Article

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Curcumin Attenuates PD-L1-Positive Neutrophil-Induced T-Lymphocyte Apoptosis and Alleviates Lung Injury During Sepsis in Rats

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Objective: This study aimed to investigate the effects of curcumin (Cur) on programmed cell death 1 ligand 1 (PD-L1) expression in neutrophils from septic rats and its regulatory influence on T-lymphocyte apoptosis and lung injury in a rat sepsis model.
Methods: Cecum ligation and puncture (CLP) experiments were conducted to establish a rat sepsis model, with the subsequent grouping of rats based on curcumin administration. Rats were monitored for 7 days to assess the 7-day survival rate. Serum, lung tissues, and thymus tissues were collected. Flow cytometry and immunohistochemistry were utilized to assess the number of PD-L1-positive neutrophils and PD-L1 positivity in both blood and lung tissues. Hematoxylin and eosin (HE) staining and Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) histochemistry were employed to examine pathological changes and cell apoptosis in lung and thymus tissues. Furthermore, a kit was employed to measure the activity of myeloperoxidase (MPO), a marker of neutrophil activation, in lung tissues. Enzyme-linked immunosorbent assay (ELISA) was utilized to determine plasma levels of inflammatory factors. Neutrophils were extracted and co-cultured with normal T lymphocytes. TUNEL assays were used to evaluate T-lymphocyte apoptosis, and Western blotting was performed to analyze the expression of PD-L1 and programmed cell death 1 (PD-1).
Results: In *in vivo* experiments, septic rats exhibited a markedly low 7-day survival rate of 12.5%, significantly elevated PD-L1 expression and positivity in blood and lung tissues, severe lung and thymus tissue damage, and significant cell apoptosis. Additionally, they had increased plasma concentrations of tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6), and decreased plasma concentration of interleukin 10 (IL-10) compared to normal and sham-operated rats ($p < 0.05$). Curcumin-treated septic rats demonstrated significantly improved 7-day survival, reduced PD-L1 expression and positivity in blood and lung tissues, mitigated lung and thymus tissue injury and cell apoptosis, lower plasma concentrations of TNF-α and IL-6, and higher plasma concentrations of IL-10 ($p < 0.05$). *In vitro* experiments showed that co-culture of T lymphocytes with neutrophils from septic rats resulted in a significantly higher rate of T cell apoptosis and increased expression of PD-L1 and PD-1 compared to co-culture with neutrophils from sham-operated rats ($p < 0.05$). Neutrophils from curcumin-treated rats exhibited a significantly lower rate of apoptosis in co-cultured T lymphocytes and decreased expression of PD-L1 and PD-1 ($p < 0.05$). The addition of PD-L1 antibodies to co-cultured neutrophils and T lymphocytes in septic rats significantly reduced T lymphocyte mortality ($p < 0.05$).
Conclusion: Curcumin effectively mitigates lung and thymus injury during sepsis and attenuates the apoptosis of rat T lymphocytes by down-regulating PD-L1 expression in centrocytes, both *in vivo* and *in vitro*.

Keywords: curcumin; neutrophil; PD-L1; T-lymphocyte apoptosis; lung injury; sepsis; cecum ligation and puncture

Introduction

Sepsis is a critical condition resulting from an imbalanced host response to infection, and its severity has been acknowledged since antiquity [1]. Recent studies indicate that globally, sepsis claims the lives of 11 million people annually, implying that one in every five deaths is linked to sepsis [2]. Although sepsis death rates have declined with advances in the healthcare system, they are still unacceptably high [2]. Premature death in sepsis is primarily attributed to organ dysfunction, prolonged inflammation, immunosuppression, and heightened susceptibility to secondary infections [3]. Sepsis is generally perceived as a biphasic disease, characterized by two concurrent phases. The initial phase involves hyperinflammation, where the innate immune system releases an overwhelming amount of inflammatory factors, potentially leading to tissue damage, which is often termed the "cytokine storm" [4]. Following this surge in inflammation, the immune system transitions into a weakened state, resulting in a period of low inflammation. During this stage, there is a depletion and demise of lymphoid and myeloid cells, compromising the

patient's immune function [5]. The extensive loss of immune cells renders patients more vulnerable to secondary infections [6].

Therefore, a comprehensive understanding of the mechanisms governing sepsis inflammation, immunosuppression, and immune cell apoptosis, coupled with the development of targeted drugs, is imperative in addressing this formidable medical challenge.

Embedded within the intricate dynamics of sepsis are two essential cell groups: neutrophils and T-cells. Neutrophils, the primary circulating leukocytes, play a pivotal role in combating pathogens [7]. Typically, they have a brief lifespan but dysregulated immature neutrophils persist during sepsis [8], leading to significant tissue damage due to delayed apoptosis and heightened adhesive properties that facilitate tissue infiltration [9]. T-cells, as lymphocytes, are crucial in orchestrating a successful adaptive immune response. CD4+T cells play a vital role in immune regulation, releasing cytokines and facilitating cellular communication. In sepsis, CD4+T cell apoptosis is maximized, contributing to immunosuppression induced by phagocyte uptake of apoptotic cells—a phenomenon closely linked to patient survival [10]. Upregulation of programmed cell death 1 ligand 1 (PD-L1) and programmed cell death 1 (PD-1) is observed in neutrophils from sepsis patients and is inversely correlated with the apoptosis rate in neutrophils [11]. PD-L1 induces apoptosis in activated T cells and stimulates interleukin 10 (IL-10) production in human peripheral blood T cells, fostering immunosuppression [12,13]. This highlights the critical role of PD-L1 in regulating neutrophil and T-cell apoptosis during sepsis, emphasizing its potential as a therapeutic target for drug development.

Curcumin (Cur), derived from Curcuma plants, is a naturally occurring compound with a proven safety profile as a phytochemical [14]. This highly versatile molecule interacts with diverse inflammatory targets, displaying antioxidant, anti-inflammatory, and anticancer properties [15]. Extensive investigations, including clinical trials, position curcumin as a promising therapeutic agent for various chronic conditions [16]. In cancer research, curcumin has shown potential in influencing the immune system by inhibiting PD-L1 expression, thereby disrupting the PD-1/PD-L1 interaction [17]. It also holds promise in restoring T-cell function through multilevel immune checkpoint inhibition [18]. While studies have explored curcumin's role in modulating the PD-1/PD-L1 pathway and T-cell function, clear evidence of its ability to alleviate sepsis-induced inflammatory responses through this pathway is still inconclusive. Investigating the potential mechanisms of curcumin in sepsis, especially its impact on the PD-1/PD-L1 pathway, is crucial for developing new therapeutic strategies to mitigate sepsis-induced inflammatory responses.

