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ORIGINAL ARTICLE

Phosphorylated Heat Shock Protein 27 Inhibits Lipopolysaccharide-Induced Inflammation in Thp1 Cells by Promoting TLR4 Endocytosis, Ubiquitination, and Degradation

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Abstract— The aims of this study were to investigate the effect of Hsp27 on LPS-induced inflammation and identify the precise mechanisms about how Hsp27 regulates LPS-induced TLR4 signaling in Thp1 cells. Thp1 cells were transfected with Flag-Hsp27 or pcDNA3.1, and then treated with LPS for indicated time. TNF-α, IL-1β, and IL-6 were determined by ELISA. The protein levels of Hsp27, p-Hsp27 (Ser15, Ser78, and Ser82), and TLR4 were measured by Western blotting. *In vitro* study showed that over-expression of Hsp27 down-regulated the release of TNF-α, IL-1β, and IL-6 and suppressed the activation of TLR4 signals after stimulated by LPS. The location of TLR4 and RAB5 was detected by confocal microscopy. Immunoprecipitation was used to determine the ubiquitination and degradation of TLR4 and interaction between Hsp27 and TLR4. Results showed that Hsp27 could promote TLR4 endocytosis and ubiquitination and degradation. Further research revealed that Hsp27 was phosphorylated after LPS, only phosphorylated Hsp27 can interact with TLR4 and inhibit the activation of TLR4 signaling, which was demonstrated by inhibition of Hsp27 phosphorylation with inhibitors or transfection of Hsp27 mutants into Thp1 cells. Phosphorylated Hsp27 reduced the release of TNF-α, IL-1β, and IL-6, and suppressed the activation of TLR4 signaling by promoting TLR4 endocytosis, ubiquitination, and degradation.

KEY WORDS: inflammation; Hsp27; phosphorylation; LPS; TLR4.

INTRODUCTION

The bacterial inflammation caused by biological factors is the most common and widespread in biological world. The main component of inflammation caused by bacterial infection is lipopolysaccharide (LPS), the major component of outer membrane of Gram-negative [1]. LPS firstly activates monocytes/macrophages, and triggers

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induction of the mitogen-activated protein kinase (MAPK)/MyD88-independent signal pathways, which plays an important role in the induction of inflammatory mediators [2, 3]. The normal inflammatory response is benefit to the body's immunity. However, excessive inflammatory reaction can cause a series of diseases, including tumor, hypertension, atherosclerosis, rheumatoid arthritis, *etc.* [4]. Thus, it is very important to explore and control excessive inflammatory reactions.

TLR receptors are first found in Drosophila; later on, they have been determined that it plays a pivotal role in the pathophysiology of inflammatory response [5]. There are 10 TLRs (TLR1–TLR10) that have been found in humans; TLRs can specifically identify and combine the pattern recognition receptor (PRR) [6, 7]. For example, TLR4 can recognize microbe-related ligands (such as LPS) and initiate a signaling cascade including myeloid differentiation primary response 88 (MyD88)-dependent pathway [8] and the TIR domain-containing adaptor-inducing IFN-β (TRIF)-dependent pathway [9]. MyD88 is common to most TLRs which can activate their downstream signaling cascades including MAPK and NF-κB [10]. While IRF3 can be transported into the nucleus by combining with the transcription factor CBP/P300 and induce the expression of interferon which leads to signal cascade amplification [11]. Many reports showed that downregulation of TLR signaling could obviously reduce the activation of inflammatory reaction [12–14], and found that MyD88 deficiency also takes effect [15].

Hsp27 is traditionally viewed as a member of the small heat shock protein family [16], which has been reported to play an important role in response to many stimulations [17, 18]. Our previous study revealed the anti-inflammatory effect of Hsp27 in LPS-induced RAW264.7 and Thp-1 cells [19]. After stimulated by LPS, Hsp27 is phosphorylated at several distinct serine residues (Ser15, Ser78, and Ser82), which could block the activation of many signal pathways including nuclear transcription factor kappa-B (NF-κB) and mitogen-activated protein kinases (MAPKs) [20]. It is reported that NF-κB could translocate into nuclear and regulate many genes expression during inflammatory responses [21]. Hsp27 can suppress NF-κB translocation from cytoplasm to nuclear by blocking the degradation of Iκβα ubiquitination [22]. The MAPKs are intracellular serine/threonine protein kinases, including extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinases (p38); the phosphorylation of ERKs, JNK, and p38 could induce the release of many pro-inflammatory factor such as TNF-α, IL-1β, and IL-6 [23]. As a result, decrease the phosphorylation of p38 and ERK, JNK is an effective way to control inflammation.

In this study, we revealed that only phosphorylated Hsp27 could suppress the activation of TLR4 signaling *via* binding with TLR4 and promoting TLR4 endocytosis and ubiquitination and degradation after LPS stimulation.

MATERIALS AND METHODS

Antibodies and Reagents

Monoclonal antibodies recognizing ERK, JNK, phospho-JNK (Thr183/Tyr185), p38, phospho-p38 (Thr180/Tyr182), IκBα, phospho-IκBα, IRF3, phospho-IRF3, and ubiquitin were purchased from Cell Signaling Technology (Beverly, MA, USA). Transfection reagent X-tremeGENE™ HP DNA and LPS (from *Escherichia coli 0111:B4*) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyclonal antibodies against β-Actin, Flag-tag, Hsp27, and phospho-Hsp27 (Ser15, Ser78, Ser82) were purchased from Bioworld Technology (Minneapolis, MN, USA). Normal mouse IgG and normal rabbit IgG were obtained from Vazyme Biotech. PF3644022 and SB203580 were purchased from Abcam (Cambridge, UK).

