Acetic acid alleviates the inflammatory response and liver injury in septic mice by increasing the expression of TRIM40

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Abstract. Sepsis remains a significant health care issue in clinical practice due to its high mortality rate and healthcare cost, despite extensive efforts to better understand the pathophysiology of sepsis. The systemic inflammatory response often leads to severe liver injury, even acute liver dysfunction and failure. Acetic acid, as a type of chemical compound, has been reported to be an emerging drug for improving metabolic syndrome and inhibiting inflammation in rats and human. To verify the effects of acetic acid in protecting the liver and reducing the inflammatory response, a septic mouse model was established by cecal ligation and puncture (CLP), and then the CLP-model mice were treated with acetic acid or PBS. Following the treatment, it was determined that, in CLP-model mice, acetic acid could alleviate the inflammatory response by decreasing the expression of cytokines including interleukin-6 and tumor necrosis factor-α. Additionally, acetic acid also alleviated the liver injury, and the levels of alanine aminotransaminase, aspartate aminotransferase, Toll-like receptor (TLR)4 and nuclear factor-κB (NF-κB) were decreased. The expression of tripartite motif-containing protein (TRIM)40 was also upregulated significantly. Therefore, the authors of the current study hypothesized that acetic acid could decrease the inflammatory response by increasing the expression of TRIM40 and TRIM40 may regulate the activity of the TLR4 signaling pathway. To further illustrate the interaction between TRIM40 and the TLR4 signaling pathway, the authors collected macrophages from the peritoneal cavity by intraperitoneally administering mice with 5 ml ice-cold normal saline. Following the collection, peritoneal macrophages were treated with acetic acid, TRIM40 small interfering RNA or PBS. It was demonstrated that acetic acid upregulated the expression of TRIM40. When TRIM40 was silenced, the protective effect of acetic acid would be reversed as well. The results suggested that TRIM40 could act on and downregulate the activity of the TLR4 signaling pathway. TRIM40 is possibly the major target for acetic acid, which may function as a protective factor in septic mice.

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Introduction

Sepsis is a dysregulated host response to infection, which could result in severe multiorgan dysfunctions and high mortality rates (1-3). Treating sepsis and sepsis-associated multiorgan failure remains challenging for clinicians and scientists (4). Even though an increasing number of studies have been conducted, the pathophysiology of sepsis in humans remains unclear (5,6). According to previous studies, sepsis influenced the prognosis of one-third of all patients on admission or during their stay in the intensive care unit and the mortality rate of these patients reached 25-35% (7,8). In the United States, sepsis is ranked as the tenth leading cause of mortality and accounts for approximately 24 billion dollars of total hospital costs every year (9). As a result, more effective drugs and new treatment methods for sepsis are imperative to reducing the morbidity and mortality rates associated with sepsis.

Systemic inflammatory response syndrome is common in sepsis and is usually initiated by severe infection or acute injury (1,10,11). This systemic inflammatory response has been revealed to often lead to dysfunction in multiple organs, including the liver, lungs, kidneys and heart (1,12). Among these organs, sepsis-associated liver dysfunction and failure are crucial to survival rate, and are connected with high mortality rate (13). According to the French EPISEPSIS (EPIdemiology of SEPSIS) study group, the incidence rate of liver dysfunction (hepatic score >0) and liver failure (hepatic score of 3 or 4) were 46.6 and 6.3%, respectively (13). However, in another clinical trial, which involved 312 patients in septic shock, the rate of acute liver failure was even reported to be 20% (14). Therefore, more attention should be paid to decreasing sepsis-associated liver dysfunction and failure.

Toll-like receptors (TLRs), a type of membrane receptor, which can recognize pathogen-associated molecular patterns and connect to the body's immune response system (15). Lipopolysaccharide (LPS) is the main component of endotoxins and can stimulate the release of multiple inflammatory mediators, which promote the progression of systemic inflammatory

response syndrome (16). Studies revealed that LPS could bind to TLR4 and TLR signaling pathways may be involved in modulating allergic immune responses (17,18). Also, TLR4 is the major pattern recognition receptor for detecting endotoxins (19). When endotoxins activate TLR4, nuclear factor-κB (NF-κB) activates (19). NF-κB is the downstream intracellular molecule, which is involved in activating the expression of many pro-inflammatory genes, including interleukin (IL)-1β and tumor necrosis factor (TNF)-α (20). As a result, reduce liver dysfunction and liver failure induced by LPS may be achieved by regulating TLR signaling pathways.

The structure of the tripartite motif-containing protein (TRIM) protein family is composed of three zinc-binding domains: RING, B-box type 1 and B-box type 2, followed by a coiled-coil region; it is also known as the RBCC family (21). The majority of TRIM proteins have been reported to be involved in the ubiquitination process (22). However, studies also revealed that some TRIM family members are involved in different cellular processes, including apoptosis, cell growth, transcriptional regulation and oncogenesis (23-25). In a past study by our group, the authors demonstrated that the TRIM family protein, TRIM40, was highly expressed in the liver tissue of cecal ligation and puncture (CLP)-model mice treated with acetic acid. Furthermore, the TLR4 signaling pathway was downregulated, and the expression levels of NF-κB, IL-6 and TNF-α were decreased. With the purpose of elucidating the molecular function of TRIM40 in the liver of CLP models, and illustrating the protective mechanism of acetic acid in liver dysfunction and liver failure, the authors decided to perform further studies. As a result, the authors identified the minimum dose of acetic acid that inhibited inflammatory responses in CLP models by upregulating the expression of TRIM40, which may regulate the activity of the TLR4 signaling pathway.

Materials and methods

Animals. All animals were fed based on the Principles of Laboratory Animal Care formulated by the National Institutes of Health (26) and guidelines of the Institutional Animal Care and Use Committee of Qingdao Municipal Hospital (Qingdao, China) (27). A total of 48 male C57BL/6 mice (6-8 weeks old) weighing 20±2 g were obtained from the School of Medicine, Qingdao University (Qingdao, China). Mice were housed in a specific pathogen-free animal facility under controlled environmental conditions of 22±2˚C and a 12 h light-dark cycle. Mice were fed with a standard pellet diet and water. The experiment was approved by the Ethics Committee in Qingdao University.

