Effects of S100A12 gene silencing on serum levels of anti-inflammatory/pro-inflammatory cytokines in septic rats through the ERK signaling pathway

Xin Wen | Xin-Rui Han | Yong-Jian Wang | Shao-Hua Fan | Zi-Feng Zhang | Dong-Mei Wu | Jun Lu | Yuan-Lin Zheng

Key Laboratory for Biotechnology on Medicinal Plants of Jiangsu Province, School of Life Science, Jiangsu Normal University, Xuzhou, P.R. China

Correspondence
Dr Dong-Mei Wu, Dr Jun Lu, and Dr Yuan-Lin Zheng, Key Laboratory for Biotechnology on Medicinal Plants of Jiangsu Province, School of Life Science, Jiangsu Normal University, No. 101, Shanghai Road, Tongshan District, Xuzhou 221116, Jiangsu Province, P.R. China. Email: wdm8610@jsnu.edu.cn (D.-M.W.); lu-jun75@163.com (J.L.); ylzeng@jsnu.edu.cn (Y.-L.Z.)

Funding information
The Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD); The Cultivate National Science Fund for Distinguished Young Scholars of Jiangsu Normal University; The Major Fundamental Research Program of the Natural Science Foundation of the Jiangsu Higher Education Institutions of China, Grant number: 13JIA180001; The National Natural Science Foundation of China, Grant numbers: 81571055, 81409092, 81271225, 31201039, 81171012, 30950031; The 2013 “Qinglan Project” of the Young and Middle-aged Academic Leader of Jiangsu College and University; The 2016 “333 Project” Award of Jiangsu Province

Abstract
We explored the effect of S100A12 gene on serum levels of anti-inflammatory/pro-inflammatory cytokines in septic rats by activating the extracellular signal-regulated kinase (ERK) signaling pathway. A total of 180 specific pathogen-free (SPF) rats were purchased to establish cecal ligation and puncture (CLP) model. Rats were assigned into the sham, model, empty vector, S100A12 siRNA, epidermal growth factor (EGF), and S100A12 siRNA + EGF groups. The expressions of S100A12, ERK-1, ERK-2, cPLA2, and NF-κB in liver tissues of rats among six groups were detected by RT-qPCR and western blotting. ELISA was used to determine serum levels of IL-1β, IL-10, TNF-α, procalcitonin (PCT), and C-reactive protein (CRP). Pearson correlation analysis was conducted to measure correlations. Cell apoptosis of rats among six groups was detected by Tunel staining. The expressions of S100A12, ERK-1, ERK-2, cPLA2, and NF-κB decreased in the S100A12 siRNA group while increased in the EGF group compared with the model group. S100A12 mRNA expression was positively correlated with mRNA expressions of related genes in the ERK signaling pathway (ERK-1, ERK-2, cPLA2, and NF-κB) in the model and empty vector groups. Expressions of IL-1β, IL-6, TNF-α, PCT, and CRP in the EGF group were higher than those in the model group, but were lower than those in the S100A12 siRNA group. Compared with the model group, cell apoptosis decreased in the S100A12 siRNA group but that increased in the EGF group. We demonstrates that S100A12 gene silencing decreases serum levels of anti-inflammatory/pro-inflammatory cytokines in septic rats by inhibiting the ERK signaling pathway.

KEYWORDS
anti-inflammatory cytokines, ERK signaling pathway, gene silencing, pro-inflammatory cytokines, sepsis, S100A12

1 | INTRODUCTION

Sepsis is thought to be a medical condition combining with more than two characteristics of systemic inflammatory response syndrome (SIRS).1 Sepsis belongs to a serious
bloodstream infection arising from many infections which could rapidly be life-threatening. Sepsis is a kind of fatal immunological disease lack of understanding of pathophysiology, and the inflammatory imbalance is a main contributor, which is a huge burden on healthcare systems and society. In spite of fast advances have been made, sepsis is still the second leading reason for death in intensive care units (ICUs) of non-coronary heart disease, and the tenth contributor to death in high-income countries over the world. The pathophysiology of sepsis is still poor to understand; therefore, only a few specific treatments can be applied to cure sepsis. Sepsis can also cause septic shock, multiple organ dysfunction syndrome and death, and contribute to activating inflammatory and procoagulant pathways. Previous study proved that the pro-inflammatory cytokines during the process of innate immune response is one of the most widely-explored aspects of the pathophysiology of sepsis, and S100A12 participate in the innate immune response associated with some autoimmune responses. Therefore, it is important to explore the effect of S100A12 gene on related pro-inflammatory and anti-inflammatory cytokines in sepsis.