The sepsis-induced acute systemic inflammatory response initiates a cascade of pathological and physiological changes, with a pronounced impact on the respiratory system [19]. As a major organ of the respiratory system, the lungs are a primary target for many pathogens during sepsis, and as an important immune organ, they are capable of inducing a robust immune response [20]. This study aims to explore the potential of curcumin in mitigating lung injury in septic rats and decipher its mechanism, particularly in the context of regulating T-lymphocyte apoptosis induced by PD-L1-positive neutrophils. Through a comprehensive analysis of the molecular regulatory mechanisms, our goal is to contribute theoretical insights for the development of safer and more effective immunotherapeutic strategies. This endeavor holds the promise of ushering in new possibilities and renewed hope for the treatment of sepsis patients.

Materials and Methods

Animals and Cecum Ligation and Puncture (CLP)-Induced Sepsis Model

Forty-four 8-week-old Sprague-Dawley (SD) male rats were procured from Hunan SJA Laboratory Animal Co., Ltd. (Changsha, China), production License No.: SCXK (Hunan) 2016–0002. Prior to commencing the experiment, the rats underwent a 3-day acclimatization period in a controlled environment with constant temperature (18–26 °C) and humidity (40–70% relative humidity). The study strictly adhered to the principles of the 3R's and was approved by the Animal Ethics Committee of Hunan Evidence-based Biotechnology Co., Ltd. (No.XZ2024013). A rat sepsis model was induced via CLP experiments [21]. Before surgery, the rats underwent a 12-hour fasting period. Following intraperitoneal injection of 3% sodium pentobarbital (40 mg/kg, P3761, SIGMA, Milwaukee, WI, USA) for anesthesia, a 1–1.5 cm incision was made in the lower left abdomen to expose the cecum. A loop ligation was performed 2/3 from the blind end of the cecum, and a small amount of feces was extruded through a puncture made 1 cm from the blind end.

The mesentery and cecum were returned to the abdominal cavity, and the incision was sutured in layers. All rats were randomly divided into four groups: Normal group (n = 11), Sham group (n = 11), CLP group (n = 11), and Cur+CLP group (n = 11). No intervention was performed in the normal group. The sham group underwent only open abdominal surgery, followed by closure of the abdominal cavity. The Cur+CLP group underwent cecum ligation and puncture experiments. Curcumin (100 mg/kg, S19245, Shyuanye, Shanghai, China) was intraperitoneally injected one hour before the CLP experiments, while an equal volume of saline was injected intraperitoneally in the sham and CLP groups [22]. Three rats were randomly selected from each group and euthanized by intraperitoneal injection of 3% sodium pentobarbital (140 mg/kg, P3761, SIGMA, Milwaukee, WI, USA) at 24 h post-surgery, and thymus and lung tissues were collected. Seven-day survival was assessed for the remaining eight rats in each group.

Flow Cytometry

Lung tissue and blood single-cell suspensions were prepared by resuspending cells in the buffer to adjust the cell number to 1×10^6 per mL. A 100 µL aliquot of the cell suspension was taken. Cells were stained with Fluorescein Isothiocyanate (FITC)-anti-Ly6G antibody (ab25024, Abcam, Shanghai, China) and allophycocyanin (APC)-anti-PD-L1 antibody (ab206967, Abcam, Shanghai, China), and then quantified by flow cytometry on a FACSCalibur instrument (HLA-B27, BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's instructions. PD-L1 neutrophils were defined as the Ly6GPD-L1 population.

Immunohistochemical Staining (IHC)

Lung tissue was fixed, dehydrated, embedded in optimal cutting temperature compound (OCT, LM82077C, LMAI Bio, Shanghai, China), and then cut into 2 µm frozen sections. Sections were washed with phosphate buffer saline (PBS, 10010023, Thermo Fisher, Waltham, MA, USA), and then blocked with PBS containing 10% bovine serum albumin (BSA, 37520, Thermo Fisher, Waltham, MA, USA) for 1 h. Subsequently, the sections were incubated overnight at 4 °C with specific primary antibodies against PD-L1 (A1645, 1:1000, ABclonal, Wuhan, China), followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000, ab6721, Abcam, Cambridge, UK) for 30 min at room temperature. Color development was achieved using 3,3-diaminobenzidine tetrahydrochloride (DAB, D5905, Merck, Shanghai, China), followed by counterstaining with hematoxylin (IH0030, Solarbio, Beijing, China). Imaging was captured using digital confocal microscopy (STELLARIS 5, Leica, Wetzlar, Germany).

Hematoxylin and Eosin Staining

After fixing the thymus and lung tissue with a 10% formaldehyde solution, the tissues were gradually dehydrated and sectioned. The sections were stained with hematoxylin and eosin (G1120, Solarbio, Beijing, China), and then pathomorphologic changes were observed under a microscope (DXR3, IQLAADGABFFAHCMAPB, Thermo Fisher, Waltham, MA, USA) after further dehydration with xylene and ethanol.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL) Staining

Lung and thymus tissues underwent a series of processing steps, including fixation, dehydration, embedding in OCT, and subsequent sectioning into 2 µm frozen sections. These sections were rinsed with PBS and blocked with PBS containing 10% BSA for 1 hour. Following this, the sections were incubated with proteinase K (P1120, Solarbio, Beijing, China) for 10 minutes. TUNEL reaction solution (T2191, Solarbio, Beijing, China) was then applied in dropwise and incubated at 37 °C for 2 hours. Subsequently, the 4',6-diamidino-2-phenylindole (DAPI) working solution (C1086, Beyotime, Shanghai, China) (1 µg/mL) was added dropwise and incubated at 37 °C for 5 minutes. Staining was achieved using DAB (D5905, Merck, Shanghai, China) for 10 minutes, followed by counterstaining with hematoxylin. The specimens were then observed under a microscope.

Myeloperoxidase (MPO) Activity

Lung tissue homogenate was prepared according to the instructions provided in the Myeloperoxidase assay kit (SEKR-0073, Solarbio, Beijing, China). After preparing the reagents, sample Optical density (OD) values were measured at a wavelength of 460 nm using a UV spectrophotometer (GENESYS 150, 840-300000, Thermo Fisher, Waltham, MA, USA), and the MPO activity was calculated.

