PDGFR kinase inhibitor protects against septic death via regulation of BTLA

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Sepsis, defined as life-threatening organ failure caused by a dysregulated host response to severe infection, is a major cause of death among intensive care unit patients. Therapies targeting on immunomodulatory is a new research field in sepsis treatment. B- and T-lymphocyte attenuator (BTLA) is an inhibitory costimulatory factor molecule of B and T lymphocytes. Studies have shown that elevated expression of BTLA in lymphocytes can reduce mortality in sepsis, but its regulatory compounds and the underlying mechanism remains to be elucidated. Here, we show that treatment with CP-673451 significantly decreases mortality of septic mouse. CP-673451 is a PDGFR kinase inhibitor which can promote the expression of BTLA, inhibit the release of chemokines such as CXCL13, and reduce first the chemotaxis of B cells to the peripheral blood and vital organs. CP-673451 also inhibits both the release of cytokines and chemokines such as IL-1β, IL-6, IL-10, TNF-α, CCL1, CCL2 and CCL7 and reduces both the chemotactic ability of T cells. This suggests that CP-673451 may prevent septic death by inhibiting lymphocyte chemotaxis and alleviating “cytokine storm”. In conclusion, our study provides a new therapeutic target and an effective compound for sepsis treatment.

sepsis, B- and T-lymphocyte attenuator, platelet-derived growth factor receptor, CP-673451, cytokine storm

INTRODUCTION

Sepsis is a main cause of death in the intensive care unit (ICU). The incidence of sepsis is as high as 535 cases per 100,000 people, and in-hospital mortality from this condition is as high as 25%–30% (Hollenberg and Singer, 2021; van der Poll et al., 2021). The American Society of Critical Care Medicine (SCCM) and the European Society of Intensive Care Medicine (ESICM) defined the diagnostic criteria of sepsis as life-threatening organ dysfunction to severe infection caused by a dysregulated host response, resulting in abnormal physiological, pathological and biochemical syndrome (Singer et al., 2016; van der Poll et al., 2016). As the clinical treatment of sepsis continues to improve and our understanding of the pathogenesis of sepsis deepens, decreases in in-hospital mortality still mainly depend on early antibiotic treatment and multiple organ support therapy (MOST), while molecular mechanism-targeted treatment focusing on septic immune dysfunction and infection is still lacking (Cecconi et al., 2018; Ferrer et al., 2008; Kaukonen et al., 2014). Sepsis patients often die of secondary infection...
after immunosuppression due to early overactivated inflammatory reactions leading to immune dysfunction (Le-lubre and Vincent, 2018). Therefore, regulating early immune dysfunction and preventing and treating sepsis-induced immunosuppression have become popular research subjects. The overactivated inflammatory response in the early stage of sepsis may be a leading cause of late immunosuppression. Early pathogen invasion activates innate immunity, and activated innate immunocytes present pathogen antigens to lymphocytes and activate cellular immunity (Chousterman et al., 2017). Immunocytes release a large number of cytokines to trigger pro- and anti-inflammatory immune responses, leading to immune regulation disorders, organ dysfunction or failure, or even death (Bone, 1996).