Currently, S100 proteins consist of a family of more than 21 low-molecular-weight acidic proteins, which are featured by presenting two calcium-binding EF-hand motifs and displaying unique properties. The S100A12, also named calcium-binding protein in amniotic fluid-1 (CAAF1s), calgranulin C or extracellular newly identified RAGE-binding protein (EN-RAGE), has pro-inflammatory qualities and is associated with various diseases including type 2 diabetes. As a mediator, S100A12 with pro-inflammatory and anti-inflammatory cytokines could be upregulated in inflammation diseases and some tumors. Besides, a number of pro-inflammatory and anti-inflammatory cytokines in human sepsis may have diagnostic values or have connections with death, suggesting that S100A12 may be essential for sepsis development. In addition, previous studies have illustrated that high S100A12 expression and increased serum levels associate with patients suffering inflammatory diseases. Moreover, some studies verified that both pro-inflammatory and anti-inflammatory cytokines in cells could be inhibited via depressing the extracellular signal-regulated kinase (ERK) signaling pathway. Meanwhile, S100A12 is proved to suppress pro-inflammatory and anti-inflammatory cytokines, indicating that S100A12 could regulate pro- and anti-inflammatory cytokines by activating the ERK signaling pathway. The above content illustrate that S100A12 gene could regulate pro-inflammatory and anti-inflammatory cytokines by activating the ERK signaling pathway, and cytokines are essential to sepsis. Therefore, the purpose of our study is to explore the role of S100A12 gene in regulating pro-inflammatory and anti-inflammatory cytokines in septic rats via activating the ERK signaling pathway.

2 | MATERIALS AND METHODS

2.1 | Ethical statement

All procedures were conducted strictly in conformity with the international guidelines and principles for Animal ethics committee. Usage of animals for experiment was strictly followed by the Care and Use of Laboratory Animals of the National Institutes of Health.

2.2 | Experimental animal and establishment of cecal ligation and puncture (CLP) model

A total of 180 specific pathogen-free (SPF) rats weighing 200-220 g were purchased by Animal Center of Hunan University of Chinese Medicine (Changsha, Hunan, China) and cultured in clean animal rooms in Hunan Normal University (Changsha, Hunan, China). Feeding conditions were as follows: room temperature of 21°C, light and dark (12:12 h) cultivation condition, animals with food and drinking freely. There were 150 rats randomly selected for preoperative fasting 12 h, and rats were conducted intraperitoneal anesthesia using 2% pentobarbital sodium (P3761, Sigma-Aldrich Co. LLC. St. Louis, MO). The establishment procedures of CLP model were as follows: Abdomen was conducted conventional disinfection and then cut about 2 cm. Then caecum was found in abdominal cavity to separate mesentery between distal cecum and large intestine. Half of distal cecum was conducted ligation by a 4-silk, the middle of which was punctured using an 18-needle. Then caecum was placed back to the original position of the abdominal cavity with abdominal suture. The rest 30 rats were as the sham group with cutting abdomen out and turning intestinal tract, but without caecum ligation and puncture, and the rest procedures were the same as the establishment process of the CLP model. After CLP surgery, blood culture of positive intestinal microorganisms was detected by 6 h intestinal tract and clinical signs of sepsis took place showing the successful model establishment.

2.3 | Construction of S100A12 RNAi lentiviral vector

S100A12 shRNA double strands of rats were purchased from Agilent Technologies (Palo Alto, CA). The fragment TTCAAGAGA was applied for loop structure. T was added into the 5′ sense strand model, and sticky-end was complemented after Hpa I enzyme fragment; AGCT was added into the 5′ antisense strand model, and sticky-end was complemented and constructed after Xho I enzyme fragment. Then S100A12 shRNA annealing was subsequently carried out. Annealing reaction system was as follows: 5 µl 10 × shDNA Annealing Buffer; 5 µl sense strand (100 µM); 5 µl antisense strand (100 µM); 35 µl ddH₂O. Annealing conditions were: 95°C for 5 min; 85°C for 5 min; 75°C for 5 min; 70°C for 5 min, and finally stored at 4°C. With GP-Supersilencing Vector linearized, the enzyme fragment
reaction system was as follows: 10 µl 10× Buffer R; 5 µl Xho I; 2 µl Hpa I; 10 µg GP-Supersilencing Vector; adding ddH2O until 100 µl. After reacted for 1 h, linearized GP-Supersilencing Vector was linked with the above shDNA, and connection system was as follows: 2 µl 10× T4 Ligation Buffer; 1 µl gpMDlg-pRRE, 7.5 µg gpMDlg-pRRE, 15 µg S100A12 siRNA lentiviral expression vector I.V. in caecum), epidermal growth factor (EGF) (CLP model rats with 1 mL EGF I.V. in caecum), S100A12 siRNA (CLP model rats with 1 mL S100A12 siRNA lentiviral expression vector I.V. in caecum), epidermal growth factor (EGF) (CLP model rats with 1 mL EGF I.V. in caecum), S100A12 siRNA + EGF (CLP model rats with 1 mL S100A12 siRNA lentiviral expression vector and 1 mL EGF I.V. in caecum) groups. Then CLP model was established, and physiological activity and survival rate of 20 rats in each group between 0 and 96 h were observed and counted after surgery, and then 2% pentobarbital sodium anesthesia was used to inject abdominal cavity of 10 rats which were randomly selected after 24 h. Blood samples of heart were collected, serum with liver tissues were selected after injection.

