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Original Article

Tocilizumab attenuates acute lung injury in rats with sepsis by regulating S100A12/NLRP3

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Abstract: Objective: To investigate the mechanism of Tocilizumab (TCZ) in attenuating acute lung injury in rats with sepsis by regulating the S100A12/NLRP3 axis. Methods: A rat model of sepsis was constructed using cecal ligation and puncture (CLP). Rats were treated with TCZ, and their lung tissue was collected. H&E staining was used to detect pathologic damage to lung tissue, and lung wet/dry (W/D) weight ratio was measured to assess pulmonary edema. Lipid oxidation assay and superoxide dismutase (SOD) activity assay kits were used to measure malondialdehyde (MDA) and SOD levels. Primary rat pulmonary microvascular endothelial cells (MPVECs) were treated with lipopolysaccharide (LPS) to construct a rat model of sepsis, which was then treated with TCZ. The mRNA and protein expressions of S100A12/NLRP3 were detected by qRT-PCR and western blot, respectively. S100A12 knockdown and overexpression plasmids, and NLRP3 knockdown plasmids were constructed and transfected into sepsis cells to intervene in the levels of S100A12/NLRP3. The apoptosis rate was detected by apoptosis assay. The levels of IL-6, TNF-α, and IL-10 in cells and tissues were analyzed by ELISA. Results: Compared to the Sham group, the CLP group had increased W/D weight ratio of lung tissue, IL-6, TNF-α, and MDA levels, lowered IL-10 and SOD levels, and more severe tissue damage (all $P<0.05$). After TCZ treatment, the above indicators were improved. The expressions of S100A12/NLRP3 cells were increased in LPS-induced MPVECs, but decreased after TCZ treatment. LPS induced apoptosis, but TCZ reduced the apoptosis, weakened the secretion levels of IL-6 and TNF-α, and enhanced IL-10 secretion levels. Transfection to cause the overexpression of S100A12 or NLRP3 plasmid partially counteracted the effect of TCZ. Knockdown of S100A12 was transfected on the basis of overexpression of NLRP3, which weakened the countervailing effect of overexpressed NLRP3 on TCZ. Conclusion: TCZ has a therapeutic effect on lung injury in rats with sepsis by reducing the expressions of S100A12/NLRP3.

Keywords: Tocilizumab, S100A12, NLRP3, sepsis, lung injury

Introduction

Sepsis is the body's imbalance immune regulation to an infection caused by bacteria, fungi, or parasites, which can induce a systemic inflammatory response. The development of sepsis will further lead to circulatory disorders and multi-organ dysfunction, and sepsis has a high mortality [1, 2]. Sepsis often occurs in severely ill patients, more susceptible to infection, such as those suffering from burns and receiving surgical procedures, and patients with aplastic anemia and chronic obstructive bronchitis. With acute onset and rapid development, sepsis quickly leads to tissue damage, organ failure, and death [3]. Lung tissue is extremely fragile, and sepsis often causes acute lung injury. At present, anti-infective therapy and immunomodulatory therapy are used for the clinical treatment of sepsis, but persistent immunoparalysis in some critically ill patients can cause the primary infection to be difficult to clear, with an unsatisfactory result [4]. Therefore, it is of great significance to explore new effective targeting molecular mechanisms for critically ill patients. The causes of acute lung injury in sepsis are numerous and complex. Currently, there are many studies on the mechanism of vascular endothelial injury and alveolar epithelial injury, but the exact mechanism still needs further proof [5]. Xie et al. believe that the pathologic process of acute lung injury in sepsis is complex, including microcirculatory disorders, endothelial dysfunction, and inflam-

matory response, and the inflammatory response is the most important trigger [6]. At present, it is known that inflammatory response and oxidative stress run through the whole process of acute lung injury and jointly promote the formation of lung injury. Dogwood acrylic ether terpene glycosides are active ingredients in dogwood with anti-inflammatory effects, which improve the symptoms of acute lung injury in rats by inhibiting the inflammatory response and oxidative stress [7]. N-acetylcysteine also protects sepsis rats from lung injury by regulating inflammatory factors and oxidative stress responses in their plasma [8]. There are several similar drugs, such as isoflavones, natural isoflavones, and mullein isoflavones [9, 10].

Tocilizumab (TCZ) is an IL-6 receptor inhibitor with anti-inflammatory effects. TCZ may be selected when treatment of rheumatoid arthritis with one or more TNF antagonists is not satisfactory [11]. In the study of Giorgio et al., it was concluded that TCZ was effective in treating pneumonia [12]. The use of the IL-6 receptor antagonist TCZ in the treatment of severe sepsis caused by severe gastrointestinal infection has also been investigated [13]. Although TCZ is known to treat acute lung injury in sepsis, the specific mechanism remains unclear. Only one study has found that TCZ may reduce acute lung and kidney injury in rat models of sepsis by downregulating NF-κB/JNK [14].

S-100 calcium binding protein A12 (S100A12) plays a great role in a variety of inflammatory diseases. For instance, S100A12 levels can assess the prevalence of primary knee osteoarthritis [15].

S100A12 is significantly enhanced in active Sjogren's syndrome, and the serum S100A12 level in patients receiving TCZ is lower than that in patients who did not receive TCZ [16]. Nishimoto Net al. pointed out that TCZ improved the expression of S100A12 in rheumatoid arthritis [17]. The above studies have revealed that S100A12 is dysregulated in immune-related diseases, and TCZ regulates the expression of S100A12, but whether TCZ affects S100A12 in sepsis or not needs further research.

Karam et al. pointed out that inflammatory mediator S100A12 could activate NOD-like receptor thermal protein domain associated protein 3 (NLRP3) to promote the secretion of mucin MUC5AC in airway epithelial cells, indicating S100A12/NLRP3 may be one of the regulatory axes for intervention in asthma and chronic obstructive pulmonary disease [18]. It has been confirmed that S100A12 is able to induce apoptosis caused by sepsis by activating the NLRP3 pathway [19]. Therefore, we conducted this study to investigate whether TZB influences the progression of sepsis by the S100A12/NLRP3 axis.

Materials and methods

Ethical statement

The local laboratory animal ethics committee has approved this animal experiment. The animal experiments were performed in strict adherence with the standards of the *Guidelines for the Care and Use of Laboratory Animals*.

Bioinformatics analysis

Peripheral blood datasets were retrieved from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov). "Sepsis" was set as a keyword, and GSE13015 and GSE28750 datasets were used. The "limma" packet in R 3.6.3 was adopted for gene differential expression analysis. GO and KEGG enrichment analysis were carried out in the DAVID database (https://david.ncifcrf.gov). The String database platform (https://string-db.org) was used to analyze protein-protein interactions (PPI).

Cecal ligation and puncture (CLP)

Thirty healthy clean Wistar rats used in the experiments were 8-10 weeks of age and weighing 200-250 g (Beijing Vital River Laboratory Animal Technology Co., Ltd., China). They were divided into a sham group (n=10), a CLP group (n=10), and a CLP + TCZ group (n=10) using a random number table method. All rats were fed to adapt to a specific sterile environment with 12 h light/12 h dark, a temperature around 24°C and a humidity of 60%-65% for 5 days. They had free access to sufficient food and water. Before surgery, rats were exposed to fasting for 12 h. Rats with neat, smooth and shiny coats that laid close to the body, and with well-proportioned limbs, clean ears, nose and mouth, were considered as healthy rats. The rats were intraperitoneally injected with 30 mg/kg pentobarbital sodium, and were fixed on the operating table in a supine position for rou-

tine skin preparation and disinfection. A 2 cm incision was made in the center of the rat's abdomen with a scalpel. Sterile toothless forceps were used to free the mesentery and cecum. The cecum was ligated circularly using sterile No. 3 silk thread at the 1/3 cecum and perforated through the blind end with an 18-gauge needle. After the intestinal contents were extruded, the peritoneum and skin were interruptedly sutured layer by layer with No. 4 silk thread. Then, 50 mL/kg of saline was immediately injected subcutaneously for anti-shock. The sham group underwent an operation using the same method, but without perforation. 2 h after the surgery, rats in the CLP + TCZ group were intraperitoneally injected with 4 mg/kg TCZ (TM-Toci-00002_6, Shanghai TheraMabs Biotechnology Co., Ltd., China) [14]. 12 h after surgery, rats in the CLP group were curled up, and their frequency of drinking, eating, and activity were decreased, showing diarrhea and tiredness with eye discharge. 24 h after molding, rats were anesthetized with 5% pentobarbital sodium for spinal dislocation, and fresh lung tissues were collected by opening the chest.