Cell Culture

Human Thp1 (mononuclear cells) were purchased from the CBCAS (Cell Bank of the Chinese Academic of Sciences, Shanghai, PR China). Cells were cultured in 1640 media containing 15% (*v*/*v*) fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) (Hyclone) at 37 °C in an atmosphere of 5% CO2.

Plasmids

Endo-free plasmids pcDNA3.0 and Flag-Hsp27 (Hsp27-WT); non-phosphorylation mutants including Flag-Hsp27-3A (S15/78/82A, Hspp27-3A); and phosphorylation mutants including Flag-Hsp27-3D and JNK-APF were preserved by laboratory. Using PCR amplifies Hsp27 mutants, and the results were sequenced.

Western Blotting

Cells were washed twice with ice-cold phosphate-buffered saline (PBS), and whole cell lysates add lysate

RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) which supplemented with phosphatase inhibitor (Roche) and protease inhibitor cocktail. The protein concentration of the lysates was determined using the BCA protein assay reagent according to the manufacturer's instructions. Every sample (20 μg) protein was electrophoresed in 12% SDS-PAGE, and then transferred the gels to polyvinylidene difluoride (PVDF). The membranes were blocked with 5% (*w*/*v*) non-fat milk in TBST for 1 h, and target proteins were incubated with indicated primary antibodies at 4 °C overnight. Then, the membranes were incubated with their respective HRP-conjugated secondary antibody at room temperature for 1 h. Target signaling was visualized by chemiluminescence method using the enhanced ECL immunoblotting system (Tanon, Shanghai, China).

Confocal Microscopy

Cells were cultured on micro-cover glass coverslips for 12 h and transfected Flag-Hsp27 or pcDNA3.1 24 h, then stimulation with LPS (500 ng/ml) for another 4 h. Cells were fixed with 4% paraformaldehyde for 20 min, and then permeabilized in 0.2% Triton X-100 in PBS, incubated in 5% bovine serum albumin in PBS to blocking solution for 1 h, and incubated separately with TLR4 (rabbit polyclonal) and RAB5 antibodies overnight at 4 °C. Fluorescent antibody, anti-rabbit, and anti-mouse were used for another 1 h. Cells were washed twice with PBS, and then were stained with DAPI for 5 min and washed twice with PBS. Images were acquired using a confocal laser-scanning microscope.

Co-immunoprecipitation and Immunoblot Analysis

Cell lysates were centrifuge for 5000 rpm, 15 min. Proteins were firstly immunoblotting with specified antibodies, flowed incubated with the Protein A/G PLUS-agarose beads for 2 h, and washed 4 times with lysis buffer. Immunoblotting was used to detected specific protein. In order to confirm specified expression of proteins, whole cell lysates were subjected to immunoblotting.

ELISA

The levels of TNF-α, IL-1β, and IL-6 in the cell culture medium were determined by corresponding ELISA kits, according to their manufacturer's instructions.

Statistical Analysis

Every experimental data analyses were repeated three times with similar trends. The data are shown as mean ± SD. Statistical analysis was assayed by Student's *t* test and one-way ANOVA. Statistical calculations were performed by SPSS13.0. In all analyses, $*p < 0.05$ means significant, n.s. means no significance.

RESULTS

Hsp27 Inhibits LPS-Induced Productions of TNF-α, IL-1β, and IL-6 in Thp1 Cells

TNF-α, IL-1β, and IL-6 are now recognized as the important pro-inflammatory factors [24, 25]. These factors play important role in inflammatory reaction. Firstly, detect whether HSP27 has anti-inflammatory effect. We transfected plasmid Hsp27 vector (pcDNA3.1-Flag-Hsp27) or empty vector (pcDNA3.1) 1 μg into Thp-1 cells, and then stimulate with LPS (500 ng/ml) for 18 h, and ELISA kits were used to detect the levels of TNF-α, IL-1β, and IL-6 in the supernatants. As shown in Fig. 1a–c, the total level of TNF-α, IL-1β, and IL-6 induced by LPS have been significantly suppressed by Hsp27.

Hsp27 Inhibits LPS-Induced Activation of TLR4 Signaling Pathways

TLR4 signals include myeloid differentiation primary response88 (MyD88)-dependent pathway and the TIR domain-containing adaptor-inducing IFN-β (TRIF)-dependent pathway. They have played important roles in LPS-induced inflammation. MAPKS consist of MKKK, MKK, MAPK, and three conservative subgroups kinases, including ERK, JNK, and P38. Activation of both signaling pathways could induce the expression of many transcription factor and lead to signal cascade amplification. We next test if Hsp27 could inhibit LPS-induced production of pro-inflammatory *via* downregulation of TLR4 signaling pathways.

