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Functional mechanism of baicalein in alleviating severe acute pancreatitis-acute lung injury by blocking the TLR4/MyD88/TRIF signaling pathway

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Summary. Severe acute pancreatitis-acute lung injury (SAP-ALI) is a disease with high mortality. This study aims to explore the mechanism of baicalein on SAP-ALI in rats by blocking toll-like receptor-4 (TLR4)/myeloid differentiation primary response gene 88 (MyD88)/TIR-domain-containing adapter-inducing interferon-β (TRIF) signal pathway. The SAP-ALI rat model was established by intraperitoneal injection of 3% pentobarbital sodium (30 mg/kg), with pancreas and intestines turned over, injected with 3.5% sodium taurocholate backward into the bile-pancreatic duct at 0.1 mL/100 g for 12h, and treated with baicalein, lipopolysaccharide (LPS), miR-182 agomir, or miR-182 antagomir. The TLR4/MyD88/TRIF pathway was activated using LPS in SAP-ALI rats after baicalein treatment. Baicalein attenuated inflammatory cell infiltration, alveolar wall edema, decreased W/D ratio and levels of TLR4, MyD88, and TRIF in the lung tissues, reduced levels of inflammatory factors in pancreatic and lung tissues and BALF, diminished ROS, and elevated GSH, SOD and CAT in pancreatic and lung tissues of SAP-ALI rats. Activation of the TLR4/MyD88/TRIF pathway partly abrogated baicalein-mediated improvements in inflammation and oxidative stress in SAP-ALI rats. miR-182 targeted TLR4. miR-182 suppressed inflammation and oxidative stress in SAP-ALI rats by targeting TLR4. Inhibition of miR-182 partly nullified baicalein-mediated attenuation on inflammation and oxidative stress in SAP-ALI rats. In conclusion, baicalein can inhibit the TLR4/MyD88/TRIF pathway and alleviate inflammatory response and oxidative stress in SAP-ALI rats by upregulating miR-182 and suppressing TLR4, thus ameliorating SAP-ALI.

Key words: Severe acute pancreatitis-acute lung injury, Baicalein, TLR4, MyD88, TRIF, Oxidative stress, miR-182, Inflammation

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Introduction

Severe acute pancreatitis (SAP) is a common acute syndrome with characteristics of pancreatic digestion by its enzymes and necrosis (Shen et al., 2019). About 10-20% of patients with acute pancreatitis develop SAP (Ye et al., 2019). The mortality rate for SAP is as high as 10% in patients with sterile pancreatic necrosis and 25% in patients with infected pancreatic necrosis (Fu et al., 2018). SAP can result in acute lung injury (ALI), which is characterized by pulmonary inflammation and edema (Reiss et al., 2012). Oxidative stress is one of the most critical pathways during this pathological process. Hydroxytyrosol reduces lipid peroxidation and oxidative stress [superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione S-transferase (GST)] in the injured pancreatic and intestinal tissues by amplifying nuclear factor erythroid-derived 2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) (Fusco et al., 2020). SAP-ALI and acute respiratory distress syndrome remain great challenges, whose mortality rate reaches 30-40%, while the potential mechanism of pancreas-associated ALI has not been fully understood (Zhou et al., 2010). Among numerous factors involved in the development of SAP-ALI, inflammatory mediators have been considered to have the dominant role in its pathogenesis (Jin et al., 2017). Reduction of the inflammatory response contributes to improvements in cerulein-induced pancreatitis and lung injury (Cordaro et al., 2020). Although some contributions have been made to

the clinical treatment of SAP-ALI, the mortality of patients with SAP-ALI is still high and remains a major problem. Therefore, more studies are required for the management of SAP-ALI. Meanwhile, novel prevention and intervention tools are in urgent need to improve the outcomes of SAP-ALI patients (Zhou et al., 2010).

Baicalein, an active component of Scutellaria baicalensis Georgi, is used traditionally for treating various diseases including bacterial and viral infections of the respiratory and gastrointestinal tract, cardiovascular diseases, and inflammation (Tsai et al., 2014; Lai et al., 2017). Baicalein produces therapeutic effects on rotenone-induced Parkinson's disease through the protection of mitochondrial function and biogenesis (Zhang et al., 2017). By reducing fungal load and suppressing TSLP-induced inflammation, baicalein prevents the occurrence of aspergillus fumigatus keratitis (Zhu et al., 2021). Myocardial hypertrophy is ameliorated in mice by baicalein through inhibition of oxidative stress and activation of myocardial autophagy (Liu et al., 2021). Baicalein can impair angiogenesis in the inflammatory microenvironment by downregulating AP-1 (Huang et al., 2017). Intra-articular injection of baicalein has an inhibitory action on cartilage catabolism and NLRP3 inflammasome signaling in a posttraumatic osteoarthritis model (Bai et al., 2021). As a negative regulator of microglia activation and immune response *in vitro* and *in vivo*, baicalein can effectively jeopardize neurodegeneration in retinal ischemia/reperfusion injury (Pan et al., 2022). Baicalein ameliorates pristane-induced lupus nephritis by activating Nrf2/HO-1 in myeloid-derived suppressor cells (Li et al., 2019). Ultramicronized palmitoylethanolamide/baicalein can decrease prostate weight and DHT production and regulate apoptotic and inflammatory pathways and oxidative stress in benign prostatic hyperplasia-induced rats (D'Amico et al., 2021). A compound containing palmitoylethanolamide and baicalein can inhibit myocardial tissue injury, neutrophil infiltration, generation of markers for mast cell activation expression (chymase, tryptase) and pro-inflammatory cytokines (TNF-α, IL-1β), proving its therapeutic efficacy on myocardial ischemia/reperfusion injury (D'Amico et al., 2019). The anti-inflammatory effect of baicalein has been illustrated in the above models. Similarly, baicalein has been shown to attenuate the pathological changes of acute pancreatitis (Pu et al., 2019). Additionally, baicalein can inhibit certain types of lipoxygenase (Deschamps et al., 2006) as an anti-inflammatory agent (Hsieh et al., 2007), exhibit antibacterial activity (Chen et al., 2016), alleviate lung injury induced by myocardial ischemia and reperfusion (Lai et al., 2017) and LPS-induced acute lung injury (Tsai et al., 2014). However, the function of baicalein in SAP-ALI is still elusive. Therefore, in this study, intraperitoneal injection of baicalein was introduced into SAP-ALI rats to investigate whether baicalein could mitigate SAP-ALI. The effects of baicalein are already addressed via Toll-like receptor 4 (TLR4)/nuclear factor-κB (NF-κB) signaling (Luo et al., 2017). TLR4 interacts with the adapter protein myeloid differentiation factor 88 (MyD88) or/and TIR-domain-containing adapter-inducing interferon-β (TRIF) to activate NF-κB, which regulates the gene expression of inflammatory mediators such as the cytokines interleukin (IL)-1α and IL-1β, tumor necrosis factor (TNF)-α, and IL-6 (Lin et al., 2012; Fang et al., 2020; Jiang et al., 2020). The excessive cytokine-mediated inflammation induced by the triggering of the TLR4 signaling pathway plays an important role in the pathogenesis of SAP (Li et al., 2016).

microRNAs (miRNAs/miRs) contain 19-25 nucleotides and are classified as highly conserved, endogenous, short non-coding RNAs (Park et al., 2018; Qin et al., 2018). It has been reported that miR-182 plays an important role in regulating inflammatory responses in ALI by targeting TLR4 (Yang et al., 2020). The role of miR-182 as a potential and useful noninvasive tumor marker for the diagnosis and prognosis of pancreatic cancer has been discussed previously (Chen et al., 2014). However, the role of miR-182 in SAP-complicated ALI is largely unknown.