Pulmonary edema

Pulmonary wet-to-dry weight (W/D) ratio was applied to assess pulmonary edema. The water and blood on the surface of fresh lung were removed. The lung was weighed using an electronic scale, and then placed in a 60°C oven for 72 h until reaching a constant weight and then weighed again. The lung W/D ratio was calculated.

H&E staining

Lung tissue was fixed with 10% neutral paraformaldehyde. After conventional dehydration, paraffin embedding, the tissue was sectioned with a microtome into 5 μm. The tissue sections were put into baths of xylene I and II (10 min for each bath), then hydrated by passing through decreasing concentrations of alcohol baths (100%, 90%, 80%, 70%, 3 min for each bath) and distilled water for 2 min. Thereafter, the tissue sections were stained with hematoxylin (G1120, Solarbio, China) for 5 min, rinsed with distilled water, differentiated with differentiation solution for 3 min and rinsed with running water twice, 2 min/time. After that, Eosin Staining Solution (G1120, Solarbio, China) was used to stain for 1 min. The sections were rinsed with distilled water for 2 s and dehydrated rapidly, then placed in increasing concentrations of alcohol I baths (75%, 85%, 95%, 100%, 3 s for each), and 100% alcohol II for 1 min. The slices were put in two xylene baths for clearing, 1 min/time, sealed with neutral gum and then observed under the microscope (× 100).

The extent of lung tissue injury was assessed based on the lung injury scoring system recommended by the ATS Pulmonary Function Committee, including alveolar edema, alveolar congestion and alveolar exudates. The scoring system was as follows. 0= no lung injury; 1= mild lung injury; 2= moderate lung injury; 3= severe lung injury.

Enzyme-linked immunosorbent assay (ELISA)

The levels of the inflammatory factors IL-6 (SP13755, Wuhan Saipei Biotechnology Co., Ltd., China), tumor necrosis factor-α (TNF-α, SP13726, Wuhan Saipei Biotechnology Co., Ltd., China), and IL-10 (SP13770, Wuhan Saipei Biotechnology Co., Ltd., China) were measured. The tissue sample was filled with 10% ice-cold phosphate-buffered saline, fully homogenized using a Polytron homogenizer and centrifuged for 10 min, then the supernatant was pipetted for detection. The cell sample was centrifuged for 10 min to remove polymers and particles before testing. The specimens were diluted in a ratio of 1:1. The biotin-labeled antibody was added to the polystyrene plate, covered with a template, shaken, and incubated at 37°C for 1 h. The solution from each well was removed. The wash buffer was added and shaken for 30 s, and the solution was removed. Affinity chain enzyme -HRP was added in each well, shaken, and incubated at 37°C for 30 min, and the solution from each well was removed. The wash buffer was added and shaken for 30 s, and the solution from each well was removed. The A and B substrates were added, shaken, and incubated at 37°C in the dark for 10 min. The stop buffer was added. The optical density value was measured at 450 nm with a microplate reader (ReadMax 1200, Shanghai Flash Spectrum Biological Technology, China).

Lipid peroxidation assay

Malondialdehyde (MDA) assay kit (S0131S, Beyotime, China) was used for experiments.

Table 1. qRT-PCR primer sequences

Gene	Sequences (5'-3')	
	Forward	Reverse
S100A12	5'-GATATACGCGTCGCCGCTTCA-3'	5'-GTAATCCGTTCGCTAGACC-3'
NLRP3	5'-TATAGCGCTTATATATGCCC-3'	5'-CCTTGAGTTAGCGCTCTC-3'
GAPDH	5'-AATACTCTTAGCGCTTAGCA-3'	5'-ACTAGCTTCTCGGCTCTAGGATC-3'

The tissue was lysed on ice by adding lysate at a ratio of 1:10 and centrifuged. The supernatant was collected to measure MDA level. Thiobarbituric acid (TBA) stock solution and MDA detection solution were prepared according to the requirements of the manual. A blank control was set up. The above different concentrations of solutions were added to make the standard curve, followed by the sample and MDA working solution. The solutions were mixed, heated and denatured in boiling water, cooled at room temperature, and centrifuged at a speed of 1,000 g for 10 min. 200 μL of supernatant was inoculated in a 96-well culture plate, and the absorbance was measured at 532 nm using the microplate reader.

Superoxide dismutase (SOD) assay

SOD assay kit (S0101S, Beyotime, China) was used to measure SOD level. 10 mg of tissue and 100 μL of SOD sample preparation were mixed, homogenized and centrifuged at a speed of 12,000 g at 4°C for 5 min. The supernatant was collected, and the protein concentration was determined with the BCA Protein Assay Kit (P0009, Beyotime, China). The protein sample was diluted with SOD detection buffer. WST-8/enzyme and reaction working fluids were prepared. Sample wells and blank control wells were set up in a 96-well plate. The sample, WST-8/enzyme working solution, SOD detection buffer, and reaction working solution were added in turn and then mixed. The absorbance was measured at 450 nm using the microplate reader.

Cell culture and transfection

Primary rat pulmonary microvascular endothelial cells (MPVECs, 184695-80-5, Hongshun Biotechnology Co., Ltd., Shanghai) were cultured in a DMEM medium containing 10% FBS at 37°C. S100A12 knockdown plasmid (si-S100A12), S100A12 overexpression plasmid (pcDNA-S100A12) and NLRP3 knockdown plasmid (si-NLRP3) were constructed. MPVECs were transfected using Lipofectamine 3000 Reagent (L3000075, Thermo Fisher, USA) and placed in an incubator for 24 h. MPVECs were taken out and stimulated with 100 ng/mL lipopolysaccharide (LPS; L8880, Solebold, Beijing) for 6 h to construct a cell damage model.

qRT-PCR

RNA from cells and tissue was extracted using Trizol reagent, and the expression level was analyzed by qRT-qPCR. UV spectrophotometer (UV5, Mettler Toledo, Switzerland) was adopted to analyze the concentration and quality of RNA sample. The first strand cDNA was reverse transacted using the SuperScript™ IV First Strand Synthesis System (18091050, Thermo Fisher, USA), and the cDNA was inactivated at 80°C. RT-PCR and Q-PCR synthesis were performed with SYBR Premix Ex Taq II kit (RR820A, Takara, Japan). GAPDH was used as an internal reference. The relative expression of mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences (Tsingke Biotechnology Co., Ltd., Beijing, China) are shown in **Table 1**.