We divide Thp1 cells into two groups, and Thp1 cells were transfected with plasmid Flag-Hsp27 and pcDNA3.1 for 48 h, respectively, and stimulated with or without LPS (500 ng/ml) for different time, and target proteins were detected by Western blotting. As shown in Fig. 2a, we found that Hsp27 have no obvious effect on the expression of ERK, JNK, and p38. However, Hsp27 inhibited phosphorylation of ERK, JNK, and p38 which indicated that Hsp27 could suppress the activation of MAPKs. Western blotting results (Fig. 2b) also showed that Hsp27 suppressed the activation

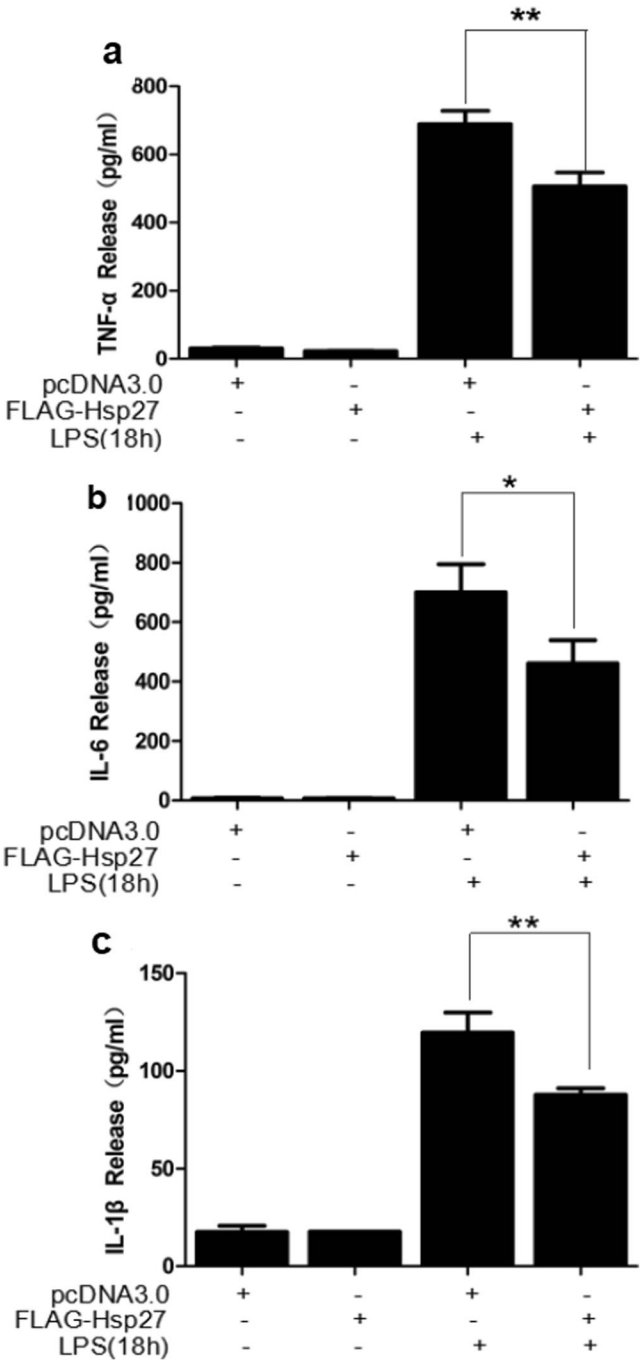

Fig. 1. Over-expression of Hsp27 inhibits the release of inflammatory factors. Thp1 cells were transient transfected with Flag-Hsp27 or pcDNA3.0 plasmids 1 μg for 48 h, and then were treated with LPS (500 ng/ml) for 18 h. The supernatants of cultured cells were collected and the release of TNF-α (**a**), IL-1 β (**b**), and IL-6 (**c**) was detected by ELISA kit, respectively. The experimental data shown are all from three independent repeated experiments, Student's *t* test was used in statistical comparisons, and the results are expressed as the mean ± standard deviation of the experimental data. $*p < 0.05$; $**p < 0.01$.

of NF-κB. Furthermore, Fig. 2c indicated Hsp27 downregulation of LPS-induced IRF3 signaling by inhibiting the phosphorylation level of Irf3. All results above showed that Hsp27 could inhibit LPS-induced TLR4 signal pathways.

