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Regulation of Lipopolysaccharide-Induced Lung Inflammation by Plasminogen Activator Inhibitor-1 through a JNK-Mediated Pathway

Patrick G. Arndt,[1]* Scott K. Young,[†] and G. Scott Worthen*[†]

The neutrophil is of undoubted importance in lung inflammation after exposure to LPS. We have shown recently that systemic inhibition of JNK decreased neutrophil recruitment to the lung after exposure to LPS, although the mechanisms underlying this inhibition are incompletely understood. As plasminogen activator inhibitor-1 (PAI-1) accentuates cell migration, with JNK activation recently shown to up-regulate PAI-1 expression, this suggested that systemic JNK inhibition may down-regulate LPS-induced pulmonary neutrophil recruitment through a decrease in PAI-1 expression. We show in this study that exposure of mice to aerosolized LPS increased PAI-1 expression in the lung and alveolar compartment, which was decreased by pretreatment with the JNK inhibitor SP600125. Exogenous, intratracheally administered PAI-1 prevented the inhibition of pulmonary neutrophil recruitment in the setting of systemic JNK inhibition, thereby suggesting a role for PAI-1 in the JNK-mediated pathway regulating LPS-induced neutrophil recruitment. In addition, PAI-1$^{-/-}$ mice had a decrease in neutrophil recruitment to the alveolar compartment after exposure to LPS, compared with wild-type controls, further suggesting a role for PAI-1 in LPS-induced lung inflammation. An increase in the intravascular level of KC is a likely mechanism for the inhibition of pulmonary neutrophil recruitment after LPS exposure in the setting of decreased PAI-1 expression, as systemic KC levels after exposure to LPS were increased in PAI-1-deficient mice and in mice pretreated with SP600125, with augmentation of intravascular KC levels inhibiting neutrophil recruitment to the lung after exposure to LPS. *The Journal of Immunology,* 2005, 175: 4049–4059.

P lasminogen activator inhibitor-1 (PAI-1)[2] is a member of the serine protease inhibitor (serpin) family. A major function of PAI-1 is the inactivation of tissue-type plasminogen (tPA) and urokinase-type plasminogen (uPA) activators, through covalent binding of PAI-1 to tPA or uPA, resulting in decreased plasmin activity and fibrinolytic potential (1–3). Through its involvement in the fibrinolytic pathway, PAI-1 has been shown to play a significant role in thrombotic-induced diseases, including venous thrombosis (4, 5) and the acute coronary syndrome, wherein serum levels of PAI-1 increase upon coronary occlusion and predict future coronary events in human subjects (6–8). More recently, however, PAI-1 has been shown to regulate cell migration, thereby suggesting that PAI-1, in addition to its role in the fibrinolytic pathway, may also modulate cellular recruitment during the acute inflammatory process (9–15).

PAI-1 regulates cell migration by three separate, although not mutually exclusive, pathways: 1) modulating extracellular matrix degradation via regulation of plasmin levels; 2) regulating cell adhesion; and 3) modifying the formation, or maintenance, of chemoattractant gradients (11–13, 16). Although many chemoattractants for human neutrophils have been described, there has been recent interest in the CXC chemokines, including IL-8, ENA-78, and growth-related oncogene α, β, and γ (17–19). Chemokines bind to glycosaminoglycan-containing proteoglycans, which are present on endothelial cells and in the extracellular matrix (20, 21). In particular, IL-8 binds to several glycosaminoglycans, including heparan sulfate and chondroitin sulfate, and has been shown recently to specifically bind to the heparan sulfate-containing proteoglycan syndecan-1 (16, 22, 23). Although the mechanisms regulating chemokine binding to proteoglycans, including syndecan-1, and the formation of haptotatic gradients are only beginning to be understood, PAI-1 has been suggested to alter these processes. Immunologic blockade of PAI-1 increased IL-8/syndecan-1 shedding from endothelial cells in vitro, which was suggested to occur through an up-regulation in plasmin activity (16). This increase in shedding of IL-8/syndecan-1 complexes decreased neutrophil migration across an activated endothelial surface (16). As previous studies have shown that increases in intravascular IL-8 levels impede neutrophil migration across endothelial cell layers in vitro and neutrophil recruitment to inflammatory foci in vivo (16, 24–28), taken together this suggests that decreases in PAI-1 levels may increase systemic chemokine levels, thereby decreasing neutrophil recruitment to inflammatory foci.

Although PAI-1 has been suggested to regulate cell migration in vitro, the role of PAI-1 in neutrophil migration in general, and more specifically its role in pulmonary neutrophil recruitment in vivo is poorly understood. Septal thickening, lung inflammation, and overall mortality were improved in PAI-1-deficient mice in a hyperoxia model of lung injury, although a direct assessment of neutrophil recruitment to the alveolar compartment was not performed in that study (29). In contrast, neutrophil recruitment to the lung was not diminished in PAI-1-deficient mice after intratracheal (IT) inoculation of *Streptococcus pneumoniae* (30), or in a model of chronic LPS exposure (31).

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[2] Abbreviations used in this paper: PAI-1, plasminogen activator inhibitor-1; BAL, bronchoalveolar lavage; IT, intratracheal; KRPD, Krebs-Ringer-buffered phosphate with dextrose; MPO, myeloperoxidase; tPA, tissue-type plasminogen; uPA, urokinase-type plasminogen; wt, wild type.

Several pathways have been described that regulate PAI-1 expression, including the MAPK family (32, 33). The MAPK JNK is activated upon exposure to a broad range of stimuli, including osmotic stress (34), heat shock (34), IL-1β (35), and TNF-α (36), and we have shown recently that JNK is activated in LPS-stimulated neutrophils (37). In addition, a role for JNK in regulating pulmonary inflammation has been suggested recently by our group and others, in that systemic inhibition of JNK decreased acute inflammation after LPS (38), stretch (39), or ischemia-reperfusion-induced lung injury (40). Activation of JNK increases PAI-1 expression in vitro (41, 42), and although other mechanisms were proposed for the decrease in acute inflammation with JNK inhibition in the above studies, the role of JNK in mediating PAI-1 expression suggests that JNK activation may increase PAI-1 expression in vivo, and that an increase in PAI-1 levels may be one mechanism regulating pulmonary neutrophil recruitment in models of lung inflammation. These possibilities are currently unexplored.

The recent findings that activation of JNK increases PAI-1 expression (41, 42), combined with our recent findings of a decrease in LPS-induced pulmonary neutrophil recruitment in the setting of systemic JNK inhibition (38), suggested to us that JNK inhibition impedes pulmonary neutrophil recruitment after exposure to LPS through a decrease in PAI-1 expression. This further suggested that PAI-1 regulates neutrophil recruitment to the lung after exposure to LPS. Finally, because PAI-1 has been shown to modulate the distribution of IL-8, and increases in intravascular levels of IL-8

inhibit neutrophil accumulation at sites of acute inflammation (25, 28), this suggested that one potential mechanism by which a decrease in PAI-1 may regulate neutrophil recruitment to the lung after LPS exposure is through an increase in the intravascular level of IL-8, or more specifically its murine homologue, KC.

Materials and Methods

Materials

All materials used were endotoxin free and are routinely tested for the presence of endotoxin by the *Limulus* amebocyte lysate test (Pyrogent; Cambrex). LPS (*Escherichia coli* 0111B:4) was obtained from Sigma-Aldrich; constitutively active PAI-1 (MPAI-191L) and the PAI-1 mutant Q123K PAI-1, which is unable to bind to vitronectin, were obtained from Molecular Innovations; and the JNK inhibitor SP600125 was obtained from EMD Biosciences. TRIzol was purchased from Invitrogen Life Technologies; Moloney murine leukemia virus-reverse transcriptase from Invitrogen Life Technologies; and Biolase Red *Taq* polymerase from Bioline. The sheep anti-mouse PAI-1 Ab used for immunohistochemistry was obtained from American Diagnostica, and the biotinylated donkey anti-sheep Ab was from Santa Cruz Biotechnology.

Animals

Female C57BL/6, PAI-1$^{-/-}$ mice, on a C57BL/6 background, and C57BL/6J (wild type (wt) controls for PAI-1$^{-/-}$ mice), 8–12 wk of age, were purchased from The Jackson Laboratory and were kept in a pathogen-free environment on a 12-h light and dark cycle with full access to food and water. All animal experiments were approved by the Animal Use and Care Committee at National Jewish Medical and Research Center.