Acquisition and culture of peritoneal macrophages. First, the mice were intraperitoneally administered with 5 ml ice-cold normal saline and after 6 h, peritoneal cell suspensions were collected with the same needle. The collected peritoneal cells suspensions were centrifuged at 1,000 x g for 3 min at 4˚C. Then the supernatant was removed and the sediment was resuspended in PBS, and centrifuge again at 1,000 x g for 3 min at 4˚C. The collected peritoneal cells were suspended in Dulbecco's modified Eagle's medium (DMEM; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 24 h at 37˚C, 50 U/ml penicillin and 50 μg/ml streptomycin. The number of cells was counted using a cell counting chamber (Wuxi Devan Scientific Ltd., Co., Wuxi, China). According to reports, after incubating the peritoneal cells for 1 h, >98% of adherent cells in culture dishes were identified as macrophages due to the observation of Fc receptor-mediated phagocytosis and the Giemsa staining performed (28,29).

RNA interference. TRIM40 small interfering (si)RNA (siTRIM40; 5'-CTTCTCTGAGGCAGTAACA-3') and negative control siRNA (siNC; 5'-TTCTCCGAACGT GTCACGT-3') were bought from Novus Biologicals, LLC (Littleton, CO, USA). Peritoneal macrophages were transfected with 50 nM siRNA or siNC using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The knockdown efficiency was assessed by RT-qPCR after 48 h of transfection.

Experimental procedures. Sepsis was induced by CLP as described by Rittirsch *et al* (30). To investigate whether acetic acid alleviates liver injury and inflammation induced caused by CLP, mice were randomly divided into four groups (n=6/group): Control, control + acetic acid, CLP + PBS and CLP + acetic acid. Animals were anaesthetized by 1.2% sevoflurane (Jiangsu Hengrui Medicine Co., Ltd., Lianyungang, China) prior to surgery. Mice in the control group were intravenously injected with PBS following a sham surgery (the abdomen was opened, but CLP was not performed), mice in the control + acetic acid group were intravenously injected with acetic acid (0.1 mmol/kg; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) following a sham surgery, mice in the CLP + PBS group were intravenously injected with 0.1 ml PBS following CLP, and mice in the CLP + acetic acid group were intravenously injected with PBS and acetic acid following CLP. A total of 12 h after the drug treatments, mice were sacrificed by cervical dislocation under anesthesia (1.2% sevoflurane) and blood was withdrawn from the heart to estimate liver injury by measuring alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (TBiL) levels. At the same time, ascites was collected, and IL-6, IL-10 and TNF-α levels in the ascites were also detected. Livers were removed and stored at -80˚C for further experiments, and partial liver specimens were submerged in 10% neutral formalin prior to being processed for histopathological analysis. To draw the survival curve, the aforementioned protocol was performed and mortality was recorded between days 1-7 after CLP, and survivors were monitored once every day for an additional 3 weeks to verify that no late mortalities occurred (10 mice/group).

To investigate the involvement of TRIM40 in the TLR4 signaling pathway, the peritoneal macrophages were divided into six groups as follows: Control, control + acetic acid, LPS, LPS + acetic acid, LPS + acetic acid + siTRIM40 and LPS + siTRIM40. Cells in the control group were treated with 0.1 ml PBS; cells in the control + acetic acid group were treated with acetic acid (0.1 mmol/ml); cells in the LPS group were treated with LPS (0.1 mmol/ml; Sigma-Aldrich; Merck KGaA); cells in the LPS + acetic acid group were treated with LPS (0.1 mmol/ml) + acetic acid (0.1 mmol/ml); cells

in the LPS + acetic acid + siRNA group were treated with LPS (0.1 mmol/ml) + acetic acid (0.1 mmol/ml) + siTRIM40 (50 nM); cells in the LPS + siTRIM40 group were treated with LPS (0.1 mmol/ml) + siTRIM40. Acetic acid and LPS were dissolved in sterile PBS. After 3 days of drug treatment in cell incubator (37˚C, 5% CO_2), the medium was collected to measure the concentration of cytokines, including TNF-α, IL-6 and IL-10. The expression levels of TRIM40, TLR4 and NF-κB in macrophages were analyzed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis and western blotting.

RT-qPCR analysis. To evaluate the mRNA levels of inflammatory mediators, mice were sacrificed under anesthesia (1.2% sevoflurane) after drug treatment for 12 h and livers were frozen. Total RNA was extracted from the liver cells using an Ultrapure RNA kit (Beijing ComWin Biotech, Co., Ltd., Beijing, China), and cDNA was synthesized using the RevertAid™ first strand cDNA synthesis kit (Thermo Fisher Scientific, Inc.). RT-qPCR was performed as previously reported (31). The reaction conditions for RT-qPCR were as follows: Pre-denaturation at 95˚C for 30 sec, and 40 cycles of 95˚C for 10 sec and 60˚C for 30 sec. The melting curve was at 95˚C for 15 sec, 60˚C for 60 sec and 95˚C for 15 sec. The primer sequences used in the current study were as follows: TRIM40, forward 5'-ATGGGCTCTCTTGACAAGGAC-3' and reverse 5'-ACTGAAGCCTTATCCATGTGC-3'; NF-κB, forward 5'-CAAGCGAGGAGGGGACGTG-3' and reverse 5'-CCCCCAGAGCCTCCACCC-3'; and TLR4, forward 5'-GCCTTTCAGGGAATTAAGCTCC-3' and reverse 5'-GATCAACCGATGGACGTGTAAA-3'; and GAPDH, forward 5'-CTGGGCTACACTGAGCACC-3' and reverse 5'-AAGTGGTCGTTGAGGGCAATG-3'.

Western blotting. Total intracellular protein was isolated using RIPA buffer supplemented with protease inhibitor cocktail (both Sigma-Aldrich; Merck KGaA). The protein concentrations were determined using a Bicinchoninic Acid Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.). Protein samples (16-20 μg/lane) separated by SDS-PAGE were loaded on 8-12% polyacrylamide gels (Beyotime Institute of Biotechnology, Haimen, China) and then transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA) using the Semi-Dry Trans-Blot Cell (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Following the transfer, the membranes were blocked by 3% bovine serum albumin (Sigma-Aldrich; Merck KGaA) in TBS for 1 h at room temperature. Blots were incubated with primary antibodies overnight at 4˚C. The primary antibodies were as follows: TLR4 (1:2,500; cat. no. IMG-5031A; Novus Biologicals, LLC), NF-κB (cat. no. SAB4501993), β-actin (cat. no. A1978; both 1:5,000; Sigma-Aldrich; Merck KGaA) and TRIM40 (1:5,000; cat. no. ab156583, Abcam, Cambridge, UK). The bands were then observed by enhanced chemiluminescent reagents (Shanghai Yeasen Biotech Co., Ltd., Shanghai, China) and exposed to x-ray films (Bio-Rad Laboratories, Inc.).