Enzyme-Linked Immunosorbent Assay (ELISA)

Following the manufacturer's instructions, ELISA kits for tumor necrosis factor-α (TNF-α) (ab236712, Abcam, Shanghai, China), IL-10 (ab214566, Abcam, Shanghai, China), and interleukin 6 (IL-6) (ab234570, Abcam, Shanghai, China) were employed to quantify TNF-α, IL-10, and IL-6.

Cell Culture and Cell Treatment

Seventy-two hours postoperative, neutrophils were isolated from blood specimens of rats using a neutrophil extraction kit (P9201, Solarbio, Beijing, China) according to the manufacturer's instructions. The isolated neutrophils were further purified using density gradient centrifugation. The success of purification was judged by observing cell morphology: the neutrophils were round, in the range of 10–15 µm in diameter, and the cells were filled with pale purplish-red granules. After successful neutrophil purification, a T-lymphocyte cell line (CL0424, Fenghbio, Changsha, China) was purchased. T lymphocytes were cultured in RPMI-1640 medium supplemented with 10% FBS (10100147C; Thermo Fisher, Waltham, MA, USA), penicillin (0.1 µmol/L), streptomycin (0.1 ng/L) (15140148; Thermo Fisher, Waltham, MA, USA), and recombinant human IL-2 (0.1 µmol/L, RP01039, ABclonal, Wuhan, China). The culture conditions were maintained at 37 °C in a 5% CO_2 atmosphere. Purified neutrophils and T-lymphocytes were co-cultured at a 1:1 ratio (1×10^6 cells/mL) in 6-well culture plates. Samples from the Cur+CLP-C (Neutrophils isolated and purified from blood specimens of rats in the CLP group in animal tests) group underwent curcumin treatment overnight at 37 °C, while other samples were treated with Dimethyl sulfoxide (DMSO) at 37 °C, using an isotype control antibody serving as the control. We used 10 µg/mL of PD-L1 antibody to stimulate the cells for 48 hours. The cells used in the experiments were characterized by short tandem repeat (STR) and mycoplasma assays, confirming no cross-infection between cells was detected.

Western Blotting

Cell lysis was performed using Radio Immunoprecipitation Assay Lysis buffer (RIPA), and protein quantification was carried out with the BCA assay kit. Proteins were separated on a 10% SDS gel and then transferred onto a Polyvinylidene fluoride (PVDF) membrane. After blocking, the membrane was incubated overnight at 4 °C with primary antibodies against β-actin (AC026, 1:1000, ABclonal, Wuhan, China), PD-L1 (A1645, 1:1000, ABclonal, Wuhan, China), and programmed cell death 1 (PD-1, A11973, 1:1000, ABclonal, Wuhan, China). The membrane was incubated with an HRP-conjugated secondary antibody (1:2000, ab6721, Abcam, Cambridge, UK) at room temperature. After washing in TBST solution for 5 minutes, BeyoECL Star (P0018AS, Beyotime, Shanghai, China) was applied for 30 seconds for detection development. Image J (V1.8.0.112, NIH, Madison, WI, USA) software was used to analyze the gray value of the bands.

Statistical Analyses

Data were expressed as mean ± standard deviation. For data obeying normal distribution, differences were tested using one-way analysis of variance (ANOVA), and multiple comparisons were performed using Bonferroni's test. Statistical analyses were conducted using SPSS 22.0 (IBM, Corp., Armonk, NY, USA) software, and data were plotted using GraphPad Prism 9.0 (Dotmatics, Boston, MA, USA). Differences were considered significant when $p < 0.05$.

Results

Curcumin Increases Survival in CLP Rats

To investigate the impact of curcumin on rat survival during sepsis, we assessed the seven-day survival rates post-surgery. Notably, no fatalities occurred within the seven-day period in the normal and Sham groups, indicating a 100% survival rate. In contrast, the CLP group experienced a notable increase in deaths during the same timeframe, resulting in a markedly reduced survival rate of only 12.5% ($p < 0.001$). Interestingly, rats in the Cur+CLP group, receiving curcumin treatment, exhibited a significantly improved seven-day survival rate compared to the CLP group ($p < 0.05$), as depicted in Fig. 1. These findings highlight the considerable lethality associated with sepsis and underscore the potential of curcumin in mitigating sepsis progression in rats, thereby reducing sepsis-induced mortality.

Cur Inhibits PD-L1 Expression in Neutrophils of CLP Rats

We investigated the expression of PD-L1 in the blood and lung tissues of rats to unveil the mechanism behind curcumin's impact on sepsis. Results showed a significant elevation in the expression of this protein in neutrophils from the CLP group compared to the normal and Sham groups

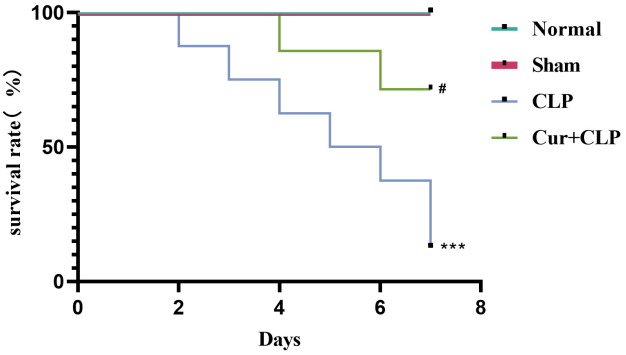

Fig. 1. Cur increases survival in CLP rats. The 7-day survival rate of rats after surgery. ***$p < 0.001$ vs Sham, $^{\#}p < 0.05$ vs CLP. CLP, cecum ligation and puncture; Cur, curcumin. n = 8.

in the blood ($p < 0.0001$). In contrast, rats in the Cur+CLP group, treated with curcumin, exhibited a reduction in the expression of this protein in neutrophils compared to the CLP group ($p < 0.01$).

Similarly, in lung tissues, the expression of this protein in the CLP group surpassed that in the normal and Sham groups ($p < 0.0001$), while the Cur+CLP group displayed a decrease in the expression of this protein compared to the CLP group ($p < 0.0001$), as illustrated in Fig. 2. These findings suggest an upregulation of PD-L1 expression in neutrophils within the blood and lung tissues of septic rats. Importantly, curcumin intervention demonstrated a downregulating effect on the expression of this protein, thus mitigating the progression of sepsis.