FIGURE 1. Exposure to aerosolized LPS increases PAI-1 expression in the lung and alveolar compartment. C57BL/6 mice (n = 5) were exposed to aerosolized LPS (300 μg/ml, 20 min), with BAL performed, blood collected, and lungs isolated at the indicated times. Total (A, B, and D) and active (C and E) PAI-1 levels were assessed by ELISA. F, PAI-1 mRNA expression in the lung after LPS exposure was assessed by RT-PCR. Gel shown is representative of one of three experiments with similar results. *, $p < 0.001$ LPS vs baseline; #, $p < 0.05$ LPS vs baseline.
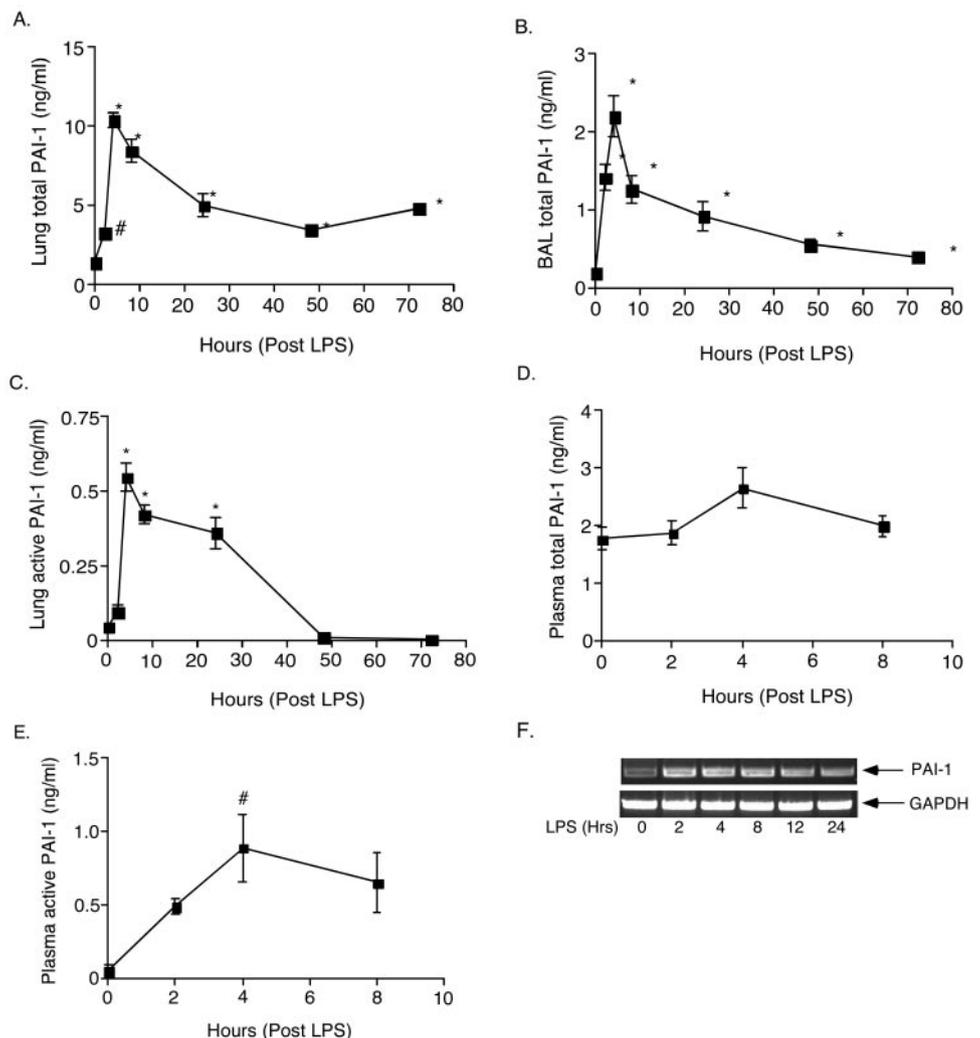
Animal models

In examining the effect of systemic JNK inhibition on the intravascular and pulmonary levels of KC and PAI-1, C57BL/6 mice were pretreated with the JNK inhibitor SP600125 (30 mg/kg, s.c.), diluted in buffer containing 30% PEG-400, 20% polypropylene glycol, 15% Cremophor EL, 5% ethanol, and 30% saline, 3 h before LPS exposure. Mice were then exposed to aerosolized LPS (300 μg/ml in 0.9% saline, 20 min), as previously described (38, 43). At set times, mice were sacrificed by exsanguination with blood collected, bronchoalveolar lavage (BAL) performed, and lungs harvested, as described previously (43). To assess the LPS-induced pulmonary neutrophil recruitment in PAI-1$^{-/-}$ mice, PAI-1$^{-/-}$ mice or C57BL/6J wt controls were exposed to aerosolized LPS (300 μg/ml, 20 min), then sacrificed 4 or 24 h after exposure to LPS, with neutrophil recruitment to the alveolar space and lung determined, as previously described (43). To determine the effect of administration of exogenous PAI-1 on neutrophil accumulation in the lung after LPS exposure in mice pretreated with SP600125, C57BL/6 mice, pretreated with SP600125 as described above, were administered constitutively active PAI-1 (MPAI-191L (10 or 50 μg/ mouse)), Q123K PAI-1 (50 μg/mouse), or saline as control, IT, followed by exposure to LPS 1 h after IT PAI-1 administration. Animals were sacrificed 4 h after exposure to LPS, with neutrophil recruitment to the alveolar space determined. Similarly, to assess the administration of PAI-1 on LPS-induced neutrophil recruitment to the lung in mice deficient for PAI-1, PAI-1$^{-/-}$ mice or C57BL/6J wt controls were administered MPAI-191L (10 or 50 μg/mouse), then exposed to LPS 1 h later, with assessment of neutrophil recruitment to the lung 4 h after LPS exposure.

For assessment of total peripheral white blood cell counts in PAI-1$^{-/-}$ or C57BL/6J wt control mice, at baseline or after LPS exposure, blood was obtained, with the number of white blood cells determined by an electronic cell counter, Model Z (Coulter Scientific Instruments). The percentage of neutrophils in the peripheral blood was determined in duplicate by a manual cell count of peripheral blood smears from PAI-1$^{-/-}$ or C57BL/6J wt control mice at baseline or at 4 or 8 h after exposure to LPS.

PAI-1 expression in RAW264.7 cells

The murine macrophage cell line, RAW264.7, was grown to 60–80% confluence in DMEM/10% FCS/1% glutamine. Cells were serum starved overnight, incubated with SP600125 (2–10 μM) or 0.1% DMSO as diluent control, for 30 min, then stimulated with LPS (100 ng/ml) or TNF-α (10 ng/ml) for 15 h. Supernatants were collected, snap frozen in an ethanol dry ice bath, and stored at $-80°$C for later determination of PAI-1 levels by ELISA. To the remaining cell culture, TRIzol was added and mRNA was extracted, as previously described (37).

ELISA

Upon collection, BAL fluid was centrifuged for 5 min at 14,000 rpm with the cell-free supernatant stored at $-70°$C. For the determination of intravascular chemokine and cytokine levels, collected blood was placed in lysis buffer (50 mM HEPES (pH 7.6), 100 mM NaCl, 2 mM EDTA, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 1% Triton X-100) and then centrifuged at 10,000 rpm for 5 min, with the supernatant then stored at $-70°$C. Plasma was isolated by placing blood, collected by exsanguination, into a 17% EDTA solution that was then centrifuged at 300 × g for 20 min, with the upper layer collected and frozen at $-70°$C until used. After isolation, the left lung was homogenized in PBS/1% Triton X-100, pH 7.4, containing aprotinin (10 μg/ml), leupeptin (10 μg/ml), and PMSF (0.6 μM/ml), then centrifuged at 5,000 rpm for 10 min, with the supernatant stored at $-70°$C. ELISAs were performed for KC, IL-1β, and TNF-α on blood, lung, and BAL fluid, as indicated, as previously described (43). Determination of total and active PAI-1 levels in plasma, lung, and BAL fluid and total PAI-1 levels in RAW264.7 supernatants was performed using ELISA kits from Molecular Innovations, with assays performed per the manufacturer's protocol.

RT-PCR

RT-PCR for murine PAI-1 was performed, as previously described (37). Murine PAI-1 primers used were 5′-CCAGCGCCTCTTCCAGT-3′ and 5′-CGGCCAGGGTTGCACTAAAC-3′, and primers were GAPDH, 5′-TCATCCATGACAACTTTGGTATCG-3′ and 5′-TGGCAGGTTTTTC TAGACGGC-3′. PCR products were resolved on 1% agarose gels, with bands identified by ethidium bromide staining.

