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INTERFERON REGULATORY FACTOR-1 MEDIATES ALVEOLAR MACROPHAGE PYROPTOSIS DURING LPS-INDUCED ACUTE LUNG INJURY IN MICE

Dongdong Wu,[*] Pinhua Pan,[*] Xiaoli Su,[*] Lemeng Zhang,[†] Qingwu Qin,[*] Hongyi Tan,[*] Li Huang,[*] and Yuanyuan Li[*]

[*]Department of Pulmonary and Critical Care Medicine, Xiangya Hospital, Central South University, Changsha, Hunan, China; and [†]Department of Thoracic Medicine, Hunan Cancer Hospital, Affiliated to Xiangya Medical School, Central South University, Changsha, Hunan, China

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ABSTRACT—Previously, we demonstrated that pyroptosis in alveolar macrophages (AMs) plays an essential role in lipopolysaccharide (LPS)-induced acute lung injury. However, the underlying mechanism remains largely unclear. Here, we show that the absence of interferon regulatory factor 1 (IRF-1) in genetic knock-out mice strongly abrogates pyroptosis in AMs and alleviates the LPS-induced lung injury and systemic inflammation. Our study demonstrates that IRF-1 contributes to caspase-1 activation and apoptosis-associated speck-like protein containing a caspase activation and recruitment domain pyroptosome formation in AMs and leads to downstream inflammatory cytokine release, including that of IL-1β, IL-18, and HMGB1. The nuclear translocation of IRF-1 is linked to the presence of toll-like receptor 4 (TLR4). Our findings suggest that pyroptosis and the downstream inflammatory response in AMs induced by LPS is a process that is dependent on TLR4-mediated up-regulation of IRF-1. In summary, IRF-1 plays a key role in controlling caspase-1-dependent pyroptosis and inflammation.

KEYWORDS—Acute lung injury, alveolar macrophage, caspase-1, interferon regulatory factor-1, pyroptosis

ABBREVIATIONS—ALI—acute lung injury; AMs—alveolar macrophages; ARDS—acute respiratory distress syndrome; ASC—apoptosis-associated speck-like protein containing a caspase activation and recruitment domain; ATP—adenosine triphosphate; BALF—bronchoalveolar lavage fluid; HMGB1—high-mobility group box 1; IFN—interferon; IRF-1—interferon regulatory factor 1; KO—knockout; LDH—Lactate dehydrogenase; LPS—lipopolysaccharide; MyD88—myeloid differentiation factor 88; NLRP3—nod-like receptor protein 3; PAMP—pathogen-associated molecular pattern; qPCR—quantitative PCR; TLR-4—toll-like receptor 4; WT—wild-type

INTRODUCTION

Acute lung injury and acute respiratory distress syndrome (ALI/ARDS) are serious clinical disorders of the lung. The mortality rates resulting from ALI/ARDS in intensive care units (ICUs) remain high at 30% to 40% (1). Sepsis is one of the main risk factors for ARDS, and many animal models of sepsis have been developed to study ALI (2). Lipopolysaccharide (LPS) models of inflammation have not reproduced the complex physiology of sepsis and our study focuses mainly on sepsis-related ARDS. So identification of novel and effective therapeutic targets and approaches is vital if the outcomes in ARDS cases are to be improved.

Alveolar macrophages (AMs) account for the 90% of the cells in bronchoalveolar lavage fluid (BALF) (3). In view of the role AMs as guardians for the alveolar–blood interface against airborne particles and microbes, their pivotal role in the pathogenesis of ALI/ARDS has recently come under close scrutiny.

Pyroptosis is a recently identified caspase-1 dependent form of cell death, which features rapid plasma membrane rupture, DNA fragmentation, and production of pro-inflammatory cytokines (4, 5). Following oligomerization of inflammasome components, the nod-like receptor protein 3 (NLRP3), interacts with the adaptor molecule apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) through an N-terminal pyrindomain (PYD) domain. The caspase-recruitment domain of ASC then assembles to nucleate the polymerization and filament formation of pro-caspase-1, leading to its self-activation and proinflammatory cytokines release (IL-1β and IL-18) (6). Inhibitors of cysteine protease caspase-1 have long been sought as a therapeutic tool because mice lacking caspase-1 are resistant to LPS-induced endotoxic shock (7). A previous study conducted by our group observed caspase-1 activation in AMs during the development of ALI/ARDS, and further, that treatment with a specific caspase-1 inhibitor has been shown to reduce LPS-induced lung injury in mice (8). Caspase-1 activation in AM is thus closely related to the occurrence of ALI/ARDS. However, the mechanism modulating caspase-1 activation during pyroptosis remains unclear.

Interferon regulatory factor 1 (IRF-1) has recently been recognized as an interferon-induced transcription factor with pro-inflammatory and pro-injury functions (9). IRF-1 promotes cell death with caspase-1 activation, which has been associated with oligodendrocyte pyroptosis in multiple sclerosis and encephalomyelitis lomyelitis (10–13). While the role of IRF-1 in AMs' susceptibility to injury is largely unknown. Inflammatory injury to lung tissue of IRF-1-knockout mice is reduced significantly during sepsis (14–16). It has been found by our research group that AMs in mice with

Address reprint requests to Pinhua Pan, PhD, Department of Pulmonary and Critical Care Medicine, Xiangya Hospital, Central South University, Changsha, Hunan 410008, China. E-mail: pinhuapan668@126.com

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LPS-induced ALI/ARDS present with activation of caspase-1, which is a characteristic of pyroptosis. Therefore, we hypothesize that IRF-1 may be involved in the pathogenesis of ALI/ARDS by regulating alveolar macrophage pyroptosis and the release of inflammatory mediators.

In the present study, we showed that the absence of IRF-1 in genetic knockout mice strongly alleviates LPS-induced lung injury and abrogates its pyroptotic effects in AMs. These findings suggest that IRF-1 plays a key role in controlling caspase-1-dependent pyroptosis and inflammation, which is, in turn, a TLR4-dependent process. Our study provides a novel perspective on the pathogenesis of ALI/ARDS.

MATERIALS AND METHODS

Reagents

LPS (*E coli* 0111:B4) and adenosine triphosphate (ATP) were obtained from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal caspase-1 P10 (M-20) antibody was sourced from Santa Cruz, CA. Rabbit polyclonal TLR4, IRF-1, IL-1β, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody were all from Cell Signaling Technology (Boston, MA). Rabbit polyclonal Histone H3 antibody and Rabbit polyclonal ASC antibody was obtained from ImmunoWay Biotechnology Co (Newark, DE). Alexa555-conjugated secondary antibody was obtained from Molecular Probes Inc (Eugene, OR).