Histological analysis. Following the sacrifice of the mice, liver tissues were sampled from the left lobe and quickly fixed in 10% neutral buffered formalin at room temperature for 24 h. Then the samples would be embedded in paraffin and serially cut into 5-μm-thick sections. Sections were stained with hematoxylin and eosin at room temperature for 30 min, then the sections were observed using a phase contrast microscope at a magnification of x200.

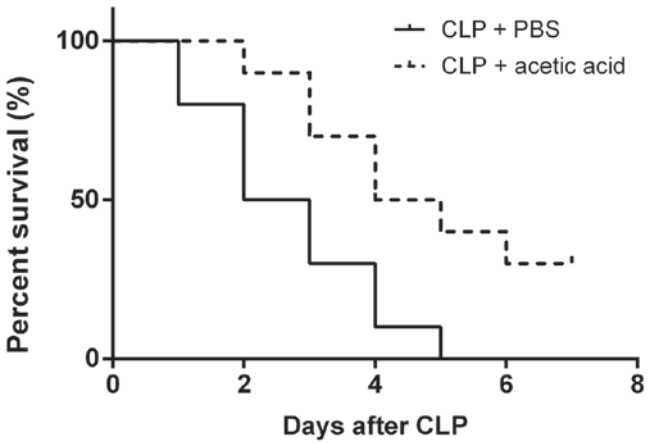

Figure 1. Acetic acid improves the survival rate of CLP-model mice. Kaplan-Meier survival curves of mice following CLP and the intravenous administration of PBS or 0.1 mmol/kg acetic acid (n=10 mice/group). CLP, cecal ligation and puncture.

ELISA and blood assays. The IL-6 (cat. no. BGK08505), IL-10 (cat. no. BGK18893) and TNF-α (cat. no. BGK06804) levels in ascites were detected using ELISA kits (PeproTech, Inc., Rocky Hill, NJ, USA) as previously reported (32). The values are recorded as ng/mg of protein. To measure ALT, AST and TBiL levels, blood assays were performed by a biochemical automatic analyzer (Vitros750; Johnson & Johnson, New Brunswick, NJ, USA).

Statistical analysis. All the statistical analyses were performed by SPSS 23.0 software (IBM Corp., Armonk, NY, USA) and graphs were drawn using GraphPad Prism 5.01 (GraphPad Software, Inc., La Jolla, CA, USA). All values are presented as mean ± standard deviation. Kaplan-Meier survival curves were generated and group comparisons were based on the log-rank test. Comparisons between different groups were conducted using the Student's t-test for two independent groups or one-way analysis of variance followed by Tukey's test for multiple independent groups. $P<0.05$ indicated that the difference between groups was statistically significant.

Results

Acetic acid protects mice against sepsis-induced mortality and alleviates inflammation induced by sepsis. To investigate the protective effects of acetic acid in reducing CLP-induced mortality, the survival rate was monitored for 7 days. In the CLP + PBS group, the survival rate on the first day of observation was 80% and all mice succumbed by day 6 after CLP ($P=0.0106$; Fig. 1). In the CLP + acetic acid group, the survival rate on day 7 was observed as 30% and three mice continued to survive for 7 days. Treatment of acetic acid improved the survival rate compared with the CLP + PBS

