Heat Shock Factor 1 Protects against Lung *Mycoplasma pneumoniae* Infection in Mice

Fabienne Gally Maisha N. Minor Sean K. Smith Stephanie R. Case
Hong Wei Chu

Department of Medicine, National Jewish Health, Denver, Colo., USA

Key Words

Heat shock factor 1 · Heat shock protein 70 · Toll-like receptor 2 · Macrophages

Abstract

Heat shock factor 1 (HSF1) is a transcriptional factor that controls the induction of heat shock proteins (e.g. HSP70) in response to stress. Bacterial infections contribute to the pathobiology of chronic lung diseases such as chronic obstructive pulmonary disease and asthma. Whether HSF1 is critical to lung bacterial infection remains unknown. This study is aimed at investigating the impact of HSF1 deficiency on lung *Mycoplasma pneumoniae* (Mp) infection and elucidating the underlying molecular mechanisms, such as Toll-like receptor 2 (TLR2) signaling. HSF1^{-/-} and HSF1^{+/+} mice were intranasally infected with Mp or saline and sacrificed 4, 24 and 72 h after treatment. HSF1^{-/-} mice had a higher lung Mp load than HSF1^{+/+} mice. Mp-induced lung TLR2, nuclear factor- κ B and associated inflammation [e.g. keratinocyte-derived chemokine (KC), neutrophils and histopathology] were delayed in HSF1^{-/-} mice as compared to HSF1^{+/+} mice. HSP70 protein levels in bronchoalveolar lavage fluid of HSF1^{-/-} mice were decreased. Furthermore, in response to Mp infection, HSF1^{-/-} alveolar macrophages had less TLR2 mRNA expression and KC production than HSF1^{+/+} counterparts. Nuclear factor- κ B activity and KC production in HSF1^{-/-} macrophages

could be rescued by addition of exogenous HSP70 protein. These data suggest that HSF1 is necessary to initiate host defense against bacterial infection partly through promoting early TLR2 signaling activation.

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Introduction

The heat shock factor 1 (HSF1) is a major transcription factor that controls the rapid induction of heat shock proteins (HSPs), in particular HSP70, in response to various environmental stressors, including pathogens [1, 2], stress, elevated temperature, heavy metals, or protein misfolding [3]. HSF1 can also regulate the expression of various genes such as cytokines. For instance, HSF1 binding to the interleukin (IL)-6 promoter is necessary for its maximal induction by lipopolysaccharide (LPS) stimulation in mouse embryo fibroblasts and peritoneal macrophages [4]. Furthermore, HSF1-deficient (HSF1^{-/-}) mice have reduced IL-6 and CCL5 production from peritoneal macrophages stimulated with LPS and interferon- γ [5]. Interestingly, HSF1 was shown to enhance *Caenorhabditis elegans* defense against *Pseudomonas aeruginosa* infection [6]. So far, little is known about the role of HSF1 in lung innate immunity against respiratory bacterial infection.

Bacterial infections including *Mycoplasma pneumoniae* (Mp) contribute to the pathobiology of chronic lung diseases such as chronic obstructive pulmonary disease (COPD) and asthma [7, 8]. However, the mechanisms responsible for increased host susceptibility to bacterial infection remain unclear. Toll-like receptors (TLRs) are pattern recognition receptors expressed on many cell types, including alveolar macrophages. TLRs recognize invading pathogens and initiate inflammatory responses by activating intracellular signaling pathways that include nuclear factor (NF)- κ B and mitogen-activated protein kinases [9]. On the other hand, excessive TLR stimulation may induce inflammatory diseases and autoimmunity [10]. Thus, the host immune system needs to balance activation and resolution of the immune response. This can be achieved by tightly regulating the expression and activation levels of TLR signaling molecules [11]. For instance, our group has previously shown that intact TLR2 signaling is critical for host defense cytokine production and Mp clearance [12]. Given the important role that HSF1 plays in mediating a variety of cellular responses, such as embryonic development and anti-inflammatory responses [13], it is likely that HSF1 may be critically involved in the regulation of TLR expression.