Animals

Male IRF-1 KO, TLR4 KO mice, and the control mice (C57BL/6J) were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were maintained in a specific pathogen-free, laminar-flow atmosphere under controlled temperature, humidity, and light. All animal protocols were approved by the Animal Care and Use Committee of the Central South University and were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

In vivo experimental design

Male IRF-1 KO, TLR4 KO, and matched C57BL/6J (8–10-week old) mice were given intraperitoneal injections of a lethal dose of LPS (20 mg/kg). Control mice received injections of sterilized phosphate buffered saline (PBS). In some experiments group survival rates of 96 h were observed. In other experiments, mice were sacrificed 16 h post-LPS. Following euthanasia, the lungs (n = 6 per group) were excised from the mice via a median sternotomy. The wet weight (*W*) of the left lung was measured using an electronic scale and then desiccated in an oven at 65°C for 72 h to determine the dry weight (D). The water content was measured by calculating W/D weight ratio. The right lung was removed and fixed in 4% paraformaldehyde for 24 h. Lungs were instilled with 1 mL PBS each time through an intratracheal catheter as descried previously (17), and lavaged >10 times by slowing withdrawing more than 0.8 mL lavage fluids each time. A total of 10 mL bronchoalveolar lavage (BAL) was withdrawn from each mouse. The 10 mL lavage fluid was centrifuged at 300 × g for 10 min to pellet AMs. Another 2 mL BALF was harvested immediately following sacrifice from other mice to detect the total protein level and cytokine level in the BALF. Cytokine (IL-1β, IL-18, and HMGB1) concentrations from 2 mL BALF and serum were detected using relevant ELISA kits.

Histological assessment

Formalin-fixed, paraffin-embedded lung tissue was cut into sections of 5-um thickness and stained with hematoxylin-eosin. Histopathological changes were viewed under a microscope by a pathologist who was blinded to the treatment. Each histological characteristic was evaluated on a scale of 0 to 3 (0 = normal; 1 = mild; 2 = moderate; 3 = severe). Total aggregate scores of lung injury were calculated according to the sum of the score for alveolar edema and hemorrhage, numbers of infiltrating of leukocytes, and thickness of alveolar walls and epithelium, as described previously (18).

Immunohistochemistry

To determine the expression of cleaved caspase-1 in the lung tissue of mice, immunohistochemistry analyses were carried out. Sections were routinely stained with antimouse caspase-1 P10 (M-20) at 1:50 (Santa Cruz, CA). The reaction products were visualized by treating the slide with 3,3′-diaminobenzidine and counterstaining with hematoxylin. For negative controls, the specific primary antibody was replaced by an equivalent volume of isotype control antibody. We observed the results under a light microscope (Olympus, Tokyo, Japan).

Cell culture and treatment

AMs were isolated by BAL as previously described (19). Then, AMs harvested from mice by lavage were cultured in RPMI 1640 (Gibico, Carlsbad, CA) supplemented with 10% FBS, 1 mmol/L glutamine, 10 mmol/L 4-(2-hydroxyethyl)-1 -piperazine ethan esulfonic acid, 50 U/mL penicillin, and 50 ug/mL streptomycin. After 3 h of adherence, the cells were washed twice, after which time whole cell lysates were harvested and subjected to western blot. As previously described (20), the viability of the AM isolate was >98%, as evaluated by trypan blue exclusion. The purity was >95%, as determined using fluorescently labeled Abs (mAbs) that specifically recognize proteins expressed by mouse macrophages (surface Ags F4/80 and CD11b). The mouse monocyte/macrophage cell line J774.A1 was purchased from American Type Culture Collection and maintained in DMEM supplemented with 10% FBS, 50 ug/mL streptomycin, and 50 U/mL penicillin.

Vector constructs and lenvirus transfection

Lentivirial constructs expressing shRNA directed against mouse IRF-1mRNA were manufactured by Hanyin (Shanghai, China). The shRNA sequences were as follows: IRF-1 shRNA: 5'- GCACTAAATGAGTCCTATTCC-3', and nonspecific shRNA: 5′- TTCTCCGAACGTGTCACGT-3′. The constructs expressing IRF-1 shRNA were infused with green fluorescent protein. The lentivirial constructs expressing mouse IRF-1 infused with puromycin resistance genes were manufactured by the same vendor (Hanyin). The IRF-1 cDNA was obtained from a commercially available source (National Center for Biotechnology Information, Bethesda, MD, USA). All lentivirial vectors were purified to a titer of 1×10^9 TU/mL. At a multiplicity of infection of 20:1 4 h after transfection, the medium was exchanged for fresh medium, and the cells were cultured for an additional 48 h. The transfection efficiency of IRF-1 shRNA was observed by fluorescence microscope. For IRF-1 expressing group, the medium was replaced and 2 ug/mL puromycin was applied for selecting for 7 days after 48 h of transfection, then IRF-1 expression of stable transfection is obtained. The effects of IRF-1 knockdown and overexpression were determined using qPCR and western blot analysis and we were able to achieve more than 80% transduction efficiency using the lentivirus delivery system (data not shown).

Quantitative real-time PCR

Total RNA was isolated from AMs using Trizol Reagent from Invitrogen (Carlsbad, CA, USA). The reverse transcript (cDNA) was synthesized from 1 ug of total RNA using the All-in-One First-stand cDNA synthesis kit (CeneCopoeia). Quantitative real-time PCR was carried out using All-in-One qPCR Mix from CeneCopoeia. The reaction mix of a total volume of 10 uL was programmed as follows: 95°C for 10 min, 40 cycles at 95°C for 10 s, 60°C for 20 s, and 72°C for 15 s. GAPDH was used as the reference gene. The sequence of primers used for quantitative PCR was as follows: TLR4 forward: 5′-TTGATACGTTTCCTTATAAG-3′, reverse: 5′-GAAATGGAGGCACCCC TT-3′; IRF 1 forward: 5′-CTCACCAGGAACCAGAGGAA-3′, reverse:5′-TGAGTGGTGTAACTGCTGTGG-3′; Caspase-1 forward: 5′-ACAAGG-CACGGGACCTATG-3′, reverse: 5′-TCCCAGTCAGTCCTGGAAATG-3′; GAPDH forward:5′-TGCACCACCAACTGCTTAGC-3′, reverse: 5′-GGCAT GGACTGTGGTCATGAG-3′.

Cellular and nuclear protein extraction

Collected cells were centrifuged at 500 g for 3 min. These pellets were then resuspended on ice with cytoplasmic extraction reagent (Vazyme, China) combined with protease inhibitor mix for 10 min, and centrifuged at 16000 g for 5 min at 4 °C to extract the cellular protein. For nuclear protein isolation, the cell pellet was lysed with nuclear extraction reagent (Nanjing, Vazyme, China) combined with a protease inhibitor mix. Protein concentration was determined by the BCA method, and 50 ug of protein per sample was mixed with sample loading buffer and boiled for 8 min.

Western blotting

Protein samples were electrophoresed in 6% to 12% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels and transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories, Berkeley, CA). After blocking with 5% nonfat milk in TBS-T for 1 h at room temperature, membranes were incubated overnight at 4°C with primary antibodies against caspase-1 P10

