The Study on the Regulation of Th Cells by MSCs Through the JAK-STAT Signaling Pathway to Protect Naturally Aged Sepsis Model Rats

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Research

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Abstract

Background: Sepsis is the leading cause of death among patients, especially elderly patients, in intensive care units worldwide. However, there is no effective treatment for sepsis in the aging population. Therefore, we designed such a study to confirm the protective effect of MSCs against sepsis in the elderly and to explore its mechanisms.

Methods: In this study, we established a sepsis model using naturally aged SD rats and injected 5×10^6 umbilical cord-derived MSCs via the tail vein. Each group of rats was analyzed for survival, examined for biochemical parameters, stained for organ histology, and analyzed for the Th cell subpopulation ratio and inflammatory cytokine levels by flow cytometry. Western blotting was performed to detect the activity of the JAK-STAT signaling pathway. We designed in vitro experiments to confirm the regulatory role of MSCs, and verified the possible mechanism using JAK/STAT inhibitors.

Results: The results revealed that the 72 h survival rate of sepsis rats treated with MSCs was significantly increased, organ damage and inflammatory infiltration were reduced, the levels of organ damage indicators were decreased, the ratios of Th1/Th2 and Th17/Treg in peripheral blood and spleen were significantly decreased, the levels of pro-inflammatory cytokines such as IL-6 were decreased, the levels of anti-inflammatory cytokines such as IL-10 were increased, and the levels of STAT1 and STAT3 phosphorylation were reduced. These results were validated in in vitro experiments.

Conclusions: Therefore, this study confirms that MSCs can control the inflammatory response induced by sepsis by regulating Th cells and inflammatory factors, and that this leads to reduction of tissue damage, protection of organ functions and ultimately improvement of survival in aged sepsis model rats. Inhibition of the JAK-STAT signaling pathway may be an important mechanism for their action.

Background

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection(1) and is the leading cause of death in ICU patients, especially among the elderly. Globally, there are 30 million cases of sepsis each year with a mortality rate of 30-40%(2, 3), and more than 60% of the deceased are elderly people over 65 years of age(4). In recent years, a combination of anti-infective, anti-shock and supportive therapy has reduced mortality from sepsis, but the severity of the disease has increased year by year(5). Recent studies have found that the immune system plays a central role during sepsis both in the early storm of inflammatory factors and in the later stages of immune suppression(6, 7). The incidence of sepsis will also continue to increase with the increasing aging of the population(8).

Mesenchymal stem cells (MSCs) interact with many types of immune cells and regulate their functions, such as metabolism, migration, cytotoxicity and inflammatory response(9). Therefore, MSCs can be used to treat a variety of autoimmune diseases, inflammation-related diseases, and other diseases triggered by an imbalance in immune system homeostasis. Currently, MSCs have been used by researchers worldwide for the treatment of sepsis. In animal experiments, MSCs have been found to reduce the systemic

inflammatory response, decrease the level of organ damage(10), and improve the survival of sepsis model animals(11).

The JAK/STAT pathway is considered one of the major signaling pathways involved in sepsis(12) and is a part of many key cytokine signaling pathways in the pathogenesis of sepsis, such as IL-4, IL-6, IL-10, IL-12, and IFN- γ (13–15). Early in the pathogenesis of sepsis, the body responds to infection with a strong immune response, manifested by a massive mobilization of pro-inflammatory cells and pro-inflammatory factors, which significantly elevates levels of pro-inflammatory cytokines such as IL-1 β and IL-6, and causes an imbalance in the Th1/Th2 and Th17/Treg ratios(16–18). A prospective observational study found that an imbalance in the Th17/Treg ratio correlated with the severity and prognosis of sepsis patients(19). The inflammatory factor storm in the early stages of sepsis causes massive depletion of lymphocytes and leads to long-term immune dysfunction(20), increased susceptibility to secondary infections, and reduced 5-year survival(21, 22). Currently, MSCs are widely used in the treatment of autoimmune diseases and acute and chronic inflammation, and a large number of studies have found that MSCs can regulate the helper T cell subsets represented by Th1 and Th17 cells.

