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Original article

Traditional Chinese medicine Pien-Tze-Huang ameliorates LPS-induced sepsis through bile acid-mediated activation of TGR5-STAT3-A20 signalling

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ABSTRACT

Pien Tze Huang (PZH), a class I nationally protected traditional Chinese medicine (TCM), has been used to treat liver diseases such as hepatitis; however, the effect of PZH on the progression of sepsis is unknown. Here, we reported that PZH attenuated lipopolysaccharide (LPS)-induced sepsis in mice and reduced LPS-induced production of proinflammatory cytokines in macrophages by inhibiting the activation of mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B (NF-κB) signalling. Mechanistically, PZH stimulated signal transducer and activator of transcription 3 (STAT3) phosphorylation to induce the expression of A20, which could inhibit the activation of NF-κB and MAPK signalling. Knockdown of the bile acid (BA) receptor G protein-coupled bile acid receptor 1 (TGR5) in macrophages abolished the effects of PZH on STAT3 phosphorylation and A20 induction, as well as the LPS-induced inflammatory response, suggesting that BAs in PZH may mediate its anti-inflammatory effects by activating TGR5. Consistently, deprivation of BAs in PZH by cholestyramine resin reduced the effects of PZH on the expression of phosphorylated-STAT3 and A20, the activation of NF-κB and MAPK signalling, and the production of proinflammatory cytokines, whereas the addition of BAs to cholestyramine resin-treated PZH partially restored the inhibitory effects on the production of proinflammatory cytokines. Overall, our study identifies BAs as the effective components in PZH that activate TGR5-STAT3-A20 signalling to ameliorate LPS-induced sepsis.

1. Introduction

Sepsis is characterized by a systemic dysregulated host response to infection. A dysregulated immune response to infection can lead to tissue and organ injury and ultimately death [1,2]. Statistics on the incidence of sepsis are mostly from high-income countries, and 2.8 million people die from sepsis each year [3]. The incidence of severe sepsis was reported to be over 750,000 cases per year in the United States (300 cases per 100,000 people) [4]. In the UK, the reported prevalence of sepsis in ICU-derived cohorts is 27 % of all ICU admissions [5]. With an ageing population, sepsis will become an even greater problem [2]. However, the pathogenesis of sepsis is still not fully understood, and there is no particularly effective treatment in the clinic. Therefore, it is important to clarify the pathogenesis of sepsis and develop safe and effective drugs to prevent and treat sepsis.

Lipopolysaccharide (LPS), which is also known as endotoxin, is made up of lipids and polysaccharides and is one of the main triggers of sepsis and septic organ dysfunction [6,7]. LPS induces inflammatory responses by binding to toll-like receptor 4 (TLR4) and then activating downstream signalling pathway such as mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B (NF-κB), eventually leading to the generation of tumour necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 [8–10]. The

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phosphorylation of NF-κB inhibitor alpha (IκBα) is critical for activation of the NF-κB signalling pathway, and this protein is marked for degradation by ubiquitin-dependent proteasomes in response to LPS stimulation, thereby releasing NF-κB dimers from the cytoplasmic NF-κB-IκB complex and allowing them to translocate to the nucleus, where they regulate the expression of target genes [11–13]. MAPK kinase kinase (MKKK) phosphorylates and stimulates the conserved serine/threonine motif of MAPK kinase (MKK) [14,15]. Subsequently, MKK activates other MAPKs, including extracellular signal-regulated kinases (ERK), C-Jun N-terminal kinases (JNK) and p38, to trigger inflammatory responses [16,17]. To prevent septic shock, it is critical to terminate the activation of the NF-κB and MAPK signalling pathway and then reduce the production of proinflammatory cytokines which can be more damaging than the primary infection [18].

Pien Tze Huang (PZH), a class-1 nationally protected traditional Chinese medicine (TCM), is mainly composed of *Musk*, *Calculus bovis* (bezoar bovis), *Snake gall* (Shedan) and *Panax notoginseng* (Sanqi) roots [19]. These natural ingredients exert anti-inflammatory and detoxifying effects and can therefore be used to treat diseases such as hepatitis, liver injury, and liver cancer. It has been reported that PZH attenuates liver fibrosis by inhibiting the NF-κB pathway, reducing inflammation, and promoting hepatic stellate cell apoptosis [20]. PZH alleviated the inflammation in collagen-induced arthritis mice by inhibiting the activation of the NF-κB signalling pathway [21]. Previous studies have shown that PZH exerted therapeutic effects on experimental autoimmune encephalomyelitis (EAE) mice by inhibiting the differentiation and function of helper T cell 1(Th1) and Th17 cells [22]. Although some biological effects of PZH has been revealed, its effects on sepsis are unclear. In this study, we demonstrated that PZH inhibited LPS-induced sepsis by blocking NF-κB and MAPK signalling pathways via the activation of the G protein-coupled bile acid receptor 1 (TGR5)-signal transducer and activator of transcription 3 (STAT3)-A20 pathway.

2. Materials and methods

2.1. Materials

LPS (*Escherichia coli* strain O111:B4) was obtained from Sigma (L2630; Sigma, St. Louis, MO, USA). PZH powder was obtained from Zhangzhou Pien Tze Huang Pharmaceutical Co., Ltd (Zhangzhou, China). The powder was dissolved in phosphate buffered solution (PBS) and ultrasonicated at 70 Hz for 40 min. Then, the supernatant was collected for *in vitro* assays, and the suspension was used for the *in vivo* assays. Stattic (Cat# HY-13818), INT-777 (Cat# HY-15677), INT-767 (Cat# HY-12434) and GW4064 (Cat# HY-50108) were purchased from MedChemExpress (Monmouth Junction, NJ, USA) and dissolved in dimethyl sulfoxide (DMSO). Cholestyramine resin was purchased from Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China). Antibodies against phospho-p38 (Cat# 4511s), p38 (Cat# 9218s), phospho-JNK (Cat# 4668s), JNK (Cat# 9252s), phospho-ERK1/2 (Cat# 4377s), ERK1/2 (Cat# 9102s), phospho-p65 (Cat# 3033s), p65 (Cat# 8242s), A20 (Cat# 5630s), and STAT3 (Cat# 9139s, CST) were purchased from Cell Signalling Technology (Boston, MA, USA). Antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cat# AC002), β-actin (Cat# AC002) and phospho-STAT3 (Cat# AC004) were obtained from ABclonal Technology (Wuhan, China). Antibodies against TGR5 (Cat# ab72608) were obtained from Abcam (Cambridge, UK).

2.2. Mice

Six- to eight-week-old male BALB/c mice were obtained from the Laboratory Animal Center of Xiamen University (Xiamen, China). The mice were randomly divided into different groups and housed under specific pathogen-free conditions with a 12/12-h light/dark cycle and a regular chow diet and water ad libitum. All animal experiments performed followed the ethics guidelines and were approved by the Laboratory Animal Center of Xiamen University (Approval number: XMULAC20230087).

2.3. Cell culture and treatment

The murine macrophage line RAW264.7 was cultured in Dulbecco's modified Eagle medium (DMEM) containing 10 % foetal bovine serum (FBS) plus 10 μg/mL streptomycin and 10 U/mL penicillin. Peritoneal macrophages (PMs) were obtained from mice after induction with 4% thioglycollate medium (T9032; Sigma) for three days. PMs and THP-1 cells were cultured in RPMI 1640 with 10 % FBS and 1 % penicillin-streptomycin. THP-1 cells were differentiated with 100 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma) for 48 h. The cells were maintained in a humidified chamber with 5% CO_2 at 37 °C. Cells were pretreated with PBS or PZH solution (1.25 mg/mL) for 2 h and incubated with LPS for 6 h, and then the supernatants and the cells were collected for enzyme-linked immunosorbent assay (ELISA) and quantitative real-time PCR, respectively. Cells pretreated with PBS or PZH solution (1.25 mg/mL) for 2 h were incubated with LPS (100 ng/mL) for 0, 20, 40 and 60 min, and then the cells were collected for Western blot analysis. Cells were pretreated with INT-777 (30 μM), INT-767 (10 μM), or GW4064 (5 μM) for 2 h and treated with LPS (100 ng/mL) for 6 h, and then the cells were collected for quantitative real-time -PCR. Cells were pretreated with INT-777 (30 μM) for 2 h and treated with LPS (100 ng/mL) for 40 min, and then the proteins were obtained for Western blot analysis. Cells were pretreated with stattic (20 μM) for 30 min and treated with PZH (1.25 mg/mL) for 2 h followed by incubation with LPS (100 ng/mL) for 40 min, and then the cells were collected for Western blot analysis.

2.4. Cholestyramine resin treatment

PZH was dissolved in PBS and ultrasonicated at 70 Hz for 40 min. Then, 0.47 g cholestyramine resin was added to the 5 mg/mL PZH solution and mixed overnight at 4 °C to remove bile acids (BAs) from the PZH solution.

2.5. LPS-induced sepsis

Mice were intragastrically pretreated with PBS or PZH (0.234 g/kg) for 2 h and then intraperitoneally (i.p.) injected with LPS (10 mg/kg). Body temperatures and survival rates were recorded. Two hours after LPS injection, the mice were anaesthetized and sacrificed, the serum was collected for cytokine assays, and the livers, lungs, and kidneys were collected for hematoxylin and eosin (H&E) staining and total RNA isolation.

2.6. Cytokine analysis by ELISA

The concentrations of TNF-α, IL-6, and IL-1β were determined using ELISA kits (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.7. Total BA analysis

The concentrations of BAs were determined (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

2.8. RNA-seq and analysis

Transcriptome sequencing of peritoneal macrophages was conducted by Bejing Genomics Institute (Shenzhen, China) according to a published procedure [23]. RNA counts were imported into R (v4.1.3), and normalization for library size and regularized-logarithmic transformation of counts were performed using DEseq2 (v1.34.0). A volcano plot was drawn by ggpolt (v3.3.6). The heatmap was drawn by pheatmap (v1.0.12) according to the gene expression in different samples.