Actin polymerization in murine peripheral blood neutrophils

Murine peripheral blood neutrophils were isolated from blood collected by exsanguination, with isolated blood placed into a 17% EDTA solution. After centrifugation for 20 min at 300 × g, the cell pellet was resuspended
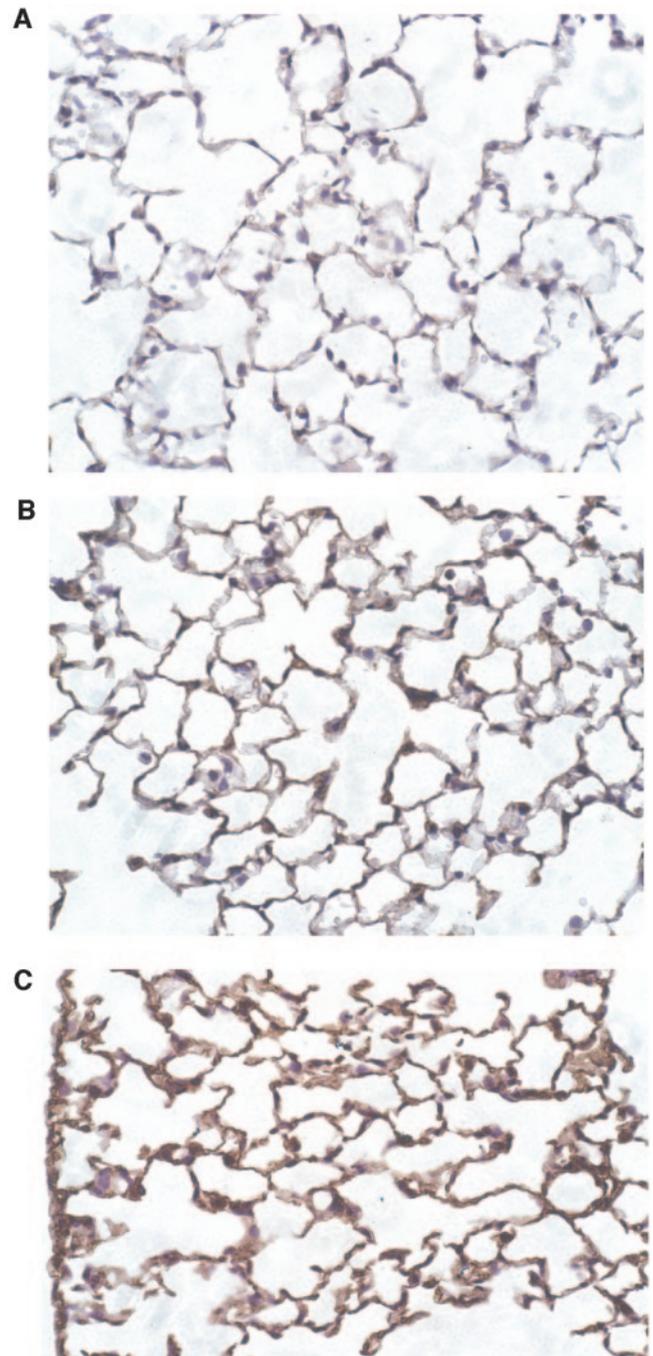
FIGURE 2. Expression of PAI-1 increases in the lung after exposure to LPS. Immunohistochemistry for PAI-1 was performed on 5-μm sections of lung tissue from C57BL/6 mice after exposure to LPS (*C*), or saline as control (*B*), as described in *Materials and Methods*. Lung sections from saline-exposed PAI-1-deficient mice (*A*) were used as negative controls. Plates are representative fields from one of three mice for each condition at ×400 magnification.

into a 6% dextran and 0.9% sodium chloride solution (dextran:sodium chloride 1:5.25), to 150% of the original volume, to allow for RBC lysis. After gravity sedimentation for 30 min, the white blood cell-rich supernatant was collected, washed once in Hank's buffered salt solution, and then layered over a Percoll gradient (78, 68, and 54%) and centrifuged for 20 min at 1200 × g. Cells were collected at the 78:68% interface, washed once in Hank's buffered salt solution, and then resuspended in Krebs-Ringer-buffered phosphate with dextrose (KRPD) at a concentration of 3 × 10⁵/ml. Cytospins of the collected cells consistently demonstrated >90% neutrophils. Isolated peripheral blood neutrophils were then exposed to KC (100 ng/ml), or PBS as control, for 10 min, centrifuged at 300 × g for 6

min, washed once in KRPD, and then resuspended at 3×10^5/ml in KRPD. Actin polymerization upon exposure to KC (100 ng/ml; 2 or 5 min), or PBS as control, was then assessed, as previously described (38).

Routine H&E staining and immunohistochemistry

After sacrifice, murine lungs from LPS-exposed PAI-1$^{-/-}$ or C57BL/6J wt controls were inflated, isolated, and fixed in 3.7% Formalin overnight at room temperature. Five-μm sections were then stained with H&E and examined microscopically. For PAI-1 immunohistochemistry, sections (5 μm) of paraffin-embedded lungs from C57BL/6 mice exposed to LPS (4 h) or saline as control, or saline-exposed PAI-1$^{-/-}$ mice, were deparaffinized with xylene and then incubated with 0.5% hydrogen peroxide for 30 min to quench endogenous peroxidase. After washing five times in PBS, nonspecific binding was inhibited by incubating slides with a rat anti-mouse CD16 Ab (BD Pharmingen) for 30 min. Slides were then incubated with a sheep anti-mouse PAI-1 Ab (1:250) (American Diagnostica) for 30 min at room temperature in a humidified chamber. After primary staining, slides were washed five times in PBS, incubated with a biotinylated donkey anti-sheep Ab for 30 min, and after extensive washing were then incubated with streptavidin HRP for 30 min. Slides were developed in a diaminobenzidine Tris buffer solution, washed, then counterstained with a 1/4 dilution of hematoxylin solution number 2, dehydrated, cleared with xylene, coverslipped, and examined microscopically.

Statistics

Statistical analysis was performed by Student's t test or one-way ANOVA with Tukey-Kramer post hoc analysis using Prism software (GraphPad). A p value of <0.05 was considered significant.

Results

Exposure to aerosolized LPS increases lung, BAL fluid, and plasma PAI-1 levels

Levels of PAI-1 are increased in BAL fluid from subjects with adult respiratory distress syndrome and in the blood, lung, heart, and kidney after i.v. LPS administration (44–49). To assess whether localized LPS exposure increases pulmonary and systemic PAI-1 expression, we measured total PAI-I levels in lung homogenates, BAL fluid, and plasma from mice exposed to aerosolized LPS. Levels of total PAI-1 increase quickly in the lung and BAL fluid, with peak levels seen 4 h after exposure to LPS, and remain above baseline for up to 72 h after LPS exposure (Fig. 1, *A* and *B*). In addition, as can be seen in Fig. 1, *A* and *B*, levels of PAI-1 increase more rapidly in BAL fluid than in lung homogenates, with levels near peak 2 h after LPS exposure in BAL fluid. Because