The experimental animals investigated in the current study were all adult laboratory animals. To our knowledge, no studies have reported on the application of MSCs to naturally aged animals, which is inconsistent with the clinical situation in which the majority of sepsis patients are elderly. In the present study, we established a sepsis model by the CLP method in naturally aged SD rats, observed the effect of MSCs on improving survival and vital organ functions in aged sepsis model rats, clarified the inhibitory effect of MSCs on the early inflammatory factor storm and the regulatory effect of helper T cell subsets in sepsis of the aged population and explored the molecular mechanisms of such effects.

Methods

Study design

This study was approved by the Ethics Committee of the Chinese PLA General Hospital. Animal experiments were approved by the Experimental Animal Welfare Ethics Committee of Chinese PLA General Hospital. The experimental animals were ninety (90) 21-month-old male SD rats (SPF (Beijing) Biotechnology, Beijing, China) weighing 801.5 ± 79.5 g. The rats were divided into a sham-operated group (n=18), sepsis model group (n=36) and sepsis+MSCs treatment group (n=36). Equal number of rats in each group were randomly assigned to 3 subgroups and samples were collected after euthanasia at 6 h, 24 h or 72 h post-operation and/or treatment. The CLP model was used as a model of sepsis, and UC-MSCs were injected into the tail vein 1 h after CLP operation to induce sepsis. Five million (5×10^6) UC-MSCs in 1 ml saline were administered to each animal in the treatment group, and the sham-operated and model groups were injected with equal amounts of sterile saline at the same time. The 72 h survival rate of the rats in each group was statistically analyzed.

In vitro experiments were performed using lymphocytes isolated from the spleen of adult SD rats. Cells were either left unstimulated (control), stimulated with LPS (LPS), or stimulated with LPS and co-cultured with MSC (LPS+MSC). In vitro experiments were performed to validate the modulation of the JAK-STAT signaling pathway by MSCs using the JAK inhibitor AZD1480 (Cat# M2044, AbMole, TX, USA), the STAT1 inhibitor fludarabine (Cat# M2028, AbMole, TX, USA), and the STAT3 inhibitor cryptotanshinone (Cat# M3982, AbMole, TX, USA).

Cell culture

Umbilical cords were obtained from the Department of Obstetrics and Gynecology, the First Medical Center, Chinese PLA General Hospital, China, and the mothers signed an informed consent. Umbilical cord-derived MSCs were isolated, cultured, and expanded using previously published protocols(23). Briefly, UC-MSCs were cultured in MEM (Gibco, Thermo Fisher Science, Cat# 12571063, MA, USA) supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Science, Cat# 10099141, MA, USA). When cells reached 70-80% confluence, they were detached with trypsin-ethylenediaminetetraacetic acid (Gibco, Cat# 25300054) and passaged. SD rat spleen lymphocytes were cultured in RPMI 1640 medium (Gibco, Thermo Fisher Science, Cat# 31870082, MA, USA) with 10% fetal bovine serum (Gibco, Thermo Fisher Science, Cat# 10099141, MA, USA).

Cecal ligation-and-perforation (CLP)

A sepsis model was established by the modified CLP method (24). The rats were anesthetized by intraperitoneal injection of 50 mg/kg sodium pentobarbital to ensure that the rats did not wake up until the surgery was completed. After the rats were anesthetized and the abdominal body hair was removed, the skin was disinfected, a 1.5-cm-long midline incision was made in the abdomen, the cecum was located and gently externalized, the cecum was ligated at 1/2 of its length with a sterile 4-gauge wire, and a small amount of intestinal contents was extruded by penetrating the cecum once at the distal end of the ligature with a 22G needle. Finally, the cecum was placed back into the abdominal cavity, and the abdominal muscles and skin were sutured layer by layer. The rats in the sham-operated group only underwent open cecum externalization without ligation or perforation.