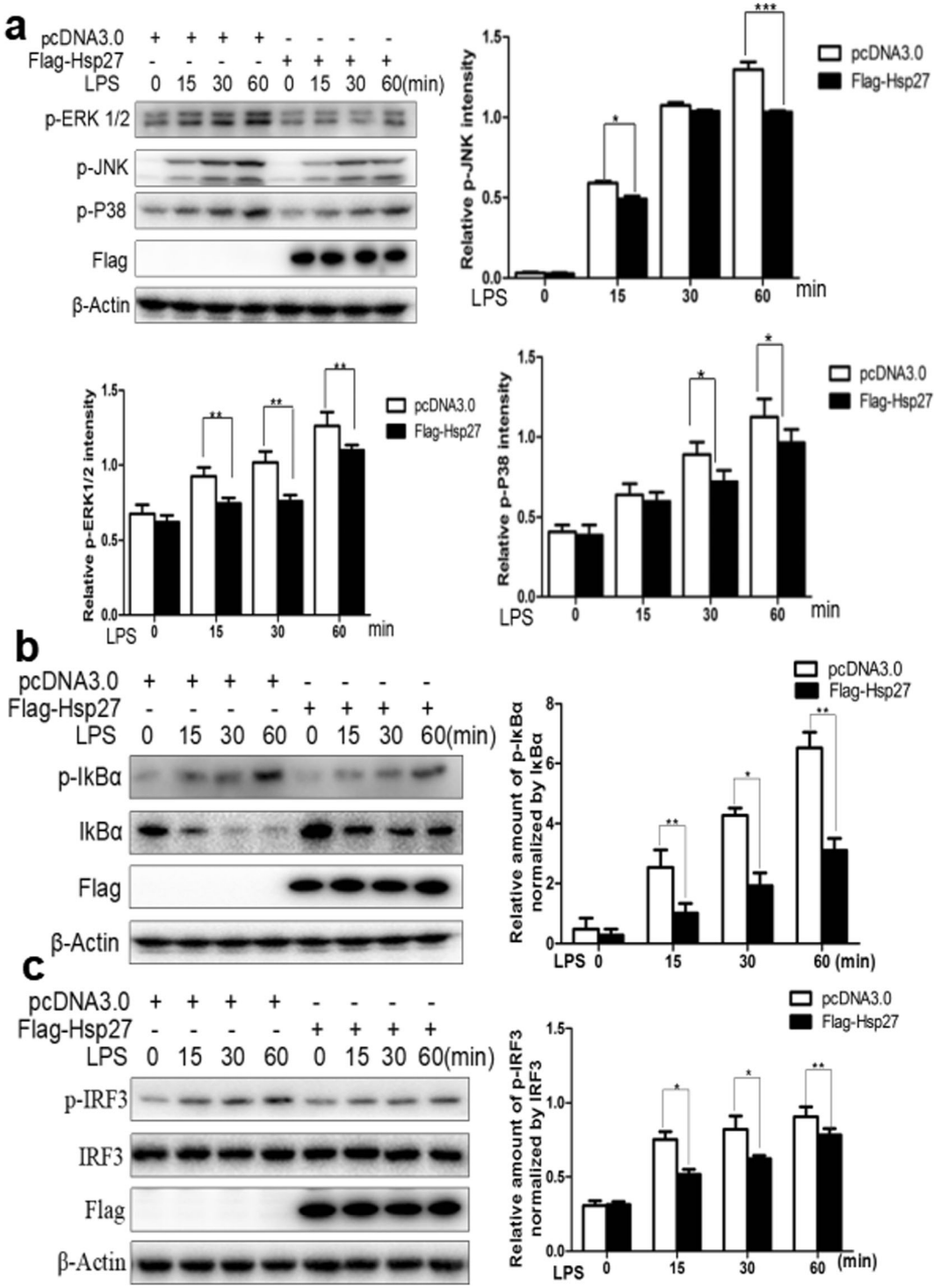

Fig. 2. Over-expression of Hsp27 regulates TLR4 signaling pathway induced by LPS. Thp-1 cells were transient transfected with Flag-Hsp27 or pcDNA3.0 plasmid for 48 h and treated with LPS (500 ng/ml) for different time (0, 15, 30, 60 min). **a** The protein levels of p-ERK, ERK, p-JNK, JNK, p-P38, and P38 were detected by Western blotting. **b** The protein levels of p-IκBα and IκBα were detected by Western blotting. **c** The protein levels of p-IRF3 and IRF3 were detected by Western blotting. The experimental data are all of from three independent repeated experiments, one-way ANOVA was used in statistical comparisons, and the results were expressed as the mean ± SD of the experimental data. $*p < 0.05$; $**p < 0.01$.

Over-expression of Hsp27 Promotes TLR4 Endocytosis, Ubiquitination, and Degradation

It has been reported that the quantity of TLR4 protein plays an important role in LPS-induced inflammation [26]. Our data showed that Hsp27 could downregulate TLR4 signaling pathways after LPS stimulation; we next want to know if Hsp27 has an effect on the total level of TLR4 protein.

As shown in Fig. 3a, over-expression of Hsp27 reduced the total amount of TLR4 protein only after LPS stimulation. Cells were pretreated with lysosome inhibitor chloroquine (10 μM) for 4 h to inhibit the degradation of protein, followed transfected with pcDNA3.1 or Hsp27, followed by stimulation with LPS. The total amount of TLR4 ubiquitination level