HSP70 engagement of TLR2 signaling is now recognized as a major route of activation of dendritic cells and other antigen-presenting cells [14, 15], and this mechanism is particularly effective for the activation of NF- κ B activity and the subsequent production of host defense cytokines [16]. HSP70 has been found to be reduced in the airway smooth muscle cells from COPD patients and rats exposed to long-term cigarette smoke [17]. However, HSP70 expression on airway epithelial cells and alveolar macrophages [18] from patients with asthma increased as compared to normal subjects. The role of HSP70 in COPD or asthma pathogenesis remains undefined. Likewise, the expression of HSF1 in any human lung diseases has not been investigated yet. Therefore, it is necessary to clarify the *in vivo* role of the HSF1/HSP70 axis in lung diseases such as bacterial infection.

In the present study, for the first time, we provide evidence that HSF1 acts as a positive regulator for TLR2 signaling by Mp via HSP70-dependant mechanism. We showed that HSF1^{-/-} mice had increased lung Mp load at all time points examined (4, 24 and 72 h after infection), but decreased host immune response as compared to HSF1^{+/+} mice during the initiation phase of Mp infection. This decreased immune response was associated with delayed TLR2 activation and reduced HSP70 pro-

tein levels in the bronchoalveolar lavage (BAL) fluid. Furthermore, HSF1^{-/-} macrophages produced less host defense cytokines (e.g. keratinocyte-derived chemokine, KC) in response to Mp infection, which could be rescued by the addition of exogenous HSP70 protein. Thus, our study provides new insights into the role of HSF1 in positively regulating host defense response by tightly controlling the expression level of TLR2 during bacterial infection.

Materials and Methods

Mp Infection in Mice

HSF1^{-/-} mice and littermate controls, obtained from Dr. Ivor Benjamin [19], on a Balb/c background (8–12 weeks old) were bred in our Biological Resources Center. We confirmed the absence of HSF1 protein in HSF1^{-/-} mice by Western blot (online suppl. fig. S1, www.karger.com/doi/10.1159/000333089). All experimental animals used in this study were covered under protocols approved by the Institutional Animal Care and Use Committee of National Jewish Health.

Mice were anesthetized by intraperitoneal injection of avertin (0.25 g/kg of body weight) and then inoculated intranasally with 50 μ l Mp (ATCC FH strain) at 1×10^8 CFU or 50 μ l saline. Four, 24 and 72 h later, mice were sacrificed to examine BAL cell profiles, chemokine KC levels, NF- κ B activity, lung Mp load, TLR2 and HSP70 expression, and lung tissue histopathology.

BAL and Lung Tissue Processing

Mice were euthanized by intraperitoneal injection of pentobarbital sodium 200 mg/kg and tracheotomized. The lungs were lavaged once with 1 ml phosphate-buffered saline (PBS). Cell-free BAL fluid was stored at -80°C for cytokine measurements and HSP70 Western blot. BAL cell cytopins were stained with the Diff-Quick Stain Kit (IMEB, Inc., San Marcos, Calif., USA) for cell differential counts. The left lung lobe was homogenized in SP-4 broth to perform Mp culture as previously described [20]. Right lung lobes were used for histology, RNA extraction and Western blot.

ELISA Procedure

KC was measured using the mouse KC Duo-Set Immunoassay (R&D Systems, Minneapolis, Minn., USA) following the manufacturer's instructions.

Lung Histopathology

Lungs were fixed in 10% phosphate-buffered formalin, dehydrated, embedded in paraffin, and cut at 4 μ m thickness. H&E-stained lung sections were evaluated in a double-blinded fashion under the light microscope using a histopathologic inflammatory scoring system as described previously in a hamster Mp infection model [21] and in mouse models of Mp infection [22–24]. A final score per mouse on a scale of 0–26 (least to most severe) was obtained based on an assessment of the quantity and quality of peribronchiolar and peribronchial inflammatory infiltrates, luminal exudates, perivascular infiltrates and parenchymal pneumonia.

Alveolar Macrophage Cultures

After the initial lavage, lungs were lavaged three more times with 1 ml PBS. Lavaged cells from the same group of mice were pooled. To test if mouse recombinant HSP70 protein (low endotoxin ADI-ESP-502, Enzo Life Sciences, Plymouth Meeting, Pa., USA) restores KC production in HSF1^{-/-} alveolar macrophages, BAL cells were plated at 3×10^5 cells/ml in 96-well plates and incubated at 37°C, 5% CO₂. After 2 h, non-adherent cells were washed, and adherent cells (alveolar macrophages) were stimulated with HSP70 protein or Mp (10 CFU/cell), or the combination of both for 24 h. We used 0.5 µg/ml of recombinant HSP70 protein, which is within physiological levels measured in human and sheep lungs [25, 26].