2.9. Gene set enrichment analysis (GSEA)

GSEA (http://www.broadinstitute.org/gsea/index.jsp) was used to identify gene sets and pathways associated with the gene expression data. GSEA of the expression data was used to assess the

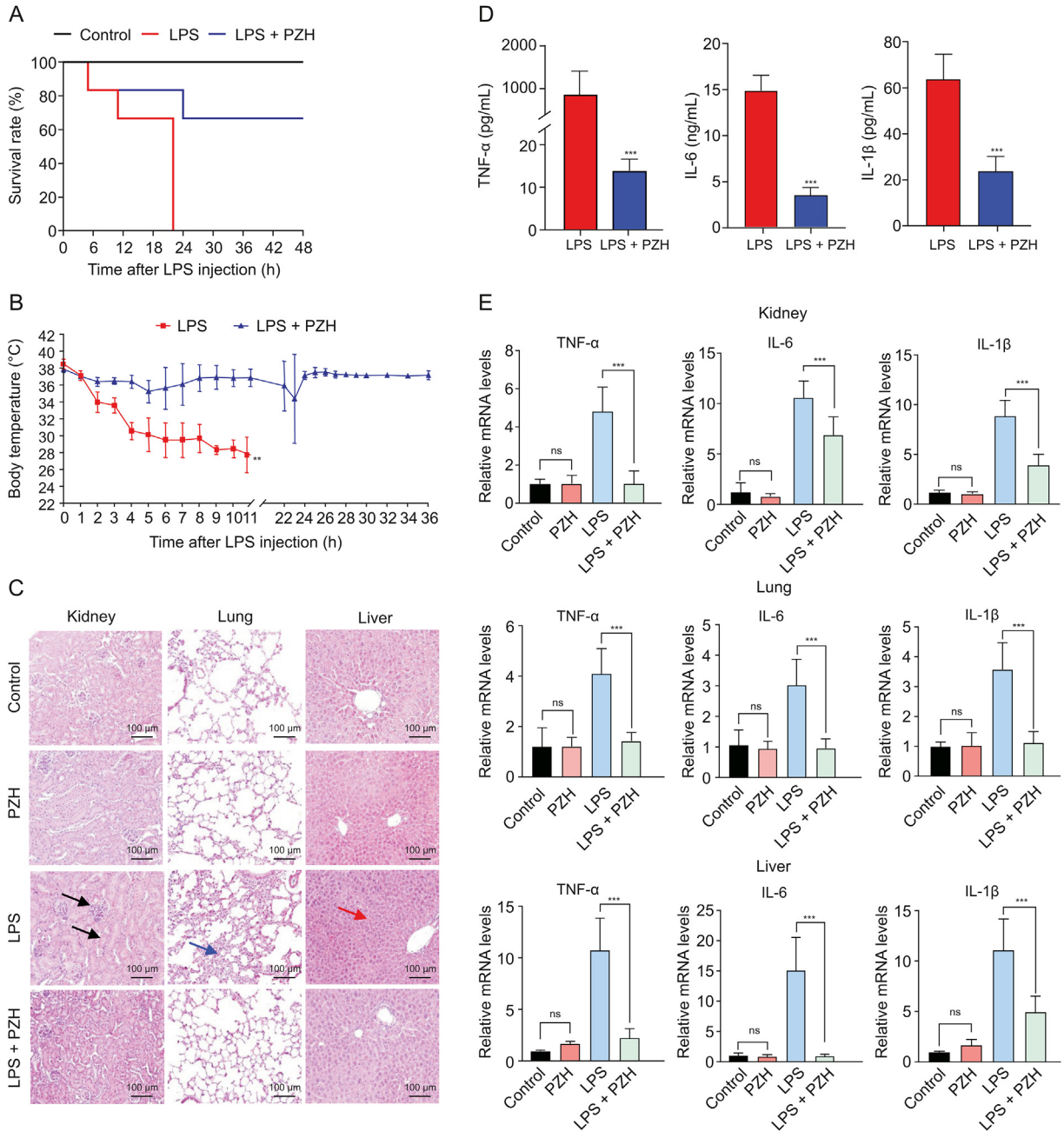
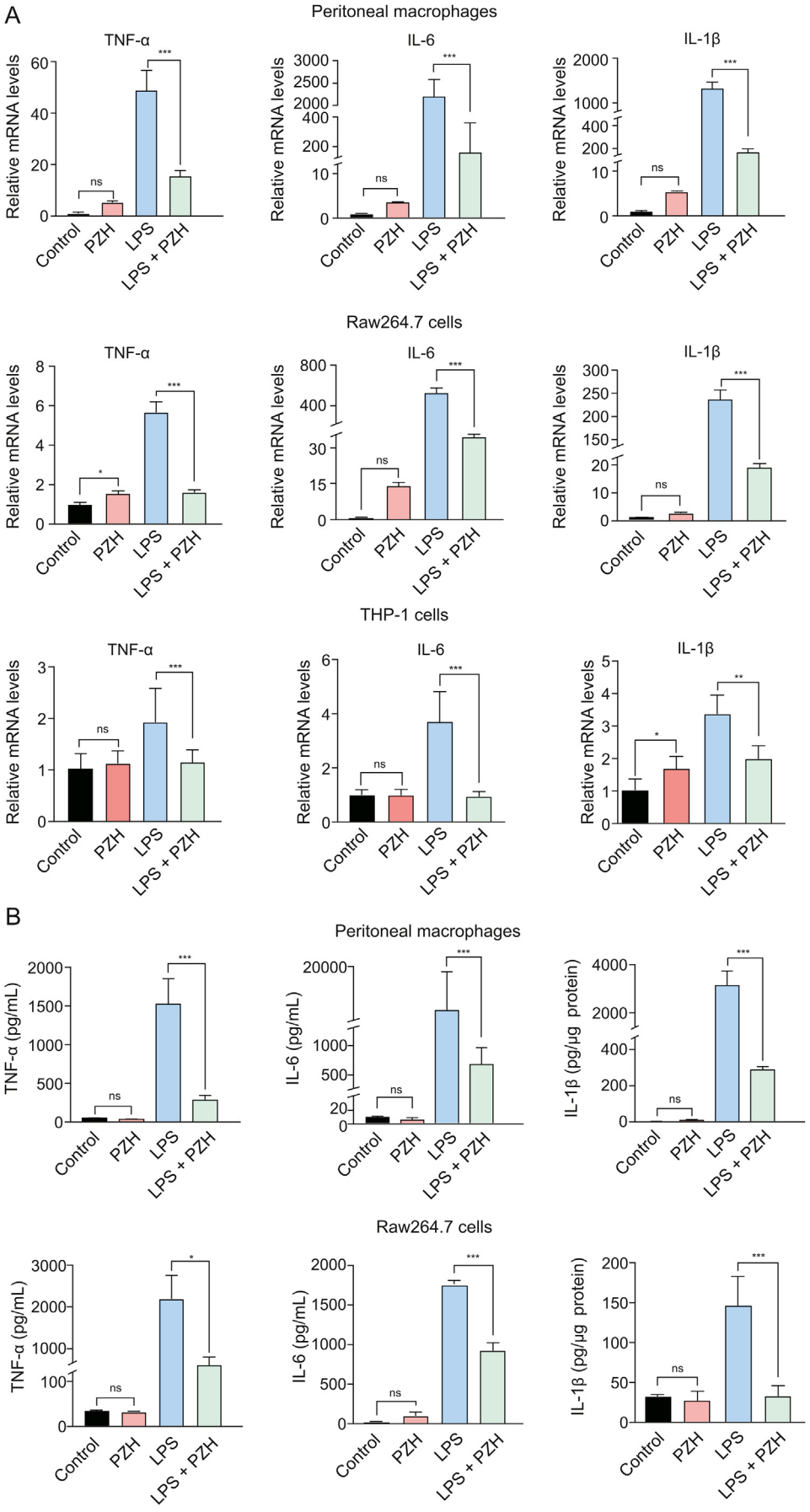


Fig. 1. Pien Tze Huang (PZH) effectively ameliorates lipopolysaccharide (LPS)-induced sepsis. (A, B) BALB/c mice were divided into two groups ($n = 6$ for each group): one group was only intraperitoneally injected with LPS (10 mg/kg), and the other group was pretreated with PZH for 2 h before lipopolysaccharide (LPS) injection. Survival rates (A) and body temperatures (B) were measured at different time points. (C) Hematoxylin and eosin-stained slides of the kidney, lung and liver sections in the different groups were examined. $n = 6$ per group. Black arrows indicate renal cystic atrophy, blue arrow indicates alveolar wall swelling, and red arrow indicates cord arrangement disorder. (D) PZH pretreatment significantly decreased the levels of interleukin (IL)-1β, IL-6 and tumour necrosis factor-α (TNF-α) in serum ($n = 6$ for each group). (E) PZH decreased the mRNA levels of IL-1β, IL-6 and TNF-α in the kidney, lung and liver induced by LPS. The data are shown as the mean ± standard deviation (SD). $^{**}P < 0.01$, $^{***}P < 0.001$, ns: no significance, based on two-tailed Student's *t*-test or one-way analysis of variance (ANOVA).


A
Peritoneal macrophages
TNF-α
IL-6
IL-1β
Relative mRNA levels
Raw264.7 cells
THP-1 cells
Control
PZH
LPS
LPS + PZH
ns
B
TNF-α (pg/mL)
IL-6 (pg/mL)
IL-1β (pg/μg protein)