Cur Alleviates Lung Injury in CLP Rats

Afterward, we investigated the influence of curcumin on the histopathological morphology, apoptosis, and MPO activity in the lungs of septic rats, as depicted in Fig. 3. Histological examination using HE staining of lung tissue sections revealed significant lesions in the CLP group compared to the normal and Sham groups. These lesions were characterized by widened alveolar walls, intense inflammatory infiltration, and localized rupture of blood vessel walls accompanied by bleeding. Notably, the Cur+CLP group, which received curcumin treatment, exhibited a reduction in the severity of lung tissue lesions compared to the CLP group.

TUNEL histochemistry of lung tissue sections highlighted an increased level of apoptosis in the CLP group compared to the normal and Sham groups ($p < 0.0001$). Conversely, the Cur+CLP group demonstrated an improvement in the extent of apoptosis compared to the CLP group ($p < 0.001$). Results from the kit assay indicated a significant elevation in MPO activity in the lung tissues of the CLP group compared to the normal and Sham groups ($p < 0.01$). In contrast, the Cur+CLP group, subjected to curcumin treatment, displayed a reduction in MPO activity compared to the CLP group ($p < 0.01$).

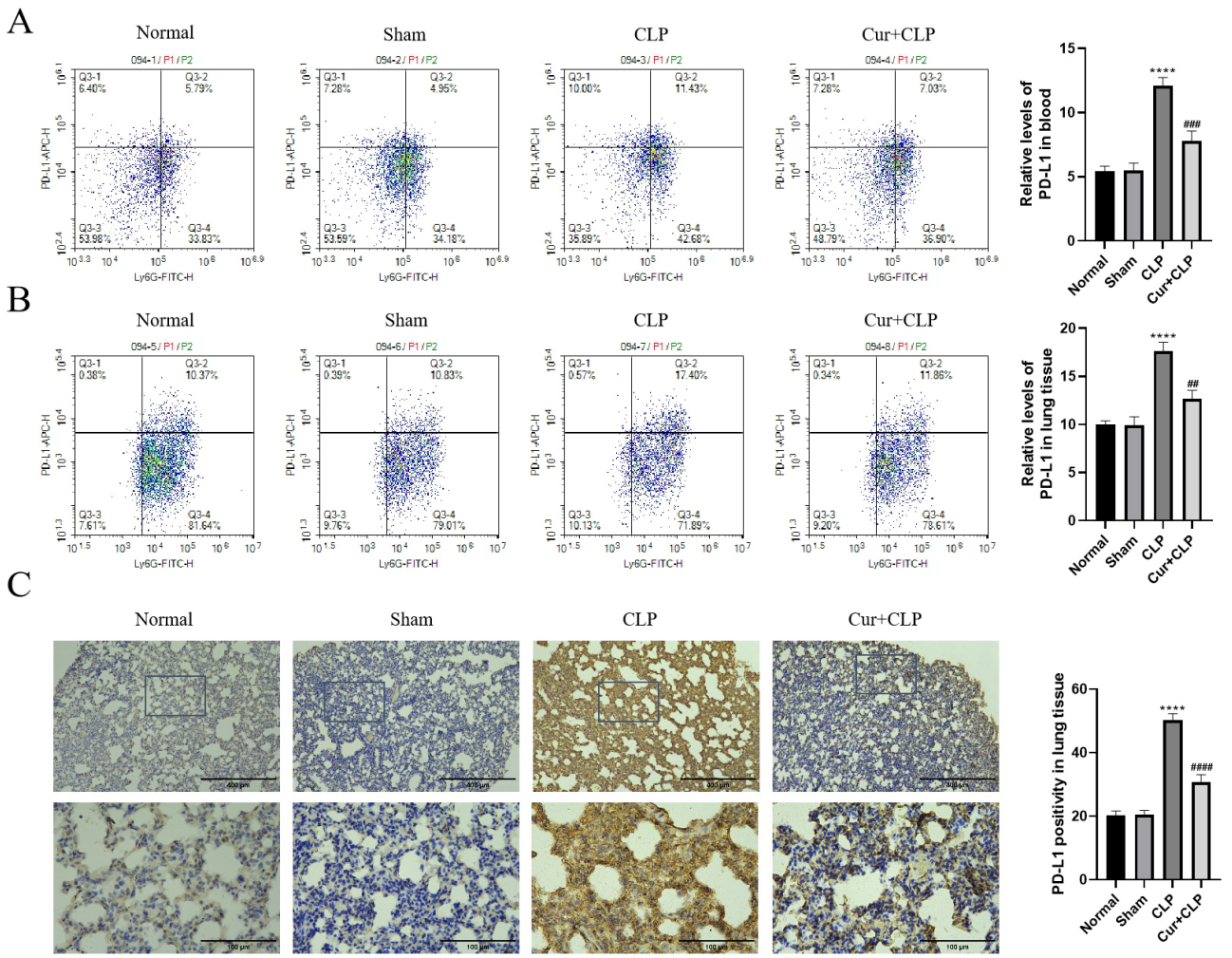

Fig. 2. Curcumin inhibits PD-L1 expression in neutrophils of CLP rats. Programmed cell death 1 ligand 1 (PD-L1) expression in blood (A) and lung tissue (B) neutrophils was measured by flow cytometry. (C) PD-L1 expression in lung tissue was detected by immunohistochemistry (100× magnification, Scale = 400 µm; 400× magnification, Scale = 100 µm). ****$p < 0.0001$ vs Sham, $^{\#\#}p < 0.01$ vs CLP, $^{\#\#\#}p < 0.001$ vs CLP, $^{\#\#\#\#}p < 0.0001$ vs CLP. n = 3.

These findings collectively suggest that sepsis induces substantial lung lesions, extensive apoptosis of lung cells, and activation of MPO in lung cells. Importantly, the administration of curcumin effectively mitigates sepsis-induced lung injury in rats.

Cur Ameliorates Thymic Tissue Damage in CLP Rats

We investigated the impact of curcumin on the pathological morphology and apoptosis of the thymus, an immunomodulatory organ in rats. As illustrated in Fig. 4, histological examination of thymus tissue sections via HE staining revealed significant differences among groups. Compared to the normal and Sham groups, the thymus tissue in the CLP group exhibited pronounced lesions characterized by reduced size of the thoracic lobules, blurred interstitial spaces, prominent proliferation of connective fibrous tissues, scattered and sparse arrangement of lymphocytes, some of which formed hollow structures by detaching from the interstitium, and others displayed conspicuous concavity upon complete detachment. Conversely, in the Cur+CLP group, where curcumin was administered, the degree of thymic tissue lesions showed improvement compared to the CLP group.