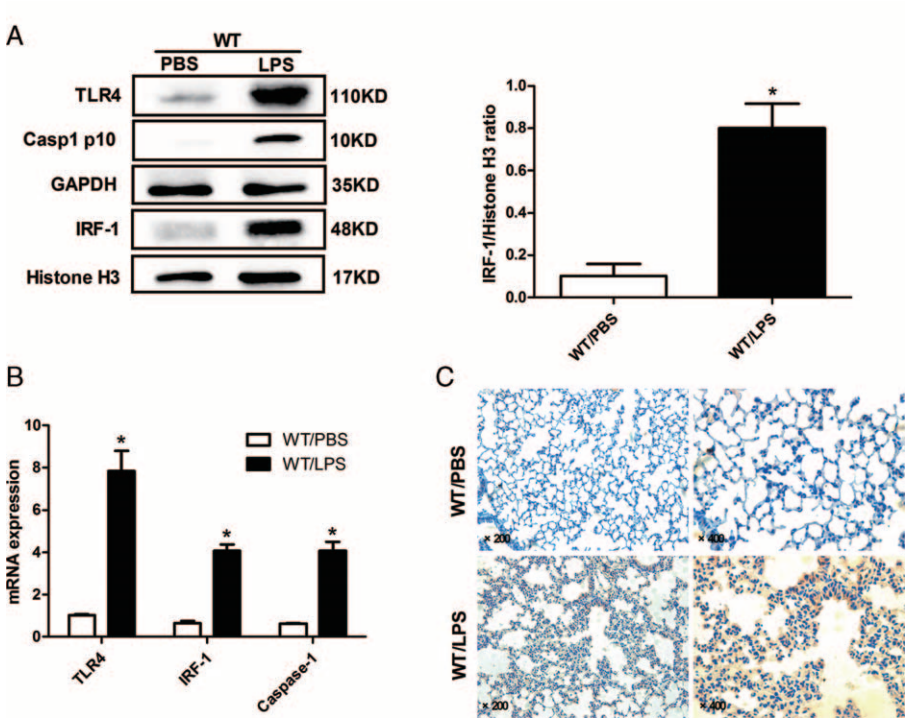

FIG. 1. **LPS induces TLR4 and IRF-1 expression and pyroptosis in alveolar macrophages *in vivo*.** Mice were treated with vehicle or LPS (20 kg/mg) by IP Injection. Lung samples and AMs were harvested from mice at 16 h post-treatment. A, Western blot analysis of the protein expression of TLR4, IRF-1, and caspase-1 p10 in AMs. B, Analysis of gene expression of TLR4, IRF-1, and caspase-1 in AMs. C, IHC staining of lung sections with caspase-1 p10 antibody (×200 and ×400). (n = 6/group, $^{*}P < 0.05$ versus the WT/PBS group). Results are representative of three independent experiments. AMs indicates alveolar macrophages; IRF-1, interferon regulatory factor 1; LPS, lipopolysaccharide; TLR4, toll-like receptor 4.

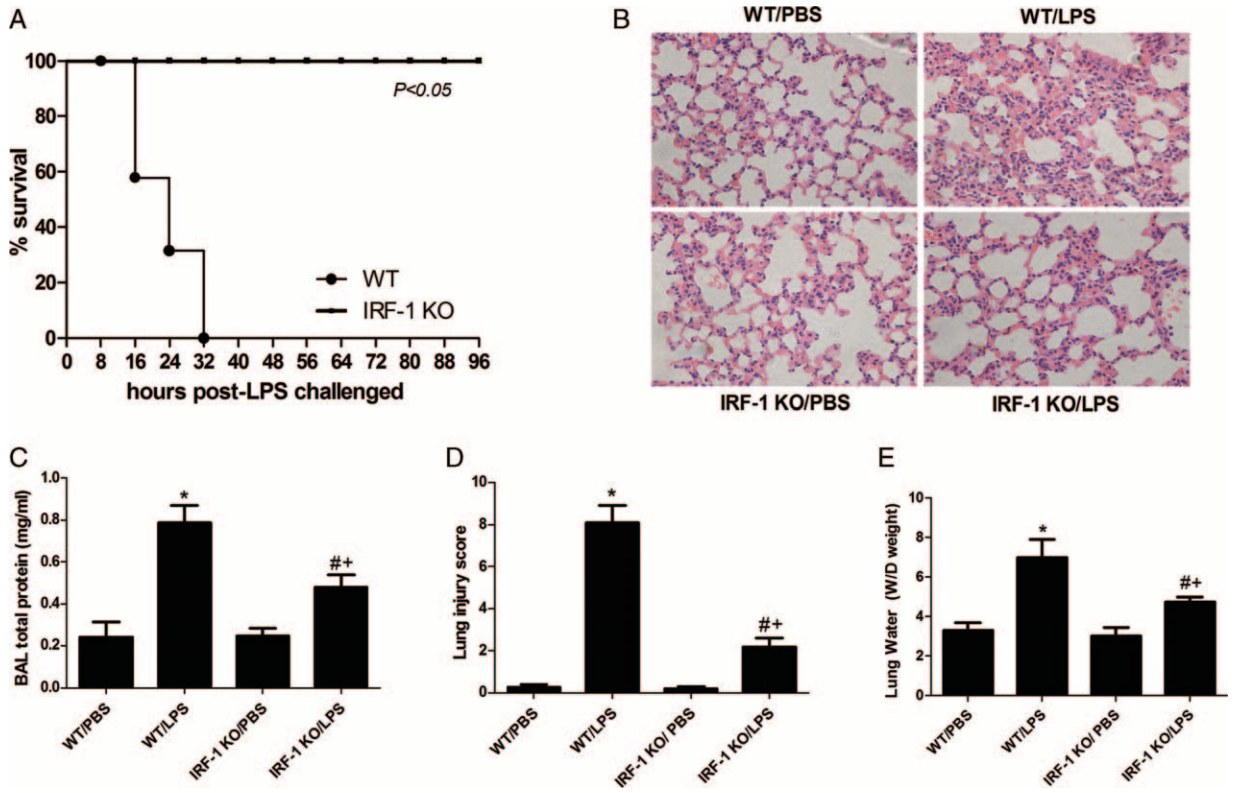

FIG. 2. **IRF-1 deletion attenuates LPS-induced acute lung injury in mice.** WT and IRF-1 KO mice were treated with vehicle or LPS (20 kg/mg) by IP injection. Lung samples and BALF were harvested at 16 h post-treatment. A, Survival curves at 96 h. B, H&E staining of lung sections (×400). C, Total protein concentration in BALF. D, Lung injury score. E, Water content of lung. (n = 6/group, $^{*}P < 0.05$ versus the WT/PBS group, $^{\#}P < 0.05$ versus the WT/LPS group, $^{+}P < 0.05$ versus the IRF-1 KO/PBS group). Results are representative of three independent experiments. BALF indicates bronchoalveolar lavage fluid; IRF-1, interferon regulatory factor 1; KO, knockout; LPS, lipopolysaccharide; PBS, phosphate buffered saline; WT, wild-type.

(1:1,000), GAPDH (1:2,000), histone H3 (1:500), IRF-1 (1:1,000), and IL-1β (1:500). After three washes, membranes were incubated with the secondary antibody conjugated with horseradish peroxidase at room temperature for 1 h. The blots were developed with Super Signal chemiluminescent substrate (Pierce Chemical Co, Rockford, IL) and exposed to film. The relative quantities of the proteins were determined with a densitometer and expressed in absorbance units (AU).

Confocal immunofluorescence imaging

J774.A1 cells were placed onto glass cover slides, fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.1% TritonX-100 for 10 min at room temperature. Cells were then blocked with 5% bovine serum albumin (BSA) in PBS for 1 h at room temperature, and then incubated with rabbit polyclonal ASC antibody (1:200 in 1% BSA in PBS) overnight at 4°C. After washing three times with PBS, the cells were stained with Alexa555-conjugated secondary antibody (diluted to 1:1,000) for 90 min at room temperature. The cells were washed three times in PBS and stained with DAPI. Slides were visualized with an Olympus Fluoroview 500 confocal microscope.

Enzyme-linked immunosorbent assay

IL-1β, IL-18, and HMGB1 1evels in the serum, BALF, and cell culture supernatant were determined using commercially available mouse IL-1β and IL-18 ELISA kits. respectively (eBioscience, San Diego, CA) according to the manufacturer's instructions. HMGB1 levels in the samples were determined by an HMGB1 detection kit (Chondrex Inc, Redmond, WA) according to the manufacturer's instructions.