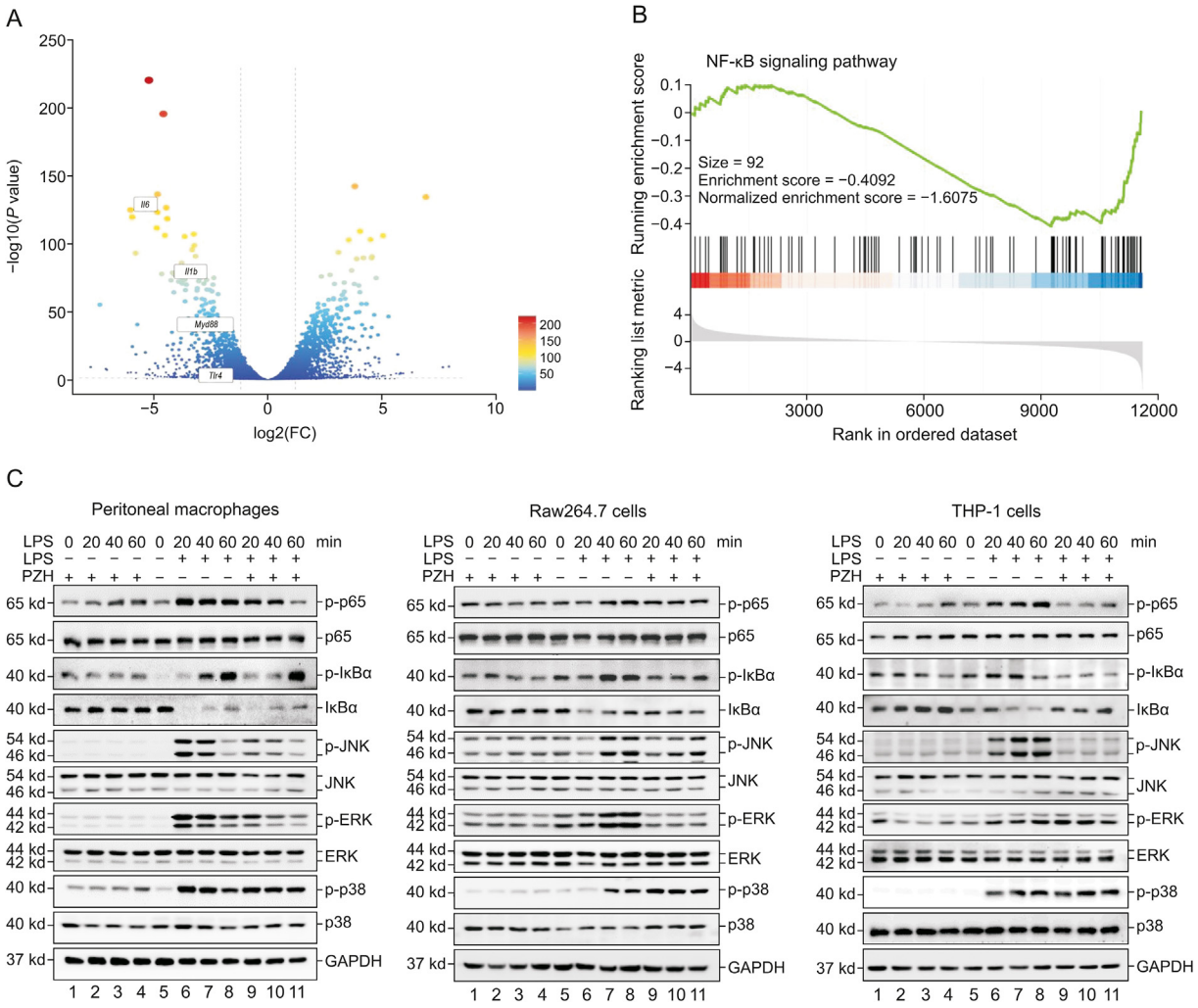


Fig. 3. Pien Tze Huang (PZH) effectively suppresses lipopolysaccharide (LPS)-induced nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) activation. (A, B) Peritoneal macrophages were treated with PZH (1.25 mg/mL) for 2 h and then incubated with or without LPS (100 ng/mL) for 6 h. (A) Volcano plot analysis showing significantly lower gene expression of *interleukin (IL)-6, IL-1b*, (*Myd88*) and *toll-like receptor 4 (Tlr4)* in the LPS + PZH group than in the LPS group. (B) Gene set enrichment analysis identified the NF-κB and MAPK signalling pathways as regulatory targets of PZH. (C) PZH (1.25 mg/mL) significantly decreased the protein expression of phospho-p65 (p-p65), phospho-nuclear factor-kappa B inhibitor alpha (IκBα), phospho-C-Jun N-terminal kinases (p-JNK) and phospho-extracellular signal-regulated kinases (p-ERK), but did not decrease the protein expression of phospho-p38 (p-p38) induced by LPS (100 ng/mL) in peritoneal macrophages, RAW264.7 cells and THP-1 cells. The results are representative of one of three experiments.

enrichment of the Kyoto Encyclopedia of Genes and Genomes (KEGG). Ggplot2 and ClusterProfiler packages were used to visualize the results.

2.10. Quantification of BAs in PZH supernatant

Quantification of BAs in PZH supernatant was performed by liquid chromatography-mass spectrometry (LC-MS) (AB SCIEX AB6500+, Foster City, CA, USA) as previously reported [24,25]. Briefly, BAs in PZH supernatant were extracted in 600 μL of methanol (−20 °C) with 100 mg of glass beads by vortexing for 60 s. The samples were then centrifuged at 12,000 rpm for 10 min at 4 °C, and 400 μL of PZH supernatant was concentrated to dryness with a vacuum concentrator. The PZH supernatant was reconstituted in 100 μL of 30% methanol, and an appropriate amount of PZH supernatant was diluted 20 times with 30% methanol and added to the LC-MS bottles for LC-MS analysis [26].

2.11. LC-MS analysis of PZH supernatant

One hundred microliters of PZH supernatant was mixed with 400 μL of methanol/acetonitrile (1:1, *V/V*) by vortexing for 30 s, and then the mixture was sonicated for 10 min at 4 °C. After 1 h incubation at −20 °C, the samples were centrifuged at 13,000 rpm for 15 min at 4 °C. The supernatant was transferred to a new tube and evaporated to dryness at 4 °C using a vacuum concentrator. The samples were then reconstituted with 100 μL of acetonitrile/H_2O solution (1:1, *V/V*) for injection analysis. The components in the PZH supernatant were

Fig. 2. Pien Tze Huang (PZH) strongly inhibits the lipopolysaccharide (LPS)-induced inflammatory response in macrophages. Cells were pretreated with phosphate buffered solution (PBS) or PZH solution (1.25 mg/mL) for 2 h and incubated with LPS (100 ng/mL) for 6 h, and then the supernatants and the cells were collected for enzyme-linked immunosorbent assay (ELISA) and quantitative real-time PCR. (A) PZH decreased the mRNA levels of interleukin (IL)-1β, IL-6 and tumour necrosis factor-α (TNF-α) in peritoneal macrophages, RAW264.7 cells, and THP-1 cells. (B) PZH decreased the production of IL-6 and TNF-α (in culture medium) and IL-1β (in cell lysates) in peritoneal macrophages and RAW264.7 cells. The data are shown as the mean ± standard deviation (SD) ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns: no significance, based on one-way analysis of variance (ANOVA).

analyzed by LC-MS. The data were collected using Analyst TF 1.6 software (SCIEX), and analyzed using MetaboAnalyst5.0, and the deconvolution and streamline criteria were used for metabolomics.

2.12. Quantitative real-time PCR

Total RNA was extracted with TRIzol reagent (AG21101; AG, Hunan, China), and 1 μg of total RNA was reversed transcribed to cDNA using RT Master Mix according to the manufacturer's protocol (Abm, Zhenjiang, China). Quantitative real-time PCR as performed using qPCR Master Mix (Abclonal Technology). Actin mRNA levels were used as the controls for normalization. The primers were as follows (5′−3′):

Tnf-α: forward, ACGTGGAACTGGCAGAAGAG; reverse, GGTCTG GGCCATAGA.

ACTGA.

Il-6: forward, AACGATGATGCACTTGCAGA; reverse, CTCTGAA GGACTCTGGC.

TTTG.

Il-1β: forward, TACAGGCTCCGAGATGAACA; reverse, AGGC CACAGGTATTTT.

GTCG.

Actin: forward, GCTGAGAGGGAAATCGTGCGTG; reverse, CCAGG GAGGAAG.

AGGATGCGG.

A20: forward, GCCAGCAGGTATATGGGAGC; reverse, TTGTTCAG CCATGGTCC.

TCG.

TGR5: forward, GCTACATGGCAGTGTTGCAG; reverse, CTGGG AAGACAGCTT.

GGGAG.

2.13. siRNA-mediated TGR5 or A20 knockdown

siRNAs targeting TGR5 and A20 were purchased from GenePharma (Shanghai, China). Mouse TGR5 siRNAs, A20 siRNAs,

