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Enhanced IL-10 inhibits proliferation and promotes apoptosis of HUVECs through STAT3 signaling pathway in sepsis

Zuohua Xie[1], Bing Lin[1], Xinju Jia[1], Ting Su[1], Ying Wei[1], Jiping Tang[1], Chengzhi Yang[2], Chuanbao Cui[3] and Jinxiang Liu[1]

[1]Department of Critical Care Medicine, The Third Affiliated Hospital of Guangxi Medical University, [2]Graduate School and [3]Department of Epidemiology, School of Public Health, Guangxi Medical University, Nanning, PR China

Summary. Aims. The present study aims to determine the expression of interleukin (IL)-10 in peripheral blood of patients with sepsis, and investigate its effects on the biological function of vascular endothelial cells.

Methods. Thirty-six sepsis patients and 20 healthy subjects were included. Peripheral blood was collected from all subjects. ELISA was used to determine IL-10 content in serum. A ratio of IL-10$^+$ T cells was determined by flow cytometry. CCK-8 assay was used to investigate proliferation. Cell cycle and apoptosis were analyzed by flow cytometry. Western blotting was used to examine the expression of phosphorylated STAT3 protein.

Results. The content of IL-10 and the ratio of IL-10$^+$ T cells were enhanced in pa-tients with sepsis. Serum from patients with sepsis inhibited the proliferation of HU-VECs, and addition of IL-10 antibody reversed this effect. IL-10 in the serum from patients with sepsis promoted the apoptosis of HUVECs. IL-10 inhibited the proliferation and promoted the apoptosis of HUVECs by enhancing the phosphorylation of STAT3.

Conclusions. The present study demonstrates that the content of IL-10 and the ratio of IL-10$^+$ T cells in peripheral blood of patients with sepsis are up-regulated, and this inhibits HUVEC proliferation and promotes HUVEC apoptosis through STAT3 sig-naling pathway. The results in this study provide a new experimental basis for further understanding the molecular mechanism of sepsis-induced vascular injury.

Key words: IL-10, Sepsis, Proliferation, Apoptosis, STAT3

Corresponding Author: Jinxiang Liu, Department of Critical Care Medicine, The Third Affiliated Hospital of Guangxi Medical University, No. 13 Dancun Road, Nanning 530000, Guangxi Zhuang Autonomous Region, PR China. e-mail: 13977128063@139.com

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Introduction

Sepsis refers to a systemic inflammatory response syndrome (SIRS) in which exoge-nous substances such as endotoxin and peptidoglycan released by invading bacteria attack tissues and organs through the peripheral circulatory system, induce inflamma-tory reaction, and then cause serious damage to the internal environment of the body (Shaver et al., 2019; Spaeder et al., 2019). With antibiotic resistance becoming more and more prominent, sepsis has become a common clinical infectious disease doing great harm. At the early stage of sepsis, patients often have clinical symptoms such as systemic fever, shortness of breath and peripheral leukocytosis (Liu et al., 2019; Mellhammar et al., 2019). If it is not treated in time, it will develop into organ dys-function, insufficient tissue perfusion, or even septic shock, eventually leading to acute multiple organ failure (Carreno et al., 2019; Shirali et al., 2019). At present, there is no radical treatment for sepsis. Treatments are mainly focused on maintaining the stability of the internal environment, and early detection and timely treatment are the main strategies to reduce the damage of sepsis. It is of great clinical significance to study the molecular mechanism of sepsis and to find new therapeutic targets.

The occurrence and development of sepsis are connected to the role of cytokines, which regulate the balance of the body in proinflammatory and anti-inflammatory processes (Nowill et al., 2019). Interleukin (IL)-10 is a type of cytokine that is mainly synthesized and secreted by a variety of immune cells, including Th cells (Hensley et al., 2019; Li et al., 2019b). IL-10 plays important roles for immune cells. For example, IL-10 can promote the differentiation and proliferation of B cells and produce anti-bodies, inhibit antigen-presenting function of macrophages, and extensively inhibit the synthesis of various proinflammatory mediators (Brakenridge et al., 2019). More and more studies also

show that IL-10 plays important roles in other diseases. For example, combined detection of IL-10 and IL-17 can help evaluate the prognosis of rheumatoid arthritis (McDonald et al., 2019). In addition, combined detection of IL-10 and IL-18 can help predict the prognosis of colon cancer patients (Li et al., 2019a). However, the role of IL-10 in sepsis still needs further studies.