Caspase-1 activity assay

Caspase-1 activity was measured using colorimetric assay (BioVision, Mountain View, CA). The assay is based on spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage by caspase-1 from the labeled substrate YVAD-pNA. The pNA light emission can be quantified using

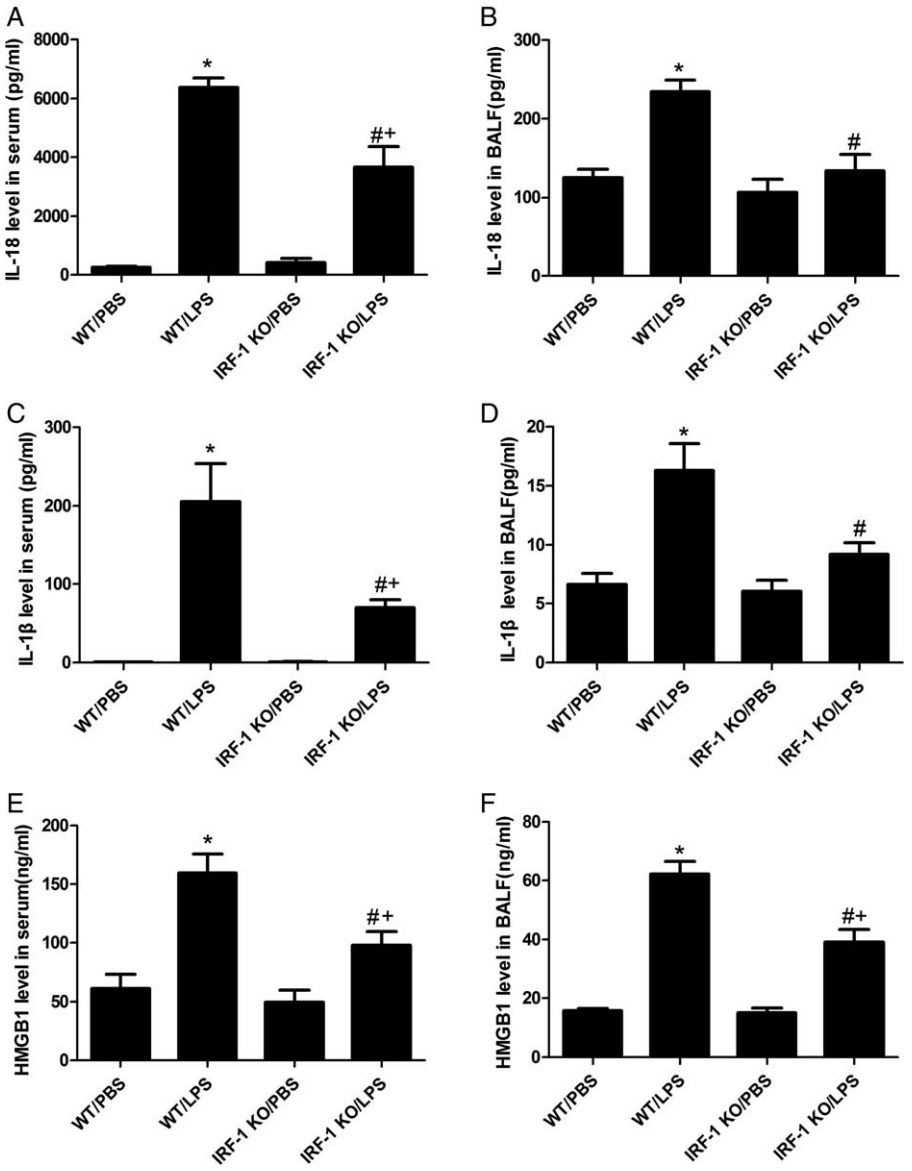

Fig. 3. **IRF-1 deletion reduced LPS-induced cytokine levels in mice.** WT and IRF-1 KO mice were treated with vehicle or LPS (20 kg/mg) by IP injection. Serum and BALF samples were harvested at 16 h post-treatment and tested for levels of IL-18, IL-1β, and HMGB1 in serum (A, C, E) and BALF (B, D, F). (n = 6/group, $^{*}P < 0.05$ versus the WT/PBS group, $^{\#}P < 0.05$ versus the WT/LPS group, and $^{+}P < 0.05$ versus the IRF-1 KO/PBS group). Results are representative of three independent experiments. BALF indicates bronchoalveolar lavage fluid; IRF-1, interferon regulatory factor 1; KO, knockout; LPS, lipopolysaccharide; PBS, phosphate buffered saline; WT, wild-type.

a spectrophotometer at 405 nm. Comparison of the absorbance of a treated sample's pNA with that of an untreated control allows determination of the fold increase in caspase-1 activity.

Determination of cell death

Lactate dehydrogenase (LDH) activity in cell-culture supernatants following LPS/ATP stimulation was determined to enable evaluation of cell death. LDH activity was quantified by spectrophotometric analysis using the Cytotoxicity Detection Kit (Roche, Basel, Switzerland), according to the manufacturer's instructions. LDH activity in 1% Triton X-100-lysed cells was determined relative to total activity at 100%. The released LDH activity was expressed as a percentage of total cellular LDH activity.

Statistical analyses

Data are expressed as mean ± SD. Statistical comparisons were performed by one-way ANOVA or two-tailed Student *t* test. Survival rates were analyzed with the Kaplan–Meier test. SPSS16.0 was used for statistical analyses. A P value <0.05 was considered to be statistically significant.

RESULTS

LPS induces TLR4 and IRF-1 expression and pyroptosis in alveolar macrophages in vivo

Previously, we demonstrated that pyroptosis occurs in AMs during LPS-induced ALI in mice (8). Here, we set out to determine the role of IRF-1 during LPS-induced ALI in mice, and the association between TLR4 and caspase-1. It has already been established that caspase-1 is a biomarker of pyroptosis. We isolated the AMs from the ALI mouse model. As shown in Figure 1A, western blot analysis demonstrated that the protein levels of TLR4, IRF-1 ($P<0.05$), and caspase-1 increased in AMs after LPS administration. We also found that mRNA expression coding for TLR4 (Fig. 1B, $P<0.05$), IRF-1 (Fig. 1B, $P<0.05$), and caspase-1 (Fig. 1B, $P<0.05$) were significantly higher when compared with the control group, a result that was consistent with our western blot analysis. To determine the levels of caspase-1 in the lung tissue, caspase-1 was detected in lung sections by immunohistochemistry staining. Higher expression levels of caspase-1 were observed in lung tissue from the ALI mouse model (Fig. 1C). These results suggest that LPS does indeed induce TLR4 and IRF-1 expression and pyroptosis in alveolar macrophages in ALI.

