Caspase-1 Activation Protects Lung Endothelial Barrier Function during Infection-Induced Stress

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Abstract

Dysregulated activation of the inflammasome–caspase-1–IL-1 β axis elicits damaging hyperinflammation during critical illnesses, such as pneumonia and sepsis. However, in critical illness models of *Salmonella* infection, burn, or shock, caspase-1 inhibition worsens outcomes. These paradoxical effects suggest that caspase-1 drives novel protective responses. Whether the protective effects of caspase-1 activation involve canonical immune cell and/or nonimmune cell responses is unknown. The objective of this study was to test the hypothesis that, in addition to its recognized proinflammatory function, caspase-1 initiates protective stress responses in nonimmune cells. *In vivo*, lung epithelial and endothelial barrier function and inflammation were assessed in mice infected with *Pseudomonas aeruginosa* in the presence or absence of a caspase-1 inhibitor. Lung endothelial barrier function was assessed *ex vivo* in isolated, perfused rat lungs infected with *P. aeruginosa* in the presence or absence of a caspase-1 inhibitor. Endothelial barrier function during *P. aeruginosa* infection was assessed *in vitro* in cultured rat wild-type pulmonary microvascular endothelial cells (PMVECs) or recombinant PMVECs engineered to decrease caspase-1 expression. We demonstrated *in vivo* that caspase-1 inhibition in *P. aeruginosa*-infected mice ameliorated hyperinflammation, but, counterintuitively, increased pulmonary edema. *Ex vivo*, caspase-1 inhibition increased pulmonary permeability in *P. aeruginosa*-infected isolated rat lungs. To uncouple caspase-1 from its canonical inflammatory role, we used cultured rat PMVECs

in vitro and discovered that genetic knockdown of caspase-1 accelerated *P. aeruginosa*-induced barrier disruption. In conclusion, caspase-1 is a sentinel stress-response regulator that initiates proinflammatory responses and also initiates novel response(s) to protect PMVEC barrier function during pneumonia.

Keywords: pulmonary endothelial cells; endothelial permeability; pulmonary edema; acute respiratory distress syndrome; vascular stress responses

Clinical Relevance

Using animal models, we demonstrate that caspase-1 inhibition during *Pseudomonas aeruginosa*-induced pneumonia leads to decreased inflammation, but, paradoxically, increased lung permeability. Furthermore, we directly demonstrate that cultured pulmonary microvascular endothelial cells (PMVECs), critical determinants of lung barrier integrity, express and activate caspase-1 in response to *P. aeruginosa* infection to initiate a barrier-protective stress response. Together, our data represent a significant conceptual advance in understanding the multifactorial consequences of caspase-1 activation during critical illness by recognizing that, in addition to an inflammatory role, caspase-1 also plays a protective role in PMVECs, a nonimmune cell phenotype.

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Living cells adapt to environmental stressors by transducing danger signals into coordinated stress responses. In the context of critical illnesses, such as pneumonia, stress signaled by localized cellular dysfunction and damage leads to systemic release of damage-associated molecular patterns (DAMPs) (1). These DAMPs, alone or in concert with pathogen-associated molecular patterns (PAMPs) released from an underlying microbial etiology, trigger the innate immune system to activate inflammation (2). Severe inflammatory responses, which devolve into systemic inflammatory response syndrome, sepsis, or septic shock, ultimately drive loss of lung epithelial and endothelial barrier function, leading to alveolar edema and evolution of the acute respiratory distress syndrome (ARDS) (3, 4).

The Inflammasome–caspase-1–IL-1 β axis is a prominent signaling pathway through which the innate immune system senses and responds to stress-released DAMPs and PAMPs (5). Thornberry and colleagues (6) first identified caspase-1 as the IL-1 β -converting enzyme, a cysteine-active site aspartate-specific protease that processes pro-IL-1 β to its active, secreted form. Caspase-1 itself is a proenzyme that, in response to DAMPs and PAMPs, assembles as part of intracellular multiprotein inflammasome complexes to initiate pro-caspase-1 processing. In addition to activating inflammation via pro-IL-1 β and pro-IL-18 processing, activated caspase-1 also initiates pyroptosis, a form of programmed cell death marked by rapid release of lactate dehydrogenase (LDH) (5). Dysregulated caspase-1 activation and IL-1 β release are well recognized pathophysiological mechanisms associated with hyperinflammation and progression of critical illness (5, 7).

The consequences of stress-induced caspase-1 activation in immune cells and in animal models present an intriguing natural paradox. For example, in mice exposed to uric acid crystal DAMPs or exposed to respiratory tract infection with *Pseudomonas aeruginosa*, caspase-1 activation leads to exacerbated, deleterious inflammatory effects (8–10). Furthermore, caspase-1 levels are elevated in critically ill patients and correlate with poor outcomes (7). Conversely, mouse models of *Salmonella enterica* infection, burn, or shock implicate caspase-1 activation as beneficial and protective to the host

(11–13). Importantly, a recent study revealed the commonly used *caspase-1*^{−/−} murine model is also deficient in noncanonical inflammatory responses (*caspase-11*^{−/−}) (14). Thus, the molecular mechanisms determining whether caspase-1 activation in response to stress elicits either beneficial or deleterious effects to the host remain unresolved. We hypothesized that disparate outcomes associated with stress-induced caspase-1 activation is indicative of unrecognized functions in nonimmune cell phenotypes. We tested this idea using a *P. aeruginosa* model of pneumonia and ARDS (15) and describe a novel, nonimmune role for caspase-1 in protecting pulmonary microvascular endothelial cell (PMVEC) barrier function. Our results identify caspase-1 as a sentinel stress-response regulator that (1) controls innate inflammation and (2) protects lung endothelial cell barrier function, which, collectively, minimize pulmonary edema and mitigate evolution of pneumonia-induced ARDS.

