

## Original Article

# Immune suppression reversal of the spleen: a promising strategy for improving the survival rate of sepsis in rats

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Received December 7, 2020; Accepted June 5, 2021; Epub August 15, 2021; Published August 30, 2021

**Abstract:** Evidence suggests that immune dysfunction exerts a central role in the morbidity and mortality of sepsis. As the spleen is the largest lymphatic tissue in the body, its influence on immune regulation during sepsis should be explored. In this study, we analysed the immune alterations of the spleen of septic rats and the effects of splenectomy at 6 h, 12 h, and 24 h following caecal ligation and puncture (CLP). Results showed declines in CD4<sup>+</sup> T cells and elevations in lymphocyte apoptosis, the percentage of Treg cells, and inflammatory cytokine levels (TNF- $\alpha$ , IL-6, and IL-10) in the spleens of CLP-induced septic rats. Moreover, splenectomy improved the survival of septic rats and bacterial clearance from peripheral blood. CLP-induced apoptosis of lymphocytes and the decreased CD4<sup>+</sup> T cell percentage in the peripheral blood could be reversed in splenectomy-treated rats. Splenectomy greatly decreased the number of white blood cells, lymphocytes, monocytes, neutrophils, and serum concentration of TNF- $\alpha$  and IL-10 after CLP. Moreover, splenectomy alleviated pathologic damage to the liver and lungs and weakened expression of CD163. These novel findings demonstrate that immune disorders of the spleen are important pathogenic factors during the course of severe sepsis. Splenectomy could alleviate apoptosis and reduction of lymphocytes induced by sepsis, and lower the level of inflammation in the body. Reversing the immune suppression of the spleen may be a novel strategy to improve sepsis survival.

**Keywords:** Splenectomy, sepsis, immune suppression, cell apoptosis, CD163

## Introduction

Sepsis is a life-threatening condition with high mortality, which begins with a systemic inflammatory response and eventually proceeds into acute infection with tissue and organ injury [1]. In sepsis, multiple inflammatory cytokines are synthesized and released from the innate immune system to defend against invading pathogens and infections [2]. However, the systemic release of tremendous amounts of pro-inflammatory cytokines during immune hyperactivation often results in widespread inflammation, multiple organ failure, and even death [3-5]. This cytokine storm is followed by a prolonged period of relative immunosuppression [6], and this immune state transition, probably to a large extent, is due to adaptive immune

dysfunction, including T and B lymphocytes [7]. Additionally, the immune cell depletion caused by lymphocyte apoptosis or programmed cell death is a critical cause of immunosuppression during sepsis [8]. Evidence suggests that immune dysfunction exerts a central role in the morbidity and mortality of sepsis.

The spleen, the largest lymphoid organ in humans and main source of circulatory pro-inflammatory cytokines, exerts a vital role in immunity [9, 10]. Immune cells from the spleen exert a crucial role in the progression of sepsis. Previously, scientists have investigated changes in the immune cells of the spleen during the development of sepsis, and thus they are often used as evaluation criteria for sepsis therapy [11]. Particularly, T cells exert a special function

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in the immune response. Because they are not only a crucial component of the adaptive immune system, but also participate in the innate immune response. It has been found that extensive apoptosis and functional loss of splenic CD4 and CD8 T cells in septic patients can lead to immunosuppression [12]. Some studies have confirmed that splenectomy is both preventive and protective with regard to sepsis [13]. Splenectomy can suppress the anti-inflammatory and anti-apoptotic activity, as well as reduce the production of inflammatory cytokines in the circulatory system. Splenectomy also exhibits protective effects on the kidney [14].

In this study, we hypothesised that the spleen was significantly immunosuppressed during sepsis and that a splenectomy could reduce both sepsis-induced apoptosis and the level of inflammatory cytokines, thus protecting the body by achieving a balanced immune response.

### Materials and methods

#### Rats

Male SD rats (weighing 200 to 250 g), were bred and housed at the Laboratory Animal Center of Xi'an Jiaotong University with standard rodent chow and water under SPF conditions (12-h light/dark cycle and temperature of 22°C). All experiments were authorized by the Institutional Animal Care and Use Committee of Xi'an Jiaotong University and were carried out in accordance with the guidelines for the Care and Use of Laboratory Animals (No. XJTULA2019-928).

#### CLP operation

Briefly, the rats were anesthetized via intraperitoneal injection of chloral hydrate (4%, 4 ml/kg). The caecum was exposed by making an approximately 1.5 cm midline incision on the anterior abdomen and was ligated with 3-0 silk that was tied below the ileocaecal valve while preserving the blood flow to the caecum. Then a double puncture with an 18-gauge needle on the caecal wall was conducted to induce polymicrobial sepsis, and a small quantity of faeces (2 mm in length) was squeezed out. The incision was closed in two layers. Following surgical procedures, the rats were resuscitated with

a 5 mL sterile physiologic saline solution, then placed under a heating lamp for 4 h to ensure a warm body temperature, and supplied with sterile diets and distilled water ad libitum.