Bone Marrow-Derived Macrophage Cultures

Monolayers of mouse bone marrow-derived macrophages (BMDM) were prepared as previously described [27]. Briefly, bone marrow cells from the tibias, femurs and pelvises of HSF1^{-/-} and HSF1^{+/-} mice were flushed with and grown in DMEM supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10% (v/v) FBS, and 10% (v/v) L929 cell-conditioned medium, as a source of macrophage colony-stimulating factor. The bone marrow cells were seeded in 6-well plates at 2×10^6 cells/well with 4 ml of medium/well for NF-κB activity assay or in 48-well plates at 0.25×10^6 cells/well with 0.5 ml of medium/well for KC production assay and cultured at 37°C under a 10% CO₂ atmosphere for 5 days. Fresh media were added on day 5. The cells were then treated with media containing 10 µM helenalin (Enzo Life Sciences, Inc., Farmingdale, N.Y., USA) for 1 h and infected with Mp (10 CFU/cell) in the presence of BSA (protein control) or recombinant HSP70 protein (0.5 µg/ml) for the indicated time points.

NF-κB Activity Assay

Lung tissues were homogenized and BMDM lysed in nuclear protein extraction buffer to extract nuclear proteins following the manufacturer's instructions (Active Motif, Carlsbad, Calif., USA). Nuclear proteins (20 µg per sample) were used to perform NF-κB p65 ELISA (Active Motif) to measure NF-κB p65 activation levels as previously reported [28]. Briefly, multiple copies of NF-κB-specific double-stranded oligonucleotide were immobilized to a 96-stripwell plate. Cell nuclear extracts (protein) were then added to the well where activated NF-κB bound specifically to the oligonucleotide at its consensus binding site. Thereafter, a primary antibody specific to the activated form of NF-κB p65 was added, followed by incubation with a horseradish peroxidase-conjugated secondary antibody and then a developing solution to provide an easily quantified, sensitive colorimetric readout (e.g. optical density). So, by using this approach, we are measuring not only NF-κB p65 nuclear translocation (since we used nuclear extract) but also its DNA-binding level.

Western Blot Analysis of Mouse TLR2 and HSP70 Proteins

Cytoplasmic extracts of lung tissues and BAL fluid were utilized to quantify TLR2 and HSP70 proteins, respectively. Briefly, 60 µg of proteins per lung sample or 30 µl of BAL fluid were electrophoresed on 10% SDS-PAGE, transferred onto nitrocellulose membrane, blocked with the Western blocking buffer, and then incubated with a rabbit anti-TLR2 antibody (H-175 sc-10739, Santa Cruz Biotechnologies, Santa Cruz, Calif., USA) or a mouse

anti-HSP70 antibody (Enzo Life Sciences, Plymouth Meeting) overnight at 4°C. After washes in PBS with 0.1% Tween-20, the membranes were incubated with anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase for TLR2 and HSP70 protein detection. Membranes were stripped and probed with a mouse anti-GAPDH antibody (6C5 sc-32233, Santa Cruz Biotechnologies). Densitometry was performed to quantify proteins of interest.

Real-Time Reverse Transcriptase PCR

Real-time reverse transcriptase PCR (RT-PCR) was performed as previously described [20]. Briefly, total RNA of lung tissue was extracted using TRIzol reagent (Invitrogen, Carlsbad, Calif., USA) and treated with DNase I (Ambion, Austin, Tex., USA). Reverse transcription was performed using 1 µg of total RNA and random hexamers in a 50-µl reaction, according to the manufacturer's instructions (Applied Biosystems, Foster City, Calif., USA). 18S rRNA was used as the housekeeping gene. The threshold cycle was recorded for each sample to reflect the mRNA expression levels. The comparative threshold cycle method was used to demonstrate the relative expression level of the gene of interest.

Statistical Analysis

Normally distributed data are presented as the means $\pm$ SEM. One-way analysis of variance was used for multiple comparisons, and Tukey's post hoc test was applied where appropriate. Student's t test was used when only two groups were compared. Non-parametric (non-normally distributed) data are expressed as means and compared using the Mann-Whitney test between the two groups. A p value <0.05 was considered statistically significant.