After pathogenic substances are released into the blood, the peripheral immune sys-tem is activated and a large number of cytokines are released as the "defense line" against these substances. Vascular injury is one of the early changes in sepsis patients. First, vascular endothelial cell dysfunction appears, and then the destruction of vascular endothelial structure occurs, eventually leading to vascular barrier and contraction dysfunction (Zhu et al., 2019; Zong et al., 2019). There are several patterns of vascu-lar injury in the occurrence and development of sepsis: i) substances like endotoxin released by pathogens directly induce vascular endothelial cell injury; ii) endotoxin causes endothelial cell secretion dysfunction, such as secretion of inflammatory fac-tors and vasoactive substances, which aggravates inflammation and ischemia injury of tissues and organs; iii) endovascular inflammatory substances directly regulate the function of vascular endothelial cells (Deshmukh et al., 2019; He et al., 2019). As an important component of the inner layer of the vascular wall, vascular endothelial cells play important roles in the structure and function of the vascular wall, but whether the cells can be regulated by IL-10 has not been reported before.

In the present study, we determine the expression of IL-10 in peripheral blood of pa-tients with sepsis, and investigate its effects on the function of vascular endothelial cells.

Materials and methods

Subjects

A total of 36 patients with sepsis who received treatments at our hospital between October 2016 and October 2018 were included in the present study. In addition, 20 healthy subjects were included in a control group. Peripheral blood (10 ml) was col-lected from all patients and healthy subjects and stored in tubes containing EDTA. The first part of the peripheral blood (3 ml) was used for lymphocyte separation and flow cytometry, and the second part of the blood (7 ml) was used for serum separa-tion. Among all patients with sepsis, 16 patients were at sepsis stage, 12 patients were at severe sepsis stage, and 8 patients were at septic shock stage. The patients were diagnosed by experienced doctors. Patients with diabetes, tumors or other immune diseases were excluded. All procedures were approved by the Ethics Committee of Guangxi Medical University. Written informed consents were obtained from all patients or their families.

Cells

Human umbilical vein endothelial cells (HUVECs; Cell Bank, Chinese Academy of Sciences, Shanghai, China) were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS). When reaching 80% confluency, the cells were trypsinized and passaged. HUVECs in logarithmic growth were used for functional experiments.

To isolate peripheral blood mononuclear cells (PBMCs), peripheral blood (3 ml) from healthy subjects was treated with Ficoll lymphocyte separation solution (GE Healthcare, Chicago, IL, USA) according to the manufacturer's manual. Then, PBMCs were mixed with 5-times volume of phosphate-buffered saline (PBS), and centrifuged at 2000xg for 2 min. After discarding the supernatant, PBMCs were resuspended with PBS and mixed thoroughly.

DMEM (250 μl) containing 10% FBS was mixed with serum from sepsis patients (sepsis group) at a ratio of 1:1, and used for incubation of HUVECs for 48 h. When reaching 70-80% confluency, the medium was replaced with fresh DMEM medium. After incubation for 24 h, culture supernatant was collected and centrifuged at 12,000 xg and 4°C for 15 min. Then, the supernatant was mixed with RPMI-1640 medium containing 10% FBS at a ratio of 1:1 to achieve conditional medium. HUVECs in negative control (NC; normal serum treatment) group were cultured with 500 μl RPMI-1640 medium supplemented with 10% FBS, HUVECs in serum group were cultured with 500 μl conditional medium, and HUVECs in serum+IL-10 ab group were cultured with 500 μl conditional medium containing IL-10 antibody. The cells were cultured for 72 h before subsequent examinations.

Enzyme-linked immunosorbent assay (ELISA)

IL-10 ELISA kit (ab46034; Abcam, Cambridge, UK) was used to determine the con-centration of IL-10. In microplates, standards (50 μl) and samples (10 μl serum and 40 μl diluent) were added into predefined wells, while blank wells were left empty. In the wells for standards and samples, horseradish peroxidase-labelled conjugates (100 μl) were added before sealing the plates for incubation at 37°C for 1 h. After washing the plates 5 times, substrates A (50 μl) and B (50 μl) were added into each well. After incubation at 37°C for 15 min, stop solution (50 μl) was added into each well, and absorbance of each well was measured at 450 nm within 15 min.