#### Splenectomy and grouping

Rats were sorted into four groups at random: control (normal), sham, CLP, and CLP+Splenectomy (SPX). Rats were anesthetised as described above. In the splenectomy group, the splenectomy was performed before CLP. Briefly, the rats were anesthetised, and a minimal incision was made in the left subcostal region of the abdomen under aseptic conditions. After ligating the corresponding arteries and veins, the spleen was excised and the abdominal incision was sutured.

#### Survival of septic rats

Sixty rats were randomly classified into the following four groups (fifteen rats each): control, sham, CLP, and CLP+SPX. The survival rates were monitored for 3 days to explore the survival times of septic rats.

#### Measurement of bacterial load

Briefly, 10 µl of peripheral blood was harvested aseptically through heart puncture at 24 h post-surgery, and diluted with 100 µl aseptic PBS. The diluted peripheral blood was then plated on tryptic soy agar plates containing 10% sterile sheep blood, and cultured at 37°C for 24 h. The number of colony forming units per millilitre (CFU/mL) in the peripheral blood was calculated.

#### Analysis of tissue histology

Tissues (spleen, liver and lung) were harvested 24 h after surgery and immersed in 10% formalin. After embedding the samples in paraffin, 3-µm-thick paraffin sections were cut and floated onto glass slides. Slides were dyed using haematoxylin-eosin (H&E) to evaluate tissue morphology. Histopathological injuries were assessed under a light microscope (Nikon, Ti-E, Japan).

#### Flow cytometry

We harvested the peripheral blood and spleens from rats at 6, 12, and 24 h post-surgery. The peripheral blood and spleens were prepared

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into single-cell suspensions, and were then incubated with APC-conjugated anti-rat CD3, FITC-conjugated anti-rat CD4 and PE-conjugated anti-rat CD8a, following the manufacturer's protocol (eBioscience, San Diego, CA, USA). Stained cells were recorded using the flow cytometer (FACSCanto II; BD Bioscience, San Jose, CA, USA).

In addition, apoptosis of the peripheral blood cells or splenocytes was examined after staining with annexin V-FITC and propidium iodide (PI) (eBioscience, San Diego, CA, USA) by the flow cytometer, in accordance with the manufacturer's instructions. The P1 gate was used to represent lymphocytes (red dots). Data were plotted and analyzed by GraphPad Prism 5.0 (San Diego, CA).

### *Complete blood count*

The fresh peripheral blood samples were stored in an anticoagulant tube containing EDTA, and a complete blood count (CBC) with differential was performed on each sample using a HEMAVET 950FS small animal blood analyser.

### *Enzyme linked immunosorbent assay (ELISA)*

The concentrations of tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ), interleukin-6 (IL-6), and interleukin-10 (IL-10) in serum samples and the spleen tissues were examined by employing commercial ELISA kits (DEKEWE, Shenzhen, China) following the instructions supplied by the manufacturer. Data were analyzed using t-test by GraphPad Prism 5.0.

### *Statistical analysis*

All statistical analyses were carried out using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA), and the results were displayed as the mean  $\pm$  standard deviation (SD). For multiple comparisons, one-way analysis of variance (ANOVA) was carried out to assess the statistical significance. *P*-value of  $<0.05$  was considered as significant.

## **Results**

### *Sepsis induced increased mortality, bacterial load, spleen index, and aggravated pathologic damage of spleen tissue in CLP-induced septic rats*

In this study, the rat model of CLP-induced sepsis was successfully constructed and the rats

in the CLP group showed typical signs of septic shock, such as hypothermia, tremor, lethargy, and bristled hair. Compared to the sham group and the control group, we found the survival rate of CLP-induced rats was extremely low (0% versus 100%) (**Figure 1A**) and the blood bacterial burden was significantly increased at 24 h after CLP surgery ( $P<0.01$ ) (**Figure 1B**). The spleen indices were increased for all subsequent time points after CLP, but were not significantly different (**Figure 1C**). In contrast, compared with the control group, the H&E stain of spleen tissue of CLP-induced rats showed obvious hyperplasia in the white pulp and lymphoid nodules. Specifically, the arterial peripheral lymphoid sheath was significantly thickened at 24 h, and separation between the white pulp and red pulp was ill-defined (**Figure 1D**).

