrix metalloproteinases, such as MMP-9, are involved in the migration of inflammatory cells across the extracellular matrix, as well as tissue remodeling (4, 5). MMP-9 was first identified in neutrophils but can also be expressed by various other cell types like monocytes/macrophages, lymphocytes and endothelial cells (5, 6). MMP-9 is not produced constitutively but needs a trigger to be expressed (5). LPS, the major constituent of the outer cell wall of Gram-negative bacteria and the principal mediator of inflammatory responses to these pathogens, induces the release of MMP-9 by neutrophils and monocytes *in vitro* (7, 8). Moreover, in mice *E.coli* LPS administration lead to a quick release of MMP-9 into the circulation, with peak values as soon as 1 hour after injection (9). In line, during experimental endotoxemia in healthy human volunteers, plasma levels of MMP-9 increased strongly, peaking at 1.5 to 3 hours after LPS injection (10). Furthermore, in a sublethal and lethal *E. coli* sepsis model in baboons, MMP-9 was found to increase in serum, again with peak levels early after the induction of the infection (11). Elevated MMP-9 levels were also found in human sepsis patients and correlated with the severity and mortality of the disease (10, 12, 13).

Previously, it was demonstrated that MMP-9 deficiency protects against mortality in an endotoxic shock model in mice and selective MMP-9 blocking was suggested as a possible new therapeutic approach for sepsis (9). However, although this study clearly established the anti-inflammatory potential of MMP-9 inhibition, the endotoxin model does not adequately mimic clinical sepsis since it lacks an infectious source from which bacteria invade the host and cause a systemic inflammatory response syndrome. To our knowledge our study is the first to investigate the role of MMP-9 in host defense against intraabdominal infection, here induced by intraperitoneal injection of *E. coli* resulting in peritonitis with rapid dissemination of the infection and sepsis. Our findings show that MMP-9 plays a pivotal protective role in the host defense against *E. coli*-induced peritonitis and indicate that blocking of MMP-9 may be harmful during abdominal sepsis.

Materials and Methods

Animals and Design

The Institutional Animal Care and Use Committee approved all experiments. MMP-9$^{-/-}$ mice with a FVB/N background and normal FVB/N wild-type (Wt) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). 8 week old female mice were used in all experiment. Peritonitis was induced by i.p.injection of 10^4 CFU *E. coli* O18:K1 as described previously

(14, 15). In one experiment 200 μg LPS (*E.coli* 0111:B4; Sigma, St. Louis, MO) was injected i.p. Peritoneal lavage fluid, blood and organs were harvested and processed for measurements of CFU, leukocyte counts, cytokines and chemokines as described (14, 15).

Evaluation of MMP-9 mRNA levels by quantitative RT-PCR

Total RNA was isolated using the RNeasy Mini Kit system (Qiagen, Venlo, the Netherlands) and treated as described before (16). RT-PCR reactions were performed on cDNA using FastStart DNA Master SYBR Green I (Roche, Woerden, the Netherlands) with 2.5 mM $MgCl_2$ in a LightCycler (Roche) apparatus. PCR conditions were 5 min 95 °C, followed by 95 °C for 15 s, 60 °C for 5 s and 72 °C for 20 s, during 40 cycles. Standard curves were constructed by PCR on serial dilutions of a concentrated cDNA and analyzed using the LightCycler software. Gene expression is presented as a ratio of the expression of the house keeping gene β2-microglobulin (17). Primers for the MMP-9 gene were 5'-TGCATTTCTTCAAGGACGGT-3' (sense) and 5'-CTGACGTGGGTTACCTCTG-3' (antisense) and for the β2 microglobulin gene 5'-TGGTCTTTCTGGTGCTTGTCT-3' (sense) and 5'-ATTTTTTTCCCGTTCTTCAGC-3' (antisense) (all Eurogentec, Seraing, Belgium).

In situ hybridization

MMP-9 specific DIG-labeled riboprobes were prepared by T7 RNA polymerase driven *in vitro* transcription from clone specific PCR products as template. Primers used were 5'-ATT TAG GTG ACA CTA TAG CAG ATG ATG GGA GAG AAG CAG -3' and 5'-TAA TAC GAC TCA CTA TAG GGG CA CCA TTT GAG TTT CAC TA-3', yielding a 563 bp product. The underlined primer regions encode the T7-promotor element. Using the probe, in situ hybridization was performed as described previously (18), using the DIG-labeled riboprobes at a concentration of 300 ng/ml. After hybridization, slides were washed and bound alkaline phosphatase activity was visualized with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate, toluidine salt (NBT/BCIP) (Roche).

Assays

Pro-MMP-9, MIP-2 and keratinocyte-derived cytokine (KC) were measured by ELISA's (R&D systems, Abingdon, UK). TNF-α, IL-6, IFN-γ and IL-10 were measured by cytometric bead array (CBA) multiplex assay (BDBiosciences, San Jose, CA). Aspartate aminotranspherase and alanine aminotranspherase were determined with commercially available kits (Sigma), using a Hittachi analyzer (Boehringer Mannheim, Mannheim, Germany). Myeloperoxidase was measured by ELISA (Hycult biotechnology BV, Uden, The Netherlands).

Phagocytosis of *E. coli* by bone marrow-derived neutrophils

Phagocytosis of *E. coli* by neutrophils was determined as previously described (19) with minor modifications. Neutrophils were isolated from bone marrow of Wt and MMP-$9^{-/-}$ mice using a discontinous Percoll gradient as described (20). The neutrophil enriched fractions consisted of 55.2 ± 5.5 % Gr1-positive cells. Cells were suspended in Hanks' balanced salt solution, plated at 0.5 x 10^6 cells/well and subsequently incubated with 12.5 x 10^6 heat-killed (HK) FITC-labeled *E.coli* (bacterium:cell ratio of 25:1). After incubation for 1 h at 37 or 4 °C, phagocytosis was stopped by immediate transfer of the cells to 4 °C and washing with ice-cold FACS buffer (phosphate-buffered saline supplemented with 0.01%

NaN_3, 0.5% bovine serum albumin, and 0.35 mM EDTA). Cells were treated with vital blue stain (Orpegen, Heidelberg, Germany) to quench extracellular fluorescence, labeled with Gr1-PE (BDBiosciences), washed with FACS buffer, and analyzed using a flow cytometer (FACScalibur, BDBiosciences). Neutrophils were gated based on forward light scatter and Gr1 positivity. Results are expressed as phagocytosis index, defined as the percentage of cells with internalized *E. coli* times the mean fluorescence intensity.

Peritoneal macrophages and whole blood ex vivo stimulation

Peritoneal macrophages from untreated MMP-$9^{-/-}$ and Wt mice were harvested by peritoneal lavage and washed and resuspended in medium (RPMI 1640 containing 10% FCS, 1 mM pyruvate, 2 mM L-glutamine, penicillin, streptomycin). Cells were then incubated in 96-well flat-bottom microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) (1×10^5 cells in 100 µl/well) for 2 h at 37 °C, 5% CO_2 and than washed with medium to remove nonadherent cells. Next, the adherent monolayer cells were stimulated for 20 hours in 100 µl of medium alone or with LPS from *E. coli* (500 ng/ml; Sigma-Aldrich) or HK-*E. coli* (1×10^7 CFU/ml) at 37 °C, 5% CO_2. Supernatants were stored at –20 °C until assayed. Whole blood was collected from untreated MMP-$9^{-/-}$ and Wt mice in heparinized tubes and diluted 1:5 with medium alone or with LPS (200 ng/ml) or HK-*E. coli* (1×10^7 CFU/ml) and incubated in polypropylene tubes for 20 hours at 37 °C, 5% CO_2. Supernatants were stored at –20 °C until assayed.

Histology

Liver and lung samples were fixed with 4% formalin, embedded in paraffin and 4 µm sections were stained with hematoxylin and eosin. All slides were scored by a pathologist without knowledge of the strain of mice according to the following parameters: 1) number of thrombi in 5 random fields, 2) presence and degree of inflammation, 3) for liver: presence and degree of necrosis. Inflammation and hepatic necrosis were rated from 0-3: 0= absent, 1= occasionally, 2= regularly, 3= massively. Granulocyte staining was performed as described previously (21).

Statistical analysis

All data are expressed as mean ± standard error (SE). Differences between groups were analyzed by the Mann-Whitney U test. Values of $P < 0.05$ were considered to represent a statistically significant difference.

Results

MMP-9 is upregulated during *E. coli* induced abdominal sepsis

To evaluate the role of MMP-9 during Gram-negative abdominal sepsis, we used a murine *E. coli* peritonitis model and compared host responses in MMP-$9^{-/-}$ and Wt mice. To confirm MMP-9 production in this model, we measured MMP-9 mRNA levels in liver and lung tissue, and pro-MMP-9 protein concentrations in plasma before and at 6 and 20 hours after the induction of peritonitis. Intraperitoneal injection of 10^4 CFU *E. coli* strongly induced MMP-9 mRNA and protein levels (all $P<0.05$ vs baseline; **figure 1**). To obtain insight into the cellular source of MMP-9 during abdominal sepsis, in situ hybridization was performed

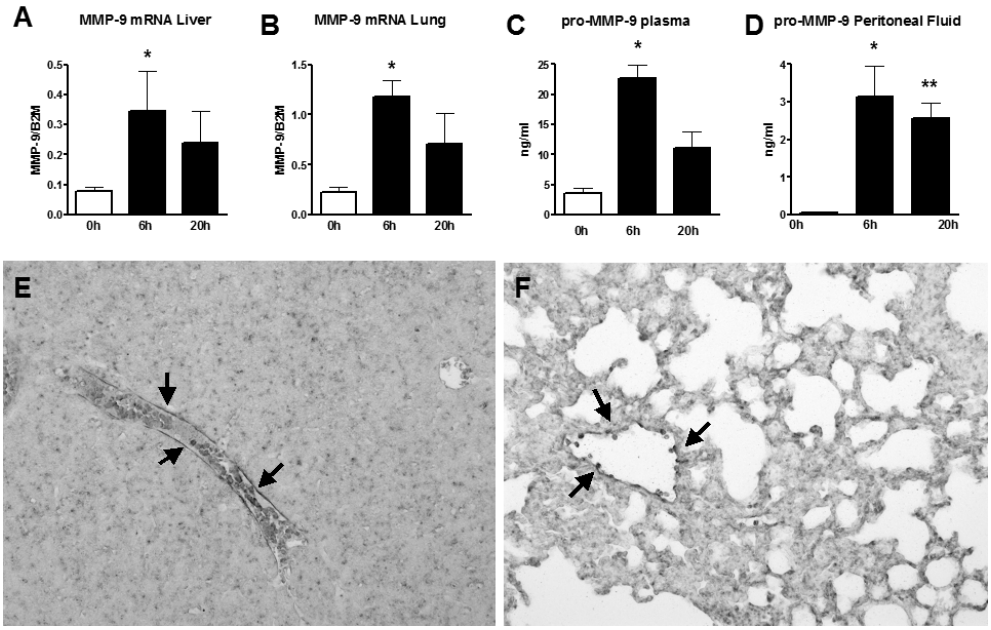

Figure 1. Enhanced MMP-9 production during septic peritonitis. Liver (A) and lung (B) MMP-9 mRNA expression and pro-MMP-9 protein levels in plasma (C) and peritoneal lavage fluid (D). Mice were injected i.p. with 10^4 CFU *E.coli* at 0 hours (h) and were sacrificed before and 6 and 20 h post-infection. Results are expressed as means ± SE of six mice per time point. $^*P < 0.05$, $^{**}P < 0.01$ versus 0h. In situ hybridization for MMP-9 was performed in liver (E) and lung (F) tissues at 6 hours after i.p. injection with 10^4 CFU *E.coli*. Wt mice showed MMP-9 mRNA localization (positive signal in blue) in leukocytes and vascular endothelium. Magnification x200.

on liver and lung tissue at 6 hours after *E. coli* injection (at the time peak pro-MMP-9 levels were detected). Liver and lung tissues both showed MMP-9 mRNA expression, which was co-localized mainly with the endothelium as well as the leukocytes adhering to the inner vessel wall **(figure 1)**.

MMP-9$^{-/-}$ mice have an enhanced bacterial outgrowth

To examine whether MMP-9 deficiency influenced the bacterial outgrowth, we established the number of *E. coli* CFU at 6 and 20 hours postinfection in peritoneal lavage fluid, blood and liver of MMP-9$^{-/-}$ and Wt mice. After 6 hours no differences in bacterial outgrowth were found between both genotypes. However, at 20 hours after infection MMP-9$^{-/-}$ mice had a significantly higher bacterial load in their peritoneal lavage fluid than Wt mice (both $P < 0.05$; figure 2). In addition, blood and liver homogenates of MMP-9$^{-/-}$ also contained more bacteria compared to Wt mice ($P < 0.05$; **figure 2**). Hence, MMP-9$^{-/-}$ mice showed a clearly increased outgrowth of *E.coli* at the primary site of infection, which was associated with an enhanced dissemination of bacteria.

Unchanged phagocytosis of *E. coli* by MMP-9$^{-/-}$ neutrophils in vitro

To investigate whether the increased bacterial outgrowth in MMP-9$^{-/-}$ mice could be the result of an intrinsic defect in the ability of MMP-9$^{-/-}$ neutrophils to phagocytose *E. coli*, we

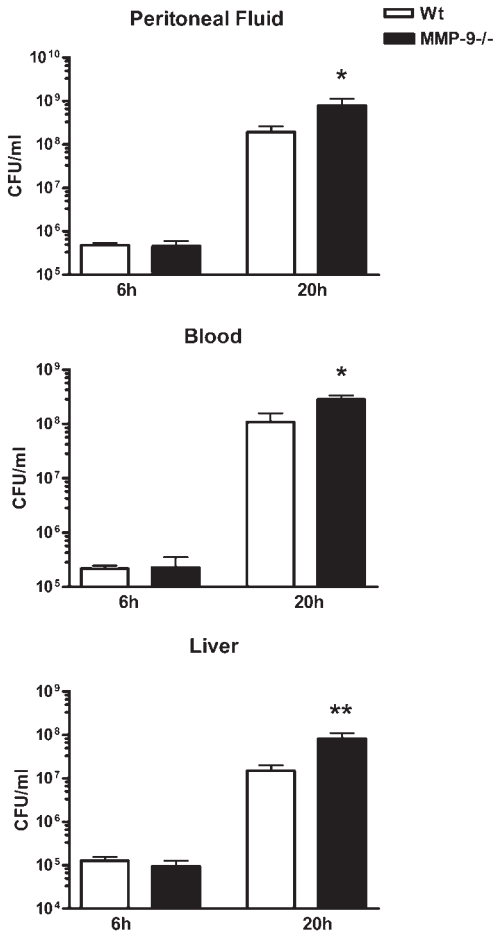

Figure 2. MMP-9$^{-/-}$ mice have an enhanced bacterial outgrowth. Bacterial outgrowth in peritoneal lavage fluid, blood and liver in Wt (open bars) and MMP-9$^{-/-}$ (black bars) mice, at 6 and 20 hours after i.p. injection with 10^4 CFU *E.coli*. Data are means ± SE of eight mice per genotype per time point. **P* < 0.05, ***P* < 0.01 versus Wt mice at the same time point.

isolated bone marrow-derived neutrophils from uninfected MMP-9$^{-/-}$ and Wt mice and compared their capacity to phagocytose HK-*E. coli ex vivo*. Neutrophils from the MMP-9$^{-/-}$ mice displayed a normal ability to phagocytose *E. coli* **(Figure 3)**.

Reduced cell influx into the peritoneal cavity in MMP-9$^{-/-}$ mice

The recruitment of leukocytes to the site of an infection is an essential part of the host defense to invading bacteria. Since MMP-9 has been implicated to play a role in cellular migration (5), probably by degradation of extracellular matrix components, we evaluated the inflammatory cell influx into the peritoneal cavity at 6 and 20 hours after injection of *E. coli* in MMP-9$^{-/-}$ and Wt mice. *E. coli* injection resulted in a profound increase in total leukocyte numbers in the peritoneal lavage fluid, mainly consisting of neutrophils and

Table 1. Cell counts and chemokine levels in peritoneal fluid

	6 h		20 h	
	Wt	MMP-9 $^{-/-}$	Wt	MMP-9 $^{-/-}$
Cells (x 10^4/ml)				
Total cells	17 ± 2.4	16.9 ± 1.9	149 ± 35	65.7 ± 9.5*
Neutrophils	5.3 ± 1.2	5.2 ± 0.7	66.9 ± 26	25.6 ± 12
Macrophages	11.2 ± 1.3	11.3 ± 1.2	82.5 ± 22	40.1 ± 6.7
Chemokines (ng/ml)				
KC	2.0 ± 0.2	1.9 ± 0.4	4.2 ± 1.5	12.7 ± 1.4**
MIP-2	0.12 ± 0.02	0.16 ± 0.04	1.2 ± 0.4	2.5 ± 0.3*

Data are means ± SEM at 6 and 20 h after intraperitoneal injection of 10^4 CFU *E.coli*.
N=8 mice per time point. * $P < 0.05$, ** $P < 0.01$ versus Wt mice at the same time-point.

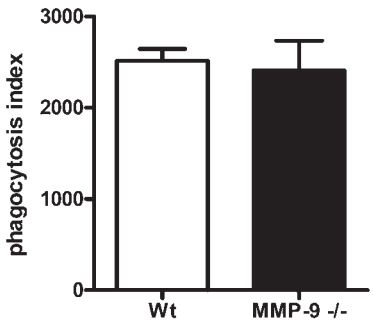

Figure 3. Unchaged phagocytosis of *E. coli* by MMP-9$^{-/-}$ neutrophils. Phagocytosis of HK-*E.coli* by MMP-9$^{-/-}$ and Wt bone marrow-derived neutrophils was compared ex vivo. There was no difference in the mean phagocytosis index between both genotypes, as defined by the percentage of cells with internalized *E. coli* times the mean fluorescence intensity. Data are means ± SE of six mice per genotype.

macrophages **(Table 1)**. MMP-9$^{-/-}$ mice showed a significantly reduced leukocyte influx at 20 hours postinfection ($P < 0.05$; **Table 1**). These data indicate that MMP-9 deficiency is associated with impaired inflammatory cell recruitment to the site of infection.

Higher chemokine and cytokine levels in MMP-9$^{-/-}$ mice

The mouse CXC chemokines KC and MIP-2 have been implicated in the attraction of neutrophils to the site of an infection (22, 23). To investigate whether a difference in local chemokine levels could have influenced the neutrophil influx, we determined MIP-2 and KC levels in peritoneal lavage fluid at 6 and 20 hours after *E.coli* injection in MMP-9$^{-/-}$ and Wt mice. The levels of both chemokines were markedly increased in both mouse strains **(Table 1)**. At 6 hours after infection no differences were found between both mouse strains.