2.4 Animal grouping

Rats were grouped into six groups, including the sham (without injection), model (CLP model rats with 1 mL normal saline intravenous injection (I.V.) in caecum), empty vector (CLP model rats with 1 mL empty vector I.V. in caecum), S100A12 siRNA (CLP model rats with 1 mL S100A12 siRNA lentiviral expression vector I.V. in caecum), epidermal growth factor (EGF) (CLP model rats with 1 mL EGF I.V. in caecum), S100A12 siRNA + EGF (CLP model rats with 1 mL S100A12 siRNA lentiviral expression vector and 1 mL EGF I.V. in caecum) groups. Then CLP model was established, and physiological activity and survival rate of 20 rats in each group between 0 and 96 h were observed and counted after surgery, and then 2% pentobarbital sodium anesthesia was used to inject abdominal cavity of 10 rats which were randomly selected after 24 h. Blood samples of heart were collected, serum with liver tissues were selected after injection.

2.5 Detection of white blood counts (WBC) and limulus amebocyte lysate (LAL)

After anesthesia, 2 mL blood of rat hearts were injected into sterile vacuum tube, and Automatic Blood Cell Analyzer (UniCel DxH 800, Beckman Coulter, Brea, CA) was applied to measure the number of WBC. The sera of rats were centrifuged at 3500g for 20 min, and LAL kit (50-650U, Chongqing Huaya Stem Cell Biotechnology Co., Ltd., Sichuan, China) was applied to detect lipopolysaccharide (LPS) concentration. The concentration of nitro-aniline was detected at 405 nm to determine LPS concentration. Then linear-regression analysis of WinKQCLTM Software was used to calculate results.

2.6 Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Liver tissues of rats in each group were selected and homogenated, and total RNA was extracted by Trizol kit (RP2401, BioTeke Corporation, Beijing, China). Then ultraviolet spectrophotometry and agarose gel electrophoresis were applied to detect RNA concentration, pureness and identify RNA integrity. Mixture system A of RNA and Oligo (dT) primers: 2 µl total RNA, 2 µl Oligo (dT), and 12 µl diethyl pyrocarbonate (DEPC) water. Reverse transcription system B: 5 µl 5× PCR buffer, 1.25 µl 10 Mm deoxynucleoside triphosphate (dNTP), 1.75 µl DEPC water, 1 µl RNase Inhibitor, and 1 µl M-MLV Reverse Transcriptase. Systems of A and B were mixed, and placed in ordinary polymerase chain reaction (PCR) instrument (37°C for 1 h, 95°C for 5 min, 4°C for forever) to get reverse transcribed cDNA (KR104, Tiangen Biotech Co., Ltd., Beijing, China). Then RT-qPCR (PH-K-100, Wuxi Puhe Biomedical Technology Co., Ltd., Jiangsu, China) was conducted, and PCR reaction system was 5 µl SYBR GEFN Mix, 1 µl Forward primer, 1 µl Reverse primer, 6 µl cDNA, 10 µl RNase Free dH2O (Table 1). PCR amplification conditions were 1 = 95°C for 30 s, 2 = 95°C for 5 s, 3 = 58°C for 5 s, 4 = go to 2 for 30 cycles, 5 = 65 − 95°C for 5 s. RT-qPCR primers were synthesized by Beijing Genomics Institute (BGI, Beijing, China). Light Cyler 480 (0501527800, Roche Ltd., Shanghai, China) software was applied to carry out quantitative analysis. Ct value in each model had a linear relationship with the logarithm of initial copy number of the model, showing the more copy numbers had smaller Ct values. Then Ct value of target gene was calculated, and threshold value method was used to compare with Ct value of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with the same sample. mRNA expression of target gene was calculated by the formula of ΔCt (target gene) = Ct (target gene) − Ct (GAPDH).