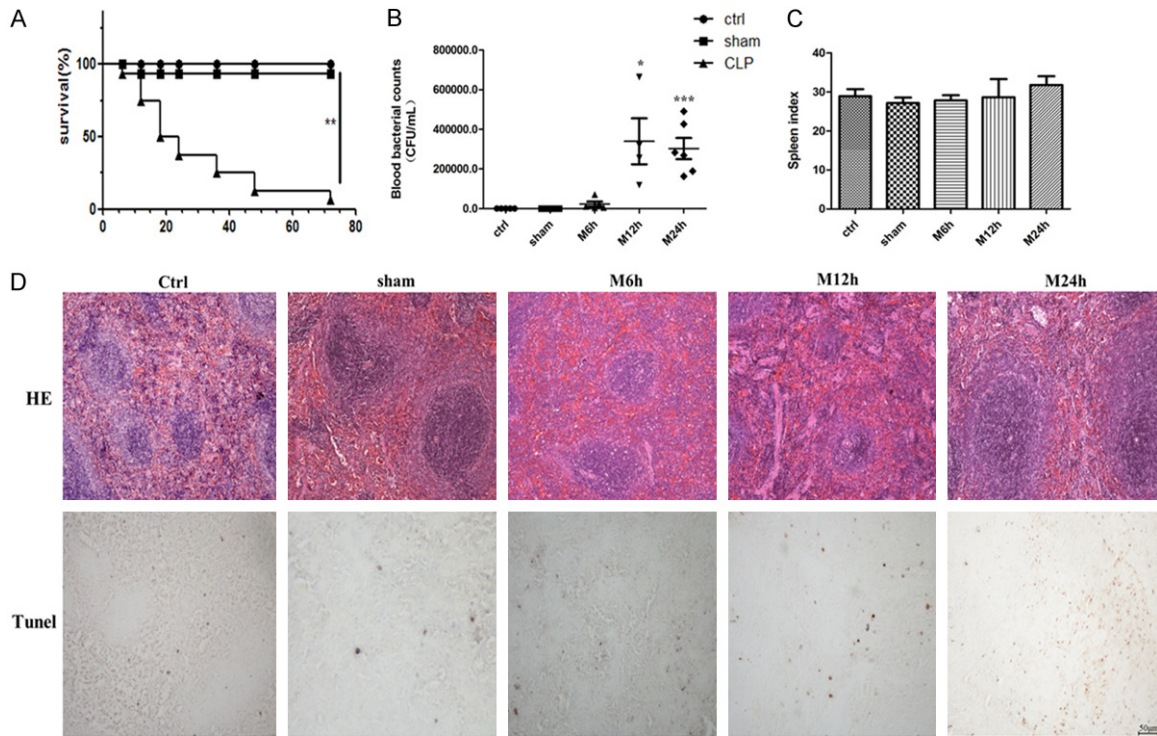
### *Sepsis elevated the percentage of apoptotic splenic lymphocytes and Treg cells, diminished the proportion of CD4<sup>+</sup> T lymphocytes, and elevated the excretion of inflammatory cytokines in the spleens of CLP rats*

First, we observed the apoptosis of splenic lymphocytes in septic rats, as shown in **Figure 2A**. The proportion of apoptotic cells in the spleens of sepsis rats was increased during the entire experimental period and significant differences were found between 12 h and 24 h, from early lymphocyte apoptosis, to late lymphocyte apoptosis, with a vast increase at 24 h ( $P<0.05$ ).

Second, we surveyed the changes in the proportion of splenic T lymphocytes in septic rats. As shown in **Figure 2B**, we found the number of CD3<sup>+</sup>CD4<sup>+</sup> helper T cells in the spleen was dramatically reduced at 6, 12 and 24 h, but the proportion of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells rose rapidly and was significantly higher in the CLP group than the control group at 24 h ( $P<0.01$ ). Unexpectedly, although the number of CD3<sup>+</sup>CD8<sup>+</sup> cytotoxic T cells increased, there was no significant difference between the control group and CLP group. As for CD4<sup>+</sup>/CD8<sup>+</sup> T cells, its proportion dramatically declined at 24 h after CLP induction ( $P<0.01$ ).

Next, we detected the concentration of inflammatory factors in the spleens of CLP rats using ELISA and found that the concentrations of pro-inflammatory cytokines TNF- $\alpha$  and IL-6 were increased. Interestingly, the concentration of anti-inflammatory cytokine IL-10 was also

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**Figure 1.** Survival rate, blood bacterial counts, spleen index, and pathologic staining of the spleen of septic rats induced by cecal ligation and puncture (CLP). A. Survival was followed for 3 days (n=15 per group). B. The blood bacterial counts were obtained by calculating the number of colony forming units (CFU) per mL of the peripheral blood (n=6 per group). C. Spleen index were calculated by (spleen weight/rat weight) × 10. D. Hematoxylin-eosin and TUNEL stain for spleen at 6 h, 12 h and 24 h after CLP operation (Original magnification, 100×). (\*P<0.05, \*\*P<0.01, \*\*\*P<0.001 compared to control group).

increased at 12 h following CLP treatment (P<0.05) (**Figure 2C**).