Up to now, the effect of baicalein on alleviating SAP-ALI has been studied but little is known about its functional mechanism and correlation with TLR4/MyD88/TRIF signaling pathway. There are few reports regarding whether baicalein can alleviate the inflammatory response via regulating upstream miR-182 and TLR4. Therefore, we are encouraged to further explore the functional mechanism of baicalein in alleviating SAP-ALI through the TLR4/MyD88/TRIF pathway to find new therapeutic targets for screening and treating SAP-ALI from the perspective of genes.

Materials and methods

Ethics statement

The present study was approved by the Ethical Committee of West China Hospital, Sichuan University (Approval no. 2021114). All procedures were strictly conducted under the code of ethics. The animal experiments were conducted based on a minimal number of animals and the least pain.

Establishment and grouping of SAP-ALI rats

Healthy male rats (aged 7-8 weeks, weighing 250-280 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The animals were kept in a specific pathogen-free animal house with a constant temperature of 22-24°C, a humidity of 50-60%, 12h artificial lighting, and free access to standard rat chow and sterile water.

A total of 96 rats were randomly assigned into sham group (N=12) (rats were intraperitoneally administered with 3% sodium pentobarbital (30 mg/kg), with pancreas and intestinal tube turned over, and then injected with

the same volume of normal saline backwards into bile-pancreatic duct in replacement of sodium taurocholate, with abdomen closed after 5 min); SAP-ALI group (n=12) [SAP-ALI rats were induced by 3.5% sodium taurocholate (Sigma-Aldrich, St. Louis, MO, USA) at 0.1 mL/100g (Wang et al., 2016)]; SAP-ALI+B (n=12) (rats were intraperitoneally injected with 200 mg/kg baicalein after SAP-ALI treatment) (Ma et al., 2018); SAP-ALI+B+antagomir-NC group (n=12) (rats were intravenously injected with 80 mg/kg antagomir-NC while injecting baicalein after SAP-ALI treatment); SAP-ALI+B+antagomir group (n=12) (rats were intravenously injected with 80 mg/kg antagomir, which is the antagonist of miR-182); SAP-ALI+B+LPS group (n=12) [rats were injected with 0.5 mg/kg lipopoly-saccharide (LPS) which is the signal pathway activator of TLR4/MyD88/TRIF] (Wang et al., 2019); SAP-ALI+ agomir-NC group (n=12) (rats were intravenously injected with 80 mg/kg agomir-NC after SAP-ALI treatment); SAP-ALI+agomir group (n=12) (rats were intravenously injected with 80 mg/kg agomir, the agonist of miR-182, after SAP-ALI treatment). The rats in the sham group and SAP-ALI groups were given the same amount of normal saline. The rats were maintained in a fasting state but had free access to sterile water after waking up. The antagomir-NC, antagomir, agomir-NC and agomir were purchased from GenePharma (Shanghai, China).

During histopathological experiments, blood samples were collected from the rats in the sham group, SAP-ALI group, and SAP-ALI+B group at 1, 3, 6, and 12h, centrifuged, and stored at -20°C. Rats were euthanized by an intraperitoneal injection of 800 mg/kg pentobarbital after 12h of blood sample collection. Fresh pancreatic tissues were removed, and pancreatic tissues of 6 rats were fixed with 4% paraformaldehyde, followed by histopathological observation. Pancreatic tissues of the remaining 6 rats were subjected to liquid nitrogen flash freezer at -80°C for enzyme-linked immunosorbent assay (ELISA). Fresh lung tissues in the middle lobe of right lung of 6 rats were placed in 4% paraformaldehyde for histopathology analysis. The remaining lung tissues were made into homogenate and stored at -80°C for reverse transcription-quantitative polymerase chain reaction (RT-qPCR), Western blot, and ELISA. The left lung was used for the measurement of lung wet-to-dry (W/D) ratio. Lung tissues of the remaining 6 rats were used to collect bronchoalveolar lavage fluid (BALF) for ELISA.

Hematoxylin and eosin (HE) staining

The pancreatic tissues and lung tissues were fixed in 4% paraformaldehyde, dehydrated in ethanol of increasing gradient concentrations, cleared in xylene, immersed in wax, and embedded in paraffin. The embedded tissues were sectioned at 4 μm, placed on the slides, baked for 2h at 60°C, and stored in dry places without light exposure. The paraffined sections were dewaxed using xylene, dehydrated using gradient ethanol, and stained using HE kit (Solarbio, Beijing, China). Then stained sections were dehydrated in ethanol of increasing gradient concentrations, cleared in xylene, and sealed using neutral gum, followed by observation under a Nikon Ti optical microscope (Tokyo, Japan) for visualising the pathological changes of the lung tissue.

The histopathological scores of pancreatic tissues were given by two independent pathologists blinded to the grouping based on Kusske criteria. Edema scores: 0, no edema; 1, interlobular edema; 2, diffuse interlobular edema; 3, acinar swelling and increased interlobular space; 4, obvious interlobular septula. Infection scores: 0, no infection; 1, infection in the margin of glandular ducts; 2, parenchymal infection<50% of lobule; 3, parenchymal infection=50-75% of lobule; 4, massive aggregation and abscessus. Bleeding scores: 0, no bleeding; 1, parenchymal bleeding=0-25%; 2, parenchymal bleeding=25-50%; 3, parenchymal bleeding=50-75%; 4, parenchymal bleeding >75%. Necrosis scores: 0, no necrosis; 1, periductal parenchymal necrosis; 2, spotty parenchymal necrosis <20%; 3, lobule deletion=20-50%; 4, lobule deletion >50%.

Lung injury was evaluated with the same blind method to observe the presence of alveolitis as follows: 0, no alveolitis; 1, mild alveolitis with local inflammatory cell infiltration <20% and intact alveolar structure; 2, moderate alveolitis=20-50%; 3, severe diffuse alveolitis >50%.

Assessment of pulmonary edema

Wet-to-dry weight (W/D) ratio was measured to evaluate the severity of pulmonary edema. At the selected time points, the lung was weighed immediately (wet weight) and then placed in a drying oven at 70°C for 48h until the weight was stable and then reweighed (dry weight). W/D ratio was calculated afterwards.