Identification of umbilical cord-derived mesenchymal stem cells

Umbilical cord MSCs were identified as previously described(25). Third passage (P3) MSCs were immunophenotyped by flow cytometry. Cells were collected and adjusted to 1×10^6 cells per sample. Cells were washed with PBS and incubated with antibodies for 15 min at room temperature protected from light. Following antibodies were obtained from eBioScience, Thermo Fisher Science (MA, USA): PE CD11b monoclonal antibody (Cat# RM2804), PE CD34 monoclonal antibody (Cat# 12-0349-41), APC CD44 monoclonal antibody (Cat#47-0441-82), APC CD45 monoclonal antibody (Cat#4 7-0441-82), APC CD45 monoclonal antibody (Cat# 47-0451-82), APC CD90 monoclonal antibody (Cat# 17-0909-41), and PE CD105 monoclonal antibody (Cat# MA5-17946). After incubation, the cells were washed with PBS and analyzed by flow cytometry using the BD Accuri C6 software system (version 1.0.264.21; BD

Biosciences). Differentiation potential was analyzed using a human mesenchymal stem cell differentiation kit (TBDscience, Tianjin, China). P3 MSCs were cultured in 6-well culture plates at a density of 10^4 cells/well, and lipogenic (Cat# TBD20190004), osteogenic (Cat# TBD20190002) and chondrogenic (Cat# TBD20190003) differentiation assays were performed and evaluated according to the kit instructions. Cell identification results are shown in Supplementary Material 1.

Histology and tissue staining

The right lung, liver, kidney, ileum and spleen of each experimental group of rats were harvested for histological observation and staining. The organs were fixed in 4% paraformaldehyde, dehydrated and paraffin embedded. Paraffin sections were analyzed by hematoxylin-eosin (HE) staining (Cat# G1120, Solarbio, Beijing, China) to examine gross histology. TdT-mediated dUTP nick-end labeling (TUNEL) staining (Cat# C1088, Beyotime, Shanghai, China) was used to label apoptotic cells in the rat spleen. The stained sections were viewed and scanned with a panoramic MIDI CaseViewer system (3DHISTECH, Hungary).

Biochemical assays

Blood samples from each experimental group of rats were centrifuged at 2000 x g for 10 min within 4 h of collection to isolate serum. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatinine (CREA) and uric acid (UA) were measured by an automated biochemical analyzer (7170-A, Hitachi, Japan). Immunocytokine concentrations in serum and spleen tissue homogenates were measured using Aimplex Multiple Immunoassays for Flow on a flow cytometer (BD Bioscience, NJ, USA) according to the manufacturer's (Aimplex Bioscience, CA, USA) instructions. IL-1 β (Cat# B311165), IL-6 (Cat# A311125), IL-17A (Cat# B311113), TNF- α (Cat# A311129), IFN- γ (Cat# A311101), IL-4 (Cat# A311121), IL-10 (Cat# A311109), IL-13 (Cat# B311177) and TGF- β (Cat# B111206) were measured.

Flow cytometry

Flow cytometry (BD Bioscience, NJ, USA) was used to analyze helper T cell subsets in peripheral blood and spleen cells suspensions according to the reagent manufacturer's (Biolegend, CA, USA) instructions. Peripheral blood was collected into containers with sodium heparin at a concentration of 1000 U/ml as anticoagulant, and spleen tissue was ground as described above. We used FITC anti-rat CD4 (Cat# 201505), anti-rat CD45 PerCP-CY5.5 (Cat# 202220) to label helper T cells, APC anti-rat CD3 (Cat# 201414) and PE anti-mouse/rat/human FOXP3 (Cat# 320008) to label Treg cells, Alexa Fluor® 647 anti-rat IFN- γ (Cat# 507810)-labeled Th1 cells, PE anti-rat IL-4 (Cat# 511906) to label Th2 cells, and Alexa Fluor® 647 anti-mouse IL-17 (Cat# 146303) to label Th17 cells. Treg and Th cells were stained intracellularly using Foxp3/transcription factor fixation/permeabilization concentrates and dilutions (Cat# 00-5521-00, eBioScience, Thermo Fisher Science, MA, USA). Th1, Th2 and Th17 cells were assayed after 5 h of stimulation with Cell Stimulation Cocktail (Cat# TNB-4975-UL100, Tonbo Bioscience, CA,

USA). Fixation, stimulation and staining were performed according to the manufacturers' instructions and analyzed by flow cytometry.