*Splenectomy protected against sepsis lethality, attenuated liver and lung injury, and alleviated apoptosis in peripheral blood*

To investigate the function of the spleen in sepsis, rats were given splenectomy or sham operation promptly before CLP treatment. Compared with the CLP group, we observed that splenectomy dramatically enhanced the survival rate of the septic rats (P<0.05) and bacterial clearance at 24 h after CLP (**Figure 3A, 3B**).

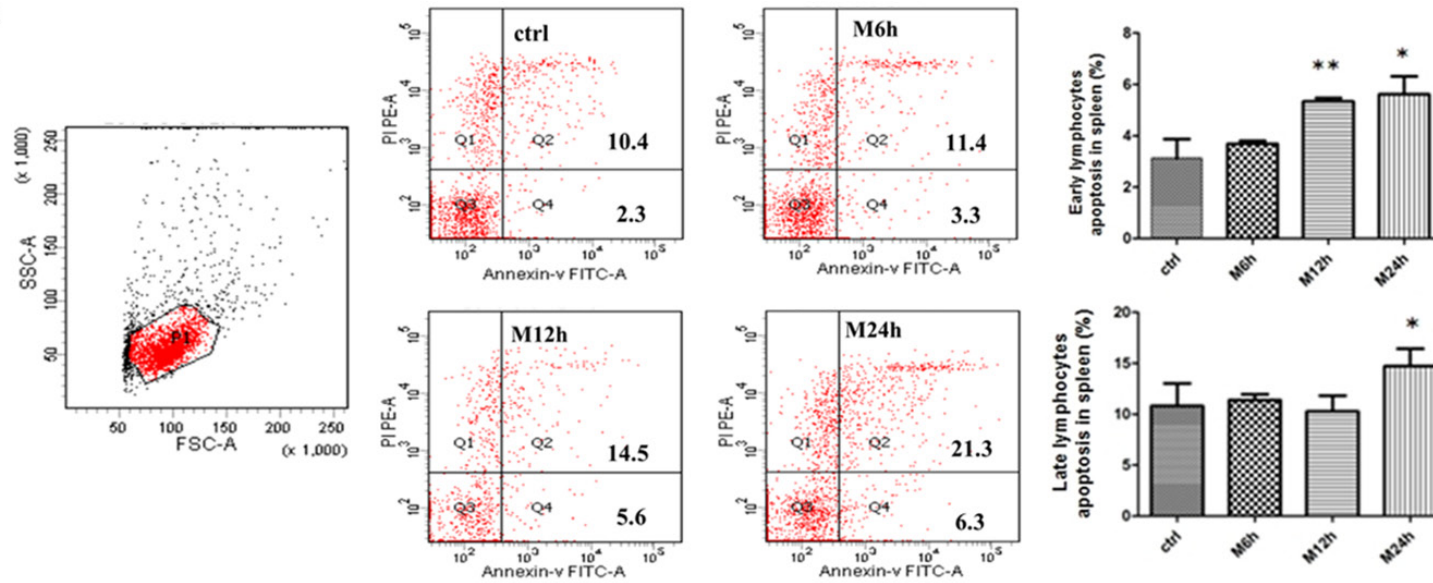
As for the peripheral blood of the CLP rats, we found that compared with the control group, the percentage of early apoptosis was increased at 12 h and 24 h (P<0.01 and P<0.001), and the percentage of late apoptosis was increased at 24 h (P<0.05) (**Figure 3C**). Splenectomy in CLP rats could greatly reduce the percentage of both early (P<0.01) and late (P<0.05) apoptosis at 24 h.

*Splenectomy decreased the quantity of immune cells, increased the quantity of platelets and the proportion of CD4<sup>+</sup> T lymphocytes, and reduced the concentration of inflammatory factors in the peripheral blood*

