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*J Immunol* 2013; 191:1393-1403; Prepublished online 1 July 2013;
doi: 10.4049/jimmunol.1300052
http://www.jimmunol.org/content/191/3/1393

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/07/01/jimmunol.1300052.DC1

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A Protective Hsp70–TLR4 Pathway in Lethal Oxidant Lung Injury

Yi Zhang,* Xuchen Zhang,‡,§ Peiying Shan,* Clayton R. Hunt,§ Tej K. Pandita,§ and Patty J. Lee*

Administering high levels of inspired oxygen, or hyperoxia, is commonly used as a life-sustaining measure in critically ill patients. However, prolonged exposures can exacerbate respiratory failure. Our previous study showed that TLR4 confers protection against hyperoxia-induced lung injury and mortality. Hsp70 has potent cytoprotective properties and has been described as a TLR4 ligand in cell lines. We sought to elucidate the relationship between TLR4 and Hsp70 in hyperoxia-induced lung injury in vitro and in vivo and to define the signaling mechanisms involved. Wild-type, TLR4−/−, and Trif−/− (a TLR4 adapter protein) murine lung endothelial cells (MLECs) were exposed to hyperoxia. We found markedly elevated levels of intracellular and secreted Hsp70 from wild-type mice lungs and MLECs after hyperoxia. We confirmed that Hsp70 and TLR4 communoprecipitate in lung tissue and MLECs. Hsp70-mediated NF-κB activation appears to depend upon TLR4. In the absence of TLR4, Hsp70 loses its protective effects in endothelial cells. Furthermore, these protective properties of Hsp70 are TLR4 adapter Trif dependent and MyD88 independent. Hsp70-deficient mice have increased mortality during hyperoxia, and lung-targeted adenoviral delivery of Hsp70 effectively rescues both Hsp70-deficient and wild-type mice. To our knowledge, our studies are the first to define an Hsp70–TLR4–Trif cytoprotective axis in the lung and endothelial cells. This pathway is a potential therapeutic target against a range of oxidant-induced lung injuries. The Journal of Immunology, 2013, 191: 1393–1403.

A critical respiratory failure is a frequent cause of hospital admissions, with >150,000 cases of acute lung injury, or its severe form, acute respiratory distress syndrome, every year in the United States (1). Oxygen therapy is a necessary and life-saving component in the treatment of these critically ill patients, but prolonged oxygen therapy at high concentrations (hyperoxia) was shown to exacerbate organ injury and potentially lead to increased mortality (2). Animal models demonstrated that hyperoxia leads to elevated levels of oxidants that damage both pulmonary epithelial and endothelial cells, thereby causing increased pulmonary capillary permeability, inflammation, and eventual respiratory demise (3, 4).

TLRs have been studied intensely in the context of microbial challenges, inflammation, and immune cells, but their role in non-infectious challenges remains an emerging area. We showed that mammalian TLR4 is required for extended survival during lethal oxidant stress resulting from hyperoxia (5, 6) or bleomycin-induced injury (7). However, the potential ligands and signaling pathways of TLR4 during hyperoxia-induced lung injury are still unknown.

Heat shock proteins (Hsps) are highly conserved proteins found in all prokaryotes and eukaryotes that maintain cellular homeostasis and, when induced, can serve as a “danger” signal by activating both innate and adaptive immunity (8, 9). In most cells, the predominant Hsps are ∼25, 70, 90, and 110 kDa (10, 11). Among these proteins, Hsp70 (also called Hsp72) is the most highly induced and conserved in all organisms from Escherichia coli to humans (12). The mouse is known to express two inducible Hsp70s, Hsp70.1 and Hsp70.3, which are products of two nearly identical genes. Interestingly, a recent gene-expression analysis of airway epithelial cells obtained from patients breathing 100% oxygen revealed increased Hsp70, suggesting a clinical relevance (13). In terms of function, overexpression of the rat inducible 70-kDa heat stress protein in a transgenic mouse increased the resistance to ischemic heart injury (14, 15). Adenoviral-mediated Hsp70 overexpression decreased ischemic injury, as well as LPS-induced acute lung injury (16, 17). Hsp70−/− mice (targeting both Hsp70.1 and 70.3) are fertile and develop normally but exhibit increased susceptibility to cardiac and lung injury (18, 19). Despite descriptions of the protective effects of Hsp70, the signaling mechanisms that underline these protective responses are poorly defined. Hsp70 was reported to be an endogenous TLR4 ligand but solely in vitro and predominantly in immune cells (20–22).

To our knowledge, our current studies show for the first time that Hsp70 acts as a TLR4 ligand in the lung and in lung endothelial cells. We detected an interaction between Hsp70 and TLR4 in vivo and in vitro. Overexpression or exogenous administration of Hsp70 using viral-expression vectors confers significant protection against hyperoxia-induced cell death and oxidant injury, Hsp70 loses its protective effect in the absence of TLR4 or Trif, a TLR4 adapter molecule. These data indicate that Hsp70 is a functionally important TLR4 ligand during lethal oxidant injury that promotes endothelial cell survival.
Materials and Methods

Isolation of primary murine lung endothelial cells and hyperoxia exposure

Isolation of murine lung endothelial cells (MLECs) from wild-type (WT), TLR4−/−, Trif−/−, MyD88−/−, and Hsp70−/− mice, as well as hyperoxic conditions, were described previously (23).

Animal hyperoxia exposure

Adult 6–8-wk-old C57BL/6 mice were obtained from The Jackson Laboratory. TLR4−/− and Hsp70−/− mice were described previously (18, 23). Trif−/− mice were provided by Dr. Masahiro Yamamoto (Osaka University, Osaka, Japan). All of the mice were backcrossed for >10 generations onto a C57BL/6 background. WT C57BL/6 mice, bred and housed in the same facilities as the knockout mice, were used as controls. Mice were exposed to 100% O2 in a Plexiglas exposure chamber and permitted food and water ad libitum. Control mice were exposed to room air. All protocols were reviewed and approved by the Animal Care and Use Committee at Yale University.