Analysis of TUNEL histochemistry in thymus tissue sections revealed a significantly higher level of apoptosis in the CLP group compared to both the normal and Sham groups ($p < 0.0001$). Moreover, the degree of apoptosis in the curcumin-treated Cur+CLP group was notably improved compared to the CLP group ($p < 0.001$). These findings suggest that sepsis induces thymic lesions and apoptosis in rats, while the administration of curcumin effectively mitigates these effects induced by sepsis in the rat thymus.

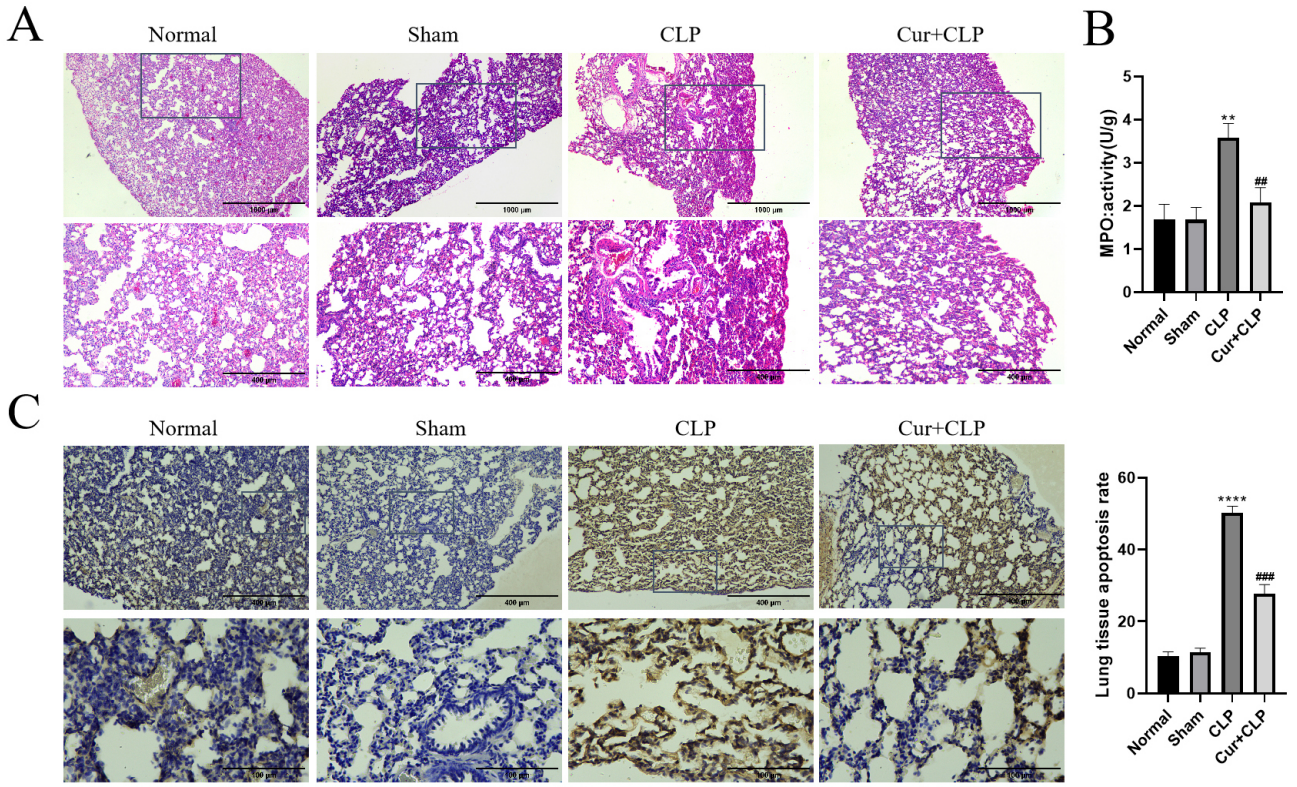

Fig. 3. Cur alleviates lung injury in CLP rats. (A) Pathological morphology of lung tissue by hematoxylin and eosin (HE) staining (40× magnification, Scale = 1000 µm; 100× magnification, Scale = 400 µm). (B) Kit to determine myeloperoxidase (MPO) in lung tissue. (C) Apoptosis of lung tissue cells by Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) histochemistry (100× magnification, Scale = 400 µm; 400× magnification, Scale = 100 µm). $^{**}p < 0.01$ vs Sham, $^{****}p < 0.0001$ vs Sham, $^{\#\#}p < 0.01$ vs CLP, $^{\#\#\#}p < 0.001$ vs CLP. n = 3.

Cur Inhibits the Inflammatory Response in Septic Rats

Examining the pivotal role of inflammatory factors in systemic immune diseases, we employed enzyme-linked adsorption assays to determine the concentrations of TNF-α, IL-6, and IL-10 in rat plasma, elucidating the impact of curcumin on inflammatory factors. Results revealed a significant abnormal elevation in the plasma concentrations of TNF-α and IL-6, along with a notably lower concentration of IL-10 in the CLP group compared to the normal and Sham groups ($p < 0.01$). However, following the administration of curcumin, all the aforementioned results were reversed ($p < 0.05$), as depicted in Fig. 5.

These findings underscore the abnormal elevation of pro-inflammatory factors and suppression of anti-inflammatory factors in rat plasma during sepsis. Importantly, curcumin intervention effectively reduced the concentration of pro-inflammatory factors and appropriately increased the concentration of anti-inflammatory factors in rat plasma. This suggests curcumin's potential to inhibit the inflammatory response in septic rats.

Cur Attenuates PD-L1-Positive Neutrophil-Induced T-Lymphocyte Apoptosis

In our final investigation, we examined the influence of curcumin on the expression of PD-L1 and programmed cell death 1 (PD-1) in neutrophils, as well as T-lymphocyte apoptosis. This involved co-culturing blood neutrophils from both the Sham and CLP groups with T cells from normal mice, as depicted in Fig. 6. TUNEL results indicated a significant increase in cell apoptosis in the CLP-C group compared to the Sham-C group ($p < 0.0001$). Importantly, compared to the CLP-C group, both the Cur+CLP-C group and the anti-PD-L1 antibody+CLP-C group exhibited a significant decrease in cell apoptosis ($p < 0.0001$).