FIGURE 3. Systemic JNK inhibition with SP600125 decreases pulmonary, but not systemic, PAI-1 expression after exposure to LPS. C57BL/6 mice (*n* = 5) were pretreated with SP600125 (30 mg/kg, s.c.), or diluent as control, 3 h before LPS exposure. Mice were sacrificed 2 or 4 h after exposure to LPS, with BAL performed, blood collected, and lungs isolated. Levels of total and active PAI-1 were measured by ELISA. *A*, Lung levels of total and active PAI-1. *B*, Total PAI-1 in BAL fluid. *C*, Intravascular levels of total and active PAI-1. C, control; SP, SP600125 only; LPS, LPS exposed; SP + LPS, SP600125 pretreated and LPS exposed. ∗, *p* < 0.001 LPS vs LPS + SP; #, *p* < 0.05 LPS vs LPS + SP.
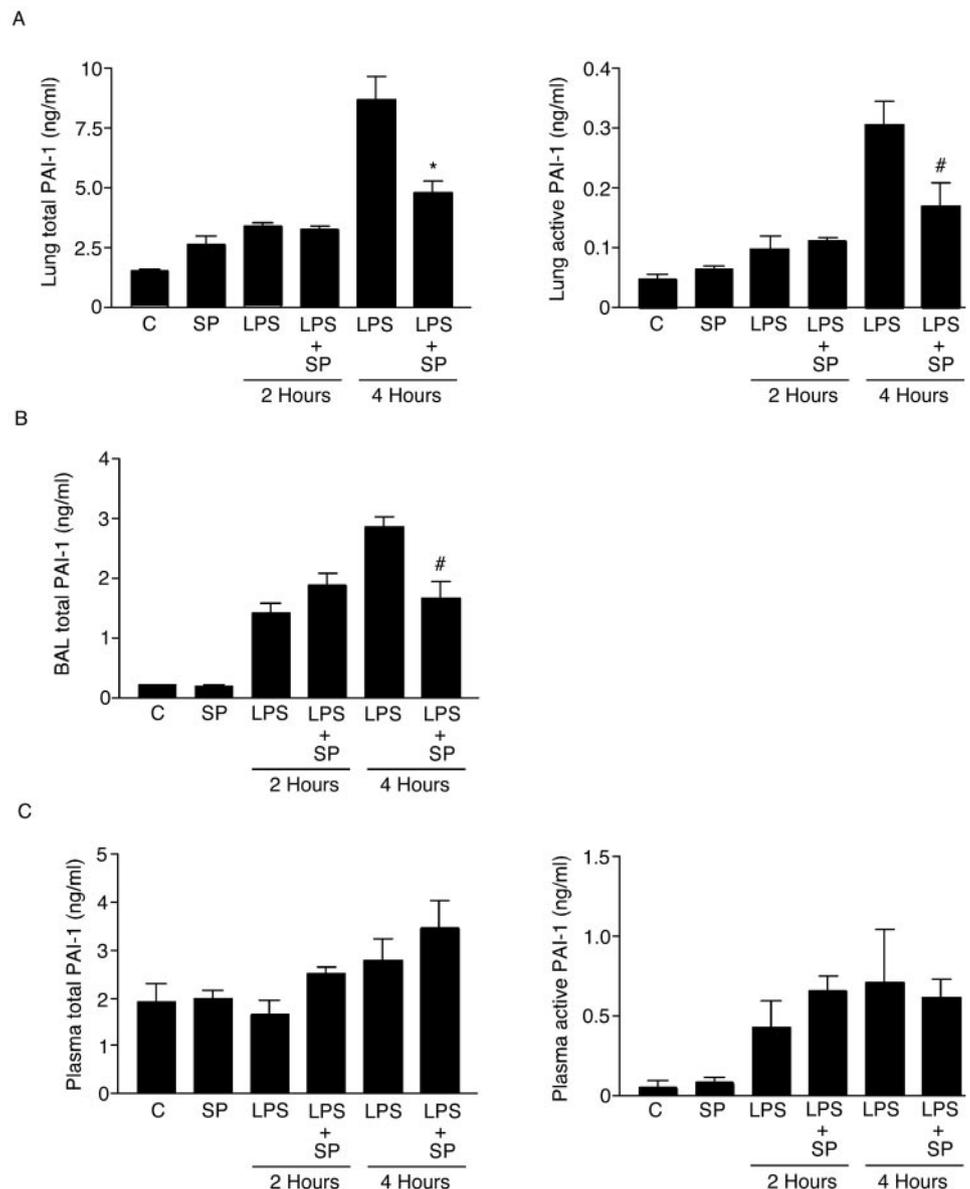
PAI-1 exists in both active and latent forms, with only the active form able to bind uPA and tPA, thereby decreasing plasmin activity, we next assessed whether LPS exposure increases levels of active PAI-1. Exposure to LPS increased active PAI-1 levels in lung homogenates, with kinetics similar to that seen for total PAI-1 (Fig. 1*C*); however, levels of active PAI-1 in the alveolar compartment after LPS exposure were below the level of detection (data not shown). In contrast to pulmonary PAI-1 expression, levels of total PAI-1 in the systemic circulation, as assessed by plasma PAI-1 levels, do not significantly increase after exposure to LPS, although systemic levels of active PAI-1 do increase after localized exposure to LPS (Fig. 1, *D* and *E*). Finally, to assess whether the increase in lung PAI-1 levels after exposure to LPS was due to an increase in PAI-1 expression, PAI-1 RNA expression was assessed by RT-PCR. Expression of PAI-1 increased in LPS-exposed lung, compared with saline control, by 4 h (Fig. 1*F*).

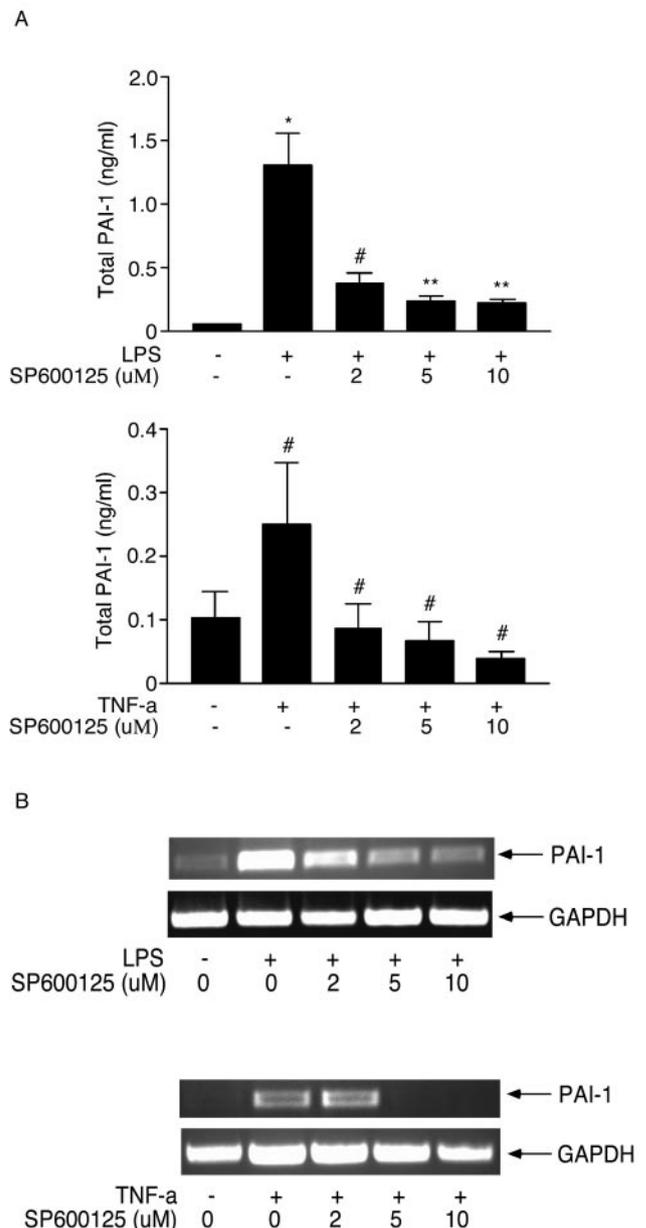*PAI-1 expression in the lung after exposure to LPS*

To confirm that aerosolized LPS up-regulated pulmonary PAI-1 levels in our model, and to begin to assess the cellular source of PAI-1 expression after localized exposure to LPS, PAI-1 expression in the lung was assessed immunohistochemically. Paraffin-embedded lung sections (5 μM) from saline- or LPS (4 h)-exposed mice were stained for PAI-1 and then examined microscopically (see *Materials and Methods*). In addition, as a negative control, lung sections from PAI-1-deficient mice were stained for PAI-1 to confirm specificity of the PAI-1 Ab. Expression of PAI-1 was increased 4 h after LPS exposure (Fig. 2*C*), by immunohistochemical staining, compared with saline-exposed lungs (Fig. 2*A*), with prominent staining of the alveolar epithelium, endothelium, and cells morphologically similar to alveolar macrophages. Faint staining for PAI-1 was present in mice deficient for PAI-1, suggesting some nonspecificity of the PAI-1 Ab used, because incubation of tissue sections in the absence of primary Ab did not reveal positive staining under any conditions (Fig. 2*A*, and data not shown).

Systemic JNK inhibition decreases LPS-induced up-regulation of PAI-1 in the lung, but not in the systemic circulation

Recently, PAI-1 expression in vitro after oxidative stress or thrombin exposure has shown to be regulated through a JNK-mediated pathway (41, 42). To examine whether JNK regulates PAI-1 expression in vivo in the lung after exposure to LPS, we measured total and active PAI-1 levels in lung, BAL fluid, and plasma from mice pretreated with the specific JNK inhibitor SP600125 (30 mg/kg) 3 h before exposure to LPS compared with those exposed to LPS only. Because PAI-1 expression was maximal at 4 h after LPS exposure in BAL fluid, lung, and plasma (Fig. 1), we chose to examine the effect of JNK activation on PAI-1 expression at 2 and 4 h after exposure to LPS. As seen in Fig. 3, pretreatment with the JNK inhibitor SP600125 decreased the LPS-induced up-regulation of total and active PAI-1 levels in the lung and total PAI-1 in BAL fluid. Systemic inhibition of JNK, however, did not alter the LPS-induced increase in the intravascular levels of total or active PAI-1 (Fig. 3*C*).