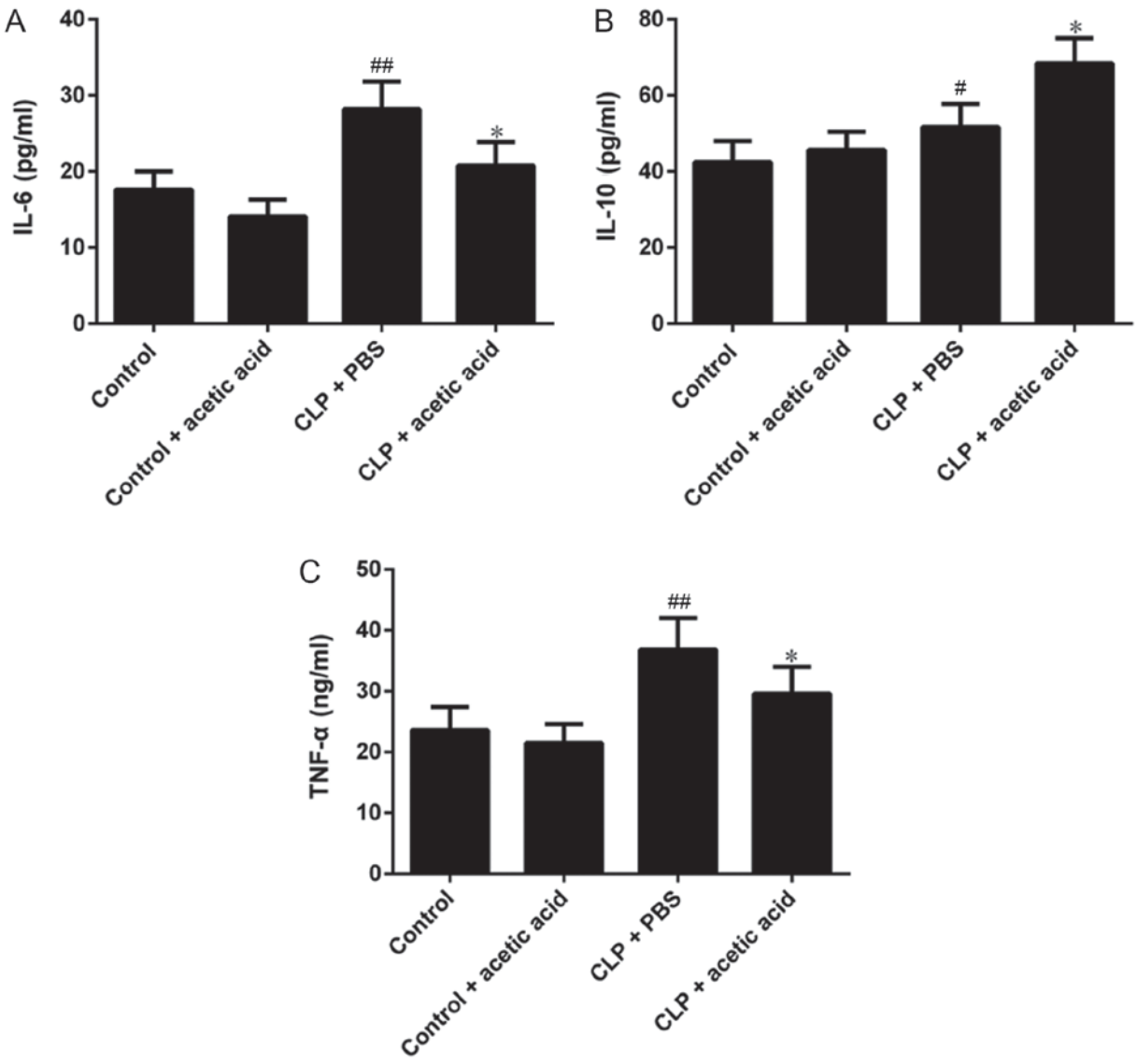

Figure 2. Acetic acid decreases the concentration of inflammatory cytokines in the serum of CLP-model mice. Serum (A) IL-6, (B) IL-10 and (C) TNF-α levels were detected by ELISA 12 h after PBS and acetic acid treatment (n=6 mice/group). Results are presented as mean ± standard deviation. *P<0.05 vs. the CLP + PBS group. $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. the control group. CLP, cecal ligation and puncture; IL, interleukin; TNF-α, tumor necrosis factor α.

group. The median survival in the CLP + PBS group was 2.5 days compared with 4.5 days in the CLP + acetic acid group. Then the authors evaluated whether acetic acid could decrease sepsis-induced inflammation by measuring serum IL-6, IL-10 and TNF-α levels. According to the results, it was determined that serum IL-6, IL-10 and TNF-α levels were 17.6±2.4 pg/ml, 42.4±5.6 pg/ml and 23.6±3.8 ng/ml in the control group, respectively (Fig. 2). CLP significantly increased serum IL-6, IL-10 and TNF-α levels by 1.6-, 1.2- and 1.6-fold compared with the control group, respectively. In the CLP + acetic acid group, serum IL-6 and TNF-α levels were significantly downregulated 26 and 20%, respectively, while the serum IL-10 level was significantly upregulated 32% compared with the CLP + PBS group. The results suggested that acetic acid decreased the synthesis of inflammatory cytokines, thus alleviating inflammation induced by sepsis. Also, no significant changes in serum concentrations were identified between the control and control + acetic acid groups.

Histological analysis of liver tissue and detection of liver injury. To explore whether acetic acid could alleviate liver injury induced by sepsis, serum ALT, AST and TBiL levels were detected. In control group, serum ALT, AST and TBiL levels were 35.5±4.6 U/l, 46.9±4.3 U/l and 7.2±0.6 μmol/l, respectively (Fig. 3). No significant differences were identified between the control + acetic acid and control groups. CLP significantly increased serum ALT, AST and TBiL levels by 1.4-, 1.4- and 2.2-fold compared with the control group, respectively. Treatment with acetic acid attenuated changes in ALT, AST and TBiL levels. In the CLP + acetic acid group, serum ALT, AST and TBiL levels decreased significantly compared with the CLP + PBS group, respectively.

Histological evaluations revealed that the livers in the control group possessed normal cell structures (Fig. 4A). In comparison with the control group, marked histopathological changes were observed in the CLP + PBS and CLP + acetic acid groups, including inflammatory cells infiltration and cell death (Fig. 4B and C). The livers from mice in the control + acetic