Western blot

The spleen was homogenized using a low-temperature high-speed grinder (Servicebio, Wuhan, China) to obtain the homogenate. Lymphocytes for in vitro experiments were collected at pre-determined time points, rinsed three times with pre-cooled PBS (Servicebio, Wuhan, China), and lysed on ice for 10 min with RIPA lysis buffer (Thermo Fisher Scientific, MA, USA). A BCA quantification kit (Solarbio, Beijing, China) was used for sample protein quantification, followed by incubation in a metal bath at 95°C for 10 min. Protein samples were transferred onto nitrocellulose membranes after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V. The membranes were blocked with Fast Blocking Buffer (NcmBlot Blocking Buffer) (Cat# P30500, New Cell & Molecular Biotech, Suzhou, China) for 10 min and incubated with primary rabbit polyclonal antibody overnight at 4°C. Next day, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Cat# 7074S, 1:3000, CST, MA, USA) at room temperature for 120 min, and washed with 1× TBST (Solarbio, Beijing, China). Protein bands were visualized and documented using an electrochemiluminescence kit (Solarbio, Beijing, China). Primary antibodies used for this study were STAT1 (Cat# 14994, 1:1000, CST), p-STAT1 (Cat# 7649, 1:1000, CST), STAT3 (Cat# 12640, 1:1000, CST), p-STAT3 (Cat# 9145, 1:2000, CST) and β -Actin (Cat# 8457, 1:2000, CST).

Statistical analysis

SPSS 22.0 statistical software was used for analysis. The measurement data were expressed as $\bar{x} \pm s$. When the data of each group conformed to normal distribution (Kolmogorov-Smirnov test) and chi-square (Levene test), one-way ANOVA was used for comparison of multiple sample means. A nonparametric test (Mann-Whitney U test) was used for pathological damage scores. The Kaplan-Meier method was used for survival analysis of each group of rats, and the statistical method was the log-rank (Mantel-Cox) test. $p < 0.05$ was considered a statistically significant difference. Graphs were plotted using GraphPad Prism 8.0 software.

Results

MSCs improved survival and biochemical parameters in aged sepsis model rats

The mean body weight of the rats in each group was not significantly different and met the weight criteria for aged rats (Fig. 1.A). A sepsis model was successfully established by the CLP method (Fig. 1.B). UC-MSCs possessed lipogenic, osteogenic and chondrogenic differentiation abilities, and the expression of surface markers met the criteria(25) (Sup. Fig. 1). The mortality rate of aged sepsis model rats was reduced after MSC treatment (Fig. 1. C-D), and the 72 h survival rate of rats in the treatment group was significantly higher than that of rats in the control-treated group ($\chi^2 = 13.56$, $P < 0.01$, Fig. 1.E). The levels

of ALT, AST, CREA, BUN and LDH were significantly higher in aged sepsis model rats than in the sham-operated group. After treatment with MSCs, the levels of these biochemical indices became significantly lower and the levels of ALP became significantly higher (Fig. 1.F-K).

MSCs ameliorate organ damage in aged sepsis model rats

MSCs were able to reduce the histopathological damage to critical organs, including the lung, liver, kidney and intestine. The alveolar structure of rats in the control-treated group were markedly disturbed, and widened septa and a large number of inflammatory cell infiltrates were observed. In contrast, rats in the treatment group showed significantly less lung damage and tissue damage scores were lower than the control-treated group (Fig. 2.A-B). The rats in the control-treated group showed massive inflammatory cell infiltration in the liver at the early stage of the disease, which was followed by hepatocellular edema, structural disorganization of liver lobules, vacuolar degeneration and debris-like necrosis, but the degree of liver injury was significantly reduced after MSC treatment (Fig. 2.C-D). Sepsis caused kidney injury in rats, which manifested as interstitial edema with massive inflammatory cell infiltration and glomerular edema. The degree of injury was significantly reduced in the MSC-treated rats (Fig. 2.E-F). In addition, the intestinal tract of rats in the control-treated group was severely damaged, the epithelial layer on both sides of the villi was markedly separated from the lamina propria, the apical part was broken, and the lamina propria was heavily infiltrated with inflammatory cells, which was significantly reduced after MSC treatment (Fig. 2.G-H). The rats in the sham-operated group showed no significant tissue damage in these organs.