Hsp70 constructs

We purchased pEGFP Hsp70 (plasmid 15215) and pEGFP Hsp70 K71E (plasmid 15216) from Addgene. The function of these constructs was reported previously (24).

Protein labeling

Human rHsp70 protein was purchased from Boston Biochem. We labeled Hsp70 with fluorescent dye using the Alexa Fluor 488 Protein Microscale Labeling kit (Molecular Probes), according to the manufacturer’s recommendations. Briefly, 100 μg Hsp70 was incubated with Alexa Fluor 488 in 0.1 M sodium bicarbonate for 15 min at room temperature. Unconjugated dye was removed by the spin filter provided in the kit. The number of dye molecules bound per protein molecule was determined by measuring the OD at 280 and 494 nm. Calculations predicted that six to nine Alexa Fluor molecules bind to each protein molecule.

Hsp70 detection and uptake

MLECs were incubated with various concentrations of Alexa Fluor 488–labeled Hsp70 or PBS for 1 h on ice for the binding studies. For the competition assays, MLECs were preincubated with unlabeled Hsp70 protein for 30 min on ice. Then labeled Hsp70 was added and incubated for another 1 h on ice. Subsequently, cells were washed with PBS/1% BSA and resuspended in PBS containing 1% paraformaldehyde. Throughout the assay, the temperature was kept at 4°C to minimize nonspecific endocytosis of exogenously added protein. The samples were evaluated using a FACSscan flow cytometer (BD Biosciences). Cell surface detection of fluorescent-labeled proteins was calculated using the geometric mean fluorescence value after subtracting the value for autofluorescence of the cells.

Confocal microscopy

MLECs were adjusted to a density of 1 × 10^5 cells/ml and seeded on poly-l-lysine–coated cover slips (BD Biosciences) in a volume of 200 μl (2 × 10^5 cells). After incubation for 24 h (37°C, 5% CO2), cells were treated with a 1:200 dilution of anti-TLR4 (Santa Cruz Biotechnology) or anti-Hsp70 (Stressgen) at 4°C overnight. After washing with PBS, cells were incubated with secondary Ab (Alexa Fluor 488 goat anti-rabbit IgG or Alexa Fluor 594 goat anti-mouse IgG at 1:300 dilution; Invitrogen) at room temperature for 1 h. Samples were washed three times by immersing in PBS for 5 min and then mounted with ProLong Gold mounting media with DAPI (Invitrogen). Labeled cells were visualized using a Nikon Ti-E Eclipse inverted microscope equipped with Perfect Focus (auto focus system), a motorized XY stage, and a Nano Focusing Piezo Stage. Images were prepared by Photoshop (Adobe) for the visualization.

EMSA

EMSA of nuclear protein isolated from MLECs were performed as previously described, with minor modifications (25). Nuclear extracts were prepared using an NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Thermo Scientific), according to the manufacturer’s protocol. The NF-κB site was synthesized as a complementary oligodeoxynucleotide strands. The sequence of NF-κB consensus oligonucleotides was 5’-AGT TGA GGG GAC TTT CCC AGG C-3’ (Santa Cruz Biotechnol-ogy). The DNA-binding ability of NF-κB in the nuclear extracts was assessed by EMSA with biotin-labeled, double-stranded NF-κB. EMSA was carried out using the LightShift Chemiluminescent EMSA Kit (Pierce). Specific binding was confirmed using 200-fold excess of unla-beled probe as a specific competitor. Protein–DNA complexes were separated using a 6% nondenaturing acrylamide gel electrophoresis and then transferred to positively charged nylon membranes and cross-linked by UV irradiation. Gel shifts were visualized with streptavidin HRP, according to standard protocols.

Measurement of lung injury markers

Mice were removed from the exposure chamber and killed after 72 h of hyperoxia exposure. Bronchoalveolar lavage was performed twice with mice (8 × 10^6 cells/1 ml; 1 ml saline, pH 7.4). Cells were counted and resuspended in PBS, and counted. The supernatant was used for bronchoalveolar lavage fluid (BALF) protein determination. The protein concentration in each sample was determined by the BCA Protein Assay Reagent (Thermo Scientific), using BSA as the standard.

Microarrays

Total RNA (100 ng/sample) from WT or TLR4−/− mice lungs was subjected to amplification, followed by labeling and hybridization to Mouse Genome 430 2.0 GeneChips (Affymetrix). Sample amplification, labeling, hybridization, and detection were performed by the W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University (West Cam-pus, Orange, CT).

Overexpression of Hsp70 in MLECs and mice

Adenovirus–CMV–Hsp70 (Ad-Hsp70) was purchased from Vector BioLabs. Adenovirus-control (CMV-null) (Ad-Ctrl), an adenovirus empty vector, was used as a control. For cell experiments Ad-Ctrl or Ad-Hsp70 was transfected at 2.5 multiplicity of infection (the average number of phage particles that infect a single cell) 24 h before hyperoxia exposure. For mouse experiments, intranasal administration of either Ad-Hsp70 or Ad-Ctrl was performed on mice (8 × 10^9 TUs/mouse); 2 wk later, hyperoxia exposure was initiated or mice were left in room air. Mice received only a single intranasal treatment for each experiment.

Hsp70 ELISA

Cell culture supernatant or mice BALF was assessed for Hsp70 protein levels by Hsp70 ELISA (R&D Systems).