BALF collection

Endotracheal intubation was performed by placing vein detained needle cricoid cartilage and fixing it. The bronchoalveolar lavage was carried out with 4, 3, and 3 mL pre-cooled PBS and the procedure was repeated 3 times. BALF was collected into the sterile centrifuge tube for centrifugation and supernatant without cells was stored at -80°C to detect the level of inflammatory factors.

RT-qPCR

Total RNA was extracted from lung tissue ground by liquid nitrogen using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and transcribed into cDNA using PrimeScript RT reagent kit (Takara, Dalian, China). Quantitative PCR was performed using an ABI7900HT

fast PCR real-time system (Applied Biosystems, Foster city, CA, USA) and SYBR® Premix Ex TaqTM II (Takara). RT-qPCR was carried out using the following conditions: pre-denaturation for 10 min at 95°C, and 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 20 s and extension at 72°C for 34 s. The $2^{-\Delta\Delta Ct}$ formula was used to analyze the data with U6 as the internal control. Gene and primer sequences are illustrated in Table 1.

ELISA

The pre-weighed pancreatic or lung tissues were added with cold phosphate buffer saline (1:9 w/v) and homogenized. After homogenization, the supernatant was centrifuged at 4°C for 10 min at 1,000×g. The levels of reactive oxygen species (ROS) and glutathione (GSH), activities of SOD and catalase (CAT), and concentrations of IL-1β, IL-6, and TNF-α were determined using the ELISA kits (R&D Systems, Minneapolis, MN, USA).

The levels of IL-1β, IL-6, and TNF-α in BALF were detected using the ELISA kits as per the instructions.

Blood sample was centrifuged for 10 min at 3000 r/min and stored at -20°C. The level of serum amylase (AMY) was detected using rat serum AMY-ELISA kit (Abcam, Cambridge, UK) following the instructions.

Western blot analysis

The lung tissues were ground in liquid nitrogen and mixed with RIPA (strong) (Beyotime, Nanjing, China) to extract the total protein. Protein concentrations were determined by bicinchoninic acid kits (Beyotime). Electrophoresis procedure was carried out as follows: electrophoresis at 60 V, then changed to 120 V when entering separating gel for 1-2h in a cold chamber at 4°C. Protein samples were then transferred onto polyvinylidene fluoride (PVDF) membranes for 2h in the cold chamber at 4°C after electrophoresis. The membranes were then blocked with 5% nonfat milk-Tris-buffered saline containing 0.05% Tween-20 (TBST) buffer and incubated at room temperature for 1-2h. The PVDF membranes were then incubated overnight at 4°C with rabbit anti-MyD88 (1:500, Abcam), rabbit anti-TRIF (1:500, Abcam), and rabbit anti-TLR4 (1:500, Abcam). After three washes in TBST, 10 min each time, the membranes were incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (1:5000, CoWin Biosciences Co., Ltd., Beijing, China) for 1h at room temperature. Membranes were washed again with TBST three times, 10 min each time. Chemiluminescence was used to expose the protein bands on the membrane for gray value analysis, using GAPDH (1:5000, CoWin Biosciences) as a control.

Dual-luciferase reporter assay

The binding sites of miR-182 and TLR4 were predicted by TargetScanHuman 7.1 (http://www.targetscan.org/vert_71/). The complementary binding sequences and mutant sequences were amplified and cloned to the pmiR-GLO luciferase vector (Promega, Madison, WI, USA). Then, wild-type plasmids TLR4-WT and mutant plasmids TLR4-MUT were constructed. The constructed vectors were mixed with mimic NC and miR-182 mimic (GenePharma) separately, and co-transferred into HEK293T cells (Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China) using Lipofectamine™ 2000 (Invitrogen) according to the instructions. Luciferase activity was determined 24h after cotransfection.

Statistical analysis

SPSS 21.0 (IBM Corp. Armonk, NY, USA) was used to analyze the data. The data are expressed as the mean ± standard deviation (SD). One-way ANOVA was used for evaluating the differences between groups, followed by Tukey's multiple comparisons test. P value was obtained using a two-sided test. Statistical significance was determined when $P<0.05$.

Results

Baicalein alleviated SAP-ALI in rats

The structures of pancreatic tissues and lung tissues of rats in the sham group appeared normal under a light microscope. Rats in the SAP-ALI group showed higher AMY levels than rats in the sham group, especially at 12h after surgery (Fig. 1A, $P<0.01$). HE staining showed obvious pancreatic edema, enlarged interlobular space, large amounts of reddish interstitial edema fluid and some island-shaped acinus (Fig. 1B), obvious inflammatory cell infiltration, and serious alveolar congestion and alveolar wall edema in the SAP-ALI group (Fig. 1D). The histopathological scores of pancreatic tissues and lung tissues were higher in the SAP-ALI group than those in the sham group (Fig. 1C,E, $P<0.01$). The AMY

Table 1. Primer sequences.

Gene	Forward 5'-3'	Reverse 5'-3'
miR-182	GAGAACAGCAGGTCCAGCAT	CTTCCTCAGCAC AGACCGAG
U6	TTCTTGGGTAGTTTGCAGTT	TTCTTGGGTAGTTTGCAGTT

level was decreased after baicalein treatment (Fig. 1A, $P<0.01$). Rats in the SAP-ALI+baicalein group showed improved conditions of pancreatic edema, infection, bleeding, and necrosis and developed slight inflammatory cell infiltration and relieved interstitial edema compared to that in the SAP-ALI group (Fig. 1B,D). Statistical results of histopathological scores displayed that injured lungs and pancreases of SAP-ALI rats treated with baicalein were improved distinctively (Fig. 1C,E, $P<0.01$).

The lung W/D ratio is the common index for lung injury (Rojas et al., 2005; Gao et al., 2011; Wang et al., 2015). As shown in Figure 1F, lung W/D ratio was comparatively increased in the SAP-ALI group compared to that in the sham group. Lung W/D ratio of groups with baicalein treatment was significantly reduced except at 1h and 3h after treatment ($P<0.01$).

Baicalein reduced inflammation and oxidative stress in SAP-ALI rats by inhibiting the TLR4/MyD88/TRIF pathway

We subsequently assessed the inflammatory response and oxidative stress in pancreatic and lung tissues and BALF of rats by ELISA, which indicated elevated levels of IL-1β, IL-6, and TNF-α in pancreatic and lung tissues and BALF ($P<0.01$, Fig. 2A-C), increased ROS, and decreased GSH, SOD and CAT activity in the pancreatic and lung tissues of SAP-ALI rats ($P<0.01$, Fig. 2D,E). Mounting evidence indicates that the activation of the TLR4/MyD88/TRIF pathway is associated with inflammatory reaction and oxidative stress (Lin et al., 2012; Fang et al., 2020; Zeng et al., 2020). Western blot showed that rats in the SAP-ALI group exhibited significantly higher expression of TLR4,