MSCs modulate systemic inflammatory responses in aged sepsis model rats

Pro-inflammatory cytokines (IL-1 β , IL-6, IL-17A, TNF- α , IFN- γ) were elevated in the control-treated rats compared to the sham-operated group; the levels of IL-1 β , IL-6 and IL-17A began to increase early in the disease onset. In comparison, circulating levels of pro-inflammatory cytokines were significantly reduced in MSCs-treated rats, and anti-inflammatory cytokines (IL-4, IL 10, IL-13, TGF- β) were elevated compared to the control-treated group (Fig. 3.A-I). In addition, the proportions of Th1 and Th17 cell subsets among helper T cells were elevated in the peripheral blood of aged sepsis model rats, and after treatment with MSCs, the proportions of Th1 and Th17 cells were significantly decreased, and the ratios of Th1/Th2 and Th17/Treg were also significantly reduced compared to the control-treated group (Fig. 3.J-P).

MSCs modulate splenic cytokines and helper T cells in aged sepsis model rats

Flow cytometry revealed that the levels of pro-inflammatory cytokines were significantly higher in the spleens of aged sepsis model rats than in the spleens of sham-operated rats, and after MSC treatment, the levels of pro-inflammatory cytokines were reduced and the levels of anti-inflammatory cytokines were significantly increased in the treated rats (Fig. 4A-I). The proportions of Th1 and Th17 CD4⁺ T cells in the spleen of aged sepsis model rats were significantly increased at the early stage of disease onset and gradually decreased thereafter, the proportions of Th1 and Th17 cells decreased after MSC treatment, and the ratios of Th1/Th2 and Th17/Treg cells were also significantly decreased (Fig. 4J-P).

MSCs reduce apoptosis in the spleen and regulate STAT signaling activation levels in aged sepsis model rats

Compared with the sham-operated group, the number of apoptotic cells in the spleen of rats in the control-treated group was significantly increased; a large number of apoptotic cells began to appear from the early stage of disease and persisted. The number of apoptotic cells in the spleen of MSC-treated rats was significantly lower than that of the control-treated group and comparable to that of the sham-operated group, and no significant apoptosis was observed (Fig. 5.A-B). Western blot assays of STAT1 and STAT3 revealed that the phosphorylation levels of STAT1 and STAT3 in the spleens of aged sepsis model rats were significantly increased at all time points compared with those in the spleens of normal rats, and maximal phosphorylation was observed at 24 h. The phosphorylation levels of STAT1 and STAT3 in the MSC-treated rats were significantly lower than those in the control-treated rats at the same time points.

MSCs ameliorate LPS-induced inflammatory responses in lymphocytes in vitro

Compared to blank controls, LPS-stimulated lymphocytes produced a large number of cytokines, of which the levels of pro-inflammatory cytokines were significantly elevated. In contrast, the levels of pro-inflammatory cytokines and anti-inflammatory cytokines in the cell supernatant of LPS-stimulated lymphocytes co-cultured with MSCs were significantly reduced. In addition, flow cytometry assays revealed that the fractions of Th1 and Th2 cells were significantly higher in LPS-stimulated lymphocytes, and the ratios of Th1/Th2 and Th17/Treg cells were also significantly higher, while the ratios of Th1/Th2 and Th17/Treg cells in the co-culture group was significantly lower than that in the LPS-stimulated group, which was consistent with the results of in vivo experiments.