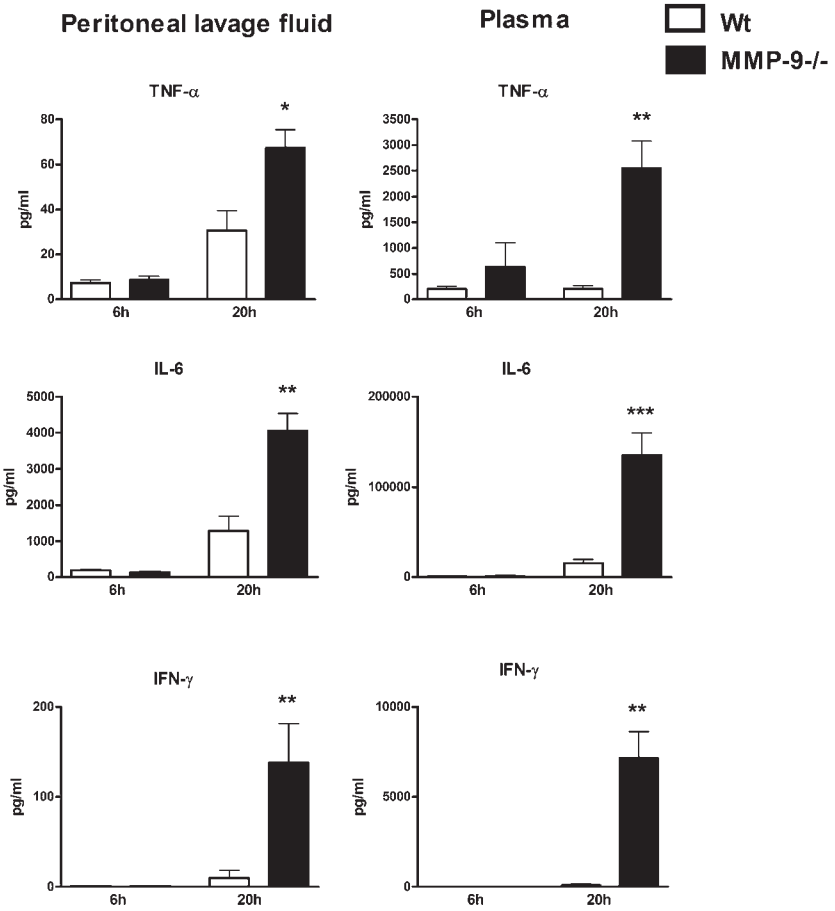

Figure 4. Higher cytokine levels in MMP-9$^{-/-}$ mice during infection. TNF-α, IL-6 and IFN-γ were measured in peritoneal lavage fluid (left panels) and plasma (right panels) at 6 and 20 hours after i.p. injection of 10^4 CFU *E.coli*. Data are means ± SE of eight mice per genotype per time point. *P<0.05, **P < 0.01, ***P < 0.001 versus Wt mice at the same time point.

However, MMP-9$^{-/-}$ mice showed significantly higher levels of MIP-2 and KC than Wt mice at 20 hours postinfection (both $P < 0.05$; **Table 1**), excluding a reduced production of neutrophil attracting chemokines as a possible explanation of the impaired neutrophil influx in MMP-9$^{-/-}$ mice. Furthermore, to determine whether MMP-9 influenced the production of cytokines during septic peritonitis, local and systemic levels of pro- and anti-inflammatory cytokines were measured **(figure 4)**. At 6 hours postinfection, TNF-α, IL-6, IFN-γ and IL-10 levels were similar in peritoneal lavage fluid and plasma of both genotypes (not shown for IL-10); however, after 20 hours, TNF-α, IL-6 and IFN-γ were significantly higher in MMP-9$^{-/-}$ mice than in Wt mice (all $P < 0.05$; **figure 4**). At this time-point the concentrations of IL-10 in peritoneal lavage fluid and plasma were very low and undetectable in most mice (data not shown).

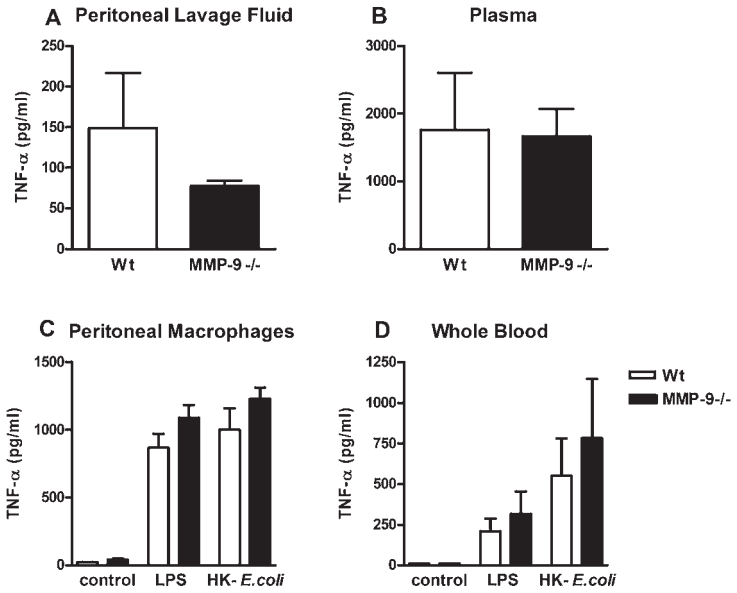

Figure 5. LPS-induced TNF-α release is unchanged in MMP-9$^{-/-}$ mice. TNF-α was measured in peritoneal lavage fluid (A) and plasma (B) at 2 hours after i.p. injection of 200 μ LPS. Ex vivo TNF-α production by peritoneal macrophages (C) and whole blood (D) from untreated MMP-9$^{-/-}$ and Wt mice was also measured at 20 hours after stimulation with LPS and HK-*E.coli*. Data are means ± SE of six mice per genotype.

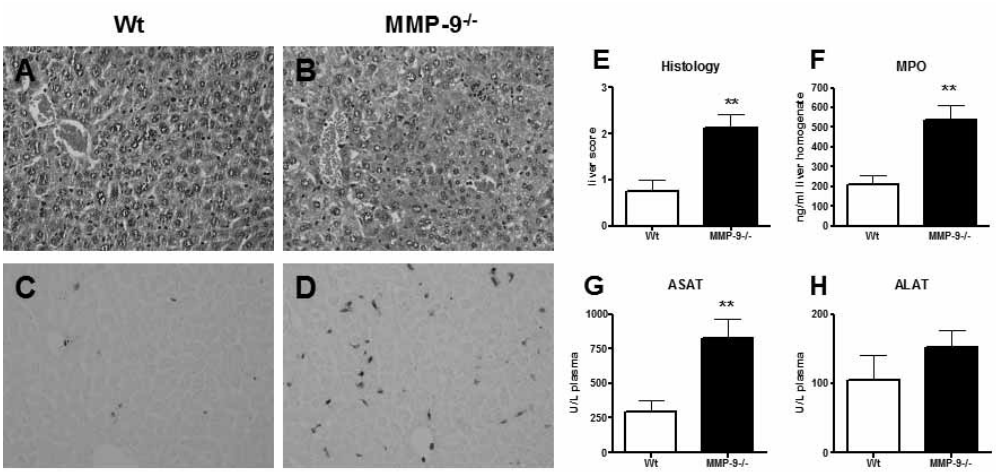

Figure 6. More sever liver damage in MMP-9$^{-/-}$ mice. Mice were sacrificed at 20 hours after i.p. injection of 10^4 CFU *E.coli*. Representative HE (A and B) and neutrophil stainings (C and D) of liver tissue of Wt (left panels) and MMP-9 $^{-/-}$ (right panels) mice. Magnification x200. (E) Graphical representation of the histology scores in Wt (open bars) and MMP-9$^{-/-}$ (black bars) determined according to the scoring system described in the methods. (F) myeloperoxidase (MPO) levels in liver homogenates. Plasma concentrations of aspartate aminotranspherase (ASAT) (G) and alanine aminotranspherase (ALAT) (H). Data are means ± SE of eight mice per genotype. $^{**}P < 0.01$ versus Wt mice.

MMP-9$^{-/-}$ mice show no difference in LPS-induced cytokine release

To investigate whether MMP-9 deficiency directly influences the induction of cytokines in vivo we compared the cytokine response to *E.coli* LPS in MMP-9$^{-/-}$ and Wt mice. LPS was injected i.p. and cytokine release was measured at 2 hours post injection. LPS-induced local (peritoneal lavage fluid) and systemic (plasma) levels of TNF-α, IL-6, IFN-γ and IL-10 injection did not differ between MMP-9$^{-/-}$ and Wt mice (as shown for TNF-α in **Figure 5**). In addition, we examined the cytokine release by MMP-9$^{-/-}$ and Wt peritoneal macrophages and whole blood upon stimulation with LPS and HK-*E.coli* ex vivo. In line, there were no significant differences in cytokine production by MMP-9$^{-/-}$ and Wt peritoneal macrophages or whole blood cultures after 20 hours of stimulation with LPS or HK-*E.coli* (shown for TNF-α in **Figure 5**).

More severe organ damage in MMP-9$^{-/-}$ mice

Our model of *E. coli* peritonitis is associated with profound liver injury (14, 15, 24). To evaluate the role of endogenous MMP-9 in liver injury during abdominal sepsis, we determined liver damage in MMP-9$^{-/-}$ and Wt mice 20 h after infection. Upon histopathological examination **(Figure 6A and B)**, 50% of the Wt mice showed signs of hepatic inflammation. In contrast, all MMP-9$^{-/-}$ mice showed inflammation of the hepatic parenchyma, as characterized by the influx of leukocytes into the interstitium. The mean total histology score of the liver (quantified according to the scoring system described in the Methods section) was significantly higher in the MMP-9$^{-/-}$ mice compared to the Wt mice ($P < 0.01$; **figure 6E**). The histological findings of more severe liver inflammation in MMP-9$^{-/-}$ mice were confirmed by granulocyte stainings of liver sections **(Figure 6C and D)** showing clearly more neutrophil infiltration in MMP-9$^{-/-}$ mice. In line, MMP-9$^{-/-}$ mice had significantly higher myeloperoxidase levels (reflecting the neutrophil content of an organ) in liver homogenates **(Figure 6F)**. Furthermore, the extent of liver injury was also determined by clinical chemistry, i.e. MMP-9$^{-/-}$ mice had significantly higher plasma levels of aspartate aminotranspherase compared to Wt mice ($P < 0.01$; **Figure 6G**). Alanine aminotranspherase levels were also higher in MMP-9$^{-/-}$ mice but the difference with Wt mice did not reach statistical significance **(Figure 6H)**. In conclusion, MMP-9 deficiency was clearly associated with more liver inflammation and damage.

To obtain insight into the role of MMP-9 in the development of inflammation in a more distant organ, lungs were harvested at 20 hours after the induction of *E. coli* infection. 62 % of the lungs of Wt mice showed signs of inflammation compared to all MMP-9$^{-/-}$ mice, as reflected by accumulation of leukocytes in the interstitium and along vessel walls **(Figure 7A and B)**. Again, the total histological scores were higher in the MMP-9$^{-/-}$ mice compared to the Wt mice ($P < 0.05$; **Figure 7E**). Neutrophil stainings showed more neutrophil influx in the lungs of MMP-9$^{-/-}$ mice **(Figure 7C and D)**. Also, myeloperoxidase levels were higher in MMP-9$^{-/-}$ mice than in Wt mice but the difference did not reach statistical significance **(Figure 7F)**.

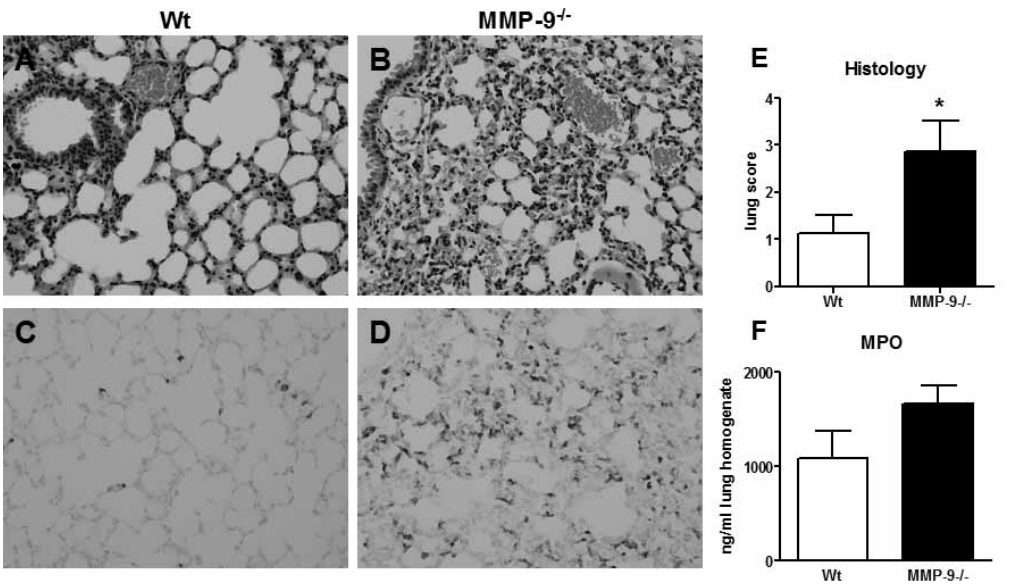

Figure 7. Increased pulmonary inflammation in MMP-9$^{-/-}$ mice. Mice were sacrificed at 20 hours after i.p. injection of 10^4 CFU *E.coli*. Representative HE (A and B) and neutrophil stainings (C and D) of lung tissue of Wt (left panels) and MMP-9 $^{-/-}$ (right panels) mice. Magnification x200. (E) Graphical representation of the degree of pulmonary inflammation and injury in Wt (open bars) and MMP-9$^{-/-}$ (black bars) mice, determined according to the scoring system described in the methods. (F) myeloperoxidase (MPO) levels were measured in lung homogenates by ELISA. Data are means ± SE of eight mice per genotype. *$P < 0.05$ versus Wt mice.

Discussion

MMP-9 has been implicated as an important enzyme during inflammation because of its ability to assist leukocyte trafficking through the extracellular matrix (5). Indeed, MMP-9 was found to stimulate leukocyte migration under the influence of chemotactic factors *in vivo* (25). During local and systemic bacterial infections inflammatory responses play a very important role in fighting the pathogens, but on the other hand can also cause tissue damage. Previously, it was demonstrated that MMP-9 deficiency protects against mortality in an endotoxic shock model in mice and selective MMP-9 blocking was suggested as a possible new therapeutic approach for sepsis (9). However, although this study clearly established the anti-inflammatory potential of MMP-9 inhibition, the endotoxin model does not adequately mimic clinical sepsis since it lacks an infectious source from which bacteria invade the host and cause a systemic inflammatory response syndrome. To our knowledge our study is the first to investigate the role of MMP-9 in host defense against intraabdominal infection, here induced by intraperitoneal injection of *E. coli* resulting in peritonitis with rapid dissemination of the infection and sepsis. The main findings of our study are that MMP-9 deficiency causes an enhanced outgrowth of *E.coli* at the primary site of infection together with increased spreading of bacteria to other body compartments and more severe organ damage. The reduced resistance against *E. coli* in MMP-9$^{-/-}$ mice did

not seem to result from a reduced phagocytosis capacity of the MMP-9$^{-/-}$ neutrophils. However, the impaired recruitment of leukocytes to the peritoneal cavity can, at least in part, explain the reduced host defense against *E.coli* in this model, exemplifying the important role of MMP-9 in leukocyte migration. These findings show that MMP-9 plays a pivotal protective role in the host defense against *E. coli*-induced peritonitis and indicate that blocking of MMP-9 may be harmful during abdominal sepsis. Of note, the earlier finding that MMP-9 deficiency protects against mortality in an endotoxic shock model (9) does not contradict the current finding. Indeed, several studies have indicated that whereas excessive systemic inflammation, such as elicited by administration of high dose endotoxin, can be harmful to the host, an adequate inflammatory response is required to eliminate bacteria in live infection models (26). Similarly, earlier findings that MMP-9$^{-/-}$ mice displayed a better outcome in other models of sterile inflammation (27, 28) does not contradict our current findings, although differences in the genetic background of mice used in different studies might have influenced the results.

MMP's form a family of enzymes which function is mainly the remodeling of extracellular matrix components. MMP-9 is a secreted multidomain enzyme, which cleaves denatured collagens (gelatins) and type IV collagen, which are present in basement membranes. This cleavage helps leukocytes to enter and leave the blood and lymph circulation (5). By the use of MMP-9$^{-/-}$ mice, more evidence has been provided for an in *vivo* role of MMP-9 in the induction of immunopathology in various non-infectious inflammation models. For instance, MMP-9 deficiency had a protective effect in mouse models of autoimmune diseases, like experimental autoimmune encephalomyelitis (27) and complement-mediated inflammation (29). In addition, MMP-9 deficiency impaired cellular infiltration during allergen-induced airway inflammation (28). Furthermore, LPS-induced lethality was reduced by MMP-9 deficiency (9). However, after intratracheal installation of LPS MMP-9$^{-/-}$ mice showed no difference in neutrophil migration to the lungs compared to Wt mice (30). Furthermore, at 4 hours after thioglycolllate-induced peritonitis, MMP-9$^{-/-}$ mice had similar neutrophil counts in their peritoneal cavity (31). These data are in contrast to our current finding that MMP-9$^{-/-}$ mice have a reduced capacity to recruit inflammatory cells to the peritoneal cavity during *E. coli* peritonitis. This discrepancy might be due to the differences between thioglycollate and *E.coli* induced peritonitis. The thioglycollate-induced peritonitis model is a very strong acute inflammatory model whereas the inflammatory cell influx during *E.coli* peritonitis develops much slower. In addition, there might be a difference in cytokine responses and systemic activation of MMP-9 release between the two models. The exact mechanism by which MMP-9 is activated during peritonitis and how it mediates the cellular migration remains to be investigated.