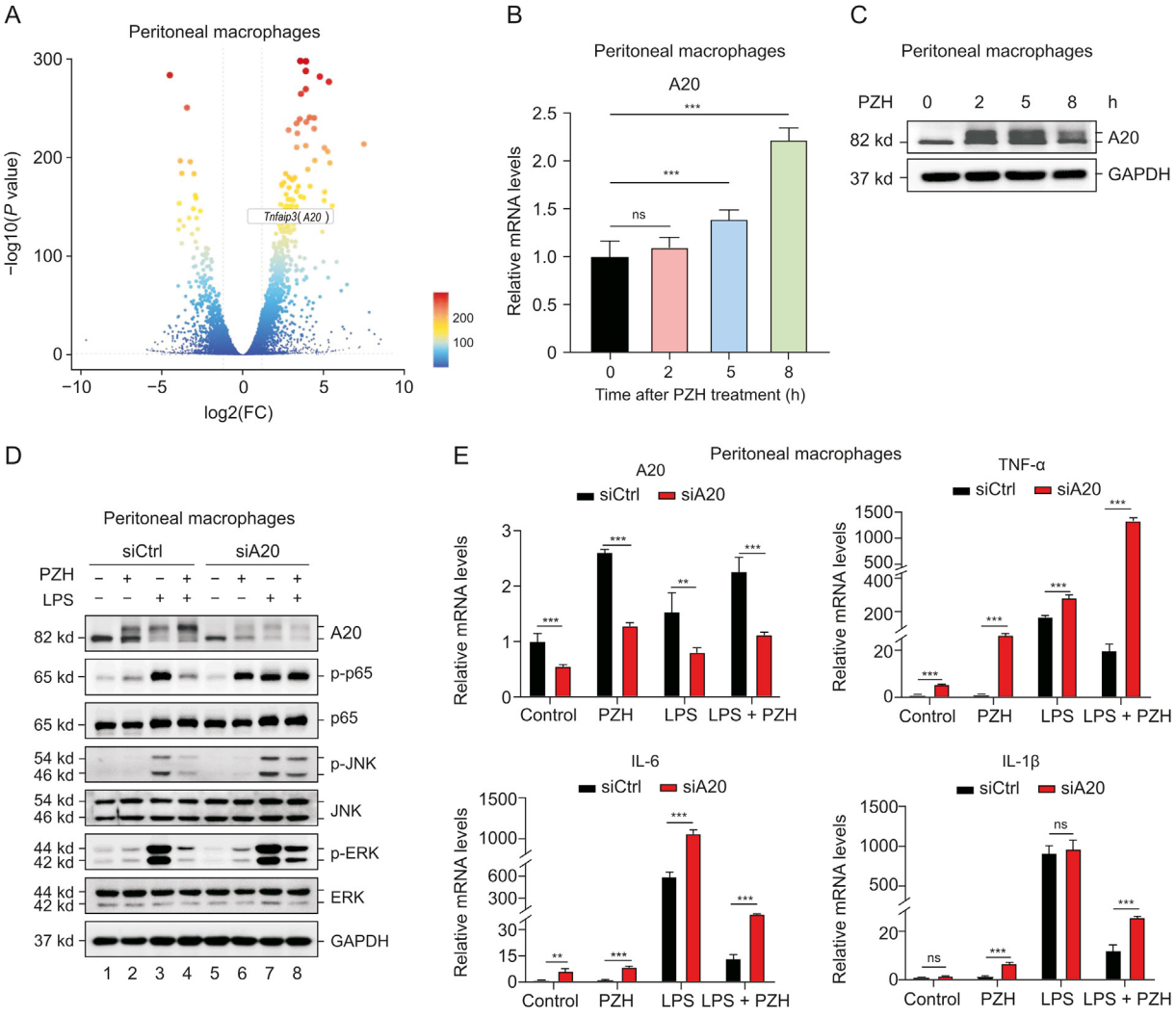


Fig. 4. Pien Tze Huang (PZH) inhibits inflammatory cytokine transcription via A20-mediated nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) inactivation. (A) Peritoneal macrophages treated with or without PZH for 8 h. Volcano plot analysis showing significantly higher *Tnfaip3* gene expression in the PZH group than in the control group. (B, C) The mRNA and protein levels of A20 were significantly increased 0–8 h after treatment with PZH (1.25 mg/mL). (D) Peritoneal macrophages were treated with PZH (1.25 mg/mL) for 2 h and then incubated with or without lipopolysaccharide (LPS) (100 ng/mL) for 40 min. In A20-knockdown peritoneal macrophages, PZH did not decrease the protein expression of phospho-p65 (p-p65), phospho-C-Jun N-terminal kinases (p-JNK) and phospho-extracellular signal-regulated kinases (p-ERK) induced by LPS. (E) Peritoneal macrophages were treated with PZH (1.25 mg/mL) for 2 h and then incubated with or without LPS (100 ng/mL) for 6 h. In A20-knockdown peritoneal macrophages, PZH did not decrease the mRNA levels of tumour necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 induced by LPS. The results are representative of one of three experiments. The data are shown as the mean ± standard deviation (SD). $^{**}P < 0.01$, $^{***}P < 0.001$, ns: no significance, based on one-way analysis of variance (ANOVA) and two tailed Student's *t*-test.

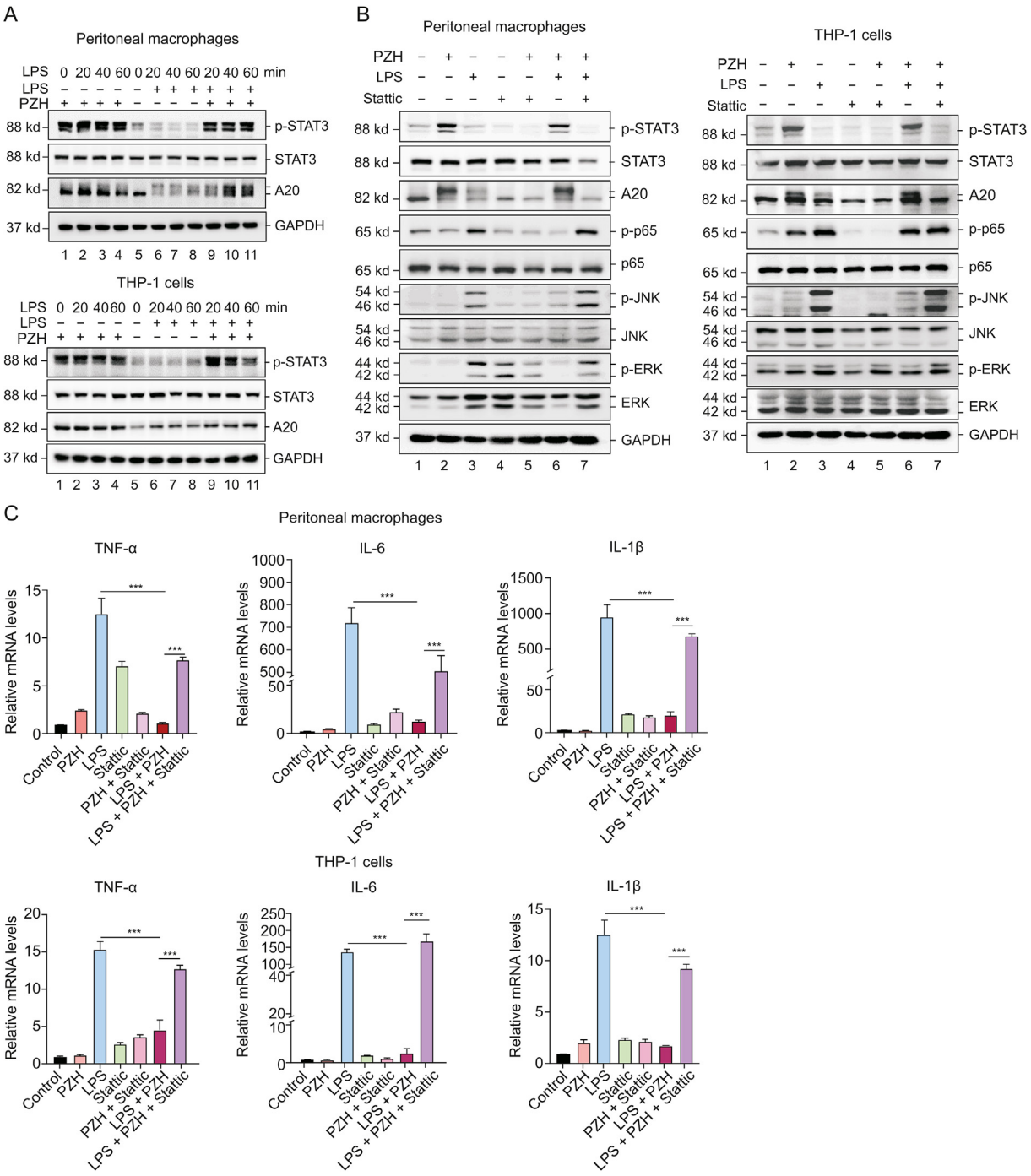
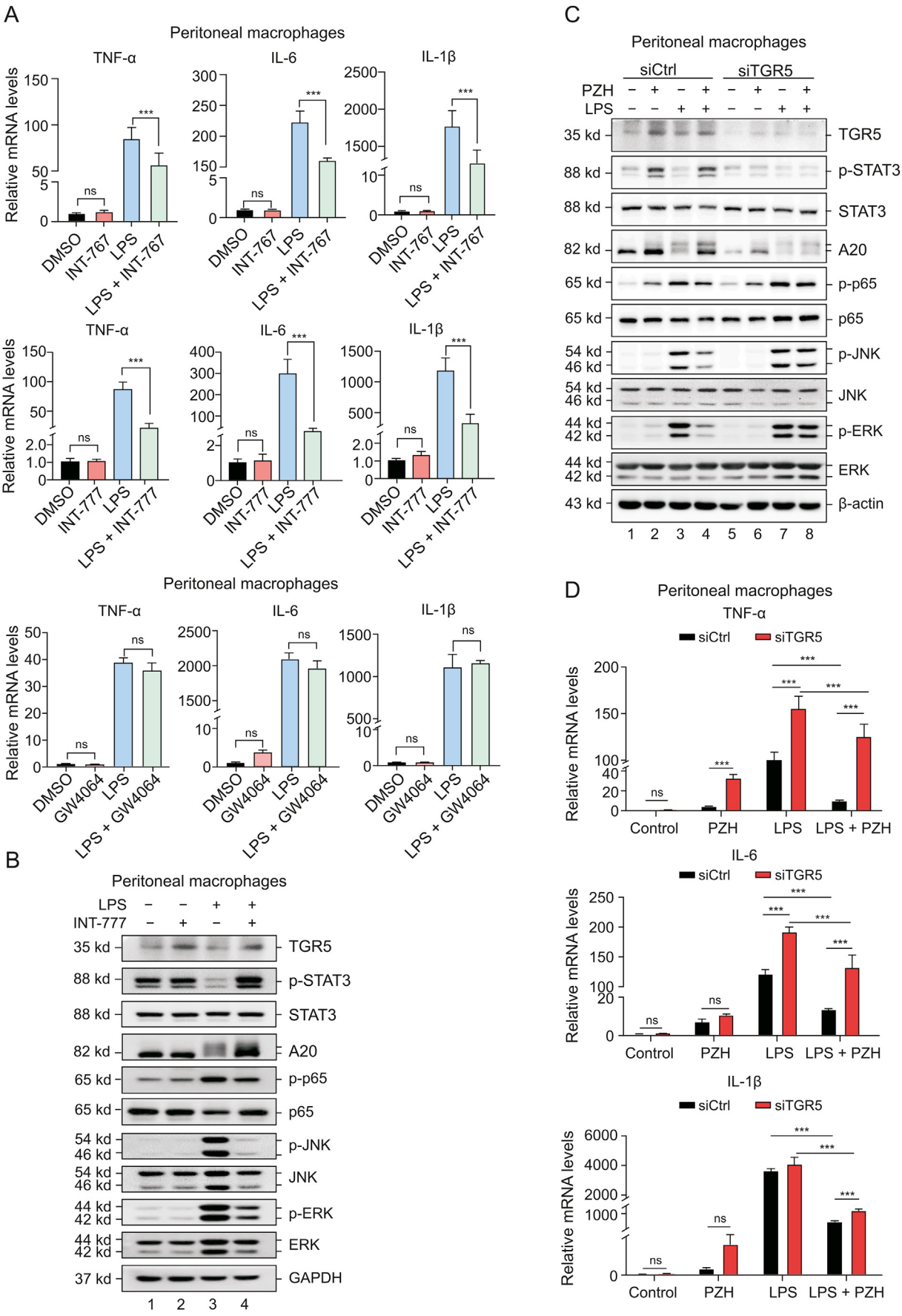


Fig. 5. Pien Tze Huang (PZH) inhibits the activation of nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) by regulating the signal transducer and activator of transcription 3 (STAT3)-A20 signalling pathway. (A) PZH (1.25 mg/mL) significantly increased the protein expression of phospho-STAT3 (p-STAT3) and A20 in peritoneal macrophages and THP-1 cells treated with or without lipopolysaccharide (LPS) (100 ng/mL). (B) Cells were pretreated with Stattic (20 μM) for 30 min and treated with PZH (1.25 mg/mL) for 2 h followed by incubation with LPS (100 ng/mL) for 40 min. Stattic treatment decreased the protein expression of p-STAT3 and A20 and increased the protein expression of phospho-p65 (p-p65), phospho-extracellular signal-regulated kinases (p-ERK), and phospho-C-Jun N-terminal kinases (p-JNK) in peritoneal macrophages and THP-1 cells treated with PZH and LPS. (C) Cells were pretreated with Stattic (20 μM) for 30 min and treated with PZH (1.25 mg/mL) for 2 h followed by incubation with LPS (100 ng/mL) for 6 h. Stattic treatment increased the mRNA levels of interleukin (IL)-6 and tumour necrosis factor-α (TNF-α) and IL-1β in peritoneal macrophages and THP-1 cells treated with PZH and LPS. The results are representative of one of three experiments. The data are shown as the mean ± standard deviation (SD). $^{***}P < 0.001$, based on one-way analysis of variance (ANOVA).

or negative control siRNAs were transfected into peritoneal macrophages using INVI DNA RNA transfection reagent according to the manufacturer's instructions (Invigentech, Irvine, CA, USA).