Results

HSF1 Deficiency Increased Mouse Lung Mp Load

We first tested whether in vivo HSF1 deficiency compromises lung bacterial clearance. Lung Mp levels were examined at 4, 24 and 72 h after infection. As shown in figure 1a, Mp levels were significantly higher in HSF1^{-/-} mouse lungs than in HSF1^{+/-} littermate controls at all time points examined. This piece of data demonstrates that HSF1 contributes substantially to host protection from Mp infection. In either strain of mice, Mp levels were similar at 4 and 24 h, but higher than those at 72 h, indicating that both HSF1^{-/-} and HSF1^{+/-} mice were starting to clear Mp by 72 h.

HSF1 Deficiency Delayed Lung Innate Immune Responses to Mp Infection

To examine whether the increased susceptibility of HSF1^{-/-} mice to Mp infection was associated with inadequate innate immune responses to invading bacteria, we examined lung leukocyte profiles and cytokine levels. In both Mp-infected HSF1^{-/-} and HSF1^{+/-} mice, BAL neutrophil numbers were increased at all time points follow-

Fig. 1. HSF1^{-/-} mice are more susceptible to Mp infection and present delayed innate immune responses. **a** Mp loads in HSF1^{-/-} or HSF1^{+/+} mouse lung tissues were quantified 4, 24 and 72 h after infection by culture onto PPLO plates (Remel, Lenexa, Kans., USA) for 7 days at 37°C, 5% CO₂. **b** Neutrophil numbers in BAL of Mp-infected HSF1^{-/-} or HSF1^{+/+} mice 4, 24 and 72 h after infection.

c KC protein level in BAL fluid of Mp-infected HSF1^{-/-} or HSF1^{+/+} mice 4, 24 and 72 h after infection. **d** Lung tissue histopathology score of Mp-infected HSF1^{-/-} or HSF1^{+/+} mice. Data are representative of at least two independent experiments for each time point. n = 4–6 mice/group. * p < 0.05, HSF1^{+/+} Mp versus HSF1^{-/-} Mp.

ing infection, as compared to saline-treated mice. Mp-infected HSF1^{-/-} mice had significantly less neutrophils after 4 h of infection than HSF1^{+/+} mice. Neutrophil numbers were similar between Mp-infected HSF1^{-/-} and HSF1^{+/+} mice 24 h after infection. However, 72 h after infection, HSF1^{-/-} mice had a higher number of neutrophils than HSF1^{+/+} mice (fig. 1b). Saline-treated HSF1^{-/-} and HSF1^{+/+} mice had similar BAL neutrophils.

Interestingly, the neutrophil chemokine KC was reduced in Mp-infected HSF1^{-/-} mice as compared to HSF1^{+/+} mice at 4 and 24 h after infection, but elevated at 72 h (fig. 1c). KC levels in all saline-treated HSF1^{-/-} and HSF1^{+/+} mice were low (<100 pg/ml) and similar.

To evaluate lung tissue inflammation, we determined the histopathology score. At 4 and 24 h after infection, Mp-infected HSF1^{-/-} and HSF1^{+/+} mice showed an increase in histopathology score versus saline control mice. Although the histology score of Mp-infected HSF1^{-/-} mice at 4 h after infection was lower than that of Mp-infected HSF1^{+/+} mice, it did not reach the statistical difference. However, HSF1^{-/-} mice exhibited greater perivascular leukocyte infiltration and parenchymal pneu-

monia 72 h after infection (fig. 1d). Both saline-treated HSF1^{-/-} and HSF1^{+/+} mice did not show any inflammatory response.

Taken together, these observations indicate that HSF1 deficiency may impair early lung innate immune responses to Mp infection, thus allowing persistence of the pathogen.