MMP-9 mRNA and pro-MMP-9 protein levels peaked at 6 hours after infection. These findings are in line with other studies showing early upregulation of MMP-9 during experimental sepsis models (9-11). In situ hybridization showed co-localization of MMP-9 mRNA with endothelium and adherent leukocytes to the inner wall of greater vessels. This is in line with previous studies which identified mRNA expression of MMP-9 in leukocytes and endothelial cells (5). Notably, we were unable to detect MMP-9 activity in peritoneal lavage fluid, liver or lung using a zymographic assay, which successfully detected MMP-9

activity in other models (32, 33). Possibly, the dilution in saline of the samples obtained (in particular peritoneal lavage fluid) was to high to enable detection. Considering the evident phenotype of MMP-9$^{-/-}$ mice in *E. coli* peritonitis, these results imply that MMP-9 activity is very low in this model, too low to allow detection by zymography yet clearly sufficient to be biologically active.

Neutrophils constitute an important component of early host defense against bacterial infection. The reduced capacity of MMP-9$^{-/-}$ neutrophils to migrate to the site of the infection was associated with an impaired local antibacterial defense and an enhanced dissemination of the infection. Earlier reports on peritonitis have documented a similar association (34-36), further indicating a protective role of migrating neutrophils. It is quite conceivable that locally produced ELR-positive CXC chemokines, such as MIP-2 and KC, contribute to this characteristic innate immune response (22, 23). In the present study, MMP-9$^{-/-}$ mice had higher local MIP-2 and KC levels during peritonitis, suggesting that even in the presence of a stronger chemotactic gradient MMP-9$^{-/-}$ neutrophils are less capable to migrate toward an intraabdominal infection. Likely, the increased MIP-2 and KC concentrations in MMP-9$^{-/-}$ mice were the result of the higher bacterial load, providing a more potent proinflammatory stimulus. Dubois et al. (9) found that at 16 hours after LPS injection, mRNA expression of cytokines in liver, spleen and kidney were similar between MMP-9$^{-/-}$ and Wt mice. In line with this study, we showed that the LPS-induced cytokine release in peritoneal lavage fluid and plasma was similar between MMP-9$^{-/-}$ and Wt mice in vivo. In addition, there were no differences in LPS and HK-*E.coli* induced cytokine production by MMP-9$^{-/-}$ and Wt peritoneal macrophages or whole blood in vitro. In contrast, in our *E. coli* peritonitis model MMP-9$^{-/-}$ mice had much higher levels of cytokines compared to Wt mice. These differences were most likely due to the increased bacterial load in the MMP-9$^{-/-}$ mice. Furthermore, the increased bacterial load in MMP-9$^{-/-}$ mice resulted in enhanced tissue inflammation and injury, as indicated by histopathology, neutrophil stainings, MPO levels and clinical chemistry. Thus, although MMP-9 deficiency protected against LPS-induced mortality probably by reducing systemic inflammatory cell responses and subsequent tissue damage, MMP-9$^{-/-}$ mice had much more severe tissue damage during *E. coli* induced abdominal sepsis compared to Wt mice. Likely, the differences in the bacterial load overruled the influence of MMP-9$^{-/-}$ deficiency on inflammation in liver and lungs. This may also explain why MMP-9$^{-/-}$ mice had more neutrophils in these organs in spite of a relatively insufficient neutrophil migration to the primary site of infection. It remains to be established why neutrophil migration into the peritoneal cavity was reduced in MMP-9$^{-/-}$ mice, whereas in liver and lungs from these animals more neutrophils accumulated. Little is known about the mechanisms involved in vivo, but our current data suggest that MMP-9 may play a role herein.

The present study is the first to document a protective role for MMP-9 in the host defense against intraabdominal infection. Taken together with the previously reported detrimental role of MMP-9 in endotoxic shock (9), these data further illustrate the existence of a delicate balance between inflammation and anti-inflammation where a certain degree of inflammation is required to combat invading pathogens and exaggerated inflammation can result in severe tissue injury.

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Chapter 9

Absence of thrombin-activatable fibrinolysis inhibitor protects against sepsis-induced liver injury in mice

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Abstract

Thrombin-activatable fibrinolysis inhibitor (TAFI), also known as carboxypeptidase R, has been implicated as an important negative regulator of the fibrinolytic system. In addition, TAFI is able to inactivate inflammatory peptides such as complement factors C3a and C5a. To determine the role of TAFI in the hemostatic and innate immune response to abdominal sepsis, TAFI gene deficient ($TAFI^{-/-}$) and normal wild type (Wt) mice received an intraperitoneal injection with *Escherichia coli*. Liver TAFI mRNA and TAFI protein concentrations increased during sepsis. In contrast to the presumptive role of TAFI as a natural inhibitor of fibrinolysis, $TAFI^{-/-}$ mice did not show any difference in *Escherichia coli*-induced activation of coagulation or fibrinolysis, as measured by plasma levels of thrombin-antithrombin complexes and D-dimer and the extent of fibrin depositions in lung and liver tissues. However, $TAFI^{-/-}$ mice were protected from liver necrosis as indicated by histopathology and clinical chemistry. Furthermore, $TAFI^{-/-}$ mice displayed an altered immune response to sepsis, as indicated by an increased neutrophil recruitment to the peritoneal cavity and a transiently increased bacterial outgrowth together with higher plasma tumor necrosis factor-α and interleukin-6 levels. These data argue against an important part for TAFI in the regulation of the procoagulant-fibrinolytic balance in sepsis and reveals a thus far unknown role of TAFI in the occurrence of hepatic necrosis.

Introduction

Abdominal sepsis is a life-threatening disease with a mortality rate of up to 60% (1). During sepsis the coagulation system is activated, which in severe cases may result in the clinical syndrome of disseminated intravascular coagulation, characterized by microvascular thrombosis and fibrin depositions in many organs (2-4). The fibrinolytic system plays an important role in preserving the microcirculation by degrading fibrin and thrombi at intra- and extravascular sites (2-4). Thrombin-activatable fibrinolysis inhibitor (TAFI), also called carboxypeptidase R, U or B, is a recently discovered inhibitor of the fibrinolytic system (5-7). TAFI is synthesized in the liver and circulates in plasma as a pro-enzyme (7). *In vitro* TAFI can be activated by thrombin (8), thrombin/thrombo-modulin complex (9), plasmin (10), trypsin (11) and elastase (12). Activated TAFI exerts its anti-fibrinolytic action by removing C-terminal lysine and arginine residues from partially degraded fibrin, thereby inhibiting the high-affinity binding of plasminogen to fibrin and the subsequent facilitated conversion into the active protease plasmin (8, 13). In addition, TAFI can also cleave lysine and arginine from inflammatory mediators like complement-derived C3a and C5a, leading to inactivation (14, 15). Complement factors C3a and C5a can induce chemotaxis, adhesion and aggregation of cells during inflammation. Therefore, TAFI could influence the outcome of severe infection not only by inhibiting fibrinolysis, but also by inactivating proinflammatory mediators (14). However, knowledge of the role of TAFI in the host response to severe infection is highly limited. TAFI plasma concentrations were decreased in patients with evidence of coagulation activation in the setting of dengue hemorrhagic fever or other infections (16, 17) and in healthy humans injected with low dose endotoxin (18). In mice and rats, however, endotoxin administration rapidly upregulated TAFI mRNA in the liver (19, 20) resulting in enhanced plasma TAFI activity after 24 hours (19). Furthermore, a DNA polymorphism that increases TAFI stability and activity was associated with a 3.1- fold increased risk to die from meningococcal disease (21). The recent generation of TAFI$^{-/-}$ mice has enabled investigations of the physiological role of this glycoprotein *in vivo* (22-24). Remarkably, TAFI$^{-/-}$ mice do not display bleeding disorders or abnormal clotting tests. In addition, TAFI deficiency did not influence endotoxin-induced lethality in mice (22, 25). However, after LPS sensitization TAFI$^{-/-}$ mice were highly susceptible to cobra venom factor, which activates and depletes the complement system, suggesting an important role of TAFI in complement inactivation *in vivo* (25). Thus far, the function of TAFI during sepsis induced by intact bacteria has not been investigated. Therefore, we here used TAFI$^{-/-}$ mice to determine the role of TAFI in the procoagulant, fibrinolytic, inflammatory and anti-bacterial host responses during abdominal sepsis caused by *Escherichia coli* (*E. coli*), one of the most frequently found pathogens in peritonitis (26).

Materials and Methods

Animals

TAFI $^{-/-}$ mice (backcrossed six times to a C57BL/6 background) were generated as described previously (24). Age and sex-matched C57BL/6 wild type (Wt) mice were purchased from Harlan Sprague Dawley Inc. (Horst, the Netherlands). The mice were kept on a 12 hour light/dark cycle and received food and water ad libitum. TAFI$^{-/-}$ mice have undetectable TAFI mRNA, protein and activity levels (24). TAFI deficiency has no effect on baseline levels of plasminogen (23), α2-antiplasmin, PAI-1 (27), fibrinogen, prothrombin time, activated partial thromboplastin time and blood cell counts (22). The Institutional Animal Care and Use Committee approved all experiments.

Induction of peritonitis

Peritonitis was induced in eight to ten week old male Wt and TAFI$^{-/-}$ mice as described previously (28-30). In brief, *E. coli* O18:K1 was cultured in Luria-Bertani medium (Difco, Detroit, Mich.) at 37°C, harvested at mid-log phase, and washed twice with sterile saline before injection. Mice were injected intraperitoneally (i.p.) with $1x10^4$ colony forming units (CFU) of *E. coli* O18:K1 in 200 µl of sterile isotonic saline.

Sample harvesting

At the time of sacrifice, mice were first anesthetized by inhalation of isoflurane (Abbott Laboratories Ltd., Kent, UK). A peritoneal lavage was then performed with 5 ml of sterile isotonic saline using an 18-gauge needle, and peritoneal lavage fluid was collected in sterile tubes (Plastipack [Becton-Dickinson, Mountain View, CA]). After collection of peritoneal fluid, deeper anesthesia was induced by i.p. injection of 0.07 ml of FFM mixture (fentanyl [0.315 mg/ml]-fluanisone [10 mg/ml] [Janssen, Beersen, Belgium], midazolam [5 mg/ml] [Roche, Woerden, the Netherlands]) per gram body weight. The abdomen was opened, and blood was drawn from the vena cava inferior into a sterile syringe, transferred to tubes containing heparin, and immediately placed on ice. Thereafter, livers and lungs were harvested and processed for RNA isolation, histology and measurements of colony forming units (CFU), cytokines and myeloperoxidase (MPO).

Evaluation of mRNA levels by quantitative RT-PCR

Total RNA was isolated using the RNeasy Mini Kit system (Qiagen) and treated with RQ1 RNase-Free DNase (Promega) and reverse transcribed using oligo (dT) primer and Moloney murine leukemia virus reverse transcriptase (Invitrogen) according to recommendations of the suppliers. RT-PCR reactions were performed on cDNA samples that were 4-fold diluted in H_20 using FastStart DNA Master SYBR Green I (Roche) with 2.5 mM MgCl2 in a LightCycler (Roche) apparatus. PCR conditions were: 5 min 95 °C hot-start, followed by 40 cycles of amplification (95°C for 15 s, 60°C for 5 s, 72°C for 20 s). For quantification standard curves were constructed by PCR on serial dilutions of a concentrated cDNA and data were analyzed using the LightCycler software as described by the manufacturer. Gene expression is presented as a ratio of the expression of the house keeping gene β2-microglobulin (31). All PCR reactions generated a single DNA product of the expected length as judged by evaluation on ethidiumbromide stained

1.2% agarose gel electrophoresis. Primers used for murine TAFI were mTAFI S633 TGGATTTCACCTGCTTTCTGT and mTAFI AS784 GGTTCTTCCTCCACATTCGAT. Primers for the house keeping gene were mB2M S74 TGGTCTTTCTGGTGCTTGTCT and mB2M AS231 ATTTTTTTCCCGTTCTTCAGC. Oligonucleatides were derived from Eurogentec, Seraing, Belgium.

Assays

TAFI and thrombin-antithrombin complexes (TATc) were measured by mouse specific ELISA´s as described previously (24, 28, 32). Protein was measured by the bicinchoninic acid (BCA) assay kit (Pierce, Rockford, Ill). D-dimer was quantitated by a sandwich-type ELISA from Asserachrom D-dimer, Diagnostica Stago, Asnières-sur-Seine, France. Fibrin deposition in lungs and liver was determined by western blot analysis as described previously (33, 34). Aspartate aminotranspherase (ASAT) and alanine aminotranspherase (ALAT) were determined with commercially available kits (Sigma, St. Louis, MO), using a Hitachi analyzer (Roche) according to the manufacturer's instructions. Levels of MPO in lung tissues were measured as described previously (35, 36). Macrophage inflammatory protein (MIP)-2 and keratinocyte-derived chemokine (KC) were measured by ELISA's according to the instructions of the manufacturer (R&D systems, Abingdon, UK). Tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-10 were measured by cytometric bead array (CBA) multiplex assay (BDBiosciences, San Jose, CA) in accordance with the manufacturer's recommendations. For these cytokine measurements livers were homogenized at 4^{0} C in 5 volumes of sterile isotonic saline with a tissue homogenizer (Biospect Products, Bartlesville, OK), which was carefully cleaned and desinfected with 70% ethanol after each homogenization. The liver homogenates were lysed in lysis buffer (300 mM NaCl, 15 mM Tris [tris(hydroxymethyl)aminomethane], 2 mM MgCl, 2 mM Triton X-100, pepstatin A, leupeptin, and aprotinin [20 ng/mL], pH 7.4) on ice for 30 minutes and spun at 1500g at 4 C for 15 min. The supernatant was frozen at – 20°C until assayed. Serum amyloid P was measured in plasma by a sandwich ELISA as described previously (37, 38).

Histology

Directly after sacrifice liver and lung samples were fixed with 4% formalin and embedded in paraffin. 4-µm thick paraffin sections were stained with hematoxylin and eosin. All slides were coded and scored by a pathologist without knowledge of the strain of mice. The liver and lungs were scored according to the following parameters: (1) the number of thrombi counted in 5 fields at a magnification of x200 (lungs) or x100 (liver), (2) the presence and degree of inflammation, which included the interstitial influx of leukocytes and the presence of endothelialitis, (3) for liver only: the presence and degree of necrosis. Inflammation and hepatic necrosis were rated from 0-3, wherein 0= absent, 1= occasionally, 2= regularly, 3= massively. Granulocyte immunostaining was performed as described previously (39, 40). Granulocytes were counted in 5 random fields (magnification x 200).

Cell counts and differentials

Cell counts were determined in peritoneal lavage fluid using a hemacytometer (Beckman Coulter, Fullerton, CA). Subsequently, the pellet was diluted in phosphate-buffered saline

to a final concentration of 10^5 cells/ml and differential cell counts were performed on cytospin preparations stained with a modified Giemsa stain (Diff-Quick; Dade Behring AG, Düdingen, Switzerland).

Determination of bacterial outgrowth

Lungs and livers were homogenized at 4 ^{0}C in 5 volumes of sterile isotonic saline with a tissue homogenizer (Biospect Products) which was carefully cleaned and desinfected with 70% ethanol after each homogenization. Serial 10-fold dilutions in sterile saline were made from these homogenates, blood and peritoneal lavage fluid, and 50 µl volumes were plated onto sheep-blood agar plates and incubated at 37^{0}C and 5% CO_2. CFU were counted after 16 hours.

Statistical analysis

Differences between groups were calculated by using the Mann-Whitney U test. Values were expressed as means ± SE. A *P* value of <0.05 was considered statistically significant.

Results

TAFI is upregulated during *E. coli*-induced abdominal sepsis

To determine whether TAFI expression changes during peritonitis, we measured TAFI mRNA in liver and lung samples at 0, 2, 6 and 20 hours after i.p. injection of *E.coli*. We chose these time-points for our experiments since they represent two stages in the clinical course of the disease; the beginning of the infection (2 and 6 hours) without clinical symptoms and the end-stage of the infection (20 hours) at which the mice are very ill. At 20 hours after infection TAFI mRNA was significantly increased in the livers of Wt mice (**Figure 1A**; $P < 0.05$); in the lungs TAFI mRNA remained undetectable throughout. To investigate whether the increased TAFI expression resulted in a rise in protein levels we measured the concentration of TAFI antigen in liver homogenates, plasma and peritoneal lavage fluid. In liver homogenates TAFI protein levels were slightly decreased at 2 hours postinfection (non significant), however at 20 hours postinfection TAFI levels were significantly upregulated (**Figure 1B**; $P < 0.05$). At 6 hours after *E. coli* injection, plasma TAFI levels showed a decrease of 20 % versus t= 0. However, after 20 hours TAFI levels were increased by 2.8-fold versus t= 0 (**Figure 1C**; $P < 0.01$). TAFI remained undetectable in peritoneal lavage fluid at all time-points.

