

Heat-shock Response Increases Lung Injury Caused by *Pseudomonas aeruginosa* via an Interleukin-10-dependent Mechanism in Mice

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ABSTRACT

Background: The heat-shock response (HSR) protects from insults, such as ischemia–reperfusion injury, by inhibiting signaling pathways activated by sterile inflammation. However, the mechanisms by which the HSR activation would modulate lung damage and host response to a bacterial lung infection remain unknown.

Methods: HSR was activated with whole-body hyperthermia or by intraperitoneal geldanamycin in mice that had their lungs instilled with *Pseudomonas aeruginosa* 24 h later (at least six mice per experimental group). Four hours after instillation, lung endothelial and epithelial permeability, bacterial counts, protein levels in bronchoalveolar lavage fluid, and lung myeloperoxidase activity were measured. Mortality rate 24 h after *P. aeruginosa* instillation was recorded. The HSR effect on the release of interleukin-10 and killing of *P. aeruginosa* bacteria by a mouse alveolar macrophage cell line and on neutrophil phagocytosis was also examined.

Results: HSR activation worsened lung endothelial (42%) and epithelial permeability (50%) to protein, decreased lung bacterial clearance (71%), and increased mortality (50%) associated with *P. aeruginosa* pneumonia, an effect that was not observed in heat-shock protein–72-null mice. HSR-mediated decrease in neutrophil phagocytosis (69%) and bacterial killing (38%) by macrophages was interleukin-10 dependent, a mechanism confirmed by increased lung bacterial clearance and decreased mortality (70%) caused by *P. aeruginosa* pneumonia in heat-shocked interleukin-10-null mice.

Conclusions: Prior HSR activation worsens lung injury associated with *P. aeruginosa* pneumonia in mice *via* heat-shock protein–72- and interleukin-10-dependent mechanisms. These results provide a novel mechanism for the immunosuppression observed after severe trauma that is known to activate HSR in humans. (**ANESTHESIOLOGY** 2014; 120:1450–62)

HEAT-SHOCK proteins are ubiquitous molecular chaperones involved in protein folding, peptide trafficking, and antigen processing under both physiologic and stress conditions. When released actively or passively into the extracellular space, heat-shock proteins function as “danger signal” mediators by mediating the transfer of antigenic peptides from stressed cells to the antigen-presenting cells or by activating toll-like receptors.¹ Activation of the heat-shock response (HSR) protects host cells and organs from sterile insults, such as oxidative stress or ischemia–reperfusion injury, *via* the inhibition of inflammatory cellular pathways.^{2–5} In humans, we have previously shown that the activation of the HSR correlates with survival after severe trauma.⁶ Furthermore, other investigators have reported that heat-shock protein (Hsp)-72 genotypes influence plasma cytokine levels and interfere with outcome after major injury in humans.⁷ Despite the evidence that the activation of the HSR may attenuate the severity of a sterile inflammation, the mechanisms by which HSR activation

What We Already Know about This Topic

- The heat-shock response, activated during trauma, appears to protect organisms from a variety of injuries

What This Article Tells Us That Is New

- The heat-shock response was activated 24 h before the experimental development of *Pseudomonas aeruginosa* pneumonia and led to worsened lung endothelial and epithelial permeability, decreased bacterial clearance, and increased mortality

would modulate lung damage and host response to a bacterial lung infection remain largely unknown.

Pseudomonas aeruginosa is an important cause of nosocomial pneumonia in critically ill patients and is associated with a high mortality rate. Host resistance to *P. aeruginosa* pneumonia requires an intact innate immune response for the clearance of bacteria from the lungs. This has been demonstrated in an experimental model of *P. aeruginosa*

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Submitted for publication July 2, 2013. Accepted for publication January 15, 2014. From the Department of Anesthesiology, University of Nice-Sophia-Antipolis, Nice, France (M.C.); French Institute of Health and Medical Research U1065, University of Nice-Sophia-Antipolis, Nice, France (J.-E.R.); Department of Anesthesiology, University of California San Francisco, San Francisco, California (M.L., J.R.); and Departments of Anesthesiology (B.M.W., K.I., D.L., C.A.R., N.A., J.-F.P.), Environmental Health Sciences (K.I.), Surgery (J.-F.P.), Medicine (J.Z.) and Cell, Developmental, and Integrative Biology, University of Alabama at Birmingham, Birmingham, Alabama (J.-F.P.).

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pneumonia⁸ and indirectly confirmed in a recent clinical study that reported that patients with large burdens of *P. aeruginosa* who did not meet clinical criteria for ventilator-associated pneumonia had an increased risk of death when compared with the risk in patients who met ventilator-associated pneumonia criteria.⁹ Because HSR activation inhibits signaling pathways, such as the nuclear factor- κ B pathway,¹⁰ that are activated by cell membrane receptors and are critical for the eradication of bacteria from the airspaces of the lung,¹¹ we first tested the hypothesis that prior HSR activation would increase the severity of lung injury in a mouse model of *P. aeruginosa* pneumonia in wild-type mice and in mice null for the inducible Hsp72, which is one of the most important heat-shock proteins expressed during HSR activation.¹²

Experimental studies indicate that the initial response to the endogenous release or exogenous administration of antiinflammatory mediators, such as interleukin (IL)-10, is associated with a more severe lung injury caused by bacterial pneumonia.^{13–21} Because the heat-shock factor 1 released during HSR activation is a transcriptional activator of IL-10 gene expression in macrophages²² and the fact that extracellular Hsp72 causes the release of IL-10 *via* the activation of the toll-like receptor-4,²³ the second aim of the study was designed to test the hypothesis that release of IL-10 during the HSR activation could be an important mechanism to explain the inhibition of the lung innate immune response after HSR activation in a mouse model of *P. aeruginosa* lung infection.

Materials and Methods

Reagents

All cell culture media were prepared by the University of California San Francisco and University of Alabama at Birmingham Cell Culture Facilities using deionized water and analytical-grade reagents. The protein concentration of cell lysates was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). The enzyme-linked immunosorbent assay kit for mouse IL-10 was purchased from R&D Systems Inc. (Minneapolis, MN). Myeloperoxidase activity was measured with a mouse myeloperoxidase kit HK210 from Cell Sciences (Canton, MA). Anti-Hsp72 antibody was purchased from Enzo Life Sciences (Farmingdale, NY). Anti-IL-10 monoclonal antibody (JES3-9D7) and isotype immunoglobulin G1 (control Ab) were obtained from Pharmingen (San Diego, CA). Geldanamycin was obtained from InvivoGen (San Diego, CA). ¹²⁵I-labeled human serum albumin (Jeanatope; ISO-TEX Diagnostics, Friendswood, TX) was used as radioactive tracer. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture

A mouse alveolar macrophage cell line (designated as MH-S cells; ATCC no. CRL-2019) was used, as we have previously published.²⁴ Cells were cultured in Roswell Park Memorial

Institute 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin and were maintained at 37°C with 5% CO₂.

Neutrophil Isolation

Mouse neutrophils were purified from bone marrow cell suspensions as previously described.²⁵ Bone marrow cells were incubated with 30 μ l of antibody cocktail specific to the cell surface markers F4/80, CD4, CD45R, CD5, and TER119 for 15 min at 4°C. Anti-biotin tetrameric antibody complexes (100 μ l) were then added to the cells and incubated for 15 min at 4°C followed by incubation with 60 μ l of colloidal magnetic dextran iron particles for 15 min at 4°C. The cell suspension was then placed into a column surrounded by a magnet. The T cells, B cells, erythrocytes, monocytes, and macrophages were captured in the column, allowing the neutrophils to pass through as a result of negative selection. Cells were then washed with Roswell Park Memorial Institute 1640 with 5% fetal bovine serum. Neutrophil purity, as determined by Wright–Giemsa-stained cytospin preparations, was consistently greater than 98%.