B- and T-lymphocyte attenuator (BTLA) is a lymphocyte inhibitory receptor that is widely expressed on the surface of immunocytes. Similar to cytotoxic T lymphocyte antigen-4 (CTLA-4), programmed death-1 (PD-1) and programmed death-ligand 1 (PD-L1), BTLA can attenuate the activation of nuclear transcription factor kappa B in activated T cells and inhibit the activity of transcription factor activator protein-1. This molecule plays a role in inhibiting the proliferation and differentiation of lymphocytes (Douna et al., 2019; Watanabe et al., 2003). Herpesvirus entry mediator (HVEM) belongs to the tumor necrosis factor receptor (TNFR) superfamily and has been identified as the only BTLA ligand. BTLA and HVEM binding can help T cells maintain a naive state, thus inhibiting T-cell differentiation and proliferation and maintaining immune homeostasis (Deng et al., 2021; Flynn et al., 2013; Miller et al., 2009). Many studies on BTLA have produced similar conclusions. Treatment with agonistic anti-BTLA antibodies can prevent graft-versus-host disease (Albring et al., 2010). BTLA inhibits lipopolysaccharide (LPS)-induced endotoxic shock, and agonistic anti-BTLA antibodies have therapeutic potential in endotoxic shock (Kobayashi et al., 2013). In allergic diseases, it was also found that after antigen inhalation, the production of IL-5 and eosinophilic inflammatory response in the airways of BTLA-deficient mice increased, indicating that BTLA can regulate T-cell death in the lungs and inhibit the aggregation of eosinophils induced by antigen in the bronchus (Tamachi et al., 2007). In addition, in a study of coronavirus disease 2019 (COVID-19), BTLA expression in CD8+ T cells of COVID-19 patients was decreased compared with that in healthy controls (Herrmann et al., 2020). Combined with the study of BTLA in sepsis, the percentage of BTLA+/CD4+ T cells in patients with sepsis was lower than that in healthy volunteers and was associated with increased mortality in patients with severe sepsis and sepsis-related death (Shao et al., 2015). At the same time, drug inhibition of BTLA expression in a sepsis animal model also increased the mortality rate (Cheng et al., 2016). These studies suggest that elevated expression of BTLA may play a protective role in sepsis treatment.

In this study, from the perspective of upregulating the expression of BTLA to regulate the immune inflammatory response, we aimed to reduce the mortality of sepsis by preventing immune dysfunction. We screened drugs that can upregulate the expression of BTLA in immune cells from a kinase inhibitor library and found that PDGFR signaling pathway inhibitors can play a protective role against sepsis by upregulating the expression of BTLA. We verified the mechanism of the protective effect of PDGFR inhibitors in a sepsis mouse model and evaluated their effects on immune cells and cytokines (chemokines and inflammatory factors). By evaluating the role of PDGFR inhibitors in innate immune regulation, we explored the potential of PDGFR inhibitors as sepsis therapy drugs.

RESULTS

Bioactive compound screening identifies that a PDGFR inhibitor upregulates BTLA expression

The expression of BTLA on immunocytes has been shown to be increased during sepsis and is assumed to be one of the main contributors leading to sepsis-induced immunocyte dysfunction (Shao et al., 2015; Shubin et al., 2013). The pathophysiological mechanism of early sepsis is multiorgan injury caused by a “cytokine storm”. Therefore, we hypothesized that increasing the expression of BTLA in immunocytes could weaken the secretory function of immunocytes during sepsis, thus attenuating the development of a “cytokine storm”. Thus, we screened a commercial library of highly selective kinase inhibitors that target 174 signaling molecules in RAW264.7 macrophages by in-cell western blotting. The results suggested that several target-selective inhibitors promoted or reduced the expression of BTLA in macrophages (Figure 1A). The top ten drugs that promoted the expression of BTLA included CP-673451 (an inhibitor of platelet-derived growth factor receptor, PDGF), SGI-7079 (an inhibitor of histone methyltransferase), PTC-209 HBr (an inhibitor of B-cell-specific Moloney murine leukemia virus insertion site 1, BMI-1), PR-619 (an inhibitor of deubiquitinylating enzymes, DUBs), oxcarbazepine (an inhibitor of sodium channels), GW2580 (an inhibitor of colony-stimulating factor-1 receptor, CSF-1R), AZD3463 (an inhibitor of anaplastic lymphoma kinase, ALK), pralatrexate (an inhibitor of dihydrofolate reductase, DHFR), LDC000067 (an inhibitor of cyclin-dependent kinase 9, CDK9), and DDR1-IN-1 (an inhibitor of discoidin domain receptor 1, DDR1) (Figure 1B). Furthermore, we observed the effect of CP-673451 on BTLA expression in immunocytes in vivo by immunofluorescence. The results showed that CP-673451 significantly promoted BTLA ex-
expression in immunocytes in the spleen. CP-673451 treatment mainly enhanced BTLA expression in CD4⁺ T cells, CD8⁺ T cells and B cells. The difference of the elevated expression of BTLA in CD4⁺ T cells and B cells after CP-673451 treatment was significant (P=0.001) (Figure 1C and Figure S1 in Supporting Information). Consistent with the enhancement of BTLA protein expression, CP-673451 also resulted in increased BTLA transcription in the spleens of CP-673451-treated cecal ligation puncture (CLP) mice relative to those of the CLP mice on the 1st and 3rd days after sepsis (Figure 1D). However, the results of cell viability test showed that CP-673451 had no significant effect on the proliferation of CD4⁺ T cells, CD8⁺ T cells and B cells in vitro (Figure S2 in Supporting Information). Furthermore, we observed the effect of CP-673451 treatment on the survival rate of CLP mice, the most accepted sepsis animal model. Treatment with CP-673451 significantly decreased CLP mouse mortality (n=25; P=0.015; Figure 1E).