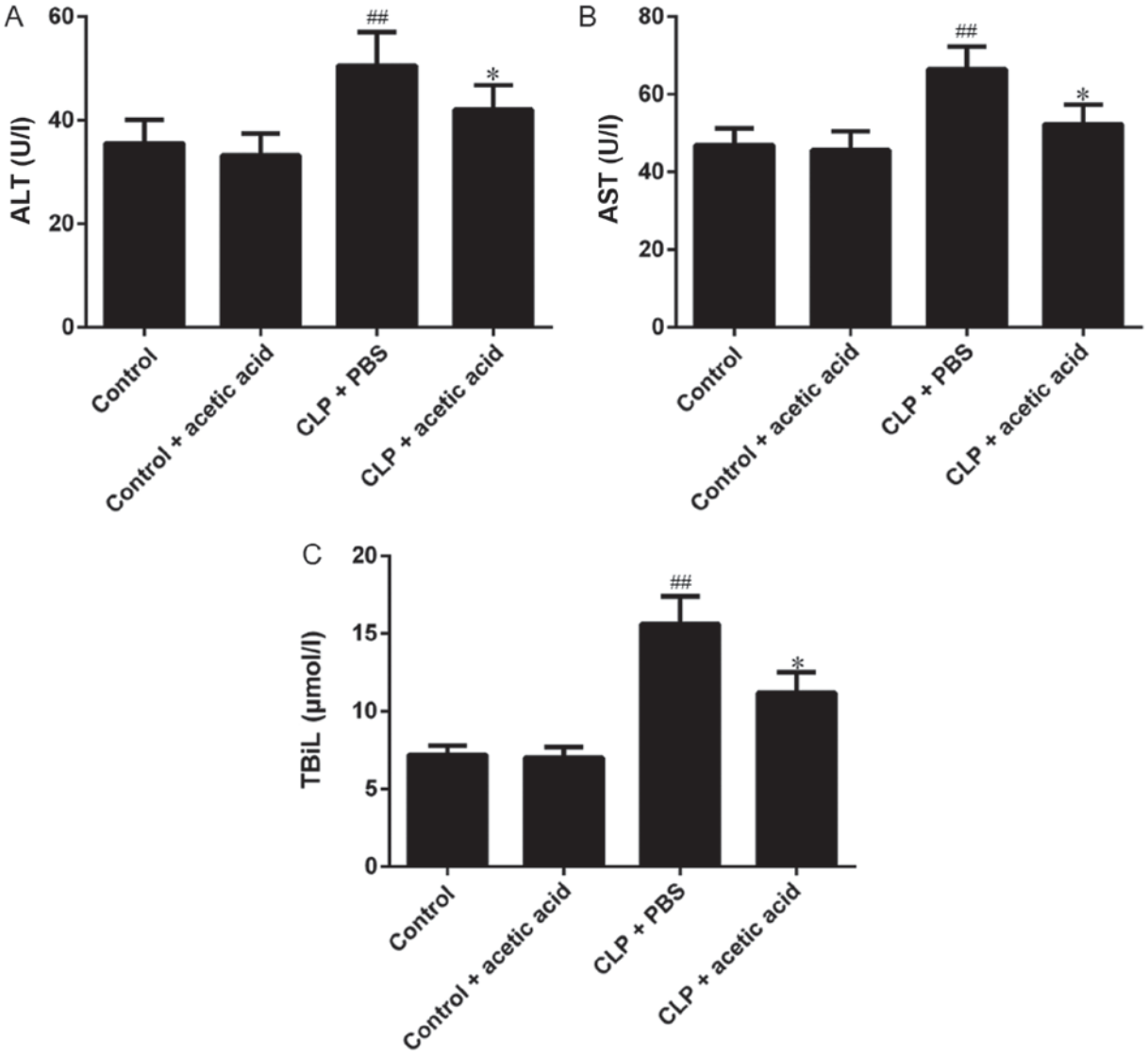

Figure 3. Acetic acid decreases the concentration of liver enzymes in the serum of CLP-model mice. Serum (A) ALT, (B) AST and (C) TBiL levels were detected by a biochemical automatic analyzer 12 h after PBS and acetic acid treatment (n=6 mice/group). Results are presented as mean ± standard deviation. *P<0.05 vs. the CLP + PBS group. ##P<0.01 vs. the control group. CLP, cecal ligation and puncture; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBiL, total bilirubin.

acid group retained normal cell structures (Fig. 4D). Acetic acid treatment attenuated the pathological changes observed in the CLP + PBS group. These results suggested that acetic acid could alleviate liver injury induced by sepsis in mice.

Acetic acid upregulates the expression of TRIM40 and inhibits the expression of NF-κB and TLR4 in liver. The authors of the current study assessed the expression levels of TRIM40, NF-κB and TLR4 in livers to verify if acetic acid could influence TRIM40 expression and the TLR4 signaling pathway. According to the results of RT-qPCR analysis and western blot analysis, it was demonstrated that mRNA levels of NF-κB and TLR4 significantly increased by 1.9- and 1.6-fold, respectively, while the mRNA level of TRIM40 significantly decreased by 28% compared with the control group (Fig. 5A). Furthermore, in the CLP + acetic acid group, the expression of TRIM40 was significantly higher while the expression levels of NF-κB and TLR4 were significantly lower compared with the CLP + PBS group. Also, CLP increased the synthesis of NF-κB and TLR4 proteins while decreasing the synthesis of TRIM40 compared with control group (Fig. 5B). In the CLP + acetic acid group, the synthesis of TRIM40, NF-κB and TLR4 proteins were upregulated compared with the control group. The results suggested that acetic acid could increase the expression of TRIM40 and decrease the expression of NF-κB and TLR4. Additionally, acetic acid may alleviate the inflammatory response in septic mice by inhibiting the activity of the TLR4 signaling pathway. No significant differences were identified between the control + acetic acid and control groups.

Acetic acid reduces supernatant inflammatory factors levels in peritoneal macrophages. To get a better understanding of how acetic acid inhibited the inflammatory response induced by sepsis, the authors of the current study investigated the inflammatory factors levels in the supernatant of peritoneal macrophages. Following the incubation with LPS,

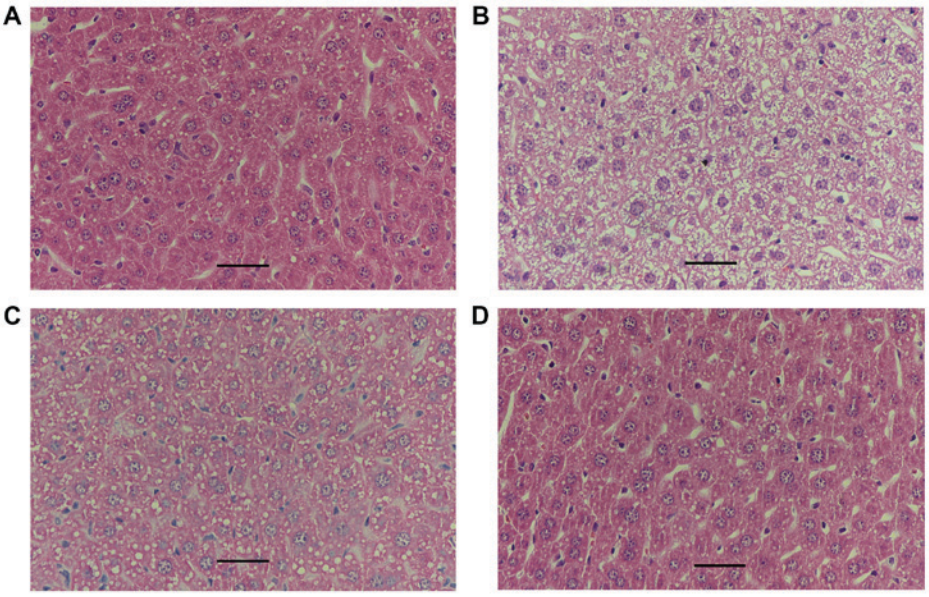

Figure 4. Acetic acid treatment attenuates pathological changes in the livers of CLP-model mice. Liver tissue in the (A) control, (B) CLP + PBS, (C) CLP + acetic acid and (D) control + acetic acid groups. Scale bar=100 μm. CLP, cecal ligation and puncture.