Western blot

1 mm PMSF was added to the RIPA buffer (R0010, Solarbio, China). The cells were collected and rinsed with PBS and serum-free medium. RIPA buffer was added and pipetted to make full contact with the sample. The lysed cell sample was centrifuged for 5 min, and the supernatant was transferred to an EP tube. The protein concentration was determined with the BCA Protein Assay Kit. The protein was heated at 70°C for 10 min. The target protein was isolated using 10% SDS-PAGE electrophoresis and transferred onto a PVDF membrane (IPFL00010, Merck, USA). Then, 50 mL PBST with 2.5 g skim milk powder was used as blocking solution. The PVDF membrane was placed into the newly configured blocking solution and sealed on a shaker for 2 h. Then, the PVDF membrane was rinsed with PBST three times, 10 min each. The membrane was incubated

with primary antibody dilution S100A12 (1:1000, ab272713, Abcam, UK), NLRP3 (1:1000, ab263899, Abcam, UK) and GAPDH (1:3500, ab8245, Abcam, UK), placed on the shaker overnight at 4°C and then rinsed with PBST three times, 10 min for each. Afterwards, secondary antibody IgG (1:2500, ab6721, Abcam, UK) was added to the membrane, incubated for 1 h with constant shaking and then rinsed with PBST three times, 10 min each. ECL chemiluminescence fluid (PE0010, Sorlarbio, Beijing) was prepared. The membrane was developed in ECL working solution and photographed on a gel imager. The grayscale value of the target band was analyzed, and the GAPDH band was set as a control for the protein sample.

Cell apoptosis assay

Flow cytometry dual staining kit (CA1020, Solarbio, China) was applied to measure cell apoptosis. The cell was centrifuged at 1,000 rpm for 5 min. The supernatant was aspirated carefully after the cell pellet. 1 mL of pre-cooled PBS at 4°C was added. The cell was resuspended, and pelleted after centrifugation. The supernatant was carefully aspirated. The Binding Buffer was diluted with deionized water at 1:9. The cell pellet was resuspended with 1 × binding buffer to 2 × 10^6/mL of concentration. Then, 100 μL of cell suspension was added to the flow cytometry tube. The reaction device was moved to a dark chamber. Another 5 μL of Annexin V/FITC was added to the flow cytometry tube, mixed and reacted for 5 min. Then 5 μL of PI and 400 μL of PBS were added, and the apoptosis was measured immediately on the flow cytometer.

RNA immunoprecipitation (RIP) protocol

RIP is an experimental protocol that studies the physical binding between individual proteins and RNA molecules. RIP kit (17-704, Merck, USA) was used. Cells were collected when they grew logarithmically. The cells were trypsinized, centrifuged at 1,000 rpm for 5 min at 4°C, and rinsed with PBS. RIP lysate was added to the collected cell pellet. The cells were placed on ice for 5 min, and incubated with anti-IgG, anti-NLRP3 and 50 μL conjugated magnetic beads for 30 min. The cell suspension was lysed and centrifuged for 10 min. Next, 100 μL of supernatant was added to the antibody-conjugated magnetic beads and incubated overnight at 4°C. After centrifugation for 10 min, the immunoprecipitated RNA was purified using Trizol reagent, and the expression level was analyzed by qRT-qPCR.

Statistical analysis

Data were statistically analyzed using SPSS 26.0. Measured data were expressed as mean ± standard deviation ($\bar{x}$ ± sd). For measured data with normal distribution, independent samples t-test and one-way analysis of variance were applied to assess the differences between and among groups, respectively. For measured data with abnormal distribution, Mann-Whitney U test was used for comparison between groups, and pairwise comparisons among groups were conducted with Bonferroni test. Statistical significance level was set at $P<0.05$.

Results

TCZ alleviated CLP-induced lung injury symptoms

H&E staining revealed that compared to the sham group, the CLP group had more severely damaged alveolar structure in lung tissue, smaller alveolar cavity, more increased collagen deposition, wider alveolar wall spacing, more obvious pulmonary interstitial fibrosis, and higher lung injury score ($P<0.0001$). Compared to the CLP group, the lung tissue in the CLP + TCZ group showed clearer alveolar structure, mild bleeding, presence of inflammatory cell infiltration, and reduced lung injury (**Figure 1A** and **1B**, all $P<0.0001$). Usually, the pulmonary edema indicated the occurrence of inflammation. Compared to the sham group, the W/D weight ratio of lung tissue in the CLP group was significantly increased; while compared to the CLP group, the W/D weight ratio was reduced in the CLP + TCZ group (**Figure 1C**, both $P<0.0001$). The above results showed that TCZ significantly alleviated the symptoms of CLP-induced lung injury in rats.

TCZ reduced CLP-induced oxidative stress and inflammatory damage

The levels of inflammatory cytokines in the lung tissue were analyzed by ELISA. Compared to the sham group, the CLP group had higher levels of IL-6 and TNF-α, but lower IL-10 levels (**Figure 2A**, all $P<0.0001$). Compared to the

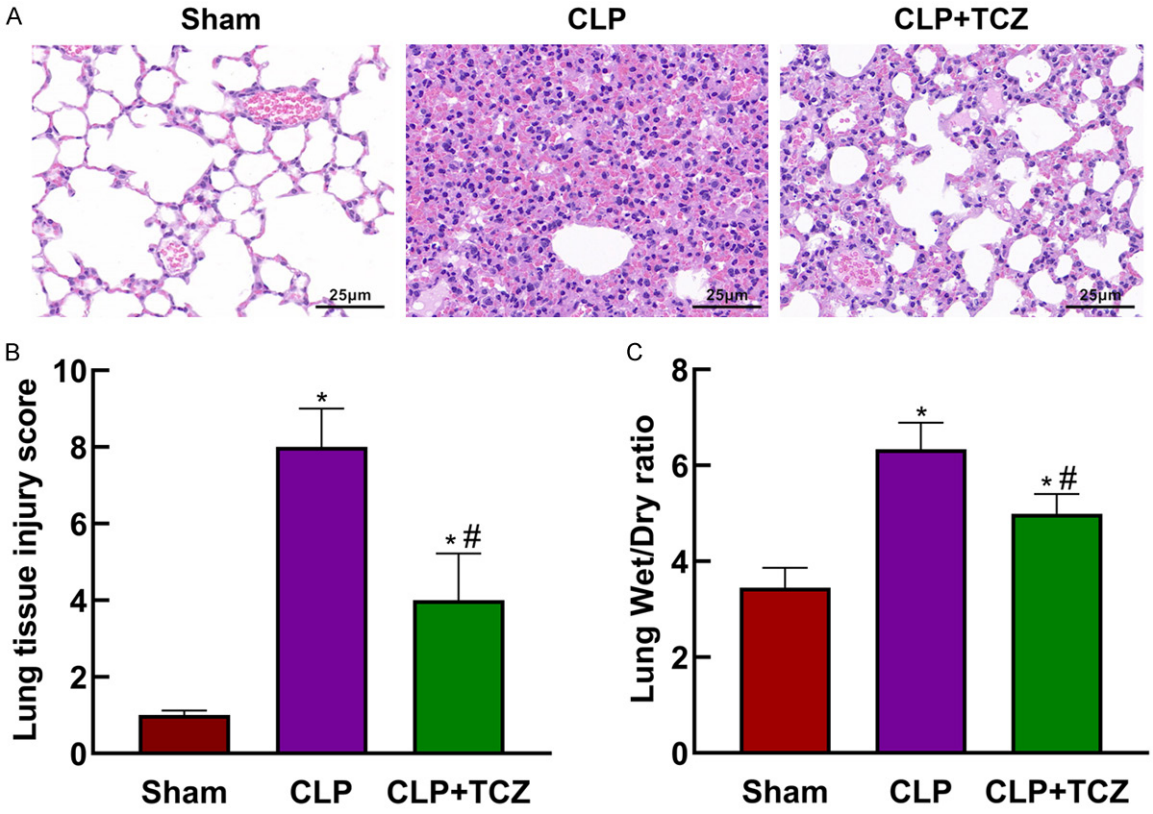

Figure 1. TCZ alleviated CLP-induced lung injury symptoms in rats. A: Outcomes from H&E staining; B: Lung injury score; C: The W/D weight ratio of lung tissue among groups. Data among multiple groups were analyzed by one way ANOVA. Compared with the sham group, *P<0.05; compared with the CLP group, #P<0.05. TCZ: Tocilizumab; CLP: Cecal Ligation and Puncture; W/D: Wet/Dry; H&E: Hematoxylin-Eosin.

