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Original Article

Methylation of TTC4 interaction with HSP70 inhibits pyroptosis in macrophages of sepsis-induced lung injury by NLRP3 inflammation

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Abstract: Acute lung injury (ALI) is an acute infectious diseases caused by a variety of factors. The function of TTC4 in sepsis-induced lung injury remains largely unknown. This study aimed to explore the critical role of TTC4 in sepsis-induced lung injury. Mice anaesthetized using pentobarbital sodium and subjected to cecal ligation and puncture (CLP) surgery. TTC4 expression levels in patients with sepsis-induced lung injury were down-regulated. The inhibition of TTC4 gene promoted lung injury in mice model of sepsis. TTC4 gene improved inflammation in vitro model and mice model. TTC4 gene reduced pyroptosis in macrophages of sepsis-induced lung injury by the inhibition of mitochondrial damage. TTC4 gene induced HSP70 expression to reduce NLRP3-induced pyroptosis in macrophages. TTC4 protein interlinked HSP70 protein. The activation of HSP70 reduced the effects of sh-TTC4 in model of sepsis-induced lung injury through mitochondrial damage. m6A-forming enzyme METTL3 reduced TTC4 stability. Our study suggests the m6A forming enzyme METTL3 control TTC4 reduced inflammation and pyroptosis in model of sepsis-induced lung injury through inhibition of mitochondrial damage by HSP70/ROS/NLRP3 signaling pathway, TTC4 gene as an represents a potential therapeutic strategy for the treatment of sepsis-induced lung injury.

Keywords: TTC4, pyroptosis, HSP70, itochondrial damage, NLRP3

Introduction

Acute lung injury (ALI) is an acute infectious diseases caused by various factors, including trauma, pneumonia, shock and septicemia [1]. ALI is characterized by the infiltration of inflammatory cells, excessive production of inflammatory mediators, diffuse pulmonary inflammation, and damage to lung parenchymal cells such as vascular macrophages and alveolar epithelial cells [2].

The damage to lung parenchymal cells results in impaired ventilation and respiratory dysfunction, leading to rapid deterioration of respiratory function. Clinical manifestations of critical illness include increased respiratory rate, respiratory distress, and refractory hypoxemia [3]. If not promptly treated, ALI can progress to acute respiratory distress syndrome [3]. Therefore, it is crucial to inhibit the inflammatory response for the treatment of ALI. Multiple immune cells and lung parenchymal cells are involved in the initiation and progression of the inflammatory response [4]. Macrophage activation plays a pivotal role in the entire process of inflammation initiation, amplification, and tissue injury, making them a crucial immune cell type that contributes to the continuous progression of inflammation and eventual loss of control [5]. Thus, inhibiting the excessive activation of macrophages and reducing the release of inflammatory factors are important strategies for controlling the inflammatory response and mitigating

ALI [6]. The activation of NLRP3 inflammatory corpuscles can be replaced by NF-κB, which in turn activates and releases the pro-inflammatory factors IL-1β/IL-18, leading to the development of inflammation in an uncontrollable direction [7]. This indicates that the activation of the NF-κB/NLRP3 signaling pathway promotes the activation and release of IL-1β/IL-18 from alveolar macrophages. These pro-inflammatory factors not only kill virus-infected cells but also cause damage to lung tissue. Therefore, it can be seen that the excessive activation of alveolar macrophages is one of the main factors contributing to ALI [8, 9].

Macrophages play a crucial role in the innate immune system of the body, acting as "guards" responsible for phagocytosis and killing of pathogenic microorganisms [10]. Their activation is beneficial for the body's defense mechanism. However, in response to more aggressive invaders, the level of macrophage activation is further increased [11]. Lipopolysaccharide (LPS), which is located in the cell wall of pathogens, is an important molecule that induces macrophage activation [11]. When the pattern recognition receptors on the surface of macrophages bind to LPS, it triggers internal signal transduction pathways, leading to the activation of downstream target proteins and exerting biological effects [12].

Methylation of m6A is a common form of RNA epigenetic modification that can regulate gene expression by affecting RNA translation and degradation, potentially influencing the occurrence and progression of diseases [13]. In recent years, there has been increasing focus on the role of m6A in lung diseases such as pulmonary fibrosis and lung cancer, and it has been shown to play a significant role in their development [14]. The enzyme complex involved in m6A methylation consists of METTL3 and METTL14, which form a heterodimer catalytic core, and a regulatory subunit called WTAP, working together to promote methylation reactions [15]. HSP70, a member of the heat shock protein family, has endogenous protective mechanisms that include stabilizing cell membranes, antioxidant effects, promoting normal protein synthesis, assisting protein folding, and inhibiting cell apoptosis [16]. Recent research findings suggest that the innate immune response mediated by HSP70 may play a crucial role in the inflammatory process through the ROS-NLRP3 signaling pathway [17, 18]. This study aimed to explore the critical role of TTC4 in sepsis-induced lung injury.

Materials and methods

Patients experiment

A total of 12 patients with sepsis and 12 normal healthy volunteers were obtained from January 2019 to May 2020 at our hospital. The written informed consents were obtained from all the subjects and this study was approved by the Ethics Committee of our hospital (No. 20200819). No patients had received chemotherapy or pre-operative radiotherapy. All the samples were centrifugated at 4°C for 10 min, serum was collected and immediately stored at -80°C.

Quantitative PCR

The total RNA was extracted from serum and cell samples using a TRIZOL reagent (Life Technologies Inc.). qRT-PCR assays were performed using Light Cycler® 480 SYBR Mix (Roche, Germany) using LightCycler® 480 real-time PCR system. The expression levels of mRNA were normalized to the GAPDH expression using the 2-ΔΔct method. Primer sequences: TTC4, 5'-GCCGTGAACTGGTGTGATGAG-3' and 5'-GCCTGATATTCCTAGCCTTGATGG-3'; HSP70, 5'-CGCTCGAGTCCTATGCCTTCA-3' and 5'-GGCACTTGTCCAGCACCTTC-3'; β-Actin, 5'-GAAGTGTGACGTGGACATCC-3' and 5'-CCGATCCACACGGAGTACTT-3'. Thermocycling conditions were as follows: 95°C for 30 s; 95°C for 5 seconds, annealing at 60°C for 35 seconds, followed by 40 cycles; 95°C for 15 s, followed by 60°C for 1 min and 95°C for 15 s.

Mice experiment

C57BL/6 (5-6 weeks old) mice were housed in barrier cages under controlled environmental conditions (22-23°C, 12/12 h of light/dark cycle, 55% ± 5% humidity). At model group, C57BL/6 mice anaesthetized using 50 mg/kg pentobarbital sodium and subjected to cecal ligation and puncture (CLP) surgery. At sham group, C57BL/6 mice were received with normal saline. After induction sepsis, all mice were anaesthetized using 50 mg/kg pentobarbital sodium and then sacrificed using cervical spon-

dylectomy. Next, after induction sepsis, mice were recorded survival rate at every day for three days. Sh-TTC4 virus (10 μg/mice, sc-154778-V, Santa Cruz Biotechnology, Inc.) was injected into mice after CLP surgery.

Immunofluorescence and western blot analysis

Cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 in PBS for 15 min at room temperature, and blocked with 5% BSA for 30 min at 37°C. Cells were treated with primary antibodies at 4°C overnight: anti-TTC4 and anti-HSP70. Cells were then incubated with Cy3-conjugated goat anti-rabbit or goat anti-mouse IgG DyLight 488-conjugated secondary antibodies for 2 h at 37°C. Nuclei were stained with DAPI and cells were observed under a fluorescent illumination microscope (Olympus IX71, Tokyo, Japan).