Preparation of *P. aeruginosa*

The wild-type PAK strain of *P. aeruginosa* and the PAK strain labeled with the green fluorescent protein (PAK-GFP) were a kind gift from Dr. Stephen Lory, Ph.D., Professor, Department of Microbiology and Molecular Genetics at Harvard Medical School, Boston Massachusetts. For each experiment, frozen bacteria were inoculated into Luria–Bertani broth (Invitrogen, Carlsbad, CA), incubated for 6 h at 37°C on a rotating platform, and then diluted 1:100 in fresh Luria–Bertani broth. After 16 to 18 h of incubation at 37°C, the stationary-phase bacteria were pelleted, washed three times in phosphate-buffered saline (PBS), and suspended in PBS to a concentration adjusted by optical density at 600 nm, as 1×10^9 colony-forming units (CFU)/ml for *in vitro* experiments or 2×10^8 CFU/ml for instillation in mice (50 μ l preparation per mouse).

Bacterial Killing Assay

The bacterial killing assay was performed, as we have previously published.²⁶ MH-S cells, a murine alveolar macrophage cell line (1×10^6 cells), were heat shocked at 43°C for 30 min, then recovered at 37°C for 1 h before adding 10^7 CFU/ml of *P. aeruginosa* PAK for 45 min at 37°C. Gentamicin (150 μ g/ml) was added and cells were incubated for 1 or 3 h at 37°C. The media were removed, the cells washed twice with sterile PBS, then lysed by adding 200 μ l of hypotonic buffer (pH 7.2), and then incubated on ice for 10 min. After adding 800 μ l of sterile water, the cell suspension was serially diluted onto agar plates, incubated for 24 h at 37°C, and the resulting colonies counted. Percent killing of intracellular bacteria was calculated using the following equation: percent killing = $(1 - \text{number of bacteria after 3 h} / \text{number of bacteria after 1 h}) \times 100$. Preliminary data

indicate that lysates from uninfected macrophages did not reveal any bacterial colonies on Luria–Bertani agar plates and that bacteria alone incubated for 1 h with gentamicin were completely killed (data not shown). In some of the experiments, MH-S cells were pretreated with a mouse IL-10-blocking antibody or its isotype control antibody 1 h before undergoing the HSR and subsequent exposure to *P. aeruginosa* PAK bacteria.

Neutrophil Phagocytosis Assay

The neutrophil phagocytosis assay was performed, as previously published.²⁷ Neutrophils were isolated, as described above. Neutrophils (10^6 cells) kept in Roswell Park Memorial Institute 1640 were exposed to 42°C for 30 min and recovered at 37°C for 1 h. Control neutrophils were kept at 37°C for 90 min. Next, neutrophils were exposed to *P. aeruginosa* PAK labeled with PAK-GFP at a multiplicity of infection 80 for 60 min. In some experiments, neutrophils were pretreated with a mouse IL-10-blocking antibody or its isotype control antibody 1 h before undergoing the HSR and subsequent exposure to *P. aeruginosa* PAK bacteria. Then, the cells were fixed, permeabilized, and stained with phalloidin at a concentration of 1:200 for 10 min. Cells were viewed under a LEICA fluorescent microscope (Leica Microsystems, Inc., Buffalo Grove, IL) and 200 cells were randomly counted three times to determine the percentage of cells containing PAK-GFP (done over three independent experiments).

Measurement of Mouse IL-10

Interleukin-10 levels in cell supernatants were measured by enzyme-linked immunosorbent assay. In brief, MH-S cells were heat shocked at 43°C or kept at 37°C for 30 min, then recovered at 37°C for 1 h. Then, 10^7 CFU/ml of *P. aeruginosa* PAK or its vehicle were added to the cell medium and the cells were kept at 37°C for 3 h. Cell supernatant was then collected and the enzyme-linked immunosorbent assay was carried out according to the manufacturer's protocol (R&D Systems Inc.).

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 myeloperoxidase kit HK210 from Cell Sciences).

Western Blot

Western blot analyses of frozen lungs were performed as we described previously.²⁸ After equal amounts of protein were loaded in each lane and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the proteins were transferred to Invitrogen iBlot polyvinylidene difluoride membranes (Invitrogen, Grand Island, NY). The membranes

were blocked for 1 h with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE), which was also used as primary and secondary antibodies incubation buffer. The primary antibody was used at dilutions of 1:1,000, incubated overnight at 4°C. Near-infrared detection was used with the IRDye[®]-conjugated secondary antibodies (LI-COR Biosciences), which were either goat anti-mouse IRDye[®] 800CW used at 1:10,000 dilution and imaged at 84- μ m resolution with the Odyssey infrared imaging system (LI-COR Biosciences). Quantification was performed with the LI-COR Biosciences analysis software.

Hsp72 Detection in the Mouse Lung

Twenty-four hours after the whole-body hyperthermia or the last administration of geldanamycin (1 μ g/g body weight intraperitoneally at 48 and 24 h before) or its vehicle (dimethyl sulfoxide), the mice were sacrificed by aortotomy and the lungs were harvested and frozen at -80°C. The expression of Hsp72 protein in lungs was determined by Western blotting.

Cell Viability Assay

Cell viability (MH-S cells, mouse neutrophils) was measured by the Alamar Blue assay after exposure to the various experimental conditions. Cell media were replaced with medium containing 10% Alamar Blue and placed at 37°C in a cell incubator for 2 h. The media were collected and read on a spectrophotometric plate reader at 530 nm.

Mice Studies

C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA) for all experiments except for the inducible Hsp72- and IL-10-null mice. IL-10-null mice and their control C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Inducible hsp72 (Hsp72-1) knockout mice (B6;129S7-*Hspa1a/Hspa1b*^{tm1Dix}/Mmcd) and their control C57BL/6J mice were purchased from the Mutant Mouse Resource Center (University of California, Davis, CA). Mice were housed in either the University of California San Francisco or University of Alabama at Birmingham Animal Care Facilities. All use of animals was according to the protocols approved by the University of California San Francisco (San Francisco, CA) and University of Alabama at Birmingham (Birmingham, AL) Animal Care and Use Committees and were conducted according to the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Pneumonia Model

The mouse pneumonia model was performed, as we have previously reported.²⁶ In brief, mice were anesthetized with tribromoethanol (250 mg/kg, intraperitoneally). The mouse was laid on a board with its head elevated at 45°. Then, 50 μ l of PBS containing 1×10^7 CFU of *P. aeruginosa* PAK was instilled into both lungs through the trachea *via* the

mouth by using a 27-gauge gavage needle. The mouse was allowed to recover for 15 min before being returned to the cage. Mice were active and appeared normal after 30 min. Four hours after the bacterial instillation, mice were euthanized with a larger dose of tribromoethanol (500 mg/kg, intraperitoneally). Blood samples were collected in a sterile manner through puncture of the inferior vena cava after laparotomy and bilateral thoracotomies were performed. The mouse lungs were removed, weighed, and homogenized for lung vascular permeability measurements. Bacterial concentration was determined by quantitative culture of homogenized lung, blood, and spleen tissue. Some mice were injected with geldanamycin or its vehicle (1 µg/g body weight intraperitoneally) 48 and 24 h before the airspace instillation of *P. aeruginosa* PAK. Pilot studies demonstrated that geldanamycin did not affect the viability of *P. aeruginosa* PAK (data not shown). For the survival studies, all mice were checked every hour during the 24 h after the instillation *P. aeruginosa* PAK bacteria into the lung until death or survival at 24 h.