**CP-673451 protects the function of vital organs of septic mice**

CP-673451 administration at 2, 24, 48 and 72 h after CLP showed that CP-673451 treatment was associated with re-
duced sepsis-induced lung injury. Compared with that of the control group, infiltration of inflammatory cells and thickened alveolar septa were significantly reduced in the CP-673451-treated group (Figure 2A). Biochemical detection of tissue enzymes also suggested that CP-673451 had protective effects on vital organ function. Specifically, the CP-673451-treated group had significantly lower enzyme release in the heart (creatine kinase, CK), kidney (blood urea nitrogen, BUN) and liver (alanine aminotransferase, ALT; aspartate transaminase, AST) on the 1st and 3rd days post-CLP (Figure 2B). Furthermore, the results of an apoptosis assay showed that CP-673451 administration after CLP also reduced immunocyte apoptosis in the thymus on the 1st day after sepsis (Figure 2C). In conclusion, these results demonstrate that CP-673451 might reduce the mortality rate of septic mice by protecting the function of vital organs and reducing the apoptosis of immune cells.

**CP-673451 treatment reduces the release of several cytokines and chemokines during sepsis**

Cytokine production and release are crucial for lymphocytes participating in the immune response. It has been shown that the peripheral blood lymphocyte count is negatively correlated with the expression levels of most cytokines during sepsis, indicating that lymphocytes are vital cells involved in the “cytokine storm” of sepsis (Espinosa, 2020). The severity of sepsis is associated with a “cytokine storm”, so it is reasonable to alleviate this “cytokine storm” to reduce self-damage caused by the host anti-infection response. BTLA plays a role in the regulation of lymphocyte activity during sepsis (Karbian et al., 2020). Therefore, we speculated that BTLA regulates lymphocyte activity by regulating the release of cytokines. We first observed the effect of CP-673451 on cytokine release in septic mice using a cytokine array kit. The results showed that the release of CXCL13, IL-1ra, IL-6, CXCL1, CCL11 and Ang-2 was elevated after CLP but significantly reduced after treatment with CP-673451. In contrast, the release of IGFBP-5, CXCL5 and IGFBP-2 was decreased after both CLP and CP-673451 treatment (Figure 3A). The results were verified by ELISA and showed that CP-673451 significantly reduced the release of CXCL13 (P<0.024 for Day 1; P<0.001 for Day 3; Figure 3B). Consistent with this observation, the concentration of CXCL13 was also elevated in septic patients relative to nonseptic patients (P<0.001; Figure 3C). We detected additional differences in cytokine expression levels by Luminex liquid chip. The release of certain cytokines, including IL-1β, IL-2, IL-4, IL-6, IL-10 and TNF-α, decreased significantly after CP-673451 treatment relative to the control group the first day after CLP. Furthermore, the release of certain chemokines, such as CCL1, CCL2, CCL7, CCL17, CCL27 and CXCL11, was also reduced significantly after treatment with CP-673451 the first day after CLP (Figure 3D and Figures S3, S4 in Supporting Information). Taken together, these data indicate that the release of several cytokines and chemokines increases during sepsis. CP-673451 treatment can reduce the release of cytokines (CXCL13, CCL1, CCL2, CCL7, CCL17, CCL27, CXCL11) and chemokines (IL-1β, IL-2, IL-4, IL-6, IL-10, TNF-α) in peripheral blood.