Total protein was extracted from lung samples or cell samples using Radio-Immunoprecipitation Assay (RIPA) and PMSF reagent (1:100, Beyotime, Beijing, China). Protein lysates were separated based on their molecular weight on SDS/PAGE gels and transferred onto a Polyvinylidene Fluoride (PVDF) membrane. The membrane was blocked with non-fat-milk (5%) for 2 h at room temperature and incubated with anti-TTC4 (1:1000, ab181194, abcam), anti-HSP70 (1:1000, ab2787, abcam), anti-NLRP3 antibody (1:1000, ab263899, abcam), anti-caspase-1 antibody (1:1000, ab207802, abcam), anti-GSDMD (1:1000, ab219800, abcam) and anti-β-actin antibody (1:10000, ab8226, abcam) at 4°C overnight. Then first antibodies were removed and TBST wash membrane using TBST. Membranes were incubated with the secondary antibody for 2 hours at room temperature. The bound antibodies were detected using enhanced chemiluminescence (ECL).

Cell culture and RNA transfection

RAW264.7 cells were cultured in Dulbecco's modified eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) in an incubator at 37°C with 5% CO_2. RAW264.7 cells were transfected with TTC4 plasmid (sc-411044, Santa Cruz Biotechnology, Inc.), si-TTC4 plasmid (sc-88730, Santa Cruz Biotechnology, Inc.) using Lipofectamine 3000. After 48 h, RAW264.7 cells treated with 200 ng/ml of LPS for 4 h and then pulsed with ATP (1 mM, Sigma-Aldrich, MO, USA) for 30 min.

ELISA kits

Tissue or cell samples in each group were collected at 2000 g for 10 min at 4°C. IFNγ, TNFα, MPO, IL-17, IL-6 and IL-1β kits were used to measure the cytokine levels.

Statistical analysis

All statistical analysis was performed in GraphPad Prism 6.0 software. Statistical significance was determined by paired sample t test for intragroup before-after comparison; was determined by one-way ANOVA followed by post hoc pairwise Bonferroni or Turkey test for between-group comparison. $P<0.05$ was considered statistically significant.

Results

TTC4 expression levels in patients with sepsis-induced ALI or mice model

We first evaluated the expression levels of the TTC4 gene in patients with sepsis-induced ALI. We observed a significant inhibition of serum TTC4 mRNA expression levels in patients with sepsis-induced ALI (**Figure 1A**). Furthermore, we found a negative correlation between serum TTC4 mRNA levels and serum IL-1β levels in patients. To assess the diagnostic value of TTC4 levels, we constructed a receiver operating characteristic (ROC) curve (**Figure 1B**, **1C**). Next, we investigated the mRNA and protein expressions of TTC4 in lung tissue of a mouse model of sepsis-induced ALI. We observed a reduction in TTC4 mRNA and protein expressions in the lung tissue of the mouse model (**Figure 1D**, **1E**). Immunofluorescence and immunohistochemistry analysis further confirmed the down-regulation of TTC4 expression in the lung tissue of the mouse model (**Figure 1F**, **1G**). Taken together, these findings suggest that TTC4 plays an inhibitory role in sepsis-induced ALI.

The inhibition of TTC4 gene promoted lung injury in mice model of sepsis

The study investigated the effects of TTC4 in a mouse model. The introduction of the Sh-TTC4

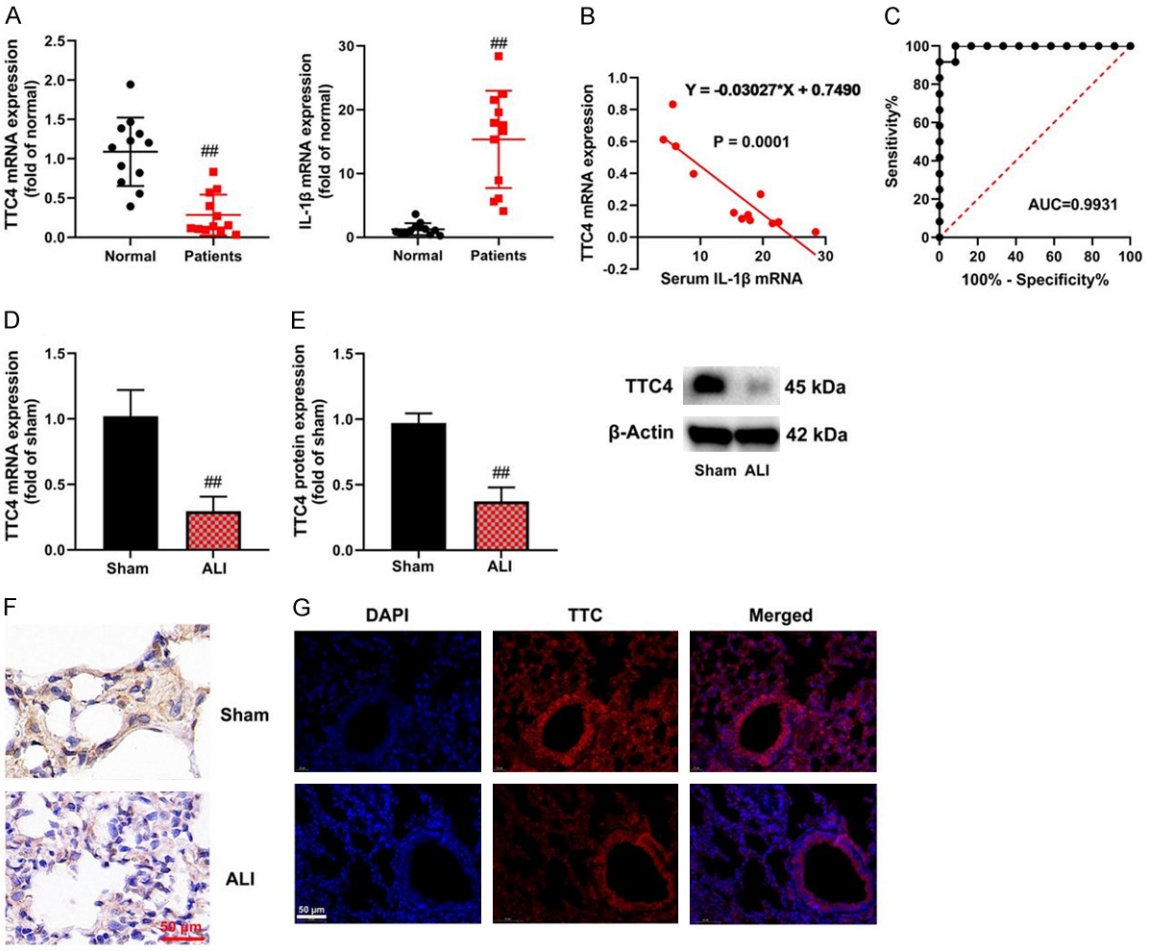

Figure 1. TTC4 expression levels in patients with sepsis-induced ALI or mice model. TTC4 mRNA expression levels (A), TTC4 was negative correlation with serum IL-1β levels (B), ROC (C) in patients, TTC4 mRNA and protein expressions (D, E), TTC4 expression (Immunofluorescence and immunohistochemistry, F and G) in mice model. ##P<0.01 compared with normal or sham control group.

virus resulted in a decrease in TTC4 mRNA expression and an increase in total cells and eosinophils in the bronchoalveolar lavage fluid (BALF). Additionally, it led to an increase in neutrophils and lymphocytes, elevated serum IgE and HDM-specific IgE levels, and aggravated lung injury in mice with sepsis (**Figure 2A-H**). The Sh-TTC4 virus also enhanced the levels of inflammation factors in the sepsis mouse model (**Figure 2I-O**). These findings suggest that the inhibition of TTC4 promotes inflammation and lung injury in mice with sepsis.

TTC4 gene improved inflammation in vitro model

Furthermore, we investigated the effect of TTC4 on inflammation in an in vitro model of sepsis. The expression of TTC4 mRNA was increased in the overexpression of TTC4 group, and downregulated in the si-TTC4 group (**Figure 3A**, **3B**). Overexpression of TTC4 resulted in a decrease in IL-1β, IL-6, INF-γ, and TNF-α levels in the in vitro sepsis model (**Figure 3C**). In contrast, downregulation of TTC4 led to a reduction in IL-1β, IL-6, INF-γ, and TNF-α levels in the in vitro sepsis model (**Figure 3D**). These findings suggest that TTC4 promotes inflammation in the in vitro sepsis model.