IRF-1 deletion attenuates LPS-induced acute lung injury and cytokine release in mice

IRF-1 KO mice were used to investigate whether IRF-1 mediates LPS-induced acute lung injury and cytokine release. To determine whether IRF-1 contributes to mortality following LPS administration, 96-h survival rates were noted. Significantly, LPS-induced mortality was 100% in the WT mice at 32 h, whereas all IRF-1 KO mice survived for 96 h postadministration (Fig. 2A). IRF-1 KO mice demonstrated significantly improved 96-h survival rates compared with the control WT mice ($P<0.05$). In a further set of experiments, four animal groups were created: WT/PBS group; WT/LPS group; IRF-1 KO/PBS group; and IRF-1 KO/LPS group. An examination of the pathology of the lung tissue showed that the WT/LPS group developed exacerbated lung inflammation, hemorrhaging and alveolar septal thickening, while lung lesions were fewer in the IRF-1 KO mice (Fig. 2B). The level of total proteins in BALF (Fig. 2C, $P<0.05$), lung injury score (Fig. 2D, $P<0.05$), and pulmonary W/D weight ratio (Fig. 2E, $P<0.05$) significantly reduced in the IRF-1 KO/LPS group when compared with the WT/LPS group, which indicated that the lung injury in IRF-1 KO mice was alleviated. For cytokine levels both in serum and BALF, we discovered that IRF-1 deletion significantly blocked LPS-induced IL-18 (Fig. 3A, $P<0.05$; Fig. 3B, $P<0.05$) as

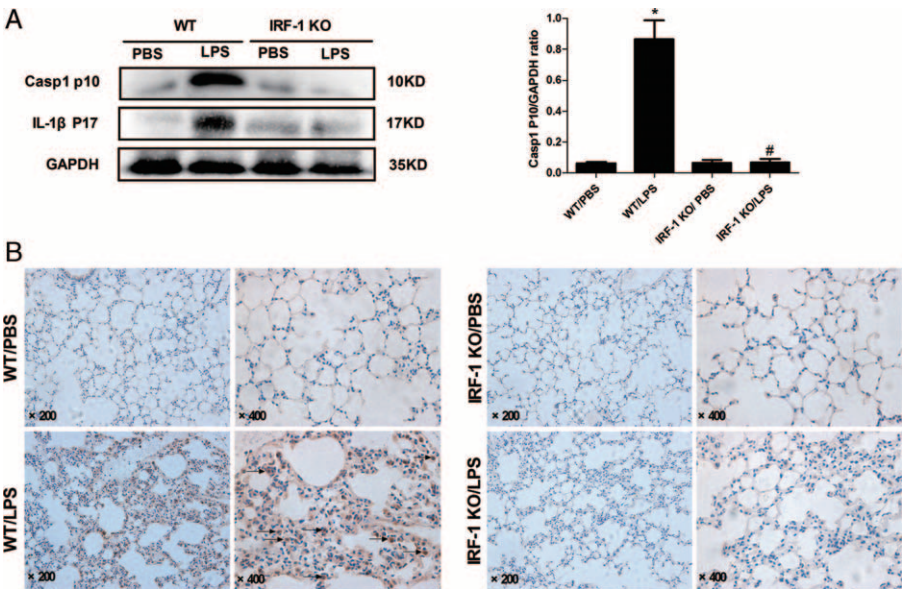

FIG. 4. **IRF-1 deletion attenuates pyroptosis in alveolar macrophages during LPS-induced acute lung injury *in vivo*.** WT and IRF-1 KO mice were treated with vehicle or LPS (20 kg/mg) by IP injection. AMs were harvested from mice at 16 h post-treatment. A, Western blotting analysis of protein expression of caspase-1 p10 and IL-1βp17 in AMs. B, IHC staining with caspase-1 p10 antibody of lung sections (×200 and ×400). The arrowheads show the positive AMs (n = 6/group, $^{*}P<0.05$ versus the WT/PBS group, $^{\#}P<0.05$ versus the WT/LPS group). Results are representative of three independent experiments. AMs indicates alveolar macrophages; IRF-1, interferon regulatory factor 1; KO, knockout; LPS, lipopolysaccharide; PBS, phosphate buffered saline; WT, wild-type.

well as IL-1β (Fig. 3C, $P<0.05$; Fig. 3D, $P<0.05$), and HMGB1 (Fig. 3E, $P<0.05$; Fig. 3F, $P<0.05$) compared with the WT/LPS group. These pyroptosis-associated cytokines have been shown to be important mediators of tissue damage. These data indicate that IRF-1 plays an important role in the pathogenesis of LPS-induced ALI.

IRF-1 deletion attenuates pyroptosis in alveolar macrophages during LPS-induced acute lung injury in vivo

Having shown that LPS-induced lung injury is associated with the pyroptosis of AMs (8), we set out to analyze whether IRF-1 deletion enhances protection by inhibiting pyroptosis in AMs. As shown in Figure 4A, LPS challenge resulted in a dramatic activation of caspase-1 ($P<0.05$) and IL-1β in AMs in WT mice. However, the levels of caspase-1 p10 and IL-1β were strongly attenuated in the AMs of IRF-1 KO mice. Moreover, caspase-1 was also detected in lung sections by immunohistochemical staining. Indeed, reduced expression of caspase-1 was observed in lung tissue in IRF-1 KO mice, as well as highly positive AMs, compared with the control group (Fig. 4B).

To determine whether IRF-1 nuclear expression and caspase-1 activation are modulated by TLR4 in LPS-induced acute lung injury, TLR4 KO mice were challenged with LPS by IP injection for 16 h. AMs were isolated from mice to perform western blotting and quantitative PCR. As shown in Figure 5, both protein level (Fig. 5A), and mRNA level (Fig. 5B) of IRF-1 and