2.7 Western blotting

The conserved liver tissues of rats in each group were fully grinded in liquid nitrogen, and lysed with protein lysate. After separation and purification, the liver tissues were separated by electrophoresis and underwent transmembrane, sealed with 5% skimmed milk and kept overnight at 4°C. The first antibodies of S100A12 (NO. sc-101347, anti-mouse, Santa Cruz Island, CA, at 1: 1000 dilution), ERK1 + ERK2 (NO. ab54230, anti-mouse, Abcam plc., Cambridge, UK, without dilution), nuclear factor-kB (NF-kB) (NO. ab16502, anti-rabbit, Abcam plc., without dilution), cytosolic form of phospholipase A2 (cPLA2) (NO. ab16502, anti-rabbit, Abcam plc., at 1: 1000 dilution), GAPDH (NO. ab8245, anti-mouse, Abcam plc., at 1: 1000 dilution) were added with TBST washing membrane (for three times each for 5 min), and the horseradish peroxidase secondary antibodies of Goat Anti-Mouse IgG(NO. ab6789, Abcam plc., at 1: 2000 dilution) and Goat Anti-Rabbit IgG(NO. ab205718, Abcam plc., at 1: 2000 dilution) were added for 1 h culture at 37°C with TBST washing membrane (for three times each for 5 min). Then 3-amino-9-ethylcarbozole (AEC) solution was added for developing, and Image J was applied for band gray analysis.
TABLE 1 The primers sequence for reverse transcription quantitative polymerase chain reaction (RT-qPCR)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S100A12</td>
<td>GGTGGTCACTAGTGCACCCCTCCGTGATG CACCTTGTGGGTTGAGG</td>
</tr>
<tr>
<td>ERK1</td>
<td>GACCCGATGTTAAAAACCTTTA TGGTTGCTGATG</td>
</tr>
<tr>
<td>ERK2</td>
<td>CATCCCTGGATACCTTTGA AGTCAGGTTGGGAACAC</td>
</tr>
<tr>
<td>NF-xB</td>
<td>TCCCCCAGAGACCTCAAGT TGGAATTTCTGATGCCG</td>
</tr>
<tr>
<td>cPLA2</td>
<td>GCAACCCAGAACAAGGGGGAACC GAGACACCTGGACTAATCGAGAC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GCATTGTGGAAGGCTCA AAGGTGGAAGAGTGGGAGT</td>
</tr>
</tbody>
</table>

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; ERK, extracellular signal-regulated kinase; NF-xB, nuclear factor-xB; cPLA2, cytosolic form of phospholipase A2.

2.8 | Enzyme-linked immunosorbent assay (ELISA)

Serums of rats in each group, ELISA kit (LDEL, Shanghai Lengton Bioscience Co., Ltd., Shanghai, China) and all reagents were placed at room temperature for 30 min. Then 30 × Wash buffer was diluted 30 times by tri-distilled water, and standard pipe was prepared to calculate well and band counts. In assay plate, each was added 50 µl standards or serum samples for 2 h at 37°C incubation. Next, buffer solution was applied to wash for four times with filter paper blotting up. And 100 µl biotin labeled antibodies were added into each well for 1 h of incubation, and buffer solution was applied to wash for four times with filter paper blotting up. Then 100 µl/well enzyme conjugate fluid was put in an incubator at 37°C for 30 min, and buffer solution was used to wash for four times with filter paper blotting up. Each well was added 100 µl tetramethyl benzidine (TMB) for 30 min dark placing; then 100 µl stop buffer was put into each well. Optical density (OD) values were determined by Microplate Reader (MK3, Thermo Fisher Scientific, Sunnyvale, CA, USA) at 450 nm, and Excel was used to draw standard curve and detect concentrations of IL-6 (NO. ab9970, anti-goat, Thermo Fisher Scientific), IL-1β (NO. ab9787, anti-rabbit, Thermo Fisher Scientific, Sunnyvale, CA, USA), TNF-α (NO. ab9755, anti-rabbit, Procalcitonin (PCT) (NO. BXC003, anti-mouse, Beijing KeyGen Gene Technology CO., Ltd., Beijing, China) and C-reactive protein (CRP) (NO. ab46820, anti-chicken, Abcam plc.).

2.9 | Flow cytometry

A 100 µl anticoagulant whole blood in each group and 20 µl fluorescein isothiocyanate (FITC) (NO. 786-059, Sangon Biotech Co., Ltd., Shanghai, China) labeled antibody of CD64 were selected for mixture. They were incubated in dark for 15 min adding 1 mL hemolysin. After incubation in dark for 12 min, they were of centrifugation at 1200 r/min for 5 min with removing liquid supernatant and adding 300 µl phosphate buffer saline (PBS). Flow cytometry (Fascan Amrican B.D Company) was used to count 10,000 cells of blood samples in each group, and the software analysis Cellquest (Becton Dickinson, Franklin Lakes, NJ) was applied to detect fluorescence intensity using 480 nm excitation waves with the length of 525 nm. The ratio of neutrophil CD64 (nCD64) fluorescence intensity and lymphocytes was the nCD64 index, and then apoptosis rate of nCD64 cell was calculated.