Materials and Methods

Detailed methods are included as online supplement.

Ethics Statement

Animal infection studies were approved by the University of South Alabama (Mobile, AL) Institutional Animal Care and Use Committee (protocol nos. 694483 and 694922).

Murine Infection Experiments

The model stress response studied here was based on pneumonia and/or sepsis induced by *P. aeruginosa* infection. We used strain PA103(UT⁺) encoding exoenzyme effectors ExoU (a phospholipase A₂) and ExoT (a dual Rho GTPase-activating protein and ADP-ribosyltransferase) (15, 16).

Effects of caspase-1 inhibition on lung inflammation and lung epithelial and endothelial barrier function in response to *P. aeruginosa* infection were determined using a mouse infection model. Control treatments consisted of intratracheal saline solution (SS) instillation and intraperitoneal DMSO injection as vehicles of infection and caspase-1 inhibition, respectively. Experimental treatments consisted of PA103(UT⁺) infection (10⁵ CFU) and caspase-1 inhibitor injection (a substrate mimetic, N-acetyl-L-tyrosyl-

L-valyl-N-[(1S)-1-(carboxymethyl)-3-chloro-2-oxopropyl]-L-alaninamide, N-acetyl-tyrosyl-valyl-alanyl-aspartyl chloromethyl ketone [YVAD], at a stock concentration of 370 μ M). Animals were allowed to recover from anesthesia and anesthetized again at either 24 or 48 hours for analyses.

Inflammation was measured as total IL-1 β in bronchoalveolar lavage (BAL) fluid by ELISA. Total BAL neutrophils were measured by staining and light microscopy. Lung tissue neutrophils were counted by staining and light microscopy of tissues taken from paraffin-embedded sections. Total CFU in homogenized lung tissues was determined by plating and counting.

Lung histology was assessed by hematoxylin and eosin staining, and light microscopy of tissues taken from paraffin-embedded sections. All lungs were prepared under identical conditions to control for effects of fixation. Lung airway compliance was measured in mechanically ventilated mice. Lung epithelial and endothelial permeability was assessed by extravasation of Evan's blue dye-labeled albumin (EBD-albumin) into the air space (collected in BAL fluid).

Isolated Rat Lung Infection Experiments

Effects of caspase-1 inhibition on lung endothelial barrier integrity in response to *P. aeruginosa* infection were determined in isolated, perfused rat lungs. Vehicle controls for infection and caspase-1 inhibition were SS and DMSO, respectively. Infections were initiated using 10⁷ total CFU of PA103(UT⁺) and caspase-1 inhibitor (YVAD) was used at 30 μ M. Experiments were initiated by addition of compounds directly to perfusate. Lung hydraulic permeability was measured and expressed as filtration coefficient (K_f), a well established and sensitive pulmonary endothelial permeability index (17, 18).

Cell Culture Infection Experiments

Effects of *P. aeruginosa* infection on caspase-1 activation were determined in cultured cells. Rat alveolar macrophages and PMVECs were infected with: (1) wild-type PA103(UT⁺); (2) isogenic mutant PA103(U⁺) expressing ExoU alone; (3) isogenic mutant PA103(T⁺) expressing ExoT alone; or (4) isogenic double-mutant PA103(Δ UT) expressing

no exoenzymes. Experiments were performed at a multiplicity of infection of 40 bacteria per host cell.

Unprocessed, full-length pro-caspase-1 was detected in cell lysates by Western blotting and normalized to β -actin. Active forms of caspase-1, IL-1 β , and IL-18 were detected in culture supernatants by Western blotting and normalized to total infection volume. Active IL-1 β was also detected in culture supernatants by ELISA. Effects of caspase-1 activation on cytotoxicity and host cell viability were measured via LDH release assay and 4-[3-(4-iodophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) metabolism assays, respectively.

Caspase-1 Knockdown and Transwell Flux Assays

Effects of caspase-1 knockdown on PMVEC barrier integrity in response to *P. aeruginosa* infection were determined using transwell flux assays. Knockdown was achieved via lentivirus-mediated expression of short hairpin RNAs (shRNAs) targeting the *caspase-1* mRNA, and controls included vector only and a scrambled shRNA. Recombinant PMVECs were infected with PA103(UT⁺) or mutant PA103(Δ UT) at a 40:1 multiplicity of infection, and barrier function was assessed as FITC-dextran (40,000 molecular weight) flux across monolayers growing on transwell filters (0.4- μ m pore size, 6.5-mm insert; Corning Inc., Corning, NY).

Statistical Analysis

GraphPad Prism v6.1 (GraphPad Software, Inc., La Jolla, CA) was used for all analyses. Data are reported as mean ($\pm$ SE). Data were assessed for normality before analysis. Pairwise comparison of parametric data was performed using two-tailed paired or unpaired *t* test to compare two groups, as indicated. One-way ANOVA was used to compare two or more groups, and Newman-Keuls *post hoc* test applied as necessary. Differences with a *P* value less than 0.05 were considered significant.