TTC4 gene reduced pyroptosis in macrophages of sepsis-induced lung injury by the inhibition of mitochondrial damage

Additionally, we investigated the impact of TTC4 on cell growth, LDH activity levels, mitochondrial function, and GSDMD protein expression in both in vitro and mice models. In the in vitro model, overexpression of TTC4 resulted in increased cell growth, decreased LDH activity

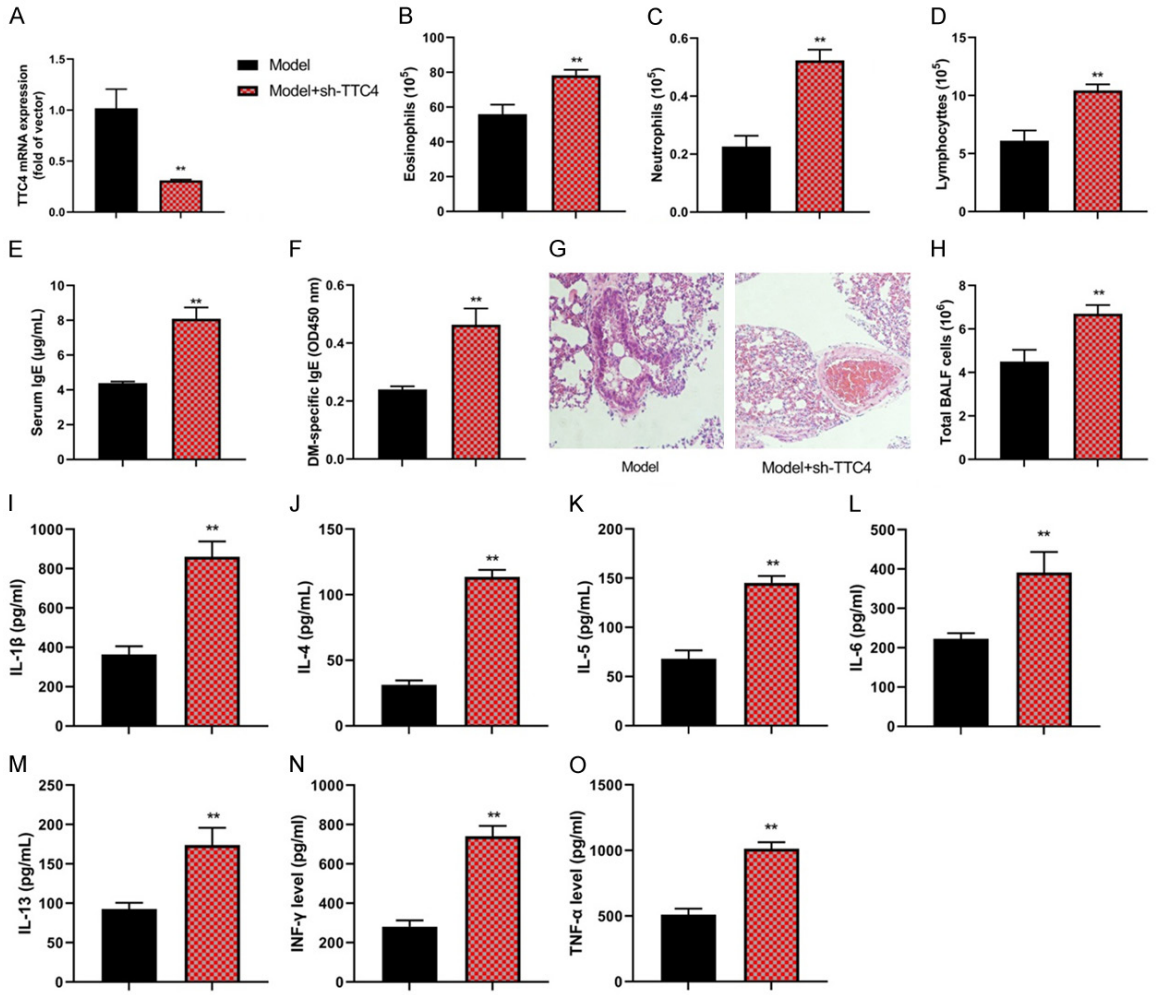

Figure 2. The inhibition of TTC4 gene promoted lung injury in mice model of sepsis. TTC4 mRNA expression (A), Eosinophils (B), neutrophils (C), Lymphocyttes (D), serum IgE (E), HDM-specific IgE levels (F), lung injury (HE staining, G), Total cells of BALF (H), IL-1β/IL-4/IL-5/IL-6/IL-13/INF-γ/TNF-α (I-O). **P<0.01 compared with model group.

levels, increased JC-1 disaggregation, and enhanced MPT (calcein AM/CoCl2 assay), indicating improved mitochondrial function (**Figure 4A-H**). Furthermore, overexpression of TTC4 suppressed the protein expression of GSDMD, while downregulation of TTC4 induced GSDMD protein expression in the in vitro model (**Figure 4I**, **4J**). In the mice model, the introduction of sh-TTC4 virus induced GSDMD protein expression (**Figure 4K**). These findings suggest that TTC4 gene reduces pyroptosis in macrophages during sepsis.

TTC4 gene induced HSP70 expression to reduce NLRP3-induced pyroptosis in macrophages

Furthermore, we investigated the potential target mechanism of TTC4 on pyroptosis in macrophages. In the in vitro model, upregulation of TTC4 led to increased mRNA expression of HSP70, while downregulation of TTC4 resulted in reduced HSP70 mRNA expression (**Figure 5A**). Moreover, upregulation of TTC4 induced protein expressions of TTC4 and HSP70, while suppressing the protein expression of NLRP3 and Caspase-1 in the in vitro model (**Figure 5B**). Conversely, si-TTC4 downregulated protein expressions of TTC4 and HSP70, while inducing protein expressions of NLRP3 and Caspase-1 in the in vitro model (**Figure 5C**). Similarly, sh-TTC4 virus downregulated protein expressions of TTC4 and HSP70, while inducing protein expressions of NLRP3 and Caspase-1 in the in vitro model (**Figure 5D**). These findings suggest that TTC4 induces HSP70 expression to reduce NLRP3 inflammasome activation in macrophages.