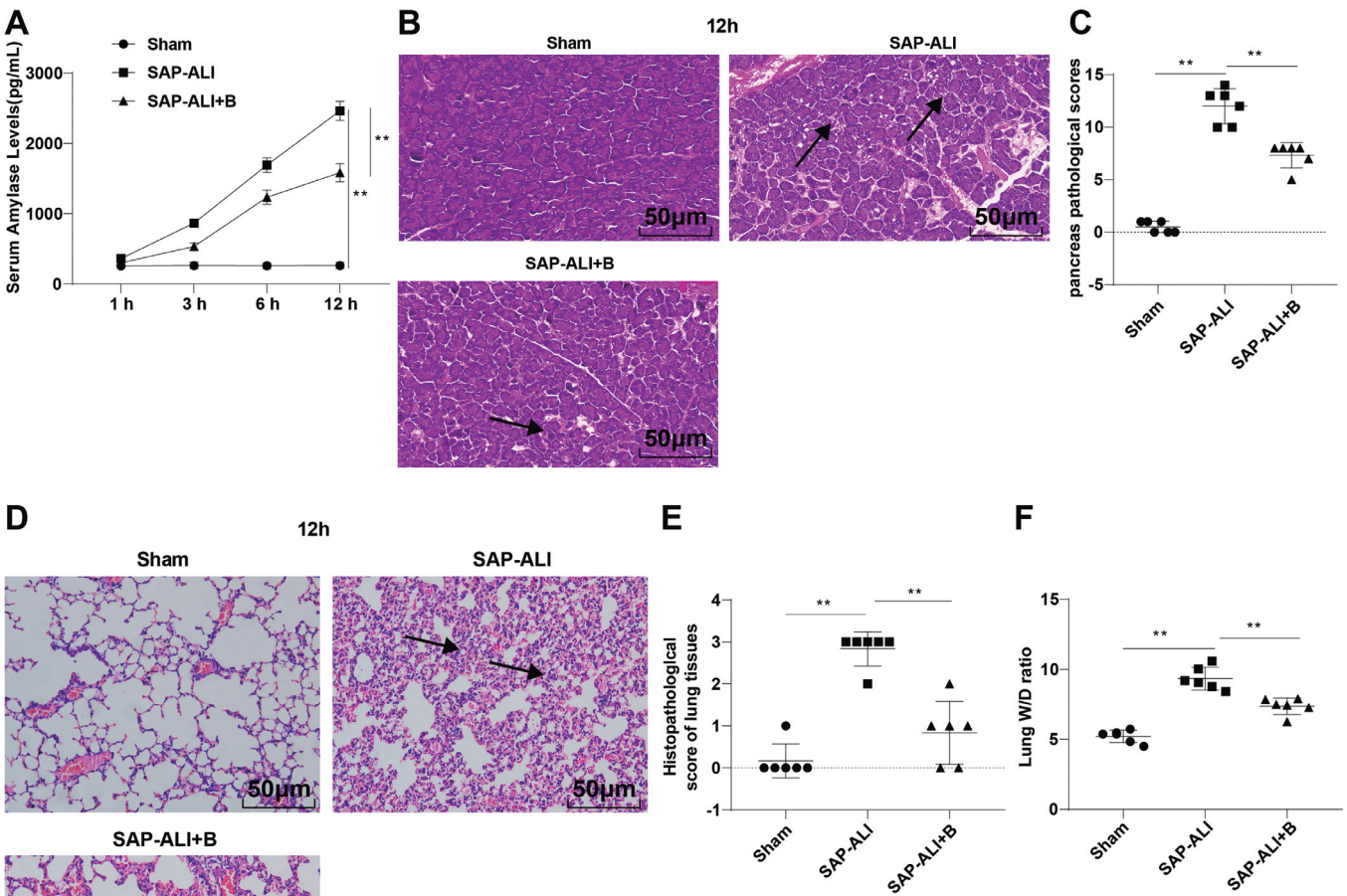

Fig. 1. Baicalein alleviated SAP-ALI in rats. SAP-ALI rat model was established by treating rats with 3.5% sodium taurocholate. After baicalein treatment, AMY level was decreased according to ELISA **(A)**, n=12; pancreatitis symptoms were relieved according to histopathological changes of pancreatic tissues observed by HE staining **(B)**, n=6; histopathological scores of pancreatic tissues were reduced **(C)**, n=6; lung injury was attenuated according to histopathological changes of lung tissues observed by HE staining **(D)**, n=6; histopathological scores of lung tissues were reduced **(E)**, n=6; lung W/D ratio was decreased in SAP-ALI rats **(F)**, n=6. Data are presented as mean ± SD (n=6). Data were analyzed using one-way ANOVA, followed by Tukey's multiple comparisons test. $^{**}P<0.01$.