JNK inhibition decreases LPS- and TNF-α-induced PAI-1 expression in the murine macrophage cell line RAW264.7

Levels of PAI-1 are increased in BAL fluid after exposure to LPS, with increased expression seen in alveolar macrophages by immunohistochemical staining (Figs. 1 and 2). Because alveolar macrophages are of undoubted importance in our aerosolized model of LPS-induced lung inflammation, we assessed whether LPS or TNF-α induces PAI-1 expression in macrophages and examined whether this up-regulation is mediated through JNK activation.

FIGURE 4. Inhibition of JNK decreases PAI-1 expression in the murine macrophage cell line RAW264.7 after exposure to LPS or TNF-α. Cultures of RAW264.7 cells were serum starved overnight, then incubated with SP600125 (2–10 μM) for 30 min. Cells were then exposed to LPS (100 ng/ml) or TNF-α (10 ng/ml) for 15 h, with supernatant collected and mRNA isolated from cultured cells. *A*, Total PAI-1 levels were measured in cell supernatants after exposure to LPS (*top*) or TNF-α (*bottom*) by ELISA. Results are mean ± SEM of three separate experiments. *B*, PAI-1 mRNA expression from RAW264.7 cells pretreated with SP600125 and then stimulated with LPS (100 ng/ml) or TNF-α (10 ng/ml) was assessed by RT-PCR, with GAPDH used as control. PCR products were resolved on 1% agarose gels and stained with ethidium bromide. Results shown are representative of three experiments with similar results. *, $p < 0.001$ LPS vs NS; **, $p < 0.001$ LPS vs LPS + SP; #, $p < 0.01$ TNF-α vs NS, LPS vs LPS + SP, and TNF-α vs TNF-α + SP.

After serum starvation overnight, cultures of the murine macrophage cell line RAW264.7 were preincubated with the JNK inhibitor SP600125 (2–10 μM) for 30 min, then exposed to LPS (100 ng/ml) or TNF-α (10 ng/ml) for 15 h, with cell culture supernatants collected, and total PAI-1 levels measured. Both LPS and TNF-α increased total PAI-1 levels in RAW264.7 supernatants, and this

up-regulation in PAI-1 levels was decreased in a dose-dependent manner by SP600125 pretreatment (Fig. 4A). To examine whether SP600125 pretreatment decreased PAI-1 mRNA expression after LPS or TNF-α exposure, thereby providing a mechanism for the observed decrease in PAI-1 levels, PAI-1 mRNA expression was examined by RT-PCR from RAW264.7 cells pretreated with SP600125 and exposed to LPS or TNF-α compared with those exposed to LPS or TNF-α alone. Pretreatment with SP600125 dose dependently decreased LPS- and TNF-α-induced PAI-1 mRNA expression (Fig. 4B).

PAI-1 expression regulates pulmonary neutrophil influx after exposure to LPS

We have shown recently that inhibition of JNK activation with SP600125 decreased pulmonary neutrophil recruitment after exposure to aerosolized LPS (38). Before assessing whether a decrease in PAI-1 expression is a mechanism for the decrease in LPS-induced pulmonary neutrophil recruitment with systemic JNK inhibition, we first determined whether PAI-1 is involved in the recruitment of neutrophils to the alveolar space after exposure to LPS. To examine the role of PAI-1 in LPS-induced lung inflammation, PAI-1-deficient mice (PAI-1$^{-/-}$) or C57BL/6J wt controls were exposed to LPS and then sacrificed 4 or 24 h after

LPS exposure. Compared with C57BL/6J wt controls, PAI-1$^{-/-}$ mice demonstrated a decrease in neutrophil influx into the alveolar compartment at both the 4- and 24-h time point (Fig. 5A). As can be seen in Fig. 5A, PAI-1 deficiency had a larger inhibitory effect on neutrophil accumulation 4 h after LPS exposure than at 24 h. In contrast, compared with the decrease in neutrophil recruitment to the alveolar compartment in PAI-1-deficient mice after LPS exposure, no change in the total neutrophil recruitment to the lung, as assessed by myeloperoxidase (MPO) and histology, after exposure to LPS was seen in PAI-1-deficient mice compared with wt controls at either time point (Fig. 5, B and C). The decrease in neutrophil recruitment to the alveolar compartment after LPS exposure in PAI-1-deficient mice was not the result of a decrease in the total white blood cell count or in the percentage of neutrophils in the peripheral circulation of PAI-1$^{-/-}$ mice, compared with wt controls, at baseline or after exposure to LPS (Fig. 5D). To confirm that a deficiency in PAI-1 expression was the mechanism for the decrease in LPS-induced neutrophil recruitment to the alveolar compartment in PAI-1$^{-/-}$ mice, constitutively active rPAI-1 (10 or 50 μg), or saline as control, was administered IT to PAI-1-deficient animals, which were then exposed to LPS and sacrificed 4 h after LPS exposure. Exogenous PAI-1 (50 μg) restored the LPS-induced neutrophil recruitment to the alveolar space in PAI-1-deficient mice (Fig. 5E).
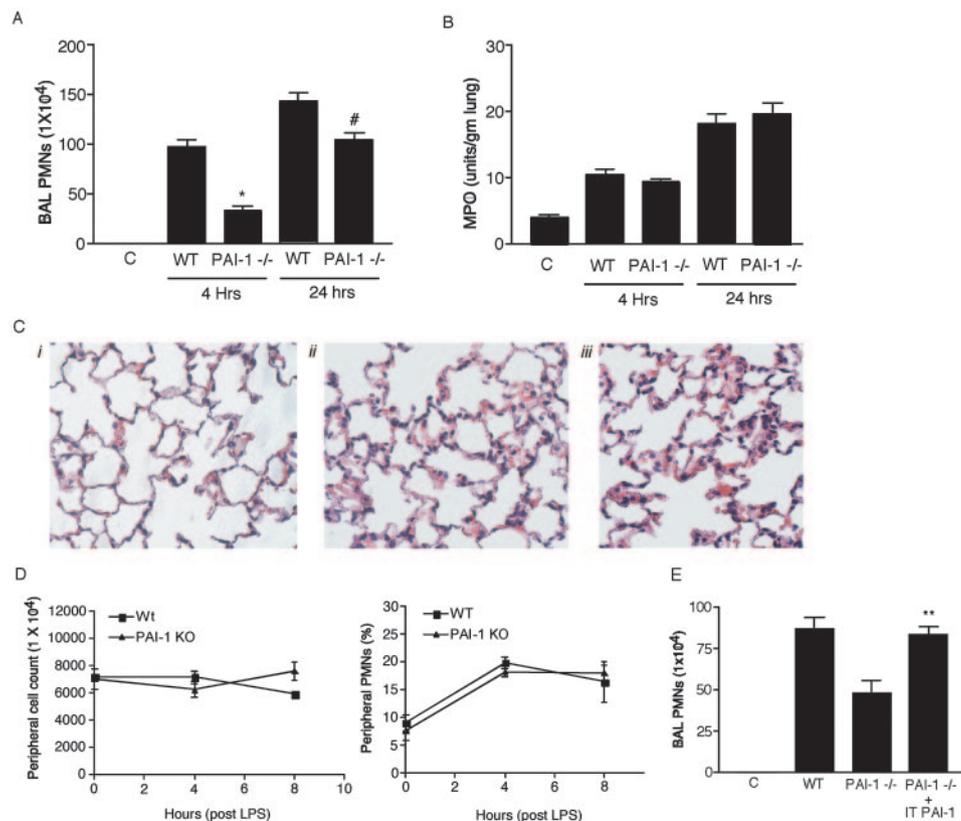
FIGURE 5. Expression of PAI-1 regulates LPS-induced pulmonary neutrophil recruitment. Mice (n = 5) deficient in PAI-1 (PAI-1$^{-/-}$) or C57BL/6J wt controls were exposed to LPS, with assessment of neutrophil accumulation at 4 or 24 h after LPS exposure in the alveolar compartment (A) and lung (B), by BAL cell counts and MPO, respectively. C, Histological assessment of LPS-induced acute lung inflammation in PAI-1$^{-/-}$ mice. Lungs from control saline-exposed PAI-1$^{-/-}$ (i), LPS-exposed PAI-1$^{-/-}$ (ii), and LPS-exposed wt mice (iii) were isolated 8 h after LPS exposure and fixed. Lung sections (5 μm) were stained with H&E. Images shown are representative fields of one of three mice at each condition at ×400 magnification. D, Total peripheral white blood cell count by Coulter (left), and percentage of peripheral circulating neutrophils (right) was assessed, as per Materials and Methods. E, PAI-1$^{-/-}$ mice (n = 5) were administered rPAI-1 (50 μg/mouse) 1 h before LPS exposure. At 4 h after exposure to LPS, mice were sacrificed and BAL was performed, with neutrophil accumulation to the alveolar compartment assessed. ∗, p < 0.001 PAI-1$^{-/-}$ vs wt; #, p < 0.01 PAI-1$^{-/-}$ vs wt; ∗∗, p < 0.05 PAI-1$^{-/-}$ + IT PAI-1 vs PAI-1$^{-/-}$.