Results

Inhibiting Caspase-1 in *P. aeruginosa*-Infected Mice Increases Pulmonary Edema

P. aeruginosa is an opportunistic pathogen and a common cause of severe pneumonia,

associated with significant morbidity and mortality (19, 20). Here, we tested the potential utility of inhibiting the inflammasome-caspase-1-IL-1 β pathway (1, 5) as an immune-modulatory therapy to treat hyperinflammation associated with *P. aeruginosa*-induced pneumonia. Considering that the available murine *caspase-1*^{-/-} model is also deficient in the proinflammatory *caspase-11* (14), we instead employed a pharmacologic approach to inhibit caspase-1 (13). In our model, murine pneumonia was established via intratracheal administration of *P. aeruginosa* strain PA103(UT⁺), which encodes two exoenzyme effectors, namely, ExoU (a phospholipase A₂) and ExoT (a dual Rho GTPase-activating protein and ADP-ribosyltransferase). Upon infection, ExoU and ExoT are directly injected into host cell cytoplasm via a type 3 secretion system, where they alter cell physiology and elicit damage (15, 16). Figure 1 shows the effects of caspase-1 inhibition on lung inflammation in response to *P. aeruginosa* infection at 24 hours after inoculation. As expected, intratracheal instillation of SS alone (infection vehicle control) did not elicit an inflammatory response in mouse lungs, as reflected by a lack of measurable IL-1 β in BAL fluid (Figure 1A, C/SS, *white bar*), lack of neutrophils in BAL fluid (Figure 1B, C/SS, *white bar*), and lack of neutrophils infiltrating the lung parenchyma and airspaces in tissue sections (Figure 1C, C/SS, *white bar*). Furthermore, instillation of SS and concomitant intraperitoneal injection of either DMSO (caspase-1 inhibitor vehicle control) or a caspase-1 small molecule inhibitor (YVAD) did not elicit inflammation (Figures 1A–1C, DMSO/SS and YVAD/SS, *white bars*, respectively). However, instillation of PA103(UT⁺) in mice concomitantly injected with DMSO elicited significant IL-1 β activation (Figure 1A, *gray bar*) as well as neutrophil infiltration into lung parenchyma and airspaces (Figures 1B and 1C, *gray bars*). As expected, concomitantly injecting PA103(UT⁺)-infected mice with a caspase-1 inhibitor (YVAD) significantly reduced IL-1 β activation (Figure 1A, *black bar*) and attenuated neutrophil infiltration into lung parenchyma and airspaces (Figures 1B and 1C, *black bars*). Importantly, administration of the caspase-1 inhibitor (YVAD) did not change the bacterial burden, as measured by total CFU grown out of lung homogenates (Figure 1D, compare *gray* and

black bars), nor did it have an effect on 48-hour mortality (data not shown). Thus, caspase-1 inhibition during *P. aeruginosa* infection ameliorates inflammation.

In our experimental model, the natural evolution of *P. aeruginosa*-induced pneumonia results in pulmonary epithelial and endothelial barrier disruption, interstitial and alveolar edema, and progression to acute lung injury (15, 21–23). Thus, we next examined the effects of caspase-1 inhibition on lung epithelial and endothelial barrier integrity, and lung function in response to *P. aeruginosa* infection. Histological analyses demonstrated that instillation of SS alone caused no overt damage to the lung, as determined by hematoxylin and eosin staining and light microscopy (Figure 2A, SS/C). Furthermore, instillation of SS and concomitant injection of either DMSO or a caspase-1 inhibitor (YVAD) did not elicit damage (Figures 2B and 2C, SS/DMSO and SS/YVAD, respectively). However, PA103(UT⁺) infection combined with DMSO injection elicited interstitial thickening and marked cellular infiltrates (Figures 2D and 2E). Unexpectedly, PA103(UT⁺) infection combined with caspase-1 inhibitor injection (YVAD) elicited interstitial (perivascular cuffing) and alveolar edema (Figures 2F and 2G), despite the expected reduction in cellular infiltrates into the lung parenchyma and airspaces (Figures 1B and 1C). All lungs were perfused and fixed in an identical manner before analyses to minimize the potential for spurious, fixation-induced fluid accumulation in the lungs. Thus, although administration of a caspase-1 inhibitor expectedly ameliorated inflammation (Figures 1A–1C), it paradoxically worsened the pulmonary epithelial and endothelial barrier-disruptive effects of *P. aeruginosa* infection (Figure 2).

We next quantified the effects of caspase-1 inhibition on pulmonary epithelial and endothelial barrier function during *P. aeruginosa* infection. As a clinically relevant assessment of lung function, we measured airway compliance, which our group has previously shown to decrease in animal models as lung permeability increases (24, 25). None of the control conditions caused an overt effect on airway compliance (Figure 3A, C/SS, DMSO/SS, and YVAD/SS, *white bars*). However, PA103(UT⁺) infection combined with DMSO injection resulted in decreased airway compliance, indicative of pulmonary

Figure 1. Caspase-1 inhibition in *Pseudomonas aeruginosa*-infected mice decreases lung inflammation. Mice were inoculated via intratracheal (I.T.→) route with 15 μ l saline solution (SS) or with 10^5 CFU of *P. aeruginosa* suspended in 15 μ l saline (PA103[UT $^+$]). Concomitantly, mice were injected via intraperitoneal (I.P.→) route with nothing (C), 50 μ l of a vehicle control (DMSO), or 50 μ l of a caspase-1 inhibitor from a 370- μ M stock solution (YVAD). Mice were analyzed at 24 hours after inoculation. (A) Effects of caspase-1 inhibition on IL-1 β levels measured in bronchoalveolar lavage (BAL) fluid, where $n = 3$ mice from each control group (SS/C, SS/DMSO, SS/YVAD), 7 mice from the PA103(UT $^+$)/DMSO experimental group, and 9 mice from the PA103(UT $^+$)/YVAD experimental group. (B) Effects of caspase-1 inhibition on numbers of neutrophils counted in BAL fluid, where $n = 7$ mice from the SS/C control group, 3 mice from the SS/DMSO control group, 3 mice from the SS/YVAD control group, 7 mice from the PA103(UT $^+$)/DMSO experimental group, and 9 mice from the PA103(UT $^+$)/YVAD experimental group. (C) Effects of caspase-1 inhibition on numbers of neutrophils present in lung parenchyma and airspaces counted in paraffin-mounted tissue sections, where $n = 4$ mice from the SS/C control group, 4 mice from the PA103(UT $^+$)/DMSO experimental group, and 5 mice from the PA103(UT $^+$)/YVAD experimental group. (D) Effects of caspase-1 inhibition on numbers of viable *P. aeruginosa* (reported as CFU) recovered from whole-lung homogenates by plating and counting, where $n = 3$ mice from the SS/C control group, 3 mice from the PA103(UT $^+$)/DMSO experimental group, and 3 mice from the PA103(UT $^+$)/YVAD experimental group. Data are presented as mean $\pm$ SEM. * $P < 0.05$ by one-way ANOVA (Newman-Keuls *post hoc* test) when comparing within the group. YVAD, N-acetyl-L-tyrosyl-L-valyl-L-N-[(1S)-1-(carboxymethyl)-3-chloro-2-oxopropyl]-L-alaninamide, N-acetyl-tyrosyl-valyl-alanyl-aspartyl chloromethyl ketone.