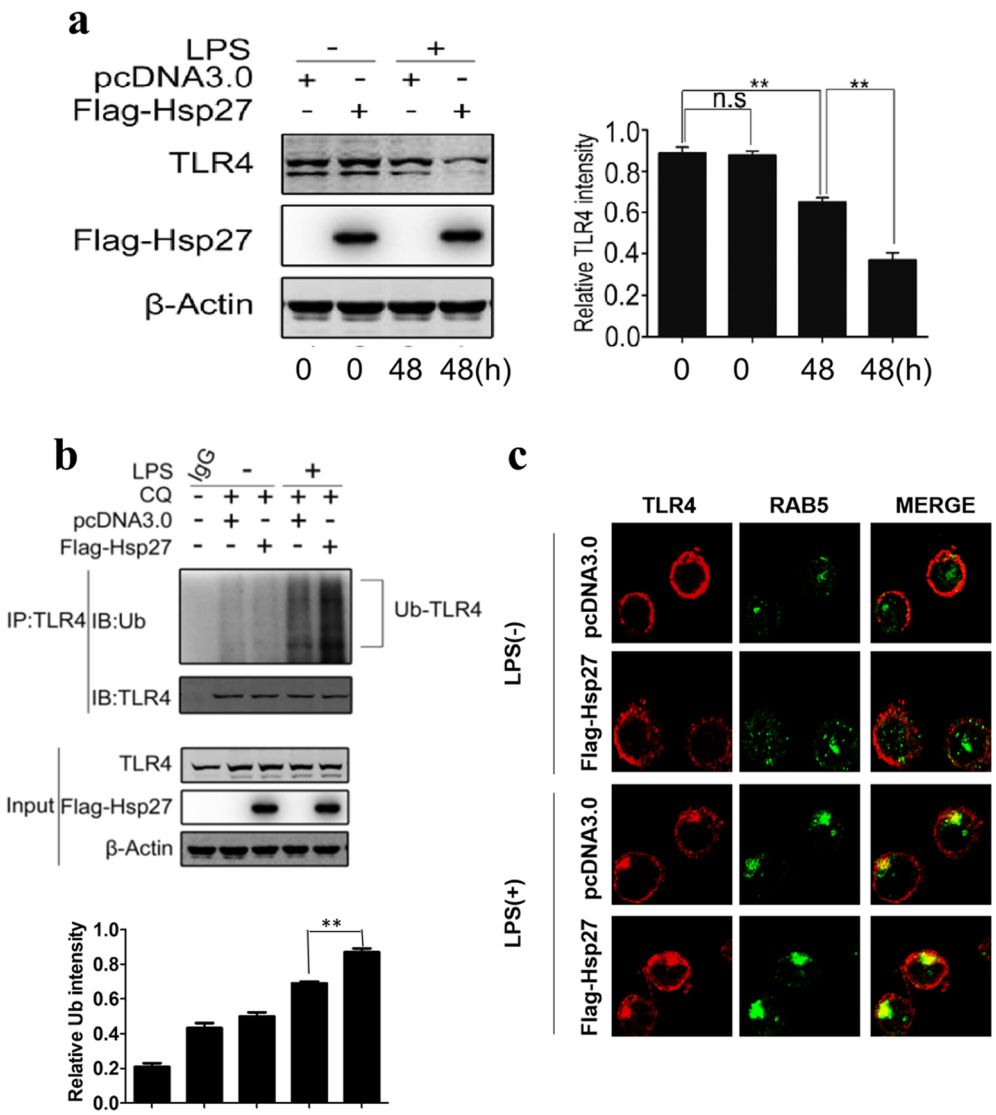

Fig. 3. Over-expression of Hsp27 promotes TLR4 endocytosis, ubiquitination, and degradation. Thp1 cells were transient transfection of Flag-Hsp27 or pcDNA3.0 plasmid for 48 h. **a** Followed stimulated with LPS (500 ng/ml) for 4 h, and then the cell lysates were collected, and subjected to Western blotting by using anti-TLR4 and anti-Flag antibodies. **b** The cells were incubated with or without CQ for 4 h, and then treated with or without LPS (500 ng/ml) for 4 h. Cell lysis was collected and Ub antibody was added for immunoprecipitation to detect the ubiquitin level of TLR4. **c** The cells were treated with LPS (500 ng/ml) for 15 min, and then co-localization of Rab5 and TLR4 was observed by confocal laser. Scale 10 μm. The experimental data are all from three independent repeated experiments, one-way ANOVA was used in statistical comparisons, and the results were expressed as the mean ± standard deviation of the experimental data. $*p < 0.05$; $**p < 0.01$.

is obviously increased after treatment with LPS (Fig. 3b). While over-expression of Hsp27 further promoted this process. Over-expression of Hsp27 could facilitate TLR4 translocating into lysosomes and increase TLR4 ubiquitination. However, TLR4 has to be translocated from the surface of cell membrane into endosome before ubiquitination [27]. As a result, we guess that Hsp27 could promote TLR4 ubiquitination by endocytosis. Rab GTPases (Rab5) is the sign of the early endosome [28]. Rab5 plays an important role in generating endocytic vesicles at the cell surface, and also can regulate endosome motility by recruiting or activating a minus end-directed microtubule motor 60 [29]. So, we use immunofluorescence and laser confocal scan microscopy to analyze the localization of TLR4 and Rab5 in Thp1 cells. As shown in Fig. 3c, the group which transfected with Hsp27 have greater number of TLR4s be internalized into endosome, as the fluorescence intensity of RAB5 is more obviously. In summary, Hsp27 can promote TLR4 ubiquitination and degradation *via* endocytosis.

Phosphorylated Hsp27 Inhibits TLR4 Signaling Pathway

The above experimental results shown that Hsp27 inhibited LPS-induced inflammation *via* promoting TLR4 endocytosis, ubiquitination, and degradation. Then, how Hsp27 participate in these processes are still unknown. Previous research showed that Hsp27 has multiple phosphorylation sites (Ser15, Ser78, Ser82), and a great deal of stimulation including injury, chemical stimulation, and heat shock could influence Hsp27 phosphorylation [30–32]. So, the next objective of this study is to explore the state of Hsp27 phosphorylation after LPS. As shown in Fig. 4a, LPS increased Hsp27 phosphorylation at Ser15 and Ser78, and has almost no effect on Ser82. The Ser15 and Ser78 phosphorylation peaked at 30 min after LPS, while total Hsp27 protein level remains unchanged.

Then, we approached the effect of Hsp27 phosphorylation on TLR4 downstream signal pathways. SB203580 and PF3644022 could suppress Hsp27 phosphorylation by inhibiting the phosphorylation of p38 MAPK and MK2, respectively [33, 34]. Thp1 cells transfected with pcDNA3.1 or Hsp27 were pretreated with SB203580 (10 μM) and PF3644022 (5 μM) for 2 h, followed stimulating by LPS for 30 min. As shown in Fig. 4b, SB203580 and PF3644022 inhibited Hsp27 phosphorylation at Ser15 and Ser78, and both SB203580 and PF3644022 does upregulate MAPK, NF-κB, and IRF3 signaling (Fig. 4c–e), which suggests that Hsp27 phosphorylation plays a key role in regulating TLR4 downstream signal pathways.