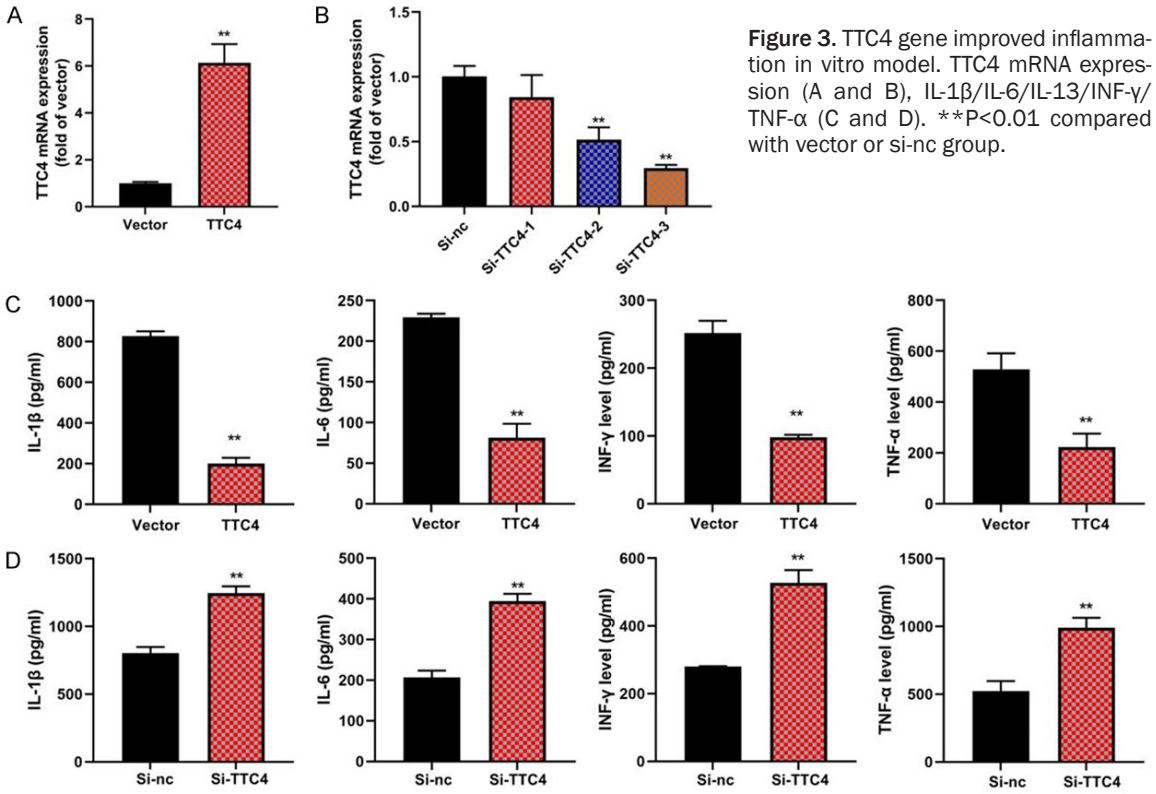

Figure 3. TTC4 gene improved inflammation in vitro model. TTC4 mRNA expression (A and B), IL-1β/IL-6/IL-13/INF-γ/TNF-α (C and D). **P<0.01 compared with vector or si-nc group.

TTC4 protein interlinked HSP70 protein

Next, we presented the 3D structures of the TTC4 protein interlinked with HSP70 protein, as well as the individual 3D structures of TTC4 and HSP70 proteins in **Figure 6A**. To further confirm their interaction, an immunoprecipitation (IP) experiment was performed. The results showed that TTC4 protein interacted with HSP70 protein, and vice versa, at the specific region of amino acids 283-286 (**Figure 6B**, **6C**). Additionally, TTC4 protein mainly interacted with HSP70 protein at amino acids 403-426, and secondarily at amino acids 473-475 or 530-604 (**Figure 6B**, **6C**). Furthermore, confocal microscopy revealed that TTC4 promoted the expression of HSP70 protein in the in vitro model (**Figure 6D**). These findings strongly suggest that TTC4 protein interacts with HSP70 protein, leading to the induction of HSP70 protein expression in the model of sepsis-induced ALI.

The activation of HSP70 reduced the effects of sh-TTC4 in model of sepsis-induced ALI through mitochondrial damage

The study investigated the mechanism by which TTC4 regulates sepsis-induced ALI through the HSP70 signaling pathway. The administration of a HSP70 agonist (TRC051384) at a dose of 20 mg/kg induced the expression of HSP70 protein and suppressed the expressions of NLRP3 and Caspase-1 proteins in a mouse model treated with sh-TTC4 virus (**Figure 7A**). The HSP70 agonist reduced the total number of cells in the bronchoalveolar lavage fluid (BALF) and decreased the levels of eosinophils, neutrophils, and lymphocytes. It also improved the serum levels of IgE and HDM-specific IgE and mitigated lung injury and inflammation in the sepsis mouse model (**Figure 7B-I**). These findings suggest that the activation of the HSP70 signaling pathway by TTC4 may be a potential therapeutic strategy for sepsis-induced ALI.

In the next set of experiments, we examined the effects of HSP70 inhibition and activation on the regulation of TTC4 in an in vitro model. We treated the cells with a HSP70 inhibitor (YK5) at a concentration of 5 μM, which led to a suppression of HSP70 protein expression and an induction of NLRP3 and Caspase-1 protein expressions in cells with up-regulated TTC4 (Figure S1A). Conversely, treatment with a HSP70 agonist (TRC051384) at a concentration of 6.25 μM induced HSP70 protein expres-

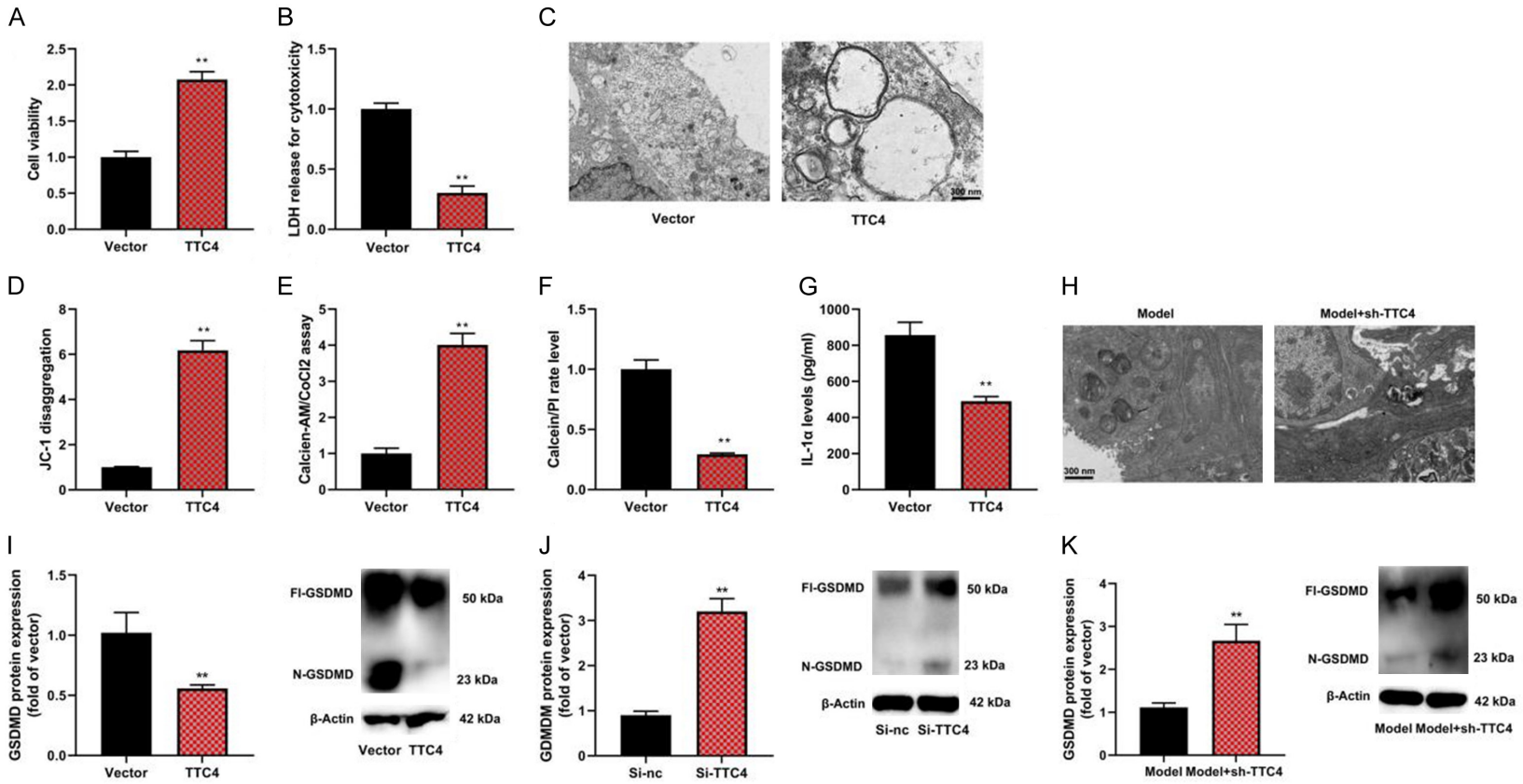

Figure 4. TTC4 gene reduced pyroptosis in macrophages of sepsis-induced lung injury by the inhibition of mitochondrial damage. Cell growth (A), LDH activity levels (B), mitochondrial damage (Electron microscope, C), JC-1 disaggregation (D), MPT (calcein AM/CoCl2 assay, E), PI level (F), IL-1α (G) in vitro model; mitochondrial damage (Electron microscope, H) in mice model; GSDMD protein expression (I-K). **P<0.01 compared with vector or si-nc or model group.