edema (Figure 3A, *gray bar*). Intriguingly, PA103(UT $^+$) infection combined with caspase-1 inhibitor (YVAD) injection resulted in a significant, further decrease in airway compliance (Figure 3A, compare *gray* and *black bars*).

As a complementary, quantitative approach, we perfused lungs with EBD-albumin and measured its extravasation into the lung interstitium and airspace via collection in BAL fluid. Instillation of SS alone caused no measurable extravasation of EBD-albumin into the airspace (Figure 3B,

white bar), whereas PA103(UT $^+$) infection in mice concomitantly injected with DMSO elicited a significant increase in EBD-albumin extravasation, indicating disruption of the pulmonary epithelial and endothelial barrier (Figure 3B, *gray bar*). Again, PA103(UT $^+$)-infected mice concomitantly injected with a caspase-1 inhibitor (YVAD) displayed a significant increase in pulmonary epithelial and endothelial barrier disruption (Figure 3B, compare *gray* and *black bars*). Taken together, our analyses strongly suggest that caspase-1 inhibition

during *P. aeruginosa* infection causes lung barrier disruption via mechanism(s) involving both an inflammatory component and a novel noninflammatory component—that is, direct epithelial and/or endothelial cell dysfunction.

Inhibiting Caspase-1 in *P. aeruginosa*-Infected Rat Lungs Increases Pulmonary Edema

We next tested the effects of caspase-1 inhibition during *P. aeruginosa* infection using an isolated, perfused rat lung (15). In this model, the heart and lungs are isolated *en bloc* and perfused in a closed circuit, allowing additions to be made directly to the pulmonary circulation via the perfusate. Thus, here we are testing the effects of inhibiting caspase-1 in the context of *P. aeruginosa* bacteremia. The lungs are hung in a force transducer and lung hydraulic permeability is measured and expressed as K_f . Any manipulations of the perfusate causing pulmonary endothelial barrier disruption result in increased K_f (17, 26). An advantage to this model is that circulating immune cells are not present, thus allowing for more refined testing of infection and its effects on pulmonary endothelial barrier function. As another advantage, this experimental protocol allows for serial repeated measures of K_f on the same isolated lung. Figure 4, segment I shows control lungs where a baseline K_f measurement was taken (segment I, *white bar*), followed by a second K_f measurement taken 60 minutes after addition of DMSO to the perfusate (inhibitor vehicle control, segment I, *gray bar*). A third K_f measurement was taken on the same lung 60 minutes after addition of SS (infection vehicle control, segment I, *black bar*). No deleterious effects on pulmonary endothelial barrier function were observed. Figure 4, segment II, shows the barrier-disruptive effect (i.e., increased K_f) of adding the wild-type PA103(UT $^+$) strain to the perfusate and taking measurements along the same time course as described for segment I (compare *black bar* to *white* and *gray bars*). Strikingly, Figure 4, segment III, shows that perfusion of a caspase-1 inhibitor (YVAD) alone had no effect on basal pulmonary endothelial permeability, but significantly worsened (i.e., further increased K_f) the effect of PA103(UT $^+$) infection (compare *black bar* to *white* and *gray bars* in segment III and to the *black*

Figure 2. Caspase-1 inhibition in *P. aeruginosa*-infected mice increases pulmonary edema. Groups of mice were inoculated intratracheally and injected intraperitoneally, as described in the legend to Figure 1. At 24 hours after inoculation, lungs were harvested under identical conditions, whereby the trachea was tied-off at the peak of inspiration, and the lungs removed and immersed in 10% neutral buffered formalin solution for 48 hours. Subsequently, paraffin-embedded sections were prepared for hematoxylin and eosin staining, and tissues analyzed by light microscopy (20× objective). For all groups, $n = 3$ mice. Representative images from each group are shown. (A) SS/C control group lungs showed normal architecture, no inflammation, and open airspaces. (B) SS/DMSO control group lungs showed normal architecture, no inflammation, and open airspaces. (C) SS/YVAD control group lungs showed normal architecture, no inflammation, and open airspaces. (D and E) PA103(UT⁺)/DMSO experimental group lungs from two different fields of view showing the bronchioarterial bundle (D) and the septal network (E). Sections displayed septal thickening and substantial cell infiltration. (F and G) PA103(UT⁺)/YVAD experimental group lungs from two different fields of view showing the bronchioarterial bundle (F) and the septal network (G). Sections displayed fluid accumulation in perivascular cuffs (asterisk) and fluid accumulation in air spaces (arrows) with a marked decrease in the number of infiltrating cells when compared with PA103(UT⁺)/DMSO experimental group lungs (D and E). Scale bars: 50 μ m. As, air space; Aw, airway; Bv, blood vessel; Lv, lymphatic vessel.

bar in segment II). To control for effects of exposure time, we performed separate experiments using the protocol described for segment III, except that no bacteria were added at the step before the final K_f measurement. Regardless of exposure time, caspase-1 inhibitor treatment alone never

caused pulmonary edema (data not shown). Thus, these data lend further support to a model in which caspase-1 activation in pulmonary endothelial cells protects their barrier function in response to *P. aeruginosa* infection.