Inhibitors validate the role of the JAK-STAT signaling pathway

The administration of JAK inhibitor (AZD1480) resulted in a significant decrease in the ratios of Th1 and Th17 in lymphocytes compared to the LPS-stimulated sample, as well as a significant decrease in the ratios of Th1/Th2 and Th17/Treg. The levels of IFN- γ (Th1-specific cytokine) and IL-17A (Th17-specific cytokine) in the supernatant of the inhibitor-treated cells were also decreased. Furthermore, the ratios of Th1 and Th17 and the levels of their specific cytokines were reduced in the cells co-cultured with MSC after the addition of JAK inhibitors compared to the inhibitor-treated cells, and the difference was statistically significant (Fig. 7.A-H). The proportion of Th1 cells and the levels of IFN- γ in lymphocytes were significantly reduced after the administration of STAT1 inhibitor, consistent with the changes in the co-culture sample. STAT1 inhibitor reduced the levels of STAT1 in lymphocytes, and the levels of p-STAT1 were significantly lower than those in the LPS-stimulated cells (Fig. 7.I-N). Similarly, the proportion of Th17 in lymphocytes and the level of IL-17A were significantly reduced after the administration of STAT3 inhibitor, consistent with the changes in the co-culture sample, and the level of STAT3 in lymphocytes was reduced after the addition of the inhibitor, and the levels of p-STAT1 were significantly lower than those in the LPS-stimulated cells (Fig. 7.O-T).

Discussion

In this study, we established a sepsis model in the elderly using naturally aged SD rats to investigate the role and possible mechanisms of MSCs in treating sepsis in the elderly, regulating the immune inflammatory status and protecting organ functions. The results showed that MSCs derived from the umbilical cord could improve the overall status and 72 h survival of aged rats with sepsis and protect vital organ functions and ameliorate histopathological damages caused by sepsis. In vivo and in vitro experiments confirmed that MSCs could inhibit the systemic inflammatory response, regulate the Th1/Th2 and Th17/Treg ratios and inflammatory cytokine levels in aged rats with sepsis, and modulation of the JAK-STAT signaling pathway may be one of the mechanisms of action.

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection(1), and the intense early inflammatory response causes a massive depletion of immune cells and immune factors, which is an important cause of immune suppression in the later stages of sepsis(26). The presence of some degree of immune decline in the elderly itself affects the progressive decline in the function of all types of immune cells of the innate and adaptive immune systems, especially CD8⁺ T cells and CD4⁺ T cells, and this results in a higher susceptibility to sepsis and mortality in the elderly(27). Currently, many studies have shown that MSCs can improve mortality and the degree of organ damage in sepsis model animals(28, 29), and the results of the latest preclinical meta-analysis have shown that MSC treatment can significantly reduce mortality in sepsis model animals. Current experimental results support a potential therapeutic effect of MSCs in clinical trials(30). To date, all three completed phase I clinical trials have confirmed the safety of MSCs applied to the treatment of patients with sepsis or endotoxemia(31–33). However, there are still no published studies on the application of MSCs in aged animals with sepsis or in elderly sepsis patients. We injected MSCs of umbilical cord origin into naturally aged rats with sepsis and found that the mental and feeding status of MSC-treated sepsis model rats improved, and that the 24 h and 72 h mortality rates were significantly reduced. Survival analysis revealed that the death of rats in the control-treated group was concentrated within 24 h after the completion of the CLP operation, which might be due to organ dysfunction or even failure caused by the outbreak of inflammatory response. In contrast, the death of rats in the MSC-treatment group occurred more randomly and the survival time was longer compared with that of the control-treated group, which indicated the important protective role of MSCs at the early stage of pathogenesis.

Current studies confirm that the application of MSCs for sepsis not only improves the survival rate of experimental animals but also improves several vital organ functions. Clinically, patients with sepsis have a high morbidity and mortality rate, and in animal experiments, CLP sepsis animals had a 7-day mortality rate of 70%(34) and a 28-day mortality rate of 80%(11). After treatment with MSCs, survival rates were significantly improved, critical organ functions such as lung, liver, and spleen were significantly protected, and pathological damages to vital organs were significantly reduced(35). However, some researchers found that MSCs did not improve mortality or systemic inflammation and stress in pigs with sepsis (36). In our study, we found that MSCs could significantly improve the pathological damage to vital organs, reduce the serum levels of ALT, AST, BUN, Cr and LDH and increase the level of ALP in aged sepsis model

rats. This indicates that MSCs possess good therapeutic effects in the aged sepsis model rats, which can effectively reduce organ damage and protect organ functions, thus MSCs improved the survival rate of aged sepsis model rats. By histological staining, we found that local infiltration of inflammatory cells was reduced after MSC treatment. Accordingly, we speculate that allaying the inflammatory factor storm in the early stage of sepsis may be an important mechanism by which MSCs exert their protective effects.