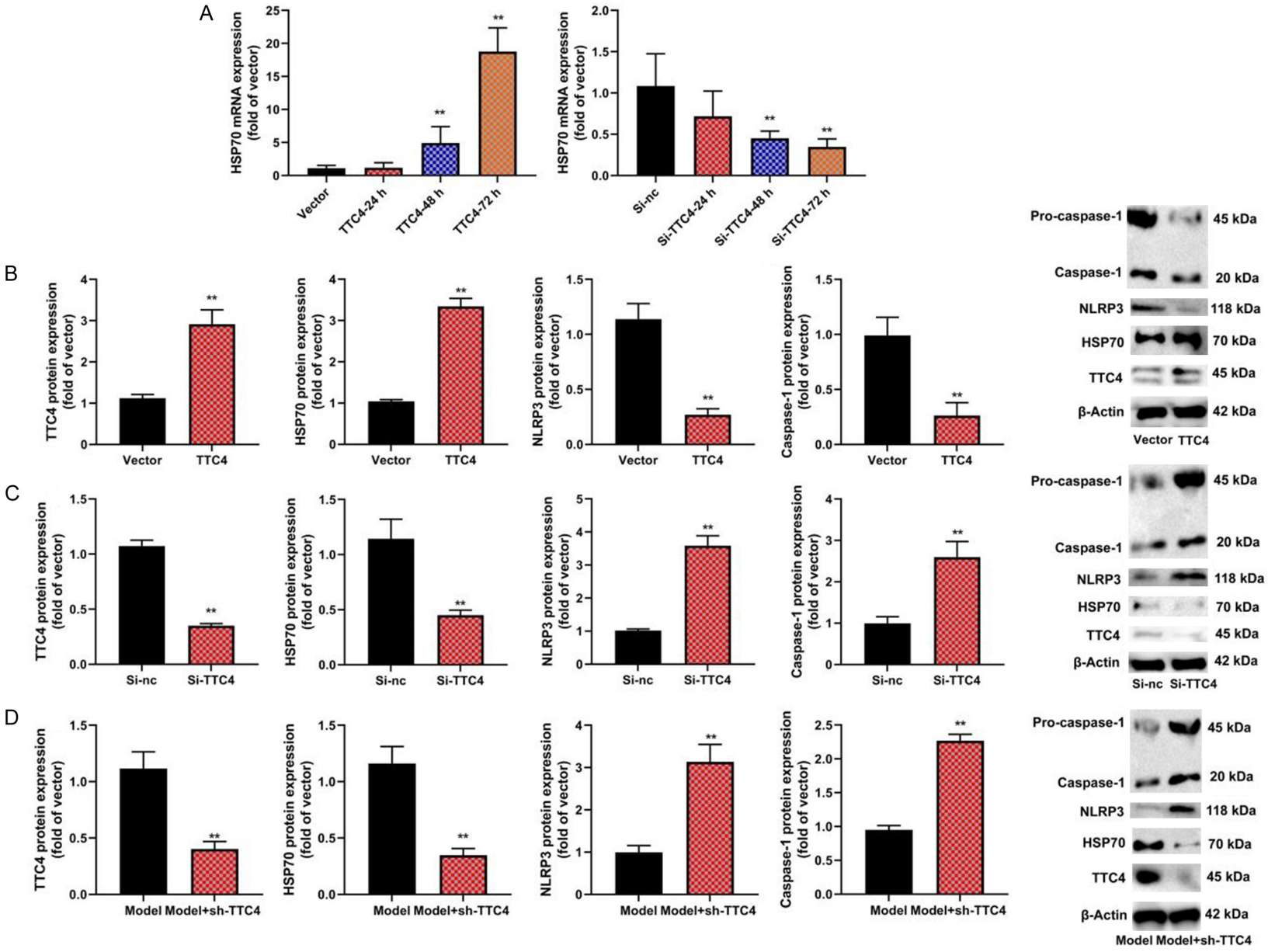

Figure 5. TTC4 gene induced HSP70 expression to reduce NLRP3-induced pyroptosis in macrophages. HSP70 mRNA expression (A), TTC4/HSP70/NLRP3/Caspase-1 protein expression (B-D). **P<0.01 compared with vector or si-nc or model group.

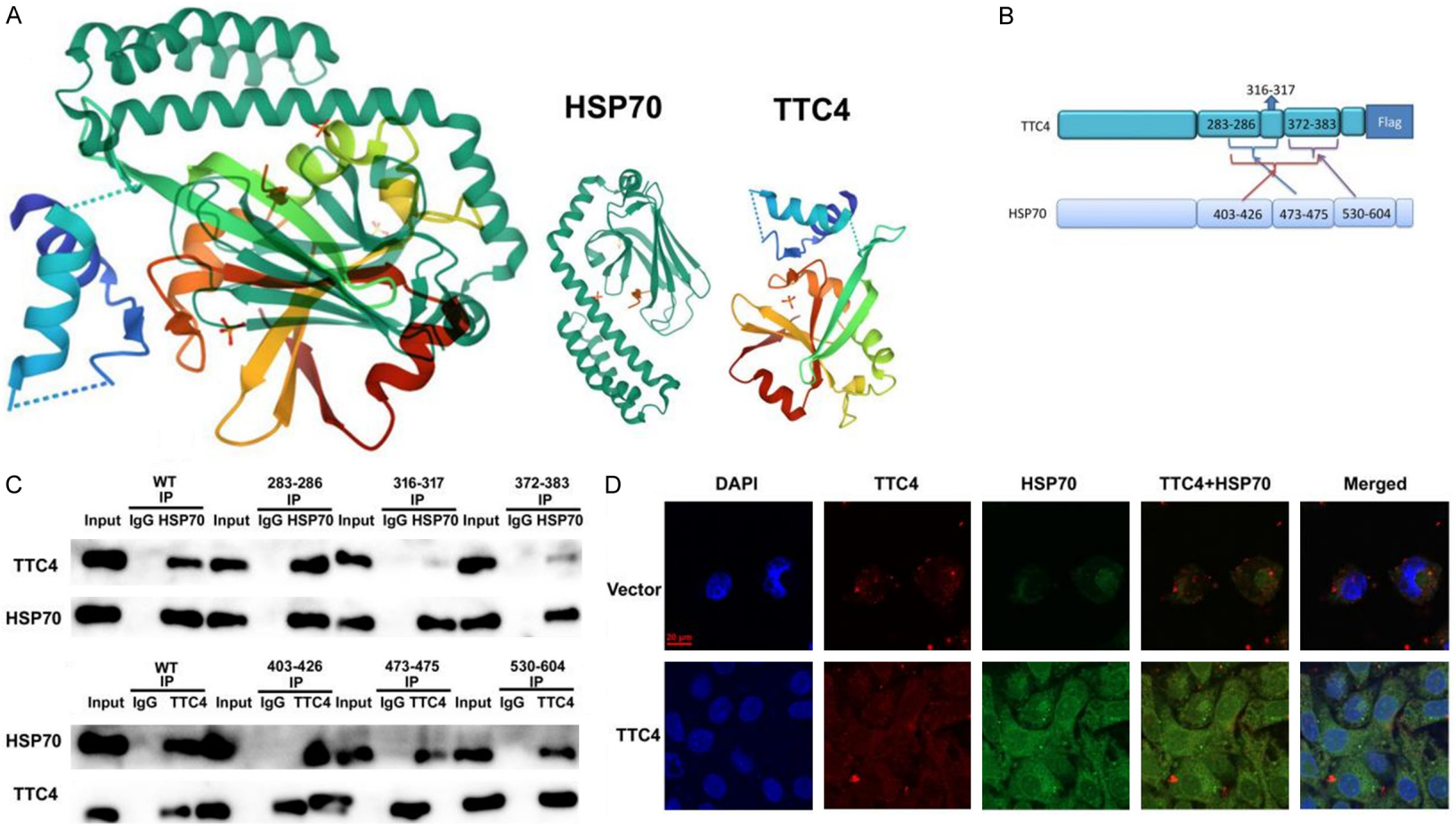

Figure 6. TTC4 protein interlinked HSP70 protein. 3D structure of TTC4 protein interlinked HSP70 protein (A), TTC4 protein interlinked HSP70 protein (B and C), TTC4 promoted HSP70 protein expression (D). **P<0.01 compared with vector or si-nc or model group.