CCK-8 assay

HUVECs were seeded at a density of 2,000/well in 96-well plates. At 0, 24, 48 and 72 h, 20 μl CCK-8 reagent (5 g/L; Beyotime, Shanghai, China) was added to the cells. At the end of the 4 time-points aforementioned, 150 μl CCK-8 reaction solution was

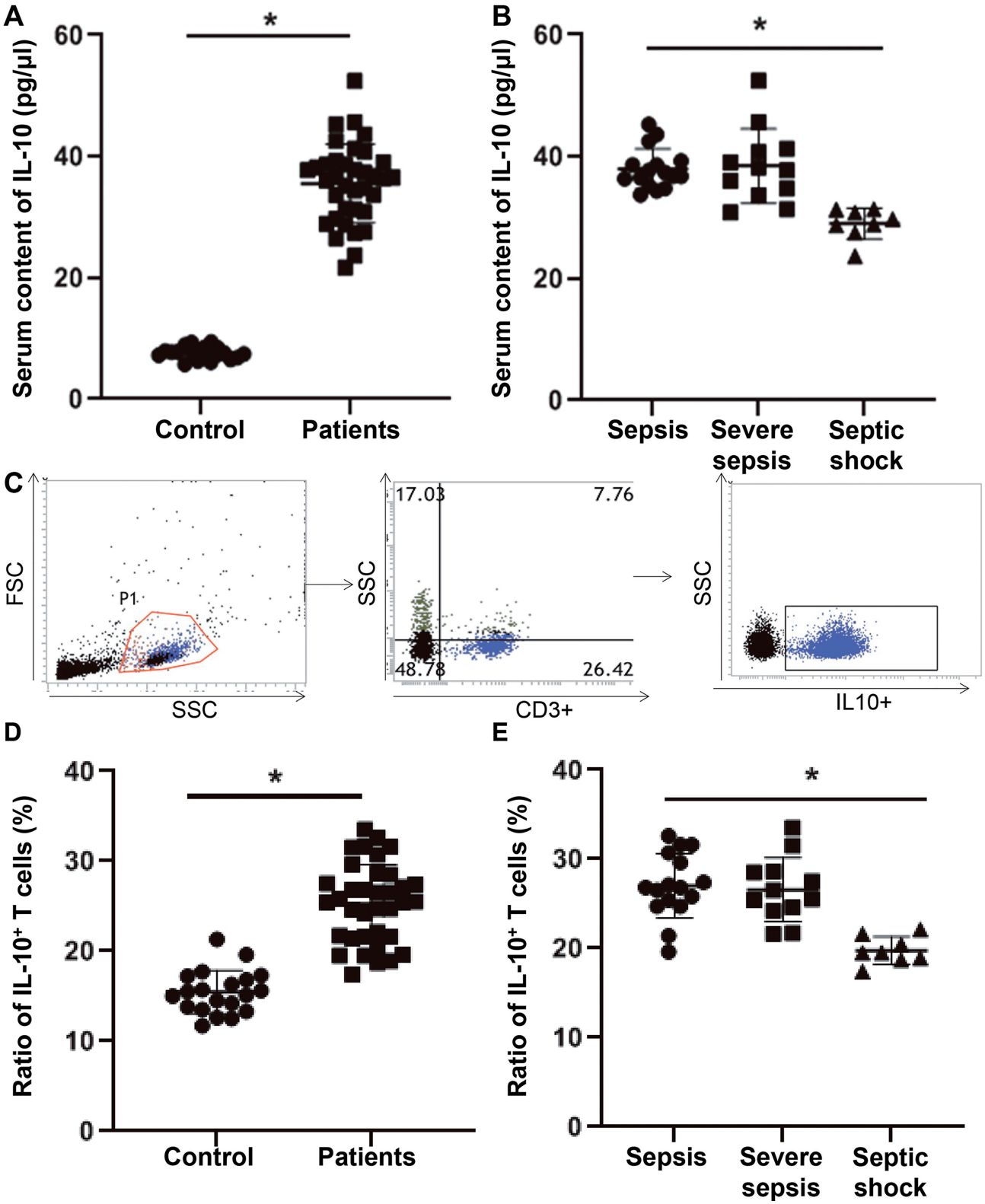


Fig. 1. Content of IL-10 and ratio of IL-10$^+$ T cells in peripheral blood from patients with sepsis. **A.** Content of IL-10 in serum from healthy subjects and sepsis patients. **B.** Content of IL-10 in serum from patients with general sepsis, severe sepsis and septic shock. ELISA was used to determine the content of IL-10 in serum. **C.** Flow cytometry showing the ratio of IL-10$^+$ T cells. **D.** Ratio of IL-10$^+$ T cells in healthy subjects and sepsis patients. **E.** Ratio of IL-10$^+$ T cells in patients with general sepsis, severe sepsis and septic shock. Flow cytometry was used to determine the ratio of IL-10$^+$ T cells. *P<0.05.

added and the cells were incubated at 37°C for 2 h. Then, the absorbance of each well was measured at 490 nm for plotting cell proliferation curves. Each group was tested in 3 replicate wells and the values were averaged.