TLR2 Signaling Activation in HSF1^{-/-} Mouse Lungs Was Impaired in the Early Phase of Mp Infection

TLR2 signaling has been shown to be critical in inducing neutrophil activation in response to various bacteria [29]. Furthermore, our group has previously shown that Mp infection in mice increases TLR2 expression and NF-κB activation [30]. To understand the mechanism of HSF1-associated neutrophil recruitment, we sought to investigate whether HSF1 deficiency impaired TLR2 signaling following Mp infection. Indeed, lung TLR2 protein levels were lower in HSF1^{-/-} mice at 4 and 24 h after infection, but higher at 72 h after infection than those in HSF1^{+/+} mice (fig. 2a, b). Lung TLR2 mRNA expression followed the same pattern as TLR2 protein (fig. 2c). TLR2

Fig. 2. Mp-induced TLR2 expression is delayed in HSF1^{-/-} mice. **a** Mp-infected HSF1^{-/-} mice demonstrated less TLR2 protein in lung tissues 4 and 24 h after infection than HSF1^{+/+} mice. The TLR2 protein level was examined using Western blot, quantified using densitometry, and normalized to GAPDH protein. **b** Representative Western blot showing TLR2 protein in Mp-infected HSF1^{-/-} and HSF1^{+/+} lung tissues 4, 24 and 72 h after infection. **c** TLR2 mRNA levels were examined by qRT-PCR in Mp-infected

ed HSF1^{-/-} and HSF1^{+/+} lung tissues 4, 24 and 72 h after infection. Data were normalized to HSF1^{+/+} saline-treated (control) mouse lung tissues. **d** NF-κB p65 nuclear activation was measured by ELISA in Mp-infected HSF1^{-/-} and HSF1^{+/+} lung tissues 4, 24 and 72 h after infection. Data are representative of at least two independent experiments for each time point. n = 4–6 mice/group. * p < 0.05, HSF1^{+/+} Mp versus HSF1^{-/-} Mp.

protein and mRNA in the lungs of saline-treated HSF1^{-/-} and HSF1^{+/+} mice were similar, but significantly lower than their Mp-infected counterparts.

To evaluate the downstream events of TLR2 signaling, we analyzed NF-κB p65 activity in nuclear protein of mouse lung tissues. NF-κB p65 activity was decreased in Mp-infected HSF1^{-/-} lung tissues 4 and 24 h after Mp infection, but was increased after 72 h (fig. 2d), as compared to HSF1^{+/+} mouse lung tissues. Those findings are in accordance with TLR2 expression in the lung and neutrophils in the BAL fluid.

HSP70 Protein Is Reduced in HSF1^{-/-} Mouse BAL Fluid following Mp Infection

Given that HSF1 controls the production of HSPs, in particular HSP70, and that bacterial infection has been shown to increase HSF1 activation [1], we examined the levels of HSP70 mRNA and HSP70 protein in Mp-infected mouse lung tissue and BAL fluid, respectively. In Mp-infected HSF1^{+/+} mouse lung tissues, HSP70 mRNA expression was significantly increased 24 h after Mp infection versus saline treatment, and returned to baseline

levels 72 h later. HSP70 mRNA expression was barely detectable in saline-treated or Mp-infected HSF1^{-/-} mouse lung tissue (fig. 3a).

HSP70 protein was significantly elevated in BAL fluid of Mp-infected HSF1^{+/+} mice at all time points compared to saline-treated HSF1^{+/+} mice. Although Mp infection increased HSP70 protein levels in the BAL fluid of HSF1^{-/-} mice over time, this change was not statistically significant (p > 0.05) (fig. 3b, c). HSP70 protein in BAL fluid of saline-treated HSF1^{-/-} and HSF1^{+/+} mice was significantly lower than their Mp-infected counterparts (fig. 3c).

Exogenous HSP70 Protein Restored NF-κB Activity and KC Production in HSF1-Deficient Macrophages

One of the primary target cells involved in Mp infection is the alveolar macrophage as it utilizes various mechanisms (e.g. KC production) to eliminate the invading pathogen [31, 32]. Since KC is not only a downstream effector of TLR2 signaling, but also one of the major neutrophil chemokine, and as neutrophil numbers were reduced 4 h after Mp infection in HSF1^{-/-} mouse BAL fluid,