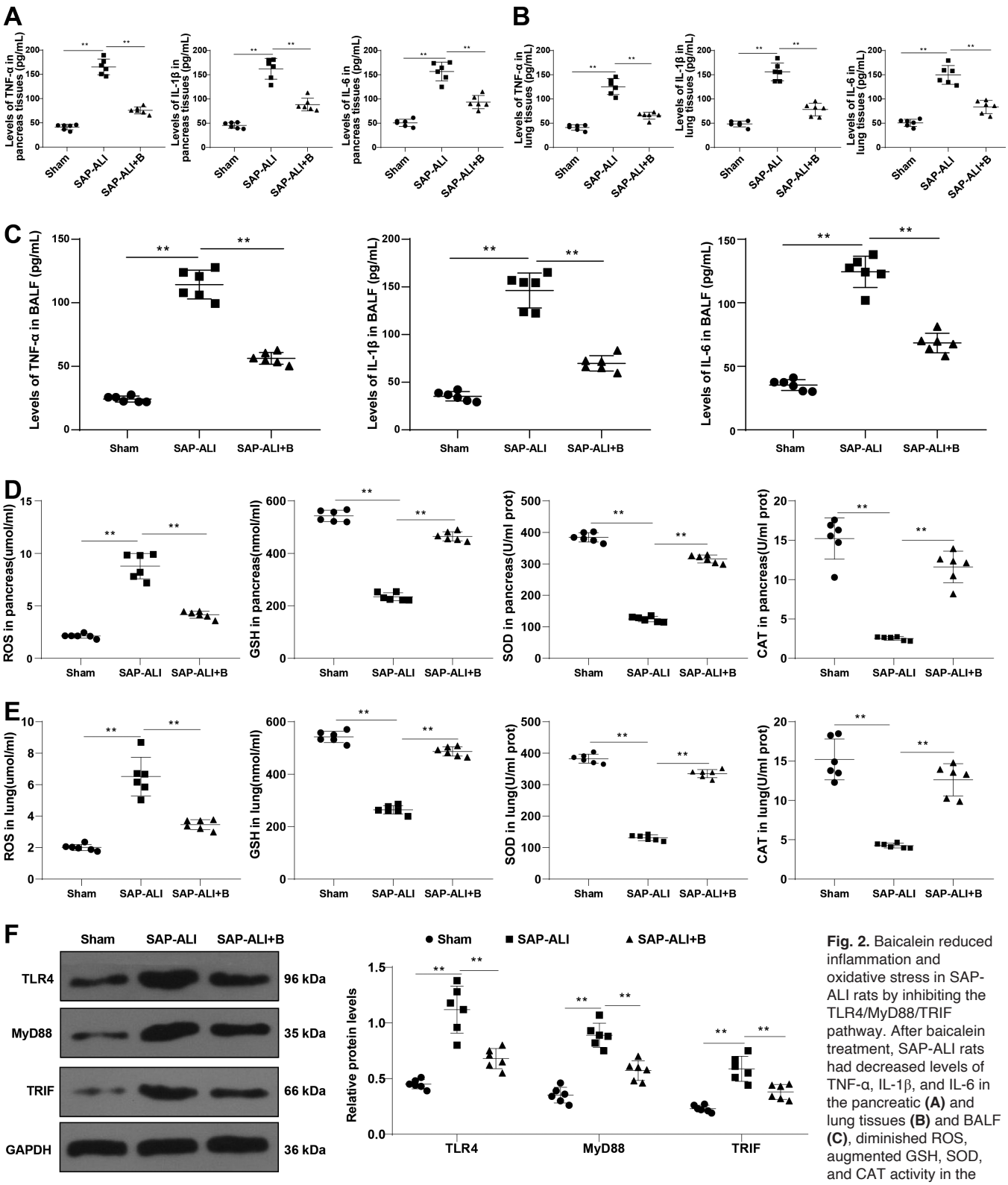

Fig. 2. Baicalein reduced inflammation and oxidative stress in SAP-ALI rats by inhibiting the TLR4/MyD88/TRIF pathway. After baicalein treatment, SAP-ALI rats had decreased levels of TNF-α, IL-1β, and IL-6 in the pancreatic **(A)** and lung tissues **(B)** and BALF **(C)**, diminished ROS, augmented GSH, SOD, and CAT activity in the pancreatic **(D)** and lung tissues **(E)**, and declined levels of TLR4 **(F)**, MyD88, and TRIF in lung tissues according to Western blot; n=6. Data are presented as mean ± SD. Data were analyzed using one-way ANOVA, followed by Tukey's multiple comparisons test. ***P*<0.01.

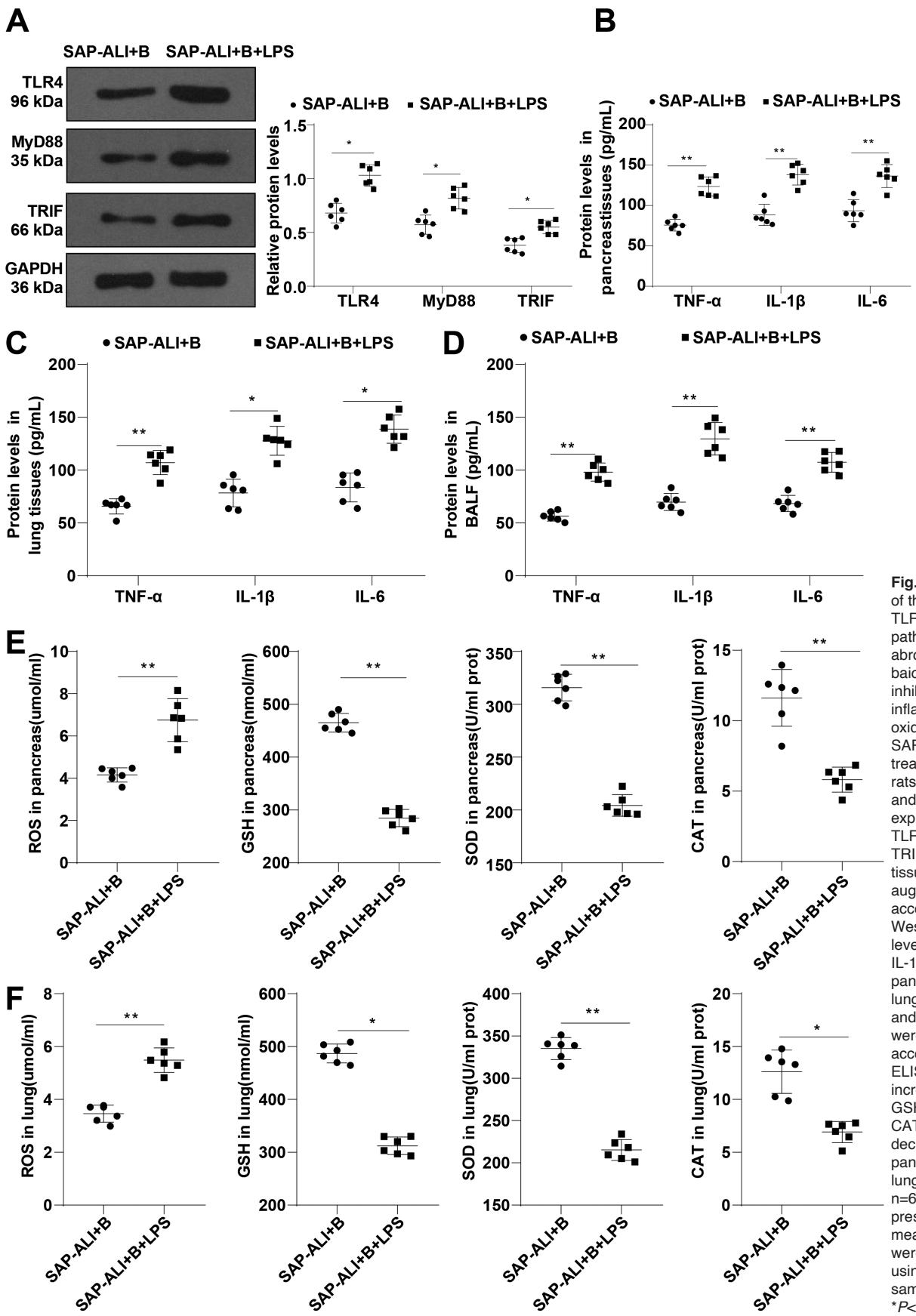

Fig. 3. Activation of the TLR4/MyD88/TRIF pathway partly abrogated baicalein-mediated inhibition on inflammation and oxidative stress in SAP-ALI rats. After treating SAP-ALI rats with baicalein and LPS, expression of TLR4, MyD88, and TRIF **(A)** in lung tissues was augmented according to Western blot. **B-C.** levels of TNF-α, IL-1β, and IL-6 in pancreatic **(B)** and lung tissues **(C)** and BALF **(D)** were elevated according to ELISA; ROS was increased, and GSH, SOD and CAT were decreased in the pancreatic **(E)** and lung tissues **(F)**. n=6. Data are presented as mean ± SD. Data were analyzed using independent sample t test. **P*<0.05, ***P*<0.01.