Western blot analysis

Lung protein analyses were performed as previously described, with minor modifications (23). Whole-lung tissues were homogenized in 62.5 mM Tris buffer, and cell pellets were lysed in 1× RIPA lysis buffer (Upstate Bio-technology). The protein concentrations of the lysates were determined by BCA Protein Assay (Thermo Scientific). Samples were electrophoresed in a 12% ready-made Tris-HCl gel (Bio-Rad Laboratories) and electrophoretically transferred onto a nitrocellulose membrane. The membranes were then incubated overnight with anti-Hsp70 (Stressgen) or β-Actin Ab (Santa Cruz Biotechnology). The membranes were incubated with HRP-conjugated goat anti-rabbit IgG Ab, followed by the detection of signal with a chemiluminescence LumiGLO detection kit (Cell Signaling Tech-nology).

Cell membrane isolation

Isolation of total cellular membrane protein of MLECs was performed using a Plasma Membrane Protein Extraction Kit (BioVision).

Apoposis assays

We used FACS to detect Annexin V–FITC labeling (BD Biosciences), according to the manufacturer’s instruction. Briefly, MLECs were washed with cold PBS and resuspended with binding buffer (10 mM HEPES/NaOH [pH 7.4], 140 mM NaCl, 2.5 mM CaCl2); a solution containing 1×10^6 cells was transferred to a 5-ml tube, and 5 μl each of annexin V and propidium iodide were added. Binding buffer was then added to each tube and analyzed by FACS (BD Biosciences). The Annexin V–FITC signal was detected by FL1 (FITC detector) at 518 nm, and the propidium
iodide signal was detected by FL2 (PE fluorescence detector) at 620 nm. Mouse lung sections were subjected to TUNEL assay using the In Situ Cell Death Detection Kit (Roche Diagnostics), as described previously (26).

**Total RNA isolation, RT-PCR amplification, and real-time RT-PCR amplification**

Total RNA from lung tissue was extracted using TRizol reagent (Invitrogen), according to the manufacturer’s instructions. First-strand cDNA was synthesized using Superscript II Reverse Transcriptase (Invitrogen) with random hexamers; conditions were 10 min at 25°C, 30 min at 48°C, and 5 min at 95°C. RT-PCR was performed using PCR Master Mix (Promega). Primers used for mouse Hsp70 were sense: 5′-CGTGGAGGAAGTTCATAGCA-3′ and antisense: 5′-CTGCTACCTGGATGACCA-3′; and for mouse β-actin were sense: 5′-GTTGGCCCGCTCTAGGCA-3′ and antisense: 5′-CTCTTTTGATGCACCCAGCATTCC-3′. Conditions for PCR were 1 cycle at 95°C for 3 min; 30 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and 1 cycle at 72°C for 5 min. Each reaction product (10 μl) was then separated on a 1% agarose gel containing 0.5 μg/ml ethidium bromide. Real-time RT-PCR reactions were carried out using the Phototope-HRP Western blot Detection System kit (Roche Diagnostics), as described previously (26).

**Immunoprecipitation**

Immunoprecipitation was performed using the Catch and Release v2.0 Kit (Upstate Biotechnology), per the manufacturer’s protocol. Briefly, lung tissue lysates were incubated with anti-TLR4 (Santa Cruz Biotechnology) or anti-Hsp70 (Stressgen) Ab (rabbit IgG was used as negative control Ab) and Ab Capture Affinity Ligand for 30 min on a rotator using Catch and Release spin columns. We washed the column three times with 400 μl 1× Wash Buffer, spinning at 5000 rpm for 15–30 s for each wash. Protein bound to the beads was eluted by Denaturing Elution Buffer containing 0.5% 2-ME. Proteins were separated by 10% SDS-PAGE and then transferred to a polyvinylidene difluoride membrane. The membrane was incubated with either Hsp70 or TLR4 Ab at 1:500 dilutions. Western blot imaging was done using the Phototope-HRP Western blot Detection System kit (Cell Signaling Technology), according to the manufacturer’s instructions.

**Caspase 3 activity**

Caspase 3 activity measurement was colored with caspases assays using the CaspACE assay system (Promega). MLEC lysates were centrifuged, and the supernatants were incubated with colorimetric substrate, Ac-Asp-Glu-Val-Asp-p-nitroanilide. The release of p-nitroanilide from Ac-Asp-Glu-Val-Asp-p-nitroanilide was measured at 405 nm using a spectrophotometer.

**Statistics**

Data are expressed as mean ± SE and were analyzed using the Student t test. A significant difference was accepted at p < 0.05.

**Results**

**TLR4 deficiency leads to increased Hsp70 mRNA and protein expression during hyperoxia in vitro**

We showed previously that TLR4 deficiency confers susceptibility to hyperoxia-induced lung and endothelial cell injury (23) and that lung-targeted activation of TLR4 confers resistance to hyperoxia-induced apoptosis (5). We were interested in identifying the relevant ligand for the protective effects of TLR4. Hsp70 was shown to be protective in various conditions (16, 17) and a potential TLR4 ligand (27). Our microarray analyses of lung lysates from WT and TLR4−/− mice exposed to 72 h of hyperoxia showed markedly elevated levels of Hsp70 in TLR4−/− mice (data not shown). We confirmed our array results with Hsp70 protein levels in MLECs. WT MLECs exposed to a course of hyperoxia showed Hsp70 protein induction by 8 h, but TLR4−/− MLECs showed consistently higher Hsp70 protein induction (Fig. 1A). We believed that extracellular or secreted Hsp70 likely serves as a TLR4 ligand and, therefore, sought to determine levels of secreted Hsp70 protein in MLEC culture media. We detected increased Hsp70 protein by ELISA after hyperoxia in MLEC culture medium (Fig. 1B). To determine whether exogenous Hsp70 protein serves a cytoprotective role, we performed cell survival studies after treating WT and TLR4−/− MLECs with human rHsp70 protein; Hsp70 decreased cell death in WT cells but not in TLR4−/− cells (Fig. 1C). These data suggested that extracellular Hsp70 has an important physiologic role and that TLR4 is involved.