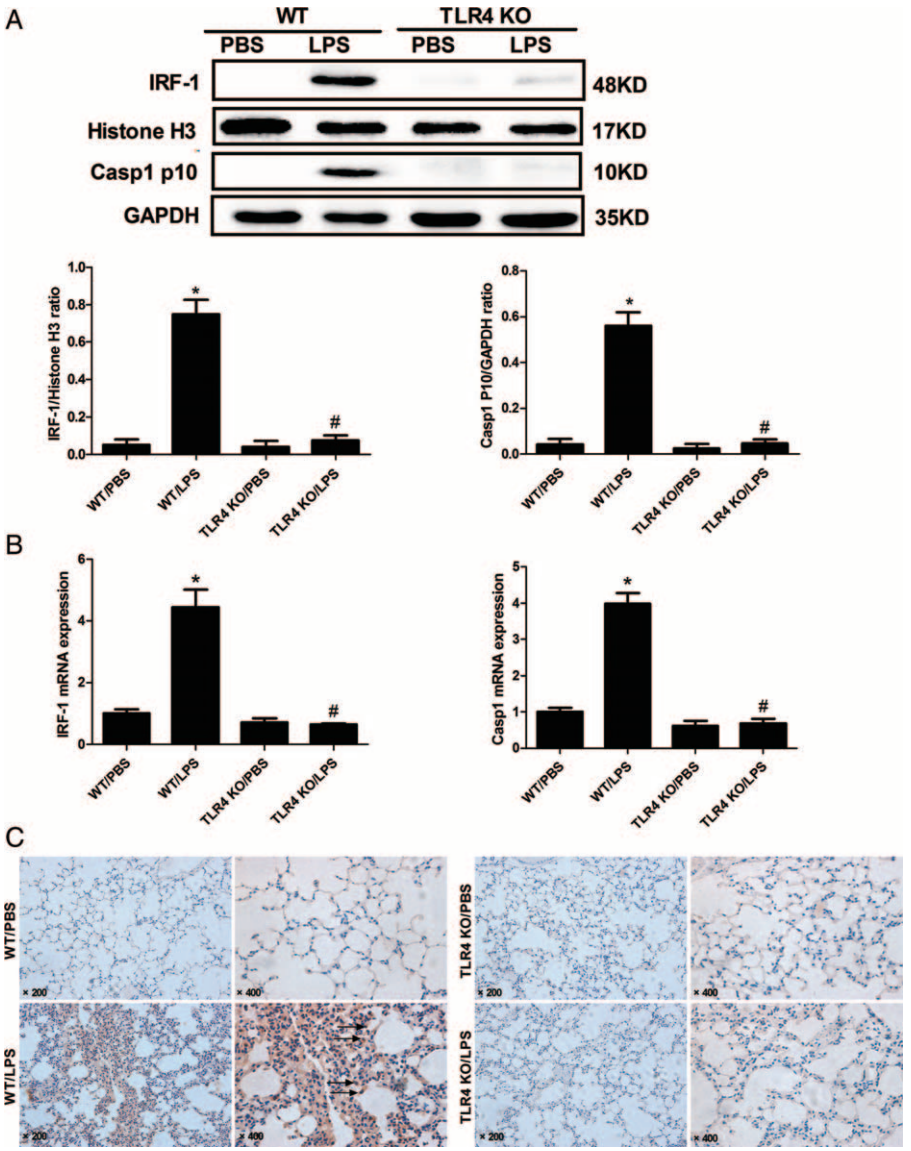

Fig. 5. **TLR4 is required for IRF-1 expression and caspase-1 activation in alveolar macrophages.** WT and TLR4 KO mice were treated with vehicle or LPS (20 kg/mg) by IP injection. Lung samples as well as BALF were harvested and AMs were sampled at 16 h post-treatment. A, Western blotting analysis of protein expression of IRF-1 and caspase-1 p10 in AMs. B, Analysis of gene expression of IRF-1 and caspase-1 in AMs. C, IHC staining with caspase-1 p10 antibody of lung sections (×200 and ×400). The arrowheads show the positive AMs (n = 6/group, $^{*}P<0.05$ versus the WT/PBS group, $^{\#}P<0.05$ versus the TLR4 KO/LPS group). Results are representative of three independent experiments. AMs indicates alveolar macrophages; BALF, bronchoalveolar lavage fluid; IRF-1, interferon regulatory factor 1; LPS, lipopolysaccharide; PBS, phosphate buffered saline; WT, wild-type.

caspase-1 were increased in AMs during LPS-induced acute lung injury, while they were both dramatically more inhibited in the TLR4 KO/LPS group than the model group ($P < 0.05$ for both). Furthermore, immunohistochemistry gave a negative result in the lung sections of LPS-treated TLR4 KO mice, but a positive result in the LPS-treated WT mice (Fig. 5C). Together, these findings demonstrate that IRF-1 expression and caspase-1 activation are dependent on intact TLR4 signaling.

LPS/ATP-induced pyroptosis and cytokine release in J744.A1 cells are IRF-1 dependent

We also studied the kinetics of IRF-1 expression in J744.A1 cell responses to LPS. The results showed that the nuclear LPS-induced IRF-1 protein expression was time dependent and reached a peak at 4 h (Fig. 6A, $P < 0.05$) and that LPS induced nuclear IRF-1 expression in a dose-dependent manner in a certain range, reaching a peak at a dose of 500 ng/mL. Continued increases of the dosage did not increase IRF-1 expression (Fig. 6B, $P < 0.05$).

The involvement of IRF-1 signaling in macrophages was examined directly in protection experiments in which IRF-1 expression was knocked down using lentivirus-delivered shRNA. J744.A1 cells were transduced with IRF-1 shRNA lentiviral vectors and a control lentiviral vector (carrying non-specific shRNA). Due to the fact that LPS combined with ATP is a powerful inducer of pyroptosis, the cells were stimulated with LPS (500 ng/mL) for 5 h and ATP (5 mM) for 1 h. Following stimulation, western blottings tests were performed to assess the IRF-1 nuclear translocation and caspase-1 activation in cytoplasm. In addition, caspase-1 activity and LDH release were also carried out post-treatment. ASC pyroptosomes were also examined by immunofluorescence.

Western blotting analysis of the protein samples confirmed that transduction of IRF-1 shRNA of J744.A1 cells with IRF-1 shRNA vector resulted in significant reduction of IRF-1 expression in both LPS/ATP-treated and untreated groups compared with controls (Fig. 7A). As a result of the reduced IRF-1 expression, the activation of caspase-1 in response to LPS/ATP was significantly suppressed compared with the controls, both in western blot analysis (Fig. 7A) and colorimetric assay (Fig. 7C, $P < 0.05$). ASC pyoptosomes were also not up-regulated in J744.A1 cells following LPS/ATP stimulation in the presence of IRF-1 shRNA (Fig. 7G).

In an effort to examine the role of IRF-1 further, we induced IRF-1 overexpression. J744.A1 cells were transduced with an IRF-1-expression lentiviral vector and a control lentiviral vector. Once this was done, the cells were stimulated with LPS (500 ng/mL) for 5 h and ATP (5 mM) for 1 h. Nuclear and cytoplasmic protein in J744.A1 cells was confirmed by western blotting (Fig. 7B). Transduction of J744.A1 cells with IRF-1 lentivirus vector resulted in significant caspase-1 activation (Fig. 7B, $P < 0.05$; Fig. 6D, $P < 0.05$) compared with the negative control in both LPS/ATP-treated and untreated groups. LDH release (Fig. 7F, $P < 0.05$) also rose progressively compared with the controls. Thus, LPS/ATP-induced macrophage pyroptosis was shown to be dependent on IRF-1.

Supernatants were also collected from the above-mentioned LPS/ATP treated J744.A1 cells in the presence of IRF-1 shRNA and IRF-1 lentivirus. As suspected, IL-18, IL-1β, and HMGB1 were heavily suppressed in the presence of IRF-1 shRNA even in the presence of LPS/ATP stimulation (Fig. 8A, $P < 0.05$; Fig. 8C, $P < 0.05$; Fig. 8E, $P < 0.05$). In the IRF-1 over expression J744.A1 cells however, these cytokines were significantly higher in supernatants compared with negative controls (Fig. 8B, $P < 0.05$; Fig. 8D, $P < 0.05$; Fig. 8F, $P < 0.05$). These findings suggest that overexpression of IRF-1 may have caused higher inflammation and that downregulation of IRF-1 can counteract severe inflammation. In sum, IRF-1 plays a key role in controlling caspase-1-dependent pyroptosis and inflammation.

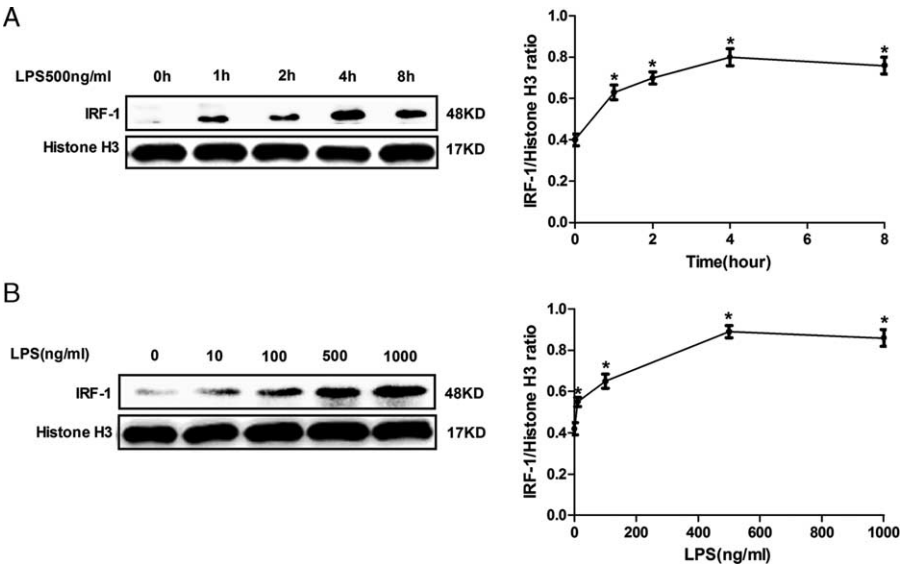

FIG. 6. **The kinetics of IRF-1 expression in J744.A1 cells response to LPS.** A, IRF-1 Western blot analysis of nuclear extracts from J744.A1 cells stimulated with LPS (500 ng/mL) for 0, 1, 2, 4, and 8 h. B, Western blot detection of IRF-1 in nuclear extracts from J744. A1 cells stimulated with LPS at 0, 10, 100, 500, and 1000 ng/mL for 4 h. $^{*}P < 0.05$ versus the unstimulated group. Results are representative of three independent experiments. IRF-1 indicates interferon regulatory factor 1; LPS, lipopolysaccharide.