2.14. Western blot analysis

Lysates for Western blot analysis were obtained from cells. The protein concentration was measured by using a bicinchoninic acid


A
Peritoneal macrophages
TNF-α
IL-6
IL-1β
Relative mRNA levels
DMSO
INT-767
LPS
LPS + INT-767
INT-777
LPS + INT-777
GW4064
LPS + GW4064
ns
***
B
Peritoneal macrophages
LPS
INT-777
35 kd TGR5
88 kd p-STAT3
88 kd STAT3
82 kd A20
65 kd p-p65
65 kd p65
54 kd 46 kd p-JNK
54 kd 46 kd JNK
44 kd 42 kd p-ERK
44 kd 42 kd ERK
37 kd GAPDH
1 2 3 4
C
Peritoneal macrophages
siCtrl
siTGR5
PZH
LPS
35 kd TGR5
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88 kd STAT3
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65 kd p65
54 kd 46 kd p-JNK
54 kd 46 kd JNK
44 kd 42 kd p-ERK
44 kd 42 kd ERK
43 kd β-actin
1 2 3 4 5 6 7 8
D
Peritoneal macrophages
TNF-α
IL-6
IL-1β
siCtrl
siTGR5
Relative mRNA levels
Control
PZH
LPS
LPS + PZH
ns
***

(BCA) Assay. Total proteins (20 μg) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Cytiva, Shrewsbury, MA, USA). The blots were incubated with appropriate specific primary antibodies overnight at 4 °C. The membranes were washed with tris buffer solution with Tween-20 (TBST), and incubated with horseradish peroxidase-conjugated secondary antibodies (Thermo Fisher Scientific, Waltham, MA, USA) and visualized by chemiluminescence.

2.15. Statistical analysis

Statistical analyses were performed by GraphPad Prism (GraphPad Software, Version 8) or SPSS (Version 18). Quantitative data were normally distributed and are presented as the mean ± standard deviation (SD). Statistically significant differences were examined using Student's *t*-test or one-way or two-way analysis of variance (ANOVA).

3. Results

3.1. PZH effectively ameliorates LPS-induced sepsis in mice by reducing the production of proinflammatory cytokines

To define the effect of PZH on sepsis, we used LPS to induce the initial phase of sepsis in mice. The results showed that 6 of 6 mice died within 22 h of LPS injection, whereas 4 of 6 mice that were pretreated with PZH survived at the end of the experiment (48 h after LPS injection) (Fig. 1A). After the administration of 10 mg/kg LPS, body temperature dropped to 28 °C at 11 h. In contrast, pretreatment with PZH effectively suppressed the drop in body temperature caused by LPS (Fig. 1B). In the absence of LPS administration, PZH-treated mice showed nonsignificant body temperature changes compared to control mice (Fig. S1A). These results suggest that PZH reduces the susceptibility of mice to the effect of LPS.

Damage to the liver, lung, and kidney occurs frequently during sepsis [27,28]. Therefore, we examined whether PZH could alleviate organ damage induced by LPS. Histopathological examination showed that PZH alleviated LPS-induced renal cystic atrophy, tubule swelling, and abnormal vacuoles in the kidney, decreased LPS-induced alveolar wall swelling and inflammatory cell infiltration in the lung, and alleviated LPS-induced cord arrangement disorder and inflammatory cell infiltration in the liver (Fig. 1C).

During infectious processes, excessive production of proinflammatory cytokines leads to a cytokine storm, thereby contributing to mortality consecutive to sepsis syndrome [29]. ELISA showed that the LPS-induced levels of TNF-α, IL-6, and IL-1β in the serum of mice that were pretreated with PZH were significantly reduced at 2 h after LPS injection (Fig. 1D). In the absence of LPS administration, PZH-treated mice showed no differences in the levels of proinflammatory cytokines compared with control mice (Fig. S1B). Furthermore, we detected the mRNA levels of TNF-α, IL-6, and IL-1β in the kidney, lung, and liver after LPS injection with or without PZH pretreatment. As shown in Fig. 1E, PZH inhibited the expression of these cytokines in the kidney, lung, and liver. These results indicate that PZH ameliorates LPS-induced sepsis by decreasing excessive proinflammatory cytokines.

3.2. PZH strongly inhibits LPS-induced inflammatory cytokine production in macrophages

Macrophages, which are part of the first lines of defence against the invasion of various pathogens, play an important role in mediating innate immune responses, including inflammation. To investigate the effects of PZH on the LPS-induced inflammatory response in macrophages *in vitro*, we detected the mRNA levels of proinflammatory cytokines in mouse PMs, the mouse macrophage line RAW264.7, and the human macrophage line THP-1. The results showed that PZH significantly decreased the mRNA levels of TNF-α, IL-6, and IL-1β induced by LPS in PMs, RAW264.7 cells, and THP-1 cells (Fig. 2A). Consistently, ELISAs showed that PZH reduced LPS-induced protein production of TNF-α, IL-6, and IL-1β in macrophages (Fig. 2B). These results suggest that PZH attenuates LPS-induced inflammatory responses by inhibiting LPS-induced inflammatory cytokine production in macrophages.

3.3. PZH effectively suppresses LPS-induced activation of the NF-κB and MAPK signalling pathways

To explore the signalling pathways by which PZH inhibits LPS-induced inflammatory cytokine production, we performed RNA-seq analysis of PMs treated with LPS with or without PZH pretreatment. Statistical analysis was performed, and the differentially expressed genes are displayed in a volcano plot (Fig. 3A). Consistent with the quantitative real-time-PCR results, the RNA-seq results showed that PZH decreased the LPS-induced expression of *Il6* and *Il1b* (Fig. 3A). In addition, *Tlr4* and *myeloid differentiation factor 88* (*Myd88*), which are responsible for LPS-induced activation of the NF-κB and MAPK signalling pathways [30], were also decreased by PZH treatment (Fig. 3A). Furthermore, GSEA showed that PZH pretreatment significantly inhibited LPS-induced activation of the NF-κB signalling pathway (Fig. 3B). Based on these data, we hypothesized that the inhibition of LPS-induced proinflammatory cytokine production by PZH involved suppression of the NF-κB and MAPK signalling pathways. To test this hypothesis, we examined LPS-induced activation of several key proteins in the NF-κB and MAPK signalling pathways with or without PZH pretreatment. As shown in Fig. 3C, PZH significantly inhibited LPS-induced phosphorylation of p65 and IκBα in PMs, RAW264.7 cells, and THP-1 cells, suggesting that PZH could suppress LPS-induced activation of the NF-κB signalling pathway. PZH also significantly inhibited LPS-induced expression of p-JNK and p-ERK in these macrophages, suggesting that PZH could suppress LPS-induced activation of the MAPK signalling pathway. Intriguingly, PZH did not suppress LPS-induced expression of p-p38 (Fig. 3C). Taken together, these results suggest that PZH inhibits LPS-induced production of

Fig. 6. Pien Tze Huang (PZH) activates signal transducer and activator of transcription 3 (STAT3)-A20 signalling via G protein-coupled bile acid receptor 1 (TGR5). (A) Cells were pretreated with INT-777 (30 μM), INT-767 (10 μM), or GW4064 (5 μM) for 2 h and treated with lipopolysaccharide (LPS) (100 ng/mL) for 6 h. INT-767 significantly decreased the mRNA levels of interleukin (IL)-6 and tumour necrosis factor-α (TNF-α) and IL-1β in peritoneal macrophages. INT-777 significantly decreased the mRNA levels of IL-6 and TNF-α and IL-1β in peritoneal macrophages. However, GW4064 did not decrease the mRNA levels of IL-6 and TNF-α and IL-1β in peritoneal macrophages. (B) Cells were pretreated with INT-777 (30 μM) for 2 h and treated with LPS (100 ng/mL) for 40 min. INT-777 significantly increased the protein expression of phospho-signal transducer and activator of transcription 3 (p-STAT3) and A20 and decreased the protein expression of phospho-p65 (p-p65), phospho-extracellular signal-regulated kinases (p-ERK), and phosphor-C-Jun N-terminal kinases (p-JNK) in peritoneal macrophages induced by LPS. (C) Peritoneal macrophages were treated with PZH (1.25 mg/mL) for 2 h and then incubated with or without LPS (100 ng/mL) for 40 min. In TGR5-knockdown peritoneal macrophages, PZH (1.25 mg/mL) did not increase the protein expression of p-STAT3, A20 and TGR5 or decrease the protein expression of p-p65, p-ERK, p-JNK induced by LPS. (D) Peritoneal macrophages were treated with PZH (1.25 mg/mL) for 2 h and then incubated with or without LPS (100 ng/mL) for 6 h. In TGR5-knockdown peritoneal macrophages, PZH did not decrease the mRNA levels of IL-6, TNF-α and IL-1β induced by LPS. The results are representative of one of three experiments. The data are shown as the mean ± standard deviation (SD). $^{***}P < 0.001$, ns: no significance, based on one-way and two-way analysis of variance (ANOVA).