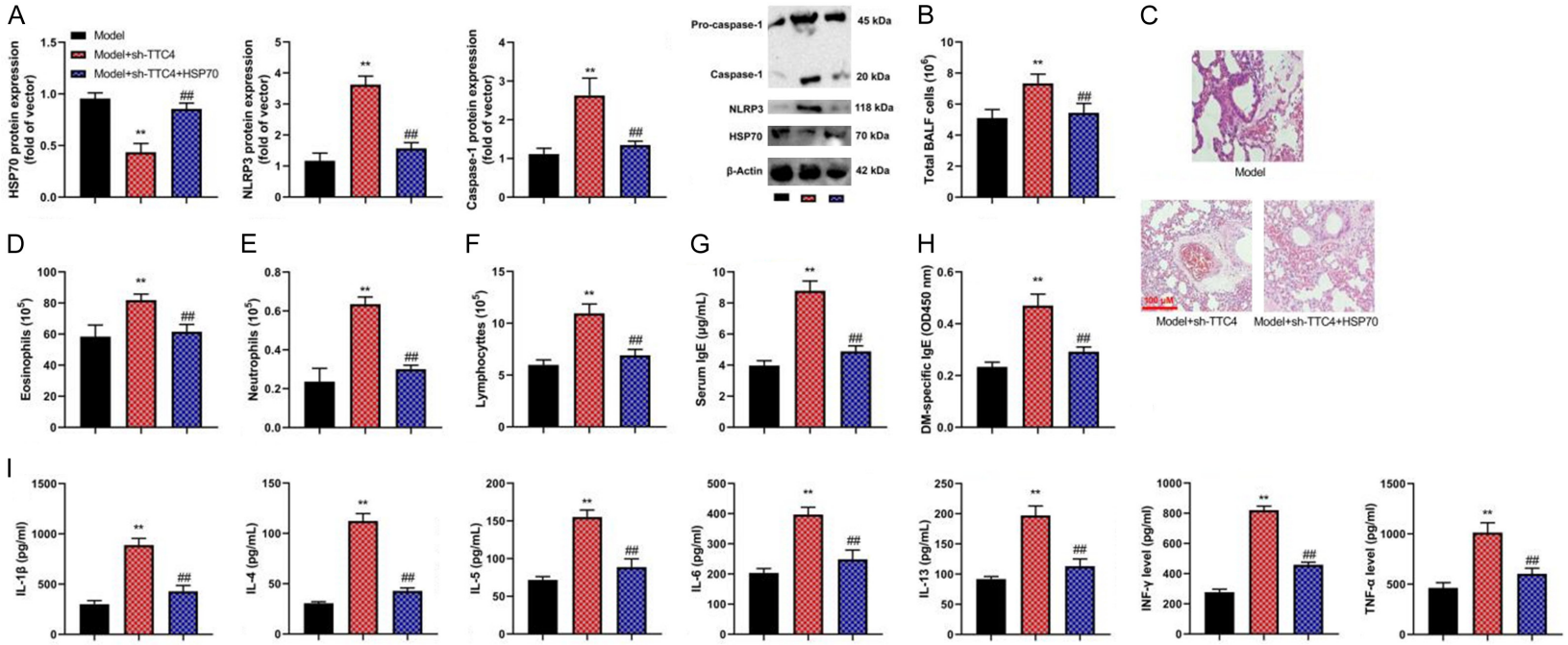

Figure 7. The activation of HSP70 reduced the effects of sh-TTC4 in model of sepsis-induced ALI through mitochondrial damage. HSP70/NLRP3/Caspase-1 protein expressions (A), total cells of BALF (B), Eosinophils (C), neutrophils (D), Lymphocyttes (E), serum IgE (F), HDM-specific IgE levels (G), lung injury (HE staining, H), IL-1β/IL-4/IL-5/IL-6/IL-13/INF-γ/TNF-α (I). **P<0.01 compared with vector or si-nc or model group, ##P<0.01 compared with model+sh-TTC4 group. HSP70, HSP70 agonist, 20 mg/kg of TRC051384.

sion and suppressed NLRP3 and Caspase-1 protein expressions in cells with down-regulated TTC4 (Figure S1B). Furthermore, the HSP70 inhibitor increased the levels of inflammation in cells with up-regulated TTC4 (Figure S1C), while the HSP70 agonist reduced inflammation in cells with down-regulated TTC4 (Figure S1D). These results provide further evidence for the involvement of the HSP70 signaling pathway in the regulation of TTC4 and its effects on inflammation.

In the subsequent experiments, we investigated the impact of inhibiting or activating HSP70 on various cellular processes in an in vitro model with up-regulated TTC4. Treatment with the HSP70 inhibitor YK5 at a concentration of 5 μM resulted in suppressed cell growth, increased LDH activity levels, reduced mitochondrial damage, and decreased JC-1 disaggregation and MPT (calcein AM/CoCl2 assay) (**Figure 8A-E**). Additionally, the HSP70 inhibitor promoted a higher rate of PI staining, indicating increased cell death, in the in vitro model with up-regulated TTC4 (**Figure 8F**). Furthermore, the HSP70 inhibitor suppressed GSDMD protein expression in the in vitro model with up-regulated TTC4 (**Figure 8G**). In a separate experiment using a mouse model, we found that treatment with an HSP70 agonist suppressed GSDMD protein expression in mice infected with sh-TTC4 virus (**Figure 8H**). These findings suggest that TTC4 induces HSP70 expression, which in turn suppresses the NLRP3/Caspase-1 inflammasome in a model of sepsis-induced ALI by inhibiting mitochondrial damage.

M6A-forming enzyme METTL3 reduced TTC4 stability

We further investigated the methylation mechanism that controls TTC4 stability in colitis. Analysis using the m6A prediction website SRAMP revealed multiple potential methylation modification sites near the stop codon of the TTC4 gene (**Figure 9A**). We observed increased m6A modification of TTC4 levels in a model of sepsis-induced ALI (**Figure 9B**). Treatment with an m6A-specific antibody suppressed the enrichment of TTC4 mRNA in an in vitro model with si-METTL3 (**Figure 9C**). Knockdown of METTL3 reduced the stability of TTC4 mRNA in the in vitro model (**Figure 9D**). We identified six m6A sites in the 3'-untranslated region (UTR) of TTC4, with significant enrichment at sites 1, 2, 3, 4, 5, and 6 (**Figure 9E**). The m6A enrichment at these sites led to decreased TTC4 levels (**Figure 9F**). Si-METTL3 reduced luciferase activity levels in cells transfected with the wild-type (WT) TTC4 construct, while the mutant (Mut) TTC4 construct showed no effect (**Figure 9F-H**). These results indicate that METTL3 regulates TTC4 expression in a mouse model of colitis.

Discussion

ALI is a clinical syndrome characterized by an excessive inflammatory reaction, increased pulmonary capillary permeability, and severe hypoxemia [19]. In severe cases, it can lead to acute respiratory distress syndrome (ARDS), which has a mortality rate of up to 50% [20]. The etiology of ALI/ARDS is complex, with causes such as severe infection, sepsis, trauma, iatrogenic lung injury from radiotherapy and chemotherapy. Among these, infection is the most common cause, with lipopolysaccharide (LPS) being the pathogenic substance of gram-negative bacteria [21]. Although significant progress has been made in the treatment of ALI/ARDS, the mortality rate remains high, highlighting the need for new treatment approaches [22]. In this study, we observed down-regulation of TTC4 expression levels in patients with sepsis-induced ALI as well as in a mouse model. Previous research by Shang et al. identified TTC4 as a positive regulator of innate immunity induced by sendai virus [23]. Therefore, our findings suggest that the down-regulation of TTC4 and its involvement in disease progression play a role in sepsis-induced ALI.