2.10 | Hematoxylin-eosin (HE) staining

Frozen rat livers of each group were selected and cut into sections, which were put into the Helly fixation Fluid (BTN131224, Beijing bioco Laibo Technology Co. Ltd., Beijing, China). Many sections were in each group and fixed 24 h with water flushing 30 min. Automatic dehydration machine was used for conventional dehydration and transparency of fixed tissues. Then paraffin embedding was carried out by LEICA EG1160 (Leica, Wetzlar, Germany) automatic embedding machine with section. Hematoxylin staining was conducted for 15 min, and then solution separation between hydrochloric acid and alcohol was for 35 s, and eosin staining was for 10 min, and 90% ethanol separation was for 40 s. The neutral balsam was applied for sealing.

2.11 | Knodell index of inflammatory activity and Chevallier semi quantitative of fibrosis

HE staining sections of rat liver tissues were observed, and Knodell scoring project was used to conduct inflammatory activity scoring. Fibrosis degree scoring of liver tissues was carried out by the project of Chevallier semi quantitative of fibrosis.

2.12 | Tunel staining

The prepared sections were for conventional dewaxing, and the following procedures were as follow: PBS rinsing was conducted three times (each for 5 min) with 50 µl 3 mL/L hydrogen peroxide.
methanol incubation for 30 min. Then PBS rinsing was carried out 3 times (each for 5 min) adding 50 µl 1 g/L Triton X-100 liquor sodii citratis with 4°C transparency for 3 min. And PBS rinsing was done for three times (each for 5 min) adding 50 µl Tunel reaction liquid with 37°C incubation for 1 h in a wet box. Next, PBS rinsing was performed for three times (each for 5 min) adding 50 µl Converter-POD with 37°C incubation for 1 h in a wet box. And PBS rinsing was conducted three times (each for 5 min) adding 50 µl DAB substrate chromogenic solution with incubation for 10 min at room temperature. Then double distilled water was used to wash for 10 min with natural air drying, and neutral balsam was applied for sealing. Subsequently, the light microscope was used for observation, counting and photographing, cell apoptotic index was calculated (the percentage of Tunel positive cells in per 100 cells).

2.13 | Statistical analysis

The SPSS 21.0 statistical software (IBM Corp. Armonk, NY) was applied to analyze statistics and measurement data were presented using the mean ± standard deviation (SD). All experiments were repeated more than three times. The comparisons among multiple groups were conducted by the one-way analysis of variance (ANOVA), and the comparisons in groups were analyzed by t test. Pearson correlation analysis was applied for correlation analysis. A value of $P < 0.05$ indicated that the difference was statistically significant, and a value of $P < 0.01$ showed that the statistics were of highly significant difference.

3 | RESULTS

3.1 | The survival rates of rats after surgery in each group

The results of survival rate of rats after surgery in each group between 0 and 96 h (Figure 1) demonstrated that in the model group, rats after surgery ate nothing in a state of drowsiness. Rats between 6 and 12 h seldom did activities with drooping spirits. Rats from 12 h started to die, and there was rat death in each period. The survival rates in the model, empty vector and S100A12 siRNA + EGF groups showed the same tendency. At 96 h after surgery, survival rates in the empty vector and S100A12 siRNA + EGF groups had no significant difference compared with the model group ($P > 0.05$). However, the survival rates in the S100A12 siRNA and EGF groups were 50% and 0%, respectively, which was obvious difference with the model group (20%) ($P < 0.05$).

3.2 | WBC, LPS, and nCD64 expression of rats in each group

The results illustrated that WBC, LPS, and nCD64 expressions of rats in the model group increased compared with the sham group (all $P < 0.05$). There was no obvious difference of WBC, LPS, and nCD64 among the model, empty vector and S100A12 siRNA + EGF groups (all $P > 0.05$). In comparison with the model group, three indexes in the S100A12 siRNA group were downregulated significantly, but those in the EGF group were upregulated (all $P < 0.05$) (Figure 2).

3.3 | mRNA expressions of S100A12, ERK-1, ERK-2, cPLA2, and NF-κB in liver tissues of rats among six groups by RT-qPCR

Figure 3 verified that mRNA expressions of S100A12, ERK-1, ERK-2, cPLA2, and NF-κB in liver tissues of other groups increased compared with the sham group (all $P < 0.05$). The model and empty vector groups had no significant difference of gene expressions (both $P > 0.05$). The S100A12 siRNA + EGF and S100A12 siRNA groups had lower S100A12 mRNA expressions than the model group (all $P < 0.05$), but no obvious difference was found in the EGF group ($P > 0.05$). There was no significant difference of mRNA expressions of ERK-1, ERK-2, cPLA2, and NF-κB between the S100A12 siRNA + EGF and model groups (both $P > 0.05$). The mRNA expressions of ERK-1, ERK-2, cPLA2, and NF-κB decreased in the S100A12 siRNA group, but those increased in the EGF group (all $P < 0.05$).