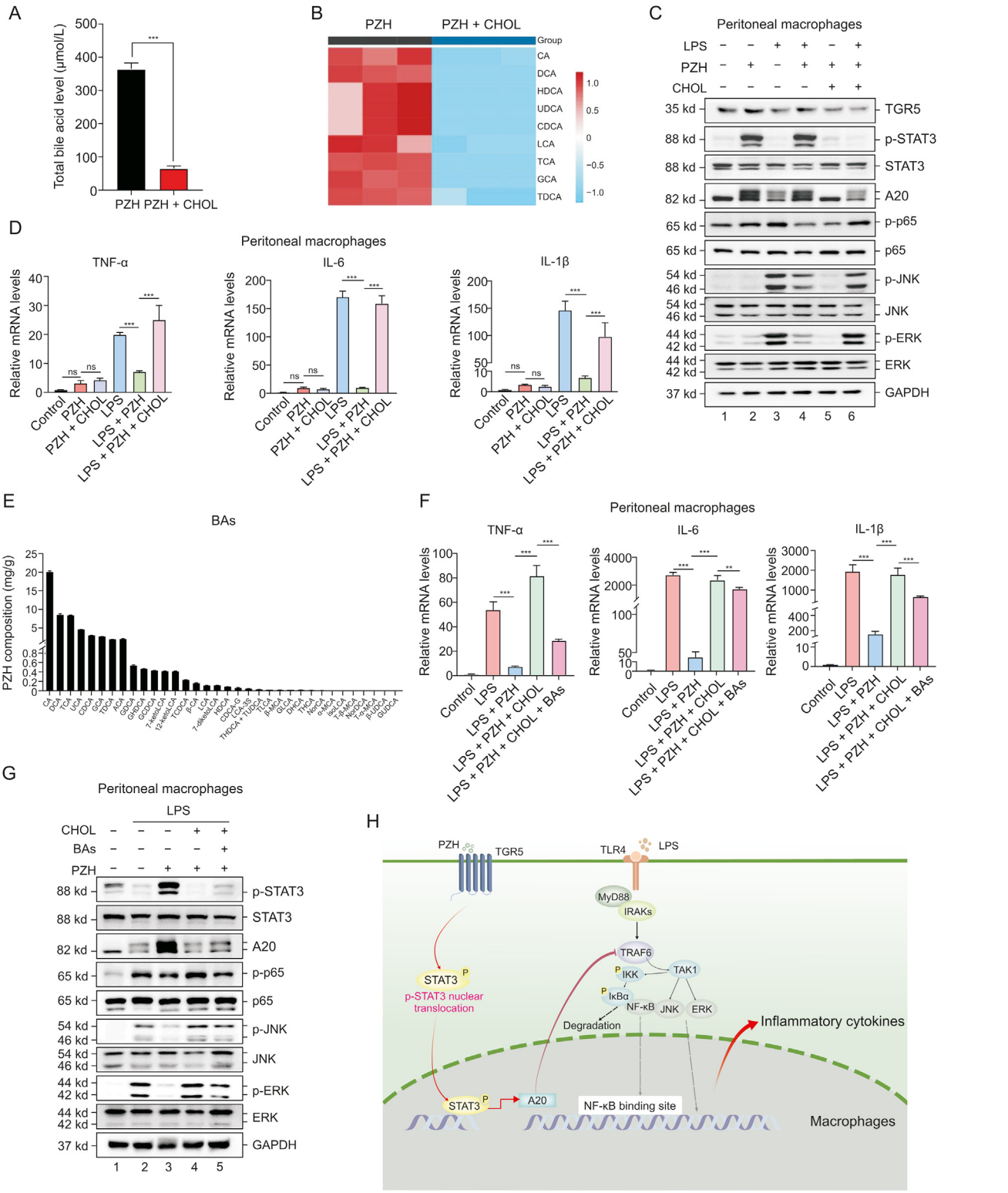

Fig. 7. Bile acid (BA), which is an important functional component in Pien Tze Huang (PZH), regulates activation of the G protein-coupled bile acid receptor 1 (TGR5)-signal transducer and activator of transcription 3 (STAT3)-A20 signalling pathway. (A) Total BA levels were detected in the PZH solution and PZH plus cholestyramine resin. (B) Liquid chromatography-mass spectrometry (LC-MS) analysis of PZH supernatant treated with or without cholestyramine resin. (C) Peritoneal macrophages were pretreated with PZH (1.25 mg/mL) or cholestyramine resin-treated PZH (1.25 mg/mL) for 2 h, and then treated with lipopolysaccharide (LPS) (100 ng/mL) for 40 min. PZH treated with cholestyramine resin did not increase the protein expression of TGR5, phospho-STAT3 (p-STAT3) and A20 or decrease the protein expression of phospho-p65 (p-p65), phospho-extracellular signal-regulated kinases (p-ERK), and phosphor-C-Jun N-terminal kinases (p-JNK) induced by LPS. (D) Peritoneal macrophages were pretreated with PZH (1.25 mg/mL) or cholestyramine resin-treated PZH (1.25 mg/mL) for 2 h and then treated with LPS (100 ng/mL) for 6 h. PZH treated with cholestyramine resin did not decrease the mRNA levels of interleukin (IL)-6, tumour necrosis factor-α (TNF-α) and IL-1β induced by LPS. (E) The composition of BAs in the PZH solution was detected by LC-MS. (F) Peritoneal macrophages were pretreated with PZH (1.25 mg/mL), cholestyramine resin-treated PZH (1.25 mg/mL), and cholestyramine resin-treated PZH (1.25 mg/mL) + BAs for 2 h, respectively, and then treated with LPS (100 ng/mL) for 6 h. Replenishing BAs including cholic acid (CA) (61.53 μM), deoxycholic acid (DCA) (27.25 μM), chenodexycholic acid (9.75 μM), taurocholic acid (TCA) (20.52 μM),

proinflammatory cytokines by suppressing the activation of the NF-κB and MAPK signalling pathways.

3.4. PZH suppresses LPS-induced activation of the NF-κB and MAPK signalling pathways by inducing A20 expression

To gain insight into the mechanism by which PZH suppresses LPS-induced activation of the NF-κB and MAPK signalling pathways, we performed RNA-seq analysis of PZH-treated and untreated control PMs. Among the upregulated genes, *Tnfaip3* (*A20*), which is a potent anti-inflammatory gene that has been reported to negatively regulate the activation of the NF-κB and MAPK pathways [31], attracted our attention (Fig. 4A). Consistent with the RNA-seq results, we observed that the mRNA and protein levels of A20 were significantly increased in PMs treated with PZH for up to 8 h (Figs. 4B and C). To determine whether PZH suppressed LPS-induced activation of the NF-κB and MAPK signalling pathways via A20 induction, we knocked down A20 in PMs and found that A20 deficiency partially abolished PZH-mediated suppression of LPS-induced p-p65, p-JNK, and p-ERK expression (Fig. 4D). Consistently, the quantitative real-time-PCR results showed that PZH fully or partially lost its ability to inhibit LPS-induced expression of TNF-α, IL-6, and IL-1β in A20-deficient PMs compared with control PMs (Fig. 4E). These results suggest that PZH suppresses LPS-induced activation of the NF-κB and MAPK signalling pathways by inducing A20 expression.

3.5. PZH induces A20 expression to suppress LPS-induced inflammatory responses in macrophages by activating STAT3

Given that the transcription factor STAT3 can induce A20 expression [32], we examined whether PZH could activate STAT3 to induce A20 expression in macrophages. We examined the effects of PZH treatment on the expression of total STAT3 and activated STAT3 (phosphorylated-STAT3 (p-STAT3)) by western blotting. The results showed that PZH treatment did not affect the expression of total STAT3 but significantly increased the protein levels of p-STAT3 and A20 in the absence or presence of LPS (Fig. 5A), suggesting that PZH could activate STAT3, which could induce A20 expression in macrophages. To confirm that PZH-induced A20 expression was dependent on STAT3 activation, we used the STAT3 inhibitor Stattic to suppress PZH-mediated activation of STAT3, and then use Western blotting to examine the expression of A20, as well as the expression of p-p65, p-JNK, and p-ERK, in macrophages in the absence or presence of LPS. As shown in Fig. 5B, Stattic effectively suppressed PZH-induced p-STAT3 expression and A20 expression, suggesting that PZH-induced A20 expression was dependent on STAT3 activation. Stattic treatment abolished the PZH-mediated reduction in LPS-induced p-p65, p-JNK, and p-ERK expression (Fig. 5B, compared Lanes 7 vs. 6), as well as the LPS-induced expression of TNF-α, IL-6, and IL-1β (Fig. 5C). Collectively, these results suggest that PZH induces A20 expression to suppress LPS-induced activation of the NF-κB and MAPK signalling pathways and LPS-induced production of inflammatory cytokines by activating STAT3.

3.6. PZH enhances STAT3-A20 signalling to suppress LPS-induced inflammatory responses in macrophages by activating the BA receptor TGR5

PZH is composed of four TCM ingredients: *Radix et Rhizoma notoginseng*, *Moschus*, *Calculus bovis*, and *Snake gall*. A total of eleven different BAs have been identified as the components of PZH [19]. Different BAs can suppress inflammatory responses by activating the nuclear receptor farnesoid X receptor (FXR) or the cell surface receptor (TGR5) depending on their characteristics [33,34]. Since BAs have been reported to activate STAT3 signalling via TGR5 [35], we hypothesized that PZH suppresses LPS-induced inflammatory responses by activating TGR5-STAT3-A20 signalling. To test this hypothesis, we first examined which BA receptors could suppress LPS-induced inflammatory responses in PMs by pretreating PMs with the dual FXR and TGR5 agonist INT-767, the TGR5-specific agonist INT-777, or the FXR-specific agonist GW4064 for 2 h and then measuring the production of TNF-α, IL-6, and IL-1β after 6 h of LPS stimulation. As shown in Fig. 6A, INT-767 and INT-777 markedly decreased LPS-induced production of TNF-α, IL-6, and IL-1β, whereas GW4064 did not show any significant impacts on inflammatory cytokine production, indicating that activation of TGR5, but not FXR suppressed LPS-induced inflammatory responses in PMs. Next, we examined whether activation of TGR5 by INT-777 could further activate STAT3 to induce A20 expression and suppress the NF-κB and MAPK signalling pathways during LPS treatment. Western blotting showed that INT-777 induced the expression of p-STAT3 and A20, but suppressed the expression of p-p65, p-JNK, and p-ERK during LPS treatment (Fig. 6B), suggesting that TGR5 suppressed LPS-induced activation of the NF-κB and MAPK signalling pathways by activating STAT3-A20 signalling. Finally, we knocked down TGR5 in PMs to determine whether TGR5 was required for PZH-mediated suppression of LPS-induced inflammatory responses. As shown in Fig. 6C, TGR5 knockdown dramatically suppressed PZH-induced expression of p-STAT3 and A20 and abolished the suppressive effects of PZH on LPS-induced expression of p-p65, p-JNK, and p-ERK. Furthermore, TGR5 knockdown partially abolished PZH-mediated suppression of LPS-induced expression of TNF-α, IL-6, and IL-1β (Fig. 6D). Taken together, these results suggest that PZH inhibits LPS-induced inflammatory responses by activating TGR5-STAT3-A20 signalling in macrophages.

