Atezolizumab alleviates the immunosuppression induced by PD-L1-positive neutrophils and improves the survival of mice during sepsis

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Abstract. Atezolizumab can reduce immunosuppression caused by T lymphocyte apoptosis in various cancer types. The current study aimed to investigate whether this drug can also alleviate immunosuppression during sepsis. For that purpose, a C57BL/6 mouse sepsis model was generated. Mice were randomly assigned to three groups: Sham, cecal ligation and puncture (CLP) and atezolizumab groups. Atezolizumab was administered *in vivo* by intraperitoneal injection. The expression of programmed death ligand-1 (PD-L1) on neutrophils and programmed death-1 (PD-1) on T lymphocytes was evaluated, and endotoxin concentration, intestinal permeability, ileum histopathological score and tight junction protein expression were assessed to determine the extent of disease in each group. The rate of T lymphocyte apoptosis was determined to assess the effects of atezolizumab on T lymphocyte apoptosis *in vivo* and *in vitro*. Survival times were also recorded to compare mouse prognosis during sepsis. In the CLP group, the proportion of PD-L1$^+$ neutrophils was significantly higher at 48, 72 and 96 h in blood, and at 24, 48, 72 and 96 h in bone marrow, compared with those of the sham group (P<0.05). The proportion of PD-1$^+$ T lymphocytes was also upregulated at 72 h in blood. In the atezolizumab group, endotoxin concentration, intestinal permeability and ileum histopathological score were lower compared with those in the CLP group (P<0.05), whereas the expression of claudin-1 and occludin proteins on ileum was higher compared with that in the CLP group (P<0.05). Both *in vivo* and *in vitro* experiments indicated that the rate of T lymphocyte apoptosis following atezolizumab treatment was lower compared with that in the CLP group (P<0.05). Survival analysis demonstrated that mice in the atezolizumab group survived longer compared with those in the CLP group (P<0.05). The current study demonstrated that treatment with atezolizumab may be an effective method for treating immunosuppression induced by sepsis.

Introduction

Sepsis is defined as serious organ dysfunction induced by a dysregulated host response to infection (1). Despite progress in therapeutic strategies, sepsis remains the leading cause of mortality of patients in intensive care units (2).

High mortality from sepsis is correlated with immune impairment, which may be caused by T lymphocyte dysfunction (3-5). Septic events trigger high levels of immune cell apoptosis, including apoptosis of T lymphocytes, resulting in suppressed immune functions, and often inducing increased susceptibility to secondary infections (6-8).

T cell apoptosis caused by sepsis is now considered to be associated with the programmed death ligand-1 (PD-L1)-programmed death-1 (PD-1) signaling axis, and the increase in PD-L1 expression during sepsis is an important indicator of immunosuppression (9). Immunotherapy targeting the PD-1/PD-L1 signaling axis is a hot topic in current research, and the tumor microenvironment is an important factor affecting the effect of this immunotherapy for numerous tumors, such as lung tumor, glioblastoma multiforme and colon cancer (10-12). Atezolizumab is an anti-PD-L1 antibody that inhibits binding of PD-L1 to its receptor PD-1, thereby restoring the suppression of T lymphocyte activity (13,14), and has shown efficacy in numerous types of cancer including locally advanced and metastatic urothelial carcinoma, non-small-cell lung cancer and metastatic renal cell carcinoma (15-17). For non-small-cell lung cancer, Fehrenbacher *et al* (16) conducted a multicenter, open-label, randomized controlled trial to evaluate the effectiveness of

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Abbreviations: CLP, cecal ligation and puncture; PD-L1, programmed death ligand-1; LPS, lipopolysaccharide; PD-1, programmed death-1; FD40S, fluorescently labeled dextran 40S; APC, allophycocyanin; H&E, hematoxylin and eosin; ZO-1, zonula occludens-1

Key words: sepsis, PD-L1, immunotherapy, survival analysis, apoptosis

atezolizumab and found that the overall survival of patients was significantly longer in the atezolizumab group than that in the docetaxel group (median overall survival, 12.6 vs. 9.7 months). Since immunosuppression of T lymphocytes also occurs in septic microenvironments (9,18), it was hypothesized that atezolizumab may alleviate T lymphocyte immunosuppression and improve prognosis during sepsis. In the present study, an animal sepsis model *in vivo* and a T lymphocyte cell line *in vitro* were used to evaluate the effects of atezolizumab during sepsis.

Materials and methods

Mouse model of sepsis and drug intervention. A total of 129 male C57BL/6 mice (age, 8-10-weeks old; weight, 20-25 g) were purchased from the Experimental Animal Center of Guangxi Medical University (Guangxi Zhuang Autonomous Region, China). All mice were housed in specific pathogen-free facilities at 25˚C and 55% humidity, under a 12-h light/dark cycle for 1 week before surgery. All mice had free access to water and food. The sepsis model was generated using the cecal ligation and puncture (CLP) procedure, according to a reported method (19). Briefly, mice were anesthetized by intraperitoneal injection of 40 mg/kg pentobarbital sodium (Sigma-Aldrich; Merck KGaA). Then, an incision was made in the lower abdomen. The cecum was ligated in the middle, and the distal cecum was punctured right through using a 21-gauge needle. A small amount of stool was squeezed into the abdominal cavity, and the abdominal incision was closed layer by layer. Mice in the control group underwent the same procedure as the mice in the sepsis group, with the exception of the CLP. All procedures were conducted under sterile conditions.

Mice were randomly assigned to three groups: Sham, CLP and atezolizumab groups. In the atezolizumab group, septic mice were treated with intraperitoneal injection of atezolizumab (100 μg on days 1 and 4; Shanghai TheraMabs Biotechnology Co., Ltd.). Mice in the other groups were treated with intraperitoneal injection of an isotype control antibody (100 μg on days 1 and 4; Shanghai TheraMabs Biotechnology Co., Ltd.).