**Regulation of CP-673451 in the transcription and release of chemokines and cytokines in vivo and in vitro during sepsis**

The results of the cytokine array kit and Illumina liquid chip both indicated that CP-673451 treatment inhibited the release of a variety of chemokines and cytokines. Therefore, we further verified the transcriptional levels of these chemokines and cytokines in vivo and in vitro. We analyzed the transcription of chemokines and cytokines by LPS-stimulated RAW264.7 macrophages in vitro, and the transcription levels of CCL1, CCL2, CCL7, CXCL11, IL-1β, IL-6, IL-10 and TNF-α were detected by real-time PCR (Table S1 in Supporting Information). CP-673451 treatment significantly reduced the transcription levels of CCL2, CCL7, IL-10 and TNF-α (Figure 4A). We further observed the effects of CP-673451 on the transcription levels of CCL1, CCL2, CCL11, CXCL13, IL-1β, IL-6, IL-10 and TNF-α in the vital organs of CLP mice the first day after CLP. The results showed that the transcription levels of CCL1, CCL2 and CXCL13 in the liver were significantly decreased after CP-673451 treatment. The transcription levels of IL-1β and TNF-α in the spleen were also reduced significantly (Figure 4B). Together, these results suggest that CP-673451 treatment reduces the transcription levels of several cytokines and chemokines during sepsis.

**CP-673451 treatment reduces the proportion of peripheral blood B lymphocytes during sepsis in vivo**

CXCL13 is a member of the chemokine family, also known as B lymphocyte chemoattractant (BLC), that can selectively cause B-cell chemotaxis. CXCL13 selectively mediates the chemotaxis of B lymphocytes after binding to the B lymphocyte surface receptor C-X-C motif chemokine receptor 5 (CXCR5) (Ansel et al., 2002; Podstawka et al., 2021). Therefore, we speculated that CP-673451 may inhibit the chemotaxis of B cells to vital organs and peripheral blood during sepsis by inhibiting the release of first CXCL13 and then cytokines during sepsis. Therefore, we observed whether the number of B cells recruited to the peripheral blood in septic patients increased. Septic patients (n=14) and healthy volunteers (n=20) were recruited for the study. The demographic and clinical characteristics of the septic patients are shown in Table 1. Briefly, respiratory tract infection and...
urinary tract infection contributed to 50% of all infections. No pathogens were identified in the blood cultures of 78.6% of the septic patients, although they had an identified site of infection. Gram-negative infection accounted for approximately 14.3%, and gram-positive infection accounted for 7.1%. The flow cytometry results showed that the proportion of B cells among the nucleated cells in the peripheral blood of septic patients was significantly higher than that of healthy volunteers on the 5th day after sepsis ($P<0.05$; Figure S5 in Supporting Information).

We further observed the effect of CP-673451 on the number of peripheral blood B lymphocytes in septic mice. Flow cytometry results showed that CP-673451 treatment reduced the proportion of B lymphocytes among the nucleated cells in the peripheral cells of septic mice ($P=0.039$ for Day 1, Figure 5). In conclusion, the proportion of peripheral blood B cells is elevated during sepsis, and CP-673451 inhibits the chemotaxis of B cells to the peripheral blood during sepsis by inhibiting the release of CXCL13. The mechanism by which CP-673451 inhibits the chemotaxis of B cells may involve inhibition of the release of CXCL13.

**DISCUSSION**

Sepsis induces the release of excessive cytokines, which causes a “cytokine storm”. This “cytokine storm” is characterized by the increased secretion of chemokines, resulting in an increase in the migration and homing of immunocytes and participation in the immune response. Excessive release of inflammatory factors leads to overactivation of the immune response, which subsequently leads to tissue and organ damage (Fajgenbaum and June, 2020; Zeng et al., 2017). It has been verified that BTLA inhibits LPS-induced endotoxic shock and the production of proinflammatory cytokines (Albring et al., 2010). BTLA also played a role in the acquired immune response. BTLA-deficient mice are prone to autoimmune hepatitis-like diseases (Oya et al., 2008). Lower BTLA expression is a risk factor for septic death (Shao et al., 2015). Thus, we intended to reduce the mortality of sepsis by preventing immune dysfunction via upregulation of the expression of BTLA to regulate the immune inflammatory response.