**Hsp70 is a TLR4 ligand during hyperoxia in vitro and in vivo**

Observations of Hsp70 as an endogenous TLR4 ligand have been made predominantly in immune cells and in vitro (e.g., monocytes, macrophages, and dendritic cells) (20–22). We sought to determine whether there were Hsp70–TLR4 interactions in MLEC membranes (Fig. 2A). We also performed coimmunoprecipitation studies to determine whether there was a physical interaction.
between TLR4 and Hsp70 in WT mice exposed to 72 h of hyperoxia. We detected Hsp70 protein using TLR4 Ab and vice versa in lung lysates (Fig. 2B). These data indicated that there was an interaction between Hsp70 and TLR4 in lung during hyperoxia in MLECs and in vivo. We determined colocalization of endogenous TLR4 and Hsp70 on the surface of WT MLECs using immunofluorescence detection of TLR4 and Hsp70 on non-permeabilized MLECs (Fig. 2C). Of note, Hsp70 and TLR4 colocalization was not detected in TLR4−/− MLECs. We also confirmed an interaction between Hsp70 and cell surface TLR4 using exogenous, rHsp70 protein labeled with Alexa Fluor 488 fluorescence dye and confocal microscopic visualization. WT and TLR4−/− MLECs were incubated either with Hsp70–Alexa Fluor 488 or PBS as a negative control (Fig. 2D). We were able to detect labeled Hsp70 in WT MLECs but not in TLR4−/− MLECs. We quantitated fluorescent Hsp70 detection on the surface of WT and TLR4−/− MLECs using flow cytometry (Fig. 2E). Cells were incubated with Hsp70–Alexa Fluor 488 (10 or 15 μg/mL) before

**FIGURE 2.** Hsp70 interacts with TLR4 in MLECs and lungs. (A) Immunoprecipitation (IP) of Hsp70 and TLR4 in WT and TLR4−/− MLEC cell membrane (CM). (B) IP of TLR4 and Hsp70 in lung lysates. (C) Colocalization of TLR4 and Hsp70 in MLECs using immunofluorescence. WT or TLR4−/− MLECs were immuno-stained for TLR4 (red) and Hsp70 (green). Nuclei were stained with DAPI (blue) and imaged by confocal microscopy. Single-immunostained images (left panels) and merged images (right panels) are shown (original magnification ×600). (D) Human rHsp70 protein was labeled with Alexa Fluor 488, incubated with WT or TLR4−/− MLECs (10 μg/mL), and imaged using laser microscopy (original magnification ×1000). (E) WT or TLR4−/− MLECs were incubated with two concentrations of Alexa Fluor 488–labeled human rHsp70 (10 and 15 μg/mL). Unlabeled Hsp70 protein (100 μg/mL) was used to “compete off” labeled Hsp70. Fluorescence intensity was measured by flow cytometry. (F) Nuclear extracts were prepared from WT and TLR4−/− MLECs, incubated with rHsp70 (10 μg/mL) for the indicated time or received no treatment (Ctrl), and mixed with a biotin-labeled oligonucleotide–containing NF-κB motif. Bound complexes were analyzed by electrophoresis. The results are representative of at least three independent experiments. (G) WT and TLR4−/− MLECs were treated with Ad-Control (C) or Ad-Hsp70 (H) and exposed to 72 h of hyperoxia. Nuclear extracts were mixed with biotin-labeled oligonucleotide–containing NF-κB motif. Competitive inhibition of NF-κB binding was performed with nonlabeled probe (original magnification ×200). Bound complexes were analyzed by electrophoresis. The results are representative of at least three independent experiments. CTRL, control; RA, room air control; RIGG, rabbit normal IgG; WCE, whole-cell extract.
cytometric detection. We also performed competition assays in which cells were preincubated with unlabeled Hsp70 protein (100 μg/ml), followed by incubation with Hsp70–Alexa Fluor 488, and then subjected to flow cytometry. Mean fluorescence values were calculated. We found a dose response, with increased Hsp70 detection at a higher dose (15 μg/ml), and found that unlabeled Hsp70 was able to decrease labeled Hsp70 detection in WT MLECs. We did not detect any exogenously added labeled Hsp70 on the surface of TLR4−/− MLECs, indicating that endogenous Hsp70 requires TLR4 expression for cell surface detection.

To confirm that extracellular Hsp70 is not only interacting with TLR4 at the cell membrane but is also signaling, we performed EMSAs using rHsp70. Hsp70 activated NF-κB in a time-dependent manner, which was abolished in TLR4−/− MLECs (Fig. 2F). Ad-Hsp70 or hyperoxia also induced NF-κB activation in MLECs (Fig. 2G). Given the previously reported concerns of rHsp70 protein and LPS contamination (28), we used virally driven Hsp70-expression constructs for subsequent studies. Next, we determined whether Hsp70 had protective effects during hyperoxia.

**Hsp70 is protective in hyperoxia**

We used Western blot and ELISA to confirm the efficacy of our Ad-Hsp70 vector in total and secreted Hsp70 protein in WT MLECs at baseline compared with cells treated with Ad-Ctrl (Supplemental Fig. 1A, 1B). We also tested for hyperoxia-induced Hsp70 with and without Ad-Hsp70 treatment. Our results show that hyperoxia induces Hsp70 secretion and mRNA in MLECs and that Ad-Hsp70 treatment augments Hsp70 expression in both WT and Hsp70−/− MLECs (Supplemental Fig. 2A, 2B). Ad-Hsp70 decreased hyperoxia-induced injury in WT MLECs, as assessed by lactate dehydrogenase (LDH) release (Fig 3A), as well as apoptosis (Fig. 3B).