Whole-body Hyperthermia in Mice

Before the treatments, the animals were stabilized at room temperature (25° ± 1°C). Whole-body hyperthermia was performed on a heating pad, with the use of a warming lamp, as we have previously reported.¹² Animals were anesthetized and maintained throughout the operation by injection of 2.5% tribromoethanol saline (20 µl/g body mass; Sigma-Aldrich). The animals were taped onto the heating pad and a rectal thermostat probe was inserted. The temperature was maintained at 42° ± 0.2°C for 20 min. During the operation, room temperature was maintained at 25° ± 1°C. The sham control group received tribromoethanol treatment but no preheat treatment. After a 24-h recovery period with free access to food and water, *P. aeruginosa* PAK bacteria were instilled into the trachea, as described in the Materials and Methods section. We have previously reported in several studies that the inhibition of the innate immunity starts within hours after activation of the HSR¹⁰ and is present at least up to 48 h after onset of heat shock.²⁴ In the current study, we used the *in vivo* protocol (exposure to bacteria 24 h after onset of the HSR) that we have previously shown to be protective effect against the sterile inflammation associated with hemorrhagic shock.³

Lung Vascular Permeability Measurement

Lung endothelial permeability to protein (%) and excess lung water (µl) was measured, as previously described.²⁶ In brief, 0.5 µCi of ¹²⁵I-albumin was injected intraperitoneally 2 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 (12,000g, 8 min) to assay the

hemoglobin concentration in the supernatant. Another fraction of homogenate, supernatant, and blood were weighed and then dried in an oven (60°C for 48 h) for gravimetric determination of the extravascular lung water. The lung wet-to-dry weight ratio (lung W/D ratio) was determined by the following standard formula:

$$\text{Extravascular lung water} = \left(\begin{array}{l} \text{lung W / D ratio}_{\text{experimental}} \\ \times \text{lung dry weight}_{\text{experimental}} \\ - \text{lung W / D ratio}_{\text{normal}} \\ \times \text{lung dry weight}_{\text{normal}} \end{array} \right) \times 1000 \text{ (}\mu\text{l)}$$

Endothelial permeability was calculated as the counts of ¹²⁵I-albumin in the blood-free lung tissue divided by the counts of ¹²⁵I-albumin in the plasma.

Bacteria Cultures from the Lungs

Lungs were collected in a sterile manner. The lungs were homogenized in sterile containers and the homogenates were serially diluted and plated in triplicate on sheep blood agar plates.

Bronchoalveolar Lavage, Cell Count, and Protein Measurement

Bronchoalveolar lavage fluid was collected by infusing 1 ml of sterile PBS (containing 5 mM EDTA) into the lungs of the mice after tracheal cannulation, as previously described.²⁶ Gentle suction was applied and approximately 85% of the fluid was withdrawn from the lungs. The collected fluid was centrifuged at 6,000 rpm for 5 min. One hundred microliter of the supernatant was immediately used for cytospin preparation. 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 microliter of bronchoalveolar lavage fluid recovered (×10⁴/ml bronchoalveolar lavage fluid). The remaining supernatant was stored immediately at -80°C for protein measurement.

Statistical Analysis

All data are summarized as mean ± SD. For the statistical analysis, we used Statview 5.0® (SAS Inc., Cary, NC) and MedCalc® 7.2.0.2 (MedCalc Software Inc., Ostend, Belgium). The normal distribution was verified using the Agostino–Pearson test. For normally distributed data, Student *t* test, one-way ANOVA, and Fisher protected least significant difference for *post hoc* comparisons were used to determine differences between experimental and control groups. To compare data that were not normally distributed, the Wilcoxon–Mann–Whitney two-sample rank sum test was used. All statistical comparison of means was

bilateral (two-tailed tests). A Kaplan–Meier analysis followed by a log rank (Mantel–Cox) test was used to compare the survival between the four experimental groups of mice at 24 h. A *P* value of less than 0.05 was considered statistically significant.

Results

Prior activation of the HSR by whole-body hyperthermia or geldanamycin increases lung injury in a mouse model of *P. aeruginosa* pneumonia. We first verified that both whole-body hyperthermia and geldanamycin, which is a benzoquinone ansamycin antibiotic and a pharmacologic activator of the HSR,²⁹ cause a significant increase in the expression of the inducible heat-shock protein Hsp72 in the mouse lung compared with controls without whole-body hyperthermia or vehicle (fig. 1, A and B). This result confirms that the Hsp72 response is due to a HSR and not merely a response to hyperthermia. Then, we tested the hypothesis that prior activation of the HSR would worsen lung injury caused by *P. aeruginosa* PAK. Airspace instillation of *P. aeruginosa* caused the development of pulmonary edema in mice which was significantly increased (as evidenced by an increase in excess lung water) by prior activation of the HSR with

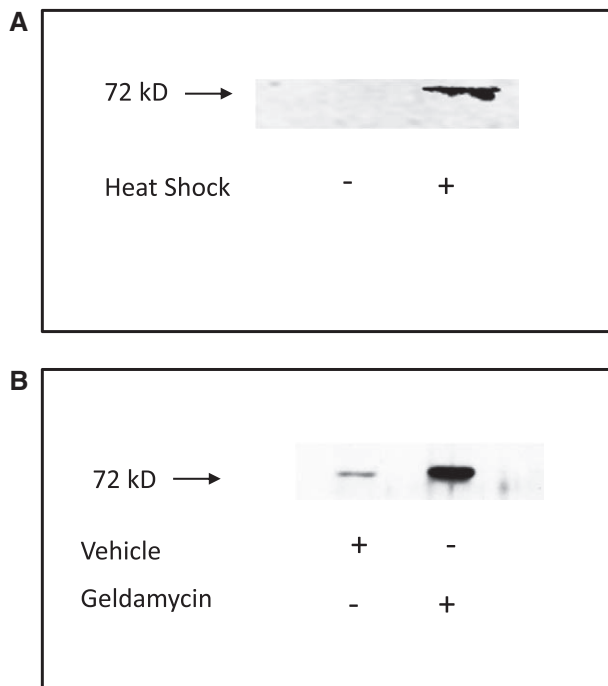


Fig. 1. Activation of the heat-shock response by whole-body hyperthermia or geldanamycin leads to an increased expression of the inducible Hsp72 protein in the mouse lung. Lungs from C57BL/6 mice were harvested 24 h in mice that underwent (A) a 20-min whole-body hyperthermia at $42^{\circ} \pm 0.2^{\circ}\text{C}$ induced by exposure heating pad and warming lamp or were kept at room temperature and (B) a pretreatment with geldanamycin or its vehicle (1 $\mu\text{g/g}$ body weight given intraperitoneally at 48 and 24 h before lung harvesting). One representative lung is shown for each experimental condition.

whole-body hyperthermia or geldanamycin (fig. 2, A–D). The increase in severity of pulmonary edema was associated with a decrease in pulmonary bacterial clearance (fig. 3A) and an increase in alveolar epithelial permeability to protein (fig. 3B), despite an increase in total cells and neutrophil recruitment into the airspaces (fig. 4, A and B). In accordance with these results, whole-lung myeloperoxidase activity (as marker of polymorphonuclear leukocytes recruitment) was strongly increased after PAK-induced pneumonia and heat shock (fig. 4C). Mice that underwent HSR and airspace instillation with vehicle 24 h later did not have any bacterial growth from their lung homogenates (data not shown). Because it has previously been demonstrated that activation of the HSR inhibits phagocytosis by neutrophils,³⁰ we next examined whether prior heat shock would decrease phagocytosis of *P. aeruginosa* PAK labeled with GFP. Our results reveal that phagocytosis of PAK-GFP was decreased by 70% in heat-shocked neutrophils compared with that in control cells (fig. 5, A and B). Furthermore, heat shock did not cause mouse neutrophil death as measured by the Alamar Blue assay (data not shown). Taken together, these results demonstrate that activation of the HSR increases lung damage associated with PAK-induced pneumonia secondary to a decrease in the clearance of *P. aeruginosa* bacteria from the alveolar space.

Exacerbation of *P. aeruginosa*-induced lung injury and mortality by prior activation of the HSR is reduced by the genetic deletion of the inducible Hsp72 protein. Prior experimental work indicates that inducible Hsp72 is one of the most important heat-shock proteins expressed during HSR activation¹² and plays an important role in the attenuation of the inflammatory response induced by immune cells.²⁴ Therefore, we tested the hypothesis that genetic deletion of Hsp72 would attenuate the exacerbation of *P. aeruginosa*-induced lung injury and mortality caused by prior HSR activation. Genetic deletion of Hsp72 was associated with suppression of the heat-shock-induced increase in lung vascular permeability (fig. 6, A and B), decrease in lung bacterial clearance (fig. 6C), and development of alveolar edema (fig. 6D). In addition, this strategy was associated with a significant increase in survival of mice subjected to heat shock followed 24 h later by airspace instillation with *P. aeruginosa* PAK although it did not affect the survival of sham mice that had similar airspace instillation with *P. aeruginosa* PAK (fig. 7).