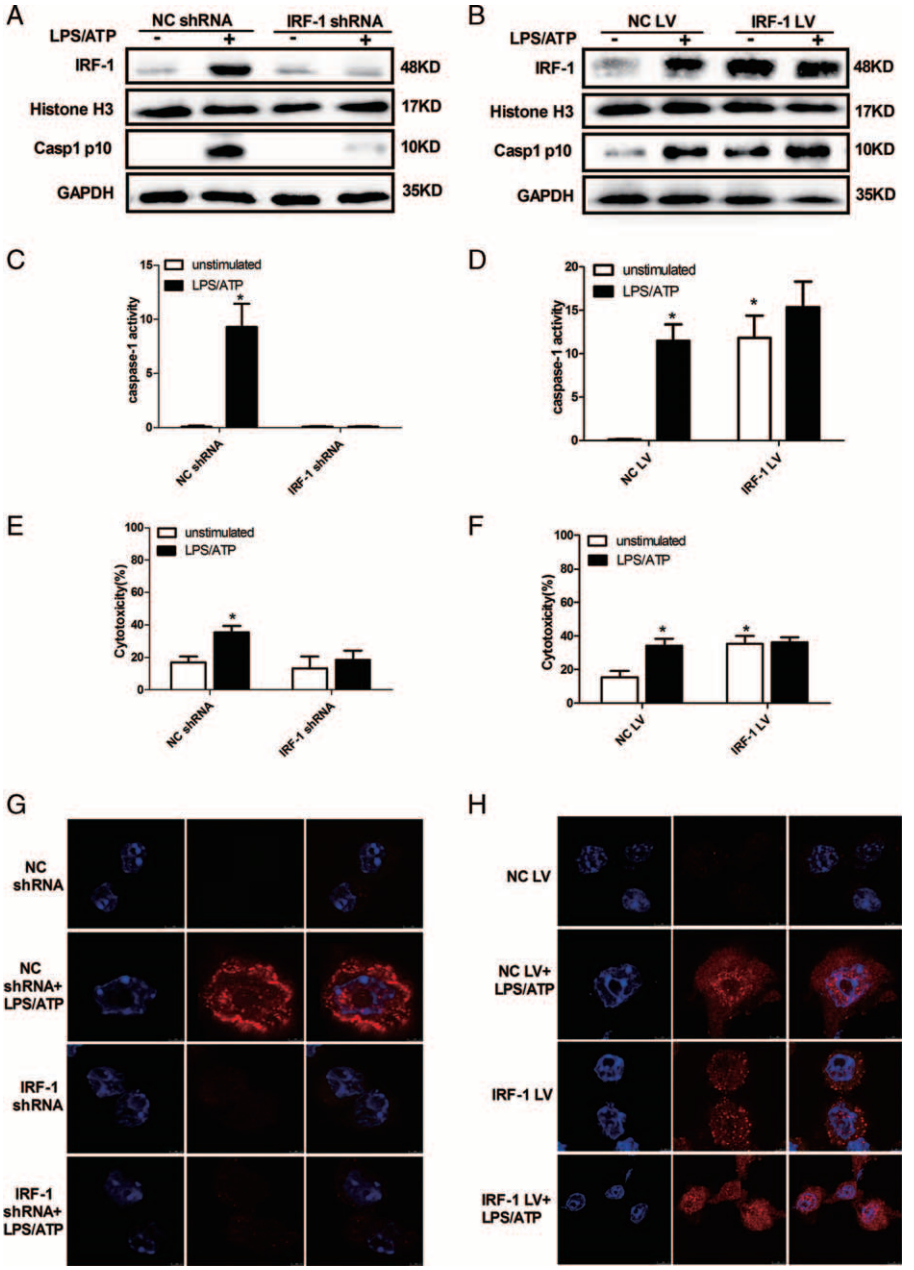

Fig. 7. **LPS/ATP-induced pyroptosis in J744.A1 cells is IRF-1 dependent.** J744. A1 cells were transfected with IRF-1 shRNA, control lentivirus vector (NC shRNA), IRF-1 lentivirus (IRF-1 LV), and control lentivirus vector (NC LV). Then J744.A1 cells were stimulated with or without LPS (500 ng/mL) for 5 h and ATP (5 mM) was added during the last hour of culture. Western blotting analysis of protein expression of IRF-1 and caspase-1 P10 in J744. A1 cells pretreated with IRF-1 shRNA (A) or IRF-1 lentivirus (B). Caspase-1 activity assay in J744.A1 cells pretreated with IRF-1 shRNA (C), or IRF-1 lentivirus (D). Assay of LDH in J744.A1 cells pretreated with IRF-1 shRNA (E) or IRF-1 lentivirus (F). ASC pyroptosomes (red) in J744.A1 cells were analyzed by immunofluorescence in the presence of IRF-1 shRNA (G) or IRF-1 lentivirus (H). $^{*}P < 0.05$ versus the NC group. Results are representative of three independent experiments. IRF-1 indicates interferon regulatory factor 1; LPS, lipopolysaccharide.

DISCUSSION

ALI/ARDS is a complex and devastating disorder of the lung associated with excessive inflammation. Pyroptosis is a form of programed cell death dependent on caspase-1 activation and cytokine release. However, the exact link between the AM pyroptosis and lung injury in ALI/ARDS remains to be elucidated.

In the present study, we identify the critical role of IRF-1 in mediating lung injury during sepsis-related ALI/ARDS induced by LPS. Indeed, as a gene noted for its involvement in the interferon (IFN) pathway, IRF-1 is often characterized as elevated in ARDS patients (21). Additionally, disruption of IRF-1 in the mouse model alleviated lung injury and protected against LPS insult. These studies indicate that IRF-1 plays an important role in mediating lung injury in ALI/ARDS.