Flow cytometry

Expression of IL-10 in $CD3^+$ T cells was detected by flow cytometry (BD Bioscienc-es, Franklin Lakes, NJ, USA). PBMC population was identified by FSC/SSC, and $CD3^+$ cell population was chosen for further analysis of IL-10 expression. Each test was repeated at least 3 times.

Western blotting

After co-culture with condition medium for 24 h, the medium of HUVECs was dis-carded, and the cells were washed with cold PBS twice before addition of Radio-Immunoprecipitation Assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) and protease inhibitor phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology, Shanghai, China) for lysis on ice for 5 min. The mixture was centrifuged at 12,000 rpm and 4°C for 10 min. The supernatant was used to determine protein concentration by bicinchoninic acid (BCA) protein concentration determination kit (RTP7102, Real-Times Biotechnology Co., Ltd., Beijing, China). The samples were then mixed with 5× sodium dodecyl sulfate loading buffer before denaturation in a boiling water bath for 10 min. Afterwards, the samples (5 μl) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 100 V. The resolved proteins were transferred to polyvinylidene difluoride membranes on ice (250 mA, 1 h) and blocked with 5% skimmed milk at room temperature for 1 h. Then, the membranes were incubated with rabbit anti-human STAT3 (1:1000; Abcam, Cam-bridge, UK), p-STAT3 (1:1000; Abcam, Cambridge, UK) or GAPDH (1:4000; Abcam, Cambridge, UK) polyclonal primary antibodies at 4°C overnight. After extensive washing with phosphate-buffered saline with Tween 20 (0.1%) 3 times for 15 min, the membranes were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:4,000; Abcam, Cambridge, UK) for 1 h at room temperature before washing with phosphate-buffered saline with Tween 20 (0.1%) 3 times for 15 min. Then, the membrane was developed with enhanced chemiluminescence detection kit (ab65623; Abcam, Cambridge, UK) for imaging. Image lab v3.0 software (Bio-Rad, Hercules, CA, USA) was used to acquire and analyze imaging signals. The relative content of target protein was expressed against GAPDH.

Statistical analysis

The results were analyzed using Graph Pad Prism 7.0 statistical software (GraphPad Software, La Jolla, CA, USA). The data were expressed as means ± standard deviations. Multigroup measurement data were analyzed using one-way ANOVA. Compar-ison between two groups was carried out using Student's t-test. $P<0.05$ indicated statistically significant differences.

Results

The content of IL-10 and the ratio of IL-10^+ T cells are enhanced in patients with sepsis

ELISA was used to determine the contents of IL-10 in serum, and flow cytometry was performed to study the ratio of IL-10^+ T cells in peripheral blood. ELISA showed that the content of IL-10 in serum from patients with sepsis (36.82±0.49 pg/ml) was sig-nificantly higher than that of control group (7.78±0.33 pg/ml) ($P<0.05$) (Fig. 1A). In addition, the content of IL-10 in serum from patients with septic shock was signifi-cantly lower than that of patients with general sepsis and patients with severe sepsis ($P<0.05$) (Fig. 1B). Flow cytometry showed that the ratio of IL-10^+ T cells in pa-tients with sepsis (26.9%±0.59%) was significantly higher than that of control group (4.78%±0.35%) ($P<0.05$) (Fig. 1C,D). Moreover, the ratio of IL-10^+ T cells in patients with septic shock was significantly lower than that of patients with general sepsis and patients with severe sepsis ($P<0.05$) (Fig. 1C,E). The results suggest that the content of IL-10 and the ratio of IL-10^+ T cells are enhanced in patients with sepsis.

Serum from patients with sepsis inhibits the proliferation of HUVECs, and addition of IL-10 antibody reverses this effect

To test the effect of serum from patients with sepsis on the proliferation of HUVECs, CCK-8 assay was carried out. The data showed that the proliferation of HUVECs treated with serum from patients with sepsis was reduced compared to that of HU-VECs treated with serum from healthy subjects ($P<0.05$ at all time-points), but addi-tion of IL-10 antibody significantly promoted the proliferation of serum-treated HU-VECs ($P<0.05$; Fig. 2A). Flow cytometry showed that the percentage of serum-treated HUVECs in G1 phase was significantly higher than that of NC group, and that of serum-treated HUVECs in S phase was significantly lower than that of NC group ($P<0.05$) (Fig. 2B). However, addition of IL-10 antibody significantly decreased the percentage of serum-treated HUVECs in G1 phase, and increased the percentage of serum-treated HUVECs in S phase ($P<0.05$) (Fig. 2B). The results indicate that se-rum from patients with sepsis inhibits the proliferation of HUVECs, and addition of IL-10 antibody reverses this effect.