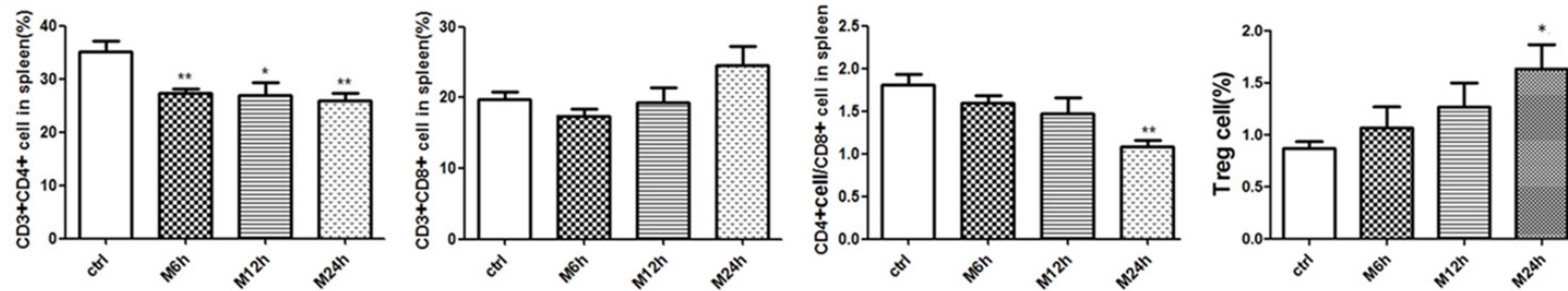
As shown in **Figure 3D**, the CLP rats showed a decrease in the quantity of leucocytes, lymphocytes, and blood platelets as compared with the control rats, whereas the number of monocytes and neutrophils were increased. We found splenectomy could raise the blood platelet count at 12 h (P<0.05) and reduce the quantity of leucocytes, lymphocytes, as well as monocytes (**Figure 3D**). We also discovered that the proportion of CD3<sup>+</sup>CD4<sup>+</sup> helper T cells and the ratio of CD4<sup>+</sup> to CD8<sup>+</sup> T cells in the peripheral blood were significantly declined in septic rats by comparison with the control group. It is interesting to note that splenectomy could increase the proportion of CD3<sup>+</sup>CD4<sup>+</sup> helper T cells and the ratio of CD4<sup>+</sup> to CD8<sup>+</sup> T cells in the peripheral blood by comparison with

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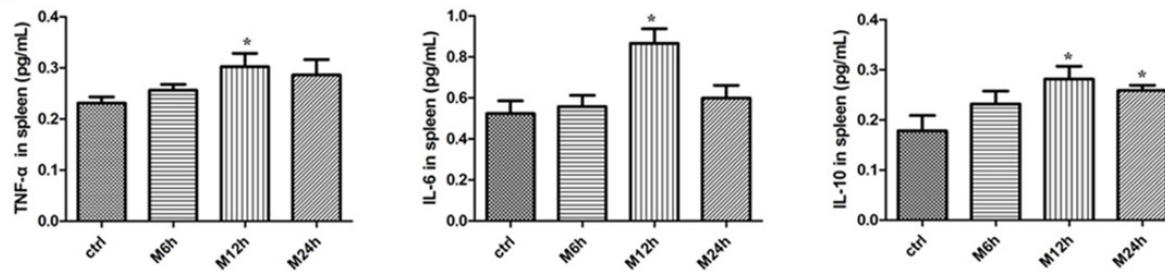
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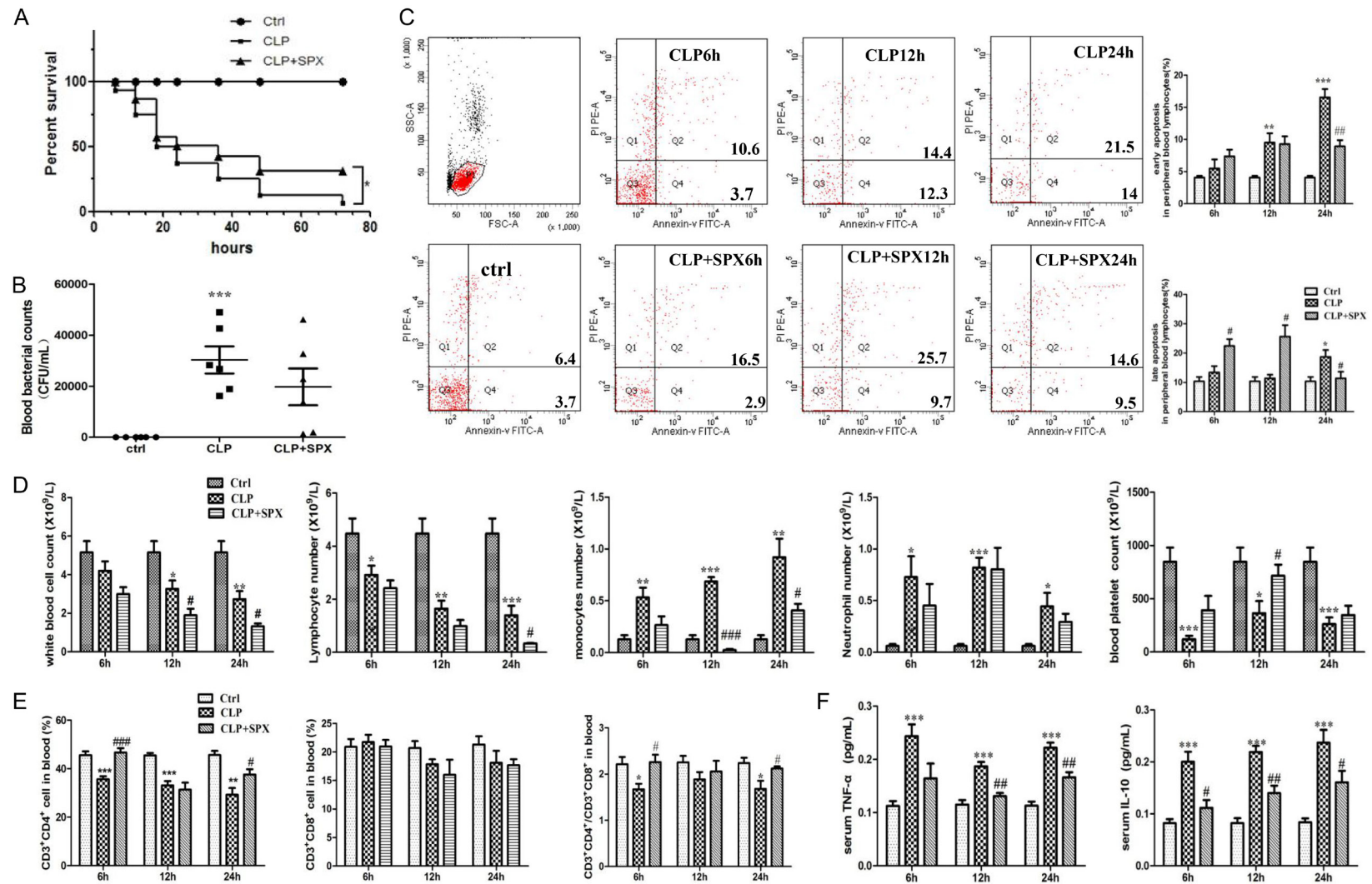