Only Phosphorylated Hsp27 Interacted with TLR4

Thp1 cells were transfected with pcDNA3.0, Hsp27-WT (wild type), Hsp27-3A (non-phosphorylated mutant), or Hsp27-3D (phosphorylated mutant) for 48 h, and then stimulated with LPS for 4 h. As shown in Fig. 5a, over-expression of Hsp27-WT or Hsp27-3D does decrease the total TLR4 protein level after LPS compared with Hsp27-3A, while Thp1 cells pretreated with SB203580 and PF3644022 did not show any decreasing of TLR4 protein level. These results suggest that only phosphorylated Hsp27 after LPS stimulated could inhibit the TLR4 signaling and decreased the total TLR4 protein level.

However, the clear mechanism about how phosphorylated Hsp27 participates in regulating TLR4 protein is still unclear. MD-2 is a soluble protein and binds to the extracellular domain of TLR4, and this complex could be identified by LPS [35]. Recent reports [36] indicate MD-2 knockout mice cannot respond to LPS and survive in endotoxic shock. These evidences indicated that MD-2 is necessary to LPS-induced inflammation. Thus, we suppose that phosphorylated Hsp27 interacting with TLR4/MD-2 complex to regulate LPS-induced TLR4 signaling. Co-immunoprecipitation was used to test this hypothesis. As shown in Fig. 5c, Hsp27 can interact with TLR4, and this interaction is obviously increased after stimulated with LPS. However, we found that KRIBB3, a specific inhibitor of Hsp27 phosphorylation, reduced complex of Hsp27/TLR4 and abolished the disruption of TLR4/MD-2. Then, we devised an innovative secondary immunoprecipitation method to accurately detect whether endogenous Hsp27 could indeed bind to TLR4 with its phosphorylated form. We use p-Hsp27 (Ser78) monoclonal antibody to pull down p-Hsp27 from cell lysates, and TLR4 was detected from this immune complex. Next Hsp27 monoclonal antibody was used to pull down Hsp27 from the supernatants of the first immunoprecipitation. However, there is no TLR4 protein was detected. These results indicated that only phosphorylated Hsp27 could interact with TLR4 (Fig. 5d) and inhibit LPS-induced inflammation.

DISCUSSION

Bacteria especially Gram-negative bacteria could activate the innate immune system by release LPS. LPS can be recognized by TLR4-MD2 complex, trigger