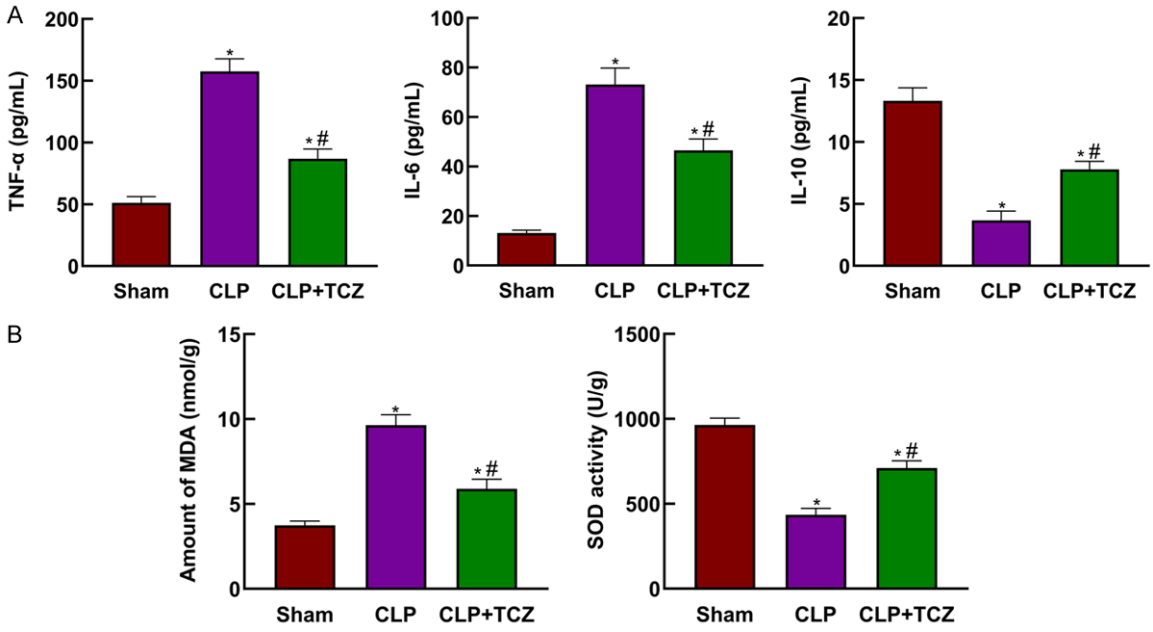

Figure 2. TCZ reduced CLP-induced oxidative stress and inflammatory damage. A: IL-6, TNF-α and IL-10 levels in lung tissues in rats detected by ELISA; B: MAD and SOD levels in lung tissues in rats detected by kits. Data among multiple groups were analyzed by one way ANOVA. Compared with the sham group, *P<0.05; compared with the CLP group, #P<0.05. TCZ: Tocilizumab; CLP: Cecal Ligation and Puncture; SOD: Superoxide Dismutase; MDA: Malondialdehyde; TNF-α: Tumor Necrosis Factor-α; IL-6: Interleukin-6; IL-10: Interleukin-10.

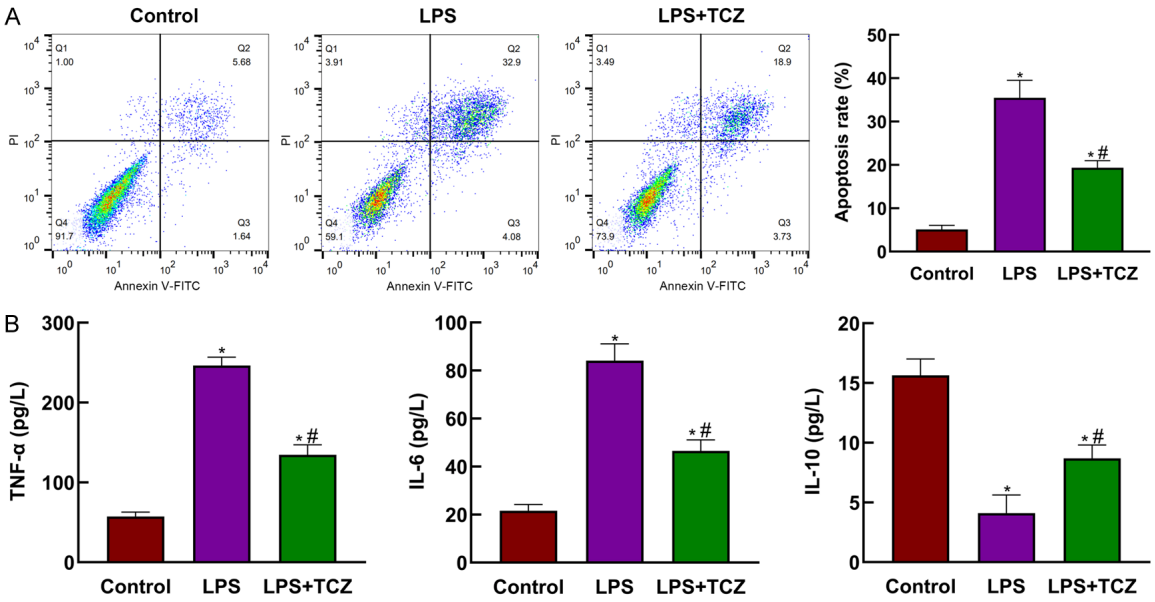

Figure 3. TCZ reduced LPS-induced inflammatory responses and cell apoptosis. A: Cell apoptosis measured with flow cytometry in each group; B: IL-6, TNF-α and IL-10 levels in lung tissues in rats detected by ELISA. Data among multiple groups were analyzed by one way ANOVA. Compared with the control group, *P<0.05; compared with the LPS group, #P<0.05. TCZ: Tocilizumab; CLP: Cecal Ligation and Puncture; LPS: Lipopolysaccharide; TNF-α: Tumor Necrosis Factor-α.

CLP group, the CLP + TCZ group showed reduced secretion levels of IL-6 and TNF-α, but an increased level of IL-10 (**Figure 2A**, all P<0.05). The oxidative stress test results showed that compared to the sham group, the CLP group had higher MDA level and lower SOD level (**Figure 2B**, all P<0.0001). The MDA level was lower but SOD level was higher in the CLP group than in the CLP + TCZ group (**Figure 2B**, all P<0.05). These findings indicated TCZ reduced CLP-induced oxidative stress and inflammatory damage.

TCZ reduced LPS-induced inflammatory responses and apoptosis

Flow cytometry results revealed that compared to the control group, the apoptosis rate in the LPS group was increased significantly, but it was decreased after TCZ treatment (**Figure 3A**, P<0.05). ELISA results revealed that compared to the control group, the LPS group showed increased levels of IL-6 and TNF-α, but a decreased level of IL-10 (**Figure 3B**, all P<0.05). LPS + TCZ group had lower levels of IL-6 and TNF-α, but higher IL-10 level than the LPS group (**Figure 3B**, all P<0.05). These suggested TCZ reduced LPS-induced inflammatory responses and cell apoptosis.