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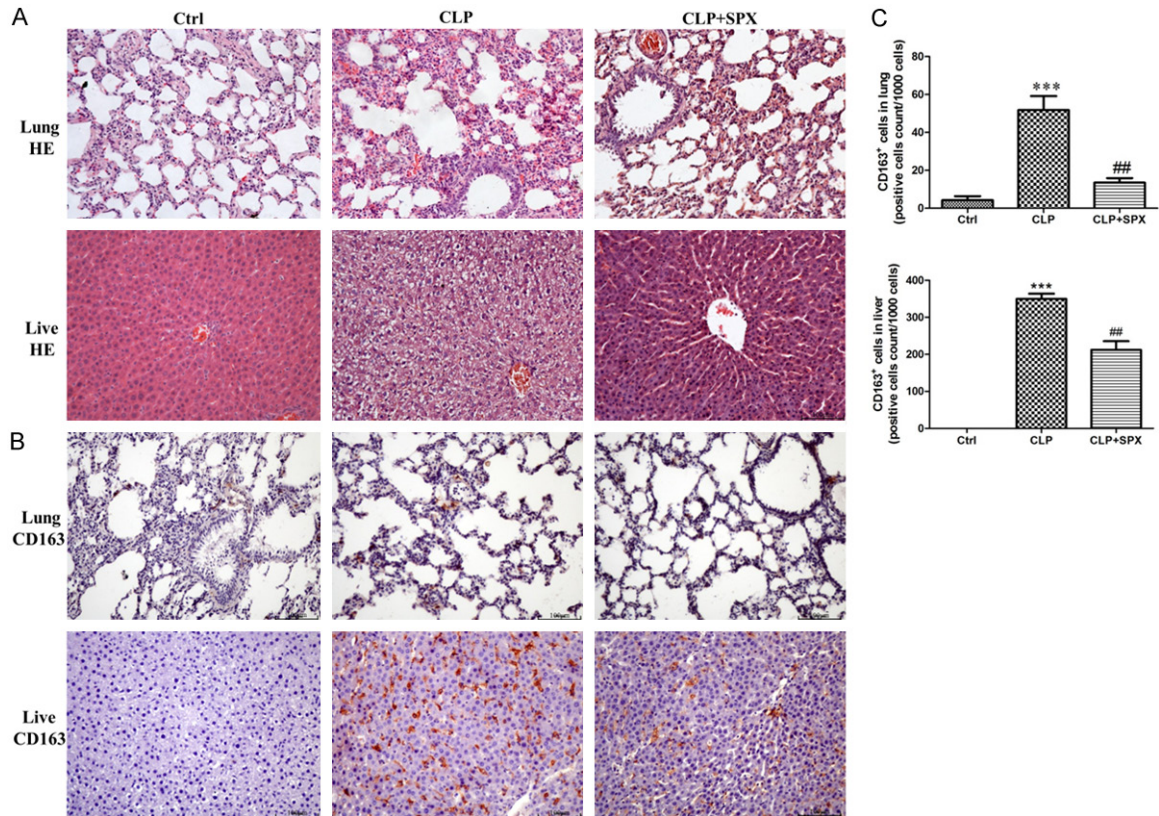
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**Figure 2.** Changes in the percents of lymphocyte apoptosis and T lymphocyte typing, and expression levels of inflammatory cytokines in spleen of septic rats at 6 h, 12 h and 24 h after CLP operation. A. Flow cytometry for the early and late lymphocyte percents of apoptosis. The P1 gate is used to represent lymphocytes (red dots). B. Flow cytometry for the percents of CD3<sup>+</sup>CD4<sup>+</sup> T cell, CD3<sup>+</sup>CD8<sup>+</sup> T cell, and Treg cell; CD3<sup>+</sup>CD4<sup>+</sup> T cell/CD3<sup>+</sup>CD8<sup>+</sup> T cell were conducted to evaluate the immune level of spleen (n=6 per group). C. ELISA assay for TNF- $\alpha$ , IL-6, and IL-10 concentrations in the spleen (n=3 per group). (\*P<0.05, \*\*P<0.01 compared to control group).