3.7. BAs are important functional components by which PZH activates the TGR5-STAT3-A20 signalling pathway to inhibit LPS-induced inflammatory responses

Because BAs are endogenous TGR5 ligands, we examined whether BAs in PZH were responsible for activating the TGR5-STAT3-A20 signalling pathway to inhibit LPS-induced inflammatory responses. We first used cholestyramine, a strong ion exchange resin that binds BAs to form insoluble complexes [36], to remove BAs from the PZH solution. The results showed that cholestyramine resin treatment significantly depleted 80% of the total BAs in the PZH solution (Fig. 7A). We also measured the levels of BAs in the

glycocholic acid (GCA) (7.466 μM) and lithocholic acid (LCA) (0.413 μM) in PZH after treatment with cholestyramine resin decreased the mRNA levels of TNF-α and IL-1β induced by LPS. (G) Peritoneal macrophages were pretreated with PZH (1.25 mg/mL), cholestyramine resin-treated PZH (1.25 mg/mL), and cholestyramine resin-treated PZH (1.25 mg/mL) + BAs for 2 h and then treated with LPS (100 ng/mL) for 40 min. Replenishing BAs including CA (61.53 μM), DCA (27.25 μM), chenodexycholic acid (9.75 μM), TCA (20.52 μM), GCA (7.466 μM) and LCA (0.413 μM) in PZH after treatment with cholestyramine resin decreased the expression of p-p65 and p-ERK induced by LPS. (H) Schematic model of the mechanism by which PZH attenuates the LPS-induced inflammatory response by activating TGR5-STAT3-A20 signalling pathway. PZH stimulates the phosphorylation of STAT3, which acts as a transcriptional activator for A20 to increase the expression of A20. A20 inhibits the activation of the nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signalling pathways to decrease the production of proinflammatory cytokines to attenuate the sepsis progression. The results are representative of one of three experiments. The data are shown as the mean ± standard deviation (SD). $^{**}P < 0.01$, $^{***}P < 0.001$, ns: no significance, based on one-way analysis of variance (ANOVA) and two-tailed Student's *t*-test. CHOL: cholestyramine resin; TRAF6: tumor necrosis factor receptor-associated factor 6; IKK: nuclear factor- kappa B kinase; TAK1: transforming growth factor beta-activated kinase 1; IκBα: nuclear factor-kappa B inhibitor alpha.

PZH solution with and without cholestyramine resin treatment by LC-MS. Consistently, cholestyramine resin treatment significantly decreased the amounts of BAs in the PZH solution (Fig. 7B). We next detected the expression of p-STAT3, A20, p-p65, p-JNK, and p-ERK, as well as the expression of TNF-α, IL-6, and IL-1β in PMs pretreated with PZH or cholestyramine resin-treated PZH in the absence or presence of LPS. The results showed that cholestyramine resin-treated PZH failed to induce the expression of p-STAT3 and A20 and failed to suppress LPS-induced expression of p-p65, p-JNK, and p-ERK compared with control PZH (Fig. 7C). Consistently, cholestyramine resin-treated PZH failed to inhibit LPS-induced expression of TNF-α, IL-6, and IL-1β to some extent (Fig. 7D). These results suggest that BAs in PZH are at least partly responsible for activating the TGR5-STAT3-A20 signalling pathway to inhibit LPS-induced inflammatory responses. To verify this finding, we performed rescue experiments by replenishing BAs in cholestyramine resin-treated PZH to determine whether the addition of BAs could restore the inhibitory effects of cholestyramine resin-treated PZH on LPS-induced inflammatory responses. To best mimic the BAs in PZH, we measured the components and concentrations of BAs in the PZH solution by LC-MS. The results showed that there were 34 BAs with different concentrations in PZH solution (Fig. 7E). Among these BAs, cholic acid (CA), deoxycholic acid (DCA), chenodexycholic acid (CDCA), taurocholic acid (TCA), glycocholic acid (GCA), and lithocholic acid (LCA) have been reported to activate TGR5 signalling [37]. We converted the concentrations of CA (61.53 μM), DCA (27.25 μM), CDCA (9.75 μM), TCA (20.52 μM), GCA (7.466 μM), and LCA (0.413 μM) to create a solution of 1.25 mg/mL PZH according to the LC-MS results and then added these BAs to cholestyramine resin-treated PZH. Next, we pretreated PMs with PZH, cholestyramine resin-treated PZH, and cholestyramine resin-treated PZH + BAs for 2 *h* and then treated PMs with LPS for 6 *h* and measured the expression of proinflammatory cytokines. The results showed that cholestyramine resin-treated PZH + BAs partially restored the inhibitory effects of cholestyramine resin-treated PZH on LPS-induced expression of TNF-α, IL-6, and IL-1β (Fig. 7F), suggesting that BAs in PZH could inhibit LPS-induced inflammatory responses. We also investigated the effects of BAs replenishment in cholestyramine resin-treated PZH on the regulation of LPS-induced activation of the NF-κB and MAPK signalling pathways. The results showed that cholestyramine resin-treated PZH + BAs restored p-STAT3 and A20 expression but inhibited LPS-induced expression of p-ERK and p-p65 compared with cholestyramine resin-treated PZH (Fig. 7G). Overall, we identified BAs as the effective components in PZH that activated the TGR5-STAT3-A20 signalling pathway to inhibit LPS-induced inflammatory responses.

Based on these results, we propose a model of the mechanism by which PZH inhibits LPS-induced inflammatory responses (Fig. 7H). Briefly, BAs in PZH bind and activate TGR5, leading to STAT3 activation to induce A20 expression. Furthermore, A20 binds to tumor necrosis factor receptor-associated factor 6 (TRAF6) to prevent TRAF6-mediated activation of the NF-κB and MAPK signalling pathways and inflammatory cytokine production.

4. Discussion

Although sepsis has a high incidence rate, high mortality, and high medical expenses worldwide, there is still a lack of safe and effective drugs to treat this condition [38]. As early as the 1980s, researchers proposed that blocking TNF-α would reduce the mortality rate of sepsis; however, subsequent clinical trials conducted on patients with sepsis showed that the use of anti-TNF-α antibody therapy increased the mortality rate of sepsis patients [39]. The anti-inflammatory drug dexamethasone can significantly reduce the mortality rate of sepsis, but it has not achieved satisfactory therapeutic effects in dozens of clinical studies due to severe side effects [40]. Some studies have shown that dexamethasone can induce diseases such as hyperglycaemia, hypertension, and myocardial hypertrophy and even increase the risk of infection for patients. In addition, various drugs, such as those that affect cytokines, bacterial virulence factors, and coagulation cascade reactions, have not achieved good therapeutic effects in clinical trials over the past 40 years [41]. In the current study, we showed that mice that were pretreated with PZH exhibited decreases in mortality rate, tissue damage, and proinflammatory cytokine production after LPS injection. Furthermore, we demonstrated that the anti-inflammatory effect of PZH was associated with activation of the TGR5-STAT3-A20 pathways to suppress LPS-induced activation of the NF-κB and MAPK signalling pathways. Our findings showed that PZH protected against LPS-induced sepsis at least in part by promoting the TGR5-STAT3-A20 pathway to suppress NF-κB- and MAPK-mediated inflammatory cytokine production. It is interesting to note that treatment with PZH alone slightly increased the expression of p-p65 in PMs, RAW264.7 cells, and THP-1 cells. We hypothesize that a small number of bacterial components in PZH (mainly derived from the component Bezoar bovis) can activate TLRs signalling on macrophages, thereby upregulating the expression of p-p65, but the intensity was not sufficient to induce significant expression of inflammatory cytokines. Pretreatment with PZH for 2 *h* could significantly increase the expression of p-STAT3 and A20, which could inhibit the activation of the NF-κB signalling pathway. However, further experiments are needed to confirm this hypothesis. As a traditional Chinese medicine, PZH has been widely used to treat hepatitis and other diseases without obvious side effects. Therefore, PZH may be a safe and effective drug for sepsis treatment.

In this study, we showed that BA-mediated activation of TGR5-STAT3-A20 signalling was required for PZH-mediated suppression of LPS-induced activation of the NF-κB and MAPK signalling pathways. The most interesting finding of the current study was that PZH induced much stronger STAT3 activation than BAs or INT-777 (Fig. S2), suggesting that other components in PZH may modulate some signal transduction pathways to cooperate with BAs to activate STAT3. It has been reported that several signalling pathways, such as the NF-κB, IL-10, phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT), Notch, and Wnt pathways, can activate STAT3 [42]. Therefore, it is important to identify which components in PZH and which signalling pathways are involved in cooperating with BAs to activate STAT3 in the future studies.

It has been reported that IL-10 can activate STAT3 to further induce some anti-inflammatory genes including *B-cell leukemia 3 (BCL3)*, *ETS variant transcription factor 3* (*ETV3*), *nuclear factor interleukin 3 (Nfil3)*, protein *strawberry notch 2 (Sbno2)*, *Zfp36*, *heme oxygenase 1 (Hmox1)*, *suppressor of cytokine signalling 3* (*Socs3*), *dual-specifity phosphatase* (*DUSP*), *tristetraprolin* (*TTP*), and *DNA damage inducible transcript 4* (*Ddit4*) [43]. Since PZH can activate STAT3, we examined whether PZH could also induce these anti-inflammatory genes to exert its anti-inflammatory effects, in addition to A20. The RNA-seq results showed that PZH could induce the expression of *BCL3*, *ETV3*, *Nfil3*, *Sbno2*, *Hmox1*, *Socs3*, and *Ddit4* (Fig. S3), suggesting that PZH exerts its anti-inflammatory effects by activating TGR5-STAT3 to induce multiple anti-inflammatory genes.

In this study, we found that although knockdown of TGR5 in PMs abolished the suppressive effects of PZH on LPS-induced activation of the NF-κB and MAPK signalling pathways, PZH could still inhibit LPS-induced production of TNF-α, IL-6, and IL-1β to a certain extent, suggesting that there are other functional components in PZH in addition to BAs taht inhibit LPS-induced inflammatory cytokine production independent of TGR5-mediated suppression of the NF-κB and MAPK signalling pathways. Indeed,

the LC-MS results showed that PZH contained panax notoginseng saponins (PNS), including notoginsenoside, ginsenoside, pseuoginsenoside, and hederagenin (Fig. S4A), which could inhibit LPS-induced production of TNF-α, IL-6, and IL-1β (Fig. S4B) but could not activate STAT3 and suppress the activation of the NF-κB and MAPK signalling pathways (Fig. S4C). Therefore, PNS may also contribute to the anti-inflammatory effect of PZH independent of the suppression of the NF-κB and MAPK signalling pathways.

In recent years, several studies have shown that the TGR5 agonist INT-777 can increase the intracellular adenosine triphosphate (ATP)/adenosine diphosphate (ADP) ratio and calcium influx to induce GLP-1 expression, which improves insulin secretion and sensitivity [44]. Other studies have shown that TGR5 agonists can reduce the release of inflammatory factors in atherosclerosis, experimental autoimmune encephalomyelitis, and inflammatory bowel disease [45]. Since PZH can strongly activate TGR5, PZH has potential application value not only in the treatment of sepsis but also in the prevention and treatment of diabetes, atherosclerosis, autoimmune encephalomyelitis, and inflammatory bowel disease.