Exacerbation of *P. aeruginosa*-induced lung injury and mortality by prior activation of the HSR is mediated by an IL-10-dependent mechanism. Previous studies have reported that IL-10 inhibits bacterial clearance from the lung in a mouse model of *P. aeruginosa*-induced bacterial pneumonia.^{13–21} Furthermore, IL-10 protein expression can be induced in response to activation of the HSR.^{22,23} Thus, in the next series of experiments, we tested the hypothesis that prior activation of the HSR would exacerbate *P. aeruginosa*-induced lung injury and mortality *via* an IL-10-dependent mechanism. We first found that prior heat shock increased by 50% the release of IL-10 by MH-S cells, a mouse alveolar

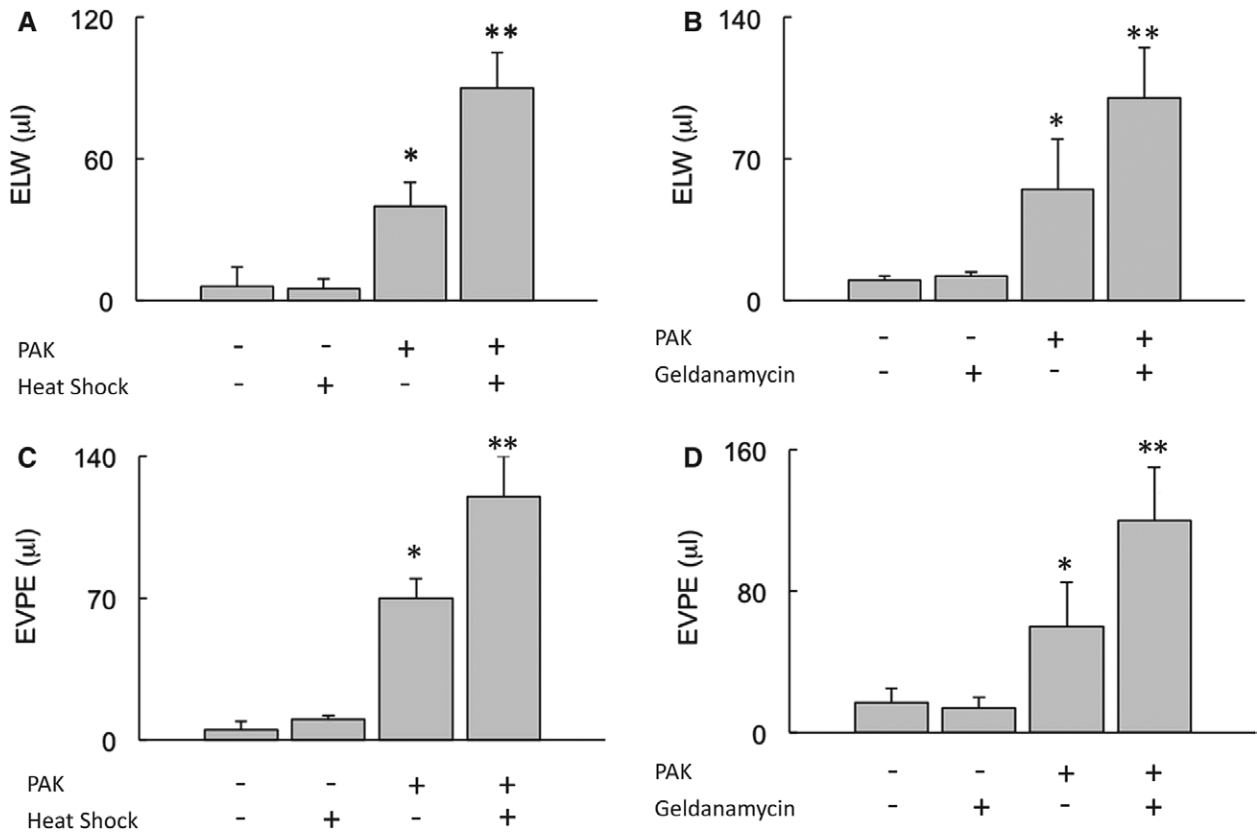


Fig. 2. Prior activation of the heat-shock response by whole-body hyperthermia or geldanamycin increases lung injury in a mouse model of *Pseudomonas aeruginosa* pneumonia. (A–D) C57BL/6 mice were left untreated or underwent a heat-shock response with whole-body hyperthermia or two intraperitoneal injections of intraperitoneal geldanamycin or its vehicle (1 μg/g body weight). Twenty-four hours later, mice had their airspaces instilled with *P. aeruginosa* bacteria (PAK strain, 1×10^7 colony-forming units [CFU]) or its vehicle. Mice were euthanized 4 h later and excess lung water (ELW) and lung vascular permeability (extravas-cular pulmonary edema [EVPE]) were measured, as described in the Materials and Methods section. For all experiments, results are shown as mean \pm SD (n = 6 mice per group); * $P \leq 0.05$ from controls; ** $P \leq 0.05$ from mice with PAK alone.

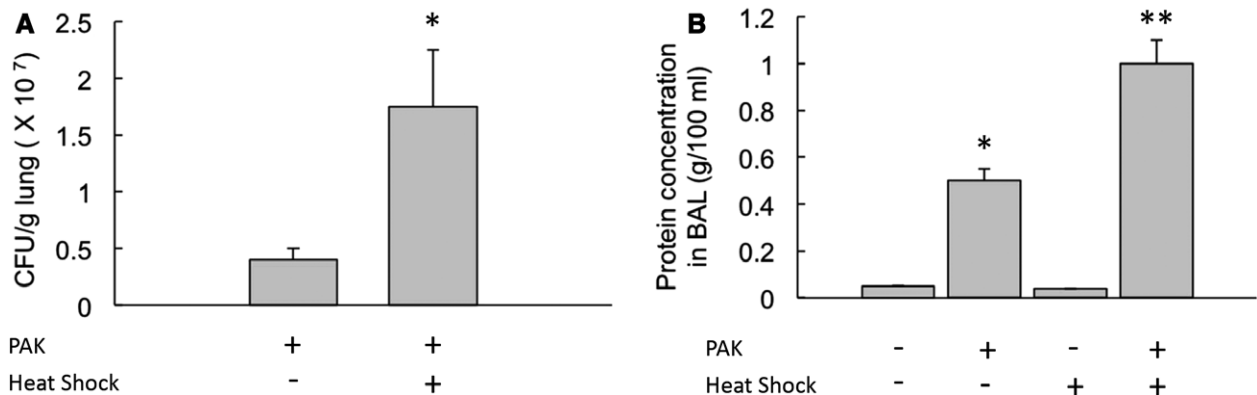


Fig. 3. Prior activation of the heat-shock response by whole-body hyperthermia decreases lung bacterial clearance and increases lung epithelial protein permeability in a mouse model of *Pseudomonas aeruginosa* pneumonia. (A and B) C57BL/6 mice were left untreated or underwent a heat-shock response with whole-body hyperthermia that was followed 24 h later by airspace instillation of *P. aeruginosa* (PAK strain, 1×10^7 colony-forming units [CFU]) or its vehicle. Mice were euthanized 4 h later and either bacteria were counted in the lung homogenates or protein concentration was measured in the bronchial alveolar lavage (BAL) fluid. For all experiments, results are shown as mean \pm SD (n = 6 mice per group); * $P \leq 0.05$ from controls; ** $P \leq 0.05$ from mice with PAK alone.