TCZ regulates S100A12 in sepsis

The possible molecular mechanisms of TCZ in sepsis were explored. The GSE13015 dataset in the GEO database included 48 patients with sepsis caused by different pathogens and 19 normal controls. The GSE28750 dataset covered 10 patients with sepsis and 20 controls. Gene expression differences were analyzed in the two datasets, and a Wayne diagram of the intersection of the different datasets was plotted, resulting in a total of 85 overlapped genes (**Figure 4A**). GO and KEGG enrichment analysis were carried out for all the overlapped genes in the DAVID database (https://david.ncifcrf.gov). Candidate genes were more enriched in immune-related GO terms, such as immune response pathway, T cell receptor pathway, and T cell activation pathway (**Figure 4B**). In KEGG analysis, some immune-related pathways were found, like Th1 and Th2 cell differentiation pathway, Th17 cell differentiation pathway and primary immunodeficiency pathway (**Figure 4C**). After that, 85 genes were analyzed for PPI (**Figure 4D**). Previous studies on Sjogren's syndrome and rheumatoid arthritis had found that TCZ regulates S100A12 [16, 17]. Compared to the sham group, the S100A12 level in the CLP

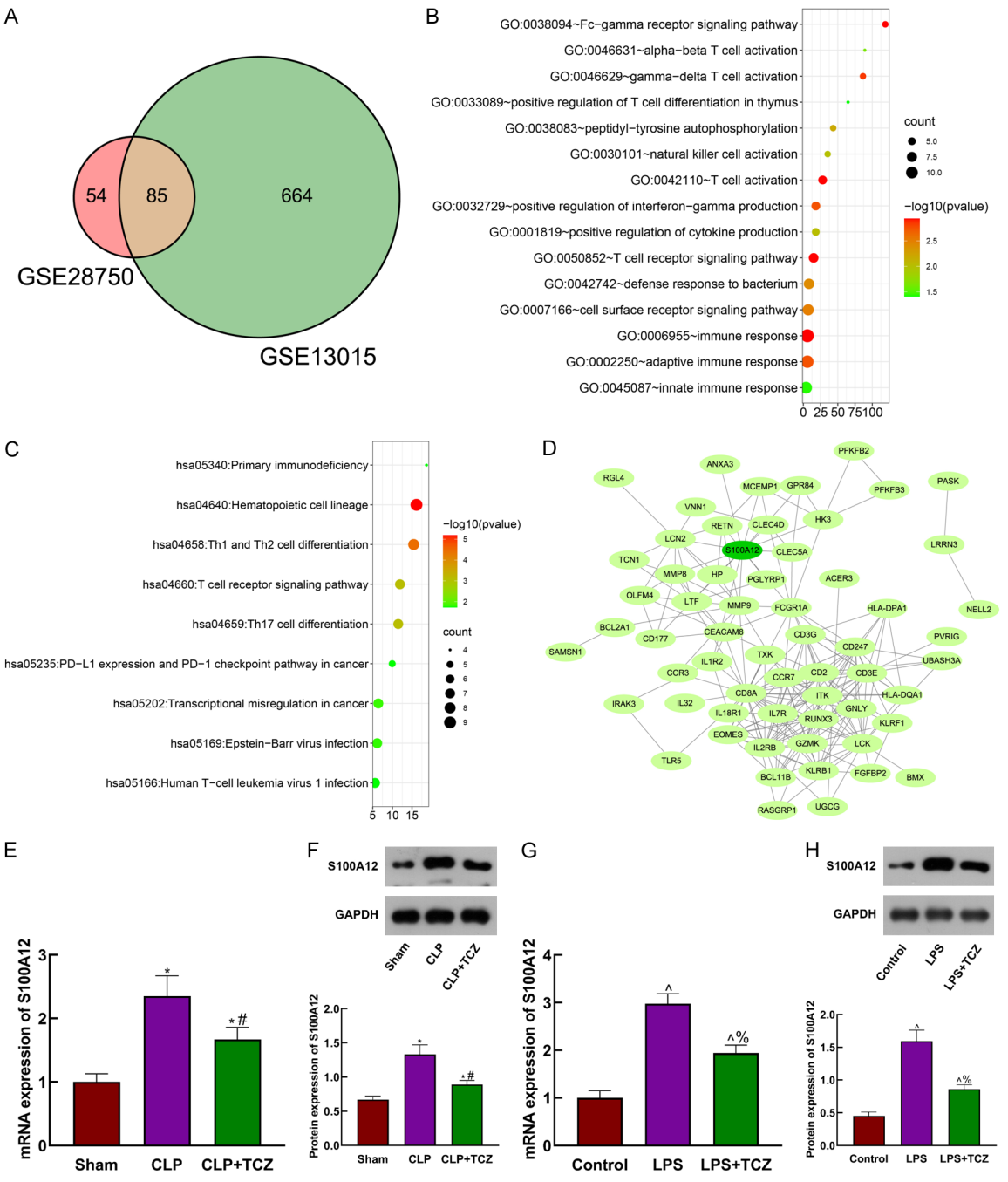

Figure 4. TCZ regulates S100A12 in sepsis. A: A Venn diagram of overlapped genes from GSE13015 and GSE28750 datasets; B: GO enrichment analysis; C: KEGG enrichment analysis; D: Protein-protein interaction analysis; E: mRNA expression of S100A12 in rat tissues; F: Protein expression of S100A12 in rat tissues; G: mRNA expression of S100A12 in rat cells; H: Protein expression of S100A12 in rat cells. Data among multiple groups were analyzed by one way ANOVA. Compared with the sham group, *P<0.05; compared with the CLP group, #P<0.05; compared with the control group, ^P<0.05; compared with the LPS group, %P<0.05. TCZ: Tocilizumab; CLP: Cecal Ligation and Puncture; LPS: Lipopolysaccharide.

group was significantly upregulated (**Figure 4E** and **4F**, P<0.05). Compared to the CLP group, the S100A12 level in the CLP + TCZ group was down-regulated (**Figure 4E** and **4F**, P<0.05). The same results were obtained in cell experiments (**Figure 4G** and **4H**, P<0.05). These experimental results confirmed that S100A12 was dysregulated in sepsis and that TCZ regulated S100A12, so TCZ may be involved in the progression of sepsis by regulating S100A12.