TAFI deficiency does not influence the hemostatic response

Thrombin is a very important activator of TAFI and its levels rise during coagulation activation. Our group previously established that this model of abdominal sepsis is associated with thrombin generation and activation of the coagulation system (28). To determine the influence of TAFI hereon, we measured TATc levels in plasma of TAFI$^{-/-}$ and Wt mice before and 6 and 20 hours after i.p. injection of *E.coli*. TATc concentrations were unchanged at 6 hours post infection when compared to uninfected mice (data not shown). At 20 hours, TATc were significantly elevated in plasma of TAFI$^{-/-}$ and Wt mice; however, TATc levels were not different between the two mouse strains **(Figure 2A)**. TAFI can

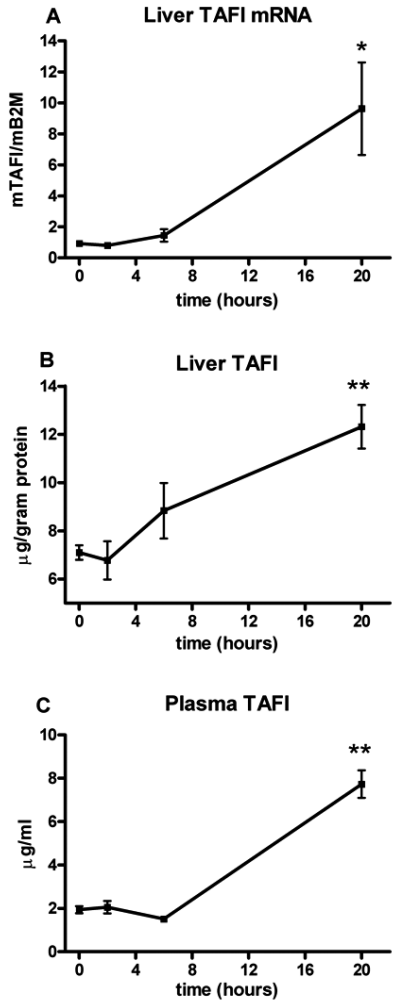

Figure 1. Enhanced TAFI production during septic peritonitis. (A) Liver TAFI mRNA expression was determined by RT-PCR. TAFI protein levels were measured by ELISA in liver homogenates (B) and plasma (C). Mice were injected i.p. with 10^4 CFU *E.coli* at t= 0 and were sacrificed before and 2, 6 and 20 hours post-infection. Results are expressed as means ± SE of four mice per time point. * $P<0.05$, ** $P<0.01$ versus t = 0.

inhibit fibrinolysis by preventing the binding of plasminogen to fibrin and the subsequent facilitated conversion into the active protease plasmin (8, 13). To investigate whether the upregulation of TAFI production during abdominal sepsis influenced the fibrinolytic activity we measured D-dimer. Plasma D-dimer levels were strongly elevated at 20 hours after *E. coli* injection, but no differences were observed between TAFI$^{-/-}$ and Wt mice **(Figure 2B)**. To measure the extent of fibrin deposition in liver and lung tissue in TAFI $^{-/-}$ and Wt mice, these organs were harvested before and at 20 hours after the induction of *E. coli* infection. Western blot analysis for fibrin showed increased fibrin accumulation in liver

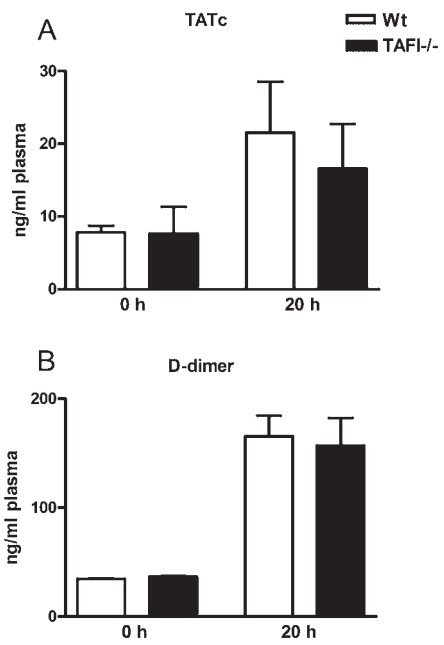

Figure 2. TAFI deficiency does not influence the hemostatic response. Plasma TATc (A) and D-dimer (B) levels were measured by ELISA at 0 and 20 hours post-infection. Mice were injected i.p. with 10^4 CFU *E.coli* at t= 0. Results are expressed as means ± SE of eight mice per time point. There were no statistical differences between both strains.

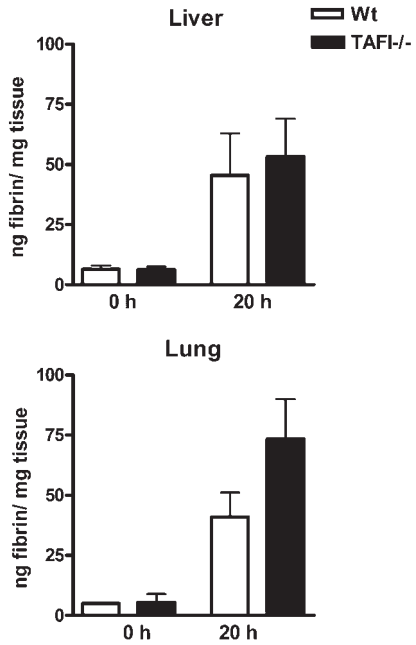

Figure 3. TAFI deficiency does not influence the extent of fibrin deposition in liver and lung tissue. Western blot was performed for fibrin deposition in liver and lung tissue of Wt and TAFI mice before and at 20 hours after *E. coli* injection. Fibrin standards were used to quantify fibrin deposition. Data are means ± SE. There were no statistical differences between both strains.

and lung tissue in animals with peritonitis, but the extent of fibrin deposition did not differ between TAFI$^{-/-}$ and Wt mice **(Figure 3)**. Together these data indicate that TAFI deficiency does not influence coagulation or fibrinolysis during *E. coli* sepsis.

TAFI deficient mice are protected from liver damage

This experimental model of abdominal sepsis is associated with profound liver injury (28-30). Considering the strong expression of TAFI in the liver and the possible role of TAFI in fibrinolytic and inflammatory responses, which probably play a role in the induction of organ damage, we examined the influence of TAFI deficiency on liver damage 20 hours post infection. Upon histopathological examination, both TAFI$^{-/-}$ and Wt mice showed mild inflammation of liver tissue as characterized by the influx of leukocytes into the hepatic parenchyma **(Figure 4A and B)**. The numbers of infiltrating granulocytes (as determined by immunostaining of liver sections) were similar in TAFI$^{-/-}$ and Wt mice **(Figure 4C)**, indicating that TAFI deficiency did not impact on the extent of hepatic inflammation.
In accordance with our previous investigations (28-30), all Wt mice showed foci of liver necrosis, which were predominantly localized in the midlobular region of the liver lobule, whereas the periportal area was unaffected **(Figure 4A, arrows)**.

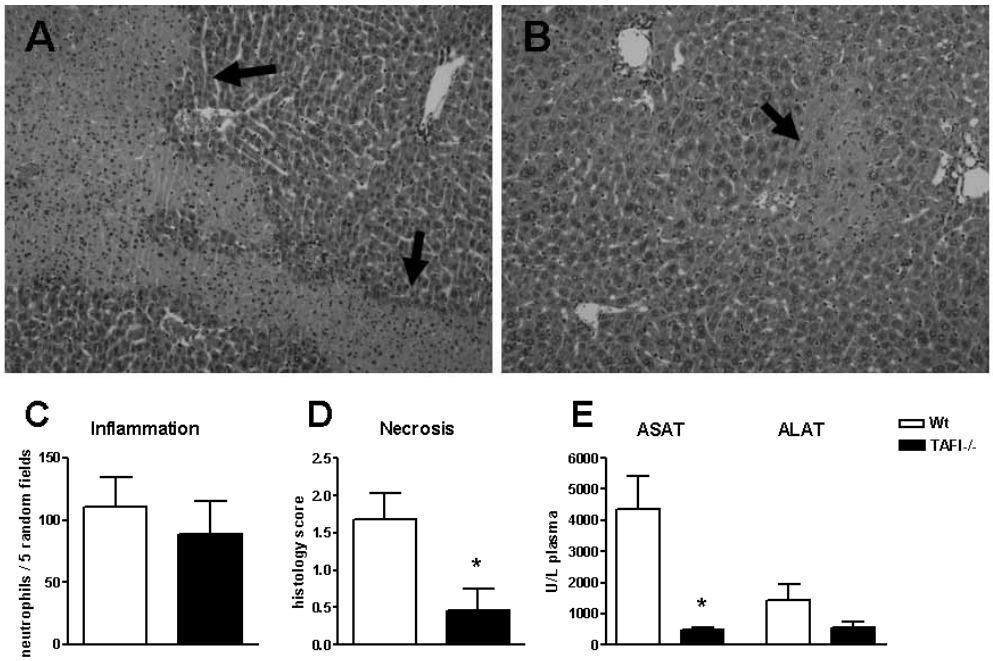

Figure 4. TAFI $^{-/-}$ mice are protected from liver necrosis. Livers were harvested at 20 hours after i.p. injection of 10^4 CFU *E.coli*. Representative HE stainings of liver sections from Wt (A) and TAFI $^{-/-}$ (B) mice are shown. Arrows point out the necrotic areas. Original magnification x100. (C) Granulocyte stainings of liver sections were performed and the numbers of neutrophils per 5 random microscopic fields were counted. Magnification x200. (D) Graphical representation of the degree of liver necrosis determined according to the scoring system described in the methods. (E) Plasma concentrations of ASAT and ALAT were measured at the same time-point. Data are expressed in units/liter (U/L). Data are means ± SE of eight mice per genotype. * P<0.05 versus Wt mice.

Remarkably, only 42% of TAFI $^{-/-}$ mice had any sign of liver necrosis **(Figure 4B, arrow)**. Furthermore, the extent of liver necrosis (quantified according to the scoring system described in the Methods section) was also markedly reduced in TAFI $^{-/-}$ mice (**Figure 4D**; $P < 0.05$). The relative protection of TAFI$^{-/-}$ mice against liver necrosis was confirmed by clinical chemistry, i.e. TAFI $^{-/-}$ mice had lower plasma levels of ALAT and ASAT compared to Wt mice **(Figure 4E)**.

TAFI deficiency does not influence lung inflammation

To obtain insight into the role of TAFI in the development of inflammation in another organ even more susceptible to inflammation-induced injury, lungs were harvested at 20 hours after the induction of *E. coli* infection. Lungs showed clear signs of inflammation in both TAFI$^{-/-}$ and Wt mice, as reflected by accumulation of leukocytes in the interstitium **(Figure 5A and B)**. Histological scores were similar in both strains **(Figure 5C)**. Granulocyte stainings of lung tissues revealed a similarly strong accumulation of neutrophils in lungs of both mouse strains (data not shown). In line, MPO levels in lung homogenates were similar in TAFI$^{-/-}$ and Wt mice before and at 6 and 20 hours postinfection **(Figure 5D)**. Thus, TAFI deficiency did not influence the inflammatory response in the lung during *E. coli* sepsis.

Table 1. TAFI deficiency results in an enhanced neutrophil recruitment

x 10^4/ml PLF	6 h		20 h	
	Wt	TAFI$^{-/-}$	Wt	TAFI$^{-/-}$
Total cells	73 ± 13	75 ± 14	394 ± 38	742 ± 101*
Neutrophils	62 ± 10	65 ± 13	272 ± 42	543 ± 72*
Macrophages	10 ± 5.4	8.6 ± 1.5	114 ± 16	183 ± 32
Lymphocytes	1.1 ± 0.4	1.3 ± 0.4	7.8 ± 3.3	14.9 ± 2.5

Data are means ± SEM at 6 and 20 h after intraperitoneal injection of 10^4 CFU *E.coli*.
N= 8 mice per time-point. PLF, peritoneal lavage fluid. *P< 0.05 versus Wt mice at the same time-point.

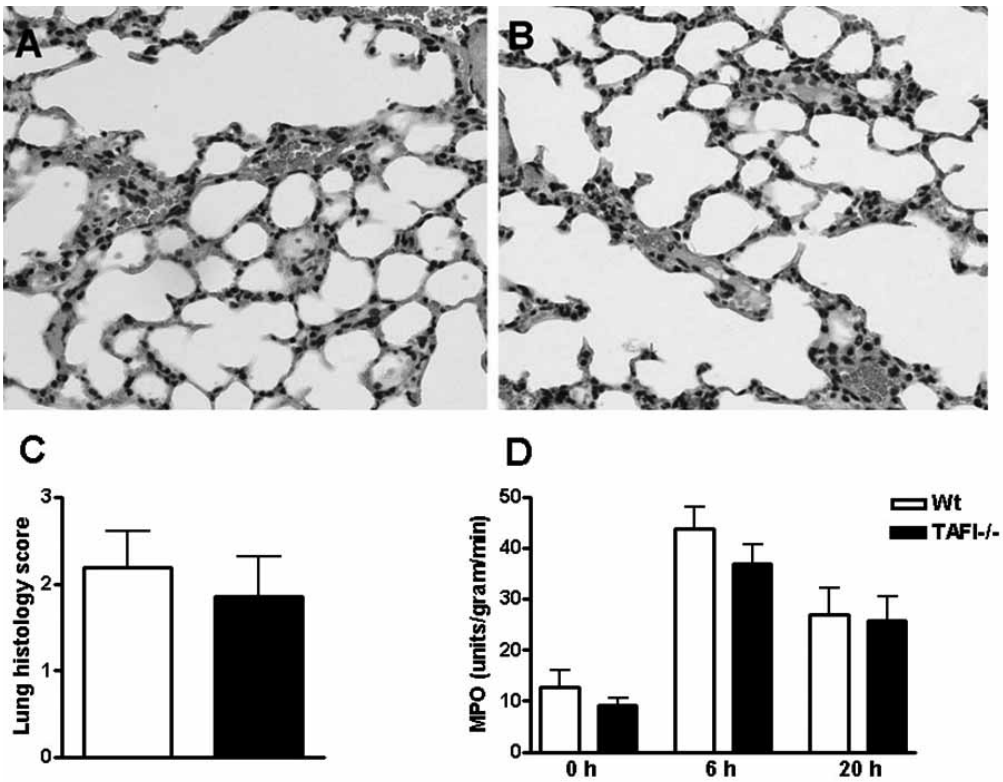

Figure 5. No difference in pulmonary inflammation. Representative HE stainings of lung tissue at 20 hours after i.p. injection of 10^4 CFU *E.coli* in Wt (A) and TAFI $^{-/-}$ (B) mice. Original magnification x200. (C) Graphical representation of the degree of lung inflammation at the same time-point, determined according to the scoring system described in the methods. (D) Myeloperoxidase (MPO) activity levels in lung tissues were determined before and at 6 and 20 hours postinfection. Data are means ± SE of eight mice per genotype. There were no statistical differences in lung inflammation between both strains.

Table 2. Chemokine and cytokine concentrations

		6h		20 h	
		Wt	TAFI $^{-/-}$	Wt	TAFI$^{-/-}$
Chemokines (ng/ml)					
MIP-2	PLF	ND	ND	4.7 ± 1.2	5.4 ± 1.4
KC	PLF	0.6 ± 0.1	0.7 ± 0.1	7.4 ± 2.1	6.5 ± 1.9
Cytokines (pg/ml)					
TNF-α	PLF	10 ± 3	20 ± 5	228 ± 47	346 ± 67
	Plasma	244 ± 52	439 ± 72*	662 ± 158	862 ± 226
	Liver	23 ± 7	32 ± 4	16 ± 3	11 ± 2
IL-6	PLF	168 ± 76	394 ± 138	16 ± 5	18 ± 6
	Plasma	474 ± 178	1064 ± 146*	67 ± 22	100 ± 34
	Liver	75 ± 13	60 ± 11	501 ± 106	592 ± 161
IL-10	PLF	22 ± 2	29 ± 3	224 ± 58	353 ± 79
	Plasma	ND	ND	30 ± 8	33 ± 18
	Liver	135 ± 34	157 ± 23	73 ± 19	91 ± 17
APP (μg/ml)					
SAP	Plasma	16 ± 2.7	16 ± 3.4	68 ± 14	182 ± 37*

Data are means ± SEM at 6 and 20 h after intraperitoneal injection of 10^4 CFU *E.coli*.
N= 8 mice per time-point. ND, not detectable. PLF, peritoneal lavage fluid. APP, acute phase protein. SAP, serum amyloid P. * $P < 0.05$ versus Wt mice.

TAFI deficiency enhances neutrophil recruitment to the peritoneal cavity

The recruitment of leukocytes to the site of an infection is an essential part of the host defense to invading bacteria. Therefore, we determined leukocyte counts and differentials in peritoneal lavage fluid 6 and 20 hours after *E.coli* injection in TAFI $^{-/-}$ and Wt mice. Peritonitis was associated with a profound rise in the number of cells in the peritoneal lavage fluid, which was mainly the result of neutrophil influx into the peritoneal cavity. TAFI $^{-/-}$ mice had two times higher neutrophil counts in their peritoneal fluid than Wt mice at 20 hours post infection (**Table 1**; $P < 0.05$).

Chemokines, cytokines and serum amyloid P

CXC chemokines play a role in the attraction of neutrophils to the site of inflammation. To investigate whether the increased neutrophil influx in TAFI$^{-/-}$ mice might be the result of an effect on local chemokine levels, the concentrations of MIP-2 and KC were measured in the peritoneal lavage fluid at 6 and 20 hours after *E. coli* injection. However, MIP-2 or

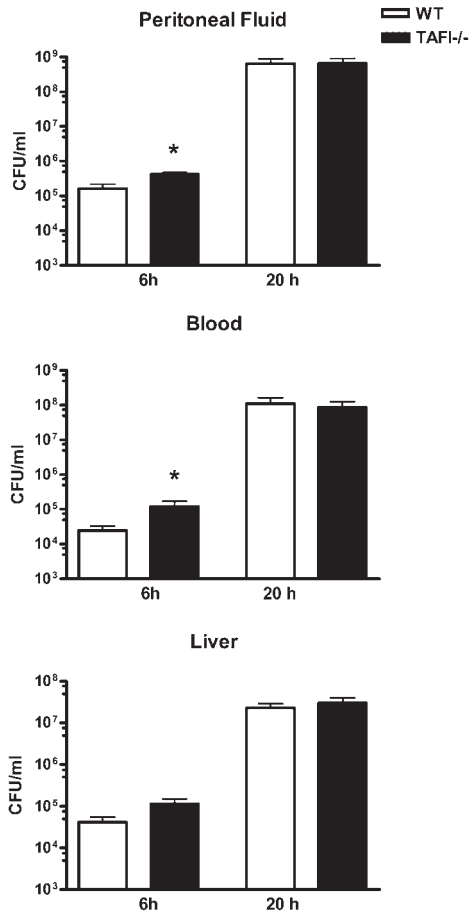

Figure 6. Bacterial outgrowth. Bacterial outgrowth in peritoneal lavage fluid, blood and liver in Wt (open bars) and TAFI$^{-/-}$ (black bars) mice, at 6 and 20 hours after i.p. injection with 10^4 CFU *E.coli*. Eight mice per genotype were used at each time point. * $P<0.05$ versus Wt at the same time point.

KC levels were similar in TAFI $^{-/-}$ and Wt mice at both time-points **(Table 2)**. To determine whether TAFI influenced the local or systemic production of cytokines during septic peritonitis, TNF-α, IL-6 and IL-10 were measured in peritoneal lavage fluid, plasma and liver homogenates. Cytokine levels in the peritoneal lavage fluid tended to be higher in TAFI $^{-/-}$ mice, but these differences did not reach statistical significance **(Table 2)**. At 6 hours post infection, TAFI $^{-/-}$ mice had significantly higher plasma levels of TNF-α and IL-6 compared to Wt mice (**Table 2**; both $P<0.05$); at 20 hours plasma cytokine levels were similar in both mouse strains. There were no differences between the two mouse strains in plasma IL-10 levels at either time point **(Table 2)**. In liver homogenates all cytokine concentrations were similar on both time-points **(Table 2)**. To examine whether the acute phase protein response was influenced by the difference in early IL-6 levels between TAFI$^{-/-}$ and Wt mice during *E. coli*-induced abdominal sepsis, we measured the murine acute phase

protein serum amyloid P in plasma at 20 hours postinfection. Circulating serum amyloid P concentrations were increased in both strains but much higher in TAFI$^{-/-}$ mice than in Wt mice **(Table 2)**.