3.4 | The protein expressions of S100A12, ERK-1, ERK-2, cPLA2, and NF-κB in liver tissues of rats among six groups by Western blotting

Figure 4 illustrated that protein expressions of S100A12, ERK-1, ERK-2, cPLA2, and NF-κB in other groups were higher than those in the sham group (all $P < 0.05$). The protein expressions of those genes did not differ obviously between the model and
empty vector groups (all $P > 0.05$). In comparison with the model group, the S100A12 siRNA + EGF and S100A12 siRNA groups had lower S100A12 protein expressions (both $P < 0.05$), in which that in the EGF group showed no significant difference ($P > 0.05$). The S100A12 siRNA + EGF and model groups were of no significant difference in protein expressions of ERK-1, ERK-2, cPLA2, and NF-$\kappa$B (both $P > 0.05$). Protein expressions of ERK-1, ERK-2, cPLA2, and NF-$\kappa$B in the S100A12 siRNA group were downregulated, but those in the EGF group were upregulated (all $P < 0.05$).

3.5 Correlations between S100A12 mRNA expression and related genes of ERK signaling pathway (ERK-1, ERK-2, cPLA2, and NF-$\kappa$B) in liver tissues of rats in each group

As shown in Figure 5, S100A12 mRNA expression was positively correlated with mRNA expressions of related genes of ERK signaling pathway (ERK-1, ERK-2, cPLA2, and NF-$\kappa$B) in the model group, suggesting that S100A12 expression could activate the ERK signaling pathway. Figure 5E verified that changes of S100A12 mRNA expression in liver tissues were consistent with the protein expression.

3.6 Concentrations of IL-1$\beta$, IL-6, TNF-$\alpha$, PCT, and CRP of rats among six groups by ELISA

Concentrations of IL-1$\beta$, IL-6, TNF-$\alpha$, PCT, and CRP in other groups increased compared with the sham group (all $P < 0.05$). There was no significant difference of inflammatory indexes that above stated in model, empty vector and S100A12 siRNA + EGF groups (all $P > 0.05$). Expressions of above inflammatory indexes in the EGF group were higher than those in the model group (all $P < 0.05$), but were lower than those in the S100A12 siRNA group (all $P < 0.05$) (Figure 6).

3.7 Pathological injury changes in liver tissues of rats among six groups by HE staining

As shown in Figure 7, in the sham group, liver cell plates had clear structures, and arranged radially in a polygonal shape with the center of the central videos, and liver cells had normal morphological structures, normal hepatic sinusoid and sinusoidal, clear interlobular artery and vein of portal area, clear cholangiole structures without inflammatory cell infiltration. In comparison with the sham group, liver cells in other groups had unclear and disorganized acini hepatitis structures, obviously inspissated nucleolus, different degrees
**FIGURE 4** Detections of protein expressions of S100A12, ERK-1, ERK-2, cPLA2, and NF-κB in liver tissues of rats among six groups by western blotting. A, Protein bands of S100A12, ERK-1, ERK-2, cPLA2, and NF-κB in liver tissues of rats among six groups; B, Detections of protein expressions of S100A12, ERK-1, ERK-2, cPLA2, and NF-κB in liver tissues of rats among six groups; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; EGF, epidermal growth factor; *Compared with the sham group, P < 0.05; #Compared with the model group, P < 0.05; ERK, extracellular signal-regulated kinase; cPLA2, cytosolic form of phospholipase A2; NF-κB, nuclear factor-κB

**FIGURE 5** Correlations between S100A12 mRNA expression and related genes of ERK signaling pathway (ERK-1, ERK-2, cPLA2, and NF-κB) in liver tissues of rats. A, \( r = 0.7680, F = 14.38, P = 0.0035 \); (B) \( r = 0.6277, F = 6.501, P = 0.0289 \); (C) \( r = 0.7963, F = 17.33, P = 0.0019 \); (D) \( r = 0.7689, F = 14.46, P = 0.0035 \); E, \( r = 0.6930, F = 9.242, P = 0.0125 \); ERK, extracellular signal-regulated kinase; cPLA2, cytosolic form of phospholipase A2; NF-κB, nuclear factor-κB
of dyeing cytoplasm, smaller or disappeared sinus hepaticus, red blood cells in hepatic sinusoid, little liver cell cathepsis and necrosis, many neutrophil cells and mononuclear cell infiltration. Compared with the model group, pathological degree in the EFG group was more serious, and that in the S100A12 siRNA was less serious with no significant difference in the empty vector and S100A12 siRNA + EGF groups.