The BAs in PZH mainly come from bezoar bovis. It is interesting that among the numerous classic formulas in traditional Chinese medicines, there are many famous formulas containing Bezoar bovis. Among the top ten classic traditional Chinese medicines, six (PZH, Angong-Niuhuang-Wan, Liu-Shen-Wan, Niuhuang-Qingxin-Wan, Niuhuang-Jiedu-Wan, and Xihuang-Wan) contain bezoar bovis, indicating that BA-mediated anti-inflammatory effects may play an essential role in the efficacy of these traditional Chinese medicines. Therefore, our current study will have a great impact on the research of traditional Chinese medicines.

5. Conclusions

In summary, our study identified BAs as the functional components in PZH that inhibit LPS-induced activation of the NF-κB and MAPK signalling pathways and the production of inflammatory cytokines by activating the TGR5-STAT3-A20 signalling pathway. Hence, PZH may be of particular value for the treatment of sepsis and other inflammation-related diseases.

CRediT author statement

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Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

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References

[1] M. Cecconi, L. Evans, M. Levy, et al., Sepsis and septic shock, Lancet 392 (2018) 75–87.
[2] J.D. Faix, Biomarkers of sepsis, Crit. Rev. Clin. Lab. Sci. 50 (2013) 23–36.
[3] N.K. Adhikari, R.A. Fowler, S. Bhagwanjee, et al., Critical care and the global burden of critical illness in adults, Lancet 376 (2010) 1339–1346.
[4] D.C. Angus, W.T. Linde-Zwirble, J. Lidicker, et al., Epidemiology of severe sepsis in the United States: Analysis of incidence, outcome, and associated costs of care, Crit. Care Med. 29 (2001) 1303–1310.
[5] F.B. Mayr, S. Yende, D.C. Angus, Epidemiology of severe sepsis, Virulence 5 (2014) 4–11.
[6] X. Chen, D. Song, LPS promotes the progression of sepsis by activation of lncRNA HULC/miR-204-5p/TRPM7 network in HUVECs, Biosci. Rep. 40 (2020), BSR20200740.
[7] W. Hu, C. Deng, Z. Ma, et al., Utilizing melatonin to combat bacterial infections and septic injury, Br. J. Pharmacol. 174 (2017) 754–768.
[8] Q. Chen, T. Chen, W. Li, et al., Mycoepoxydiene inhibits lipopolysaccharide–induced inflammatory responses through the suppression of $TRAF_6$ polyubiquitination [corrected, PLoS One 7 (2012), e44890.
[9] Y. Lu, W.C. Yeh, P.S. Ohashi, LPS/TLR4 signal transduction pathway, Cytokine 42 (2008) 145–151.
[10] M. Tanaka, Y. Kishimoto, M. Sasaki, et al., *Terminalia bellirica* (gaertn.) roxb. extract and Gallic acid attenuate LPS-induced inflammation and oxidative stress via MAPK/NF-κB and akt/AMPK/Nrf2 pathways, Oxid. Med. Cell. Longev. 2018 (2018), 9364364.
[11] W. Zhao, L. Ma, C. Cai, et al., Caffeine inhibits NLRP3 inflammasome activation by suppressing MAPK/NF-κB and A2aR signaling in LPS-induced THP-1 macrophages, Int. J. Biol. Sci. 15 (2019) 1571–1581.
[12] J.E. Thompson, R.J. Phillips, H. Erdjument-Bromage, et al., I kappa B-beta regulates the persistent response in a biphasic activation of NF-kappa B, Cell 80 (1995) 573–582.
[13] L. Wang, X. Qiao, S. Zhang, et al., Porcine transmissible gastroenteritis virus nonstructural protein 2 contributes to inflammation via NF-κB activation, Virulence 9 (2018) 1685–1698.
[14] J. Meng, H. Gao, W. Zhai, et al., Subtle regulation of cotton resistance to *Verticillium* wilt mediated by MAPKK family members, Plant Sci. 272 (2018) 235–242.
[15] J. Bai, Y. Zhang, C. Tang, et al., Gallic acid: Pharmacological activities and molecular mechanisms involved in inflammation-related diseases, Biomed. Pharmacother. 133 (2021), 110985.
[16] S. Zhou, M. Chen, Y. Zhang, et al., *OsMKK3*, a stress-responsive protein kinase, positively regulates rice resistance to *Nilaparvata lugens* via phytohormone dynamics, Int. J. Mol. Sci. 20 (2019), 3023.
[17] M. Jiang, Z. Chu, Comparative analysis of plant *MKK* gene family reveals novel expansion mechanism of the members and sheds new light on functional conservation, BMC Genomics 19 (2018), 407.
[18] L. Ulloa, K.J. Tracey, The "cytokine profile": A code for sepsis, Trends Mol. Med. 11 (2005) 56–63.
[19] Z. Chen, Pien Tze Huang (PZH) as a multifunction medicinal agent in traditional Chinese medicine (TCM): A review on cellular, molecular and physiological mechanisms, Cancer Cell Int. 21 (2021), 146.
[20] H. Zheng, X. Wang, Y. Zhang, et al., Pien-Tze-Huang ameliorates hepatic fibrosis via suppressing NF-κB pathway and promoting HSC apoptosis, J. Ethnopharmacol. 244 (2019), 111856.
[21] Y. Deng, H. Luo, J. Shu, et al., Pien Tze Huang alleviate the joint inflammation in collagen–induced arthritis mice, Chin. Med. 15 (2020), 30.
[22] X. Qiu, Q. Guo, X. Liu, et al., Pien tze Huang alleviates relapsing-remitting experimental autoimmune encephalomyelitis mice by regulating Th1 and Th17 cells, Front. Pharmacol. 9 (2018), 1237.
[23] Q. Chen, S. Zhuang, Y. Hong, et al., Demethylase JMJD2D induces PD-L1 expression to promote colorectal cancer immune escape by enhancing $IFNGR1$-$STAT3$-IRF_1 signaling, Oncogene 41 (2022) 1421–1433.
[24] T. Hu, Z. An, C. Shi, et al., A sensitive and efficient method for simultaneous profiling of bile acids and fatty acids by UPLC-MS/MS, J. Pharm. Biomed. Anal. 178 (2020), 112815.
[25] T. Yang, T. Shu, G. Liu, et al., Quantitative profiling of 19 bile acids in rat plasma, liver, bile and different intestinal section contents to investigate bile acid homeostasis and the application of temporal variation of endogenous bile acids, J. Steroid Biochem. Mol. Biol. 172 (2017) 69–78.
[26] P. Bhargava, M.D. Smith, L. Mische, et al., Bile acid metabolism is altered in multiple sclerosis and supplementation ameliorates neuroinflammation, J. Clin. Invest. 130 (2020) 3467–3482.
[27] X. Wang, X. Meng, J.R. Kuhlman, et al., Knockout of *Mkp-1* enhances the host inflammatory responses to gram-positive bacteria, J. Immunol. 178 (2007) 5312–5320.
[28] L. Li, Y. Liu, H.Z. Chen, et al., Impeding the interaction between Nur77 and p38 reduces LPS-induced inflammation, Nat. Chem. Biol. 11 (2015) 339–346.

[29] J.M. Cavaillon, Exotoxins and endotoxins: Inducers of inflammatory cytokines, Toxicon 149 (2018) 45–53.
[30] R. Li, Y. Guo, Y. Zhang, et al., Salidroside ameliorates renal interstitial fibrosis by inhibiting the TLR4/NF-κB and MAPK signaling pathways, Int. J. Mol. Sci. 20 (2019), 1103.
[31] D. Priem, G. van Loo, M.J.M. Bertrand, A20 and cell death-driven inflammation, Trends Immunol. 41 (2020) 421–435.
[32] X. Huang, Z. Feng, Y. Jiang, et al., VSIG4 mediates transcriptional inhibition of *Nlrp3* and *Il-1β* in macrophages, Sci. Adv. 5 (2019), eaau7426.
[33] O. Chávez–Talavera, A. Tailleux, P. Lefebvre, et al., Bile acid control of metabolism and inflammation in obesity, type 2 diabetes, dyslipidemia, and nonalcoholic fatty liver disease, Gastroenterology 152 (2017) 1679–1694.e3.
[34] W. Jia, G. Xie, W. Jia, Bile acid–microbiota crosstalk in gastrointestinal inflammation and carcinogenesis, Nat. Rev. Gastroenterol. Hepatol. 15 (2018) 111–128.
[35] N.S. Nagathihalli, Y. Beesetty, W. Lee, et al., Novel mechanistic insights into ectodomain shedding of EGFR ligands amphiregulin and TGF-α: Impact on gastrointestinal cancers driven by secondary bile acids, Cancer Res. 74 (2014) 2062–2072.
[36] H.S. Schadt, A. Wolf, J.A. Mahl, et al., Bile acid sequestration by cholestyramine mitigates FGFR4 inhibition-induced ALT elevation, Toxicol. Sci. 163 (2018) 265–278.
[37] E. Ibrahim, I. Diakonov, D. Arunthavarajah, et al., Bile acids and their respective conjugates elicit different responses in neonatal cardiomyocytes: Role of Gi protein, muscarinic receptors and TGR5, Sci. Rep. 8 (2018), 7110.
[38] J.E. Gotts, M.A. Matthay, Sepsis: Pathophysiology and clinical management, BMJ 353 (2016), i1585.
[39] C.J. Fisher Jr., J.M. Agosti, S.M. Opal, et al., Treatment of septic shock with the tumor necrosis factor receptor: Fc fusion protein, N Engl J. Med. 334 (1996) 1697–1702.
[40] V.S. Madamsetty, R. Mohammadinejad, I. Uzieliene, et al., Dexamethasone: Insights into pharmacological aspects, therapeutic mechanisms, and delivery systems, ACS Biomater. Sci. Eng. 8 (2022) 1763–1790.
[41] T.F. Yeh, Y.J. Lin, W.S. Hsieh, et al., Early postnatal dexamethasone therapy for the prevention of chronic lung disease in preterm infants with respiratory distress syndrome: A multicenter clinical trial, Pediatrics 100 (1997), E3.
[42] T. Xia, M. Zhang, W. Lei, et al., Advances in the role of STAT3 in macrophage polarization, Front. Immunol. 14 (2023), 1160719.
[43] M. Saraiva, P. Vieira, A. O'Garra, Biology and therapeutic potential of interleukin-10, J. Exp. Med. 217 (2020), e20190418.
[44] P. Comeglio, A. Morelli, L. Adorini, et al., Beneficial effects of bile acid receptor agonists in pulmonary disease models, Expert Opin. Investig. Drugs 26 (2017) 1215–1228.
[45] Z. Cai, S. Yuan, Y. Zhong, et al., Amelioration of endothelial dysfunction in diabetes: Role of takeda G protein-coupled receptor 5, Front. Pharmacol. 12 (2021), 637051.