IL-10 in the serum from patients with sepsis promotes the apoptosis of HUVECs

To examine apoptosis, flow cytometry was

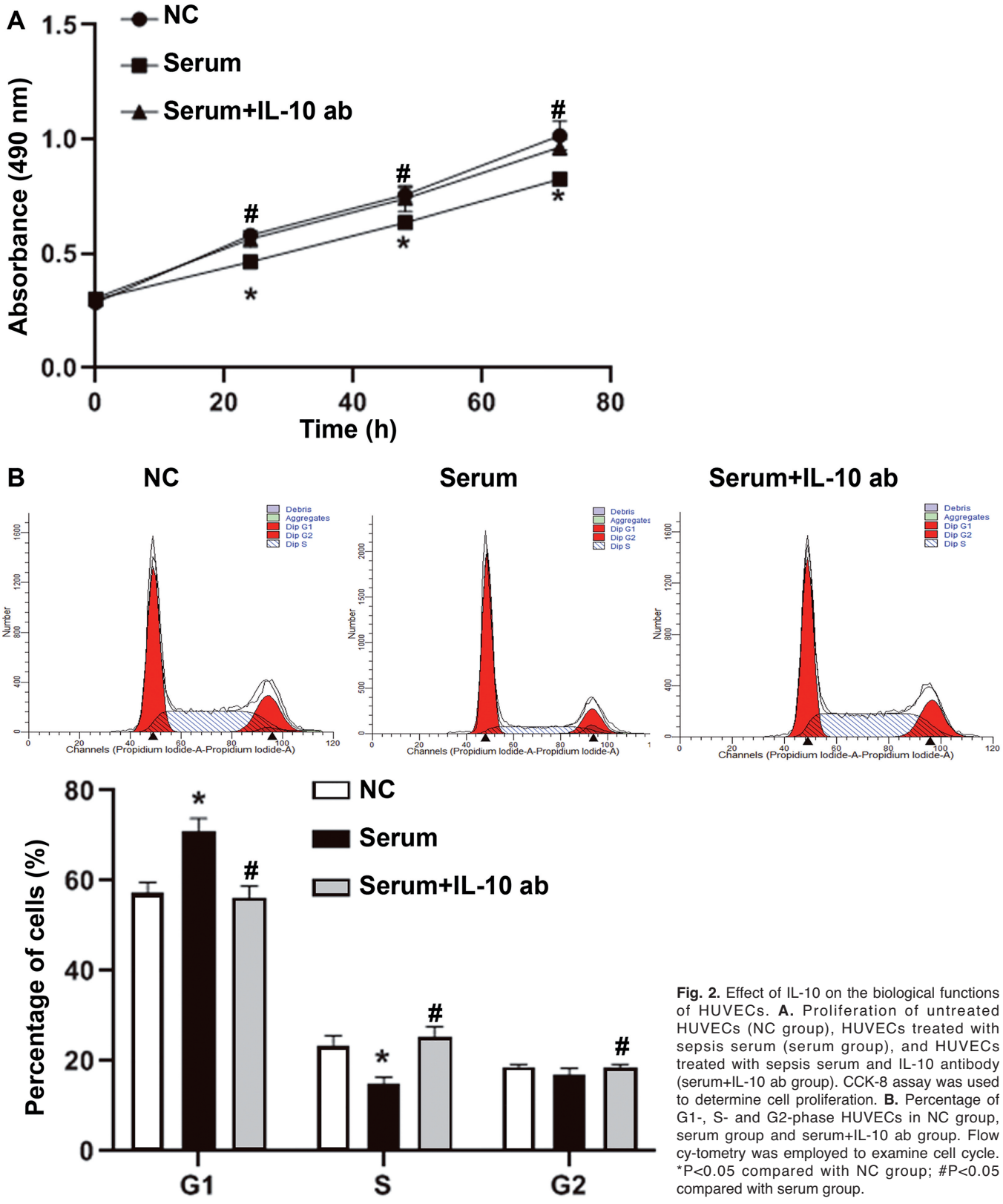


Fig. 2. Effect of IL-10 on the biological functions of HUVECs. **A.** Proliferation of untreated HUVECs (NC group), HUVECs treated with sepsis serum (serum group), and HUVECs treated with sepsis serum and IL-10 antibody (serum+IL-10 ab group). CCK-8 assay was used to determine cell proliferation. **B.** Percentage of G1-, S- and G2-phase HUVECs in NC group, serum group and serum+IL-10 ab group. Flow cy-tometry was employed to examine cell cycle. *P<0.05 compared with NC group; #P<0.05 compared with serum group.

employed. The data showed that the apoptotic rate of HUVECs in serum group (34.7±2.72%) was significantly higher than that in NC group (12.56±1.23%) (P<0.05), but addition of IL-10 antibody decreased the apoptotic rate of HUVECs treated with sepsis serum (12.2±2.3%) (P<0.05) (Fig. 3). The result suggests that IL-10 in the serum from patients with sepsis promotes the apoptosis of HUVECs.