Caspase-1 Is Expressed in *In Vitro*-Cultured PMVECs, and *P. aeruginosa*-Secreted Type 3 Exoenzyme Effectors Inhibit Caspase-1 Activation

Our *in vivo* airway infection and *ex vivo* bacteremia data indicate that caspase-1 inhibition during *P. aeruginosa* infection exacerbated pulmonary epithelial and/or endothelial barrier disruption, raising the intriguing prospect that caspase-1 plays a protective role in these cells. PMVECs cover a vast surface area, interface with the entire cardiac output, and are the major contributors to lung endothelial barrier permeability. Thus, to uncouple caspase-1 from its canonical inflammatory role, we used *in vitro*-cultured PMVECs (27) to elucidate the role of caspase-1 during *P. aeruginosa* infection.

We first confirmed that *in vitro*-cultured rat PMVECs expressed *caspase-1* mRNA (RT-PCR data not shown). Next, we used our previously described infection model (15), in which cultured rat alveolar macrophages (positive control cell line) or PMVECs were inoculated with saline as a control (C) or independently infected with: (1) wild-type PA103(UT⁺); (2) isogenic mutant PA103(UT⁺) encoding only ExoU; (3) isogenic mutant PA103(T⁺) encoding only ExoT; or (4) isogenic double-mutant PA103(Δ UT), where both exoenzyme genes are deleted. Cultures were harvested for assay at 3 hours after treatment. Western blots and densitometry in Figures 5A–5D show that pro-caspase-1 (inactive form) was detectable both in macrophage and PMVEC whole-cell lysates under all conditions tested. Intriguingly, infection with the double-mutant PA103(Δ UT) strain resulted in the highest observable levels of activated caspase-1 assessed in culture supernatants (Figures 5A and 5C, bottom panel, labeled “Super”) (28). Infection with PA103 mutants expressing either secreted exoenzyme effector (UT⁺ only or T⁺ only) resulted in modest caspase-1 activation (Figures 5A and 5C, bottom panel, labeled “Super”). Infection with wild-type PA103(UT⁺) did not elicit detectable caspase-1 activation at 3 hours after infection (Figures 5A and 5C, bottom panel, labeled “Super”).

To determine whether infection with wild-type PA103(UT⁺) permanently suppressed caspase-1 activation, we

Figure 3. Caspase-1 inhibition in *P. aeruginosa*-infected mice decreases lung compliance and increases lung permeability. Groups of mice were inoculated intratracheally and injected intraperitoneally, as described in the legend to Figure 1, and analyzed 24 hours after inoculation. (A) Effects of caspase-1 inhibition on lung airway compliance, where $n = 6$ mice from the SS/C control group, 3 mice from the SS/DMSO control group, 3 mice from the SS/YVAD control group, 6 mice from the PA103(UT⁺)/DMSO experimental group, and 6 mice from the PA103(UT⁺)/YVAD experimental group. (B) Effects of caspase-1 inhibition on lung permeability measured as the extravasation of systemically perfused Evan's blue dye conjugated to albumin (EBD-albumin) into the air space, where $n = 5$ mice from the SS/C control group, 5 mice from the PA103/DMSO experimental group, and 5 mice from the PA103/YVAD experimental group. Ventilated mouse lungs were perfused, *in situ*, with EBD-albumin, and lungs were subsequently washed and BAL fluid collected and measured by spectrophotometry, with spectrophotometric absorbance measured at a wavelength of 620 nm (EBD-BAL A_{620 nm}). Data are presented as mean $\pm$ SEM. * $P < 0.05$ by one-way ANOVA (Newman-Keuls *post hoc* test) for experimental conditions (gray and black bars) compared with control conditions (white bars). ** $P < 0.05$ by one-way ANOVA (Newman-Keuls *post hoc* test) when comparing within a group.

extended the infection time course. Interestingly, the extended time course revealed that caspase-1 activation in response to PA103(UT⁺) infection occurred, but was delayed compared with infection with the double-mutant PA103(Δ UT) strain (Figures 5E and 5F). As

a secondary indicator of caspase-1 activation in PMVECs, we assayed for its canonical function, namely, IL-1 β activation and secretion to the culture medium. Figures 5G and 5H show infection with either PA103(UT⁺)- or PA103(Δ UT)-stimulated release of activated IL-1 β to the

culture medium with PA103(UT⁺)-infected PMVECs, demonstrating delayed activation. We also observed that PA103(UT⁺) and PA103(Δ UT) both stimulated IL-1 β activation at 3 hours after infection (data not shown). The *P. aeruginosa* ExoU and ExoS exoenzyme effectors have been previously reported to inhibit caspase-1 activation in macrophages (2, 10), and ours is the first report that the same is true of ExoT. The observed delay in caspase-1 activation in response to infection with *P. aeruginosa* expressing ExoU was also reported previously in a murine macrophage model (10). It is known that ExoU- and ExoS-mediated inhibition of caspase-1 activation requires their respective enzymatic activities, but the underlying mechanisms remain unknown. Inhibiting caspase-1 activation may confer an evolutionary advantage to the pathogen when colonizing a eukaryotic host by dampening inflammation and remodeling the lung endothelial growth niche.

Caspase-1 Activation in *In Vitro*-Cultured PMVECs Results in Modest IL-1 β and IL-18 Activation and Does Not Induce Pyroptotic Cell Death

In addition to stimulating processing of pro-IL-1 β , the other major consequences of caspase-1 activation in immune cells are processing of pro-IL-18 into its active form, and initiation of rapid programmed cell death (pyroptosis) (29). Figure 6A shows ELISA data comparing the levels of IL-1 β activation displayed by *P. aeruginosa*-infected PMVECs versus macrophages at 3 hours after treatment. Regardless of *P. aeruginosa* strain background, the absolute levels of IL-1 β activation by infected PMVECs were negligible when compared with infected macrophages.