**The protective effect of Hsp70 depends upon a TLR4–Trif pathway**

Ad-Hsp70 decreased hyperoxia-induced MLEC injury, as assessed by LDH release (Fig. 4A), and it decreased cell death in WT MLECs (Fig. 4B). Next, we wanted to determine whether Hsp70 was dependent upon TLR4. We found that Ad-Hsp70 lost its protective effects in TLR4−/− MLECs, indicating that Hsp70 was dependent upon TLR4 to decrease hyperoxia-induced cell injury and death. To test whether secreted Hsp70 was responsible for the observed effects of Ad-Hsp70, we treated MLECs with methyl β-cyclodextrin (MBC) (29) before administering Ad-Hsp70. We found that MBC completely blocked the ability of Ad-Hsp70 to decrease hyperoxia-induced LDH release and apoptosis in WT MLECs, leading to levels of injury and death equivalent to those of TLR4−/− MLECs (Supplemental Fig. 3A, 3B). As further evidence that secreted (or extracellular) Hsp70 conferred antiapoptotic effects, we were also able to block the effects of Ad-Hsp70 with anti-Hsp70–neutralizing Ab (Supplemental Fig. 3C).

MyD88 and Trif are the major adapter proteins for TLR4 signaling. To determine the involvement of each, we isolated MLECs from Trif−/− and MyD88−/− mouse lungs. Of note, Trif−/− MLECs exhibited increased levels of injury and cell death compared with WT MLECs (Fig. 4C, 4D), whereas MyD88−/− MLECs were not significantly different from WT MLECs (Supplemental Fig. 4). Importantly, Ad-Hsp70 lost its protective effects in Trif−/− MLECs (Fig. 4C, 4D) but not in MyD88−/− MLECs (Supplemental Fig. 4). These data indicated that Hsp70 was dependent upon Trif for its protective effects.

**Hsp70-ATPase mutant still had a protective effect in MLECs**

There is the possibility that Hsp70 acts as an extracellular chaperone for TLR4. To investigate whether the protective actions of Ad-Hsp70 decreased hyperoxia-induced MLEC injury, as assessed by LDH release (Fig 4A), and it decreased cell death in WT MLECs (Fig. 4B). Next, we wanted to determine whether Hsp70 was dependent upon TLR4. We found that Ad-Hsp70 lost its protective effects in TLR4−/− MLECs, indicating that Hsp70 was dependent upon TLR4 to decrease hyperoxia-induced cell injury and death. To test whether secreted Hsp70 was responsible for the observed effects of Ad-Hsp70, we treated MLECs with methyl β-cyclodextrin (MBC) (29) before administering Ad-Hsp70. We found that MBC completely blocked the ability of Ad-Hsp70 to decrease hyperoxia-induced LDH release and apoptosis in WT MLECs, leading to levels of injury and death equivalent to those of TLR4−/− MLECs (Supplemental Fig. 3A, 3B). As further evidence that secreted (or extracellular) Hsp70 conferred antiapoptotic effects, we were also able to block the effects of Ad-Hsp70 with anti-Hsp70–neutralizing Ab (Supplemental Fig. 3C).

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**Hsp70-ATPase mutant still had a protective effect in MLECs**

There is the possibility that Hsp70 acts as an extracellular chaperone for TLR4. To investigate whether the protective actions of
of its chaperone activities. The WT Hsp70 and mutant K71E Hsp70 constructs did not have any protective effects on TLR4−/− MLECs, confirming that Hsp70 uses TLR4 for its cytoprotective actions.

Hsp70 protects hyperoxia-induced MLEC injury and caspase 3 activation through TLR4

Our previous study showed that activation of caspase 3 plays an important role in hyperoxia-induced cell death (30). We sought to determine whether Hsp70 had the ability to inhibit caspase 3 activation and the role of TLR4, if any. We overexpressed Hsp70 using Ad-Hsp70 in WT and TLR4−/− MLECs and found that Ad-Hsp70 significantly protected MLECs from hyperoxia-induced caspase 3 activation in WT MLECs but not in TLR4−/− MLEC, suggesting that Hsp70-mediated antiapoptotic effects are mediated by TLR4 (Fig. 6).

TLR4 deficiency leads to increased Hsp70 mRNA and protein expression during hyperoxia in vivo

We confirmed increased Hsp70 expression using RT-PCR and quantitative (real-time) RT-PCR analyses in lung tissue. Lung lysates from WT mice exhibited Hsp70 mRNA induction during hyperoxia,
but this induction was markedly exaggerated in TLR4−/− mice (Fig. 7A, 7B). We also detected increased Hsp70 protein by ELISA after hyperoxia in BALF (Fig. 7C).

**Hsp70 is protective in hyperoxia in vivo**

To demonstrate the impact of Ad-Hsp70 in vivo, we treated WT and Hsp70−/− mice with intranasal, and thus, lung-targeted, Ad-Ctrl or Ad-Hsp70 prior to 72 h of hyperoxia, a time point of maximal lung injury. We confirmed that hyperoxia induces Hsp70 protein secretion in BALF and Hsp70 mRNA in lung lysates (Supplemental Fig. 2C, 2D, respectively). Intranasal Ad-Hsp70 administration markedly induced Hsp70 expression in BALF and lung lysates in WT and Hsp70−/− mice. Hyperoxia increased BALF leukocytes and protein (an indication of increased lung permeability) in WT and Hsp70−/− mice, which improved with Ad-Hsp70 treatment compared with Ad-Ctrl treatment (Fig. 8A, 8B, respectively). Mice treated with Ad-Hsp70 showed markedly less BALF LDH activity (Fig. 8C) and lung apoptosis (Fig. 8D) than did controls after 72 h of hyperoxia. Hyperoxia is a highly lethal model, and strategies that improve survival by even several hours are significant. We tested the impact of Ad-Hsp70 on survival by treating WT and Hsp70−/− mice (Fig. 8E) with Ad-Ctrl or Ad-Hsp70 prior to hyperoxia. Hsp70−/− mice were significantly more susceptible to hyperoxia than were WT mice, but they were successfully rescued with Ad-Hsp70 treatment. WT mice given Ad-Hsp70 vector achieved the highest level of protection against lethal hyperoxia, likely due to augmented Hsp70 expression above that induced by hyperoxia alone. Taken together, these studies show that Hsp70 administration in vitro and in vivo has significant protective properties against lethal hyperoxic injury.