3.8 | Cell apoptosis of rats among six groups by tunel staining

Tunel staining results under microscope (400×) demonstrated that sedimentable particles in visible nucleus were brown, and non-apoptotic nucleus was blue after the color reaction (brown) between DNA fragmentation of apoptotic cells and stains. The apoptosis rates of each group were the sham (3.52 ± 0.99)% , model (48.25 ± 1.32)% , empty vector (49.54 ± 1.56)% , S100A12 siRNA + EGF (51.24 ± 2.10)% , S100A12 siRNA (32.50 ± 1.24)% , EGF (77.52 ± 4.25)% . The other groups had higher apoptosis rate than the sham group (all P < 0.05). No obvious difference of apoptosis rate was found in the model, empty vector and S100A12 siRNA + EGF groups (all P > 0.05). In comparison with the model group, cell apoptosis decreased in the S100A12 siRNA group but that increased in the EGF group (all P < 0.05) (Figure 8).

3.9 | Evaluation results of inflammatory activities and fibrosis degrees among six groups

Evaluation of liver pathological changes in sepsis proved that other groups showed different degrees of pathological changes compared with the sham group (all P < 0.05). Inflammatory activities and fibrosis degrees in the model, empty vector and S100A12 siRNA + EGF groups were of partial but unobvious difference, belonging to the second stage and grade in general (P > 0.05). Histopathology results in the S100A12 siRNA group were the first stage and grade, but pathological changes in the EGF group were obviously

**FIGURE 6** Concentrations of IL-1β, IL-6, TNF-α, PCT, and CRP among six groups by ELISA. A, Concentration of IL-1β, IL-6, and TNF-α; B, concentration of CRP; C, concentration of PCT; EGF, epidermal growth factor; *Compared with the sham group, P < 0.05; #Compared with the model group, P < 0.05; PCT, procalcitonin; CRP, C-reactive protein; IL, interleukin; TNF, tumor necrosis factor; ELISA, enzyme-linked immunosorbert assay
upregulated compared with the model group, belonging to the third stage and fourth grade in general (all \( P < 0.05 \)) (Table 2).

## 4 DISCUSSION

Sepsis is a serious bloodstream infection caused by many infections which is life-threatening and the second leading contributor for death in ICUs.\(^2\) S\(100A12\) expression with some pro-inflammatory and anti-inflammatory cytokines increased in inflammation diseases, and those cytokines in sepsis show diagnostic value and associations with death, indicating that \(S100A12\) is significant for sepsis occurrence.\(^9\) Therefore, the purpose of our study is to explore the \(S100A12\) effect on pro-inflammatory and anti-inflammatory cytokines in sepsis by way of activating the ERK signaling pathway.

In our study, we demonstrated that silencing \(S100A12\) gene could reduce expressions of WBC, LPS, and nCD64 cells so as to increase survival rate, but decrease apoptosis rate of septic rats. Also, silencing \(S100A12\) gene could alleviate the pathological changes of liver tissues in septic rats. Previous study verified that WBC was the essential independent factor associated with plasma \(S100A12\) concentration.\(^11\) Similarly, \(S100A12\) expression had positive connection with a clinical history of inflammation markers, like WBC and IL-6, demonstrating that silencing \(S100A12\) could decrease WBC.\(^21\) Moreover, a study found that LPS could induce \(S100A12\), and meanwhile \(S100A12\) mRNA expression could be upregulated obviously via LPS stimulation due to the mediation of immune responses, which was consistent with our study.\(^22\) Besides, Sonis et al. proved that nCD64 expression was thought to be beneficial for neonatal sepsis diagnosis, which could be an early indicator of neonatal sepsis.\(^23\) Meanwhile, nCD64 was proven to be essential to the pathophysiologic process and innate immune, which showed the similar effect of \(S100A12\).\(^24\) Consistently, high serum levels of WBC and nCD64 were found in the sepsis group, and WBC could be inhibited by silencing \(S100A12\) as above mentioned, indicating that inhibiting \(S100A12\) could also decrease nCD64 expression.\(^25\) Also, inflammatory markers were proven to participate in sepsis pathophysiology with diagnostic value during the process of innate immune response, and high expressions of inflammatory markers were found in sepsis.\(^9\) Therefore, decreased WBC, LPS and nCD64 by silencing \(S100A12\) may increase survival rate to reduce inflammation response of sepsis. Moreover, a previous study demonstrated that liver is an early manifestation of inflammation, and liver injury was a kind of common response in patients suffering sepsis.\(^26\) Because silencing \(S100A12\) could benefit sepsis, pathological changes liver injury could be relieved in sepsis.