The present study was approved by the Animal Care Committee of Guangxi Medical University (approval no. 201901006), and all experiments were conducted in accordance with the Laboratory Animal Guideline for Ethical Review of Animal Welfare issued by the National Standard GB/T35892-2018 of the People's Republic of China. Blood was collected by cardiac puncture from mice anesthetized by pentobarbital sodium intraperitoneal injection in this study. Mice were euthanized following blood collection, or when they met criteria for humane endpoints, including loss of crawling ability and loss of eating ability after surgery. Mice were euthanized with CO_2 followed by cervical dislocation, and a flow rate of 30% displacement of chamber volume per minute of CO_2 was used for euthanasia. The confirmation criteria for mouse death was the arrest of breathing and heartbeat.

Detection of PD-L1 in blood and bone marrow neutrophils by flow cytometry. Blood and bone marrow were harvested from the hearts and thighs of mice at 24, 48, 72 and 96 h after surgery. Mice were euthanized before collection of bone marrow. Red blood cells in whole blood cells were lysed using Red Blood Cell Lysis Buffer (Beijing Solarbio Science & Technology Co., Ltd.). Cells were stained with allophycocyanin (APC)-anti-Ly6G and PE-anti-PD-L1 antibodies (BD Pharmingen; BD Biosciences), and then quantified by flow cytometry on a FACSCalibur instrument (BD Biosciences), according to the manufacturer's instructions. PD-L1$^+$ neutrophils were defined as the Ly6G$^+$PD-L1$^+$ population, and data was analyzed using FlowJo software (version 10; FlowJo, LLC).

Detection of PD-1 expression in T lymphocytes in blood by flow cytometry. Blood samples were harvested from the hearts of mice at 72 h post-surgery, ~1 ml blood was collected from each mouse. Red blood cells in whole blood cells were lysed using Red Blood Cell Lysis Buffer (Beijing Solarbio Science & Technology Co., Ltd.). Cells were stained with APC-anti-CD3 (eBioscience; Thermo Fisher Scientific, Inc.) and PE-anti-PD-1 (eBioscience; Thermo Fisher Scientific, Inc.) antibodies, and then quantified by flow cytometry on a FACSCalibur instrument (BD Biosciences) according to the manufacturer's instructions. Data was analyzed as aforementioned.

Detection of T lymphocyte early/late apoptosis in vivo by flow cytometry. Blood was harvested from the hearts of mice at 72 h after surgery. Red blood cells were lysed using Red Blood Cell Lysis Buffer (Beijing Solarbio Science & Technology Co., Ltd.). Cells were stained with APC-anti-CD3 (eBioscience; Thermo Fisher Scientific, Inc.) antibody, FITC-Annexin V and PI (MultiSciences Biotechnology Co., Ltd.). T lymphocyte apoptosis was detected by flow cytometry as aforementioned, according to the manufacturer's instructions. CD3$^+$ Annexin V$^+$ cells were defined as apoptotic T lymphocytes in blood. Data was analyzed as aforementioned.

Detection of blood endotoxin and intestinal permeability. Serum was collected from mice in the sham, CLP and atezolizumab groups at 24, 48 and 72 h after surgery using pyrogen-free tubes. Endotoxin levels were measured using an End-point Chromogenic *Tachypleus amebocyte* Lysate kit (Xiamen Bioendo Technology Co., Ltd.) according to the manufacturer's instructions, and calculated from a standard curve by reading the absorbance at 405 nm using an ELISA instrument (BioTek Instruments, Inc.). All experiments were performed in triplicate.

Intestinal permeability was measured at 24, 48 and 72 h after surgery using FITC-dextran (FD40S; Sigma-Aldrich; Merck KGaA), according to a reported method (20). FD40S (0.5 ml, 22 mg/ml) was administered to mice by gavage, 5 h before sacrifice. Blood was harvested from the mice hearts at the time of sacrifice. The extracted plasma was diluted using the same volume of PBS, and then the concentration of FD40S was measured using a fluorescence spectrophotometer (Shimadzu Corporation), with excitation and emission wavelengths of 470 and 515 nm, respectively. A standard curve was drawn using measurements from serial samples at different concentrations. All experiments were performed in triplicate.

Histological examination of the ileum by hematoxylin and eosin (H&E) staining. At 72 h after modeling, distal ileum

specimens (~1 cm) were collected, fixed in 4% paraformaldehyde (Wuhan Boster Biological Technology, Ltd.) for 24 h at room temperature and embedded in paraffin. Then, 5-μm thick sections were cut and stained with H&E. The sections were stained with hematoxylin for 5 min and then dehydrated with 70 and 90% ethanol, followed by staining with eosin for 3 min; Images were obtained using a microscope (Olympus BX53; Olympus Corporation). Histopathology was assessed according to the Chiu grading system, and histopathological scores (0-5 scale) were calculated following observation by light microscopy at x200 magnification. The Chiu scoring standard of the ileal mucosa is divided into six levels according to the severity of injury: i) 0, the intestinal mucosal epithelium is normal without injury; ii) 1, the intestinal mucosal subepithelial space is widened and the capillaries have hyperemia; iii) 2, the intestinal mucosal subepithelial space is further extended and elevated, and separation of the epithelium and lamina propria occurs; iv) 3, part of the villous epithelium of the intestinal mucosa falls off; v) 4, the intestinal mucosal villous epithelium completely falls off, and capillary hemorrhages can be observed; and vi) 5, the intestinal mucosal lamina propria disintegrates, and mucosal bleeding ulcer occurs (21,22).