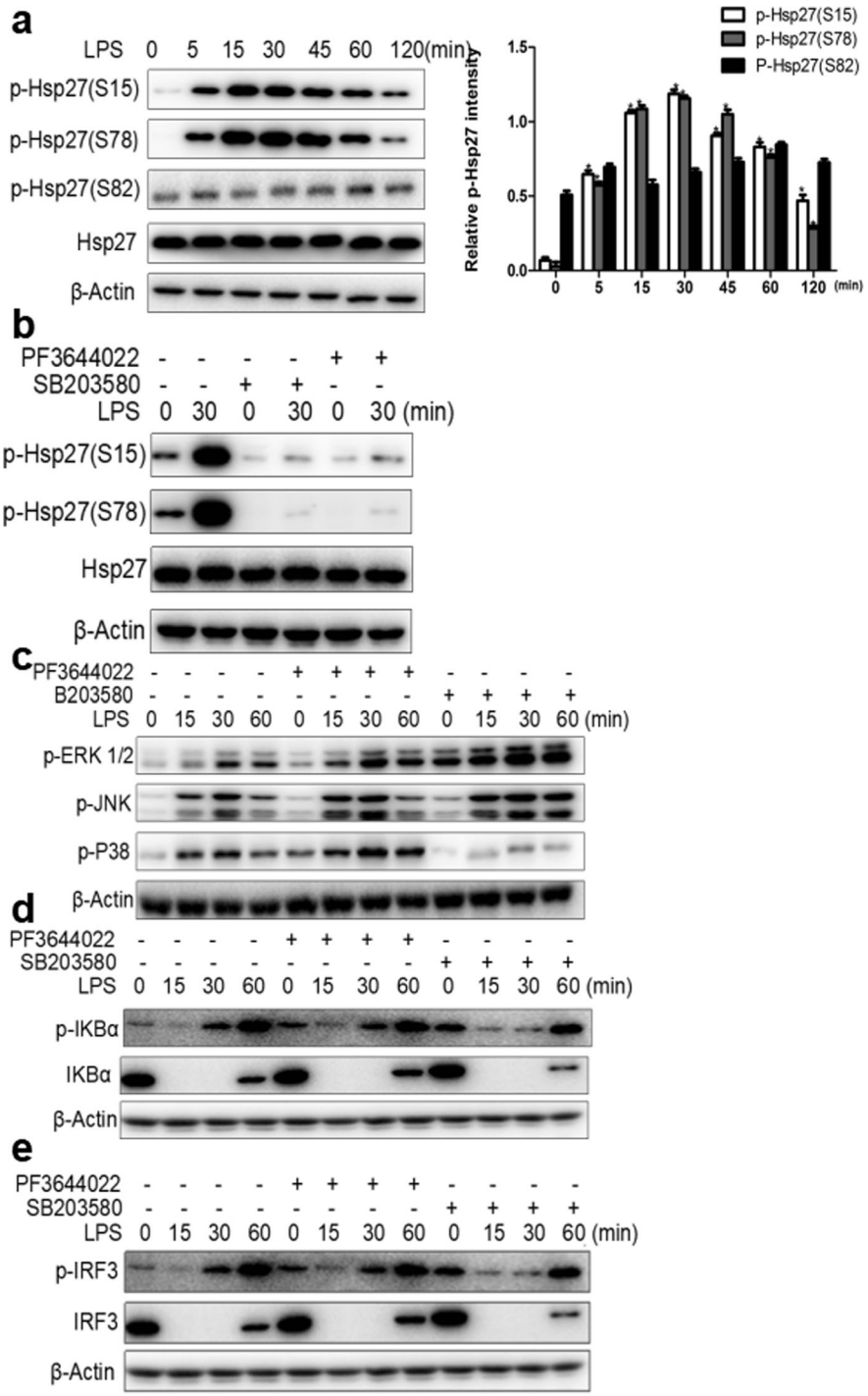

Fig. 4. Phosphorylated Hsp27 participate in TLR4 signaling. **a** THP-1 cells were treated with LPS at different time points. The protein levels of p-Hsp27 (S15), p-Hsp27 (S78), and p-Hsp27 (S82) were detected by Western blot. **b** Thp-1 cells were incubated with SB203580 (10 μM) or PF3644022 (5 μM) for 2 h, and then treated with LPS (500 ng/ml) for 30 min, and the protein level of phosphorylated Hsp27 was detected by Western blot. **c**–**e** Thp-1 cells were incubated with SB203580 (10 μM) or PF3644022 (5 μM) for 2 h, and then cells were treated with LPS (500 ng/ml) for different time and the cell lysis was collected; immunoblotting analysis showed the total p-ERK1/2, p-JNK, p-P38, p-IκBα, IκBα, p-IRF3, and IRF3. The experimental data are obtained from three independent repeated experiments, one-way ANOVA was used in statistical comparisons, and the results are expressed as the mean ± SD of the experimental data. $*p < 0.05$; $**p < 0.01$.