Importantly, there were no observable differences in PMVECs when infections were performed with or without LPS pretreatment (overnight at 100 ng/ml final concentration), a critical priming signal known to increase expression of ILs and inflammasome components in macrophages (Figure 6A, compare No LPS to LPS treatment). In addition to stimulating IL-1 β activation, *P. aeruginosa* infection of PMVECs also stimulated IL-18 processing and secretion (Figure 6B).

We next determined whether infection-mediated caspase-1 activation in PMVECs induced pyroptotic cell death. At

Figure 4. Caspase-1 inhibition in *P. aeruginosa*-infected rat lung increases permeability. Endothelial barrier function in isolated rat lungs was measured as hydraulic permeability (filtration coefficient [K_f]). The schematic on the right depicts the serial treatment protocol for lungs assayed in each segment. *Segment I*: serial K_f measurements taken at baseline (white bar), 1 hour after addition of DMSO (inhibitor vehicle control) to the perfusate at a final concentration of 0.3% (gray bar), and 1 hour after addition of 200 μ l saline (infection vehicle control) to the perfusate (black bar). *Segment II*: serial K_f measurements taken at baseline (white bar), 1 hour after addition of DMSO to the perfusate (gray bar), and 1 hour after addition of 10⁷ CFU of PA103(UT⁺) suspended in 200 μ l saline to the perfusate (black bar, infected control). *Segment III*: serial K_f measurements taken at baseline (white bar), 1 hour after addition of a caspase-1 inhibitor (YVAD) to the perfusate at a final concentration of 30 μ M (gray bar), and 1 hour after PA103(UT⁺) infection (black bar, experimental). For each segment, $n = 3$ rat lungs. Data are presented as mean $\pm$ SEM. * $P < 0.05$ by one-way ANOVA (Newman-Keuls *post hoc* test) when comparing within segment III.

Figure 5. Caspase-1 is activated in cultured rat pulmonary microvascular endothelial cells (PMVECs) in response to *P. aeruginosa* infection, and exoenzyme effectors ExoU and ExoT delay onset of caspase-1 activation. Rat alveolar macrophages or PMVECs growing in culture were untreated or independently inoculated with different *P. aeruginosa* strains at a multiplicity of infection (MOI) of 40 bacteria per host cell. At indicated time points after inoculation, cell lysates and clarified culture supernatants ("Super") were prepared and analyzed by Western blot. Cell lysates were probed with an antibody against caspase-1 and subsequently stripped and reprobed with an antibody against β -actin. Clarified culture supernatants were probed with an antibody against caspase-1 or an antibody against IL-1 β . (A) Effects of *P. aeruginosa* infection on caspase-1 activation in macrophages at 3 hours after inoculation. Macrophages were untreated (C) or inoculated with wild-type PA103(UT⁺), mutant PA103(U⁺) expressing only ExoU, mutant PA103(T⁺) expressing only ExoT, or double-mutant PA103(Δ UT) expressing no exoenzyme effectors. The *top panels* show expression of the inactive pro-caspase-1 enzyme in whole-cell lysates. The *middle panels* show β -actin levels as a loading control. The bottom

3 hours after infection, overall PMVEC health was largely unchanged by infection with either the PA103(UT⁺) or PA103(Δ UT) strain when assayed for cytotoxicity via LDH release to the culture medium (Figure 6C) or metabolism of WST-1 (a redox indicator of cell viability; Figure 6D). The low, but significantly different, amount of LDH release elicited by wild-type PA103(UT⁺) was most likely due to the documented cytolytic effects of ExoU phospholipase A₂ activity (30, 31), and was not likely the result of pyroptosis, as reported in macrophages (5). These data indicate that the observed lung barrier-disruptive effects of caspase-1 inhibition during *P. aeruginosa* infection are not likely related to the canonical, inflammatory outcomes commonly associated with caspase-1 activation.

Caspase-1 Knockdown in PMVECs Increases the Rate of Barrier Disruption Caused by *P. aeruginosa* Infection

In vivo and *ex vivo*, caspase-1 inhibition reduced inflammation, but worsened the barrier-disruptive effects of *P. aeruginosa* infection; however, the specific cell type(s) affected were unknown. The data presented in Figures 5 and 6 show caspase-1 expression and activation in *P. aeruginosa*-infected PMVECs. Thus, we tested caspase-1 for a novel role in protecting PMVEC barrier function during *P. aeruginosa* infection. To assay PMVEC barrier function, cells were grown as confluent monolayers on transwell filters to experimentally mimic their *in vivo* function of forming barriers that limit the flux of large molecules across the monolayer (15). However, we have previously determined that the addition of DMSO (caspase-1 inhibitor vehicle control) adversely affects the integrity of PMVEC monolayers, thus precluding testing of the caspase-1 inhibitor in this functional permeability assay (unpublished data). Therefore, we used shRNA knockdown as an alternative genetic approach to the pharmacological inhibitor (YVAD). To this end, we used lentivirus-mediated transduction and generated three recombinant PMVEC lines: (1) expressing green fluorescent protein 2 from the copepod *Pontellina plumata* (copGFP) reporter alone (GFP); (2) coexpressing copGFP and a control, scrambled shRNA (shScr); and (3) coexpressing copGFP and two shRNAs targeting the *caspase-1* mRNA (shCas-1).