TAFI deficiency is associated with a transiently enhanced bacterial outgrowth

To determine whether TAFI influences antibacterial host defense, we compared the bacterial load in peritoneal lavage fluid, blood and liver of TAFI $^{-/-}$ and Wt mice at 6 and 20 hours post infection. At 6 hours TAFI $^{-/-}$ mice had more bacteria in their peritoneal lavage fluid and blood than Wt mice (**Figure 6**; $P < 0.05$), whereas the bacterial loads in liver tended to be higher in TAFI$^{-/-}$ mice (not significant). After 20 hours bacterial loads had increased strongly in both mouse strains and no significant differences in the number of *E. coli* CFU existed anymore between TAFI $^{-/-}$ and Wt mice **(Figure 6)**.

Discussion

The clinical syndrome of sepsis is the result of a systemic response of the host to a severe infection, characterized by the concurrent activation of various mediator systems. TAFI is a carboxypeptidase B-type enzyme that may function as an important regulator at the cross road of coagulation, fibrinolysis and inflammation. We here determined the role of endogenous TAFI in the host response to abdominal sepsis induced by i.p. injection of *E. coli*. In contrast to its presumptive role as a natural inhibitor of fibrinolysis, TAFI appeared to have no part in the procoagulant or fibrinolytic response to Gram-negative sepsis. Instead, the present investigation links the local production of TAFI in the liver with the occurrence of hepatic necrosis, as indicated by abundant expression of TAFI mRNA and protein in the livers of Wt mice and a strongly reduced occurrence and severity of hepatic necrosis in TAFI$^{-/-}$ mice. Furthermore, we show for the first time a role for TAFI in inflammatory cell recruitment to the site of infection *in vivo*, since the TAFI$^{-/-}$ mice had a markedly increased number of inflammatory cells in their peritoneal cavity.

TAFI mRNA is strongly expressed in the liver of humans, mice and rats (7, 19, 20). Hepatic TAFI mRNA expression rapidly increased after endotoxin administration to rodents (19, 20) and TAFI plasma levels were elevated during peritonitis induced by cecal ligation and puncture in rats (41). In line with these previous studies we found that TAFI mRNA was expressed in the liver and became upregulated during *E. coli* induced peritonitis, which was accompanied by elevated TAFI protein levels in liver and plasma. In humans, TAFI protein levels vary in different diseases. In contrast to murine sepsis, decreased TAFI levels have been found during infections in humans (16, 17), which might be the consequence of enhanced consumption. Furthermore, in healthy humans exposed to low dose endotoxin circulating TAFI levels decreased 16 % relative to baseline (18). We found a decrease of 20% in the early phase after infection. However, later on plasma TAFI levels showed a profound rise of 2.8-fold relative to baseline. Possibly, the low-grade human endotoxemia model was too mild to induce this secondary increase in TAFI concentrations; otherwise it is possible that the response differs between humans and mice. Of note, TAFI plasma levels were elevated and correlated with acute phase proteins in other non infectious

human diseases, including inflammatory diseases like inflammatory bowel diseases, Reiter's syndrome, Behcet's syndrome and celiac disease (42, 43), and also during coronary artery disease (44) and ischemic stroke (45).

In line with previous findings (28), *E. coli* peritonitis was associated with activation of the coagulation system as reflected by a rise in TATc concentrations in plasma, and increased fibrin deposition in liver and lungs. Although TAFI deficiency is not expected to influence TATc levels, it would impact on fibrin deposition if TAFI plays a significant role as an inhibitor of fibrinolysis in sepsis *in vivo*. *In vitro*, activated TAFI can inhibit plasmin formation by removing the plasminogen binding sites on fibrin, an effect by which it might promote thrombus formation. In accordance, previous *in vivo* studies have shown that activated TAFI plays an important role in the susceptibility of a clot to lysis, i.e. inhibitors of active TAFI increased endogenous thrombolysis in experimental jugular vein and arterial thrombolysis models in mice and rabbits (46-48). Furthermore, increased TAFI antigen levels are associated with a mild risk for thrombosis (48). Surprisingly, TAFI$^{-/-}$ mice did not show any differences in venous or arterial thrombosis models or in mortality induced by acute pulmonary thromboembolism or LPS-induced disseminated intravascular coagulation (22-24). In line, our study demonstrates that during abdominal sepsis TAFI$^{-/-}$ mice display an unaltered activation of the fibrinolytic system as reflected by the plasma levels of D-dimer and did not show a decreased tendency to form fibrin depositions in either liver or lungs. Together these data strongly argue against an important role for endogenous TAFI as regulator of the procoagulant-fibrinolytic balance in sepsis.

Surprisingly, TAFI$^{-/-}$ mice showed significantly less and smaller foci of liver necrosis and decreased plasma transaminase levels indicating less hepatocellular injury. These data suggest that TAFI deficiency protects against sepsis-induced liver necrosis and possibly explain the distribution pattern of foci within the liver of Wt mice, which coincides with the lobular distribution pattern of TAFI mRNA, showing highest expression in the pericentral and midlobular area of the liver unit (49). The periportal area, in which TAFI expression is low or even absent, was protected from sepsis-induced liver injury. Since TAFI deficiency was not accompanied with changes in fibrinolytic or coagulant responses in our model, these differences in liver necrosis seem independent of TAFI's anti-fibrinolytic properties. Campbell et al. established that TAFI can remove arginine from the carboxyterminus of C3a and C5a, which inactivates these complement factors (14, 15). Interestingly, recent studies using C3a and C5a gene-deficient mice have shown that these complement factors play a very important protective role in acute liver necrosis induced by carbon tetrachloride or partial hepatectomy (50-52). Since TAFI deficient mice might have a higher C3a and C5a activity, this could play a role in their relative protection from liver necrosis in our model. To directly demonstrate an effect of TAFI deficiency on complement analytical separation of intact C5a and C3a from C5a and C3a that lack an arginine residue would be required; mere measurement of these complement factors by ELISA would not suffice. Unfortunately, at present the technique required for such analyses is not operational in our laboratory. In addition, we are currently not able to reliably measure complement activity in mice. Besides complement products cytokines, including TNF and

IL-6, have been implicated as mediators of liver inflammation and injury (53); however the concentrations of TNF and IL-6 did not differ in liver homogenates prepared from TAFI$^{-/-}$ and Wt mice after induction of abdominal sepsis. More studies are needed to investigate the mechanism by which TAFI impacts on liver injury during sepsis.

Peritonitis is characterized by recruitment of leukocytes to the site of infection (28-30). TAFI is able to inactivate complement-derived inflammatory peptides C5a and C3a, which are involved in leukocyte chemotaxis (14, 15). Furthermore, an increased number of leukocytes was found in the peritoneal cavity after i.p. injection of thioglycollate in TAFI$^{-/-}$ mice superimposed on a partial plasminogen deficient setting (23). We found that neutrophil influx into the peritoneal cavity was markedly increased in TAFI$^{-/-}$ mice at 20h after infection. Besides neutrophils C5a is also able to attract macrophages and lymphocytes (54, 55). In our study the numbers of macrophages and lymphocytes were also increased in TAFI$^{-/-}$ mice compared to Wt mice, however this difference was not significantly different. This is the first *in vivo* evidence of a specific role of TAFI in the attraction of leukocytes to the site of an infection. It has been suggested that TAFI might play a protective role during Gram-negative sepsis by preventing hyper-reactivity of the inflammatory response and subsequent septic shock (15). However, we found no effect of TAFI deficiency on the inflammatory responses in the liver and lungs.

Since the inflammatory response of phagocytic cells can influence anti-bacterial host defense, we investigated the bacterial outgrowth in both genotypes. We found an increased bacterial load in peritoneal lavage fluid and blood in TAFI$^{-/-}$ mice at 6 hours after *E. coli* injection. A clear explanation for this finding is lacking; it cannot be explained by differences in the inflammatory response because the cellular influx to the peritoneal cavity was similar at that time point. Possibly, TAFI contributes to fibrin-mediated intraperitoneal adhesion formation, which can wall off the infection and prevent early local and systemic bacterial spread (56-58). The difference in bacterial load disappeared after 20 hours, probably due to the overwhelming local and systemic bacterial outgrowth overruling the modest effect of TAFI. Considering that there is no evidence that TAFI can influence cytokine production, the elevated plasma levels of TNF-α and IL-6 in TAFI$^{-/-}$ mice at 6 hours postinfection were most likely the consequence of the presence of an increased bacterial load in blood, providing a more potent proinflammatory stimulus.

Intraperitoneal administration of *E. coli* results in a paradigm that resembles the clinical condition commonly associated with septic peritonitis, with diaphragmatic lymphatic clearance, systemic bacteremia and profound activation of coagulation and inflammation (28-30, 59). To our knowledge this is the first study to show an *in vivo* role for TAFI in a bacterial sepsis model. We demonstrate that TAFI does not play a role in facilitating the development of fibrin depositions in organs during *E. coli*- induced septic peritonitis. Rather, the enhanced production of TAFI in the liver during abdominal sepsis significantly contributed to the occurrence of hepatocellular necrosis. These data point to a thus far undiscovered function of endogenous TAFI in the liver during severe bacterial infection.

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Chapter 10

Urokinase-type plasminogen activator receptor plays a role in neutrophil migration during LPS induced inflammation, but not during *Escherichia coli* peritonitis

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Abstract

Urokinase-type plasminogen activator receptor (uPAR) is expressed on many different cells, including leukocytes. uPAR has been implicated to play a role in the migration of neutrophils to sites of inflammation. To determine the role of uPAR in neutrophil recruitment in response to bacterial products or intact bacteria, uPAR gene-deficient ($uPAR^{-/-}$) and wild-type (Wt) mice were injected intraperitoneally with either *Escherichia coli* or lipopolysaccharide (LPS) derived from this bacterium. $uPAR^{-/-}$ mice demonstrated a reduced LPS-induced influx of neutrophils into peritoneal lavage fluid, while the chemokine and cytokine response was unaltered. In contrast, during *E. coli* peritonitis $uPAR^{-/-}$ mice showed a normal neutrophil influx to the primary site of infection. The unaltered neutrophil trafficking in $uPAR^{-/-}$ mice during bacterial infection was corroborated by histology of liver and lung tissue and myeloperoxidase measurements in tissue homogenates. $uPAR^{-/-}$ mice displayed modestly but significantly lower bacterial loads in the peritoneal cavity together with a reduced dissemination to the circulation early in the infection. These data suggest that uPAR in part mediates neutrophil migration into the peritoneal cavity upon local instillation of LPS, but that this function of uPAR can be compensated for during abdominal infection caused by intact *E. coli*.

Introduction

Effective recruitment of inflammatory cells to the site of an infection is of crucial importance for an adequate host defense against invading microorganisms. The urokinase type plasminogen activator receptor (uPAR; CD87) has been implicated to play an important role in this process (1, 2). UPAR is a high affinity receptor for urokinase-type plasminogen activator (uPA), which is expressed by many different cell types including leukocytes, endothelial cells and tumor cells. The expression of uPAR on monocytes and neutrophils becomes upregulated upon their exposure to bacteria or lipopolysaccharide (LPS) (3-5). UPAR can influence cellular movement in different ways. The binding of uPA by uPAR results in the formation of plasmin at the leading edge of cells, thereby facilitating cell migration by pericellular proteolysis of extracellular matrix proteins (2, 6). Besides functioning as a proteinase receptor, uPAR also affects cellular migration, adhesion, differentiation and proliferation through intracellular signaling, which in part occurs independent of the proteolytic activity of uPA. Since uPAR is glycosylphosphatidylinositol (GPI)-linked and lacks an intracellular domain, uPAR needs to become part of functional transmembrane units with other molecules in order to transduce signals into the cellular interior. UPAR has been found to form such functional units with several components, including members of the integrin adhesion receptor superfamily (7, 8).

Recent studies have demonstrated a role for uPAR in the immune response to bacterial infection. UPAR gene deficient ($uPAR^{-/-}$) mice displayed a strongly reduced neutrophil recruitment to the pulmonary compartment after induction of *Pseudomonas* or pneumococcal pneumonia, which was associated with an impaired antibacterial defense (9, 10). Moreover, in $uPAR^{-/-}$ mice cerebrospinal fluid pleocytosis was significantly attenuated during experimental pneumoncoccal meningitis (11). Thus far, the contribution of uPAR to leukocyte trafficking toward the peritoneal cavity has only been studied in the model of sterile peritonitis induced by intraperitoneal administration of thioglycollate, revealing a profoundly reduced neutrophil influx in $uPAR^{-/-}$ mice (12). In the present study we sought to determine the contribution of uPAR in neutrophil migration into the abdominal cavity during peritonitis elicited by bacterial products or intact bacteria. Considering that *Escherichia (E.) coli* is the most common pathogen involved in intraabdominal infections in humans (13) we here investigated the role of uPAR in the inflammatory response to intraperitoneally administered *E. coli* LPS and in host defense against peritonitis induced by intact *E. coli* by comparing inflammatory cell influx, chemokine and cytokine responses, bacterial outgrowth and organ damage in $uPAR^{-/-}$ and normal wild type (Wt) mice.

Materials and Methods

Animals

$uPAR^{-/-}$ mice on a C57BL/6J background (backcrossed at least six times) were purchased from The Jackson Laboratory (Bar Harbor, Maine) and bred in the institutional animal facilities. Normal Wt C57BL/6J mice were purchased from Harlan, Horst, the Netherlands.

All experiments were done with 8-10 weeks old female mice. The Committee on Use and Care of Animals of the Academic Medical Center approved all experiments. Animal experimentation guidelines were followed in all experiments.

Induction of LPS-induced inflammation

uPAR$^{-/-}$ and Wt mice received an intraperitoneal injection with 200 µg LPS (*E. coli*, serotype 0111:B14; Sigma, St. Louis, MO) and were sacrificed before and at 3, 6 and 20 hours after injection. In one experiment mice received 500 ng LPS intraperitoneally and were sacrificed at 20 hours after injection.

Induction of *E. coli* peritonitis

Peritonitis was induced as described previously (14-16). In brief, *E. coli* O18:K1 was cultured in Luria-Bertani medium (Difco, Detroit, Mich.) at 37°C, harvested at mid-log phase, and washed twice with sterile saline before injection. Mice were injected intraperitoneally with 1x10^4 colony forming units (CFU) of *E. coli* O18:K1 in 200 µl of sterile isotonic saline.

Sample harvesting

At the time of sacrifice, mice were first anesthetized by inhalation of 2% isoflurane (Abbott Laboratories Ltd., Kent, UK) / 2 liters of O_2. A peritoneal lavage was then performed with 5 ml of sterile isotonic saline using an 18-gauge needle, and peritoneal lavage fluid was collected in sterile tubes (Plastipack [Becton-Dickinson, Mountain View,). After collection of peritoneal fluid, deeper anesthesia was induced by i.p. injection of 0.07 ml of FFM mixture (fentanyl [0.315 mg/ml]-fluanisone [10 mg/ml] [Janssen, Beersen, Belgium], midazolam [5 mg/ml] [Roche, Woerden, the Netherlands]) per g. The abdomen was opened, and blood was drawn from the vena cava inferior into a sterile syringe, transferred to tubes containing heparin, and immediately placed on ice. Thereafter, livers and lungs were harvested and processed for measurements of CFU and chemokines and cytokines and histology as described below.

Cell counts and differentials

Cell counts were determined in peritoneal lavage fluid using a hemacytometer (Beckman Coulter, Fullerton, CA). Subsequently, the pellet was diluted in phosphate-buffered saline to a final concentration of 10^5 cells/ml and differential cell counts were performed on cytospin preparations stained with a modified Giemsa stain (Diff-Quick; Dade Behring AG, Düdingen, Switzerland).

Assays

TNF-α, IL-6 and IL-10 levels were determined in peritoneal lavage fluid and plasma using a commercially available cytometric beads array multiplex assay (BDBiosciences, San Jose, CA) in accordance with the manufacturer's recommendations. Macrophage inflammatory protein (MIP)-2 and keratinocyte derived chemokine (KC) levels were measured using commercially available ELISA kits (R&D systems, Abingdon, UK). Myeloperoxidase (MPO) was also measured by ELISA (Hycult biotechnology BV, Uden, The Netherlands).

Determination of bacterial outgrowth

Lungs and livers were homogenized at 4°C in 5 volumes of sterile isotonic saline with a tissue homogenizer (Biospect Products) which was carefully cleaned and desinfected with 70% ethanol after each homogenization. Serial 10-fold dilutions in sterile saline were made from these homogenates, blood and peritoneal lavage fluid, and 50 µl volumes were plated onto sheep-blood agar plates and incubated at 37°C and 5% CO_2. CFU were counted after 16 hours.

Histology

Directly after sacrifice liver and lung samples were fixed with 4% formalin and embedded in paraffin. 4 µm thick paraffin sections were stained with hematoxylin and eosin. All slides were coded and scored by a pathologist without knowledge of the strain of mice. The liver and lungs were scored according to the following parameters: (1) the number of thrombi counted in 5 fields at a magnification of x100 (liver) or x200 (lungs), (2) the presence and degree of inflammation, which included the influx of leukocytes and the presence of endothelitis, (3) for liver only: the presence and degree of necrosis. Inflammation and hepatic necrosis were rated from 0-3, wherein 0= absent, 1= occasionally, 2= regularly, 3= massively. Granulocyte staining was performed as described previously (10, 16). Granulocytes were counted in 5 random fields at a magnification of x200 (liver) or x400 (lungs).

Statistical analysis

Data are expressed as means ± SEM, unless indicated otherwise. Comparisons between groups were conducted using the Mann-Whitney *U* test. A value of $p < 0.05$ was considered to represent a statistically significant difference.