IL-10 inhibits the proliferation and promotes the apoptosis of HUVECs by en-hancing the phosphorylation of STAT3

To determine the phosphorylation level of STAT3 stimulated by IL-10, Western blot-ting was used. The data showed that the expression of p-STAT3 in HUVECs treated with sepsis serum was significantly higher than that in NC group (P<0.05), and addi-tion of IL-10 antibody decreased the expression of p-STAT3 in HUVECs treated with sepsis serum (P<0.05) (Fig. 4A). CCK-8 assay and flow cytometry showed that addition of STAT3 phosphorylation inhibitor Stattic significantly enhanced the proliferation and G1/S transition of HUVECs treated with sepsis serum (P<0.05) (Fig. 4B,C). Moreover, addition of Stattic significantly inhibited the apoptosis of HUVECs treated with sepsis serum (P<0.05) (Fig. 4D). These results indicate that IL-10 inhibits the proliferation and promotes the apoptosis of HUVECs by enhancing the phosphorylation of STAT3.

Discussion

Sepsis is a clinically common acute and severe disease. The main reason that affects the prognosis of patients is the damage of tissues and organs caused by uncontrolled systemic inflammatory responses (Szabo et al., 2019). The barrier dysfunction and vascular dysfunction caused by vascular injury play important roles in this process (Teo et al., 2019). Maintaining the integrity of vascular function and structure is help-ful to improve the perfusion of tissues and organs, to prevent