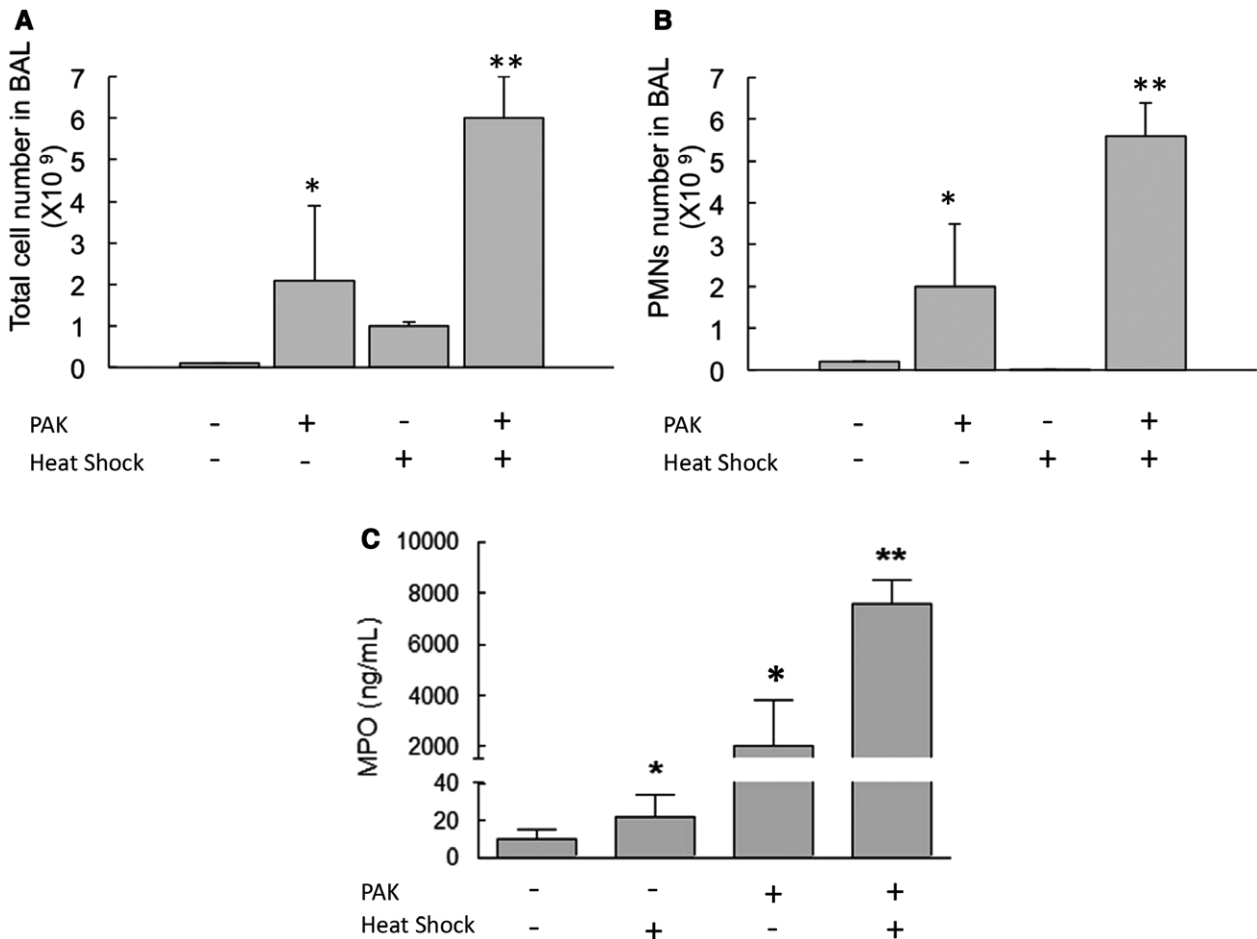


Fig. 4. Prior activation of the heat-shock response by whole-body hyperthermia increases total cell and neutrophil recruitment into the airspaces in a mouse model of *Pseudomonas aeruginosa* pneumonia. (A–C) C57BL/6 mice were left untreated or underwent a heat-shock response with whole-body hyperthermia that was followed 24 h later by airspace instillation of *P. aeruginosa* (PAK strain, 1×10^7 colony-forming units [CFU]) or its vehicle. Mice were euthanized 4 h later and bronchial alveolar lavage (BAL) was performed and cells counted. In separate experiments, 4 h after *P. aeruginosa* instillation, lungs were removed and frozen in liquid nitrogen for measurement of myeloperoxidase (MPO) activity as described in the Materials and Methods section. For all experiments, results are shown as mean \pm SD ($n = 6$ mice per group); * $P \leq 0.05$ from controls; ** $P \leq 0.05$ from mice with PAK alone. PMN = polymorphonuclear leukocytes.

macrophage cell line (fig. 8A). Heat shock did not cause MH-S cell death as measured by the Alamar Blue assay (data not shown). Furthermore, heat-shocked MH-S cells that were subsequently exposed to *P. aeruginosa* PAK bacteria released significantly more IL-10 compared with IL-10 released by the cells exposed to the *P. aeruginosa* but kept at 37°C (fig. 8A). We next demonstrated that the decrease in intracellular bacterial killing observed in heat-shocked MH-S cells was dependent on IL-10, because it was blocked by pretreatment with a specific blocking mouse IL-10 antibody, but not by its isotype control antibody (fig. 8B). In the next series of experiments, we found that exposure to increasing doses of mouse IL-10 (0.1 to 10 ng/ml) caused a progressive decrease in the phagocytosis of *P. aeruginosa* labeled with the PAK-GFP by mouse neutrophils (fig. 8C). Furthermore, HSR-mediated inhibition of neutrophil phagocytosis of *P. aeruginosa* bacteria was completely inhibited by pretreatment with IL-10-blocking antibody, but not with an isotype control antibody (fig. 8D).

Interestingly, pretreatment with IL-10 (10 ng/ml) did not further decrease the uptake of *P. aeruginosa* bacteria by heat-shocked neutrophils (fig. 8D). Finally, the results of these *in vitro* experiments were confirmed in our *in vivo* mouse model of heat shock and subsequent *P. aeruginosa* pneumonia. Decreased lung bacterial clearance and excess of 24-h mortality caused by *P. aeruginosa* pneumonia in heat-shocked wild-type mice were significantly attenuated in IL-10-null mice (fig. 8, E and F). Taking together, these results demonstrate an important mechanistic role for IL-10 in decreasing lung bacterial clearance by inhibiting intracellular *P. aeruginosa* killing by macrophages and bacterial phagocytosis by neutrophils and thus increasing mortality after activation of the HSR in a mouse model of *P. aeruginosa* pneumonia.

Discussion

In this study, we demonstrate that (1) prior HSR activation worsens the development of protein-rich pulmonary edema,