Expression of tight junction protein in the ileum is detected by immunohistochemistry. At 72 h after modeling, a section of the ileum was cut under anesthesia with intraperitoneal injection of 40 mg/kg pentobarbital sodium (Sigma-Aldrich; Merck KGaA). The procedure for obtaining pathological slices was the same as that described above. Next, the slices were blocked with 5% goat serum (Beijing Solarbio Science & Technology Co., Ltd.) for 15 min at room temperature, then incubated overnight at 4˚C with anti-claudin-1 (1:100; cat. no. Ab15098; Abcam), anti-occludin (1:100; cat. no. 168986; Abcam) and anti-zonula occludens-1 (ZO-1; 1:100; cat. no. 21773-1-AP; ProteinTech Group, Inc.) antibodies. After a wash with PBS, tissue was incubated with horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibody (1:100; cat. no. abs20002ss; Absin, Inc.) at 37˚C for 30 min, according to the manufacturer's instructions. Images were obtained using a microscope (Olympus BX53; Olympus Corporation; magnification, x200). The expression of these proteins was calculated using ImageJ software (version 1.8.0; National Institutes of Health).

Purification of neutrophils and co-culture with a T lymphocyte cell line. Neutrophils were purified from blood samples from the CLP or sham groups 72 h after surgery using a Neutrophil Extraction kit (Beijing Solarbio Science & Technology Co., Ltd.), according to the manufacturer's instructions.

The CTLL-2 mouse T lymphocyte cell line (Fenghbio Co., Ltd.) was used for *in vitro* experiments. T lymphocytes were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 ng/ml streptomycin and 100 U/ml recombinant human IL-2 (all Beijing Solarbio Science & Technology Co., Ltd.) at 37˚C under a 5% CO_2 atmosphere.

Purified neutrophils and CTLL-2 cells were co-cultured in 6-well culture plates at a 1:1 ratio ($1x10^6$ cells/ml) under the culture conditions described above. Samples from the atezolizumab group were treated with 10 μg/ml atezolizumab overnight at 37˚C, while those in the other two groups were treated with 10 μg/ml isotype control antibody overnight at 37˚C as a control.

Detection of T lymphocyte early/late apoptosis by flow cytometry after co-culture. After co-culture of T lymphocyte CTLL-2 cells with neutrophils isolated from septic mice for 24 h, cells were collected for staining with APC-anti-CD3 antibody, FITC-Annexin V and PI (MultiSciences Biotech Co., Ltd.). T lymphocyte apoptosis was detected by flow cytometry as aforementioned and the apoptosis rate was calculated by FlowJo software, according to the manufacturer's instructions. Cells that stained positive for Annexin V were considered apoptotic.

Detection of PD-1 expression in T lymphocyte cell lines by flow cytometry after co-culture. To confirm that CTLL-2 cells expressed PD-1, the positive cells were detected by flow cytometry. After co-culture of CTLL-2 cells with neutrophils isolated from septic mice for 24 h, cells were collected for staining with PE-anti-PD-1 antibody (eBioscience; Thermo Fisher Scientific, Inc.). PD-1 expression in CTLL-2 cells was detected by flow cytometry and analyzed as aforementioned, according to the manufacturer's instructions.

Calculation of survival time of mice after operation. After operation, the survival times of mice in the CLP and atezolizumab groups were recorded. Mouse death was determined by physical examination, and death was confirmed when there was no respiration and heartbeat. Survival rates were monitored continuously for 144 h, and 12 mice were studied in the CLP and atezolizumab groups. Mice were monitored once an hour. After 144 h of monitoring, the living mice were sacrificed under anesthesia by intraperitoneal injection of sodium pentobarbital.

Statistical analysis. Continuous, normally distributed variables are presented as the mean ± standard deviation, while non-normally distributed data are presented as the mean (minimum, maximum). A Student's t-test was applied for comparisons between two groups. If the data were not normally distributed between two groups, a Mann-Whitney U test was used. Comparisons among three groups were conducted using ANOVA followed by an LSD test. Mouse survival times were evaluated by Kaplan-Meier analysis, and statistical significance was determined by the log-rank test using SPSS version 17.0 (SPSS, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Sepsis mouse model is successfully established by the CLP method. After recovery from the CLP procedure, mice with sepsis showed mental depression, erection of whole-body hair, trembling and slow crawling. In addition, bloody or purulent ascites, and intestinal tube swelling and expansion could be observed in their abdominal cavity, and their distal cecum was black and necrotic with various degrees of adhesion around it (Fig. 1A-C).

Expression of PD-L1 in neutrophils is increased in blood and bone marrow during sepsis. PD-L1 expression in blood and bone marrow neutrophils was measured at various time points by flow cytometry. In blood, compared with that of the sham

Figure 1. CLP sepsis mouse model establishment and evaluation. (A) Surgical procedure of CLP. (B) External manifestation of sepsis in mice. (C) Intra-abdominal manifestation of sepsis in mice. CLP, cecal ligation and puncture.

