Online methods supplement

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

1. Ganter MT, Roux J, Su G, et al. Role of small GTPases and alphavbeta5 integrin in Pseudomonas aeruginosa-induced increase in lung endothelial permeability. *Am J Respir Cell Mol Biol* 2009;40:108-118.

2. Frank J, Roux J, Kawakatsu H, et al. Transforming growth factor-beta1 decreases expression of the epithelial sodium channel alphaENaC and alveolar epithelial vectorial sodium and fluid transport via an ERK1/2-dependent mechanism. *J Biol Chem* 2003;278:43939-43950.

3. Pittet JF, Griffiths MJ, Geiser T, et al. TGF-beta is a critical mediator of acute lung injury. *J Clin Invest 2001*;107:1537-1544.

4. Czekay RP, Aertgeerts K, Curriden SA, et al. Plasminogen activator inhibitor-1 detaches cells from extracellular matrices by inactivating integrins. *J Cell Biol* 2003;160:781-791.

5. Carles M, Lafargue M, Goolaerts A, et al. Critical Role of the Small GTPase RhoA in the Development of Pulmonary Edema Induced by *Pseudomonas aeruginosa* in Mice. *Anesthesiology*. (In press)

6. Ganter MT, Roux J, Miyazawa B, et al. Interleukin-1beta causes acute lung injury via alphavbeta5 and alphavbeta6 integrin-dependent mechanisms. *Circ Res* 2008;102: 804-12.

7. Su G, Hodnett M, Wu N, et al. Integrin alphavbeta5 regulates lung vascular permeability and pulmonary endothelial barrier function. *Am J Respir Cell Mol Biol* 2007;36:377-86.

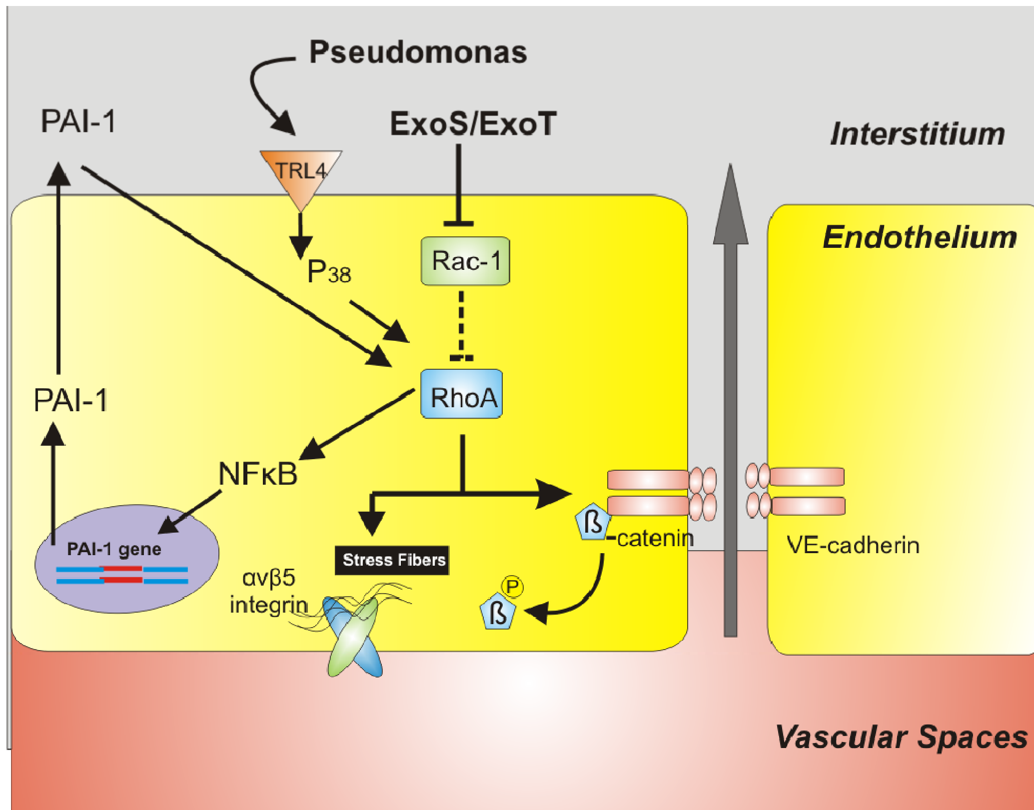

Figure 1. Schematic of the mechanisms by which *P. aeruginosa* increases PAI-1 expression via a RhoA-NFkappaB-dependent mechanism and by which PAI-1 released in the extracellular space further increases RhoA activation in lung endothelial cells. *P. aeruginosa* via the intracellular injection of ExoS/ExoT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