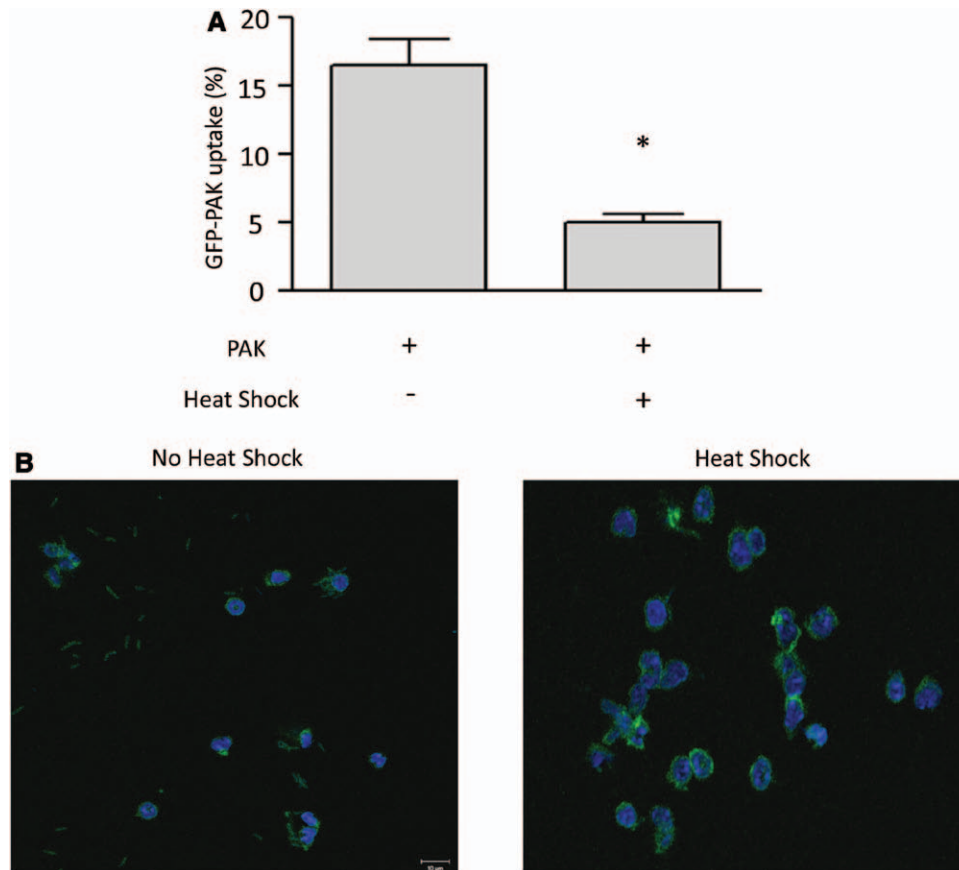


Fig. 5. Activation of the heat-shock response inhibits phagocytosis of *Pseudomonas aeruginosa* by mouse neutrophils. (A) Primary mouse neutrophils were isolated, heat-shocked at 42°C for 30 min, and then exposed 60 min later to *P. aeruginosa* expressing green fluorescent protein (PAK-GFP) for 30 min. After cell fixation, permeabilization, and staining with phalloidin, the number of intracellular PAK-GFP was counted, as described in the Materials and Methods section. Results are shown as mean \pm SD ($n = 3$ experiments repeated in triplicate); * $P \leq 0.05$ from non-heat-shocked cells. (B) This provides an example of the inhibition of PAK-GFP bacteria phagocytosis by primary mouse neutrophils that underwent prior heat shock activation. Please note that although PAK-GFP bacteria are stuck to the outside of heat-shocked neutrophils, they have not been endocytosed.

decreased lung bacterial clearance, and increased mortality associated with *P. aeruginosa* pneumonia; (2) this HSR effect was not observed in mice null for the inducible Hsp72, one of the most important heat-shock proteins expressed during HSR activation,¹² thus confirming the specificity of this observation; and (3) IL-10 plays an important mechanistic role in mediating HSR-mediated decrease in bacterial killing by MH-S cells, which is a mouse lung macrophage cell line, and decrease in neutrophil bacterial phagocytosis. These *in vitro* results were confirmed by the increased lung bacterial clearance and decreased mortality caused by *P. aeruginosa* pneumonia in heat-shocked IL-10-null mice.

Heat-shock response activation has been shown to attenuate cell and organ response to inflammatory stimuli induced by several pathological conditions, particularly associated with sterile inflammation.²⁻⁵ However, the effect of HSR activation on the innate and adaptive immune response to lung bacterial infection remains unknown. We found in the current study that HSR activation by heat or a pharmacologic inhibitor of Hsp90 increased lung injury caused by *P.*

aeruginosa bacteria in mice. These results are in accordance with the fact that HSR activation inhibits cellular pathways, such as the nuclear factor- κ B pathway,¹⁰ that are activated by cell membrane receptors and are critical for the eradication of bacteria from the airspaces of the lung.¹¹ Interestingly, HSR activation inhibited lung bacterial clearance despite an increase in the number of neutrophils migrating into the airspaces of the lungs in response to the bacterial challenge. Previous work has demonstrated that there are functional heat-shock elements on the promoter of IL-8, a critical neutrophil chemokine, that result in an increased protein expression of this chemokine.^{31,32} However, the increased number of airspace neutrophils in the heat-shocked mice that had their airspaces instilled with *P. aeruginosa* was not associated with an increase, but with a decrease in bacterial clearance from the lung of these animals. It has been previously reported that HSR activation inhibits neutrophil phagocytosis.³⁰ We also found in our study that HSR activation decreased the ability of neutrophils to phagocytose *P. aeruginosa* bacteria by 70% (fig. 5).

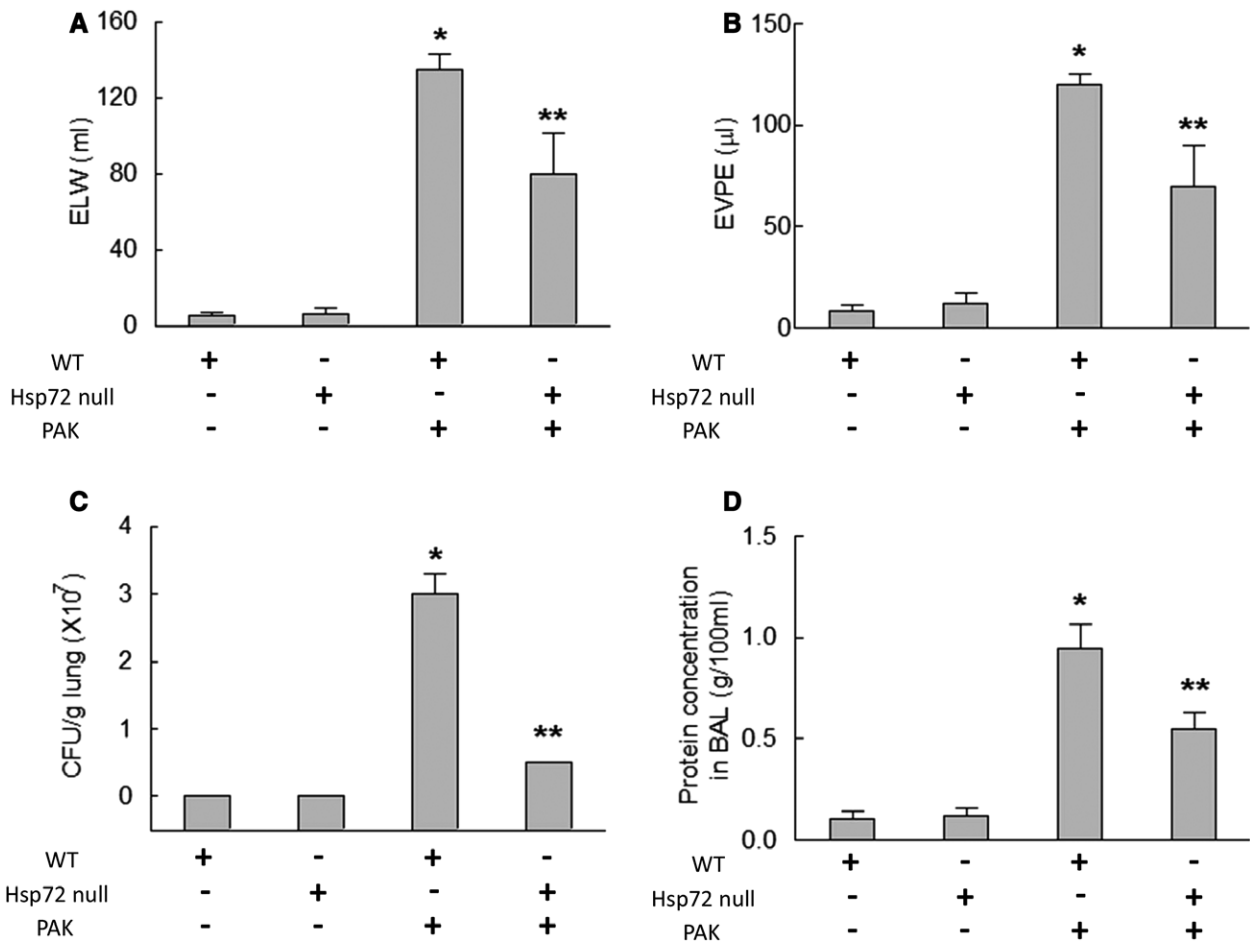


Fig. 6. Genetic deletion of Hsp72 attenuates the effect of the heat-shock response on lung vascular permeability and bacterial clearance in a mouse model of *Pseudomonas aeruginosa* pneumonia. (A–D) C57BL/6 wild-type (WT) or Hsp72-null mice were left untreated or underwent a heat-shock response with whole-body hyperthermia that was followed 24 h later by airspace instillation of *P. aeruginosa* (PAK strain, 1×10^7 colony-forming units [CFU]) or its vehicle. Mice were euthanized 4 h later and excess lung water (ELW) and lung vascular protein permeability (EVPE) were measured, as described in the Materials and Methods section. In separate experiments, bronchial alveolar lavage (BAL) was performed and cells counted and BAL fluid protein concentration measured. For all experiments, results are shown as mean \pm SD ($n = 6$ mice per group); * $P \leq 0.05$ from controls, ** $P \leq 0.05$ from mice with PAK alone.