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**Figure 3.** Effects of splenectomy on septic rats. A. Survival was followed for 3 days (n=15 per group). B. The blood bacterial counts were obtained by calculating the number of colony forming units (CFU) per mL of the peripheral blood. C. Flow cytometry for the early and late lymphocyte percents of apoptosis at 6 h, 12 h, and 24 h. D. Routine blood tests for the number of white blood cell, lymphocytes, monocytes, neutrophils and blood platelets in peripheral blood (n=3 per group). E. Flow cytometry for the percents of CD3<sup>+</sup>CD4<sup>+</sup> T cells, CD3<sup>+</sup>CD8<sup>+</sup> T cells and CD3<sup>+</sup>CD4<sup>+</sup> T cells/CD3<sup>+</sup>CD8<sup>+</sup> T cells were conducted to evaluate the immune level (n=6 per group). F. ELISA assay for serum TNF- $\alpha$  and IL-10 (n=3 per group). (\*P<0.05, \*\*P<0.01, \*\*\*P<0.001 compared to control group; #P<0.05, ##P<0.01, ###P<0.001 compared to CLP group).



**Figure 4.** Effects of splenectomy on liver and lung in septic rats. Hematoxylin-eosin stain (A) and the expression of CD163 by immunohistochemical staining (B) on liver and lung were shown at 24 h after CLP operation (Original magnification,  $\times 200$ ). (C) The statistical results are expressed as number of positive cells per 1000 cells in the liver or lung. (\*\*\*P<0.001 compared to control group; ##P<0.01 compared to CLP group, n=4 per group).

the CLP group, displaying statistical significance at 6 h and 24 h (P<0.05). However, as for the proportion of CD3<sup>+</sup>CD8<sup>+</sup> cytotoxic T cells, no dramatic changes were found (Figure 3E). Additionally, the ELISA results indicated that splenectomy significantly reduced the concentration of serum TNF- $\alpha$  at 12 h and 24 h (P<0.01), and the anti-inflammatory factor IL-10 at 6 h, 12 h, and 24 h (P<0.05 or P<0.01) when compared to the CLP rats (Figure 3F).

*Splenectomy attenuated liver and lung injury and decreased the expression of CD163 in the lung and liver of the CLP rats*

Histological analysis (Figure 4A) revealed that the lungs of the CLP group showed a strong interstitial reaction, accompanied by obvious inflammation, a shrink in the alveolar interval, thickening of the septum, as well as vascular congestion. The livers of rats subjected to sepsis by CLP displayed an aggravated hepatocyte

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necrosis, inflammatory cell infiltration and bleeding. By comparison with the CLP rats, the group pre-treated with splenectomy demonstrated a better morphology of the lung parenchyma and pathologic injury of the liver.

Elevated expression of CD163 was used as an indicator of the severity of sepsis. As shown in **Figure 4B, 4C**, CD163 level was markedly increased in the liver ( $P < 0.001$ ) and lung ( $P < 0.001$ ) of septic rats 24 h after the CLP operation. However, in the CLP+SPX group, the expression of CD163 in the lung ( $P < 0.01$ ) and liver ( $P < 0.01$ ) was dramatically decreased at 24 h, compared with the CLP group.