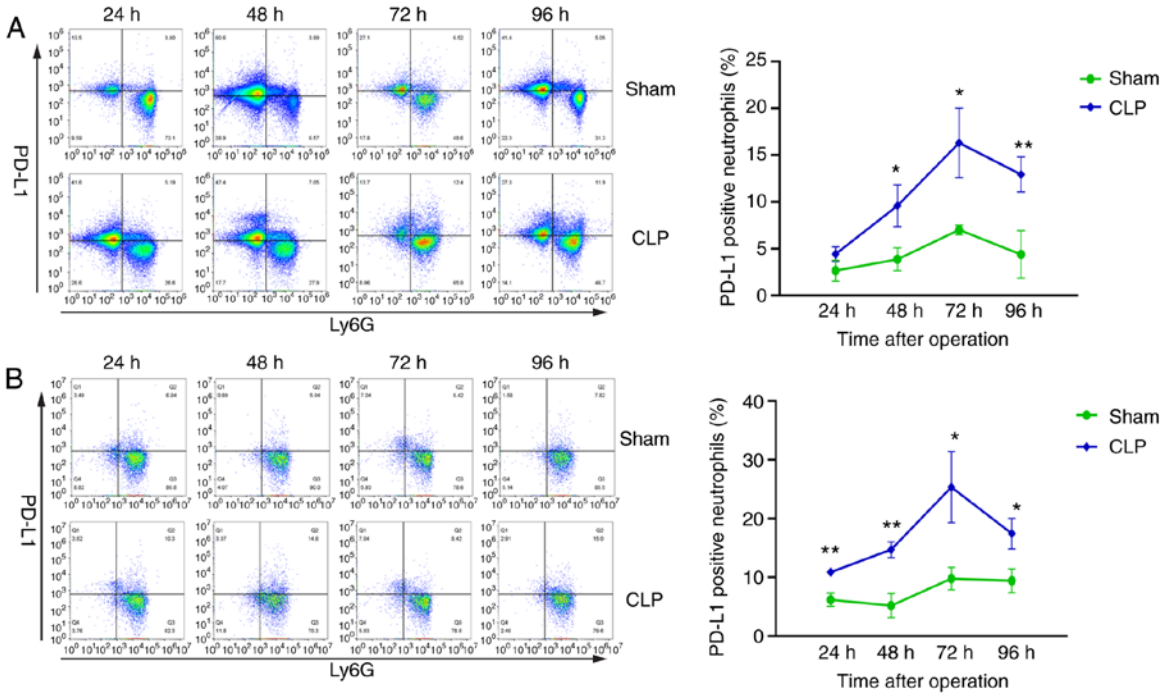

Figure 2. Proportion of PD-L1$^+$ neutrophils in the sham and CLP mice. (A) PD-L1 expression in blood neutrophils (n=12 mice per group). (B) PD-L1 expression in bone marrow neutrophils (n=12 mice per group). *P<0.05, **P<0.01. PD-L1, programmed death ligand-1; CLP, cecal ligation and puncture.

group, the proportion of PD-L1$^+$ neutrophils in the CLP group was 4.433±0.77% (vs. 2.647±1.117%, P=0.085), 9.583±2.222% (vs. 3.867±1.22%, P=0.017), 16.3±3.716% (vs. 7.003±0.512%, P=0.013) and 12.933±1.877% (vs. 4.387±2.531%, P=0.009) at 24, 48, 72 and 96 h, respectively (Table SI). Hence, the proportion of PD-L1$^+$ neutrophils was significantly higher in the CLP group compared with that in the sham group at 48, 72 and 96 h (P<0.05; Fig. 2A). In the bone marrow, compared with that of the sham group, the proportion of PD-L1$^+$ neutrophils in the CLP group was 10.867±0.493% (vs. 6.197±1.127%, P=0.003), 14.7±1.353% (vs. 5.2±2.05%, P=0.003), 25.367±6.037% (vs. 9.8±1.928%, P=0.013) and 17.433±2.616% (vs. 9.417±2.029%, P=0.014) at 24, 48, 72 and 96 h, respectively (Table SII). These data demonstrated that the proportion of PD-L1$^+$ neutrophils was significantly higher in the CLP group than in the sham group at all the time points tested (P<0.05; Fig. 2B).

Atezolizumab treatment reduces endotoxin levels and intestinal mucosal permeability in septic mice. The blood endotoxin levels measured at 24, 48 and 72 h after surgery were 0.224±0.008, 0.241±0.128 and 0.23±0.064 EU/ml, respectively, in the sham group; 0.496±0.102, 0.742±0.153 and 0.956±0.162 EU/ml, respectively, in the CLP group; and 0.477±0.069, 0.488±0.145 and 0.564±0.128 EU/**ml, respec**tively, in the atezolizumab group (Table SIII). Compared with those in the sham group, the endotoxin levels were significantly increased at 24, 48 and 72 h in the CLP group (P<0.01; Fig. 3A). Furthermore, relative to those in the CLP group, the endotoxin levels were significantly decreased at 48 and 72 h in the atezolizumab group (P<0.05; Fig. 3A).

The degree of intestinal permeability was indicated by the concentration of FD40S in the blood. The concentrations of FD40S at 24, 48 and 72 h after surgery were 1.754±0.575, 2.758±0.636 and 3.473±1.434 μg/ml, respectively, in the sham group; 8.919±2.681, 25.024±3.68 and 32.283±3.456 μg/ml, respectively, in the CLP group; and 8.519±2.335, 13.1±4.098 and 17.981±3.211 μg/ml, respectively, in the atezolizumab group (Table SIV). Compared with that in the sham group,

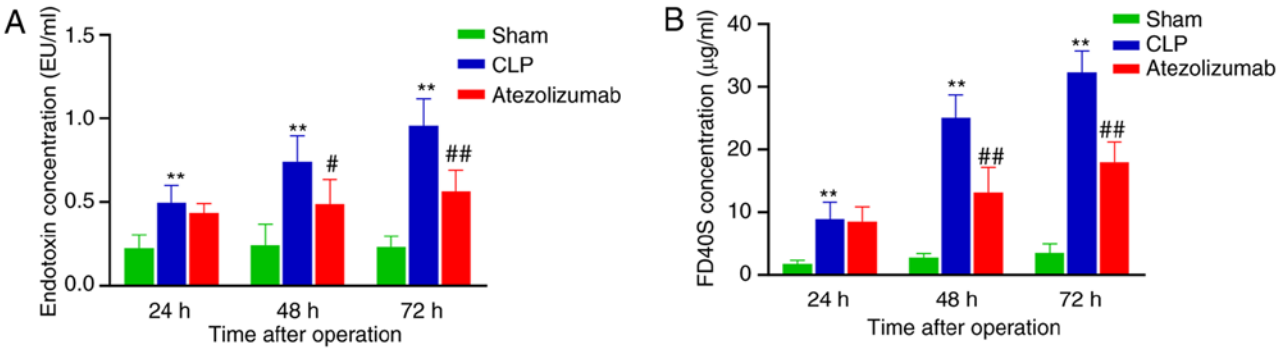

Figure 3. Endotoxin and FD40S concentrations in the sham, CLP and atezolizumab groups. (A) Endotoxin concentrations in each group (n=15 mice per group). (B) FD40S concentrations in each group (n=15 mice per group). **P<0.01 vs. sham groups; #P<0.05, ##P<0.01 vs. CLP groups. CLP, cecal ligation and puncture; FD40S, FITC-dextran 40S.