We, therefore, have investigated the role of IRF-1 in mediating AM pyroptosis during the development of ALI/ARDS. We showed that tissue-derived alveolar macrophages from LPS-stimulated IRF-1 KO mice exhibit less pyroptosis than their WT counterparts (Fig. 4A). Furthermore, we found that overexpression of IRF-1 in J744.A1 cells alone is a sufficient

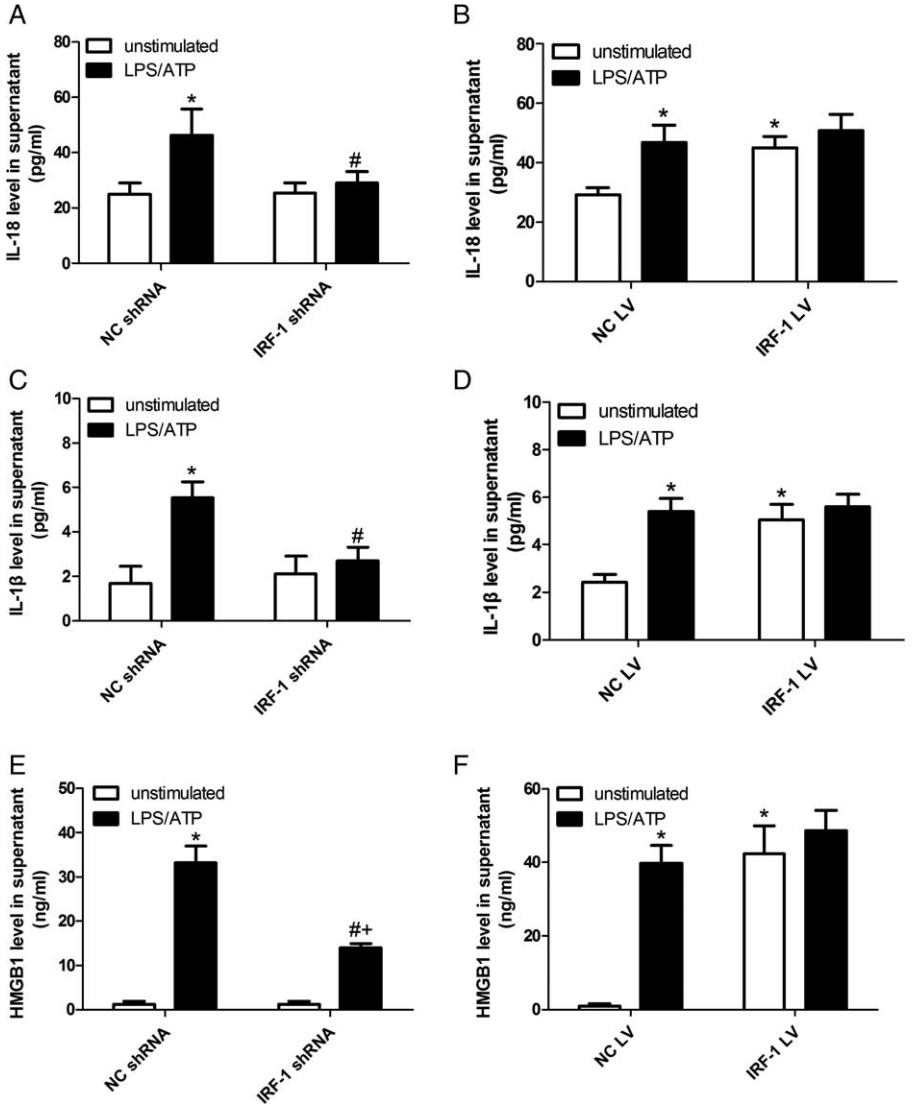

FIG. 8. **IRF-1 mediates LPS/ATP-induced cytokine release in J744.A1 cells.** Transduced J744.A1 cells were stimulated with or without LPS (500 ng/mL) for 5 h and ATP (5 mM) was added during the last hour of culture in the presence of IRF-1 shRNA or IRF-1 lentivirus. Two milliliters of the supernatants were harvested and tested for levels of pro-inflammatory cytokines. Knockdown of IRF-1 in J744.A1 cells inhibited the expression of IL-18 (A), IL-1β (C), and HMGB1 (E) in the supernatants. Overexpression of IRF-1 in J744.A1 cells boosted the expression of IL-18 (B), IL-1β (D), and HMGB1 (F) in the supernatants. $^{*}P < 0.05$ versus the NC group; $^{\#}P < 0.05$ versus the NC+LPS/ATP group; $^{+}P < 0.05$ versus the IRF-1 shRNA group. Results are representative of three independent experiments. IRF-1 indicates interferon regulatory factor 1; LPS, lipopolysaccharide.

stimulation to induce pyroptosis. Consequently, our data provides evidence that IRF-1 activation induces pyroptosis by modulating ASC pyroptosome formation and caspase-1 activation (Fig. 7). Indeed, it has been shown that IRF-1 is an essential transcription factor involved in pyroptotic cell death. Previous studies have proved that the caspase-1 gene contains an IRF-1-binding element (IRE), and that caspase-1 expression cannot be up-regulated in oligodendrocyte progenitor cells stimulated by IFN without the presence of IRF-1 (22, 23). IRF-1 can also induce the pyroptosis of oligodendrocyte in the development of multiple sclerosis, promote the inflammatory reaction, and lead to inflammatory demyelination (9). The presence of IRE as a gene promoter of caspase-1 makes it possible that IRF-1 directly regulates caspase-1 activity and alveolar macrophage pyroptosis from the structure.

IRF-1 may be also the actor controlling the release of inflammatory mediators. The present study demonstrates that IL-18, IL-1β, and HMGB1 are significantly reduced both in serum and BALF in IRF-1 KO mice during LPS-induced acute lung injury (Fig. 3). We also validated that overexpression of IRF-1 serves as a strong promoter of the above-mentioned inflammatory cytokine release (Fig. 8). Currently, caspase-1- mediated tissue damage is thought to require the pro-inflammatory caspase-1 substrates IL-1β and IL-18, while the existence of another caspase-1-dependent mediator of endotoxemia, HMGB1, has been described. Reduced HMGB1 levels in caspase-1-deficient mice correlated with their resistance to LPS, while the neutralization of HMGB1 with Abs protected mice deficient in IL-1β and IL-18 from succumbing to a lethal dose of LPS (7). Furthermore, IRF-1

mediates the release of HMGB1 by post-translational modification through acetylation (15). These results imply that IRF-1 could play a key role in pyroptosis-associated cytokines.

Toll-like receptor 4 (TLR4) functions in pathogen-associated molecular pattern recognition and initiation of immune responses and inflammatory processes. It has been confirmed that administration of LPS up-regulates lung TLR4 expression and gene silencing of TLR4 attenuates inflammatory response and lung injury (24). Collectively, these data support an emerging concept that the quantity of TLR4 expressed on AMs will modulate IRF-1 activation to modify pyroptotic inflammatory signaling and elicit cytokine expression.

We also identified TLR4 as necessary for the activation of IRF-1 (Fig. 5). However, the mechanism by which TLR4 mediates IRF-1 expression in AMs compromised by LPS remains to be elucidated. The myeloid differentiation factor 88 (MyD88) adaptor protein has been shown to recruit members of the IRF-1 family of transcription factors to evoke TLR target genes. Indeed, IRF-1 has been shown to be a participant in MyD88 signaling and migrates to the nucleus more effectively after stimulation with various TLR ligands (25). Our experiments demonstrate that IRF-1 serves as a link between the initial upstream LPS-induced TLR4 signal and downstream pathological mechanisms by activating genes with pro-pyroptotic functions.

Based on these observations, we conclude that that IRF-1 plays a key role in controlling caspase-1-dependent pyroptosis and inflammation. LPS-induced pyroptosis in AMs is a process that is dependent on TLR4-mediated upregulation of IRF-1. By identifying the molecular mechanisms of IRF-1-mediated caspase-1 signaling in macrophage pyroptosis and the associated inflammation, our study provides a novel therapeutic approach to management of ALI/ARDS.

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