We screened a highly selective kinase inhibitor library and found that CP-673451 can upregulate BTLA expression.
on immunocytes and reduce sepsis-related mortality. CP-673451 is a potent pharmacologically selective PDGFR kinase inhibitor that inhibits PDGFR-beta phosphorylation in many kinds of tumors, selectively inhibits PDGF-BB-stimulated angiogenesis and significantly inhibits tumor growth in multiple mouse tumor models (Roberts et al., 2005). PDGFs and PDGFRs have been recognized as prototypes for growth factor and receptor tyrosine kinase function for over 20 years. Studies have revealed the roles of PDGFR-alpha signaling in many vital organs and in vessel formation and hematopoiesis (Andrae et al., 2008). PDGF signaling is implicated in a range of diseases, such as leukemias, gliomas and sarcomas (Buhletal., 2020; Jitariu et al., 2018). The relationship between PDGFs and sepsis is unclear. Biochemical detection of tissue enzymes also suggested that CP-673451 had protective effects on vital organ function. CP-673451 administration can significantly reduce enzyme release in the heart, kidney and liver in septic mice. Furthermore, CP-673451 administration also reduced immune cell apoptosis. That is, CP-673451 might reduce the mortality rate of septic mice by protecting the function of vital organs and reducing the apoptosis of immune cells.

To further elucidate the mechanism by which CP-673451 reduces mortality in sepsis, we observed its effect on cytokine release. The serum concentration of IL-1β, IL-2, IL-4, IL-6, IL-10 and TNF-α decreased significantly after CP-673451 treatment in septic mice. Furthermore, the release of chemokines, such as CCL1, CCL2, CCL7 and CXCL13, was also reduced significantly after treatment with CP-673451 in septic mice. In the early stage of sepsis, these cytokines and chemokines were mainly produced and released by myeloid cells such as macrophages, neutrophils and monocytes. Indicated that myeloid cells would be part of the mechanism underlying CP-673451’s inhibitory effect on “cytokine storm” during sepsis. CXCL13 is a B lymphocyte chemoattractant that can selectively cause B-cell chemotaxis. CXCL13 reduced the chemotaxis of B cells to the peripheral blood and vital organs. Our results showed that CXCL13 was significantly increased during sepsis and its expression was significantly reduced after treatment with CP-673451. This might be the cause of CP-673451 treat-
Figure 4  Regulation of CP-673451 on the transcription and release of chemokines and cytokines in vivo and in vitro during sepsis. A, Real-time PCR analysis of CCL1, CCL2, CCL7, CXCL11, IL-1β, IL-6, IL-10 and TNF-α mRNA expression in RAW264.7 macrophages treated with or without CP-673451 (20 μg mL⁻¹) and stimulated with LPS (100 ng mL⁻¹) for 8, 16 and 24 h. B, Real-time PCR analysis of CCL1, CCL2, CXCL11, CXCL13, IL-1β, IL-6, IL-10 and TNF-α mRNA expression in liver or spleen tissues of CLP mice with or without CP-673451 treatment. Error bars indicate the SD. *, P<0.05; **, P<0.01, one-way ANOVA.
Figure 5. CP-673451 treatment reduces the proportion of peripheral blood B lymphocytes in mice during sepsis. A, Flow cytometry detected the proportions of B lymphocytes in the peripheral blood of the control, CLP, and CLP+CP-673451 groups on the 1st, 3rd, and 5th days after sepsis. B, Statistical histogram of flow cytometry analysis. Error bars indicate the SD. *, $P<0.05$; **, $P<0.01$, one-way ANOVA.

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ment reducing the proportion of B lymphocytes in septic mice. T-cell chemotaxis to the peripheral blood and vital organs was reduced by CCL1, CCL2 and CCL7, further reducing the release of cytokines such as IL-10 (Figure 6).

Our study indicated that the release of several cytokines and chemokines increased during sepsis, while CP-673451 treatment reduced the release of these cytokines and chemokines. This might be the mechanism by which CP-673451 alleviates the cytokine storm during sepsis, thus protecting against septic death.