The cytokine storm is an excessive immune response induced by various stimuli, which is the most important pathophysiological feature of early sepsis and the main cause of multi-organ dysfunction and long-term immune suppression(37). Immune cells play a crucial role in the initiation phase of a cytokine storm. When the organism is stimulated by pathogenic microorganisms, activated CD4⁺ T cells differentiate into different subpopulations to play different roles; they are mainly divided into pro-inflammatory cell subpopulations represented by Th1 and Th17 and anti-inflammatory cell subpopulations represented by Treg and Th2. Sepsis is a dysregulation of the pro-inflammatory/anti-inflammatory response caused by severe infection, as evidenced by an imbalance in the ratios of Th1/Th2 and Th17/Treg cells(38, 39). In the early stages of infection, a variety of inflammatory cells are recruited and activated, and they release large amounts of inflammatory cytokines and chemokines, such as TNF- α and IL-1 β , which are rapidly secreted and reach a peak within a few hours. Under normal conditions, the body regulates the degree of inflammatory response by secreting anti-inflammatory factors to remove harmful substances and maintain intracellular homeostasis(40, 41). However, when this balance is disrupted, early reactive cytokines trigger a cascade of additional cytokines, which results in the activation and release of a large number of inflammatory factors, including IL-6, IL-12, and macrophage inflammatory protein (MCP)-1 α , and induces an uncontrolled systemic inflammatory response(42, 43). MSCs have been found to reduce serum levels of inflammatory factors in adult sepsis model animal studies(30, 44), and this has been confirmed in aged sepsis model animals. In our study, we found that the ratios of Th1/Th2 and Th17/Treg cells were significantly elevated in the peripheral blood of aged sepsis model rats, which indicates that helper T-cell subsets play a role in the systemic inflammatory response. We also found that the ratios of Th1/Th2 and Th17/Treg cells were significantly reduced after treatment with MSCs and approached the levels comparable to the sham-operated group. Furthermore, cytokines associated with helper T cells, such as IFN- γ , IL-17, IL-4 and IL-10, showed the same trend as the change in the ratio of immune cells, which also confirmed that MSCs regulated the secretory function of Th cells, and kept the excessive inflammatory response under control. In addition, MSCs were able to reduce the levels of pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF- α and elevate the levels of anti-inflammatory cytokines such as IL-10 and TGF- β in the circulation of aged sepsis model rats. This prevented the initiation and development of cytokine storms to a certain extent. Based on the in vivo experiments, we established an in vitro sepsis model by adding LPS to rat lymphocytes cultured in vitro. The results showed that, compared with the LPS-stimulated sample, the cells co-cultured with MSCs produced significantly less pro-inflammatory factors and significantly more anti-inflammatory factors at all time points, and that the ratios of Th1/Th2 and Th17/Treg cells were decreased in the culture. From these results, we can conclude that MSCs can regulate the inflammatory response in vitro and in vivo by regulating the number and function of Th cell subpopulations.