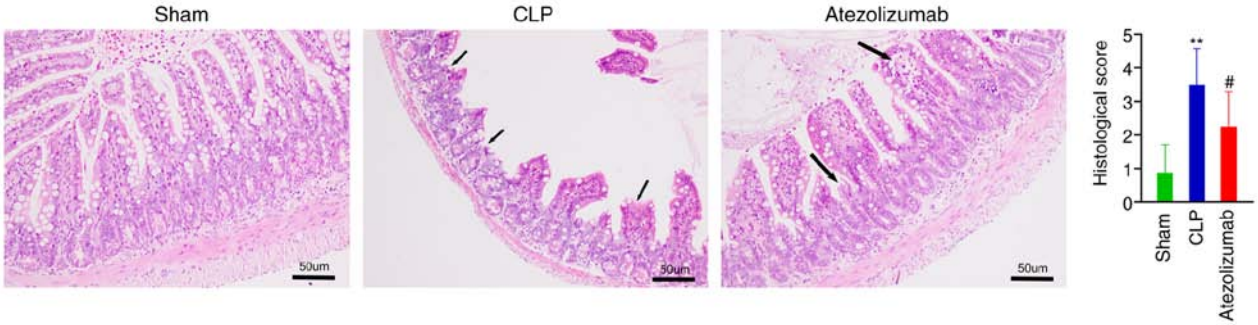

Figure 4. Ileum histopathological scores in sham, CLP and atezolizumab mice (n=8 mice per group). Small arrows show villous epithelial shedding, bleeding and ulcers; large arrows show widening of subcutaneous space and shedding of villi epithelium. Scale bar, 50 μm. **P<0.01 vs. sham groups; #P<0.05 vs. CLP groups. CLP, cecal ligation and puncture.

FD40S was significantly increased in the CLP group at 24, 48 and 72 h (P<0.01; Fig. 3B). Furthermore, relative to that of the CLP group, the FD40S concentration was significantly decreased in the atezolizumab group at 48 and 72 h (P<0.01; Fig. 3B).

Atezolizumab treatment decreases ileum histological scores in septic mice. The ileal mucosa of the CLP group showed increased villous epithelial shedding, bleeding and ulcers. In the atezolizumab group, the ileal mucosa exhibited varying degrees of widening of the subcutaneous space and shedding of villi epithelium, but ulcers of the mucosa were rare. Evaluation of histopathological scores by H&E-stained distal ileum samples indicated that the mean score in the CLP group was significantly higher than that in the sham group (3.5±1.069 vs. 0.875±0.835, P<0.01), while the mean score in the atezolizumab group was significantly lower than that in the CLP group (2.25±1.035 vs. 3.5±1.069, P<0.05) (Fig. 4; Table SV).

Atezolizumab treatment increases the expression of tight junction proteins in the ileum during sepsis. The expression of three tight junction proteins in the ileum was assessed by immunohistochemistry (Fig. 5A-C; Table SVI). The expression of claudin-1 protein in the sham, CLP and atezolizumab groups was 1.03±0.061, 0.652±0.17 and 0.846±0.127, respectively. The expression of claudin-1 protein in the CLP group was significantly lower than that in the sham group (P<0.05), while the expression of claudin-1 protein in the atezolizumab group was higher than that in the CLP group (P<0.05). The expression levels of occludin protein in the sham, CLP and atezolizumab groups were 1.02±0.125, 0.68±0.194 and 0.888±0.122, respectively. Compared with that in the sham group, the expression of occludin protein in the CLP group was significantly lower (P<0.05), while the expression of occludin protein in the atezolizumab group was significantly higher than that in the CLP group (P<0.05). The expression levels of ZO-1 protein in the sham, CLP and atezolizumab groups were 1.01±0.137, 0.794±0.124 and 0.896±0.092, respectively. The ZO-1 protein expression in the CLP group was significantly lower than that in the sham group (P<0.05). The ZO-1 protein expression in the atezolizumab group was higher than that in the CLP group, but the difference between the two groups was not statistically significant (P>0.05).

Atezolizumab treatment decreases T lymphocyte apoptosis during sepsis in vivo and in vitro. *In vivo*, 72 h after surgery, the apoptosis rates of T lymphocytes in the sham, CLP and atezolizumab groups were 9.735±1.966, 26.158±3.342 and 18.014±7.415%, respectively. Compared with the that in the sham group, the apoptosis rate of T lymphocytes in the CLP group was significantly increased (P<0.01; Fig. 6), while the apoptosis rate of T lymphocytes in the atezolizumab group was significantly decreased relative to that in the CLP group (P<0.01; Fig. 6).