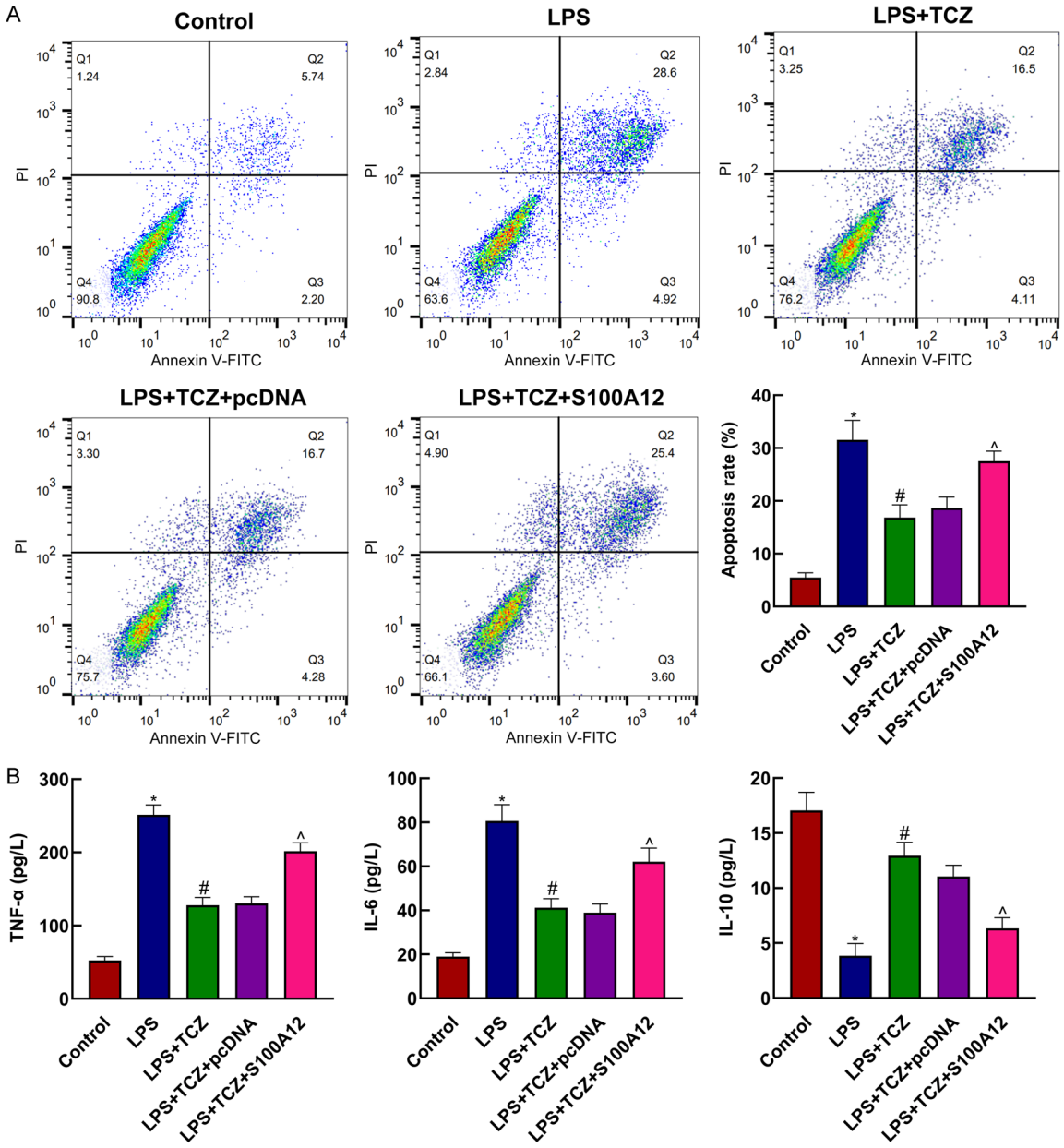

Figure 5. TCZ affected cell apoptosis and inflammatory response in sepsis by regulating S100A12. A: Cell apoptosis detected by flow cytometry; B: Expression levels of IL-6, TNF-α, and IL-10 in rat lung tissues measured with ELISA. Data among multiple groups were analyzed by one way ANOVA. Compared with the control group, *P<0.05; compared with the LPS group, #P<0.05; compared with the LPS + TCZ + pcDNA group, ^P<0.05. TCZ: Tocilizumab; CLP: Cecal Ligation and Puncture; LPS: Lipopolysaccharide; TNF-α: Tumor Necrosis Factor-α; ELISA: Enzyme-Linked Immunosorbent Assay.

TCZ affected cell apoptosis and inflammatory response in sepsis by regulating S100A12

In order to investigate whether TCZ affected the progression of sepsis through S100A12, S100A12 was overexpressed in a cell model of sepsis, and cell apoptosis and expression levels of inflammatory factors were detected. The results manifested that compared to the control group, the LPS group had increased cell apoptosis, IL-6 and TNF-α levels, but decreased IL-10 level (all P<0.05). Compared to the LPS group, the LPS + TCZ group had a decrease in apoptosis, IL-6 and TNF-α levels, and an increase in IL-10 level (all P<0.05). Compared to the LPS + TCZ + pcDNA group, the apoptosis, IL-6, and TNF-α levels were increased, and IL-10 level was decreased in the LPS + TCZ + S100A12 group (**Figure 5A** and **5B**, all P<0.05). Findings confirmed that TCZ affected cell apop-

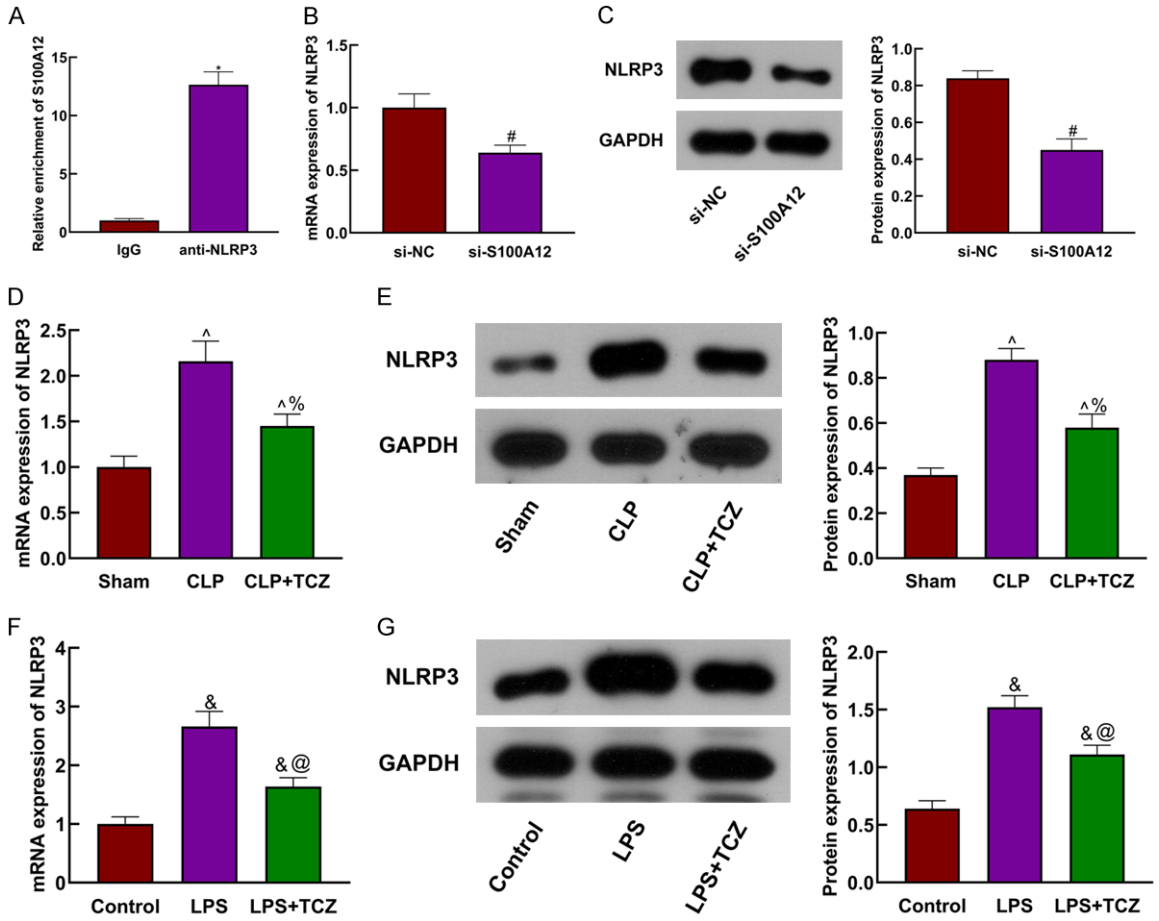

Figure 6. TCZ regulated the S100A12/NLRP3 axis. (A) Binding between S100A12 and NLRP3 analyzed by the RIP assay; (B) NLRP3 mRNA expression after knockdown of S100A12 detected with qRT-PCR; (C) NLRP3 protein expression after knockdown of S100A12 detected with western blot; (D-F) NLRP3 mRNA expression in rat tissues and cells detected with qRT-PCR; (E-G) NLRP3 protein expression in rat tissues and cells detected with western blot. Independent samples t-test was applied to assess the differences between two groups (A-C). Data among multiple groups were analyzed by one way ANOVA (D-G). Compared with the IgG group, *P<0.05; compared with the si-NC group $^{\#}$P<0.05; compared with the sham group, $^{\wedge}$P<0.05; compared with the CLP group, $^{\%}$P<0.05; compared with the control group, $^{\&}$P<0.05; compared with the LPS group, $^{@}$P<0.05. TCZ: Tocilizumab; CLP: Cecal Ligation and Puncture; LPS: Lipopolysaccharide; NLRP3: NOD-Like Receptor Thermal Protein Domain Associated Protein 3.

tosis and inflammatory response in sepsis by regulating S100A12.