Figure 6. Caspase-1 activation in PMVECs results in modest IL-1 β and IL-18 activation and does not induce pyroptotic cell death. Rat alveolar macrophages or PMVECs growing in culture were untreated or independently inoculated with either *P. aeruginosa* PA103(UT⁺) or the isogenic double-mutant PA103(Δ UT) strain at a MOI of 40 bacteria per host cell. Cells were analyzed at 3 hours after inoculation. (A) Culture supernatants from control (C) PMVECs and macrophages were collected and IL-1 β activation/secretion was measured by ELISA. Note that the absolute levels of IL-1 β secretion by PMVECs were negligible, even in the presence of LPS pretreatment (100 ng/ml, overnight incubation), a critical priming signal known to increase IL-1 β and inflammasome component gene expression in macrophages. (B) Clarified culture supernatants from control (C) or infected PMVECs were prepared and analyzed for IL-18 levels by Western blot. (C) Clarified culture supernatants from control (C) or infected PMVECs were analyzed for lactate dehydrogenase (LDH) release into the culture medium as an indicator of cell death. Data represent percent released as normalized to total LDH assayed in the cell monolayer. (D) Control (C) or infected PMVECs were analyzed for metabolism of WST-1 as an indicator of cell viability. Normalized WST-1 conversion was measured spectrophotometrically at A_{440nm} after 1 hour of incubation and corrected for background at A_{620nm}. (A–D) $n = 3$ experiments. All data represent mean $\pm$ SEM. * $P < 0.05$ by one-way ANOVA (Newman-Keuls *post hoc* test) when compared within the group. Macs, macrophages; WST-1, 4-[3-(4-iodophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolol]-1,3-benzene disulfonate.

Figures 7A and 7B show Western blots for pro-caspase-1 in whole-cell lysates from all three recombinant PMVEC lines under uninfected (SS) or *P. aeruginosa*-infected (3-h infection) conditions. The shRNA directed against *caspase-1* mRNA readily knocked down pro-caspase-1 levels.

There were no measureable effects of *P. aeruginosa* infection in the pro-caspase-1 expression levels. Intriguingly, the caspase-1 knockdown PMVECs still exhibited IL-1 β activation in response to *P. aeruginosa* infection (data not shown), indicating that only very low levels of activated caspase-1

are sufficient to activate IL-1 β and/or that PMVECs possess redundant (or compensatory) pathways for IL-1 β activation.

We next tested the cultured recombinant GFP and shRNA targeting the *caspase-1* mRNA-1 PMVEC lines in the transwell macromolecular flux assay (15). Figure 7C shows that caspase-1 knockdown did not affect PMVEC basal permeability rate, as measured using a fluorescently labeled 40-kD dextran reporter. However, in response to infection with wild-type PA103(UT⁺), caspase-1 knockdown PMVEC monolayers demonstrated a left shift in the dextran flux permeability profile compared with control (Figure 7D), indicating that caspase-1 knockdown increased the rate of barrier disruption. Moreover, in response to infection with the PA103(Δ UT) mutant deleted for both exoenzymes, caspase-1 knockdown PMVECs were also more prone to barrier disruption compared with control PMVECs (Figure 7E). Together, these data suggest that caspase-1 activation in PMVECs is protective by delaying the onset of barrier disruption in response to *P. aeruginosa* infection.

Discussion

In this prospective, experimental study, we elucidated a novel nonimmune role for caspase-1 as a protective factor of pulmonary barrier function in the setting of *P. aeruginosa*-induced pneumonia and ARDS. Although administration of a caspase-1 inhibitor to *P. aeruginosa*-infected mice provided an expected amelioration of hyperinflammation, we also identified an unexpected deleterious effect on lung epithelial and endothelial barrier function. Specifically, *P. aeruginosa* infection and concomitant inhibition of caspase-1 increased pulmonary edema: histological findings were complemented by quantitative analyses of airway compliance and

Figure 5. (Continued). panels show activated caspase-1 released to the culture medium. MW, molecular weight markers. (B) Representative densitometric analysis of macrophage pro-caspase-1 was normalized to β -actin for cell lysates. Active caspase-1 was normalized to sample volume. Data are expressed as relative density units (R.D.U.). (C) Effects of *P. aeruginosa* infection on caspase-1 activation in PMVECs at 3 hours after inoculation under the untreated and infection conditions described for A above. (D) Representative densitometric analysis of PMVEC pro-caspase-1 was normalized to β -actin for cell lysates. Active caspase-1 was normalized to sample volume. (E) Effects of *P. aeruginosa* infection on caspase-1 activation 4 and 6 hours after inoculation in PMVECs that were untreated (C) or inoculated with either wild-type PA103(UT⁺) or double-mutant PA103(Δ UT). (F) Representative densitometric analysis of PMVEC active caspase-1 was normalized to sample volume. (G) Effects of *P. aeruginosa* infection on IL-1 β activation 4 and 6 hours after inoculation in PMVECs that were untreated (C) or inoculated with either wild-type PA103(UT⁺) or double-mutant PA103(Δ UT). (H) Representative densitometric analysis of PMVEC active IL-1 β was normalized to sample volume. (A–H); $n = 3$ experiments; representative blots and corresponding densitometry are shown.