Results

uPAR$^{-/-}$ mice show a diminished LPS-induced neutrophil influx into the peritoneal cavity

Previous work has implicated uPAR as a regulator of cell recruitment to sites of inflammation (1, 2, 9-12). Therefore, we wanted to investigate whether uPAR deficiency affects cell influx during *E. coli* LPS-induced peritoneal inflammation. Leukocyte counts and differentials in peritoneal lavage fluid were determined after intraperitoneal injection of a low (500 ng) and a high dose (200 µg) of LPS. Both doses induced an influx of immune cells, especially neutrophils, into the peritoneal cavity at 20 hours after injection. UPAR$^{-/-}$ mice showed a reduction in the number of neutrophils relative to Wt mice of 47% after the low dose and 39% after the high dose of LPS (both $P < 0.05$; **Table 1**).

uPAR$^{-/-}$ mice have a normal chemokine and cytokine response to LPS

Since, ELR^{+} CXC chemokines have been implicated in the attraction of neutrophils to the site of an infection (17, 18), we measured the main mouse ELR^{+} CXC chemokines MIP-2 and KC in peritoneal fluid. LPS injection enhanced abdominal MIP-2 and KC concentrations in

Table 1. LPS-induced cell influx into the peritoneal cavity

	500 ng LPS		200 µg LPS	
x10⁴/ml PLF	Wt	uPAR$^{-/-}$	Wt	uPAR$^{-/-}$
Total cells	58.0 ± 11.2	41.7 ± 2.6	59.3 ± 4.7	37.3 ± 7.7*
Neutrophils	20.5 ± 2.6	10.9 ± 3.1*	56.6 ± 4.9	34.5 ± 7.1*
Macrophages	31.7 ± 7.3	26.8 ± 3.7	2.1 ± 0.5	2.2 ± 0.5
Others	5.7 ± 2.1	4.1 ± 1.6	0.7 ± 0.3	0.6 ± 0.2

Data are means ± SE (N = 8 mice per group for each time point) after i.p. administration of 500 ng or 200 µg *E. coli* LPS. PLF, peritoneal lavage fluid. Statistics by Mann-Whitney *U* test. *$P < 0.05$ versus Wt mice at the same dose.

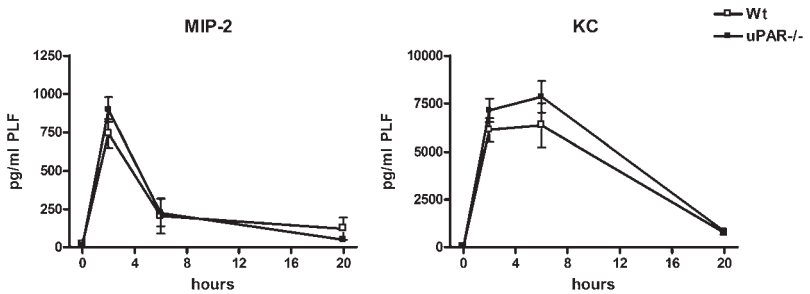

Figure 1. Unaltered LPS-induced chemokine release into peritoneal fluid in uPAR$^{-/-}$ mice. MIP-2 and KC levels in peritoneal fluid of uPAR$^{-/-}$ (closed symbols) and Wt (open symbols) mice at 0, 3, 6 and 20 hours after intraperitoneal injection of 200 µg LPS. Data are expressed as means ± SE. N= 8 per group per time point.

both Wt and uPAR$^{-/-}$ mice. The levels of both chemokines were similar in the two genotypes **(Figure 1)**, ruling out the possibility that reduced levels of these neutrophil attractants were responsible for the decreased neutrophil influx in uPAR$^{-/-}$ mice.
Furthermore, to investigate whether uPAR deficiency might influence the local or systemic cytokine response to *E. coli* LPS, we measured the concentrations of TNF-α, IL-6 and IL-10 in peritoneal fluid and plasma. TNF-α and IL-6 were increased in peritoneal fluid at 2, 6 and 20 hours after LPS injection, whereas IL-10 remained undetectable here; there were no differences in peritoneal fluid cytokine levels between uPAR$^{-/-}$ and Wt mice **(Figure 2)**. In plasma, LPS induced a strong rise in all cytokine concentrations measured, however none of the cytokine levels differed between uPAR$^{-/-}$ and Wt mice **(Figure 2)**. These data indicate that uPAR$^{-/-}$ mice are able to mount a normal local and systemic cytokine response to *E. coli* LPS induced inflammation.

uPAR does not mediate neutrophil trafficking during *E. coli* peritonitis

Having established that uPAR$^{-/-}$ mice showed a reduced neutrophil response to LPS injection, we next investigated whether uPAR also mediates neutrophil recruitment to the

Table 2. Leukocyte counts in peritoneal lavage fluid during *E. coli*-peritonitis

	6 h		20 h	
x10^4/ml PLF	Wt	uPAR$^{-/-}$	Wt	uPAR$^{-/-}$
Total cells	110 ± 11.3	73.7 ± 5.25*	270 ± 121	243 ± 46.9
Neutrophils	73.3 ± 8.63	53.6 ± 5.46	209 ± 99.6	165 ± 34.8
Macrophages	32.8 ± 5.38	19.3 ± 5.87	43.8 ± 17.8	44.9 ± 9.40
Others	3.50 ± 0.67	1.53 ± 0.54	0.66 ± 0.21	1.01 ± 0.33

Data are mean ± SE (N = 8 mice per group for each time point) at 6h or 20h after i.p. administration of *E. coli* (10^4 CFU). PLF, peritoneal lavage fluid. Statistics by Mann-Whitney *U* test. $^*P < 0.05$ versus Wt mice at the same time-point.

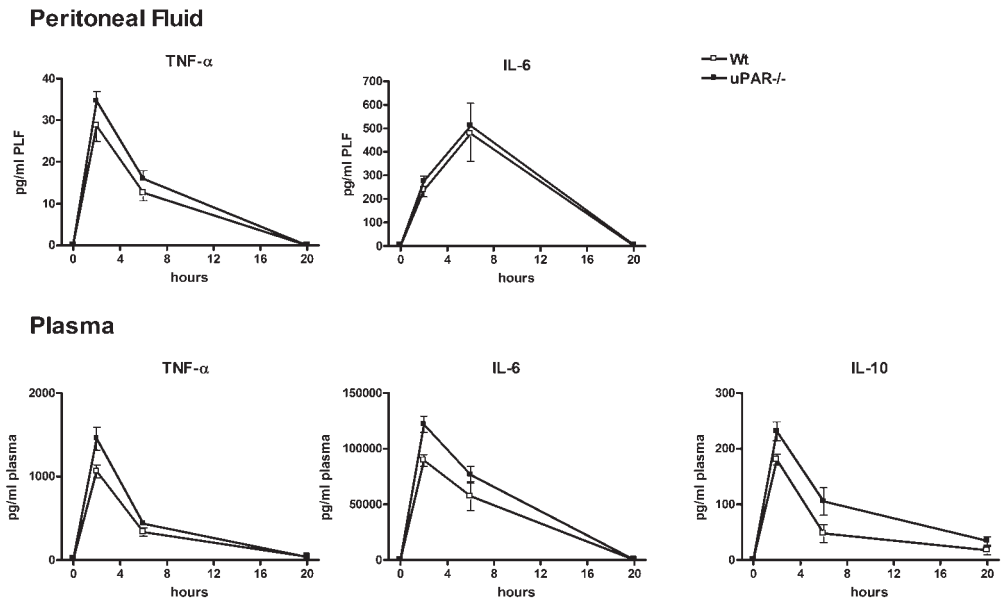

Figure 2. Unaltered LPS-induced cytokine response in uPAR$^{-/-}$ mice. TNF-α, IL-6 and IL-10 levels in peritoneal fluid and plasma of uPAR$^{-/-}$ (closed symbols) and Wt (open symbols) mice at 0, 3, 6 and 20 h after intraperitoneal injection of 200 μg LPS. Data are expressed as means ± SE. N= 8 per group per time point.

abdominal cavity during peritonitis induced by intact *E. coli*. To this end we injected Wt and uPAR$^{-/-}$ mice intraperitoneally with 10^4 CFU *E. coli*. Intraabdominal infection with *E. coli* resulted in a strong influx of leukocytes into the peritoneal fluid and like during LPS-induced abdominal inflammation, neutrophils were the main cells recruited to the peritoneal cavity **(Table 2)**. Remarkably, at both 6 and 20 h postinfection, neutrophil counts in peritoneal fluid harvested from uPAR$^{-/-}$ and Wt mice were similar **(Table 2)**. To obtain further proof for the finding that uPAR does not contribute to neutrophil trafficking during

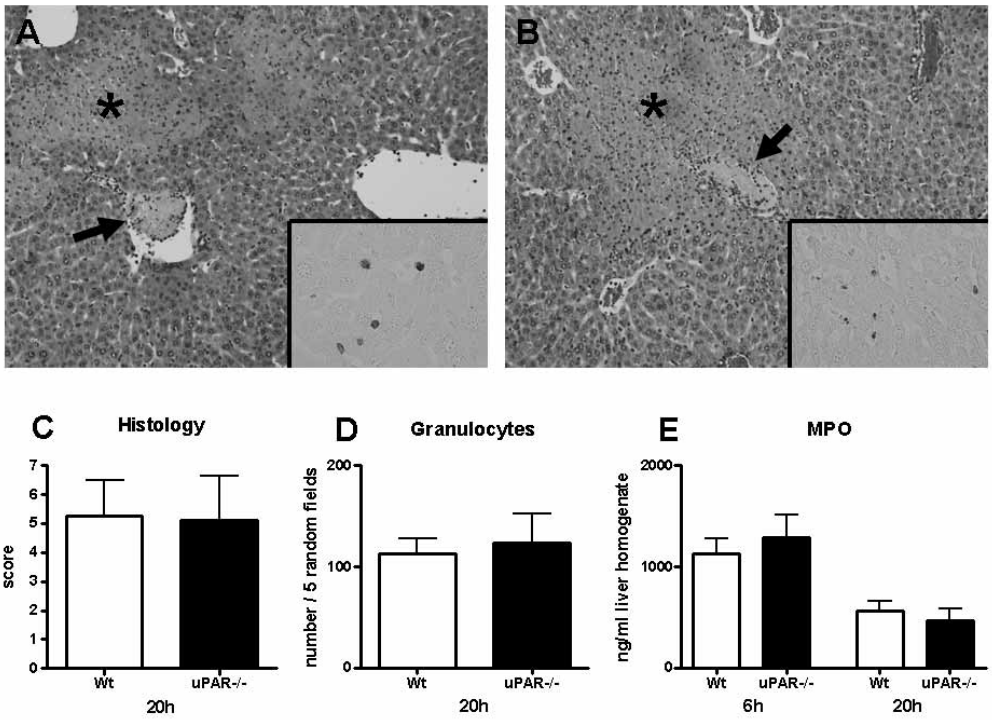

Figure 3. uPAR$^{-/-}$ mice display unaltered neutrophil migration to the liver during *E. coli* peritonitis. Representative HE stainings (microscopic magnification x100) with inserts of representative granulocyte stainings (microscopic magnification x500) of liver tissue at 20 hours after intraperitoneal injection of 10^4 CFU *E.coli* in Wt (A) and uPAR $^{-/-}$ (B) mice. Livers of WT and uPAR $^{-/-}$ mice show inflammation, tissue necrosis (∗) and thrombi (arrows). (C) Histology scores of liver tissue were obtained as described in the Methods section, (D) the number of granulocytes per 5 random fields were determined and (E) myeloperoxidase levels were measured in uPAR$^{-/-}$ (closed bars) and Wt (open bars) mice. Data are means ± SE. N=8 mice per genotype.

bacterial peritonitis, we performed histological analyses with granulocyte stainings of liver tissue and measured MPO levels in liver homogenates **(Figure 3)**. Upon histopathological examination, both uPAR$^{-/-}$ and Wt mice showed inflammation of the hepatic parenchyma as characterized by the influx of leukocytes into the interstitium. In addition, both genotypes of mice showed liver tissue necrosis and intrahepatic thrombi. Histology scores of the livers (quantified according to the scoring system described in the Methods section) did not differ between Wt and uPAR$^{-/-}$ mice. Granulocyte stainings revealed that the number of granulocytes in 5 random microscopic fields, determined in all liver sections, did not differ between Wt and uPAR$^{-/-}$ mice. These findings were confirmed by MPO measurements in liver homogenates, which showed no difference between Wt and uPAR$^{-/-}$ mice at 6 or 20 hours after *E.coli* injection. Finally, to obtain insight into the role of uPAR in neutrophil migration to a more distant organ during peritonitis, lungs were harvested at 20 hours after the induction of *E. coli* infection. Lungs showed clear signs of inflammation in both Wt and uPAR$^{-/-}$ mice, as reflected by accumulation of leukocytes in the interstitium **(Figure 4)**. In addition, lungs of both Wt and uPAR$^{-/-}$ mice contained multiple thrombi. Although the

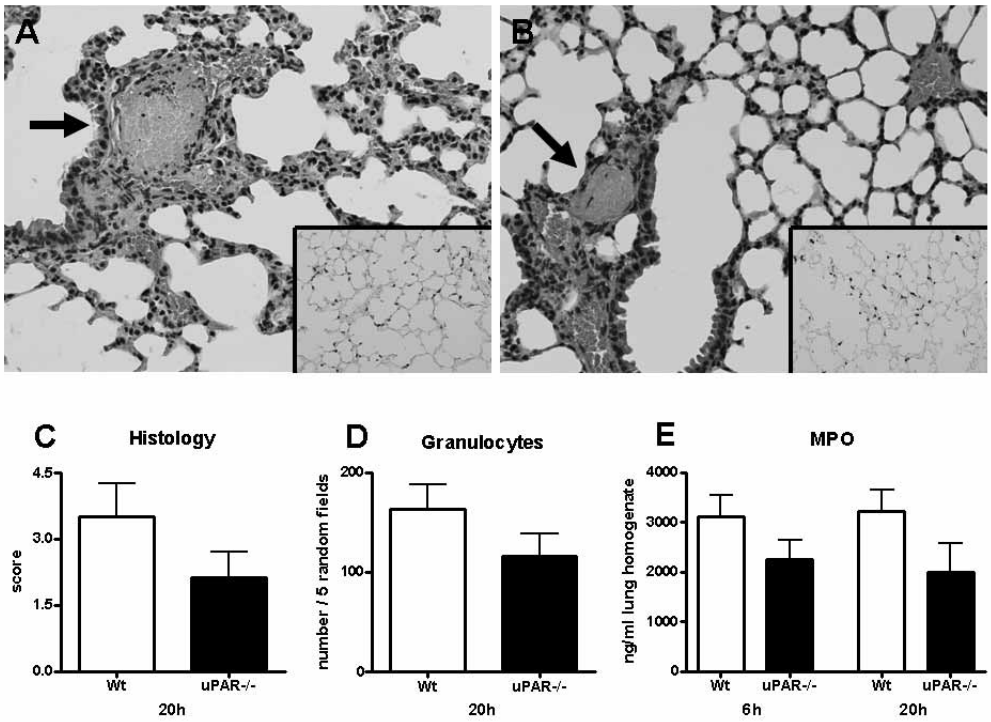

Figure 4. uPAR$^{-/-}$ mice display unaltered neutrophil migration to the lungs during *E. coli* peritonitis. Representative HE stainings with inserts of representative granulocyte stainings (microscopic magnification x200) of lung tissue at 20 hours after intraperitoneal injection of 10^4 CFU *E.coli* in Wt (A) and uPAR $^{-/-}$ (B) mice. Lungs of WT and uPAR $^{-/-}$ mice show inflammation and thrombi (arrows). (C) Histology scores of lung tissue were obtained as described in the Methods section, (D) the number of granulocytes per 5 random fields were determined and (E) myeloperoxidase levels were measured in uPAR$^{-/-}$ (closed bars) and Wt (open bars) mice. Data are means ± SE. N=8 mice per genotype.

mean total histological score tended to be lower in uPAR$^{-/-}$ mice, the difference with Wt mice did not reach statistical significance (P = 0.19). Granulocyte stainings were performed and granulocytes/5 random microscopic fields were counted in the lung sections, showing a slightly lower number of neutrophils (not significant) in uPAR$^{-/-}$ mice. In line, MPO concentrations in lung homogenates, measured at 6 and 20 hours after infection, also tended to be lower in uPAR-/- mice (non significant). Altogether these data indicate that uPAR does not importantly contribute to neutrophil trafficking during *E. coli* induced peritonitis.

Bacterial outgrowth and chemokine/cytokine response

In theory uPAR deficiency can impair host defense against bacterial infection in at least two ways. First, an impaired recruitment of neutrophils to the site of infection is expected to facilitate the outgrowth of bacteria, and second uPAR has been implicated to play a role in bacterial phagocytosis (19). In case uPAR$^{-/-}$ mice have higher bacterial loads in their peritoneal cavity this could have masked a relatively impaired neutrophil influx, i.e. the

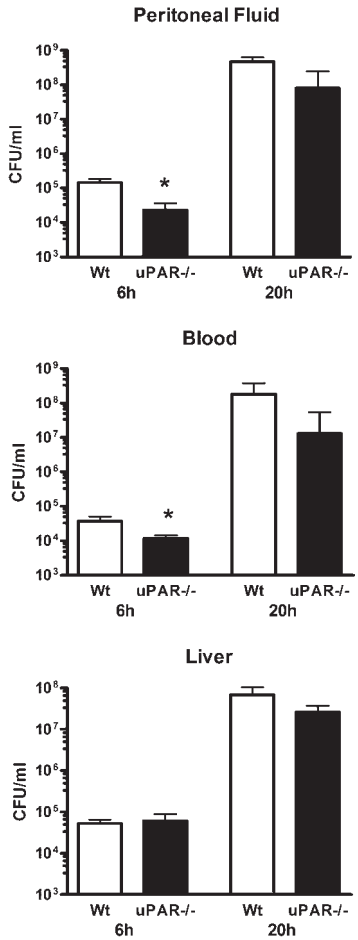

Figure 5. Bacterial outgrowth. Numbers of *E. coli* CFU in peritoneal lavage fluid, blood and liver at 6 and 20 hours after intraperitoneal injection of 10^4 CFU *E.coli* in uPAR$^{-/-}$ (closed bars) and Wt (open bars) mice. Data are expressed as means ± SE. N= 8 per group per time point. * $P < 0.05$ versus Wt mice. Statistics by Mann-Whitney *U* test.