IT administration of PAI-1 reverses the inhibition of LPS-induced pulmonary neutrophil recruitment by systemic JNK inhibition through a vitronectin-binding independent mechanism

To determine whether a decrease in PAI-1 levels was a mechanism for the decrease in LPS-induced pulmonary neutrophil recruitment with systemic JNK inhibition, constitutively active rPAI-1 (10 or 50 μg), or saline as control, was administered IT to mice pretreated with SP600125, followed by exposure to LPS and assessment of neutrophil recruitment to the alveolar space 4 h after exposure to LPS. IT administration of PAI-1 alone did not induce neutrophil accumulation into the alveolar compartment, nor did PAI-1 synergize with LPS to increase pulmonary neutrophil recruitment compared with LPS exposure alone (Fig. 6A). Administration of PAI-1, however, did significantly reverse the inhibition of pulmonary neutrophil recruitment to the alveolar space in the setting of systemic JNK inhibition in a dose-dependent manner (Fig. 6A). To begin determining the importance of PAI-1 binding to vitronectin in regulating LPS-induced pulmonary neutrophil recruitment in the setting of systemic JNK inhibition, a mutant of PAI-1 unable to bind to vitronectin, Q123K PAI-1, was administered IT (50 μg/mouse) in mice pretreated with SP600125, followed by exposure to LPS. Administration of Q123K PAI-1 completely reversed the inhibitory effect of SP600125 on LPS-induced pulmonary neutrophil recruitment similar to constitutively active PAI-1 (Fig. 6B),
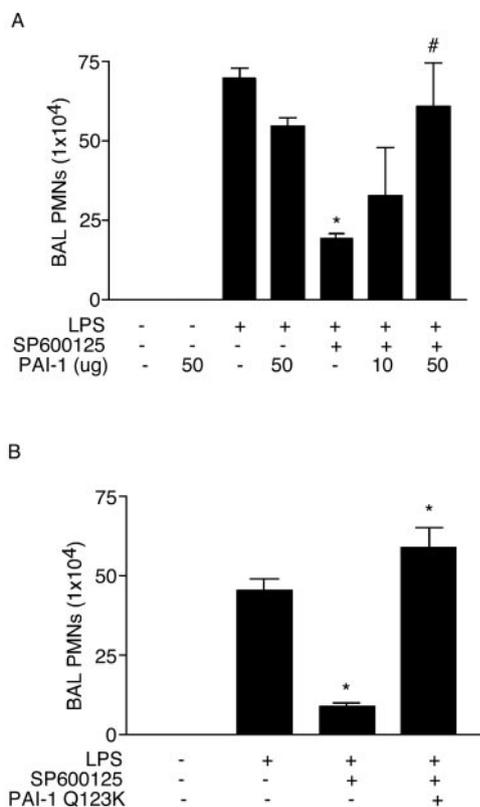
FIGURE 6. Repletion of PAI-1 in mice pretreated with SP600125 reverses the inhibition in LPS-induced neutrophil recruitment to the lung in a vitronectin-binding independent manner. *A*, Constitutively active rPAI-1 (MPAI-191L) (10 or 50 μg/mouse), or *B*, a PAI-1 mutant unable to bind to vitronectin (Q123K PAI-1) (50 μg/mouse) was administered IT to mice, which were pretreated with SP600125 (30 mg/kg, s.c.) 3 h before IT PAI-1 administration. Mice were then exposed to LPS 1 h after IT PAI-1, with assessment of neutrophil accumulation in the alveolar compartment 4 h after exposure to LPS by BAL cell counts and differentials. *, $p < 0.001$ LPS + SP vs LPS and LPS + SP + PAI-1 Q123K vs LPS + SP; #, $p < 0.05$ LPS + SP + PAI-1 vs LPS + SP.

suggesting that the ability of PAI-1 to bind to vitronectin is not necessary in the JNK-mediated pathway regulating neutrophil recruitment to the lung after exposure to LPS.

Deficiency of PAI-1 increases intravascular KC levels after LPS exposure, with no change in BAL levels of KC, TNF-α, or IL-1β

To determine whether PAI-1 regulates the level of neutrophil chemoattractants in vivo, as a mechanism for the decrease in neutrophil recruitment, intravascular, BAL fluid, and lung KC levels were measured 4 h after exposure to LPS, which corresponds to the peak in KC levels in our model (43). Intravascular KC levels are increased in PAI-1-deficient mice compared with C57BL/6J wt controls (Fig. 7A, *left*); however, KC levels in BAL fluid and lung were no different in PAI-1$^{-/-}$ mice compared with wt controls (Fig. 7, *A* and *B*). In addition, BAL fluid and systemic levels of TNF-α and IL-1β are unchanged after LPS exposure in PAI-1$^{-/-}$ mice compared with wt controls (Fig. 7C, and data not shown). Because systemic levels of KC were increased in PAI-1$^{-/-}$ mice after exposure to LPS, with a deficiency of PAI-1 most likely increasing pulmonary plasmin activity, this suggested to us that increased plasmin levels in the lung may increase systemic KC levels. To examine this possibility, plasmin (10–1000 ng), or saline as control, was administered IT to mice 1 h after exposure to LPS, with animals sacrificed 5 h after LPS exposure. Systemic KC levels were augmented in mice administered IT plasmin after LPS exposure compared with LPS exposure alone (Fig. 7D, *left*), with lung KC levels also increased in the setting of IT plasmin administration (Fig. 7D, *right*).

Systemic JNK inhibition increases systemic KC levels

Our recent finding of a decrease in LPS-induced neutrophil recruitment to the lung and alveolar compartment with systemic inhibition of JNK was paradoxically associated with an increase in BAL fluid levels of KC and MIP-2 (38). Based on our findings of an increase in systemic KC levels in PAI-1$^{-/-}$ mice after LPS exposure (Fig. 7A) and the role of JNK in regulating LPS-induced pulmonary PAI-1 expression (Fig. 3), we questioned whether systemic levels of KC are elevated in mice in which JNK is systemically inhibited before LPS exposure. To investigate this possibility, intravascular KC levels were assessed at baseline and after exposure to LPS from mice pretreated with the JNK inhibitor SP600125 and exposed to LPS compared with those exposed to LPS alone. Intravascular KC levels were increased in mice administered SP600125 and exposed to LPS, compared with those exposed to LPS alone, at baseline and 4 h after LPS exposure (Fig. 8). In contrast, lung KC levels were unchanged in SP600125-pretreated mice compared with those pretreated with vehicle alone (Fig. 8).

Augmentation of systemic KC levels decreases pulmonary neutrophil recruitment after aerosolized LPS exposure most likely through an inhibition of KC-induced actin polymerization