The emergence and resolution of inflammation is a natural process orchestrated by the immune system to remove pathogenic substances [24]. Controlled inflammatory reactions are conducive to the removal of these substances [25]. However, uncontrolled inflammatory reactions can cause fatal damage to the body, as seen in excessive inflammation during ALI [26]. Our study establishes that the inhibition of TTC4 gene promoted inflammation and lung injury in a mouse model of sepsis. He et al. have previously concluded that TTC4 suppresses vascular endothelial cell apoptosis

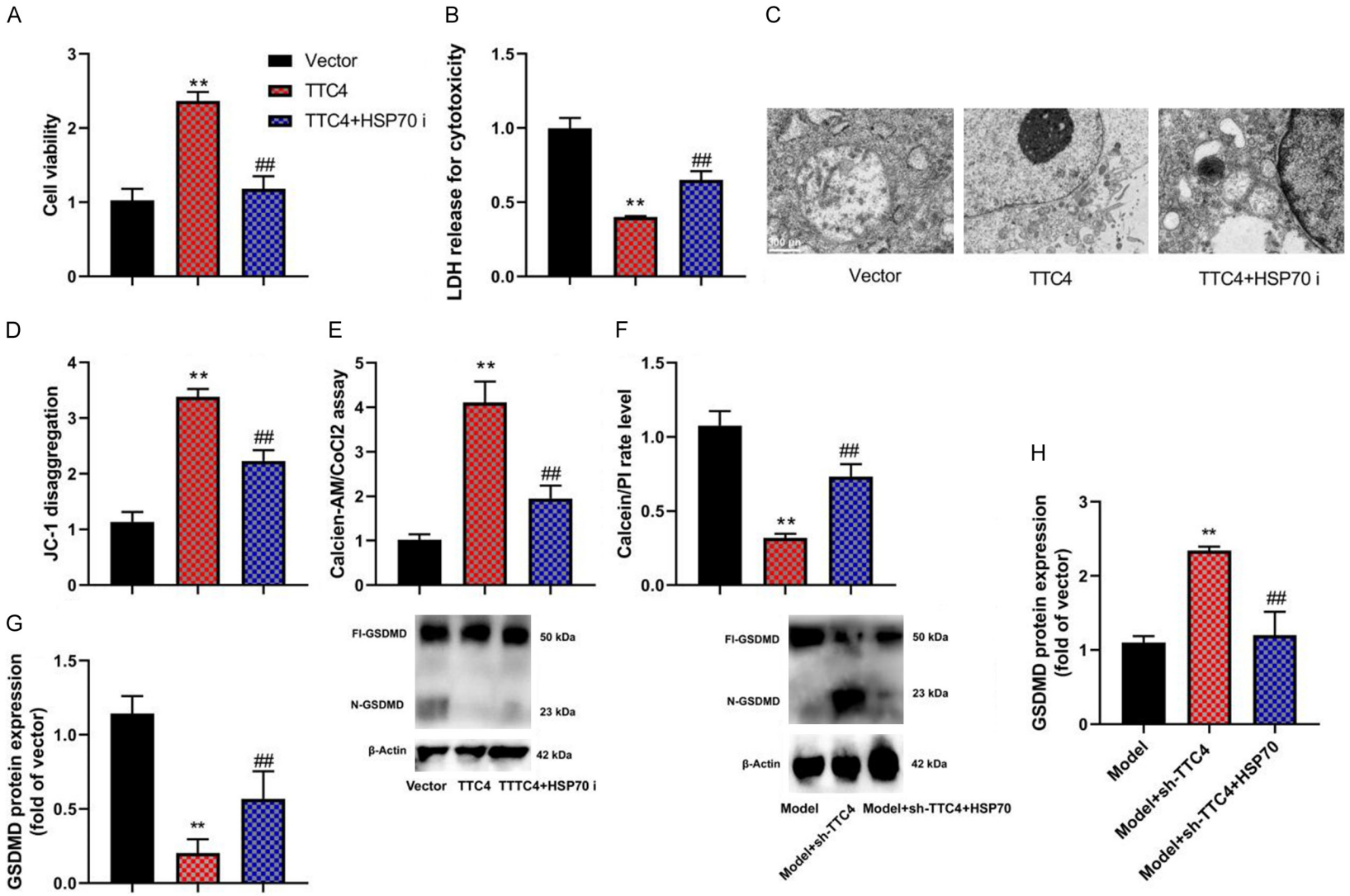

Figure 8. HSP70 controls the effects of TTC4 on mitochondrial damage. Cell growth (A), LDH activity levels (B), mitochondrial damage (Electron microscope, C), JC-1 disaggregation (D), MPT (calcein AM/CoCl2 assay, E), PI level (F) in vitro model; GSDMD protein expression (G and H). **P<0.01 compared with vector or model group, ##P<0.01 compared with TTC4 or model+sh-TTC4 group. HSP70 i, HSP70 inhibitor, 5 μM of YK5; HSP70, HSP70 agonist, 6.25 μM of TRC051384.

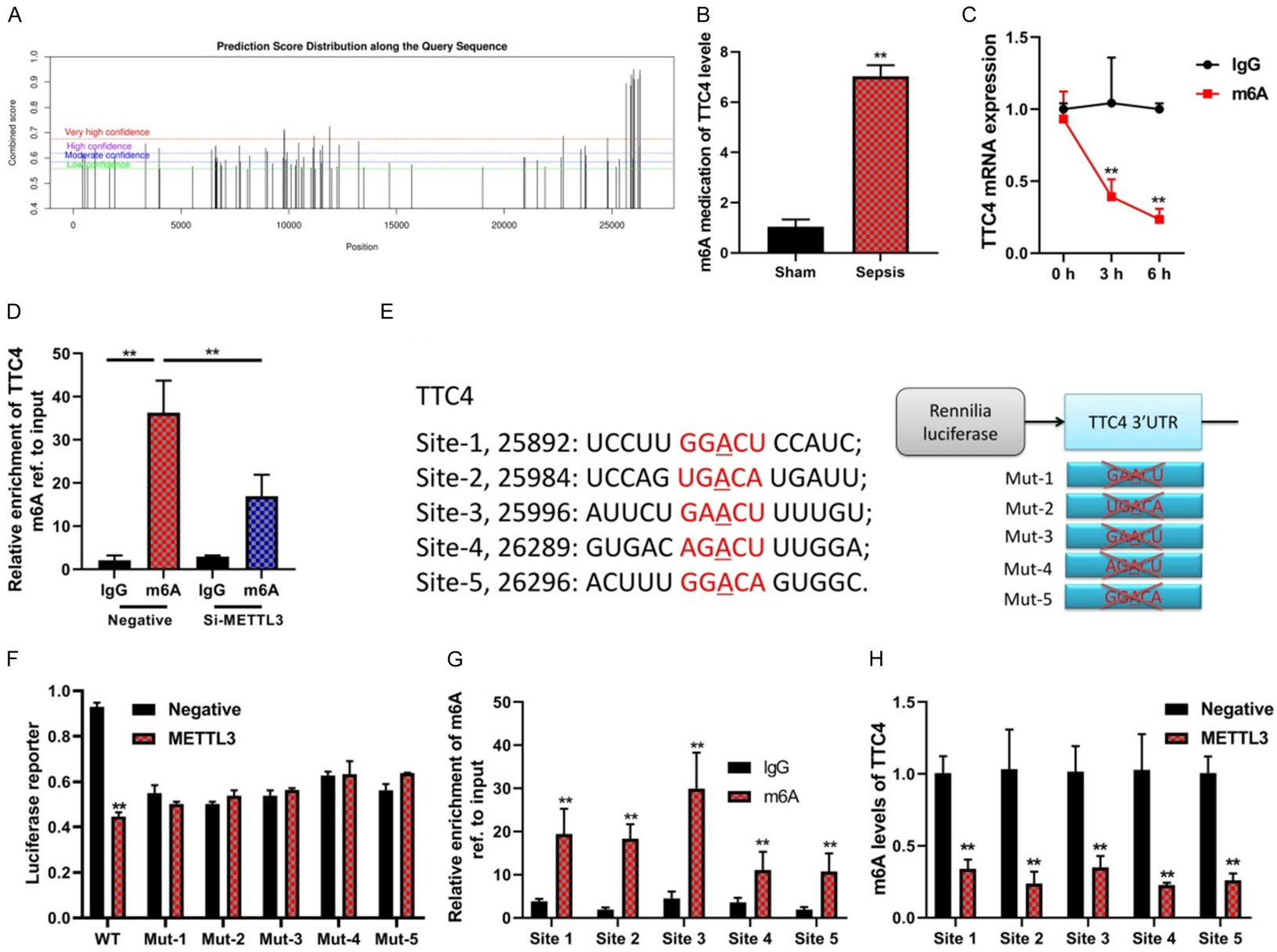

Figure 9. M6A-forming enzyme METTL3 reduced TTC4 stability. m6A modification site of TTC4 (A), m6A medication of TTC4 level (B), METTL3-mediated UAF1 m6A modifications (C and D), the position of m6A motifs within TTC4 transcript sequence (E), luciferase reporter activity level (F), m6A levels of UAF1 (G and H). **P<0.01 compared with vector or negative or IgG group.