Fig. 3. HSP70 expression is reduced in Mp-infected HSF1^{-/-} mouse BAL fluid. **a** HSP70 mRNA relative levels were examined by qRT-PCR in saline-treated and Mp-infected HSF1^{-/-} and HSF1^{+/+} lung tissues 4, 24 and 72 h after saline or infection. **b** HSP70 protein was detected by Western blot in BAL fluid (30 μ l/lane) of Mp-infected HSF1^{-/-} and HSF1^{+/+} mice 4, 24 and 72 h after infection. **c** Mp-infected HSF1^{-/-} mice demonstrated less HSP70 protein in BAL fluid than HSF1^{+/+} mice. The HSP70 protein level was quantified using densitometry and normalized to the total protein concentration present in each sample. Data are representative of at least two independent experiments for each time point. $n = 4-6$ mice/group. * $p < 0.05$, HSF1^{+/+} Mp versus HSF1^{-/-} Mp.

we tested if ex vivo HSF1^{-/-} and HSF1^{+/+} alveolar macrophages differed in their production of TLR2 mRNA and KC protein in response to Mp infection. As shown in figure 4a, Mp infection did not increase TLR2 mRNA expression in HSF1^{-/-} alveolar macrophages. On the contrary, TLR2 mRNA was significantly induced in HSF1^{+/+} alveolar macrophages 24 h after Mp infection. Likewise, Mp infection did not significantly change KC production in HSF1^{-/-} macrophages (about 20% increase; $p > 0.05$), but markedly increased KC production in HSF1^{+/+} macro-

Fig. 4. Recombinant HSP70 protein restores HSF1^{-/-} alveolar macrophage KC production. **a** TLR2 mRNA levels were examined by qRT-PCR in Mp-infected HSF1^{-/-} and HSF1^{+/+} alveolar macrophages 24 h after infection. **b** KC in cultured HSF1^{-/-} or HSF1^{+/+} alveolar macrophage supernatants was measured 24 h after Mp infection (10 CFU/cell) in the absence or presence of recombinant HSP70 protein (0.5 μ g/ml). Data are representative of three independent experiments. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$.

phages (about 300%; fig. 4b). This set of data was supportive of mouse BAL fluid KC data, as shown in figure 1c.

Because extracellular HSP70 is capable of augmenting TLR2 signaling in macrophages [16, 33, 34], we hypothesized that decreased HSP70 protein in HSF1^{-/-} mouse BAL fluid (fig. 3b) may limit alveolar macrophage activation such as KC production. To test this hypothesis, we treated HSF1^{-/-} and HSF1^{+/+} alveolar macrophages with recombinant HSP70 protein alone or with the combination of HSP70 and Mp infection for 24 h. HSP70 alone had no significant effect on KC production. However, the combination of both HSP70 protein and Mp greatly enhanced KC production in HSF1^{-/-} macrophages to the levels seen in HSF1^{+/+} alveolar macrophages (fig. 4b).

We next explored whether recombinant HSP70 could enhance NF- κ B activation. Since the number of alveolar macrophages is very limited, we used BMDM to perform nuclear protein extraction and NF- κ B activity assay. Table 1 shows that in HSF1^{-/-} BMDM, Mp infection in the presence of BSA (protein control) moderately increases

NF- κ B activation. However, the combination of Mp and recombinant HSP70 in HSF1^{-/-} BMDM further increases NF- κ B activation levels that are comparable to those in Mp-infected HSF1^{+/+} BMDM. HSP70 alone had no effect on NF- κ B activation. To address if HSP70-induced NF- κ B activation is responsible for KC production following Mp infection, we measured KC production in Mp-infected BMDM in the presence or absence of helenalin, a well-known NF- κ B inhibitor [35]. Table 2 indicates that like our data in alveolar macrophages, HSF1^{-/-} BMDM KC production following Mp infection was further increased by the addition of exogenous HSP70. NF- κ B inhibition abrogated HSP70- as well as Mp-induced KC production in both HSF1^{+/+} and HSF1^{-/-} BMDM.

Discussion

In this report, we demonstrate for the first time a critical role of HSF1 in lung defense against bacterial (e.g. Mp) infection that contributes to the pathobiology of several prominent lung diseases, including community-acquired pneumonia, asthma and COPD. Specifically, we found that HSF1 deficiency increased lung Mp load, which was linked to dampened early activation of TLR2 signaling such as TLR2 expression, NF- κ B activity and neutrophil recruitment. This dampening of TLR2 signaling was likely mediated by impaired induction of HSP70 following bacterial infection.