Because LPS-induced intravascular KC levels were increased in PAI-1$^{-/-}$ mice and in mice pretreated with the JNK inhibitor SP600125, which was associated in both settings with a decrease in pulmonary neutrophil recruitment, we investigated the effect of augmenting intravascular KC levels on neutrophil recruitment to the alveolar compartment after exposure to aerosolized LPS. KC (1.2 μg/kg) was administered i.v. to C57BL/6 mice, followed by exposure to LPS, or saline as control, 2 h later, with neutrophil accumulation in the alveolar compartment assessed 4 h after exposure to LPS. Administration of i.v. KC alone did not induce pulmonary neutrophil recruitment; however, i.v. injection of KC before LPS exposure decreased pulmonary neutrophil recruitment
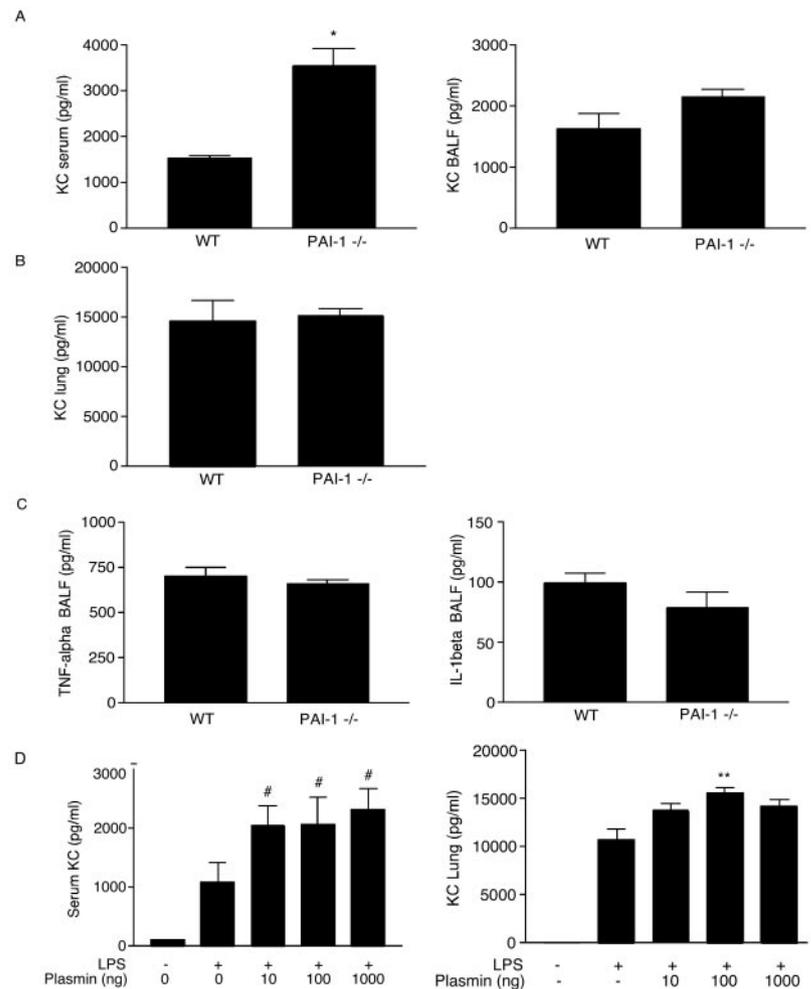
FIGURE 7. Deficiency of PAI-1 increases serum KC levels after LPS exposure without affecting BAL levels of KC, TNF-α, or IL-1β. Intravascular, lung, and BAL levels of KC (*A* and *B*), and BAL levels of TNF-α (*C*, *left*) and IL-1β (*C*, *right*) were assessed in PAI-1$^{-/-}$ or wt mice 4 h after exposure to LPS by ELISA. *D*, C57BL/6 mice ($n = 4$) were exposed to LPS; administered plasmin (10–1000 ng/mouse) IT, or saline as control; and then sacrificed 5 h after LPS exposure. Intravascular and lung KC levels were measured by ELISA. ∗, $p < 0.001$ PAI-1$^{-/-}$ vs wt; #, $p < 0.05$ LPS + plasmin vs LPS; ∗∗, $p < 0.01$ LPS + plasmin vs LPS.
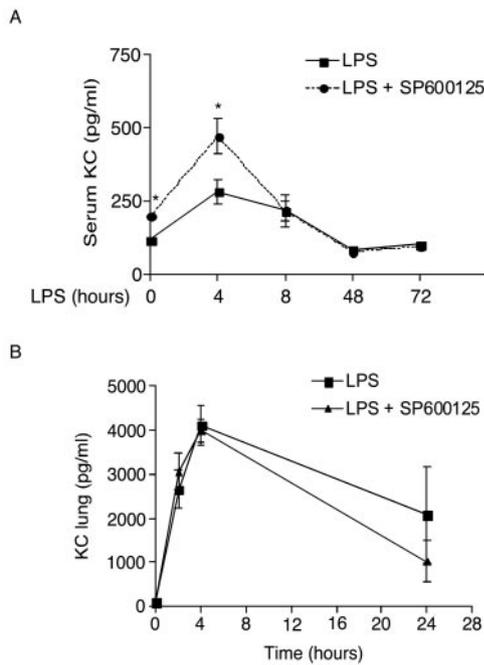
to the alveolar space compared with LPS exposure alone (Fig. 9*A*). In contrast, total lung neutrophil recruitment, as assessed by MPO, was unchanged in mice administered i.v. KC and exposed to LPS compared with those exposed to LPS alone (Fig. 9*B*). To confirm that systemic KC levels increased with i.v. KC administration, intravascular KC levels were measured by ELISA 4 h after exposure to LPS. Intravascular KC levels were almost 2-fold higher in mice administered KC and exposed to LPS compared with those exposed to LPS alone (Fig. 9*C*). To identify a mechanism for the decrease in pulmonary neutrophil recruitment with augmentation of systemic KC levels, KC-induced actin polymerization was assessed in isolated peripheral blood murine neutrophils pre-exposed to exogenous KC, or buffer as control. Peripheral blood murine neutrophils pre-exposed to KC demonstrated a decrease in actin polymerization upon subsequent re-exposure to KC, compared with peripheral blood murine neutrophils pre-exposed to buffer and then exposed to KC (Fig. 9*D*).

Discussion

Although PAI-1 expression is known to increase in the lung in vivo after systemic LPS administration (47, 49); in alveolar macrophages (50), alveolar epithelium (51, 52), and endothelial cells (53) in vitro following LPS stimulation; and in the BAL fluid of human subjects with adult respiratory distress syndrome (44–46), our results now extend those findings by showing that PAI-1 expression increases in the lung and BAL fluid in an animal model of localized LPS-induced lung inflammation. In addition, our findings suggest that PAI-1 is involved in the acute inflammatory process

by showing that PAI-1 regulates LPS-induced pulmonary neutrophil recruitment to the alveolar compartment (Fig. 5*A*). This effect of PAI-1 expression on LPS-induced pulmonary neutrophil accumulation appears to predominate early, because the degree of inhibition in alveolar neutrophil recruitment in PAI-1$^{-/-}$ mice, compared with C57BL/6J wt controls, is greater 4 h after LPS exposure than at 24 h (Fig. 5*A*). The reason for this preferential effect of PAI-1 on neutrophil accumulation in the alveolar compartment at earlier time points is unclear, but may only reflect that the peak in PAI-1 expression after LPS exposure is at 4 h.

Although previous studies have shown a decrease in inflammation in PAI-1$^{-/-}$ mice in a murine model of Ag-induced arthritis (54) and after hyperoxic-induced lung injury (29), to our knowledge this is the first study showing a decrease in neutrophil recruitment to the lung in a model of acute lung inflammation in mice deficient for PAI-1. Our findings, however, are in contrast to those of Rijneveld et al. (30), which found no decrease in alveolar neutrophil recruitment in PAI-1$^{-/-}$ mice administered *S. pneumoniae*. At least two potential possibilities explain the differences in our findings compared with Rijneveld et al. First, assessment of pulmonary neutrophil influx was only examined 48 h after *S. pneumoniae* inoculation in the study by Rijneveld et al., thereby possibly missing an early effect of PAI-1$^{-/-}$ on neutrophil recruitment, as seen in our study (30). Second, the inflammatory mediators used in the two studies are different. Whereas we assessed the effect of PAI-1 deficiency on pulmonary neutrophil recruitment after exposure to LPS, a component of Gram-negative bacteria and which signals through TLR4, the study by Rijneveld

FIGURE 8. Systemic inhibition of JNK with SP600125 increases serum KC levels. C57BL/6 mice ($n = 5$) were pretreated with SP600125, or diluent as control, 3 h before LPS exposure. At the selected times, mice were exsanguinated and lungs were isolated. Intravascular (*A*) and lung (*B*) KC levels were determined by ELISA. *, $p < 0.01$ LPS + SP600125 vs LPS.

epithelial cells after thrombin exposure and after oxidative stress in GH4 cells (41, 42). Although the role of JNK activation in PAI-1 expression in vivo, and in particular in the lung after LPS exposure, has not been examined previously, these studies suggested to us that LPS-induced PAI-1 expression in the lung may be regulated through the activation of JNK. We show in this study that systemic inhibition of JNK decreased LPS-induced PAI-1 expression in the lung and BAL fluid. In addition, because the alveolar macrophage is an important source of PAI-1 expression after LPS exposure (50), we additionally show that inhibition of JNK activation decreased LPS- and TNF-α-induced PAI-1 expression in the murine macrophage cell line RAW264.7, suggesting that one mechanism for the decrease in PAI-1 levels in the BAL fluid in the setting of systemic JNK inhibition is due to a decrease in PAI-1 expression in alveolar macrophages (Fig. 4).