The next set of experiments demonstrated that the HSR inhibition of the bacterial clearance after a lung challenge with *P. aeruginosa* was specific to the HSR because Hsp72-null mice did not show the increase in lung injury and inhibition of lung bacterial clearance associated with HSR in their wild-type littermates. These *in vivo* results are in accordance with previous experimental work that has shown that intracellular Hsp72 directly inhibits the nuclear factor- κ B pathway^{33–35} which is critical for the eradication of *P. aeruginosa* bacteria.¹¹ It should be pointed out that HSR activation might not be detrimental in established sepsis. Indeed, if a brisk inflammatory response to infecting organism is essential for pathogen containment and eradication. However, systemic spread of inflammation beyond the focus of bacterial infection leads to organ injury and mortality.³⁶ Thus, it is not surprising that HSR activation with Hsp90 inhibitor, such as a derivative of the geldanamycin used in our

study prolonged survival, attenuate systemic inflammation and reduce lung injury in murine sepsis.³⁷ In addition, the results of clinical studies confirm that patients with a genetic variation on Hsp72 that causes low expression of that protein in mononuclear cells have higher risk for septic shock.^{38,39} Taken together, our data and the results of previous studies indicate that prior induction of the HSR increases lung damage caused by bacterial pneumonia because of the HSR-mediated decrease in bacterial clearance from the airspaces of the lung. Furthermore, genetic deletion of Hsp72 completely reverses the deleterious effect of HSR activation on lung injury caused by *P. aeruginosa* bacteria. This result demonstrates that the increased lung expression of Hsp72 associated with HSR activation may regulate the intensity of the inflammatory response to bacterial products within the lung. On the basis of the results of previous work, it also suggests that in the presence of established systemic inflammatory

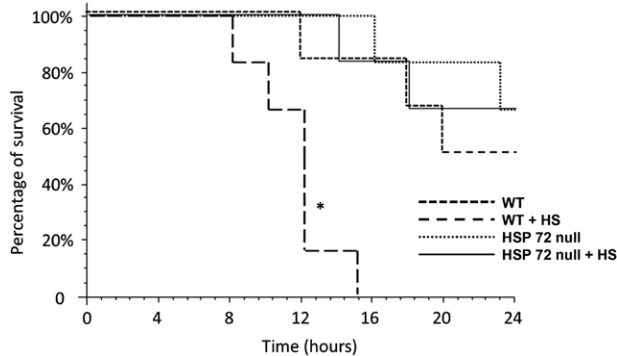


Fig. 7. Genetic deletion of Hsp72 attenuates the increased mortality associated with a prior heat-shock response in a mouse model of *Pseudomonas aeruginosa* pneumonia. C57BL/6 wild-type (WT) or Hsp72-null mice were left untreated or underwent a heat-shock response with whole-body hyperthermia that was followed 24 h later by airspace instillation of *P. aeruginosa* (PAK strain, 1×10^7 colony-forming units [CFU]) or its vehicle. Kaplan-Meier survival analysis was performed ($n = 10$ mice per group). WT but not Hsp72-null mice that underwent a heat-shock response and had their airspaces instilled with *P. aeruginosa* PAK died significantly earlier ($*P = 0.05$) than the WT mice that did not undergo a heat-shock response before the onset of *P. aeruginosa* pneumonia.

response secondary to sepsis, Hsp72 may attenuate a systemic exuberant inflammatory response by the host that may cause end-organ damage.

The second important aim of the study was to determine potential mechanisms that could explain the inhibitory effect of HSR activation on the bacterial clearance by immune cells. For example, previous work has shown that the initial response to the endogenous release or exogenous administration of antiinflammatory mediators, such as IL-10, is associated with more severe lung injury caused by bacterial pneumonia.^{13–21} Furthermore, the heat-shock factor 1 released during HSR activation is a transcriptional activator of the IL-10 gene expression in macrophages²² and the fact that extracellular Hsp70 causes the release of IL-10 *via* the activation of the toll-like receptor-4²³ and enhances immunosuppressive function of CD4⁺CD25⁺FoxP3⁺ T regulatory cells that release IL-10.⁴⁰ In the current study, heat-shocked alveolar macrophages released more IL-10 than that released by the cells kept at 37°C. Furthermore, HSR activation inhibited intracellular killing of *P. aeruginosa* bacteria by alveolar macrophages *via* an IL-10-dependent mechanism despite the fact that it is known that HSR activation and Hsp70 both increase phagocytosis by these cells.^{41–43} These results are in accordance with previous work from our laboratory that has shown that macrophages play an important role in the airspace clearance of *P. aeruginosa*.^{44,45} Furthermore, exposure of alveolar macrophages to *P. aeruginosa* caused the release of IL-10 that impaired the ability to kill this bacterium, and this defect was partially reversed by neutralization of that cytokine.¹⁵

Does HSR activation affect phagocytosis and intracellular killing of *P. aeruginosa* by neutrophils? Neutrophils are

critical for the eradication of *P. aeruginosa* from the lungs.⁸ Furthermore, a low airspace bacterial inoculum is cleared more efficiently than a larger one, suggesting that granulocyte killing of *P. aeruginosa* is saturable within the airspaces of the lungs.⁴⁶ Interestingly, despite an increase in neutrophil migration into the lungs induced by HSR activation, lung bacterial clearance was reduced in heat-shocked mice. This apparent paradox may in part be explained by the fact that HSR activation has been shown to inhibit neutrophil phagocytosis.³⁰ We found a significant 70% inhibition of *P. aeruginosa* phagocytosis caused by HSR activation in neutrophils, an effect blocked by pretreatment with an IL-10-blocking antibody. Furthermore, we also found that exposure of mouse neutrophils to low concentrations of IL-10 (5 to 10 ng/ml) resulted in the comparable inhibition of the phagocytosis of live *P. aeruginosa* bacteria. These results are in accordance with previous work that has shown that IL-10 inhibits neutrophil phagocytic activity of *Escherichia coli* and *Staphylococcus aureus* bacteria *in vitro*.^{47,48} Our *in vitro* results were confirmed in our *in vivo* mouse model of heat shock and subsequent *P. aeruginosa* pneumonia. Decreased lung bacterial clearance and excess of 24 h mortality caused by *P. aeruginosa* pneumonia in heat-shocked wild-type mice were attenuated in IL-10-null mice. Taken together, our data and the results of previous studies indicate an important mechanistic role for IL-10 in mediating the inhibitory effect of HSR activation on the bacterial clearance by immune cells.

The current study has several limitations. First, we examined here the HSR effect on bacterial pneumonia caused by one bacterium, *P. aeruginosa*. Previous work has shown that the host response to bacterial pneumonia is organism dependent.⁴⁹ Thus, additional work will be needed to determine whether the effect of HSR is comparable in experimental models of bacterial pneumonia induced by other microorganisms. Second, we only examined the effect of HSR on bacterial eradication by alveolar macrophages and neutrophils. We did not examine the effect of HSR activation on several other immune cells that participate in the elimination of *P. aeruginosa* from the distal airspaces of the lungs. In particular, intratracheal dendritic cells appear to modulate the balance between IL-12 and IL-10 expression in response to *P. aeruginosa* pneumonia in mice.⁵⁰ Furthermore, Rag2-null mice deficient in lymphocytes showed impaired bacterial clearance in response to *P. aeruginosa* pneumonia⁵¹ although it is known that HSR activation enhances immunosuppressive function of CD4⁺CD25⁺FoxP3⁺ T regulatory cells that release IL-10.⁴⁰ Finally, although our data indicate an important mechanistic role of IL-10 to explain the impaired lung bacterial clearance associated with HSR activation in our experimental model of *P. aeruginosa* pneumonia, IL-10 release after the onset of bacterial infection may play an important function to attenuate parenchymal tissue destruction when the pathogen elicits a strong local and systemic inflammatory response (reviewed in the study by Cyktor and Turner⁵²).