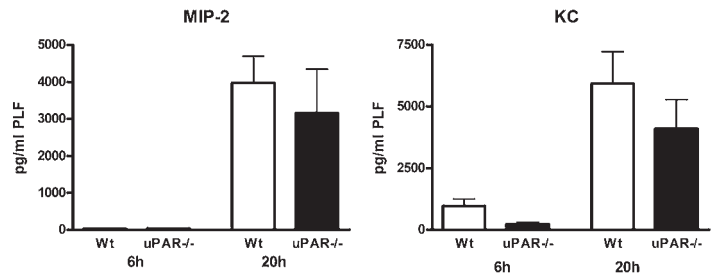

Figure 6. uPAR$^{-/-}$ mice show unaltered peritoneal chemokine levels after *E. coli* infection. MIP-2 and KC levels in peritoneal fluid of uPAR$^{-/-}$ (closed bars) and Wt (open bars) mice at 6 and 20 hours after intraperitoneal injection of 10^4 CFU *E.coli*. Data are expressed as means ± SE. N= 8 per group per time point.

higher bacterial load would have provided a more potent proinflammatory stimulus overcoming the deficient recruitment of uPAR$^{-/-}$ neutrophils. Therefore, we compared the number of *E.coli* CFU at 6 and 20 hours after infection in peritoneal lavage fluid; in addition, blood and liver were cultured at the same time points **(Figure 5)**. Remarkably, at 6 hours post infection uPAR$^{-/-}$ mice had slightly but significantly fewer bacteria in their peritoneal cavity and blood than Wt mice (both $P < 0.05$). After 20 hours, uPAR$^{-/-}$ mice still showed lower mean bacterial counts in both compartments but the differences was not statistically significant anymore. The bacterial counts in the liver did not differ between the two genotypes at either time point. To examine whether uPAR deficiency influences chemokine or cytokine responses we measured chemokine levels in peritoneal fluid and cytokine concentrations in both peritoneal fluid and plasma. uPAR$^{-/-}$ and Wt mice displayed similar MIP-2 and KC levels at the site of infection at both 6 and 20 h post infection **(Figure 6)**. In peritoneal fluid uPAR$^{-/-}$ mice had lower cytokine levels at 6 h after infection, significantly so for IL-10 **(Figure 7)**. In plasma, uPAR$^{-/-}$ mice tended to have lower TNF, IL-6 and IL-10 concentrations at both time points, but the difference with Wt mice never reached statistical significance **(Figure 7)**.

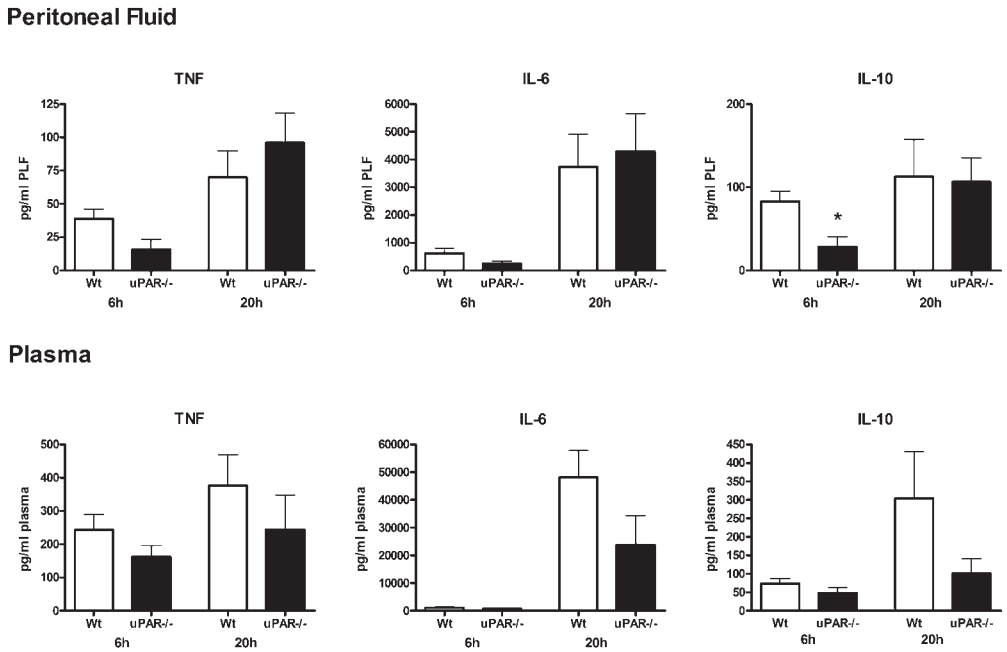

Figure 7. E.coli-induced cytokine response. TNF-α, IL-6 and IL-10 levels in peritoneal fluid and plasma of uPAR$^{-/-}$ and Wt mice at 6 and 20 h after intraperitoneal infection with 10^4 CFU *E.coli*. Data are expressed as means ± SE. N= 8 per group per time point. * P < 0.05 versus Wt mice at the same time-point. Statistics by Mann-Whitney *U* test.

Discussion

Peritonitis is the second most common cause of sepsis (20), with *E. coli* as one of the major pathogens involved (13). UPAR has been implicated as an important receptor for the regulation of leukocyte trafficking to sites of inflammation and infection (1, 2, 6). The requirement for uPAR for cell invasion into the peritoneal cavity has been demonstrated previously in a non-infectious inflammation model (12). To our knowledge our study is the first to investigate the role of uPAR in neutrophil migration into the peritoneal cavity in response to locally instilled LPS or *E. coli*. We here demonstrate that uPAR participates in neutrophil migration to the abdomen upon intraperitoneal injection of LPS, but that this function of uPAR is compensated for during abdominal infection caused by intact *E. coli*.

uPAR is a multifunctional protein involved in different inflammatory responses, including cell-associated proteolysis, cell adhesion, chemotaxis, cell migration and proliferation (1, 2, 6). UPAR, which lacks an intracellular domain and therefore is not able to directly activate intracellular pathways, can induce cellular responses by interacting with other molecules such as vitronectin, caveolin and integrins. Much research has been done on the functional and physical interaction between uPAR and β2 integrins, among which LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18). In a previous study, $uPAR^{-/-}$ mice displayed a 50% reduction in leukocyte counts in their peritoneal lavage fluid after intraperitoneal administration of thioglycollate; treatment with a blocking anti-LFA-1 antibody strongly reduced leukocyte influx in Wt mice while only marginally influencing peritoneal leukocyte counts in $uPAR^{-/-}$ mice, suggesting that the deficiency of $uPAR^{-/-}$ leukocytes to migrate was due to a perturbed LFA-1 function (12). During *Pseudomonas* pneumonia, anti-CD11b treatment profoundly inhibited neutrophil recruitment into bronchoalveolar lavage fluid of Wt mice, but did not further diminish the already strongly reduced neutrophil influx in $uPAR^{-/-}$ mice (9). Although these data clearly establish a biologically relevant interaction between uPAR and β2 integrins, the functional consequence of the association between uPAR and individual members of the integrin family likely depends on the site of inflammation and the nature of the inflammatory stimulus. Indeed, in contrast to CD11a, CD11b does not play a significant role in neutrophil recruitment to the peritoneal cavity during thioglycollate-induced peritonitis (21, 22), however, during polymicrobial peritonitis induced by cecal ligation and puncture, CD11b gene deficient mice demonstrated a reduced neutrophil influx into the abdominal cavity (23). Moreover, neutrophil recruitment during pneumococcal pneumonia, which occurs by a CD11b/CD18 independent mechanism (24, 25), is largely dependent on the presence of uPAR (10). Our current finding that $uPAR^{-/-}$ mice have a reduced capacity to recruit neutrophils to the site of inflammation after LPS injection is in line with the earlier findings during thioglycollate-induced peritonitis. The mechanism by which uPAR mediates this response remains to be investigated, although an interaction with one or more members of the integrin family seems likely. It is quite conceivable that locally produced ELR^{+} CXC chemokines, such as MIP-2 and KC, contribute to this characteristic innate immune response (17, 18). In the present study, $uPAR^{-/-}$ and Wt mice had similar peritoneal MIP-2 and KC levels after LPS injection, indicating that these chemokines could not have contributed to the differences in neutrophil recruitment between these strains.

Neutrophils constitute an important component of early host defense against bacterial infection. A reduced capacity of neutrophils to migrate to the site of an infection is likely associated with an impaired local antibacterial defense, indicating a protective role of migrating neutrophils (23, 26, 27). Since we found an impaired LPS-induced neutrophil migration to the peritoneal cavity in uPAR$^{-/-}$ mice, we hypothesized that uPAR-/- mice would also show a reduced neutrophil recruitment during abdominal *E. coli* infection and as a consequence thereof an impaired antibacterial host defense. Surprisingly, we did not find a significant difference in neutrophil influx into peritoneal lavage fluid at 6 and 20 hours after *E. coli* infection. The notion that uPAR does not participate in neutrophil trafficking to a significant extent during *E. coli* peritonitis was further supported by the fact that the number of neutrophils in liver and lungs, as determined by neutrophil staining and measurement of MPO concentrations, did not differ between uPAR$^{-/-}$ and Wt mice. A possible explanation for the discrepant roles of uPAR in LPS and *E. coli* induced neutrophil influx may lie in the fact that *E. coli* provided a much stronger and more long-lived proinflammatory stimulus, as shown by higher numbers of total neutrophils and higher concentrations of MIP-2, TNF- and IL-6 in the peritoneal lavage fluid when compared to cell numbers and mediator levels found after LPS injection. Thus during *E. coli* peritonitis the effect of uPAR deficiency on neutrophil recruitment to the site of infection possibly was compensated for by other potent chemotactic mediators, like complement factors and CXC chemokines (18, 28).

Gyetko et al. showed that neutrophils from uPAR$^{-/-}$ mice had a diminished bacterial phagocytosis capacity in vitro (19). In contrast, we found that the bacterial outgrowth was attenuated in uPAR$^{-/-}$ mice early in the infection. Furthermore, the uPAR$^{-/-}$ mice showed lower cytokine levels in their peritoneal lavage fluid at 6 hours postinfection. This latter finding was most likely the result of a lower inflammatory stimulus due to the lower bacterial load in the peritoneal cavity. We have no clear explanation for the differences in bacterial loads between uPAR$^{-/-}$ and Wt mice at 6 h post infection. The fact that all differences disappeared during a more advanced stage indicates that they did not have a great impact on the course of infection.

uPAR is considered to play an important role in neutrophil migration to sites of inflammation and infection. Thus far the role of uPAR during bacterial infection had only been demonstrated in experimental models of pneumonia and meningitis, which clearly revealed the contribution of this receptor to neutrophil influx into the lungs and the cerebrospinal fluid respectively (9-11). We here show that although uPAR is involved in the influx of neutrophils into the peritoneal cavity in response to local administration of LPS, it is not indispensable for an adequate neutrophil recruitment during peritonitis induced by intact *E. coli*. These data suggest that the role of uPAR in neutrophil trafficking may depend on the type of infection.

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Summary

Summary

More and more evidence shows that mediators of the fibrinolytic system have properties that reach beyond fibrinolysis. The fibrinolytic system is activated during severe infection and inflammation. Therefore, in this thesis, we focused on the role of the mediators of the fibrinolytic system in inflammatory responses in mice and man. We used different experimental models to study inflammatory processes and host defense. Sterile inflammation was induced by endotoxin (LPS), Staphylococcal enterotoxin B (SEB) or turpentine injection. In addition, live bacterial infection models (pneumonia, peritonitis, sepsis) were induced by common Gram-negative bacteria (*Klebsiella pneumoniae, Escherichia coli*). **Chapter 1** is a general introduction. It describes the different mediators of the fibrinolytic system, the inflammatory responses and the infectious diseases relevant for the studies presented in this thesis.

In **chapter 2**, we studied the role of plasmin, the end-product of the fibrinolytic system, in the induction of LPS-induced inflammatory pathways in healthy human volunteers. The recent findings that plasmin is able to induce several cellular proinflammatory responses *in vitro*, including activation of p38 MAPK, led us to hypothesize that plasmin may also influence inflammatory pathways *in vivo*. Previous studies showed that tranexamic acid, an inhibitor of plasmin activity, potently inhibited plasmin-induced proinflammatory responses *in vitro*. Therefore, we studied the effect of tranexamic acid infusion on activation of coagulation, granulocytes, endothelial cells and the cytokine network in healthy humans injected with a single dose of LPS. LPS injection into healthy humans induced a strong increase in the plasma levels of D-dimer, indicative for plasmin activation, which was significanty attenuated by pre-treatment with tranexamic acid. However, tranexamic acid did not influence LPS-induced coagulation activation, granulocytosis, neutrophil activation or degranulation, endothelial cell activation or cytokine release. Thus, these data argue against a role for early plasmin generation in the subsequent activation of other inflammatory pathways during human endotoxemia.

In the following studies of this thesis we used mice with a targeted gene deletion (-/- mice) encoding for a fibrinolytic mediator to investigate the influence of these mediators on inflammatory processes. Inflammation or infection was induced in these gene-deficient mice and their responses were compared with normal wild type (WT) mice. First, we investigated the role of an important mediator of the fibrinolytic system, plasminogen activator inhibitor (PAI-1), in different models of inflammation. PAI-1 is a major inhibitor of fibrinolysis by virtue of its capacity to inhibit urokinase type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). Systemic inflammation is invariably associated with elevated circulating levels of PAI-1. However, knowledge about the functional role of PAI-1 in a systemic inflammatory response syndrome was highly limited. Therefore, in **chapter 3**, we determined the role of endogenous PAI-1 in cytokine release induced by administration of LPS or SEB. Surprisingly, PAI-$1^{-/-}$ mice demonstrated strongly elevated plasma interferon (IFN)-γ concentrations after injection of either LPS or SEB. In addition, PAI-$1^{-/-}$ splenocytes released more IFN-γ after incubation with LPS or SEB than WT splenocytes *ex vivo*. These results show that endogenous PAI-1 serves to inhibit IFN-γ

release during LPS-induced inflammation. Furthermore, this effect of PAI-1 does not seem to depend on its interaction with uPA/uPA-receptor (uPAR) or tPA since the LPS-induced IFN-γ release in mice gene-deficient for uPA, the uPAR or tPA was not different from WT mice. To investigate the role of PAI-1 in a more chronic sterile inflammation model and the acute phase response, we next used the well-established model of turpentine-induced tissue injury and compared local and systemic inflammatory responses in PAI-$1^{-/-}$ and WT mice (**chapter 4**). Subcutaneous turpentine injection elicited strong increases in PAI-1 protein concentrations in plasma and at the site of injury. PAI-1 deficiency enhanced the early influx of neutrophils to the site of inflammation. Furthermore, PAI-$1^{-/-}$ mice showed a reduced early interleukin (IL)-6 induction with subsequently lower acute phase protein levels and a much slower recovery of body weight loss. Together these findings suggest that PAI-1 is not merely a marker of inflammation but plays a functional role in local and systemic inflammatory responses.

Bacterial pneumonia is a serious illness associated with a high morbidity and mortality. During severe pneumonia several mediator systems become activated, resulting in a profound inflammatory response together with increased procoagulant activity and suppression of the fibrinolytic system by upregulation of PAI-1. However, whether PAI-1 plays a role in antibacterial host defense or inflammatory responses during pneumonia was still unknown. Therefore, in **chapter 5**, we investigated the role of PAI-1 in inflammatory responses and host defense during severe pneumonia caused by the common respiratory pathogen *K. pneumoniae*. We found that PAI-1 deficiency impaired host defense as reflected by enhanced lethality and increased bacterial outgrowth and dissemination. Moreover, 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. These data show that PAI-1 is essential for host defense against severe Gram-negative pneumonia and suggest that PAI-1 protects the host by promoting neutrophil recruitment to the pulmonary compartment.

Next, we investigated whether tPA, an activator of the fibrinolytic system, could also influence inflammatory responses or host defense during severe Gram-negative pneumonia. Therefore, in **chapter 6**, we examined the effect of pulmonary tPA overexpression and tPA deficiency on host defense against *Klebsiella* pneumonia. We show that tPA overexpression results in a reduced mortality during *K. pneumoniae*-induced pneumonia, which was associated with a markedly reduced bacterial outgrowth in the lungs, a decreased dissemination of the infection and less distant organ damage. Furthermore, transgenic tPA expression resulted in significantly decreased TNF-α and IL-6 levels. However, tPA$^{-/-}$ mice had a normal antibacterial host defense against *Klebsiella* pneumonia. These data show that local transgenic tPA overexpression in the lungs improves host defense against *K. pneumoniae*. The mechanism behind this protective effect of tPA during pneumonia still needs to be elucidated. Another severe bacterial infection with a profound inflammatory response is peritonitis, which is a life-threatenig disease that can lead to sepsis and death. Abdominal sepsis results in the systemic activation of various host mediator systems

including the cytokine network and the coagulation and fibrinolytic systems. Sepsis is associated with enhanced production of tPA. Therefore, in **chapter 7**, we investigated the function of endogenous tPA in the immune responses to *E. coli*-induced peritonitis. $tPA^{-/-}$ mice demonstrated an impaired host defense against *E. coli* peritonitis. The protective function of tPA was independent of plasmin since plasminogen gene-deficient mice were indistinguishable from WT mice. Furthermore, $tPA^{-/-}$ mice seemed to have a reduced neutrophilic migratory response to the peritoneal cavity and adenoviral overexpression of tPA caused an enhanced cell migration to the peritoneal cavity during *E. coli* peritonitis. These data again suggest a pro-inflammatory and protective role for tPA during host defense against a Gram-negative infection which seems independent of its role in the generation of plasmin. Recent studies showed that tPA is able to activate matrix metalloproteinase (MMP)-9, which is involved in extracellular matrix degradation and leukocyte migration. One can hypothesize, that in our peritonitis model tPA activates MMP-9 and this mechanism might be responsible for the differences in cell migration and host defense between $tPA^{-/-}$ and WT mice. Next, we investigated whether MMP-9 plays a role in the inflammatory response and host defense in the *E. coli* peritonitis model (**chapter 8**). Indeed, $MMP\text{-}9^{-/-}$ mice showed a reduced resistance against *E. coli* peritonitis, as indicated by an enhanced bacterial outgrowth in the peritoneal cavity, increased dissemination of the infection and more severe distant organ damage. $MMP\text{-}9^{-/-}$ mice also showed much higher peritoneal chemokine and cytokine levels compared to WT mice, although the cytokine response to LPS was not influenced by MMP-9 deficiency. These data indicate that the higher cytokine levels were probably the consequence of the higher pro-inflammatory stimulus provided by the higher bacterial load. However, despite the increased local bacterial load and chemokine concentrations, $MMP\text{-}9^{-/-}$ mice displayed a diminished recruitment of leukocytes to the site of infection, indicating that, cellular migration was strongly impaired. Moreover, $MMP\text{-}9^{-/-}$ neutrophils showed a normal phagocytosis of *E.coli in vitro*. These data suggest that MMP-9 is an essential component of an effective host response to *E. coli* peritonitis by inducing cellular migration to the peritoneal cavity.