We propose that increases in systemic KC levels after exposure to LPS are a mechanism for the decrease in pulmonary neutrophil recruitment in the setting of PAI-1 deficiency. Neutrophil recruitment to areas of ongoing inflammation requires the tight regulation of neutrophil-chemokine interactions. Exposure of circulating peripheral blood neutrophils to augmented levels of systemic chemokines decreases subsequent neutrophil recruitment to regions of acute inflammation via down-regulation of cell surface L-selectin and chemokine receptor expression (58–60). Specifically for IL-8, augmentation of systemic IL-8 levels decreased neutrophil recruitment to the lung and s.c. tissue (25, 28). Although the complex mechanisms by which chemoattractant and haptotactic gradients are formed and maintained after localized inflammation, and the manner by which serum chemokine levels are regulated, are only beginning to be understood, the recent observation that inhibition of PAI-1 increased IL-8 shedding and impeded neutrophil migration across an activated endothelial surface (16) suggested to us that PAI-1 may regulate chemokine partitioning in vivo, thereby influencing neutrophil recruitment to the lung.

Our results presented in this work confirm previous studies of the effect of augmenting systemic IL-8 levels on neutrophil recruitment to sites of acute inflammation, as we show that modulation of systemic KC levels decreased pulmonary neutrophil recruitment after localized exposure to LPS, most likely through a decrease in KC-induced actin polymerization (Fig. 9). In addition,

et al. used a complete Gram-positive organism, components of which include lipoteichoic acid and peptidoglycan, which signal through TLR2 (55–57). Therefore, the role of PAI-1 in acute lung inflammation may differ after exposure to whole organisms, in which several inflammatory components are present concurrently, or with inflammatory agents preferentially signaling through TLR2 or TLR4. Further investigation is necessary to evaluate these possibilities.

Inhibition of JNK activation, by SP600125, has been shown recently to decrease PAI-1 expression in proximal renal tubular

FIGURE 9. Intravenous KC decreases LPS-induced neutrophil recruitment to the alveolar compartment, and pre-exposure to KC inhibits subsequent KC-induced actin polymerization in peripheral murine neutrophils. *A–C*, C57BL/6 mice ($n = 5$) were administered KC (1.2 μg/kg), or saline as control, i.v. 1 h before exposure to aerosolized LPS (300 μg/ml, 20 min). Neutrophil recruitment to the alveolar space was assessed by BAL cell count, and differential (*A*) and total lung neutrophil accumulation by MPO assay (*B*). Intravascular KC levels were determined by ELISA (*C*). C, Control; KC, i.v. KC only; LPS, LPS only; KC + LPS, i.v. KC, followed by LPS exposure. *, $p < 0.001$ LPS vs LPS + KC. *D*, Peripheral blood murine neutrophils were isolated and pre-exposed to KC (100 ng/ml, 10 min) (KC), or KRPD as control (C), as per *Materials and Methods*. KC-induced (100 ng/ml, 2 or 5 min) actin polymerization was then assessed, as previously described (38), with KC-induced actin polymerization normalized to actin polymerization from unstimulated cells giving a relative fluorescence index (RFI). #, $p < 0.05$ KC vs C.
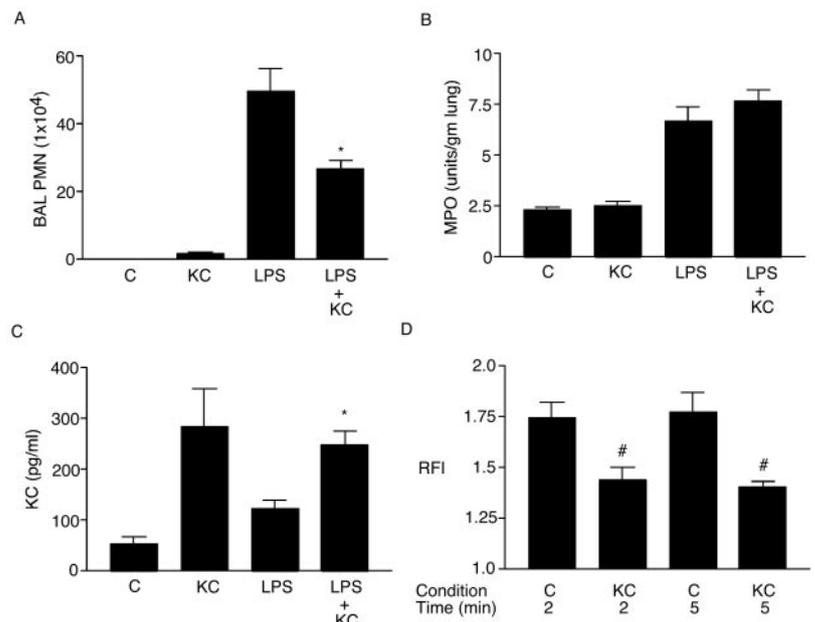
we now extend those previous findings by showing in this study that a deficiency of PAI-1 increases systemic KC levels after exposure to LPS (Fig. 7*A*), suggesting that PAI-1 regulates systemic KC levels after pulmonary LPS exposure and, by extension, that an increase in systemic KC levels is one mechanism by which a deficiency of PAI-1 limits neutrophil accumulation in the lung after exposure to LPS. The finding of an increase in LPS-induced systemic KC levels after the IT administration of plasmin provides additional evidence that PAI-1 regulates systemic KC levels (Fig. 7*D*). Finally, because systemic JNK inhibition increased systemic KC levels, with a corresponding decrease in pulmonary PAI-1 expression after LPS exposure, this further suggests that an increase in the systemic levels of KC is a mechanism for the decrease in LPS-induced pulmonary neutrophil recruitment in the setting of systemic JNK inhibition, and that this occurs in a PAI-1-dependent manner. Although not directly examined in this study, recent studies would suggest that the mechanism by which a deficiency in PAI-1 increases systemic chemokine levels is through an increase in chemokine shedding from proteoglycan binding sites in the lung (16). Alternatively, the possibility exists that a deficiency in PAI-1 increases pulmonary KC expression; however, this is unlikely, as there was no increase in lung KC levels in PAI-1-deficient mice compared with C57BL/6J wt controls (Fig. 7*B*).

Although we propose that an increase in systemic KC levels is a mechanism for the decrease in LPS-induced pulmonary neutrophil recruitment in PAI-1$^{-/-}$ mice, other mechanisms may also be involved. PAI-1 is known to disrupt the binding of cell surface uPAR to vitronectin, which allows for cell detachment and subsequent migration before adhesion is re-established (9–11). Therefore, in the setting of PAI-1 deficiency, neutrophils may remain inappropriately adherent to the extracellular matrix, with an inability to migrate into the alveolar compartment. Our findings of a decrease in alveolar neutrophil recruitment, with no change in total pulmonary neutrophil accumulation, as assessed by MPO and lung histology, in PAI-1$^{-/-}$ mice after LPS exposure support this hypothesis. In contrast, administration of the PAI-1 mutant unable to bind vitronectin also reversed the inhibition of LPS-induced neutrophil recruitment to the lung in SP600125-pretreated mice (Fig. 6*B*), suggesting that the interaction of PAI-1 with vitronectin is not necessary in the JNK-mediated pathway regulating pulmonary neutrophil recruitment after exposure to LPS.

In examining the role of JNK activation in LPS-induced PAI-1 expression in the lung and cell cultures, we were obligated to use the specific pharmacologic inhibitor of JNK activation, SP600125. In in vitro experiments, we carefully used concentrations of SP600125, which have been shown previously to be specific for JNK inhibition by us (37) and others (61). In addition, the majority of the effect of SP600125 on inhibiting PAI-1 expression in RAW264.7 cells after exposure to LPS or TNF-α occurred at the lowest concentration used, thereby further implying specificity of SP600125 for the inhibition of JNK (Fig. 4). Finally, additional confirmation of our results of the effect of systemic JNK inhibition on LPS-induced PAI-1 expression in the lung is restricted by the lack of viability of mice deficient in both major isoforms of JNK, JNK1 and JNK2. Although JNK1$^{-/-}$ and JNK2$^{-/-}$ mice are viable, results using these mice are difficult to interpret due to the potential confounding effect of the other JNK isoform.

Although our findings suggest that PAI-1 regulates pulmonary neutrophil recruitment after LPS exposure via modulation of systemic KC levels, the mechanisms by which this occurs are still unknown. We hypothesize that a decrease in pulmonary PAI-1 levels leads to an increase in plasmin activity that induces KC shedding from bound sites in the lung and KC efflux into the systemic circulation. Further studies, however, are necessary to in-

vestigate this possibility, and to examine how PAI-1 regulates the formation and maintenance of chemotatic and haptotatic gradients in the complex environment of the lung.

Disclosures

The authors have no financial conflict of interest.

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