The protective effect of Hsp70 depends upon a TLR4–Trif pathway in vivo

We previously reported that TLR4−/− mice are more susceptible to hyperoxia-induced death and are successfully rescued by adenoviral delivery of another known cytoprotective Hsp, HO1 (23). In contrast, Ad-Hsp70 did not improve the survival of TLR4−/− mice exposed to hyperoxia (Fig. 8F). These data suggested that Hsp70 had specific TLR4-dependent therapeutic effects that are distinct from those of other protective Hsps. To confirm that Trif is involved in vivo, we administered Ad-Hsp70 or Ad-Ctrl to WT and Trif−/− mice before subjecting them to 72 h of hyperoxia, a time point of maximal lung injury. We first confirmed that hyperoxia and Ad-Hsp70 induced Hsp70 in both WT and Trif−/− lungs (data not shown). We found that hyperoxia-exposed Trif−/− mice have increased BALF cell influx, protein leak, and LDH release compared with WT mice, and Ad-Hsp70 completely lost its ability to decrease lung injury in the absence of Trif (Fig. 9). Taken together, these studies indicate that Hsp70 ameliorates hyperoxic injury via Trif in vitro and in vivo.

**FIGURE 7.** Hsp70 is induced and secreted in lungs during hyperoxia. (A) Hsp70 mRNA expression in WT and TLR4−/− mice lung lysates was measured by real time (quantitative) RT-PCR after 24, 48, or 72 h of hyperoxia. (B) Hsp70 mRNA expression in lung lysates was measured by RT-PCR after 72 h of hyperoxia. (C) Hsp70 ELISA in BALF of WT mice after 72 h of hyperoxia. Data are mean ± SD. *p < 0.05 versus RA, †p < 0.05 versus corresponding WT hyperoxia (n = 5/group). RA, Room air control.

**Discussion**

A protective role for Hsp70 has been found in models of sepsis-related lung injury, such as the cecal ligation and puncture (CLP) model. Rats administered vector-driven porcine Hsp70 prior to CLP had significantly decreased lung inflammation and edema, as well as reduced mortality (17). Singleton and Wischmeyer (19) subjected Hsp70-knockout mice to CLP and found prolonged NF-kB activation, increased inflammatory cytokine release, and injury in lung tissue that was associated with increased mortality. Hsp70 was also implicated in glutamine-mediated attenuation of lung injury during sepsis and in hypoxia-induced preconditioning to combat high altitude–associated lung injury in rats (31, 32). To our knowledge, our studies show for the first time that Hsp70 is a critical protective response in sterile, oxidant-induced lung injury incurred during hyperoxia exposure.

Our current studies also build upon previous reports of Hsp70-associated cytoprotection by elucidating the signaling mechanisms. Specifically, we identified Hsp70 as a functionally important TLR4 ligand in vitro and in vivo. We previously reported that TLR4 signaling is necessary to prevent hyperoxia-induced lung structural cell injury and mortality (23). Prior to our report, TLR4 deficiency was thought to be generally protective against noninfectious challenges, such as endotoxin, ischemia-reperfusion, and ozone (33–35). We confirmed the central role of TLR4 in maintaining lung structural integrity in a chronic model when TLR4 knockout mice were allowed to age. We detected increased levels of lung and systemic oxidants in TLR4-knockout lungs and circulation, eventually resulting in age-related, spontaneous emphysema (36). However, the relevant TLR4 ligand remained elusive. We explored the role of endogenous and damage-associated TLR ligands.

Multiple endogenous TLR2 or TLR4 ligands have been described. These ligands range from damage-associated molecular pattern (DAMP) molecules and matrix turn over to inflammatory mediators and lipids. Of the endogenous TLR ligands described, five are intracellular, six are matrix components, four are modified lipids or lipoproteins, and eight fall into other categories (37). DAMPs generated from injured cells and damaged matrix can activate TLR4 signaling in the absence of microbial-derived molecules (38). Less is known about endogenous TLR4 ligands in the lung. In premetastatic lungs, S100A8 and serum amyloid A were described as endogenous TLR4 ligands (32). In hemorrhagic
shock-resuscitation injury, oxidant-induced neutrophil activation served an important signaling function in mediating alveolar macrophage priming and lung inflammation. The endogenous TLR4 ligand, HMGB1, was thought to mediate neutrophil oxidant activation in the lung, leading to inflammation and tissue injury (39).

Hsps are endogenous DAMPs that are released by cells during stress and injury. For example, Hsp60 and Hsp70 are thought to regulate bacteria-induced inflammation when released (40). Extracellular Hsp70 was shown to be released by virally infected lung airway epithelial cells and to activate neutrophils via TLR4 (41). Hsp70 was shown to be released and biologically active in human and mouse BALF and regulate airway epithelial cell cytokine expression in a TLR4- and NF-κB-dependent manner (42). In the heart, cardiomyocytes secrete Hsp70 and mediate the expression of cardiodepressant cytokines via a TLR4-dependent mechanism (43). Ischemia-reperfusion injury of the liver led to increased Hsp70 in the circulation, which signaled via TLR2 and TLR4 on hepatocytes, with subsequent NF-κB activation and MIP-2 induction (44). To the best of our knowledge, Hsp70–TLR4 interactions in the lung or in vivo have not been explored, and we show for the first time a TLR4–Hsp70 protective pathway both in lungs and endothelial cells.