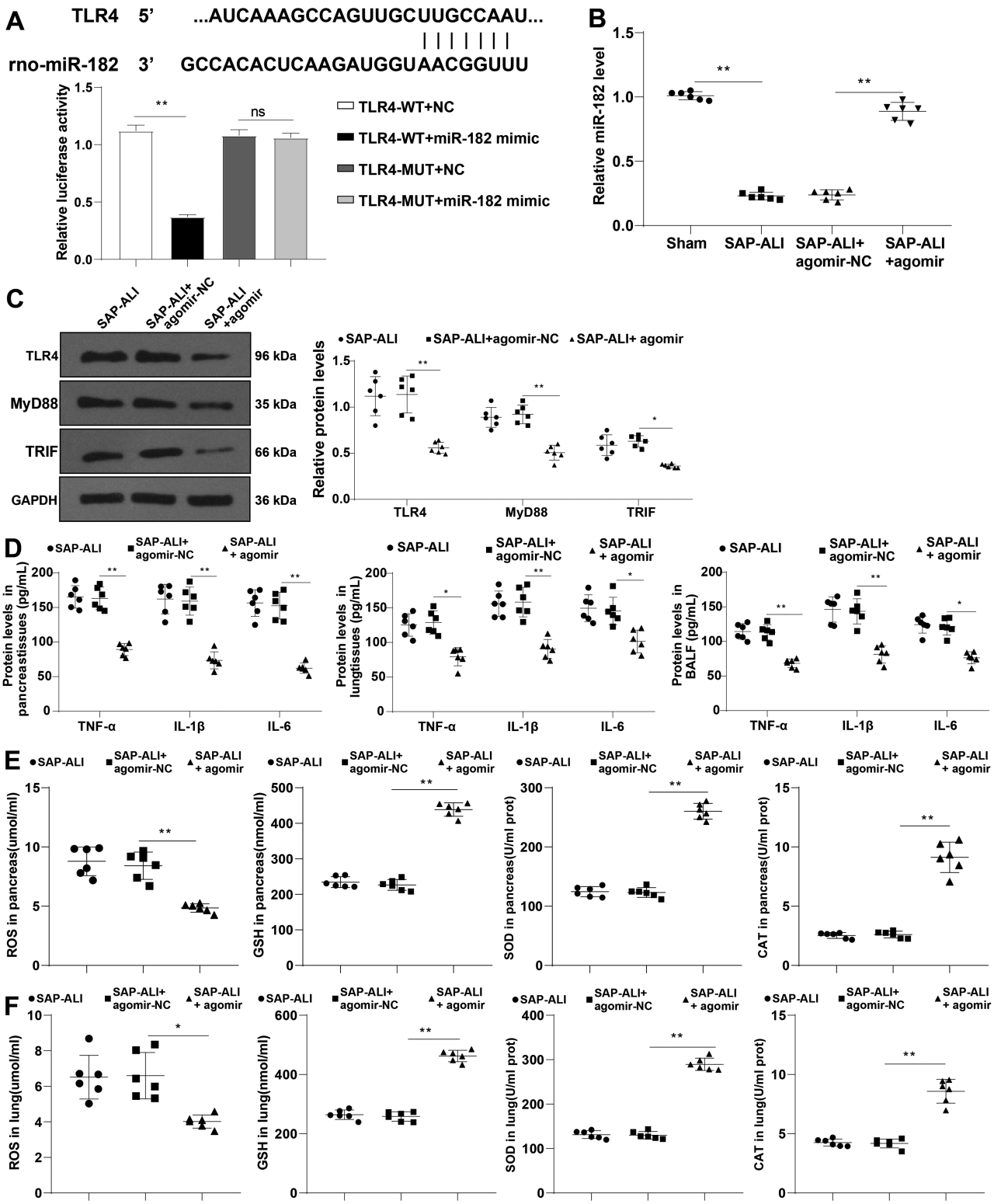

Fig. 4. miR-182 reduced inflammation and oxidative stress in SAP-ALI rats by targeting TLR4. binding site of miR-182 and TLR4 was predicted on TargetScan database and target relationship was verified using dual-luciferase assay **(A)**; miR-182 expression was decreased in lung tissues of SAP-ALI rats according to RT-qPCR. After treating SAP-ALI rats with miR-182 agomir **(B)**, miR-182 expression was promoted according to RT-qPCR **(B)**; expression of TLR4, MyD88, and TRIF in lung tissues was decreased according to Western blot **(C)**; levels of TNF-α, IL-1β, and IL-6 in lung tissues were declined in pancreatic and lung tissues and BALF according to ELISA **(D)**; ROS was diminished, and GSH, SOD, and CAT were augmented in pancreatic **(E)** and lung tissues **(F)**. n=6. Data are presented as mean ± SD. Data in panel A were analyzed using t test and the data in panels B-F were analyzed using one-way ANOVA, followed by Tukey's multiple comparisons test. **P*<0.05, ***P*<0.01.