[27]. Thus, our findings suggest that TTC4 serves as a reparative factor in inflammation and lung injury associated with sepsis-induced ALI.

As a pro-inflammatory form of programmed cell death, pyroptosis plays a role in caspase-1 or caspase-11 mediated cleavage of the GSDMD protein [28]. It induces swelling and lysis of inflammatory cells, leading to the release of a large amount of inflammatory substances, thereby triggering a strong inflammatory response [29, 30]. Our study demonstrates that the TTC4 gene reduces pyroptosis in macrophages during sepsis-induced lung injury by inhibiting mitochondrial damage. This finding is consistent with the previous study by He et al., which showed that TTC4 inhibits apoptosis in vascular macrophages [27]. Thus, it is possible that TTC4 may regulate cell growth and pyroptosis in macrophages during sepsis-induced ALI.

Recent studies have revealed a close relationship between the NLRP3 signaling pathway and the inflammatory response in ALI induced by various factors [31, 32]. Stimulation with LPS leads to the production of ROS, which can activate NLRP3. The activated NLRP3, in turn, activates Caspase-1, resulting in the maturation of IL-1β and IL-18 and triggering a robust inflammatory response, thereby exacerbating lung injury [33]. Inhibition of the NLRP3 signaling pathway has been shown to significantly improve lung inflammation in ALI mice [33]. In our study, we found that the TTC4 gene induces the expression of HSP70, which in turn reduces NLRP3-induced pyroptosis in macrophages. Additionally, He et al. demonstrated that TTC4 interacts with HSP70 to inhibit apoptosis in vascular macrophages [27]. Taken together, our findings suggest that TTC4 interacts with HSP70 to attenuate NLRP3-induced pyroptosis in macrophages during sepsis-induced ALI.

However, some studies have found that HSP70 can also be found outside the cell [34]. Particularly, under specific stress conditions, HSP70 can stimulate the proinflammatory response of primary airway epithelial cells, leading to the release of inflammatory cytokines. Some researchers have observed increased expression of HSP70 in lung tissue from patients with chronic obstructive pulmonary disease, indicating a correlation with disease severity [35]. When HSP70 is secreted outside the cell, it can trigger an inflammatory reaction [36]. Several studies have demonstrated that HSP70 can activate the expression of pro-inflammatory genes in airway epithelial cells, as well as stimulate their NLRP3 inflammasome and ATP release, thereby worsening the condition of ALI patients and affecting prognosis [37-39]. Importantly, we have shown for the first time that the M6A-forming enzyme METTL3 reduces TTC4 stability.

Our study revealed that the m6A-forming enzyme METTL3 controls TTC4 to reduce inflammation and pyroptosis in a model of sepsis-induced acute lung injury (ALI) by inhibiting mitochondrial damage through the HSP70/ROS/NLRP3 signaling pathway. TTC4 gene shows promise as a potential therapeutic target for the treatment of sepsis-induced lung injury. Furthermore, the regulatory roles of m6A/METTL3 control TTC4 in sepsis-induced ALI or other inflammatory diseases warrant further investigation.

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Disclosure of conflict of interest

None.

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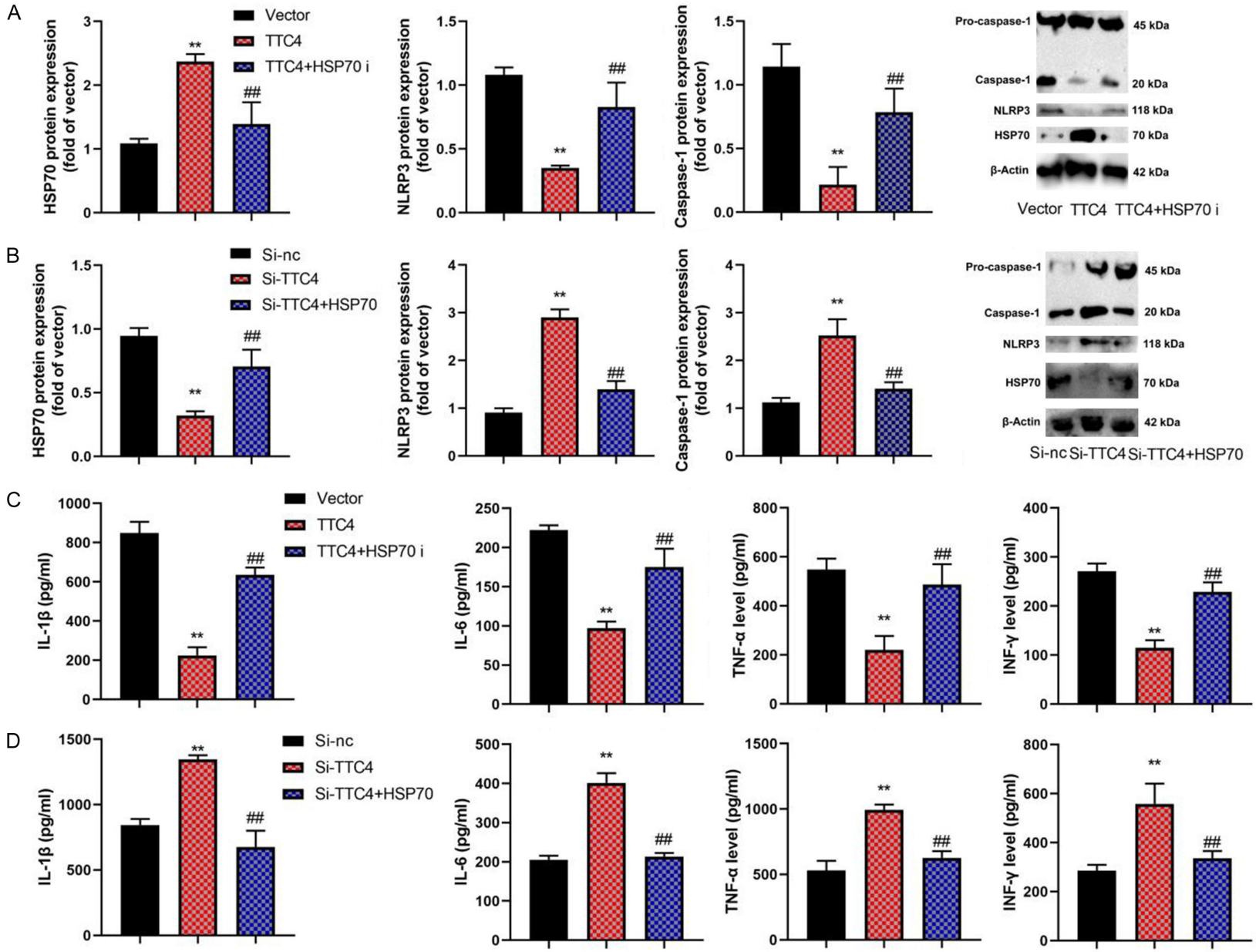

Figure S1. HSP70 controls the effects of TTC4 on inflammation levels. HSP70/NLRP3/Caspase-1 protein expression (A and B), IL-1β/IL-6/INF-γ/TNF-α (C and D). **P<0.01 compared with vector or model group, ##P<0.01 compared with TTC4 or si-TTC4 group.