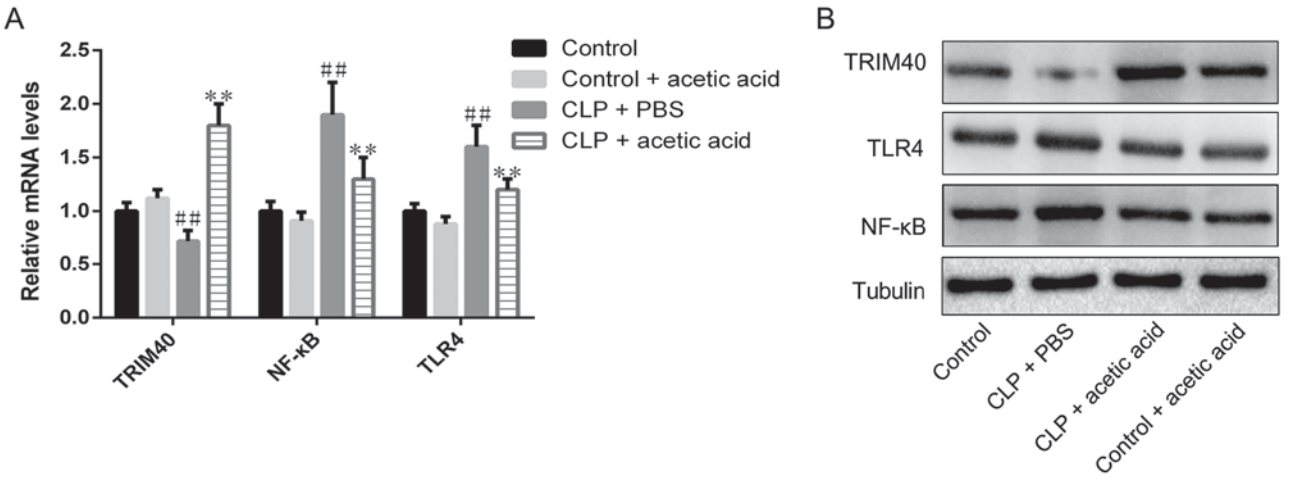

Figure 5. Acetic acid decreases NF-κB and TLR4, and increases TRIM40 levels in CLP-model mice. The (A) mRNA levels and (B) protein levels of NF-κB, TLR4 and TRIM40 in the livers of CLP-model mice. Results are presented as mean ± standard deviation. **P<0.01 vs. the CLP + PBS group. $^{\#\#}$P<0.01 vs. the control group. CLP, cecal ligation and puncture; TRIM40, tripartite motif-containing protein 40; NF-κB, nuclear factor-κB; TLR4, Toll-like receptor 4.

the secretion of IL-6, IL-10 and TNF-α increased 159, 123 and 146%, respectively, compared with the control group (Fig. 6). The results demonstrated that LPS may stimulate the synthesis and secretion of cytokines. IN the LPS + acetic acid group, the secretion of IL-6 and TNF-α significantly decreased by 30 and 36%, respectively, and the secretion of IL-10 significantly increased by 53%, compared with the LPS group. The results suggested that inflammation induced by LPS was attenuated by acetic acid and that acetic acid may regulate the secretion of cytokines in macrophages. However, acetic acid had no significant effects on normal macrophages. When peritoneal macrophages were treated with LPS + acetic acid + TRIM40 siRNA, the secretion of IL-6 and TNF-α significantly decreased while the secretion of IL-10 significantly increased compared with the LPS group.

Acetic acid upregulates the expression of TRIM40 and TRIM40 may inhibit the activity of TLR4 signal pathway in peritoneal macrophages. To assess the role of TRIM40 in the TLR4 signaling pathway, the mRNA levels and protein expression levels of TRIM40, NF-κB and TLR4 were measured. The results of the RT-qPCR analysis demonstrated that the transfections were successful as incubating cells with siTRIM40 significantly decreased the mRNA expression of TRIM40 compared with the control group (Fig. 7A). In the LPS group, the expression of TRIM40 significantly decreased while the expression of NF-κB and TLR4 significantly increased compared with the control group (Fig. 7B). It was also demonstrated that the mRNA level of TRIM40 of LPS-stimulated cells significantly increased by 1.9-fold when treated with acetic acid, while levels of NF-κB and TLR4 significantly decreased by 42 and 33%, respectively,