After co-culture with neutrophils from septic mice for 24 h, the apoptosis rates of T lymphocytes in the sham, CLP and

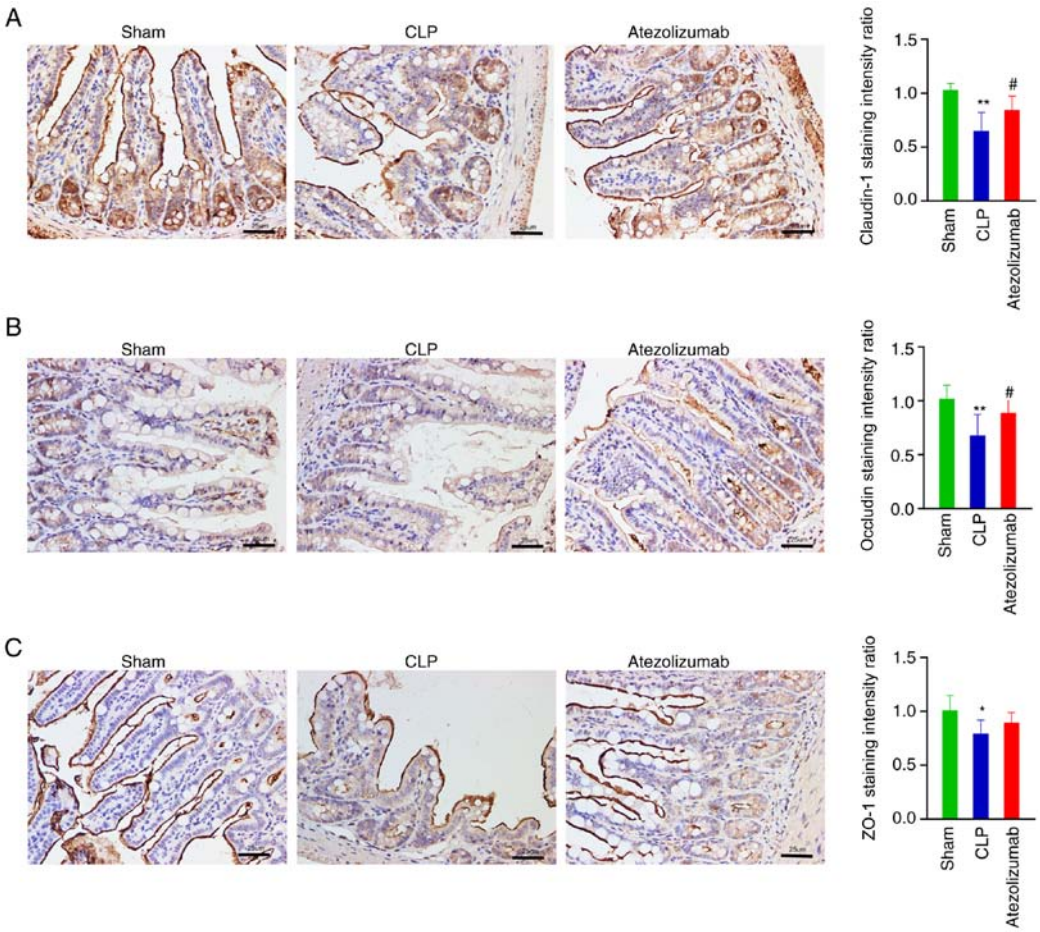

Figure 5. Tight junction protein expression in the ileum of mice. (A) Expression of claudin-1 protein in ileum (n=5 mice per group). (B) Expression of occludin protein in ileum (n=5 mice per group). (C) Expression of zonula occludens-1 protein in ileum (n=5 mice per group). Scale bar, 25 μm. *P<0.05, **P<0.01 CLP vs. sham groups; $^{\#}$P<0.05 vs. CLP groups. CLP, cecal ligation and puncture.

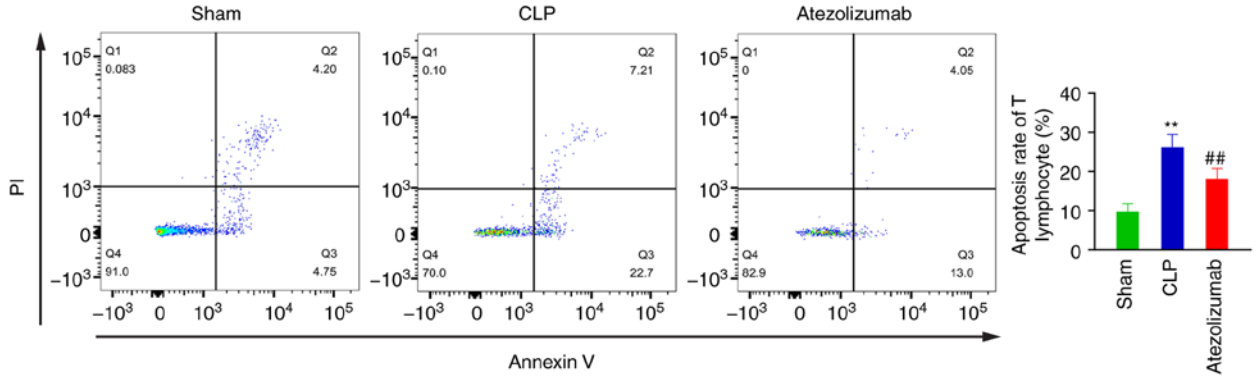

Figure 6. Apoptosis rates of T lymphocytes *in vivo* during sepsis (n=4 mice per group). **P<0.01 vs. sham groups; $^{\#\#}$P<0.01 vs. CLP groups. CLP, cecal ligation and puncture.

atezolizumab groups were 8±1.638, 27±2.855 and 12.93±2.15%, respectively (Table SVII). Compared with that in the sham group, the apoptosis rate of T lymphocytes in the CLP group was significantly increased (P<0.01; Fig. 7), while the apoptosis rate of T lymphocytes in the atezolizumab group was significantly decreased relative to that in the CLP group (P<0.01; Fig. 7).

Confirmation of PD-1 expression in T lymphocytes in vivo and T lymphocytes cell line in vitro. At 72 h after surgery, the PD-1 expression rates in blood T lymphocytes in the sham and CLP groups were 18.65±3.483 and 26.388±3.2%, respectively. The difference between two groups was statistically significant (P<0.05; Fig. 8A).

Following co-culture with neutrophils from septic mice for 24 h, PD-1 was found to be expressed in ~17.105±1.878 and 17.763±2.263% of CTLL-2 cells in the sham and CLP groups, respectively. No significant difference between these two groups was found (P>0.05; Fig. 8B).