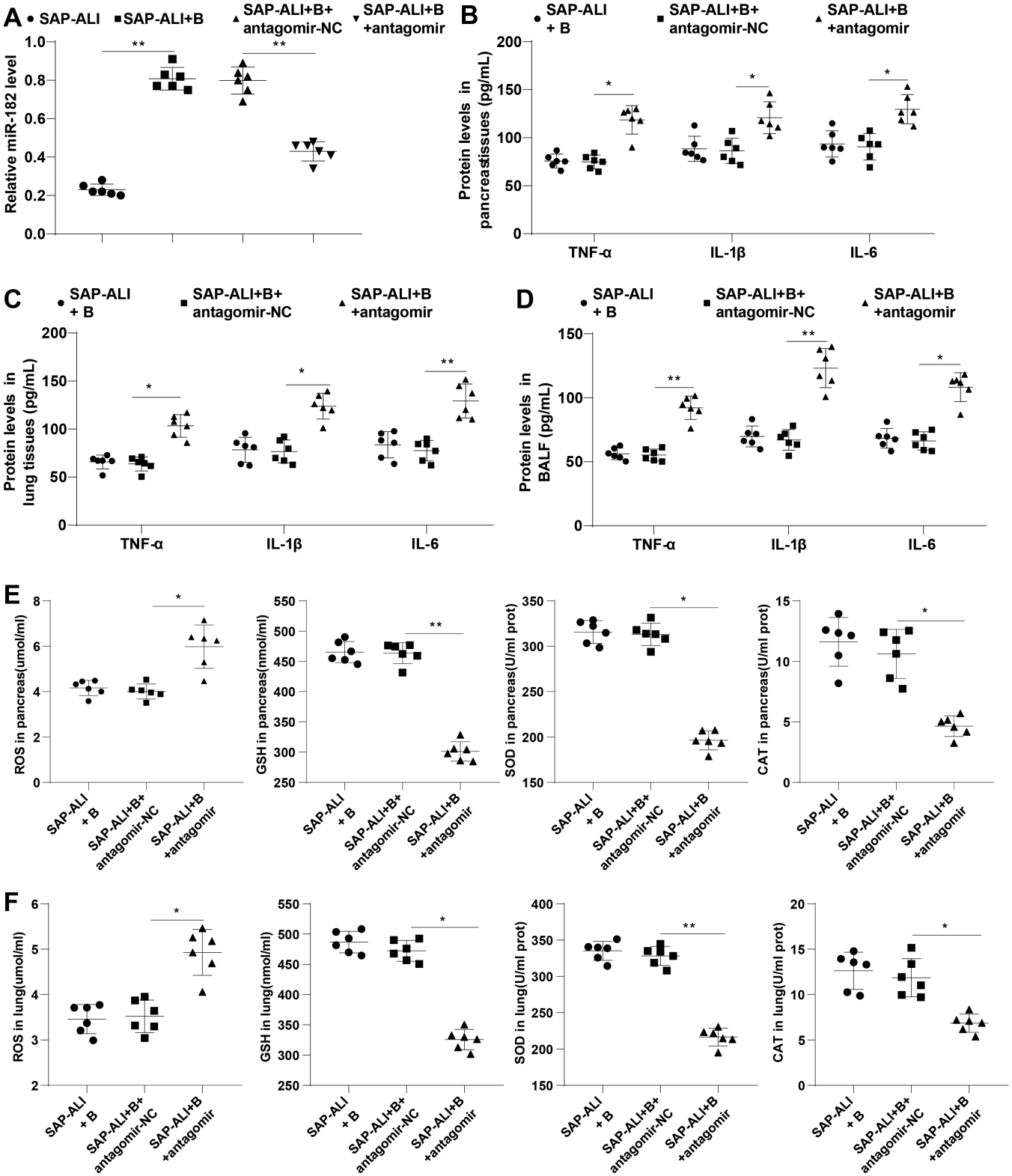

Fig. 5. Inhibition of miR-182 partially annulled baicalein-mediated alleviation effect on inflammation and oxidative stress in SAP-ALI rats. After treating SAP-ALI rats with baicalein and miR-182 antagomir, miR-182 expression **(A)** was decreased in the lung tissues according to RT-qPCR; levels of TNF-α, IL-1β, and IL-6 in pancreatic **(B)** and lung tissues **(C)** and BALF **(D)** were elevated according to ELISA; ROS was increased, and GSH, SOD, and CAT were decreased in pancreatic **(E)** and lung tissues **(F)**. n=6. Data are presented as mean ± SD. One-way ANOVA was used for evaluating differences within groups, followed by Tukey's multiple comparisons test. **P*<0.05, ***P*<0.01.

MyD88, and TRIF than the sham group (P<0.01), while rats in the SAP-ALI+B group exhibited lower expression than in the SAP-ALI group (P<0.01, Fig. 2F). The levels of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α were decreased in the pancreatic and lung tissues and BALF after baicalein treatment (Fig. 2A-C). The SAP-ALI+B group had decreased ROS and increased GSH, SOD, and CAT in the pancreatic and lung tissues (P<0.01, Fig. 2D,E). These results indicate that baicalein reduced inflammation and oxidative stress in SAP-ALI rats by inhibiting the TLR4/MyD88/TRIF pathway.

Activation of the TLR4/MyD88/TRIF pathway partly abrogated baicalein-mediated inhibition on inflammation and oxidative stress in SAP-ALI rats

To further test the hypothesis that baicalein reduces inflammation and oxidative stress in SAP-ALI rats by inhibiting TLR4/MyD88/TRIF signaling pathway, we treated SAP-ALI rats with baicalein and used the known inducer, LPS to explore whether the alleviation effect of baicalein on SAP-ALI changes after activating the TLR4/MyD88/TRIF signaling pathway. The result suggested that the levels of TLR4, MyD88, and TRIF were elevated in the lung tissues (P<0.05, Fig. 3A), levels of TNF-α, IL-1β, and IL-6 were increased in the pancreatic and lung tissues and BALF (P<0.05, Fig. 3B-D), ROS was augmented, and GSH, SOD, and CAT were diminished in the pancreatic and lung tissues of the SAP-ALI+B+LPS group compared with the SAP-ALI+B group (P<0.05, Fig. 3E,F). These results showed that the activation of the TLR4/MyD88/TRIF pathway can partially invalidate baicalein-mediated suppression on inflammation and oxidative stress in SAP-ALI rats.

miR-182 reduced inflammation and oxidative stress in SAP-ALI rats by targeting TLR4

Previous studies have stated poor expression of miR-182 in ALI and its correlation with pulmonary inflammation (Park et al., 2018; Yang et al., 2020). RT-qPCR demonstrated decreased miR-182 expression in lung tissues of SAP-ALI rats (P<0.01, Fig. 4B). Taken together, we speculated that miR-182 might target TLR4. Next, the binding site of miR-182 and TLR4 was predicted by TargetScanHuman 7.1 (Fig. 4A). Dual-luciferase reporter assay suggested that luciferase activity in WT plasmid was decreased in the TLR4-WT+miR-182 mimic group compared to that in the TLR4-WT+NC group (P<0.01), while luciferase activity in MUT plasmid showed no significant difference between the TLR4-MUT+miR-182 mimic group and TLR4-MUT+NC group (P>0.05), which proved their targeting relation (Fig. 4A). SAP-ALI rats were then treated with miR-182 agomir to upregulate miR-182. RT-qPCR showed that the expression of miR-182 was significantly increased in the lung tissues after treatment with miR-182 agomir (P<0.01, Fig. 4B), and Western blot demonstrated decreased protein levels of TLR4, MyD88, and TRIF (P<0.05, Fig. 4C), suggesting that miR-182 targeted TLR4 and subsequently inhibited the TLR4/MyD88/TRIF pathway. ELISA showed that the levels of IL-6, IL-1β, and TNF-α in pancreatic and lung tissues and BALF were clearly decreased (P<0.05, Fig. 4D), ROS was decreased, and GSH, SOD, and CAT were increased in the pancreatic and lung tissues in the SAP-ALI+B+miR-182 agomir group (P<0.05, Fig. 4E,F). These results indicated that miR-182 reduced inflammation and oxidative stress in SAP-ALI rats by targeting TLR4.

Inhibition of miR-182 partially annulled baicalein-mediated alleviation effect on inflammation and oxidative stress in SAP-ALI rats

Based on the above results, we speculated that baicalein may play its role in the inflammatory response by upregulating miR-182 expression. To test our hypothesis, we detected miR-182 expression in SAP-ALI rats after baicalein treatment, and the result showed that miR-182 expression in SAP-ALI+B group was significantly increased compared to that in the SAP-ALI group (P<0.01, Fig. 5A). Next, we performed the combined experiment of baicalein and miR-182 to test if silencing miR-182 changes the alleviation effect of baicalein on inflammation and oxidative stress in SAP-ALI rats. The result showed that miR-182 was downregulated in the SAP-ALI+antagomir group relative to that in the SAP-ALI+antagomir-NC group (P<0.01, Fig. 5A). After treating SAP-ALI rats with baicalein and miR-182 antagomir, ELISA showed upregulation of IL-6, IL-1β, and TNF-α in pancreatic and lung tissues and BALF (P<0.05, Fig. 5B-D), elevation of ROS, and downregulation of GSH, SOD, and CAT in the pancreatic and lung tissues (P<0.05, Fig. 5E,F). In summary, inhibition of miR-182 partly invalidated baicalein's alleviation action on inflammation and oxidative stress in SAP-ALI rats.