Given reports that Hsp70 is a stress-response gene that can act as a “marker” for the degree of injury (45), we theorized that the exaggerated levels of Hsp70 induction during hyperoxia in TLR4−/− mice were due to the increased oxidant stress found in the setting of TLR4 deficiency, as we described previously (36). In addition, we hypothesized that, despite elevated levels of Hsp70, a putative TLR4 ligand, there is inadequate Hsp70-protective

**FIGURE 8.** Hsp70 protects against lethal hyperoxia and is TLR4 dependent. WT and Hsp70−/− mice were treated with Ad-Ctrl or Ad-Hsp70 and exposed to 72 h of hyperoxia. (A) Lung inflammation was detected by BALF cell counts. (B) Lung permeability was assessed by BALF protein content. (C) Lung injury was measured by LDH activity in BALF. (D) Lung cell death was assessed by TUNEL staining on lung sections, and the number of TUNEL+ cells was quantitated and expressed as a percentage of the total number of lung cells counted on each section. Data are mean ± SD. *p < 0.05 versus RA Ad-Ctrl WT mice, **p < 0.05 versus hyperoxia Ad-Ctrl WT mice, ***p < 0.05 versus corresponding Ad-Ctrl mice (n = 5/group). WT and Hsp70−/− mice (E) or WT and TLR4−/− mice (F) were treated with intranasal Ad-Ctrl or Ad-Hsp70 and exposed to hyperoxia. Survival proportions were compared among the four groups. *p < 0.05 versus WT Ad-Ctrl mice, **p < 0.05 versus WT Ad-Hsp70 mice, ***p < 0.05 versus Hsp70−/− Ad-Ctrl mice [n = 10/group in (E); n = 5–6/group in (F)]. RA, Room air control.
signaling in the absence of TLR4. We confirmed this by showing that exogenous Hsp70 protein or Ad-Hsp70 can delay apoptosis and prolong survival of WT and Hsp70-deficient cells and mice. Furthermore, the dependence of Hsp70 on TLR4 seems specific to Hsp70. Adenoviral overexpression of another well-known Hsp, HO1, had protective effects even in TLR4/−/− mice (23). In general, we found that the impact of silencing Hsps may be more dramatic than overexpression, depending on the specific protein. This may be a function of protein distribution, kinetics, level of induction achieved by intranasal adenoviral administration, the fact that hyperoxia is so toxic (100% lethal), or because there is a threshold effect of specific heat shock overexpression in lungs.

We have delineated an Hsp70–TLR4 signaling pathway that serves important cytoprotective functions using both in vitro and in vivo models of oxidant injury. Cultured lung endothelial cells and mice exposed to hyperoxia exhibit significant injury and, ultimately, death. Lung endothelial cell injury and death are critical features of hyperoxia-induced lung failure and eventual demise. We found that Hsp70 is not only induced during hyperoxia exposure, it is also necessary to protect against excessive oxidant generation and caspase 3–mediated cell death in endothelial cells and lungs. Hsp70-deficient mice or endothelial cells exhibited increased injury, oxidant generation (as measured by lipid peroxidation), and death. Using adenoviral Hsp70 overexpression constructs, we were able to rescue Hsp70-deficient mice and cells from hyperoxia-induced mortality and cell death. Even WT mice, which retain the ability to induce endogenous Hsp70 during hyperoxia challenge, demonstrated prolonged survival when Hsp70 is overexpressed compared with mice given control vector. Binding to the coxsackievirus and adenovirus receptor mediates adenovirus endocytosis (46). Unlike epithelial cells, MLECs lack the coxsackievirus and adenovirus receptor. Lung epithelial cells have higher efficiency of infection with adenovirus (47) and likely play a major role in Ad-Hsp70–mediated protection. We chose to focus on MLECs based on our previous hyperoxia studies in which MLEC signaling is important; however, we recognize that epithelium, as a source or target of Hsp70, is likely involved as well.

To our knowledge, we describe for the first time increased baseline levels of Hsp70 mRNA and protein expression in the lungs and endothelial cells of TLR4-knockout mice. This indicated that TLR4 is not necessary for basal Hsp70 expression and that the increased Hsp70 expression observed in TLR4-deficient cells and animals is likely a stress response. As we showed previously, TLR4-deficient lungs and mice have increased levels of oxidant stress, even in an unchallenged state, due to increased NADPH oxidase activity, leading to systemic stress and, ultimately, lung destruction (36). This is distinct from that found in the colon, where TLR4 is known to be required for Hsp70 induction (48). We used loss-of-function and gain-of-function approaches to confirm the specific roles of Hsp70 in hyperoxic lung injury and TLR4-dependent mechanisms. Hsp70 expression

**FIGURE 9.** Hsp70-mediated protection is Trif dependent in mice. WT or Trif−/− mice were treated with Ad-Ctrl or Ad-Hsp70 and exposed to 72 h of hyperoxia. (A) Lung inflammation was detected by BALF cell counts. (B) Lung permeability was assessed by BALF protein content. (C) Lung injury was assessed by LDH activity in BALF. (D) Hsp70 ELISA in BALF. (E) Lung cell death was assessed by TUNEL staining on lung sections, and the number of TUNEL+ cells was quantitated and expressed as a percentage of the total number of lung cells counted on each section. Data are mean ± SD. (F) Summary of Hsp70–TLR4 signaling during hyperoxia. We postulate that endothelial TLR4 is required for an adaptive mechanism that delays hyperoxic apoptosis and prolongs survival. Hyperoxia induces Hsp70 secretion from MLECs (white circle labeled Hsp70) or other cell types (gray circle labeled Hsp70). Extracellular Hsp70 interacts with TLR4 and transduces signal through the Trif–NF-κB pathway. Induced expression of Bcl-2 and decreased activation of caspase 3 inhibit cell apoptosis and prolong survival. *p < 0.01 versus RA WT Ad-Ctrl mice, **p < 0.01 versus corresponding Ad-Ctrl mice, †p < 0.01 versus corresponding WT mice (n = 5/group). RA, Room air control.
and induction are robust in both WT and TLR4-knockout lung tissue and endothelial cells during hyperoxia, but there is a complete lack of Hsp70-mediated cytoprotection in the absence of TLR4.