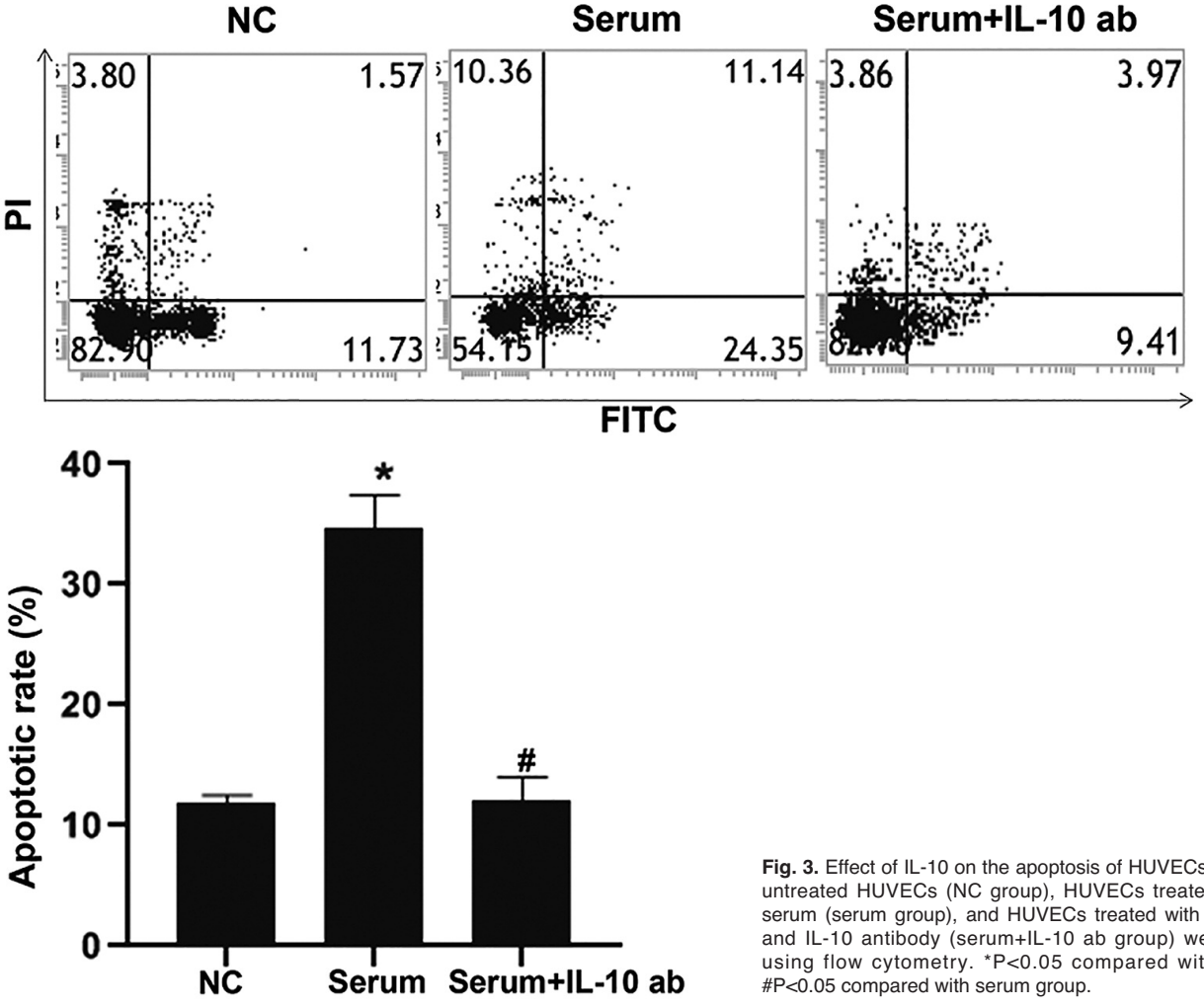
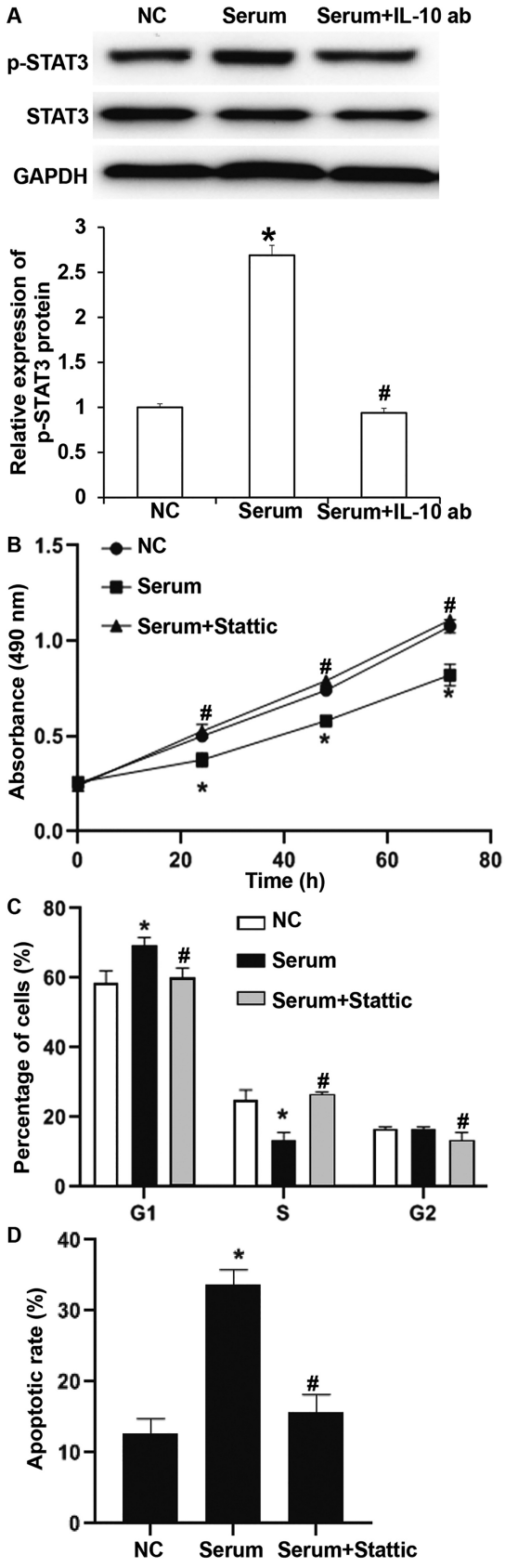


Fig. 3. Effect of IL-10 on the apoptosis of HUVECs. Apoptosis of untreated HUVECs (NC group), HUVECs treated with sepsis serum (serum group), and HUVECs treated with sepsis serum and IL-10 antibody (serum+IL-10 ab group) were examined using flow cytometry. *P<0.05 compared with NC group; #P<0.05 compared with serum group.