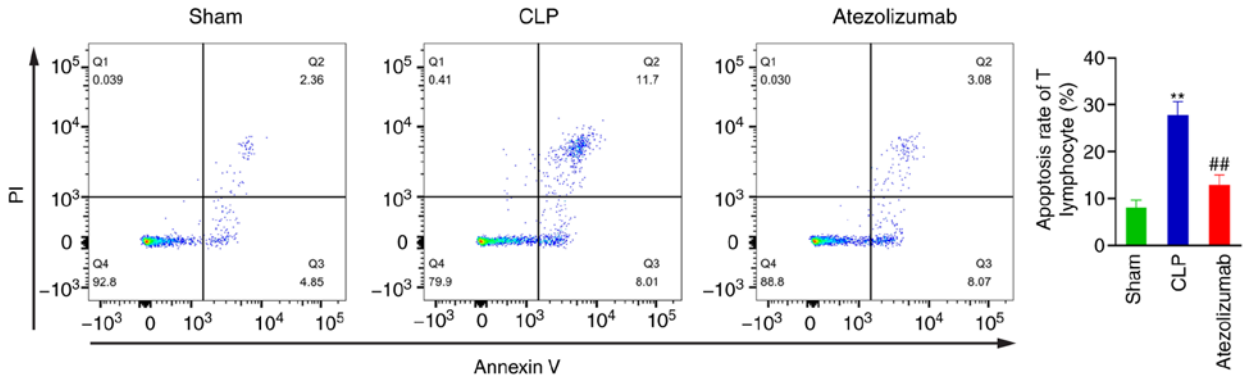

Figure 7. Apoptosis rates of T lymphocytes after co-culture with septic neutrophils *in vitro* (n=4 mice per group). **P<0.01 vs. sham groups; ##P<0.01 vs. CLP group. CLP, cecal ligation and puncture.

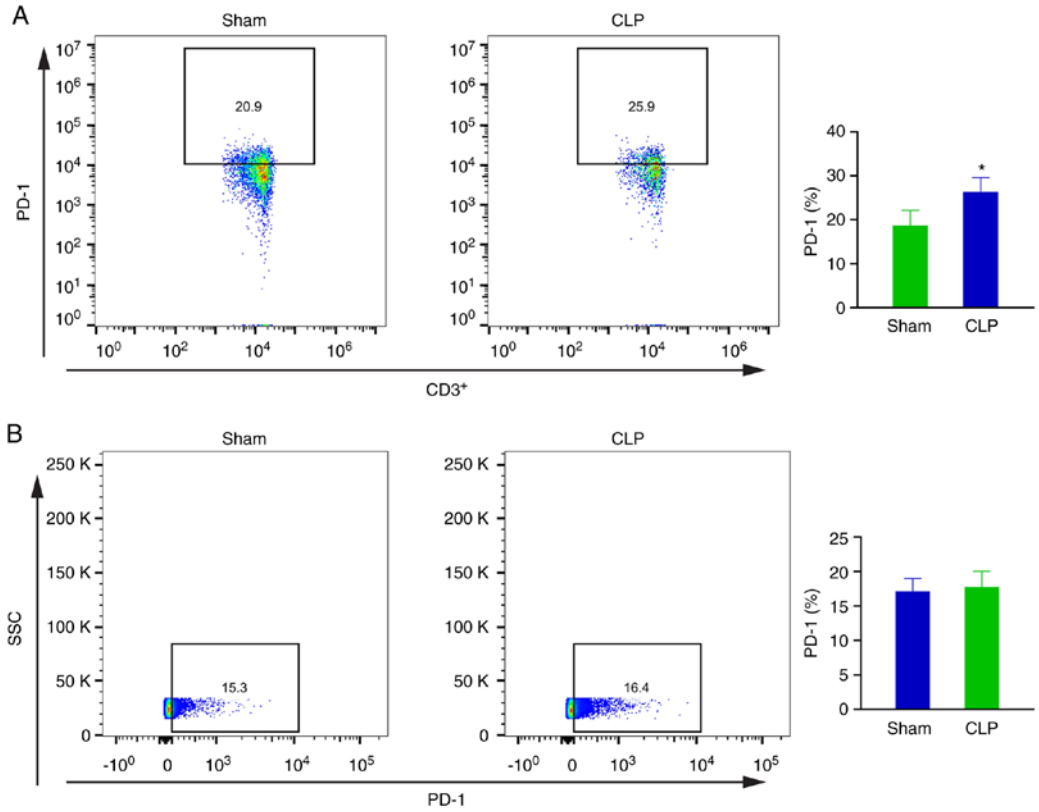

Figure 8. Proportion of PD-1+ T lymphocytes *in vivo* and *in vitro*. (A) PD-1 expression in blood T lymphocytes (n=4 mice per group). (B) PD-1 expression in a T lymphocyte cell line (n=4 mice per group). *P<0.05 vs. sham group. PD-1, programmed death-1; CLP, cecal ligation and puncture.

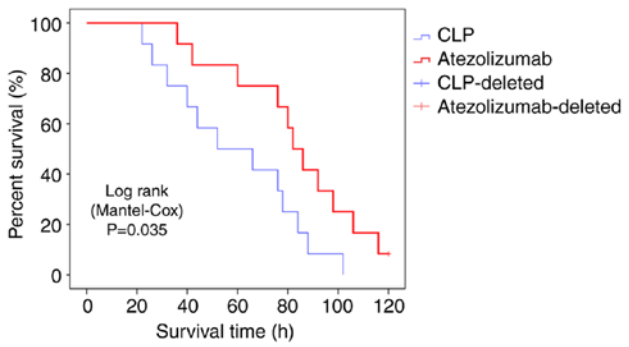

Figure 9. Survival time in CLP and atezolizumab mice (n=12 mice per group). Mice in the atezolizumab group survived longer than those in the CLP group (P<0.05). CLP, cecal ligation and puncture.