In the current study, we found that rHsp70 itself has protective effects and was dependent on TLR4. Hsp70 appears to be an extracellular ligand of TLR4, although the chaperone function of Hsp70 is also an important consideration. Hyperoxia induced Hsp70 secretion and interaction with TLR4. Given that Hsp70 is released by a nonclassical protein-transport pathway and that intact surface membrane lipid rafts are required for efficient stress-induced Hsp70 release, we used MBC to test the role of secreted Hsp70 (49). MBC and soluble Hsp70 Ab abolished the protective effects of Hsp70, which implicated extracellular Hsp70, rather than intracellular Hsp70, in our model. Extracellular Hsp70, known as a “chaperokine,” was reported to be a danger signal produced and released when cells are under stress and as activators of the immune system (49). Our data show an alternative, protective role for extracellular Hsp70.

Hsp70’s best-known function is as a molecular chaperone: it selectively binds to denatured or partially unfolded domains in polypeptides. Hsp70s are weak ATPases, and they cycle through low- and high-affinity substrate-binding states driven by nucleotide hydrolysis: ATP-bound Hsp70 binds substrates with low affinity, and ADP-bound and nucleotide-free Hsp70 exhibits tight binding to substrate (50). The Hsp70 K71 mutation abolishes the ATPase function and loses affinity to substrate (24, 51). We used Hsp70 WT and Hsp70 K71E constructs to show that the adaptive functions of Hsp70 were independent of its chaperone activities.

Although we demonstrate that Hsp70 binding to MLEC’s requires the expression of TLR4, we did not specifically address the role of coreceptors or binding proteins. The mechanism of TLR4 activation is quite complex and (unlike other TLRs) involves several auxiliary proteins (LPS-binding protein, CD14), as well as a coreceptor (MD-2) (52). TLR4 ligands, such as LPS, HMGB1, hyaluronan, and biglycan, were shown to require CD14 and LPS-binding proteins (31, 53); Hsp70 likely has similar requirements. Both Hsp70 and Hsp90 were reported to associate with TLR4 on the cell surface after bacterial stimulation and are thought to serve as endogenous regulators of the innate immune response (53, 54). Our coimmunoprecipitation and coimmunofluorescence studies demonstrate binding and proximity between TLR4 and Hsp70; however, additional studies, such as fluorescence resonance energy transfer, chromatography, and specific targeting of coreceptors, would be necessary to confirm the role of TLR4-associated complexes.

Moreover, we found that Hsp70-dependent TLR4 signaling requires the TLR4 adapter Trif but not the well-characterized MyD88 adapter pathway. TLR4 is the TLR known to activate two distinct signaling pathways in the production of proinflammatory cytokines or type I IFNs. MyD88 and TIRAP/Mal are essential for both (55). Little is known about Trif-mediated TLR4 signal transduction in response to ligands other than LPS. Imai et al. (56) reported that avian flu-induced lung injury is triggered by the signaling of oxidized phospholipids through TLR4 and Trif in lung macrophages. Similarly, oxidized phospholipid, OxPAPC, promoted avian flu-induced lung injury and inflammation by activating lung macrophages via TLR4–Trif (57). Notably, both of these reports which show that TLR4–Trif activation promotes lung injury, unlike our findings that Hsp70–TLR4–Trif is lung protective, are viral infection models, whereas hyperoxia is a nonmicrobial lung challenge. As we reported previously, TLR4 has a distinct role in microbial and nonmicrobial challenges of the lungs. In addition, the type of infection also determines the role that TLR4–Trif activation plays. For example, rapid Trif-dependent TLR4 activation provides important host immunity against Gram-negative bacteria (58). There appears to be tissue specificity as well. In liver Kupffer cells, MyD88-independent TLR4–Trif signaling promoted alcoholic steatohepatitis, whereas in liver stellate cells, TLR4 activation promoted fibrosis (59).

Despite these reports of a generally injurious, proinflammatory TLR4–Trif pathway, emerging evidence suggests the presence of a protective role as well. LPS preconditioning provides neuroprotection against subsequent cerebral ischemic injury through activation of TLR4, and investigators invoked a neuroprotective capacity of Trif/IRF3 signaling (60). Trif was found to contribute to oitis media pathogenesis, as well as recovery (61). Similarly, targeting a Trif/IRF3 pathway ameliorated liver dysfunction associated with chronic EtOH (62). We previously performed survival studies of Trif−/− mice and found that they are more susceptible to hyperoxia compared with WT mice (data not shown).

We summarized our proposed Hsp70–TLR4–Trif signaling in MLECs and lungs during hyperoxia (Fig. 9F). To our knowledge, we show for the first time that Hsp70 in lung endothelial cells activates a Trif-dependent pathway to promote antiapoptotic and anti-inflammatory responses. The distinct results in TLR4–Trif activation likely are a function of the type and degree of injury, as well as the specific cells and tissues involved.

In conclusion, we delineated a novel Hsp70–TLR4–Trif therapeutic axis against lethal oxidant lung injury. Both in vivo and in vitro studies using gain-of-function and loss-of-function approaches demonstrate the ability of Hsp70 to ameliorate injury and death via TLR4 and Trif. Hsp70 activates NF-kB via TLR4 and significantly modulates cell death, as well as inflammatory cell recruitment and oxidative damage, which are critical to the pathogenesis of oxidant-induced respiratory failure. By demonstrating the pivotal roles for Hsp70 and TLR4 signaling during nonmicrobial challenges, we expanded our understanding of the critical, noninfectious roles that these innate immune molecules play in the lung and provide potentially new therapeutic targets against a range of lung diseases.

Acknowledgments

We thank Dr. Evan Eisenberg (Laboratory of Cell Biology, National Heart, Lung, and Blood Institute, National Institutes of Health) for the Hsp70 K71E mutant construct.

Disclosures

The authors have no financial conflicts of interest.

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