In **chapter 9**, we investigated the role of thrombin-activatable fibrinolysis inhibitor (TAFI) in the hemostatic and innate immune response to *E.coli*-induced abdominal sepsis. TAFI is a negative regulator of the fibrinolytic system. In addition, TAFI might play a role in the inflammatory response since it is able to inactivate inflammatory peptides such as complement factors C3a and C5a, involved in chemotaxis. We found no differences in *E. coli*-induced activation of coagulation or fibrinolysis between $TAFI^{-/-}$ and WT mice. Thus, these data argue against an important part for TAFI in the regulation of the procoagulant-fibrinolytic balance in sepsis. However, $TAFI^{-/-}$ mice did show increased neutrophil recruitment to the peritoneal cavity. Furthermore, $TAFI^{-/-}$ mice were strongly protected from liver necrosis revealing a thus far unknown role of TAFI in the occurrence of hepatic necrosis.

uPAR is the receptor for the plasminogen activator uPA and is expressed on many different cells, including leukocytes. uPAR has been implicated to play a role in the migration of neutrophils to sites of inflammation. In **chapter 10**, we investigated the role of uPAR in neutrophil recruitment in response to bacterial products (LPS) or intact bacteria (*E. coli*).

uPAR$^{-/-}$ mice demonstrated a reduced LPS-induced influx of neutrophils into peritoneal lavage fluid, while the chemokine and cytokine response was unaltered. In contrast, during *E. coli* peritonitis uPAR$^{-/-}$ mice showed a normal neutrophil influx to the primary site of infection. These data suggest that uPAR in part mediates neutrophil migration into the peritoneal cavity upon local instillation of LPS, but that this function of uPAR can be compensated for during abdominal infection caused by intact *E. coli*.

Together these data indicate that mediators of the fibrinolytic system have properties that reach beyond the regulation of fibrin degradation. Especially neutrophil migration seems to be influenced by different proteins of the fibrinolytic system, which has a major impact on the outcome of various Gram-negative infections in mice. However, the exact mechanisms by which these fibrinolytic proteins mediate the inflammatory responses still remain to be elucidated.

Samenvatting voor niet-ingewijden

Samenvatting voor niet-ingewijden

Fibrinolyse is de naam voor het oplossen (lyseren) van bloedstolsels (fibrine). Dit gebeurt door plasmine, een eiwit dat normaal gesproken in het bloed aanwezig is als plasminogeen maar na activatie verandert in plasmine, het eindproduct van het fibrinolytische systeem. Dit eiwit staat onder invloed van andere "regulerende" eiwitten, waardoor het kan worden gestimuleerd (tPA en uPA) of geremd (PAI-1) om stolsels op te ruimen. Al deze eiwitten bij elkaar vormen het fibrinolytische systeem.

Infectieziekten vormen nog steeds een grote dreiging wereldwijd. Infectieziekten zijn ziekten die ontstaan door de invasie van micro-organismen in het lichaam, zoals bacteriën, virussen (zoals bij griep en HIV), schimmels (zoals bij candidiasis) of parasieten (zoals bij malaria). De infectie met een micro-organisme leidt meestal tot een ontstekingsreactie van het lichaam, hetgeen dient als verdedigingsmechanisme. Dit proefschrift beschrijft met name onderzoek naar bacteriële infecties van de longen (pneumonie) en de buikholte (peritonitis), die beide vaak voorkomen, in het bijzonder bij mensen met een verminderde afweer, zoals bij ernstig zieke patiënten. Tijdens ontstekingsprocessen en infecties bij dieren of mensen treedt een zeer snelle activatie van de fibrinolyse op (door verhoging van tPA en uPA), die gevolgd wordt door een remming van dit systeem (door verhoging van PAI-1). In de tijd worden deze veranderingen gevolgd door activatie van ontstekingsmechanismen zoals de ontstekingscellen (witte bloedcellen ofwel leukocyten) en het cytokine- en chemokine-netwerk (bestaande uit een complex geheel van ontstekingsregulerende eiwitten die worden geproduceert door o.a. leukocyten). In de studies die in dit proefschrift zijn beschreven is onderzocht of de eiwitten die behoren tot het fibrinolytische systeem deze ontstekingsmechanismen kunnen beïnvloeden.

De leukocyten zijn de cellen die ons verdedigen tegen bacteriën en andere micro-organismen die proberen binnen te dringen in ons lichaam. Leukocyten worden gealarmeerd door chemokines en cytokines (de boodschapper-eiwitten) als er ergens in het lichaam een infectie of ontsteking is ontstaan. Vervolgens treden deze cellen uit de bloedbaan naar de plek van ontsteking waardoor er ter plekke een "ontstekingsinfiltraat" ontstaat: een hele hoop ontstekingscellen en bacteriën door elkaar. Het belangrijkste doel van de leukocyten is om alle bacteriën ter plekke te doden, en om het uitbreiden van de infectie naar andere organen van het lichaam te voorkomen. Als dit niet lukt kan er een situatie ontstaan van sepsis, ook wel bloedvergiftiging genoemd, waarbij bacteriën zich via het bloed verspreiden over vele plaatsen in het lichaam, hetgeen gepaard gaat met een ernstige algehele ontstekingsreactie. Hierdoor kunnen multipele organen tegelijk in hun functie falen (zoals de lever en de nieren) en kan er een levensbedreigende situatie ontstaan.

Antibiotica zijn goede hulpmiddelen wanneer het eigen afweermechanisme tekort schiet. Maar er is een duidelijke toename van resistentievorming onder bacteriën, een mondiaal probleem. Daarom is het belangrijk om te blijven zoeken naar andere mogelijkheden voor de bestrijding van bacteriële infectieziekten. Dit is de belangrijkste reden om onderzoek, zoals in dit proefschrift beschreven, te blijven doen, onderzoek naar wat er nou precies

gebeurt met alle cellen en eiwitten op het moment van een infectie en naar de interacties tussen verschillende systemen. Door middel van dit soort onderzoek wordt er gezocht naar eiwitten in het lichaam die helpen in de strijd tegen de infectie en eiwitten die de strijd juist tegenwerken. In theorie zouden we in de toekomst deze eiwitten van buitenaf kunnen manipuleren om zo infecties op een nieuwe manier te kunnen bestrijden.

In dit onderzoeksproject hebben wij onderzocht in welke mate eiwitten behorend tot het fibrinolytische systeem bijdragen aan ontstekingsprocessen (cytokinen en chemokines en activatie van leukocyten) die relevant zijn voor het ontstaan van infectieziekten en andere ontstekingsziekten. Wij hebben dit onderzocht door ontstekingsreacties in normale muizen (ook wel wild-type muizen genoemd) en in muizen die een van de fibrinolytische eiwitten missen (PAI-1, tPA, plasminogeen, TAFI, of uPAR,de zogenaamde knock-out muis) met elkaar te vergelijken. Ook hebben wij bij muizen juist sterk verhoogde concentraties van tPA of PAI-1 in de longen of in het bloed geïnduceerd door toediening van een adenovirale vector die tPA of PAI-1 produceert. Als model voor acute ontsteking bij de mens hebben wij bij gezonde jonge mannelijke vrijwilligers een ontsteking geïnduceerd door toediening van lipopolysaccharide (LPS, endotoxine). LPS is een bestanddeel van de wand van Gram-negatieve bacteriën, dat na injectie in de bloedbaan een sterke ontstekingsreactie teweegbrengt. Verder hebben we ontstekingsprocessen geïnduceerd in muizen door toediening van terpentijn (leidt tot chronische ontsteking), LPS (leidt tot acute ontsteking) of levende bacteriën.

Hoofdstuk 1 is een algemene introductie. Het beschrijft de verschillende fibrinolyse-mediatoren, de ontstekingsreacties en de infectieziekten die zijn gebruikt in dit proefschrift.

In **hoofdstuk 2** hebben we onderzoek gedaan naar de rol van plasmine in de ontstekingsreacties die ontstaan tijdens acute ontsteking, geïnduceerd door injectie van LPS in de bloedbaan van gezonde mensen. Tegelijk met de LPS-injectie kreeg de helft van de jonge mannelijke vrijwilligers een placebo-injectie en de andere helft kreeg een medicijn, tranexaminezuur, dat de activiteit van plasmine remt. Het toedienen van deze plasmineremmer bleek geen effect te hebben op de door LPS-geïnduceerde stijging van het aantal leukocyten in het bloed of hun activatie, noch op vaatwandcel (endotheel) activatie of cytokine-concentraties in het bloed. Deze resultaten pleiten derhalve tegen een effect van plasmine op acute ontstekingsreacties opgewekt door LPS-injectie.

In de volgende experimenten hebben we gebruik gemaakt van knock-out muizen. Eerst hebben we gekeken naar de rol van PAI-1 bij verschillende ontstekingsprocessen. De bloedspiegel van PAI-1 is sterk verhoogd tijdens ontstekingen en infecties in muizen en mensen, maar de rol van dit eiwit in afweermechanismen is onbekend. In **hoofdstuk 3** hebben we daarom de rol van PAI-1 onderzocht bij acute ontsteking door o.a. LPS injectie. Hieruit bleek dat PAI-1 een sterk remmend effect heeft op het cytokine interferon-gamma (IFN-γ), want dit was in veel hogere concentraties in het bloed van de PAI-1 knock-out muis te vinden dan in de wild-type muis. Het effect hiervan op ontstekingziekten en infecties waarbij IFN-γ een rol speelt en het mechanisme waardoor PAI-1 IFN-γ remt moet nog verder uitgezocht worden.

Voor het onderzoek, beschreven in **hoofdstuk 4** hebben we gebruik gemaakt van een chronisch ontstekingsmodel van de achterpoten (door injectie van terpentijn onder de huid van de achterpoten van de muis) waarbij muizen ook een systemische acuut fase reactie ontwikkelen (een aspecifieke afweerreactie gemedieerd door de cytokines interleukine(IL)-1 en IL-6). In dit model bleek PAI-1 ook sterk verhoogd aanwezig te zijn. De afwezigheid van PAI-1 bij de knock out muizen versterkte het aantal leukocyten in het begin van de ontstekingsreactie in de achterpoten. Verder hadden deze knock-out muizen lagere concentraties van het IL-6 in het bloed met hierbij ook lagere concentraties van acuut fase eiwitten (aspecifieke afweereiwitten die worden geproduceerd door de lever na activatie door IL-6). Deze bevindingen suggereren dat PAI-1 niet alleen een marker voor ontsteking is maar ook een functionele rol heeft bij lokale en systemische ontstekingsprocessen.

Een bacteriële longontsteking (pneumonie) is een ernstige ziekte met een hoge kans op sterfte, in het bijzonder bij patiënten met een pre-existent verlaagde afweer. Tijdens een pneumonie worden verschillende afweermechanismen geactiveerd waardoor er een uitgebreide ontsteking ontstaat. Hiernaast krijg je activatie van bloedstolling en remming van fibrinolyse door verhoogde concentraties van PAI-1 in de longen. De rol van PAI-1 in antibacteriële afweermechanismen of ontstekingsreacties in de long tijdens pneumonie was nog onbekend. In **hoofdstuk 5** werd onderzoek gedaan naar de rol van PAI-1 tijdens pneumonie. Er werd ontdekt dat bij PAI-1 knock-out muizen veel meer bacteriën en minder leukocyten in de longen aanwezig waren tijdens de infectie dan bij de wild-type muizen. Verder gingen de PAI-1 knock-out muizen ook veel eerder dood. Andere experimenten met juist heel hoge concentraties van PAI-1 in de longen van de muizen (door toediening van een virale vector via de neus, die vervolgens PAI-1 in de long produceert) lieten zien dat deze muizen met veel PAI-1 juist veel leukocyten en veel minder bacteriën in hun longen en een veel betere overleving hadden dan de controle muizen. Deze resultaten laten zien dat PAI-1 waarschijnlijk een essentiële rol speelt bij de verdediging tegen ernstige Gram-negatieve pneumonie en suggereren dat PAI-1 de gastheer beschermt door het stimuleren van de migratie van leukocyten naar de longen.

Vervolgens, hebben we in **hoofdstuk 6** onderzocht of tPA overexpressie (m.b.v. een adenovirale vector) ook effect had op de ontstekingsreacties tijdens Gram-negatieve pneumonie. tPA overexpressie resulteerde in een lagere mortaliteit, hetgeen was geassocieerd met verminderde bacteriële uitgroei, minder verspreiding van de bacteriën door het lichaam en minder orgaanschade (lever). Deze muizen hadden ook meer leukocyten in de longen in het begin van de infectie. Echter, tPA-knock-out muizen lieten een normale afweer tegen dezelfde infectie zien. Het exacte mechanisme achter het beschermende effect van tPA tijdens pneumonie moet nog achterhaald worden.

Een andere levensbedreigende infectie die gepaard gaat met een sterke ontstekingsreactie is buikvliesontsteking (peritonitis), die kan leiden tot sepsis en de dood. Sepsis en peritonitis zijn geassocieerd met een verhoogde productie van tPA. Daarom hebben we in **hoofdstuk 7** onderzoek gedaan naar de functie van tPA in de immuun reactie tegen peritonitis, veroorzaakt door de bacterie *E. coli*. De tPA knock-out muizen hadden een slechtere afweer tegen *E. coli* peritonitis dan wild-type muizen. Dit bleek onafhankelijk van het effect van

tPA op plasmine, want plasminogeen knock-out muizen hadden geen verslechterde afweer tegen dezelfde infectie. Ondanks dat ze veel meer bacteriën in hun buikholte hadden vertoonden de tPA knock-out muizen evenveel leukocyten t.o.v. de wild-type muizen. Adenovirale overexpressie van tPA in het bloed veroorzaakte een verhoogde leukocyten migratie naar de buikholte tijdens *E. coli* peritonitis. Deze resultaten laten zien dat tPA mogelijk een stimulerende rol heeft in de ontstekingsrespons en beschermt tegen Gram-negatieve bacteriële infectie. Het mechanisme waarop dit effect van tPA berust is nog onduidelijk, maar lijkt in ieder geval onafhankelijk te zijn van plasmine. Recente studies hebben laten zien dat tPA niet alleen plasmine maar ook matrix metalloproteinase (MMP)-9 kan activeren, een eiwit dat betrokken is bij extracellulaire matrix degradatie, oftewel het open maken van de vaatwand zodat de leukocyten makkelijker kunnen migreren naar een plek van infectie. Het is mogelijk dat in ons peritonitismodel tPA MMP-9 activeert en dat tPA op deze manier de celmigratie en daardoor ook de anti-bacteriële afweer beinvloed. We hebben in **hoofdstuk 8** onderzocht of MMP-9 inderdaad een rol speelt in de leukocyten migratie naar de buikholte in het *E. coli* peritonitis model. En inderdaad, MMP-9 knock-out muizen vertoonden minder leukocyten in de buik en meer bacteriën. In theorie zou dit een verklaring kunnen zijn voor de verschillen in de tPA knock out muizen en de wild-type muizen, maar dit moet nog nader worden onderzocht.

In **hoofdstuk 9** hebben we onderzoek gedaan naar de rol van het eiwit thrombin-activatable fibrinolysis inhibitor (TAFI) in ontstekingsreacties en afweer tijdens peritonitis. TAFI is een eiwit dat, net als PAI-1, de fibrinolyse kan remmen. TAFI kan naast de fibrinolyse ook complement factoren C3a en C5a remmen. Het complementsysteem bestaat ook weer uit vele eiwitten en speelt een grote rol bij het aantrekken van leukocyten tijdens ontsteking. De afwezigheid van TAFI in de muis had een geen effect op de fibrinolyse-activatie. Wel vonden we een versterkte leukocyten migratie naar de buikholte, mogelijk t. g.v. minder remming van de complement-activatie. Hiernaast liet TAFI ook een verrassend beschermend effect zien op leverschade tijdens bloedvergiftiging die ontstond bij de peritonitis.

De uPA (wat naast tPA ook een activator is van de fibrinolyse)-receptor (uPAR) is een eiwit dat op de buitenkant van een cel vast zit en signalen van buiten de cel naar binnen de cel zendt. De uPAR is aanwezig op verschillende soorten cellen, inclusief leukocyten. Studies hebben laten zien dat uPAR mogelijk een rol speelt bij de migratie van leukocyten. In **hoofdstuk 10** hebben we onderzoek gedaan naar de rol van uPAR bij de migratie van leukocyten als reactie op een injectie van LPS of *E. coli* bacteriën in de buikholte. De uPAR knock-out muizen hadden na toediening van LPS minder cellen in hun buikholte terwijl ze gelijke concentraties chemokines and cytokines hadden vergeleken met de wild-type muizen. Maar tijdens een echte bacteriële *E. coli* peritonitis lieten uPAR knock-out muizen een normaal aantal leukocyten in de buikholte zien. Deze resultaten suggereren dat uPAR deels de leukocyten migratie naar de buikholte als reactie op LPS medieert, maar dat voor deze functie van uPAR gecompenseerd wordt tijdens een echte buikholte- infectie door *E. coli*.

Concluderend is vastgesteld dat verschillende eiwitten van het fibrinolytische systeem, met name PAI-1, tPA, TAFI en uPAR, een rol spelen bij ontstekingsprocessen. Vooral de migratie

van de leukocyten naar de plaats van een infectie of ontsteking wordt sterk beïnvloed door de verschillende mediatoren van het fibrinolytische systeem, hetgeen een grote invloed heeft op de overleving van de bacteriën en de uitkomst van de ziekte. De manier waarop deze eiwitten hun invloed op de leukocyten en andere processen uitoefenen zal in de toekomst nog verder onderzocht moeten worden.

Curriculum Vitae

Curriculum Vitae

Rosemarijn Renckens was born on January 10th 1974 in Ndola, Zambia. She grew up in Haarlem and Hoorn where she graduated from highschool in 1992 at the Rijks Scholen Gemeenschap. After studying psychology and medical informatics, both for one year, she started medical school in 1994 at the Academic Medical Center (AMC) of the University of Amsterdam.

In 1998, she did a research internship of six months at the Department of Infectious Diseases and Microbiology of the Imperial College School of Medicine (st Mary's hospital) in London, UK. She studied the effect van Macrocyclon on mycobacterial viability and cytokines in human blood in the laboratory of prof. dr. D.B. Young, under the guidance of dr. B. Kampmann.

She received her medical degree in 2001, after which she started her PhD project at the Laboratory of Experimental Internal Medicine/ Center for Infection and Immunity Amsterdam (CINIMA) under supervision of prof. dr. T. van der Poll, at the AMC in Amsterdam. She studied the role of the fibrinolytic system in inflammation and infection (most of this research is presented in this thesis) and the effect of a pre-existent sterile acute phase response on host defense during pneumonia.

In 2005 she started her residency in Internal Medicine, which she performs at the Departments of Internal Medicine of the Flevoziekenhuis in Almere (head: dr. S.H.A. Peters), and of the AMC in Amsterdam, under supervision of prof. dr. P. Speelman. In 2009 she hopes to start her specialization at the Department of Infectious Diseases, Tropical Medicine & AIDS at the AMC, Amsterdam, under supervision of dr. J. Prins.

Dankwoord

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