Western blot results revealed a substantial elevation in the expression of PD-L1 and PD-1 in the co-cultured cells of the CLP-C group compared to the Sham-C group ($p < 0.001$). Intriguingly, the Cur+CLP-C group reversed this phenomenon ($p < 0.05$), as depicted in Fig. 6. These findings underscore the crucial role of curcumin in inhibiting the expression of PD-L1 and PD-1 in neutrophils of septic mice, thereby suppressing T-lymphocyte apoptosis through the regulation of these protein expressions.

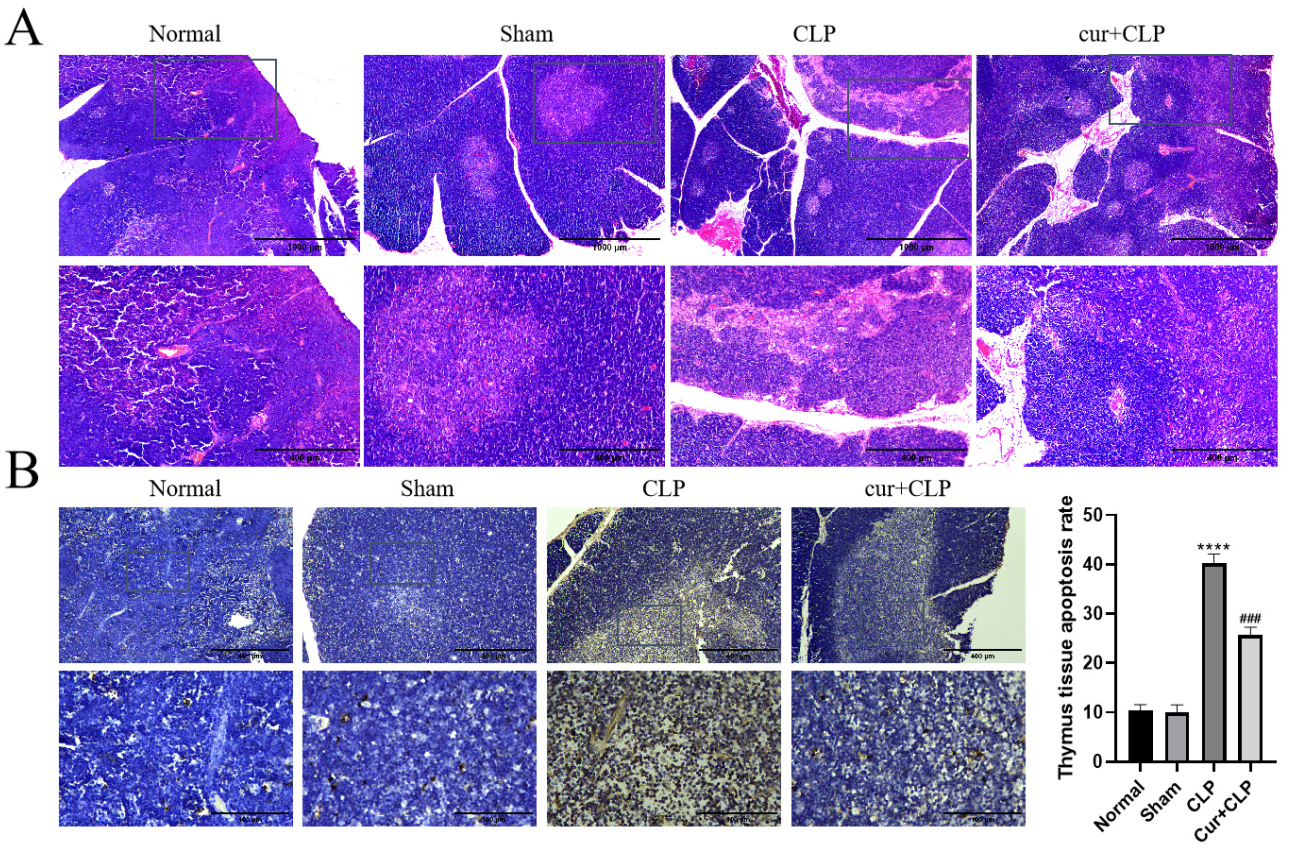

Fig. 4. Cur ameliorates thymic tissue damage in CLP rats. (A) Pathological morphology of thymus tissue detected by HE staining (40× magnification, Scale = 1000 μm; 100× magnification, Scale = 400 μm). (B) Apoptosis of thymus tissue cells detected by TUNEL histochemistry (100× magnification, Scale = 400 μm; 400× magnification, Scale = 100 μm). ****$p < 0.0001$ vs Sham, $^{###}p < 0.001$ vs CLP. n = 3.