S100A12 bonded to NLRP3 and promoted NLRP3 expression

RIP protocol showed that NLRP3 antibody was significantly enriched in relation to S100A12 (**Figure 6A**, t=17.98, P<0.0001). Knockdown of S100A12 in cells was achieved with plasmid transfection. Compared to the si-NC group, the expressions of mRNA (t=4.98, P=0.0076) and protein (t=9.37, P=0.0007) levels of NLRP3 were reduced in the si-S100A12 group (**Figure 6B** and **6C**, both P<0.05). After detecting the expression of NLRP3 in sepsis tissue, we found that compared to the sham group, the CLP group had higher NLRP3 mRNA expression (**Figure 6D** and **6E**, P<0.05). The mRNA and protein expressions of NLRP3 were downregulated in the CLP + TCZ group when compared to those in the CLP group (**Figure 6D** and **6E**, both P<0.05). In the cells, the LPS group had upregulated mRNA and protein expressions of NLRP3 than the control group (**Figure 6F** and **6G**, both P<0.05). These experimental results suggested that NLRP3 expression was upregulated in sepsis, and that S100A12 was able to bind to NLRP3 and promote NLRP3 expression.

TCZ regulated the S100A12/NLRP3 axis to reduce inflammatory response and cell apoptosis

Rescue experiments were designed to demonstrate the involvement of the S100A12/NLRP3

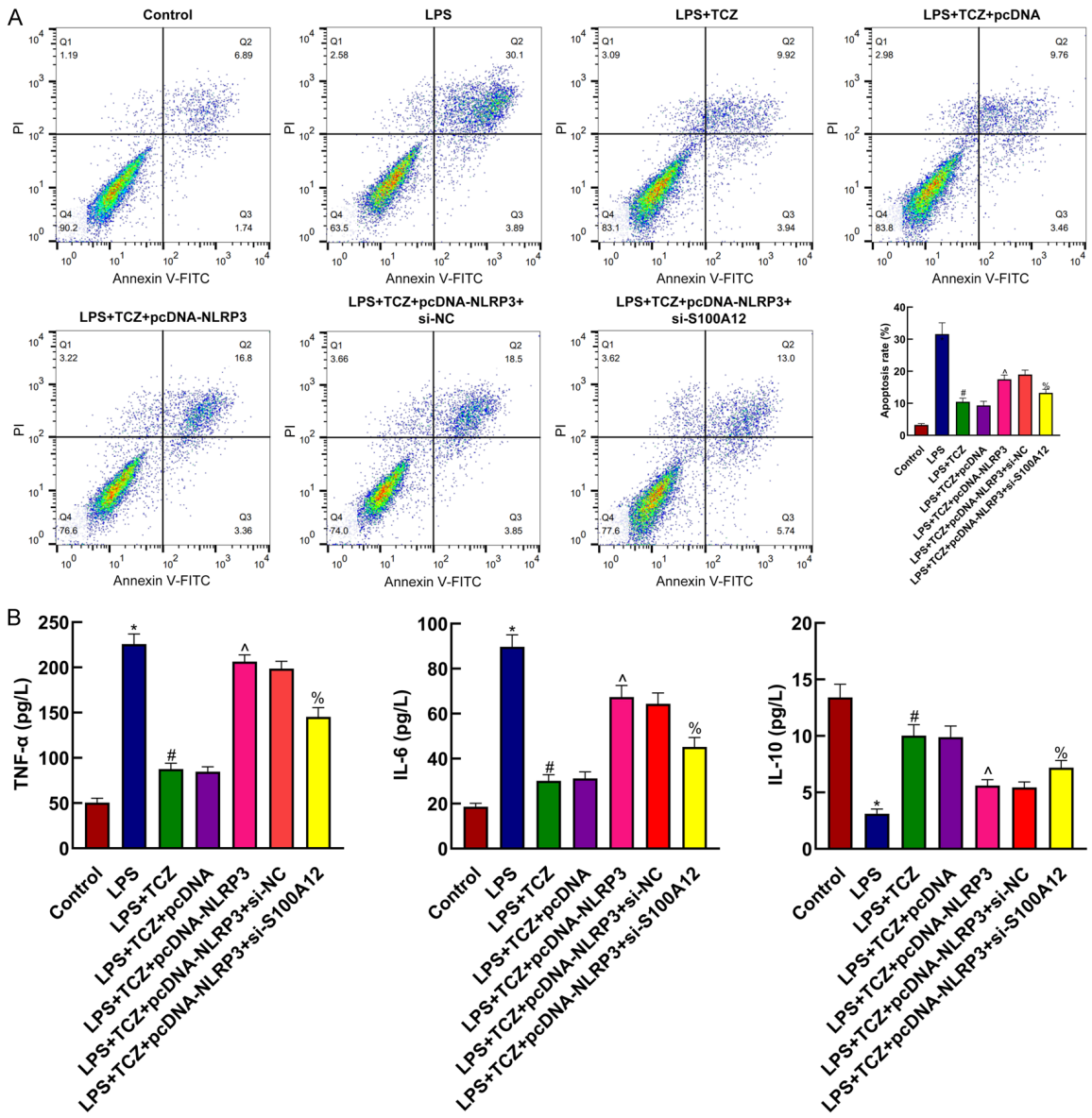

Figure 7. TCZ regulated the S100A12/NLRP3 axis to reduce the inflammatory response and cell apoptosis. A: Cell apoptosis detected by flow cytometry; B: Expression levels of IL-6, TNF-α, and IL-10 in rat lung tissues measured with ELISA. Data among multiple groups were analyzed by one way ANOVA. Compared with the control group, *P<0.05; compared with the LPS group, #P<0.05; compared with the LPS + TCZ + pcDNA group, ^P<0.05; compared with the LPS + TCZ + pcDNA-NLRP3 + si-NC group, %P<0.05. TCZ: Tocilizumab; CLP: Cecal Ligation and Puncture; LPS: Lipopolysaccharide; TNF-α: Tumor Necrosis Factor-α; NLRP3: NOD-Like Receptor Thermal Protein Domain Associated Protein 3; ELISA: Enzyme-Linked Immunosorbent Assay.

molecular axis in TCZ treatment. The cell apoptosis in LPS-induced cells was reduced after TCZ treatment. The apoptosis was increased after transfection with NLRP3 overexpression plasmid. Knockdown plasmid for S100A12 was transfected in cells, and the apoptosis was reduced compared to before (**Figure 7A**, P<0.05). From the results by ELISA, IL-6 and TNF-α levels were reduced and IL-10 level was increased in TCZ-treated cells. After transfection in cells with NLRP3 expression, the effect of TCZ was weakened. After transfection of pcDNA-S100A12, IL-6 and TNF-α secretions were weakened and IL-10 secretion was enhanced compared to the TCZ-treated and NLRP3-overexpressing cells (**Figure 7B**, all P<0.05). The above outcomes revealed that TCZ regulated the S100A12/NLRP3 axis to reduce the inflammatory response and cell apoptosis.