Atezolizumab treatment improves the survival of septic mice. To investigate the role of atezolizumab in mouse survival, the death rates of mice in the CLP and atezolizumab groups were recorded (Table SVIII). As shown in Fig. 9, within 90 h, ~50% of mice in the CLP group had died, while the corresponding rate was 25% for the atezolizumab group. Hence, septic mice treated with atezolizumab clearly survived for longer than those treated with isotype control antibody (P<0.05; Fig. 9).

Discussion

There are currently two main mouse models for studying sepsis at the animal level. One is induced by lipopolysaccharide (LPS) and the other is under CLP procedure. CLP is

a method to create an animal model of polymicrobial sepsis. As the CLP model usually induces a longer-lasting systemic inflammatory response, it is suitable for experimental observation of longer-term treatment (23). Moreover, the CLP model is also superior to the LPS model in studying physiological functions (24).

The cause of high mortality in sepsis has always been a concern. Boomer *et al* (25) conducted autopsies of patients following sepsis and found that the main cause of death was immunosuppression rather than the excessive inflammatory response. Lymphocyte apoptosis is now considered an important step in the development of immunosuppression in patients with sepsis, in which T lymphocyte apoptosis is a key factor (8,26,27). In addition to T lymphocyte apoptosis, PD-1 and PD-L1 are also indicators of immunosuppression (9,28,29).

The interaction of PD-L1 and PD-1, which are expressed on the surface of T lymphocytes, results in immunosuppression. Hence, an antibody specifically targeting PD-L1 is suitable for alleviation of the suppression of effector T lymphocytes (30). Atezolizumab is a monoclonal antibody targeting the immunosuppressive receptor PD-L1, and its effectiveness has been verified in numerous diseases, such as urothelial and renal cell carcinoma and non-small-cell lung cancer (15-17).

Immunotherapy with anti-PD-1 or anti-PD-L1 antibodies target the PD-1-PD-L1 pathway. Anti-PD-1 antibodies are reported to alleviate immunosuppression of T lymphocytes and improve the survival rate of septic mice (31). Since neutrophils are the most abundant white blood cells in the blood, the inhibitory effect of PD-L1$^+$_neutrophils on T lymphocytes via the PD-L1-PD-1 axis may be considerable.

In the present study, it was found that the population of neutrophils expressing PD-L1 markedly increased during sepsis. Septic mice treated with atezolizumab had less endotoxin burden, reduced damage of the intestinal mucosa and intestinal permeability, higher expression of intestinal tight junction proteins and survived for a longer period. Finally, both *in vivo* and *in vitro* experiments confirmed that atezolizumab can protect T lymphocytes against the effects of PD-L1$^+$ positive neutrophils.

The gut is often considered to be the driver of multiple serious diseases, including sepsis (20). The intestinal mucosal barrier is important to guard against the bacterial and endotoxin attacks experienced in the gut. The morphology of this barrier mainly comprises intestinal epithelial cells and tight junction proteins (32,33). Sepsis often leads to intestinal epithelial cell apoptosis (34) and tight junction protein destruction (20,35), resulting in increased permeability (36,37). Damage to the intestinal mucosal barrier leads to bacterial and endotoxin translocation into circulation, which exacerbates sepsis (38), and may even cause multiple organ failure (39,40). Therefore, the intestinal mucosal barrier, intestinal permeability and endotoxin levels are important indicators reflecting the severity of sepsis.

A large number of endotoxins appear in the blood circulation during the onset of sepsis, and endotoxin is an important factor that disrupts the intestinal mucosal barrier (41). The mechanism of atezolizumab to protect the intestinal mucosal barrier may be that it can enhance the function of T cells in the body, thereby reducing blood circulation endotoxin, which demonstrates its ability to protect the intestinal mucosal barrier. The detailed underlying mechanism is not yet clear, and further research is needed to determine it.

To date, no studies on atezolizumab for the treatment of sepsis have been published. There are few reports of other anti-PD-L1 antibody reagents used in the treatment of sepsis, but often performed only *in vitro* or *in vivo* experiments. In the present study, it was verified that treatment with atezolizumab protected T lymphocytes during sepsis via *in vivo* and *in vitro* experiments, and demonstrated that it also improves the prognosis of septic mice *in vivo*. Zhang *et al* (42) reported that PD-L1 blockade decreased lymphocyte apoptosis *in vivo*, and PD-L1 expression in blood neutrophils was significantly increased during sepsis. It was presented in the present study that in addition to the increase in PD-L1 expressed by blood neutrophils, PD-L1 expressed by bone marrow neutrophils also increased during sepsis. This suggested that PD-L1 expression may occur in multiple organs at the same time during the onset of sepsis.

Apoptosis during sepsis is triggered by numerous pathways, with multiple cell death stimuli involved. Hence, it is difficult to prevent T lymphocyte apoptosis by blockade of a single factor (18). Although multiple factors contribute to cell death, the PD-1-PD-L1 axis is an important pathway affecting T lymphocytes (42,43). Immunotherapy targeting this pathway can decrease T lymphocyte apoptosis, thereby alleviating immunosuppression during sepsis. This type of immunotherapy is expected to become an important method for the treatment of sepsis in the future.

However, the present study had some limitations. First, although T lymphocyte apoptosis is an important factor in the development of immunosuppression, other factors also contribute to this process. Whether these factors are also affected by atezolizumab requires further evaluation. Second, animal models of sepsis cannot completely replicate sepsis onset in humans; hence, the effects of atezolizumab on humans may differ from those on mice. Future studies on atezolizumab administration in patients with sepsis warrants consideration. In conclusion, treatment with atezolizumab may be an effective method for the immunosuppression induced by sepsis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JC drafted the manuscript and performed the experiments with the assistance of RC. SH and BZ performed the data

analysis. SZ designed the study and corrected the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Animal Care Committee of Guangxi Medical University (approval no. 201901006).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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