A large number of preclinical and clinical studies have shown that the immune inflammatory response plays a pivotal role in sepsis (Patil et al., 2017). Targeting therapy with immune checkpoints such as PD-1, PD-L1, CTLA-4 and BTLA, which may reverse the overactivated innate and adaptive immune systems during sepsis, has emerged as a new immune therapy strategy for sepsis. In this study, we showed that the selective PDGFR kinase inhibitor CP-673451 increased the expression of BTLA by inhibiting the activation of PDGFR kinase, inhibits the release of chemokines such as CXCL13, reduces the chemotaxis of B cells to the peripheral blood and vital organs, and then reduces the production of cytokines such as IL-10 in these cells. CP-673451 also inhibits the release of chemokines such as CCL1, CCL2 and CCL7, reduces T-cell chemotaxis to peripheral blood and vital organs, and further reduces the release of IL-10.

### MATERIALS AND METHODS

#### In-cell western blotting

The intracellular target proteins were detected using a quantitative immunofluorescence assay. Briefly, RAW264.7 cells were cultured in DMEM (HyClone™, high glucose, diluted 1000-fold) on 96-well plates and incubated in the absence or presence of one compound (200 μL, diluted 1000-fold) from the Target-selective Inhibitory Library (#L3500, Selleck) for 8 h. Next, the cells were fixed in 3.7% paraformaldehyde and incubated at room temperature for 20 min. The cells were then permeabilized with 10% Triton X-100 and incubated with 150 μL of blocking fluid (donkey serum diluted 10-fold in PBS) at room temperature for 1.5 h. The cells were then permeabilized with 10% Triton X-100 and incubated with 150 μL of blocking fluid (donkey serum diluted 10-fold in PBS) at room temperature for 2 h. Finally, they were incubated with secondary antibodies (IRDye 800cw goat anti-rabbit, 1:1000, Abcam) at room temperature for 1 h. The cells were then incubated with primary antibodies (BTLA/CD272, 1:1000, #ab212089, Abcam) at room temperature for 2 h. They were then incubated with secondary antibodies (IRDye 680LT donkey anti-mouse, 1:1000, #925-68022, LI-COR) and protected from light for 1 h. An Odyssey CLX (CLX-1730, LI-COR) imaging system was used in the exposure.

#### Sepsis mouse model

Sepsis was induced surgically in male C57BL/6 mice (Charles River, 7 weeks old, 22±1 g). Briefly, 3% and 2% isoflurane were used to induce and maintain anesthesia, respectively. Small incisions were made in the midline of the
abdomen of the mice. Their cecal were placed outside, and their distal ileocecal valves were ligated using 4.0 silk thread. Then, 16-gauge needles were used to penetrate the cecal at the ligation sites, after which their abdomens were closed in two layers. Finally, the mice were subcutaneously injected with 1 mL of Ringer’s solution. The mice were not administered antibiotics after CLP. The mice were randomly divided into CLP and CLP+CP-673451 groups. Administration group was given CP-673451 (200 mg kg$^{-1}$) through gavage at 2, 24, 48, and 72 h after CLP. The mice in the CLP group were administered equal volumes of phosphate-buffered saline (PBS) (pH 7.2–7.4).

Immunofluorescence

Fresh spleen tissues were isolated from mice, opened through transverse incision and washed with PBS several times. Then, they were fixed with 4% formaldehyde solution for 24 h and embedded in paraffin. Paraffin-embedded tissues were cut into 3-μm sections. The sections were deparaffinized in xylene and hydrated in graded alcohols, followed by antigen retrieval. The sections were then blocked with 10% donkey serum for 1 h and incubated with primary antibodies (anti-CD272/BTLA, ab212089, Abcam) overnight at 4°C. Next, the sections were incubated with secondary antibodies (IRDye680RD donkey anti-Rabbit, 1:1000 [926-68073, LI-COR]) and protected from light for 1 h at room temperature. Nuclei were stained with DAPI.

Quantitative real-time polymerase chain reaction

Total RNA was extracted using TRIzol reagent (#15596026, Ambion) according to the manufacturer’s instructions. First-strand cDNA was synthesized according to the GoScript™ Reverse Transcription Mix using a random primer protocol. Briefly, 20-μL reactions were prepared by combining 4 μL of nuclease-free water, 4 μL of GoScript™ Reaction Buffer-Random Primer, 2 μL of GoScript™ Enzyme Mix, and 10 μL of RNA diluted in RNase-free water. The cDNA from various cell samples and tissue homogenates was then amplified using real-time quantitative PCR with gene-specific primers in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, #1855195).