inflammatory factors from infiltrating local tissues, and to reduce organ damage (Hirano et al., 2019; Zhang and Niu, 2019). There are more than 18 million severe sepsis cases every year in the world and 750 thousand sepsis patients in the United States every year, and the incidence is still rising at a rate of 1.5-8.0% every year (Ling et al., 2019; Sharma et al., 2019). The prognosis of sepsis patients is poor and the mortality rate is high. About 14,000 people die of sepsis complications every day in the world, and about 215 thousand people die of sepsis every year in the United States (Zonnenberg et al., 2019). The mortality of sepsis is even higher than that of myocardial infarction, and it is the main cause of death for noncardiac patients in intensive care units. Sepsis is mainly driven by immune inflammation caused by infection, in which the synthesis and secretion of a large number of cytokines play important roles (Jiang et al., 2019; Aviram et al., 2020). For example, blocking PD1 induces the down-regulation of IL-10 expression and the up-regulation of IFN-γ, preventing the occurrence of post-sepsis aspergillosis (Vu et al., 2020). Transgenic mice with interleukin-32γ can inhibit LPS-induced sepsis (Kim et al., 2014). Of note, IL-10 has a strong anti-inflammatory ef-fect. The expression of IL-10 in the peripheral blood of patients with sepsis is higher than that in healthy people, but significantly lower than that in patients with SIRS (Potjo et al., 2019). In the present study, we found that the expression of IL-10 in serum of patients with sepsis or severe sepsis was significantly higher than that in the septic shock group, and IL-10 expression in all sepsis patients was significantly higher than that in the control group. Similar trends were observed for the ratio of IL-10$^+$ T cells. These results suggested that IL-10 level was reduced at the advanced stage of sepsis, possibly being associated with the inhibition of anti-inflammation.

In addition to its strong anti-inflammatory effect, IL-10 can also act on tissues and cells to exert its biological functions (Thompson et al., 2019). It is reported that IL-10 plays a synergistic effect via a variety of mechanisms, through which human amniotic mesenchymal stem cells with the modification of IL-10 gene are able to promote wound healing (Xiao et al., 2019). IL-10 is related to metastasis formation and tumor size of lung cancer (Karlicic et al., 2016). In the present study, we discovered that IL-10 inhibited HUVEC proliferation

Fig. 4. IL-10 exerts its biological function via STAT3 signaling pathway. **A.** Expres-sion of phosphorylated STAT3 protein in untreated HUVECs (NC group), HUVECs treated with sepsis serum (serum group), and HUVECs treated with sepsis serum and IL-10 antibody (serum+IL-10 ab group) as determined by Western blotting. **B.** Pro-liferation of HUVECs in NC group, serum group, and serum+IL-10 ab group as ex-amined by CCK-8 assay. **C.** Cell cycles of HUVECs in NC group, serum group, and serum+IL-10 ab group as tested by Flow cytometry. **D.** Apoptosis of HUVECs in NC group, serum group, and serum+IL-10 ab group as studied by Flow cytometry. *P<0.05 compared with NC group; #P<0.05 compared with serum group.

and promoted HUVEC apoptosis. This suggested that significantly elevated expression of IL-10 at the early stage of sepsis promoted the injury of vascular endothelial cells. Studies show that IL-10 can activate the STAT3 signaling pathway (Chen et al., 2019; Zhang et al., 2019). In order to further analyze the biological function of IL-10, we studied the changes of the STAT3 signal-ing pathway. Our results showed that IL-10 was involved in the activation of the STAT3 signaling pathway. By inhibiting the STAT3 signaling pathway, we found that the inhibition of HUVEC proliferation and the promotion of HUVEC apoptosis by IL-10 were reduced. This also suggested that IL-10 affected HUVEC function through the STAT3 signaling pathway. A limitation of the present study is that we only verified the changes of IL-10-STAT3 signaling pathway at the cellular level. Whether it can be established at the animal level remains to be further studied. We did not use sepsis peripheral serum to stimulate T cells from healthy people and observe the changes of IL-10. Therefore, it is unclear what factors stimulate the high expression of IL-10 in T cells from sepsis patients. These problems need further studies.

In conclusion, the present study demonstrates that the content of IL-10 and the ratio of IL-10$^+$ T cells in peripheral blood of patients with sepsis are up-regulated, and this inhibits HUVEC proliferation and promotes HUVEC apoptosis through the STAT3 signaling pathway.

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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.

Author contributions. ZX and JL contributed to the design of the study. ZX, BL, XJ, TS, YW, JT, CY and CC performed the experiments. ZX and BL analyzed the data. ZX and JL interpreted results and prepared the manuscript. The final version of the manuscript has been read and approved by all authors.

Ethical approval and consent to participate. All procedures performed in the current study were approved by the Ethics Commit-tee of Guangxi Medical University. Written informed consent was obtained from all patients or their families.

Consent for publication. Written informed consents for publication of any associated data and accompanying images were obtained from all patients or their parents, guardians or next of kin.

Competing interests. The authors declare that they have no competing interests.

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