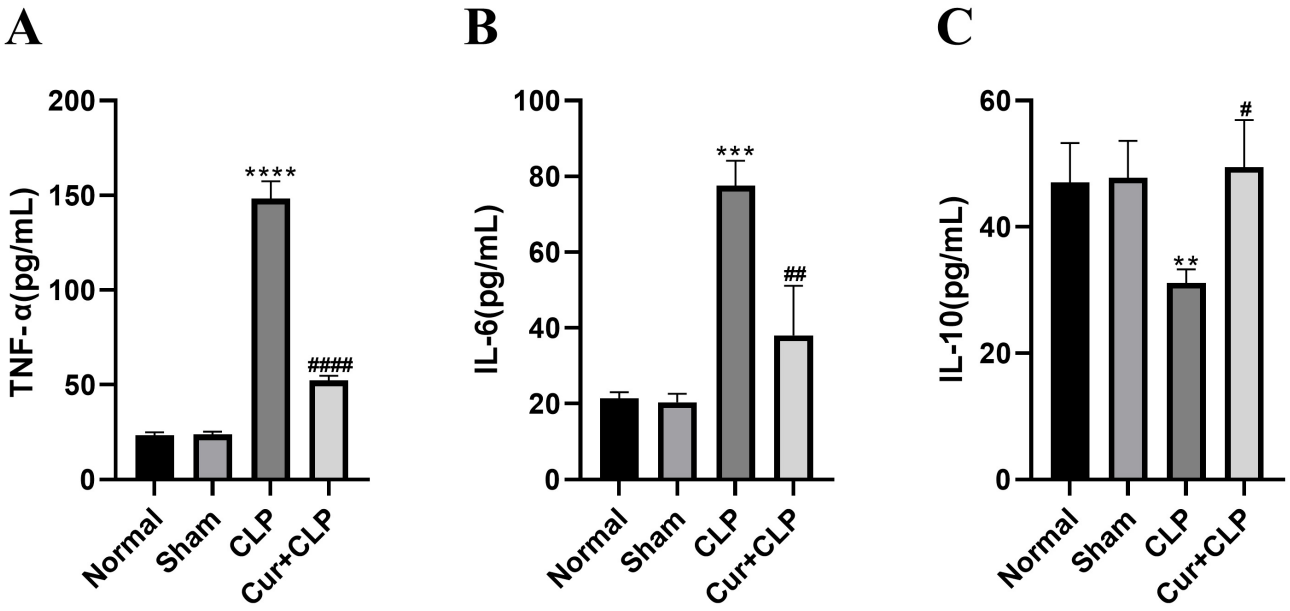

Fig. 5. Curcumin attenuates the inflammatory response in septic rats. Plasma concentrations of tumor necrosis factor-α (TNF-α) (A), interleukin 6 (IL-6) (B), and interleukin 10 (IL-10) (C) were assessed via Enzyme-linked immunosorbent assay. **$p < 0.01$ vs Sham, ***$p < 0.001$ vs Sham, ****$p < 0.0001$ vs Sham, $^{#}p < 0.05$ vs CLP, $^{##}p < 0.01$ vs CLP, $^{####}p < 0.0001$ vs CLP. n = 3.

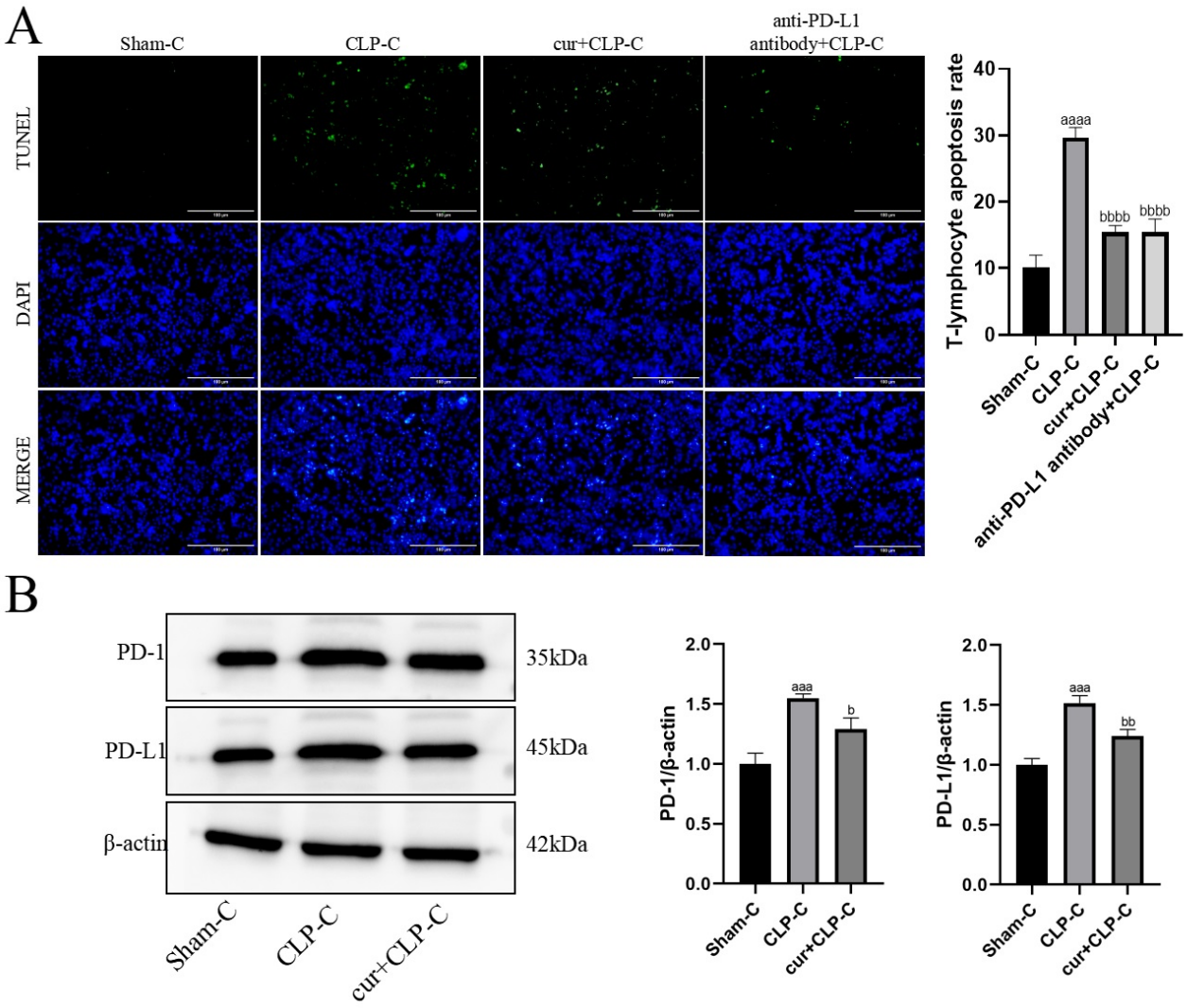

Fig. 6. Cur attenuates PD-L1-positive neutrophil-induced T-lymphocyte apoptosis. (A) T-lymphocyte apoptosis detected by TUNEL (400× magnification, Scale = 100 µm). (B) PD-L1, programmed cell death 1 (PD-1) expression detected by WB. $^{aaa}p < 0.001$ vs Sham-C, $^{aaaa}p < 0.0001$ vs Sham-C, $^{b}p < 0.05$ vs CLP-C, $^{bb}p < 0.01$ vs CLP-C, $^{bbbb}p < 0.0001$ vs CLP-C. n = 3. CLP-C, Neutrophils isolated and purified from blood specimens of rats in the CLP group in animal tests.