Biochemical assay

Blood samples were analyzed according to the manufacturer’s instructions. Briefly, the blood samples were kept at room temperature for 2 h and subsequently centrifuged (1000×g, 10 min, 4°C). The light-yellow supernatants were extracted and stored on ice. Next, the blood samples were diluted with normal saline (1:10), and AST (#011059, IDEXX), CK (#010838, IDEXX), ALT (#011052, IDEXX), BUN (#011195, IDEXX) reagent tablets were added according to the manufacturer’s instructions. The results were determined using a catalyst I (IDEXX) automatic biochemical analyzer.

Hematoxylin and eosin staining

Fresh lung tissues were isolated from mice and opened transversely. Then, they were fixed with 4% formaldehyde solution for 24 h, embedded in paraffin, and cut into 3-μm sections. The sections were deparaffinized in xylene and hydrated in graded alcohols. The tissue sections were then stained with hematoxylin and eosin after dehydration and were used for histopathological assessment.

Cytokine assays

The Proteome Profiler Mouse XL Cytokine Array Kit (#ARY028, R&D) is a membrane-based sandwich immunoassay that simultaneously detects 111 mouse cytokines. Briefly, capture antibodies were spotted in duplicate on nitrocellulose membranes to bind to specific target proteins present in the sample. The captured proteins were then detected with biotinylated detection antibodies and visualized using chemiluminescent detection reagents. The signal produced was proportional to the amount of analyte bound. The relative concentrations of chemokines in the sera were assessed using a mouse chemokine assay kit (#10000061997, Bio-Rad) according to the manufacturer’s instructions. For data acquisition and analysis, Bio-Plex Manager software (version 6.0) was used.

TUNEL staining

Fresh thymus tissues were isolated from mice and opened transversely. Then, they were fixed with 4% formaldehyde solution for 24 h, embedded in paraffin, and cut into 3-μm sections. The tissue sections were deparaffinized in xylene and hydrated in graded alcohols, followed by antigen retrieval. After 0.2% Triton X-100 treatment, a one-step TUNEL apoptosis assay kit (C1086, Beyotime) was used according to the manufacturer’s protocols.

Cell culture and establishment of an LPS-stimulated cell inflammation model

RAW264.7 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (#ST30-3302, PAN) and penicillin-streptomycin (#C0222, Beyotime). The cells were maintained in a 5% CO$_2$ incubator at 37°C. To construct the LPS-stimulated cell inflammation model, RAW264.7 cells (10$^5$ cells) were incubated with 100 ng mL$^{-1}$ LPS for 8, 16 and 24 h.
Enzyme-linked immunosorbent assay (ELISA)

CXCL13 concentrations in serum were measured using a human CXCL13/BLC/BCA-1 Quantikine ELISA kit (#DCX130, R&D). According to the manufacturer’s protocol, the optimum assay range was 7.8-500 pg mL⁻¹ heparin plasma. A fluorescence reader was used to detect excitation at a wavelength of 460 nm.

Patient samples

Plasma samples from patients with sepsis (n=14) and healthy controls (n=20) were collected from the Department of Critical Medicine, Daping Hospital, Chongqing, China. Sample collection was approved by the institutional review board. Sepsis was defined according to the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3) (Singer et al., 2016).

Statistical analysis and image processing

Unpaired Student’s t tests were used to compare the means of two groups. One-way analysis of variance (ANOVA) was used for comparisons among several groups. When ANOVA yielded significant differences, post hoc testing of differences between groups was performed using the least significant difference (LSD) test. The Kaplan-Meier method was used to compare differences in mortality rates between groups. Statistical significance was set at P values<0.05. GraphPad Prism 7.0 software was used for statistical analysis, and statistical charts were generated. Adobe Illustrator CC (version 18.0.0, 32-bit) and Adobe Photoshop CS6 software (version 13.0.1, 64-bit) were used to construct mechanism diagrams and reasonably adjusted pictures.

Compliance and ethics The author(s) declare that they have no conflict of interest.

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SUPPORTING INFORMATION

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