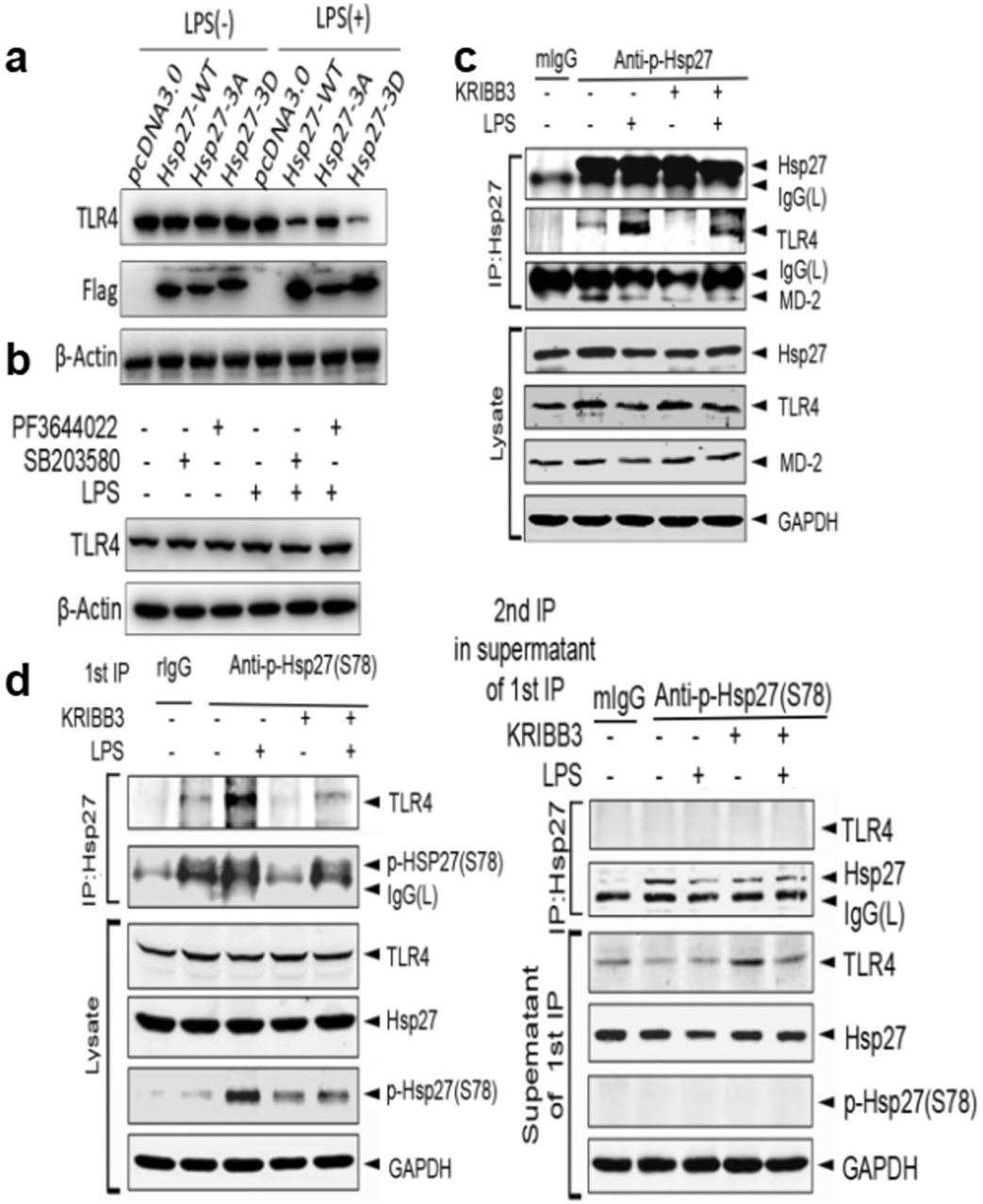

Fig. 5. Phosphorylated Hsp27 interacted with TLR4. **a** Hsp27-WT, Hsp27-3A, Hsp27-3D, and empty plasmid pcDNA3.0 were transfected into the Thp-1 cells, followed treated with LPS (500 ng/ml) for 4 h, the level of TLR4 protein was detected by Western blot. **b** Thp-1 cells were incubated with PF3644022 or SB203580 for 2 h, followed treated with or without LPS (500 ng/ml) for 4 h, the level of TLR4 protein was detected by Western blot. **c** Thp1 cells were pretreated with KRIBB3 (1 μM) for 1 h (Lane 4 and 5), and lysed after LPS (500 ng/ml) challenge for 30 min. Cell lysates were subject to immunoblot analysis with anti-Hsp27, anti-TLR4, anti-MD-2, and anti-GAPDH. The same lysates were immunoprecipitated with anti-Hsp27 monoclonal antibody followed by immunoblot analyzed with anti-Hsp27, anti-TLR4, and anti-MD-2 antibodies. **d** 1st IP: Thp-1 cells were pretreated with KRIBB3 for 2 h, and then treated with LPS (500 g/ml) for 4 h. Cell lysates were subject to immunoblot analysis with anti-TLR4, anti-Hsp27, anti-p-Hsp27 (Ser78), and anti-GAPDH. The same lysates were immunoprecipitated with anti-p-Hsp27 (Ser78) monoclonal antibody followed by immunoblot analyzed with anti-TLR4 and anti-p-Hsp27 (Ser78) antibodies. 2nd IP: Supernatant of first IP was subject to immunoblot analysis with anti-TLR4, anti-Hsp27, anti-p-Hsp27 (Ser78), and anti-GAPDH. The same supernatant was immunoprecipitated with anti-Hsp27 monoclonal antibody followed by immunoblot analyzed with anti-TLR4 and anti-p-Hsp27 (Ser78) antibodies. The experimental data are obtained from three independent repeated experiments; one-way ANOVA was used in statistical comparisons, $*p < 0.05$; $**p < 0.01$.

inflammatory pathways, and induce the activation of MyD88-dependent/MyD88-independent pathways. Meanwhile, a series of pro-inflammatory factors (cytokines, chemokines, or costimulatory molecules) are released, which can led to sepsis, systemic inflammatory, and even death. It is known that LPS could induce endocytosis of TLR4, which is essential for its signaling function. And Hsp27 has been determined as an anti-inflammatory factor [37–39]. Therefore, we highlight effect of Hsp27 in LPS-induced inflammation, and try to elucidate the precise molecular mechanism utilized by Hsp27.

Firstly, we want to know whether Hsp27 can inhibit the accumulation and amplification of inflammatory signals. *In vivo* studies showed that LPS stimulated upregulated the release of TNF-α, IL-1b, and IL-6. And the group that over-expression of Hsp27 markedly inhibit that. Further study shown over-expression of Hsp27 inhibited TLR4 downstream signaling including ERK1/2, JNK, and p38, which plays important role in LPS-induced inflammation.

TLR4 is the upstream of these signals, and its quantity determines the strength of downstream signals [40]. It has been reported that Toll-like receptor 4 deficiency increases resistance in sepsis-induced immune dysfunction [41]. In resting state, TLR4 was distributed in the cell membrane and Golgi body. In response to LPS stimulation, TLR4 is activated as a receptor of LPS and transported into the inner body for ubiquitination and degradation [42], and then activates downstream signaling. So, we want to know if Hsp27 could affect the total TLR4 protein level. Result shown that over-expression of Hsp27 decreased the total number of TLR4 *via* ubiquitination and degradation, and laser confocal results further proved this conclusion. As a result, we confirmed that Hsp27 does negatively regulate the inflammatory response *via* decrease the total level of TLR4 and its downstream signaling.

Secondly, we want to know how Hsp27 inhibits LPS-induced inflammation. It has been reported that phosphorylated Hsp27 participates in regulating many physiological responses. For example, research in our laboratory found phosphorylated Hsp27 could regulate JNK and NF-κB signaling pathways, and even could promote lipid clearance in hepatic cells [43, 44]. In this study, we found LPS increased Hsp27 phosphorylation at Ser15 and Ser78. As a result, we guess that Hsp27 phosphorylation is important in LPS-induced inflammation. In order to verify this conjecture, Hsp27 phosphorylation inhibitors, SB203580 and PF3644022, were used to pretreat Thp1 cells after transfection with pcDNA3.1 or Hsp27 independently. Results showed that both SB203580 and PF3644022 can block the inhibitory effect of Hsp27 on TLR4 downstream signaling induced by LPS. Based on the above results, we believe that phosphorylation of Hsp27 could regulate LPS-induced inflammation.

At last, we want to investigate the more detailed molecular mechanism of how phosphorylated Hsp27 functions as negative regulator of inflammation. We speculate whether phosphorylated Hsp27 can also affect the total TLR4 protein level. So, we explored the effect of phosphorylated Hsp27 on the total protein of TLR4 by adding Hsp27 phosphorylation inhibitors or over-expression of Hsp27-3D (a phosphorylation Hsp27 mimic mutant). Results show that only phosphorylated Hsp27 can interact with TLR4 and promote TLR4 endocytosis.

In summary, this study indicated that phosphorylated Hsp27 is involved in regulating LPS-induced inflammation through inhibiting both MyD88 and IRF3 signaling. We also proved that phosphorylated Hsp27 exhibit its anti-inflammation function at least in part through interacting with TLR4 and promoting TLR4 endocytosis followed by ubiquitination and degradation. This study elucidates a novel mechanism of how Hsp27 regulates TLR4 signal pathways and suppresses LPS-induced inflammation in Thp1 cells.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

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