Discussion

Sepsis, a globally recognized life-threatening inflammatory syndrome, presents a significant challenge due to its elevated fatality rates. Ongoing research efforts into sepsis are resulting in updates to clinical definitional criteria and a clearer comprehension of its immunopathology and pathogenesis [23]. Current therapeutic strategies can be broadly classified into six groups: interventions targeting injury-associated molecular patterns, interventions targeting pathogen-associated molecular patterns, modulation of inflammatory mediators, immune-preventive modulation, stabilization of endothelial barriers, and restoration of vascular anticoagulant properties [24].

Presently, two primary types of animal models are utilized for sepsis studies: those induced by lipopolysaccharide (LPS) and the CLP model. The CLP model, implemented through cecum ligation and puncture, offers a versatile approach for creating sepsis animal models including a broad spectrum of microorganisms. Notably, CLP models often provoke a more prolonged systemic inflammatory response, making them particularly well-suited for investigations requiring extended therapeutic experimental observations [25]. Moreover, the CLP model has demonstrated superiority over the LPS model when studying physiological functions [26]. In our study, the CLP model was successfully established through cecum ligation and puncture, resulting in a significantly higher 7-day mortality rate in the

CLP model group. This observation validates the successful establishment of the sepsis model in our experimental setup. The primary cause of death in patients with sepsis is often related to immunosuppression rather than an overwhelming inflammatory response [27].

In the context of sepsis, the apoptosis of lymphocytes, especially T-lymphocytes, is recognized as a crucial step in the progression of immunosuppression [28]. PD-L1 and programmed cell death 1 (PD-1), present on the surface of T-lymphocytes, engage in interactions that lead to the apoptosis of these lymphocytes [29]. Neutrophils undergo an upregulation of PD-L1 expression during sepsis [30]. Given the abundance of neutrophils in the blood, the inhibitory impact of PD-L1-positive neutrophils on T-lymphocytes is considered particularly significant. Therefore, pharmaceutical interventions specifically targeting PD-L1 may hold the potential to alleviate the consequences of T-lymphocyte apoptosis. Curcumin, as demonstrated in various cancer studies, has shown an ability to modulate the immune system by influencing the interaction between PD-1 and PD-L1, achieved through the inhibition of PD-L1 expression [17]. In our present study, a noticeable increase in the population of PD-L1-expressing neutrophils was observed in the blood and lung tissues of septic rats. This increase was significantly reduced in septic rats treated with curcumin. Additionally, results from *in vitro* experiments provided further confirmation that curcumin acts to protect T lymphocytes from the adverse effects of PD-L1-positive neutrophils.

Sepsis has the potential to induce acute injuries across multiple organs in the body, with the lungs often being the initial site of impact. As the primary organ in the respiratory system, the lungs are directly exposed to the external environment and have the largest epithelial surface. Serving as an immune organ, the lungs can mount robust immune responses [20]. The systemic inflammatory response triggered by sepsis can cause severe damage to alveolar epithelial cells, accompanied by the infiltration of inflammatory cells. This cascade of events may progress to acute lung injury or even evolve into acute respiratory distress syndrome, thereby increasing the risk of dysfunction in other organs [31].

In our current study, the rat model of sepsis exhibited pronounced lesions and apoptosis in the lungs and thymus, accompanied by a significant elevation in the pneumonia marker MPO. Conversely, septic rats treated with curcumin showed an improvement in the extent of lung and thymus lesions and apoptosis, accompanied by a significant reduction in MPO levels.

Until now, research on the use of curcumin for sepsis treatment has primarily focused on the early stages of the condition, with a primary emphasis on targeting inflammatory mediators [32]. There has been a notable absence of reports on curcumin's potential in treating sepsis by specifically targeting PD-L1 to regulate immunosuppression. Our study fills this gap by substantiating, through both in vivo and in vitro experiments, that curcumin treatment effectively restrains PD-L1 expression during sepsis. This inhibition, in turn, safeguards T lymphocytes from apoptosis and concurrently demonstrates a mitigating effect on lung injury in septic rats *in vivo*. While existing literature has indicated a significant increase in PD-L1 expression in blood neutrophils during sepsis [33], our present findings diverge by revealing not only an elevation in PD-L1 expression in blood neutrophils during sepsis episodes but also an augmentation in PD-L1 expression in neutrophils within lung tissues. This observation suggests a simultaneous occurrence of heightened PD-L1-positive neutrophils across multiple organs during sepsis episodes.

In the intricate landscape of sepsis-induced apoptosis, numerous pathways come into play, activated by a diverse array of cell death stimuli. Consequently, preventing apoptosis in T lymphocytes by targeting a single factor proves to be a formidable challenge [34]. Amidst the multitude of contributing factors, the PD-1-PD-L1 axis emerges as a pivotal pathway exerting a substantial influence on T lymphocytes [34,35]. Immunotherapies designed to intervene in this pathway show promise in mitigating T-lymphocyte apoptosis, thereby offering a potential avenue for alleviating immunosuppression during sepsis. This form of immunotherapy holds significant potential as a crucial treatment approach for sepsis in the foreseeable future.

Nonetheless, certain limitations exist within the scope of this study. Firstly, while T-lymphocyte apoptosis stands out as a crucial factor in the evolution of immunosuppression, it is crucial to acknowledge the potential involvement of other contributing factors. Further comprehensive investigations are warranted to ascertain whether curcumin equally impacts these additional factors. Secondly, the animal models employed for sepsis, though valuable, do not entirely replicate the intricacies of sepsis pathogenesis in humans. Consequently, the effects of curcumin observed in rats may diverge from those in human subjects. Subsequent studies should prioritize exploring the applicability of curcumin in sepsis patients. In conclusion, beyond curcumin's demonstrated capacity to target diverse inflammatory factors for alleviating sepsis-induced systemic inflammatory responses, it also emerges as a promising contender for addressing sepsis-induced immunosuppression.

Conclusion

Curcumin effectively alleviates lung and thymus injury during sepsis and attenuates apoptosis of rat T lymphocytes by down-regulating PD-L1 expression in centrocytes both *in vivo* and *in vitro*.

Availability of Data and Materials

The data used and analyzed during the current study are available from the corresponding author.

Author Contributions

JZ designed the research study and provided help and advice on the experiments. YQ performed the research and analyzed the data. JG contributed to formal analysis, ensuring accurate data interpretation, while GH was key in investigation, handling experimental setup and data collection. All authors were involved in the drafting and critical revision of the manuscript. All authors have read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was approved by the Animal Ethics Committee of Hunan Evidence-based Biotechnology Co., Ltd. (No.XZ2024013).

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Conflict of Interest

The authors declare no conflict of interest.

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