Our work has significantly improved our understanding of the role of HSF1 in lung infection as previous studies of HSF1 in lung diseases are inconclusive, and research findings vary depending on the diseases examined [17, 36]. Unlike previous findings [37], our study actually indicates that HSF1 acts as a positive regulator for host defense, possibly by enhancing TLR2 signaling in respiratory bacterial infections [38, 39]. Mp infection significantly increased HSF1 protein levels (online suppl. fig. S1). Moreover, our finding of delayed Mp clearance from HSF1^{-/-} mouse lungs is supportive of a recent paper showing impaired clearance of a systematic low dose of *Listeria monocytogenes* in HSF1^{-/-} mice [1]. We realize that the role of HSF1/HSP70 in human lung diseases remains unclear and even controversial. For instance, HSP70 is downregulated in patients with COPD [17]. Since bacterial infection is a major cause of COPD exacerbations, decreased HSP70 may predispose the COPD patients to bacterial infections. Thus, appropriate enhancement of HSF1/HSP70 may be beneficial in the prevention of bacterial infections in COPD airways. However, HSP70 ex-

Table 1. NF- κ B p65 activation was measured by TransAM® ELISA in Mp-infected HSF1^{-/-} and HSF1^{+/+} BMDM after 30 min of infection

Genotype	Treatment	Mp infection	NF- κ B p65 OD at 450 nm
HSF1 ^{-/-}	BSA	no	0.030 ± 0.010
		yes	0.109 ± 0.003
	HSP70	no	0.031 ± 0.017
		yes	0.187 ± 0.024**
HSF1 ^{+/+}	BSA	no	0.051 ± 0.012
		yes	0.202 ± 0.009
	HSP70	no	0.039 ± 0.009
		yes	0.195 ± 0.018

Data are representative of three independent experiments.

** p < 0.001, HSP70 + Mp versus BSA + Mp in HSF1^{-/-} BMDM.

Table 2. KC (pg/ml) in supernatants of cultured HSF1^{-/-} or HSF1^{+/+} BMDM was measured 24 h after Mp infection (10 CFU/cell) in the presence of BSA (protein control) or recombinant HSP70 protein (0.5 μ g/ml)

Geno-type	Treat-ment	Mp infection	0.1% DMSO	10 μ M helenalin in 0.1% DMSO
HSF1 ^{-/-}	BSA	no	32 ± 6	ND
		yes	25,368 ± 1,131	ND
	HSP70	no	0.5 ± 0.1	ND
		yes	55,480 ± 2,992**	11 ± 2
HSF1 ^{+/+}	BSA	no	59.7 ± 2.1	ND
		yes	48,892 ± 6,345	ND
	HSP70	no	ND	ND
		yes	63,595 ± 1,856	7 ± 1

Data are representative of three independent experiments.

ND = Not detected. No significant differences were detected in BSA- or HSP70-treated Mp-infected HSF1^{+/+} BMDM.

** p < 0.001, HSP70 + Mp versus BSA + Mp in HSF1^{-/-} BMDM.

pression is increased in airway epithelium and alveolar macrophages of patients with asthma as compared to normal subjects [18], and HSP70 expression levels correlated with the severity of asthma and the percentage of eosinophils in BAL fluid. In the same study, patients with chronic bronchitis did not have an increase in HSP70 expression, indicating that HSP70 upregulation may be somehow specific to asthma inflammation. HSP70 is also upregulated in alveolar macrophages of patients with acute respiratory distress syndrome [40].

One key question in our study is the mechanisms by which HSF1 promotes lung defense against bacterial infection. We have underlined the importance of HSP70 expression during bacterial infection since extracellular HSP70 has been shown to enhance the innate immune system and exert immunoregulatory effects possibly by interacting with TLRs [16, 33]. We found that the HSP70 protein level in BAL fluid of Mp-infected HSF1^{-/-} mice was significantly lower than in wild-type mice, suggesting the contribution of HSP70 to lung innate immune responses to Mp infection. Since TLR2 signaling can be activated by HSP70 [14, 16, 34], and has been shown to be critical to lung defense against Mp infection [12], we analyzed the time course of lung tissue TLR2 signaling. We showed that early TLR2 signaling activation (e.g. TLR2 upregulation, NF- κ B activation and cytokine production) was significantly dampened in the absence of HSF1. Together, our results suggest that lack of timely production of HSP70 may account for dampened or delayed TLR2 signaling in HSF1-deficient mice. To test this possibility, we determined if exogenous HSP70 would rescue TLR2 signaling in HSF1^{-/-} alveolar macrophages since they represent the first line of host defense against Mp [32]. We found that, in agreement with our in vivo results, HSF1^{-/-} macrophages ex vivo had reduced NF- κ B activity and KC production in response to Mp infection. However, NF- κ B activity and KC production were restored by exogenous recombinant HSP70. Interestingly, HSP70 protein alone did not stimulate KC production, suggesting that a physiological dose of HSP70 is not sufficient to activate TLR2 signaling, but may act as a costimulus of TLR2 signaling.