In addition, \(S100A12\) mRNA expression in liver of septic rats positively correlated with mRNA expressions of ERK1, ERK2, NF-\(\kappa\)B, cPLA2, showing \(S100A12\) could mediate the...
ERK signaling pathway in regulation of serum inflammatory response in septic rats. Besides, Silencing S100A12 could reduce serum inflammatory indexes of IL-1β, IL-10, TNF-α, PCT, and CRP. A study demonstrated that S100A12 was shown to be associated with inflammatory diseases, and increased inflammation reaction combined with some inflammatory factors. Kang et al. found that S100A12 activate airway epithelial cells to produce MUC5AC, and NF-κB and

**FIGURE 8** Cell apoptosis of rats among six groups by TUNEL staining. A, Electrophoretogram of cell apoptosis of rats under microscope (400x); (B) Cartogram of cell apoptosis rate of rats among six groups; EGF, epidermal growth factor; *Compared with the sham group, P < 0.05; **Compared with the model group.
ERK pathways were able to mediate MUC5AC expression, showing that S100A12 had close connections with NF-κB and ERK. Besides, NF-κB is proven to be an inflammatory central transcription factor activated by receptor for advanced glycation end product (RAGE) with S100A12 gene, and S100A12 was proven to activate the ERK pathway, showing the positive connection among them. Moreover, a previous study showed that inhibition of cPLA2 expression might be a novel target for sepsis, indicating that silencing S100A12 could also inhibit cPLA2 with the positive association. Consistently, S100A12 could activate and represent a variety of signal transduction pathways in combination with Ca²⁺. Also, S100A12 is a ligand receptor for RAGE, and advanced glycation end products (AGES) were involved in the activation of p21ras with correlation with the ERK signaling pathway, indicating that S100A12 could regulate the ERK signaling pathway. High serum levels of PCT, CRP were found in the sepsis group, therefore silencing S100A12 is helpful to reduce sepsis could depress PCT and CRP. Besides, Ayumu Nakashima verified that S100A12 and CRP, IL-6, and IL-10 showed closely positive connection. And Huang and Tu proved that expressions of pro-inflammatory factors like IL-10, IL-6, and TNF-α were decreased when S100A12 expression was silenced, similar to our study.

In conclusion, our study proved that S100A12 gene silencing decreases serum levels of anti-inflammatory/pro-inflammatory cytokines in septic rats by inhibiting the ERK signaling pathway, through which it might provide a new target for sepsis treatment. Still, inner mechanisms between S100A12 and those pro-inflammatory and anti-inflammatory cytokines were unclear, and further studies need to be carried out to confirm the S100A12 role for sepsis treatment.

ACKNOWLEDGMENTS

This work was supported by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), the 2016 “333 Project” Award of Jiangsu Province, the 2013 “Qinglan Project” of the Young and Middle-aged Academic Leader of Jiangsu College and University, the National Natural Science Foundation of China (81571055, 81400902, 81271225, 31201039, 81171012, and 30950031), the Major Fundamental Research Program of the Natural Science Foundation of the Jiangsu Higher Education Institutions of China (13KJA180001), and grants from the Cultivate National Science Fund for Distinguished Young Scholars of Jiangsu Normal University. We would like to acknowledge the reviewers for their helpful comments on this paper.

CONFLICTS OF INTEREST

None.

ORCID

Dong-Mei Wu http://orcid.org/0000-0002-5363-7676

REFERENCES


### TABLE 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Inflammation activity score (G)</th>
<th>Fibrosis score (S)</th>
<th>Grade (G) Stage (S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0</td>
<td>0</td>
<td>0 (G) 0 (S)</td>
</tr>
<tr>
<td>Model</td>
<td>8.33 ± 1.21</td>
<td>6.00 ± 1.55</td>
<td>II (G) II (S)</td>
</tr>
<tr>
<td>Empty vector</td>
<td>9.33 ± 1.97*</td>
<td>6.83 ± 1.47*</td>
<td>II (G) II (S)</td>
</tr>
<tr>
<td>S100A12 siRNA + EGF</td>
<td>10.17 ± 2.14*</td>
<td>7.67 ± 1.86*</td>
<td>II (G) II (S)</td>
</tr>
<tr>
<td>S100A12 siRNA</td>
<td>5.33 ± 1.03*</td>
<td>3.67 ± 0.82*</td>
<td>I (G) I (S)</td>
</tr>
<tr>
<td>EGF</td>
<td>17.50 ± 2.88*</td>
<td>11.83 ± 2.48*</td>
<td>IV (G) III (S)</td>
</tr>
</tbody>
</table>

EGF, epidermal growth factor; *Compared with the model group, P < 0.05.


27. Kang JH, Hwang SM, Chung IY. S100A8, S100A9 and S100A12 activate airway epithelial cells to produce MUC5AC via extracellular signal-regulated kinase and nuclear factor-kappaB pathways. *Immunology*. 2015;144:79–90.