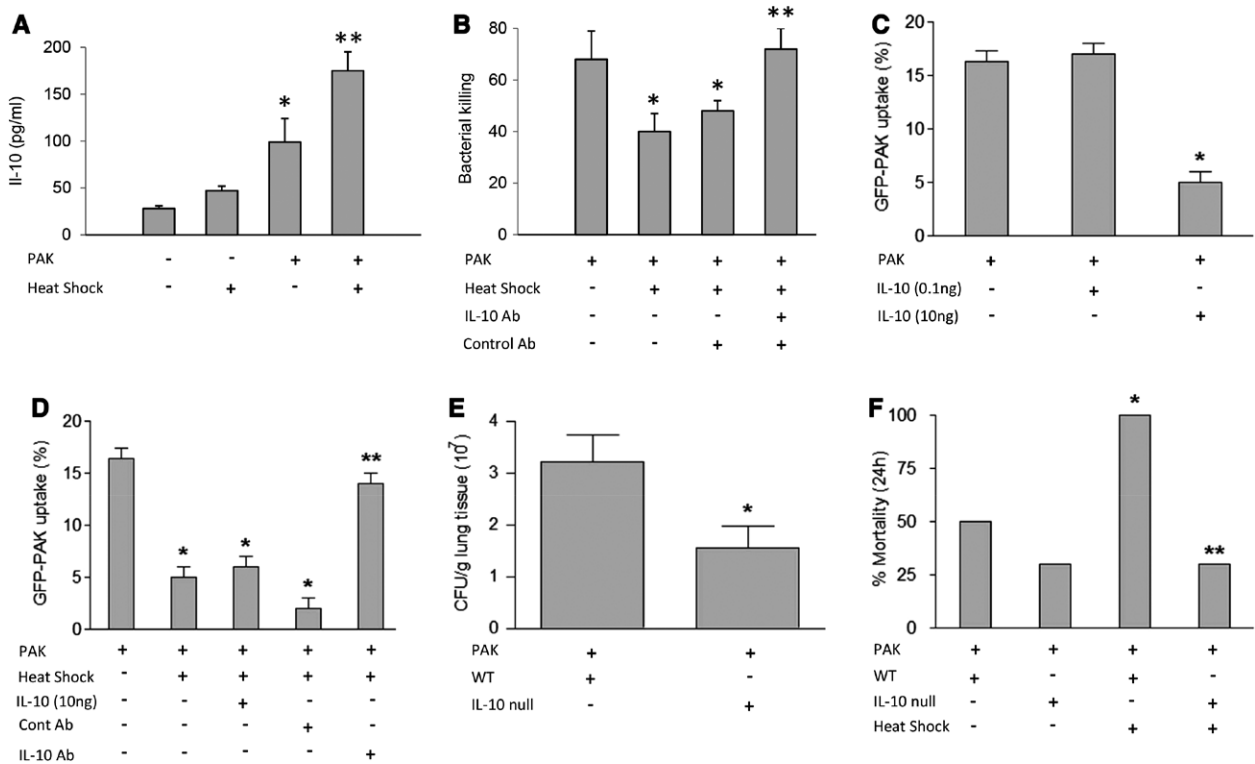


Fig. 8. Increased interleukin (IL)-10 release induced by the activation of the heat-shock response is associated with a decreased intracellular killing by alveolar macrophages, decreased bacterial phagocytosis by neutrophils, and increased mortality in a mouse model of *Pseudomonas aeruginosa* pneumonia. (A) MH-S cells, a mouse alveolar macrophage cell line (1×10^6 cells), were left untreated or heat shocked with heat for 30 min at 43°C , then exposed 1 h later to *P. aeruginosa* PAK (2×10^7 colony-forming units [CFU]/ml; multiplicity of infection [MOI] = 10:1) or its vehicle for 4 h. Enzyme-linked immunosorbent assay of IL-10 was carried out according to the manufacturer's protocol. Results are shown as mean \pm SD ($n = 5$ experiments repeated in triplicate); * $P \leq 0.05$ from control cells; ** $P \leq 0.05$ from cells treated with PAK alone. (B) MH-S cells were heat shocked at 43°C for 30 min or kept at 37°C , then recovered at 37°C for 1 h before adding 10^7 CFU/ml of *P. aeruginosa* PAK for 45 min at 37°C . Gentamicin ($150 \mu\text{g}/\text{ml}$) was added and cells were incubated for 1 or 3 h at 37°C . After cell lysis, percent of intracellular bacterial killing was calculated, as described in the Materials and Methods section. In some experiments, MH-S cells were pretreated with a mouse IL-10-blocking antibody or its isotype control antibody 1 h before undergoing the heat-shock response and subsequent exposure to *P. aeruginosa* bacteria. Results are shown as mean \pm SD ($n = 5$ experiments repeated in triplicate); * $P \leq 0.05$ from non-heat-shocked cells; ** $P \leq 0.05$ from heat-shocked cells pretreated with an isotype control antibody. (C) Primary mouse neutrophils were isolated, exposed to increasing concentrations of mouse IL-10 (0.1–10 ng/ml), then exposed 60 min later to *P. aeruginosa* expressing green fluorescent protein (PAK-GFP) for 30 min. After cell fixation, permeabilization, and staining with phalloidin, the number of intracellular PAK-GFP was counted, as described in the Materials and Methods section. Results are shown as mean \pm SD ($n = 3$ experiments repeated in triplicate); * $P \leq 0.05$ from neutrophils exposed to IL-10 vehicle. (D) Primary mouse neutrophils were isolated as described in the Materials and Method section. Cells were heat shocked at 43°C for 30 min or kept at 37°C , then exposed 60 min later to *P. aeruginosa* PAK-GFP for 30 min. After cell fixation, permeabilization, and staining with phalloidin, the number of intracellular PAK-GFP was counted, as described in the Materials and Methods section. Results are shown as mean \pm SD ($n = 3$ experiments repeated in triplicate); * $P \leq 0.05$ from non-heat-shocked cells; ** $P \leq 0.05$ from heat-shocked cells pretreated with an isotype control antibody. (E and F) C57BL/6 wild-type (WT) or IL-10-null mice underwent a heat-shock response with whole-body hyperthermia that was followed 24 h later by airspace instillation of *P. aeruginosa* (PAK strain, 1×10^7 CFU) or its vehicle, as described in the Materials and Method section. Number of live bacteria in the lung homogenates and mortality at 24 h are reported. Results are shown as mean \pm SD ($n = 6$ mice per group for lung bacterial clearance and $n = 8$ mice per group for mortality); * $P \leq 0.05$ from non-heat-shocked mice, ** $P \leq 0.05$ from wild-type mice.

In summary, our studies demonstrate that HSR activation significantly increases lung injury caused by *P. aeruginosa* in mice. This effect is mediated in large part by IL-10-dependent mechanisms that inhibit airspace clearance of *P. aeruginosa* bacteria by alveolar macrophages and neutrophils. These results also suggest that if HSR activation may have a protective effect early after the onset of sterile inflammation,

such as severe trauma,^{3,12} it may also facilitate the later development of immunosuppression and bacterial lung infection in these patients.

Acknowledgments

The authors kindly thank Marybeth Howard, Ph.D. (Department of Anesthesiology, University of California San Fran-

cisco, San Francisco, California), for her technical assistance on this project.

This study was supported in part by a grant (no. RO1 GM-62188) from the National Institutes of Health (Bethesda, Maryland) (to Dr. Pittet) and a Research Fellowship Grant from the Foundation for Anesthesia Education and Research (Rochester, Minnesota) (to Dr. Wagener).

Competing Interests

The authors declare no competing interests.

Correspondence

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