Discussion

This study focused on the specific mechanism of the IL-6 receptor blocker TCZ in the treatment of lung injury induced by sepsis through the S100A12/NLRP3 axis. The cecal ligation and puncture (CLP) method, which is well known for modeling sepsis, has been widely used in animals for sepsis research [20]. Our study found that lung tissue damage was improved significantly after TCZ treatment in a rat model of sepsis. When the body is stimulated, there is an imbalance between oxidation and antioxidants. A large neutrophil inflammatory infiltrate occurs. This phenomenon is called oxidative stress, which has a negative impact on the body. Low SOD level causes severe oxidative stress, but TCZ increases SOD activity and decreases the MDA level in rats with sepsis. It has been reported that a decrease in SOD activity leads to severe oxidative stress [21]. Besides, it has been confirmed that TCZ significantly reverses reduced SOD activity and increased the MDA level induced by fructose [22]. This would be consistent with the findings in the current study.

TCZ was initially used in the treatment of rheumatoid arthritis. Studies have shown that in autoimmune diseases such as rheumatoid arthritis, IL-6 improves the activation of Th17 cells, which promotes the release of TNF and IL-6, forming an inflammatory storm [23]. Sepsis is a systemic inflammatory response syndrome caused by infection, and the inflammatory response plays an important role in the progression of its course. TCZ has been shown to inhibit diseases manifested as inflammatory storms, such as systemic inflammatory response syndrome and macrophage activation syndrome, and inhibits excessive activation of cytokines [24]. TCZ has been confirmed to inhibit the expression of IL-6 in lung and kidney tissues of rats with septic lung and kidney injuries [14]. In addition, TCZ promotes the expression of anti-inflammatory factor IL-10 and inhibits the expression of TNF-α and IL-1β, which can improve the prognosis of mice with sepsis and reduce multi-organ damage [25]. It has been revealed that Shionone inhibits inflammatory factors such as IL-6, TNF-α, and IL-1β, promotes IL-10 levels, and regulates macrophage polarization to improve lung damage caused by sepsis [26]. In this study, TCZ was used to intervene in CLP model rats, and the expression of inflammatory factors in rat lung tissue was detected. Compared to the sham group, the expression of inflammatory factors (IL-6, TNF-α) in the lung tissue of CLP rats was enhanced, and the anti-inflammatory factor IL-10 was inhibited. The above situation was reversed after TCZ was added, suggesting that TCZ can inhibit the inflammatory response of septic rats, and this effect may be achieved by destroying the function of the IL-6 signaling pathway conduction and inhibiting a variety of cytokines.

In this study, the therapeutic effect of TCZ was further analyzed at a cellular level by constructing a cell damage model using LPS-induced MPVECs, which has been used in several studies on sepsis [27]. We found that LPS induced a cellular inflammatory response, but TCZ could attenuate the LPS-induced inflammatory response and reduced cell apoptosis. The role of S100A12 was found from the bioinformatic analysis of sepsis. Wu et al. pointed out that compared to healthy people, patients with sepsis had significantly increased S100A12 levels, and non-survivors had higher S100A12 levels, which suggested that the S100A12 level might influence the development of sepsis [28]. In this study, it was verified that the expression of S100A12 was elevated in rat models of sepsis and LPS-induced pulmonary microvascular endothelial cells (MPVECs). In addition, S100A12 has previously been shown to activate NLRP3 inflammasomes and accelerate the progression of asthma and chronic obstructive pulmonary disease [18]. The mechanism by which S100A12 is involved in sepsis progression by regulating NLRP3 has also been partially revealed [19]. Liu et al. found that the inhibition of the macrophage NLRP3/caspase-1 signaling cascade reduced liver damage in mice with sepsis [29]. Another study demonstrated that symptoms could be improved by inhibiting the activation of NLRP3 inflammasomes by HSF1 in sepsis-induced acute lung injury [30]. In this study, S100A12 promoted the expression of NLRP3. The levels of S100A12 and NLRP3 were increased in CLP animal models and LPS cell models. The expressions of both were decreased after TCZ treatment, which showed that TCZ downregulated S100A12/NLRP3 in a targeted manner. In the rescue experiment, we found that after increas-

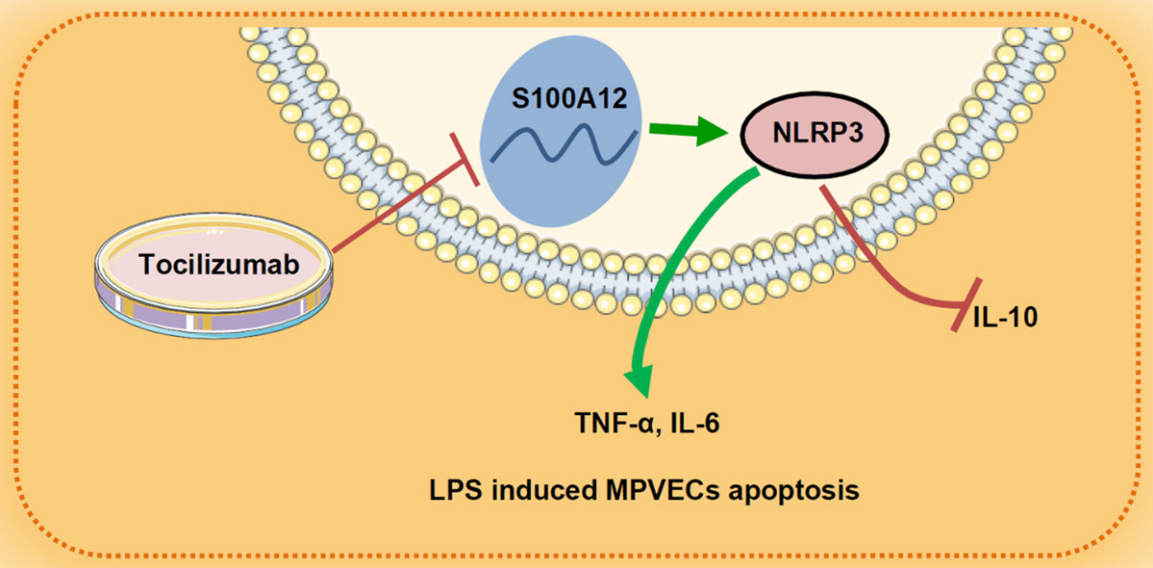

Figure 8. Tocilizumab affects the mechanism of lung injury in rats with sepsis by regulating S100A12/NLRP3. LPS: Lipopolysaccharide; NLRP3: NOD-Like Receptor Thermal Protein Domain Associated Protein 3; MPVECs: Primary Rat Pulmonary Microvascular Endothelial Cells; TNF-α: Tumor Necrosis Factor-α.

ing the expression of NLRP, the anti-inflammatory and anti-apoptosis effects of TCZ were partially eliminated. After inhibition of S100A12, the resistance of NLRP3 to TCZ was eliminated, which further confirmed that S100A12 regulated NLRP3.

However, there are some limitations in this study. There was no further exploration of the mechanism by which TCZ affects S100A12 expression, and no clinical samples were collected for experiments. Therefore, whether TCZ can treat acute lung injury in patients with sepsis by the S100A12/NLRP3 pathway still needs to be verified by a large clinical trial.

In summary, TCZ has an effect on improving acute lung injury in sepsis. This study innovatively confirms that TCZ plays a role by reducing the S100A12/NLRP3 axis, providing evidence for targeted molecular therapy for TCZ in the treatment of acute lung injury in sepsis. The mechanism of this study is shown in **Figure 8**.

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Disclosure of conflict of interest

None.

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