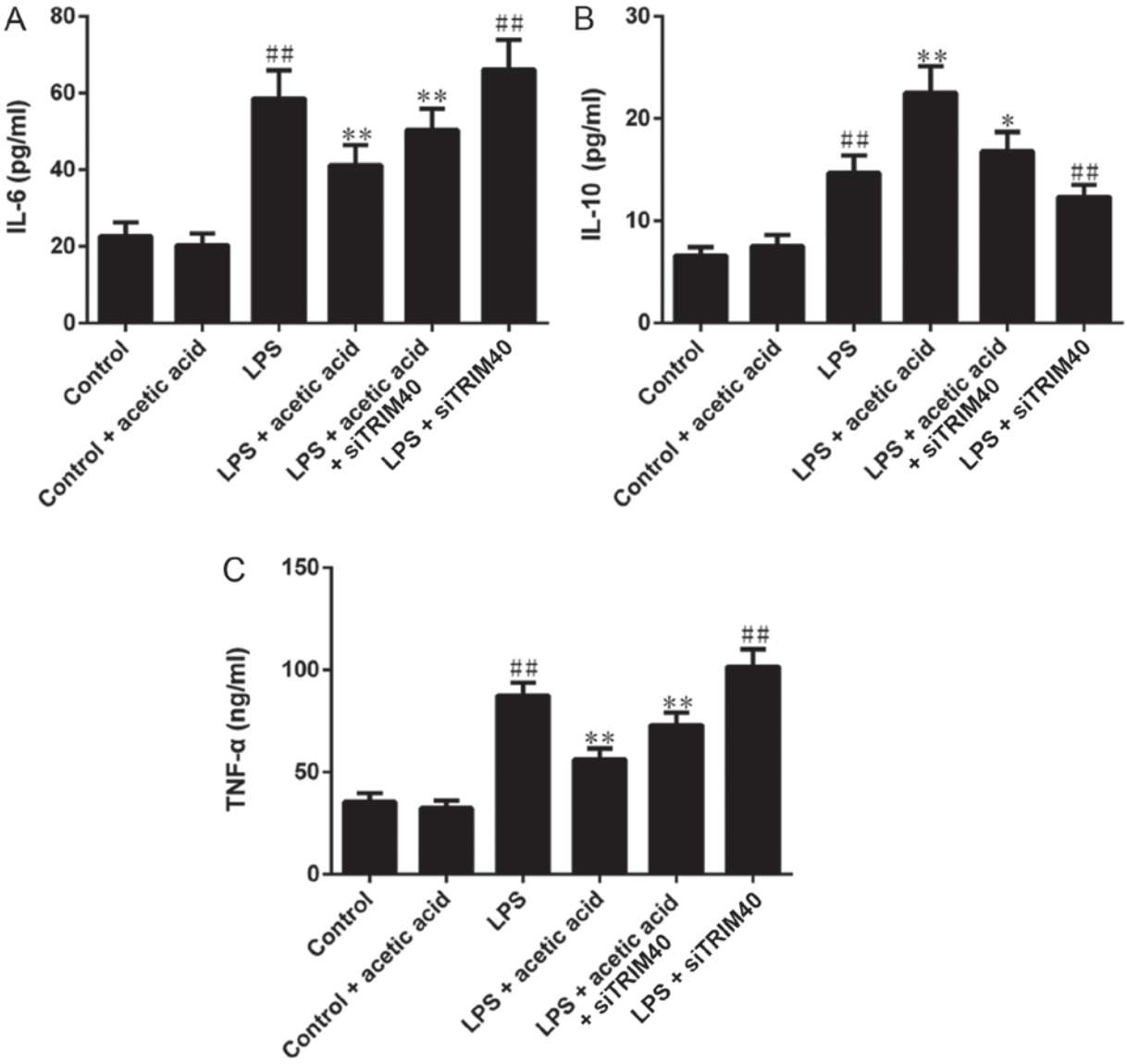

Figure 6. Acetic acid reduces supernatant inflammatory factors levels in peritoneal macrophages. Protein concentrations for (A) IL-6, (B) IL-10 and (C) TNF-α were measured using ELISA kits (n=6 mice/group). Results are presented as mean ± standard deviation. *P<0.05, **P<0.01 vs. the LPS group. $^{\#\#}$P<0.01 vs. the control group. IL, interleukin; TNF-α, tumor necrosis factor α; LPS, lipopolysaccharide; TRIM40, tripartite motif-containing protein 40; si, small interfering RNA.

compared with the LPS group. When LPS-stimulated peritoneal macrophages were treated with acetic acid and siTRIM40, the expression of TRIM40 significantly reduced compared with the LPS group. Additionally, the expression levels of NF-κB and TLR4 increased by 1.6- and 1.3-fold, respectively, compared with the LPS + acetic acid group. When compared with the LPS group, the expression levels of TLR4 and NF-κB were significantly increased in the LPS + siTRIM40 group. All these results suggested that acetic acid could promote the expression of TRIM40 and that TRIM40 may inhibit the activity of the TLR4 signaling pathway in peritoneal macrophages. Additionally, the protein expression trends were similar to the mRNA levels (Fig. 7C). The level of TRIM40 in the LPS + acetic acid group was increased, while the levels of NF-κB and TLR4 decreased compared with the LPS group. After treating the LPS + acetic acid group with siTRIM40, the levels of NF-κB and TLR4 markedly increased.

Discussion

In the current study, it was demonstrated that acetic acid could alleviate the inflammatory response and liver injury in CLP-model mice by increasing the expression of TRIM40, and TRIM40 could regulate the activity of the TLR4 signaling pathway. To illustrate the interaction between TRIM40 and the TLR4 signaling pathway, LPS-induced peritoneal macrophages were observed following the alteration of TRIM40 levels. The results suggested that TRIM40 may act on as decreasing TRIM40 expression upregulated the activity of the TLR4 signaling pathway; the protective effect of acetic acid was reversed when TRIM40 was silenced. As a result, the authors of the current study hypothesized that acetic acid could inhibit inflammation and protect liver in septic mice mainly by enhancing the expression of TRIM40.

Acetic acid, as a type of common chemical compound, has been gradually receiving substantial attention and interest in

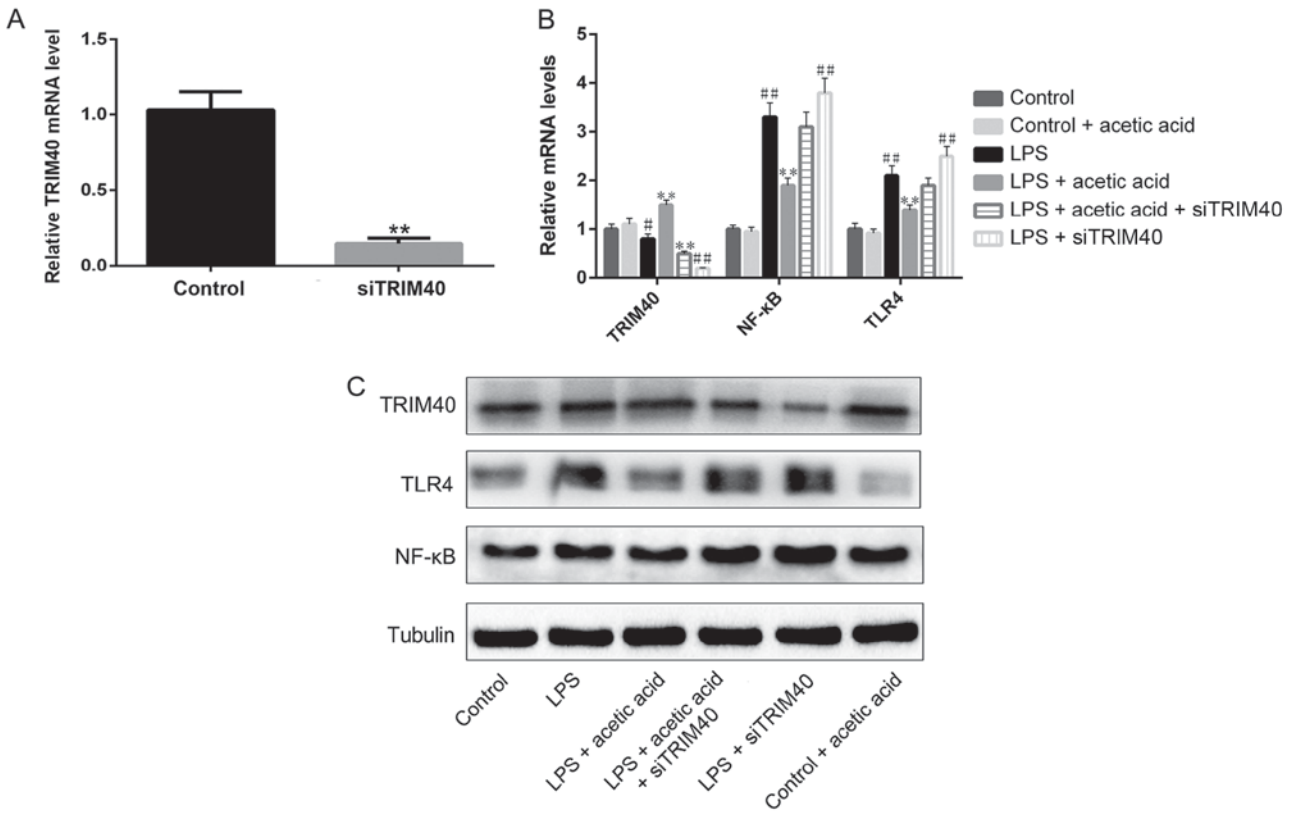

Figure 7. Acetic acid upregulates the expression of TRIM40 and TRIM40 may inhibit the activity of the TLR4 signaling pathway in peritoneal macrophages. (A) Relative mRNA expression of TRIM40. (B) Relative mRNA and (C) protein expression levels of TRIM40, NF-κB and TLR4 in peritoneal macrophages. Results are presented as mean ± standard deviation. **P<0.01 vs. the LPS group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. the control group. TRIM40, tripartite motif 40; NF-κB, nuclear factor-κB; TLR4, Toll-like receptor 4; si, small interfering RNA.