### Discussion

Due to the lack of therapeutic options, sepsis is still a major challenge to clinicians. Currently, it is known that the malfunction of T lymphocytes may bring about the progression of infection and even death [15, 16]. As the main reservoir of lymphocytes, the spleen is likely to exert a crucial function in the development of sepsis. In the present study, we found polymicrobial sepsis could trigger an increased bacterial load in the blood, severe mortality, and higher spleen indices. In addition, hyperplasia of the white pulp and lymphoid nodules occurred, and the arterial peripheral lymphoid sheath was significantly thickened during sepsis. These observations explicitly demonstrated that spleen was injured during sepsis.

CD4 T cells, also called as T helper cells, can regulate the activities of many other immune cells. Here, we discovered that the proportion of splenic CD4 helper T cells was significantly diminished, indicating that the sustained hyperactivation of innate immune cells (i.e., monocytes or macrophages) is difficult to achieve. An increase in Treg cells is injurious during sepsis, and it is related to the decreased proliferation and loss of function of effector T cells [17] and other innate immune cells. Our findings showed that rats treated with CLP surgery experienced an elevation in the quantity of splenic apoptotic cells throughout the entire experimental period. It has been demonstrated that apoptosis is responsible for the decrease in peripheral and splenic lymphocytes during sepsis [12]. In addition, we discovered that the expression levels of effective inflammatory cytokines TNF- $\alpha$ , IL-6 and IL-10 in the spleen were also elevated after

CLP. These findings suggested that immune disorders of the spleen (including T-lymphocytes apoptosis, and inflammatory cytokine secretion) exert critical functions in sepsis-induced immune dysfunction.

To determine the precise immunologic role of the spleen during the development of sepsis in rats, we removed the spleen at the same time as the CLP operation. Many studies have shown that splenectomy patients are exposed to greater hazards of fatal septic shock caused by overwhelming bacterial infection [18]. Controversially, some studies indicate that splenectomy could inhibit the anti-inflammatory and anti-apoptosis processes and protect the kidney [14]. In our study, in the splenectomy group, we observed a significant improvement in the mortality rate, bacterial load, and pathologic damage to the liver and lungs that is caused by sepsis.

Earlier studies have confirmed that the spleen is critical in immune suppression during inflammatory diseases such as sepsis [19]. This study further revealed the weakening effect that a splenectomy has on sepsis immunosuppression. First, T cell depletion and dysfunction are the main factors leading to increased mortality in patients with non-burn sepsis. Immunologic suppression resulting from dysfunction of adaptive immune system has been recognised as a significant secondary effect during sepsis [20]. However, splenectomy significantly reduced the number of early and late apoptotic lymphocytes in the peripheral blood of sepsis rats. The quantity of CD4<sup>+</sup> T cells and the CD4<sup>+</sup>/CD8<sup>+</sup> ratio were dramatically increased, which may be responsible for the improved outcomes of septic mice [21]. These results added to the evidences that splenectomy could improve survival by effectively reducing sepsis-induced immunosuppression [22, 23]. Secondly, splenectomy also brought about a decline in the quantity of immune cells in the peripheral blood of septic rats, such as leukocyte, lymphocytes, monocytes, and neutrophils. The number of blood platelets was increased. This may help to reduce the immune response in the peripheral blood to achieve a new immune balance. The serum concentration of both pro-inflammatory cytokine TNF and anti-inflammatory cytokine IL-10 were significantly reduced at each time point in the splenectomy group, indicating this



new balance. In addition, the reduction of pro-inflammatory factors caused by splenectomy also improved neurobehavioral and infarct outcomes after stroke [24]. Many prior studies have demonstrated that CD163, as a marker of activated neutrophils and matured phagocytic cells (M2) [25], can regulate the expression of anti-inflammatory molecules, involving interleukin-10 and haem oxygenase-1 [26]. In the present study, we uncovered splenectomy could markedly decrease the level of CD163 in liver and lung tissue at 24 h in rats that had undergone the CLP operation, thereby reducing the activation of neutrophils and the polarization of M2 macrophages.

It is worthwhile to mention that there are several limitations to this study, including a lack of analysis on the changes in neutrophils, macrophages, Treg cells and other immune cells. A second limitation is that this study does not provide detailed changes in macrophage typing and cytokine expression.

In conclusion, immune disorders of the spleen play an important role during severe sepsis. Splenectomy could alleviate apoptosis and lymphocyte depletion induced by sepsis and reduce the level of inflammation in the body. Effectively reversing the immune suppression of the spleen may be a novel way to improve survival of those suffering from this lethal disease.

### Acknowledgements

This study was subsidized by grants from Shaanxi Province Natural Science Foundation (2017SF-123 and 2020JM-411).

### Disclosure of conflict of interest

None.

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