Discussion

SAP-ALI is a serious disease with high mortality (Hu et al., 2022). There is evidence that baicalein is a promising therapeutic agent for the treatment of ALI (Chen et al., 2019). Therefore, it is important to explore the functional mechanism of baicalein in SAP-ALI treatment. Our study found that baicalein can alleviate inflammation and oxidative stress in SAP-ALI rats by upregulating miR-182 and blocking the TLR4/MyD88/TRIF signaling pathway.

Elevated AMY level is one of the diagnostic criteria of SAP (Ismail and Bhayana, 2017). The histopathological scores of pancreatic and lung tissues and AMY levels were reduced in SAP-ALI rats after baicalein treatment. Baicalein has been reported to be able to attenuate intestinal ischemia-reperfusion (I/R) injury-induced ALI by inhibiting inflammation (Chu et al., 2017). Moreover, a recent study has demonstrated

that baicalein can attenuate symptoms including lung edema (Tsai et al., 2014). Our study found that the severe symptoms of inflammatory cell infiltration, lung W/D ratio, and interstitial edema in SAP-ALI rats were relieved significantly after baicalein treatment. This result suggested that baicalein can alleviate SAP-ALI in rats.

The pathogenesis of ALI has tight connections with inflammation and oxidative stress (Sarma and Ward, 2011). IL-6 is a commonly measured cytokine in pulmonary inflammation (Reiss et al., 2012). TNF-α and IL-1β are key pro-inflammatory cytokines in SAP pathogenesis (Vonlaufen et al., 2007). SAP-ALI rats exhibited decreased levels of IL-6, TNF-α, and IL-1β in the pancreatic tissues, lung tissues, and BALF after baicalein treatment. Research has found that innate immunity and inflammatory responses may contribute to neurological deficits possibly through the release of endogenous ligands, which exert functions largely through TLRs (Lin et al., 2012). Upon recognition of respective PAMPs (pathogen-associated molecular pattern), TLRs recruit a specific set of adaptor molecules that harbor the TIR domain, such as MyD88 and TRIF, and initiate downstream signaling events including NF-κB that induces the expression of inflammation-associated molecules and cytokines (Kawai and Akira, 2010). TLR4 interacts with the TRIF to activate NF-κB, which regulates the gene expression of inflammatory mediators such as the cytokines IL-1β, TNF-α, and IL-6 (Miyake, 2004; Takeda and Akira, 2004). TLR4 can activate the mononuclear phagocyte system and contribute to inflammatory reactions through MyD88 and TRIF pathways (Lucas and Maes, 2013). In SAP-ALI rats treated with baicalein, the expression pattern of TLR4, MyD88, and TRIF were consistent with that of IL-6, TNF-α, and IL-1β. SOD, CAT, and GSH are pivotal antioxidant enzymes, whose activity can reflect the ability of cells to eliminate ROS and resist oxidative stress (Boysen, 2017). We observed decreased ROS and increased GSH, SOD, and CAT in pancreatic and lung tissues of SAP-ALI rats after baicalein treatment. TLR4 is an innate receptor involved in oxidative stress, and TLR4 stimulation provokes the activation of the MyD88- or TRIF-dependent pathways (Imai et al., 2008; Hosoki et al., 2018). Baicalein reduces inflammation and oxidative stress to ameliorate myocardial ischemia through the TLR4/MyD88/MAPKS/NF-κB pathway (Li et al., 2022). Collectively, baicalein can alleviate inflammation and oxidative stress in SAP-ALI rats by inhibiting the TLR4/MyD88/TRIF pathway.

To further validate the involvement of the TLR4/MyD88/TRIF pathway in the baicalein-mediated alleviation effect, we activated the TLR4/MyD88/TRIF pathway using the known inducer LPS. MyD88 and TLR4 engage in local and systemic control over bacterial growth in pneumonia (Sanchez-Tarjuelo et al., 2020). TLR4 activation can strengthen the oxidative status of intestinal epithelial cells (Latorre et al., 2014). In the same way, the activation of the TLR4/MyD88/TRIF pathway led to reduction of IL-6, TNF-α, and IL-1β, elevation of ROS, and decline of GSH, SOD, and CAT in the pancreatic and lung tissues of SAP-ALI rats. The suppressed TLR4/MyD88/TRIF/NLRP3 pathway is implicated in the reno-protective effects of 5-O-methyldihydroquercetin and cilicicone B by contributing to the reduction of inflammation and oxidative stress (Zeng et al., 2020). Equally, the activation of the TLR4/MyD88/TRIF pathway partly abolished baicalein-mediated alleviation on inflammation and oxidative stress in SAP-ALI rats.

TLR4 is validated as a target of miR-182 (Zhao et al., 2019). The miR-182-5p/TLR4 axis is implicated in oxidative stress and inflammation in mice with cadmium-induced hepatotoxicity (Hao et al., 2021). The target relationship between miR-182 and TLR4 was verified in our study, and we overexpressed miR-182 using agomir and tested the expression of related inflammatory factors. The expression levels of TLR4, MyD88, and TRIF, and levels of IL-6, IL-1β, and TNF-α were reduced in SAP-ALI rats after treatment with miR-182 agomir, while ROS level was increased and GSH, SOD, and CAT activities were decreased. Another study has revealed that overexpression of miR-182-5p attenuated H_2O_2-induced inflammation via the TLR4/NF-κB pathway, which is reflected by a reduction in pro-inflammatory cytokines in C8-D1A cells (Zhang and Wu, 2018). One recent study showed that miR-182-5p can repress oxidative stress in atherosclerosis by targeting TLR4 (Qin et al., 2018). The results further indicated that miR-182 reduced inflammation and oxidative stress in SAP-ALI rats through inhibition of TLR4 expression. To further test this conclusion, we suppressed miR-182 expression in SAP-ALI rats using antagomir after baicalein treatment. After silencing miR-182, the levels of IL-6, IL-1β and TNF-α in lung tissues and BALF of SAP-ALI rats treated with baicalein were increased, and GSH, SOD and CAT activities were diminished while ROS was augmented, suggesting that the alleviation effects of baicalein on SAP-ALI rats were partially averted after silencing miR-182.

This study focused on the regulatory function of baicalein in SAP-ALI rats and discovered that baicalein affected the activation of the downstream TLR4/MyD88/TRIF pathway by upregulating miR-182, thus reducing oxidative stress and inflammation in SAP-ALI rats. The functional mechanism of the TLR4/MyD88/TRIF pathway in SAP-ALI was superficially explored, which is one of the limitations, and shall be addressed in future investigations. The focus of future studies should be placed on whether miR-182 and TLR4/MyD88/TRIF pathway play roles in other pathological reactions in SAP-ALI, and whether they can become the biomarkers for earlier screening for SAP-ALI. The protein expression levels of TLR4/MyD88/TRIF were detected in this study, yet their interactions remained unclear, which represents another limitation to be addressed in the future. In future studies, we shall also pay attention to the regulation mechanism of miR-182/TLR4/

MyD88/TRIF from the perspective of histopathology and early screening of biomarkers.

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

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