recent years. Acetic acid is the major ingredient in vinegar, and has been reported to be helpful for preventing metabolic syndrome in rats and human (33). In addition, vinegar has also revealed to be effective in anti-hypercholesterolemia, anti-hyperglycemia, anti-hypertension, anti-cancer and anti-inflammation treatment (34-36). Beh *et al* (33) revealed that synthetic acetic acid vinegar and Nipa vinegar could potentially alleviate obesity by altering inflammation, lipid metabolism and gut microbe composition in high-fat-diet-induced obese mice. According to the aforementioned study, synthetic acetic acid vinegar and Nipa vinegar treatments significantly suppressed the expression of inducible nitric oxide synthase (iNOS) in the liver, and reduced the serum lactate dehydrogenase level when compared with the untreated obese group. Additionally, Nipa vinegar contributed to improved anti-inflammation treatment by suppressing the expression of NF-κB and iNOS (37). These results may be attributable to the upregulation of adiponectin by Nipa vinegar, as adiponectin inhibits inflammation by suppressing the activation of NF-κB (37).

Yamashita (34) also determined that exogenously-administered acetic acid influenced lipid metabolism in the liver and skeletal muscles, and alleviated obesity and obesity-linked type 2 diabetes. To illuminate the protective effect of pineapple vinegar, Mohamad *et al* (35) determined that oral administration of pineapple vinegar restored liver antioxidant levels, and reduced serum enzyme biomarker levels, the expression of inflammatory factors and the liver protein, cytochrome P450, in mice with paracetamol-induced liver damage. As the major ingredient in different types of vinegars, acetic acid may have potential in anti-inflammation treatment and thus protect of liver. Therefore, the authors investigated the possibility of using acetic acid to inhibit the inflammatory response and decrease liver injury in septic mice, and the effectiveness of acetic acid was verified in studies by our group.

The current study demonstrated that the expression of TRIM40 was downregulated in septic mice and this phenomenon could be reversed with intravenous injections of acetic acid. Noguchi *et al* (38) revealed that TRIM40 was highly expressed in the normal gastrointestinal tract and bound to Nedd8, which was conjugated to target proteins by neddylation. The aforementioned study also determined that TRIM40 promoted the neddylation of NF-κB essential modulator, which is a crucial regulator in NF-κB activation. As a result, TRIM40 could inhibit the activity of NF-κB. This finding was also verified in the current study. Additionally, Noguchi *et al* (38) observed that the expression of TRIM40 was downregulated in the gastrointestinal tract and chronic inflammatory lesions of the gastrointestinal tract. The results suggested that, in the gastrointestinal tract, TRIM40 could prevent inflammation-associated carcinogenesis. In comparison, the expression of TRIM40 was decreased in CLP-model mice and LPS-treated peritoneal macrophages in the current study. However, the expression of TRIM40 increased significantly following acetic acid treatment. Therefore, the authors

of the current study hypothesized that acetic acid may alleviate inflammation by upregulating the expression of TRIM40. Then, the authors performed further experiments to elucidate the relationship between TRIM40 and the TLR4 signaling pathway. When TRIM40 was silenced, the protective effect of acetic acid in reducing inflammation was severely weakened and the expression levels of inflammatory cytokines were increased. The results suggested that TRIM40 could be the major target for acetic acid to function as protective factor in septic mice.

Several cytokines have been correlated with the severity of sepsis (39). For example, IL-6 and TNF-α are the major inflammatory mediators that are significantly elevated in sepsis (10,40). Excessive generation of cytokines can be regulated by the activation of certain signaling pathways, including TLR4 and p38 mitogen-activated protein kinase (40,41). The TLR4 signaling pathway has been demonstrated to upregulate in sepsis and the expression of NF-κB also increases, since it is the downstream intracellular molecule of the TLR4 signaling pathway (42). The overexpression of cytokines demonstrated that they caused liver injury by activating complex signaling cascades (43). Thus, the excessive inflammatory response and associated liver injury may be ameliorated by suppressing the generation of cytokines and associated signaling pathways in sepsis. Several studies previously had demonstrated the role of TLRs in the production of cytokines and the activation of inflammatory cascades in sepsis (44,45).

A number of studies investigated the involvement of NF-κB in sepsis-induced liver injury (40,41). Feng *et al* (46) demonstrated that the degradation of NF-κB inhibitor α (IκB-α) and the accumulation of NF-κB in the nucleus increased in septic mice, and roflumilast alleviated liver injury by inhibiting the expression of NF-κB and degradation of IκB-α. Those results suggested that NF-κB is closely associated with liver injury in septic mice and liver injury was ameliorated by inhibiting the expression of NF-κB. In the current study, the authors revealed that liver injury was reduced following acetic acid treatment and the expression of NF-κB was markably decreased. In conclusion, acetic acid may alleviate liver injury in septic mice by suppressing the production of cytokines and inhibiting the TLR4 signaling pathway.

In summary, the experiments in the current study indicate the effectiveness of acetic acid in attenuating inflammation and decreasing liver injury in septic mice by upregulating the expression of TRIM40. TRIM40 suppressed the production and secretion of cytokines, including IL-6 and TNF-α, increased the expression of IL-10, alleviated liver damage, inhibited the activity of TLR4 signal pathway and improved the survival rate in septic mice. Acetic acid treatment reduced inflammation and decreased the expression levels of inflammatory cytokines. When TRIM40 was silenced, the protective effects of acetic acid were reversed. TRIM40 is possibly the predominant target of acetic acid, allowing acetic acid to function as protective factor in septic mice. However, further studies are required to illustrate the mechanism of alleviation of inflammation by acetic acid and whether other factors are affected by alterations in TRIM40 expression. The findings in the current study provide novel insights into the pharmacological activities of acetic acid as a novel strategy for the treatment of sepsis.

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Availability of data and materials

Data and materials can be obtained from corresponding author upon reasonable request.

Authors' contributions

HY and LM performed the cell studies and wrote the manuscript. DA, NH and HL performed the animal studies. XS performed the analysis of the data. XP designed the study.

Ethics approval and consent to participate

The experiment was approved by the Ethics Committee in Qingdao University (Qingdao, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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