Figure 7. Genetic knockdown of caspase-1 in cultured rat PMVECs increases the rate of barrier disruption during *P. aeruginosa* infection. Lentivirus-mediated transduction was used to generate three recombinant PMVEC lines: (1) expressing copGFP reporter alone (GFP); (2) coexpressing copGFP and a control, scrambled shRNA (shScr); and (3) coexpressing copGFP and two shRNAs targeting the *caspase-1* mRNA (shCas-1). Recombinant PMVECs grown in culture were untreated (SS) or independently inoculated with either *P. aeruginosa* PA103(UT⁺) or the isogenic double-mutant PA103(ΔUT) strain at an MOI of 40 bacteria per host cell. Cells were analyzed after inoculation as described subsequently here. (A) At 3 hours after inoculation, cell lysates were collected and probed with an antibody against caspase-1. Blots were subsequently stripped and reprobed with an antibody against β-actin as a loading control. (B) Representative densitometric analysis of recombinant PMVEC pro-caspase-1 levels was normalized to β-actin. Data are expressed as R.D.U. Representative blots and corresponding densitometry are shown. (C) The recombinant control and shCas-1 PMVECs were grown as monolayers on transwell filters, and transendothelial flux of FITC-dextran was measured over a 180-minute time course. Caspase-1 knockdown did not affect PMVEC basal permeability. (D) Transendothelial FITC-dextran flux of recombinant control and shCas-1 PMVECs was assayed during infection with wild-type PA103(UT⁺). Compared with control PMVECs, caspase-1 knockdown PMVECs displayed an increased rate of dextran flux when infected with wild-type PA103(UT⁺), as evidenced by a left shift in the flux curve profile. (E) Transendothelial FITC-dextran flux of recombinant control and shCas-1 PMVECs was assayed during infection with PA103(ΔUT). Compared with control PMVECs, caspase-1 knockdown PMVECs displayed an increased rate of dextran flux when infected with PA103(ΔUT), as evidenced by a left shift in the flux curve profile. (A–E) *n* = 3 experiments. All data represent mean ± SEM. copGFP, green fluorescent protein 2 from the copepod *Pontellina plumata*; shRNA, short hairpin RNAs.

epithelial/endothelial permeability, which collectively indicated a marked loss of lung barrier function. Moreover, reductionist cell culture and recombinant genetics approaches determined that caspase-1 in PMVECs is activated in response to *P. aeruginosa* infection, and that caspase-1 is protective of PMVEC barrier function in a cell death-independent manner. Together, these data strongly suggest that caspase-1 activation in response to *P. aeruginosa* infection mounts an inflammatory response and an inflammation-independent, PMVEC barrier-protective response. A limitation of our study includes the selection of a pharmacological approach to inhibit caspase-1 in animals, which was necessitated by the existing genetic models harboring confounding mutations in proinflammatory pathways (i.e., *caspase-1*^{−/−} *caspase-11*^{−/−} double mutant) (14). Furthermore, potential protective roles for caspase-1 in airway epithelial or endothelial cells in other vascular segments (e.g., pulmonary artery, pulmonary vein) were not tested. Finally, mechanism(s) underlying caspase-1-mediated protection of PMVEC barrier function in response to *P. aeruginosa* infection remain unresolved.

Interestingly, our studies indicate that caspase-1 activation in PMVECs may protect barrier function via mechanism(s) working independently of the known, canonical caspase-1 pathways, namely, activation of IL-1β, IL-18, and pyroptosis (5). In support of this idea, IL-1β is known to elicit endothelial barrier disruption (32) and, thus, caspase-1 inhibition should have been protective rather than deleterious under the conditions tested in the present study. Moreover, it seems unlikely that IL-1β/IL-18 play protective roles, because infection of PMVECs with either wild-type PA103(UT⁺) or the attenuated virulent PA103(ΔUT) double mutant resulted in modest, but comparable, levels of IL-1β/IL-18 activation (Figures 5G, 5H, and 6B), whereas only wild-type PA103(UT⁺) elicited dramatic PMVEC barrier disruption (compare Figures 7D and 7E, infection of the GFP-control PMVEC line).

Alternatively, we postulate that the protective mechanism(s) of caspase-1 activation in PMVECs involve novel cellular caspase-1 cleavage substrates. Recent proteomics and bioinformatics studies globally identified enzymes involved in diverse physiological processes, such as glycolysis, as potential substrates for caspase-1 (33, 34). Intriguingly, the global

proteomics studies of Shao and colleagues (33) showed cultured macrophages responding to *Salmonella* infection by up-regulating glycolysis, and that activated caspase-1 cleaved glycolytic enzymes to reduce the amounts of glucose consumed and lactate produced. However, the consequences of caspase-1 acting as a governor of cellular glycolytic flux were not determined. In our vertically integrated model of stress-induced ARDS (15), *P. aeruginosa* infection stimulates increased glycolytic flux in PMVECs (unpublished data). Moreover, inhibitors of eukaryotic glycolysis partially protect PMVECs from *P. aeruginosa* infection-induced barrier disruption (unpublished data). Thus, our future studies on PMVEC stress responses will focus on elucidating the protective role of caspase-1 as a governor of cellular glycolytic flux in the context of infection and adaptive response to injury (35).

In a broader context, our studies raise the intriguing notion that caspase-1 activation in response to stress and its

consequences to the host involve inflammatory and noninflammatory pathways. It is highly likely that caspase-1 activation during critical illnesses, such as pneumonia and ARDS, is a complex and dynamic process, as highlighted by the fact that elevated caspase-1 is detected in critically ill patients (7). Indeed, the nucleotide oligomerization domain-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome complex has been associated with pleiotropic effects that are either beneficial or deleterious (36). Thus, akin to hormesis, we postulate that caspase-1-mediated stress responses in the lung are governed by the goldilocks effect, whereby too much or too little caspase-1 activation in either the inflammatory and/or noninflammatory pathway ultimately drives deleterious outcomes (37–39). This idea of optimal response windows or response thresholds has been offered as an explanation for the failure of immunomodulatory approaches to treat critical illnesses, such as sepsis and septic shock, whereby too much

inflammation is damaging, but too little inflammation impairs pathogen clearance (40). Ultimately, caspase-1 in PMVECs may represent a novel sentinel stress-response regulator, the activation of which protects lung barrier function during critical illness, such as *P. aeruginosa*-induced ARDS. Future studies engineering molecular control over the magnitude of caspase-1 responses are required to further elucidate the protective window of caspase-1 activation in PMVECs, and other nonimmune cell phenotypes. ■

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