Given the facts that HSP70 is an established target gene of HSF1 [41] and lower HSP70 levels are detected in HSF1^{-/-} mice compared to HSF1^{+/+} mice, there may be additional mechanisms involved in mediating HSP70 accumulation. For instance, NF- κ B has been shown to regulate HSP70 and contribute to cardioprotection after permanent coronary occlusion [42]. Furthermore, HSP70 can be upregulated during infection, including adenovirus type 5 [43], herpes simplex virus type 1 [43], human cytomegalovirus [44] and *L. monocytogenes* [45]. We realize that although our data strongly suggest a critical role of HSF1 in regulating TLR2 signaling through HSP70, HSF1 may utilize other mechanisms to regulate TLR2 signaling. For example, HSF1 represses tumor necrosis factor (TNF)- α production in Raw 264.7 macrophages [46]. As TNF- α normally enhances TLR2 signaling [47], this suggests that HSF1 may use both positive and negative mechanisms to balance TLR2 activation.

Fig. 5. Proposed role of HSF1 and HSP70 in innate immune response to respiratory Mp infection. Upon bacterial infection, HSF1 is activated, leading to transcription and secretion of HSP70. By binding to TLR2, HSP70 stimulates KC production in lung cells (e.g. alveolar macrophages) through NF- κ B nuclear translocation, thus eliciting neutrophil recruitment and bacterial clearance.

Given our results of HSF1-associated activation of TLR2 signaling, one may ask whether TLR2 is a direct target gene of HSF1. Using the web-based software tool TESS (Transcription Element Search Software), we searched for putative HSF1 transcription factor binding sites in the promoter sequence of the TLR2 gene (5,000-bp 5'-flanking region upstream of TLR2 starting codon). We found 23 putative binding sites for HSF1 in the TLR2 gene promoter. However, the characterization of those binding sites is beyond the scope of this publication and awaits future studies.

We understand the controversies regarding the role of HSF1 in regulating inflammatory cytokine production. Our current study suggests that HSF1 may promote KC production following bacterial infection. But, HSF1 deficiency has been reported to amplify LPS-induced inflammatory responses by promoting NF- κ B and TNF- α activity/expression [13]. Furthermore, HSF1 has been shown to repress pro-IL-1 β gene in LPS-stimulated human monocytes [48], through physical interaction with the nuclear factor of IL-6 [49]. It is quite possible that HSF1 function may vary depending on the insult, the timing, as well as the tissue or cells involved.

Our work will provide new opportunities for studying the HSF1/HSP70 axis in bacterial infections under disease conditions such as asthma, COPD and cystic fibrosis. Furthermore, our work can be extended to other microbial organisms (e.g. *Pseudomonas aeruginosa*) and associated signaling pathways (e.g. TLR4). Now that HSP70 recombinant protein can be stably delivered in vivo using nanoparticles loaded with HSP70 [50], elucidation of the role of HSF1/HSP70 axis in lung diseases will likely lead to novel and attainable therapeutics to eliminate persistent bacterial infections in the airways.

In summary, we have found that HSF1 deficiency reduces Mp-induced TLR2 expression, NF- κ B activation and subsequent production of KC in the lung, including alveolar macrophages, which may eventually impair lung bacterial clearance. This cascade was associated with the reduction of extracellular HSP70 protein (fig. 5).

Acknowledgements

The authors thank Dr. Ivor Benjamin at the University of Texas Southwestern Medical Center, Dallas, for providing HSF1^{+/-} mice, Linda Remigio for her help in the preparation of BMDM, and Di Jiang for the technical assistance. This work was funded by National Institutes of Health grants NIH/NHLBI R01 HL088264 and NIH/NIAID R01 AI070175.

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