The spleen, as the largest peripheral immune organ, accumulates a large number of lymphocytes and is an important site for the specific immune response of the organism. Sepsis induces increased apoptosis of B lymphocytes and effector T cells, which results in immune paralysis of the organism against subsequent infections. Current studies have confirmed that sepsis causes apoptosis of a large number of lymphocytes in the spleen, and the number of apoptotic splenocytes and lymphocytes is closely related to the severity of inflammation and patient prognosis(45). In patients with sepsis, the rate of lymphocyte apoptosis was significantly higher among those who died within 28 days after disease onset than those who were alive on day 28 (46). Using TUNEL staining of spleen tissues of aged sepsis model rats in each experimental group, we found that the proportion of apoptotic cells was significantly higher in the spleen of aged sepsis model rats, and that the number of apoptotic cells was significantly reduced after treatment with MSCs. This indicates that MSCs can inhibit lymphocyte apoptosis induced by sepsis and suggests that MSCs have a positive effect on preventing immune dysfunction and long-term immunosuppression. To further investigate the source of changes in circulating Th cells and inflammatory factors, we examined the levels of inflammatory factors and the ratios of Th cell subpopulations in the spleen and found that the overall trends of changes in inflammatory factors and Th cell subpopulations in the spleen of rats in each group were consistent with those in peripheral blood. This indicates that MSCs can regulate the ratio of Th cells in the spleen and maintain the Th1/Th2 and Th17/Treg balance, which in turn regulates the levels of inflammation-related cytokines and controls the inflammatory response. After treatment with MSCs, the activation of Th1 and Th17 cells in the spleen of aged sepsis model rats was reduced, and the number of activated cells released into the peripheral blood was correspondingly reduced. This regulated the inflammatory response and exerted a protective effect on all organs of the body.

The JAK-STAT pathway is capable of mediating cell proliferation and apoptosis(47), which is thought to be involved in sepsis-induced multi-organ dysfunction(12). The JAK-STAT signaling pathway is activated by both pro-inflammatory cytokines (IFN- γ , IL-12, and IL-27) and anti-inflammatory cytokines (IL-4, IL-10, and IL-13), which indicates that individual STAT family members differentially regulate the balance of Th cell subsets. In the helper T-cell subpopulation, Th1 cell differentiation is mainly regulated by STAT1 and STAT4(48), while STAT6 and STAT3 regulate the differentiation of the immunoregulatory Th2 and Th17 cells, respectively(49). One study showed that STAT1 knock-out mice were significantly resistant to LPS-induced endotoxemia(50) and CLP-induced septic shock(51). This was attributed to the altered balance of the Th1/Th2 immune response, which resulted in significantly reduced organ damage in the liver and kidney. Another study showed that JAK2 inhibitors improved survival after CLP operation in mice and rats by reducing the expression of pro-inflammatory mediators such as TNF- α , IL-6 and high mobility group box-1 (HMGB-1)(52). To explore the molecular mechanism of Th cell regulation by MSCs, we examined the relevant STAT signaling pathways in the spleen of aged sepsis model rats. We found that the phosphorylation levels of STAT1 and STAT3 were significantly increased in the spleen of sepsis model rats, the phosphorylation levels of both molecules were significantly reduced after MSC treatment, and the expression levels of STAT1 and STAT3 were comparable to those of normal rats. In the in vitro experiments, we administered inhibitors of the JAK, STAT1 and STAT3 signaling pathways to the LPS-

stimulated cells and LPS-stimulated cells co-cultured with MSC. The results showed that the proportion of Th cell subpopulations in the LPS-stimulated sample changed after treatment with individual inhibitor, and the overall trend of the changes was consistent with that in the LPS+MSC cells. This indicated that MSCs and JAK-STAT inhibitors exerted the same effect, while the results of LPS+MSCs treated with inhibitors showed that MSCs and inhibitors could function synergistically to some extent. In addition, we evaluated the levels of IFN- γ and IL-17, which represent Th1 and Th17 cells, respectively, in each sample. We found that JAK inhibitors were able to reduce the levels of both IFN- γ and IL-17, while STAT1 inhibitor reduced the levels of IFN- γ and STAT3 inhibitor reduced the levels of IL-17. The above results confirm that MSCs regulate the quantity and function of the relevant Th cell subpopulations by inhibiting the JAK-STAT signaling pathway to control the inflammatory response. If this is the case, can JAK inhibitors be used directly to treat sepsis? The answer is uncertain. The MSCs used in this study have more diverse regulatory effects than the single effect of JAK-STAT pathway inhibitors. The mechanisms of immunomodulatory effects of MSCs are complex; they include the synthesis and secretion of multiple mediators, direct interaction with target cells, and regulation by certain antigen-presenting cells. In MSCs, no single pathway controls the entire process(53), and their regulatory effects are influenced by the microenvironment and immune status. Thus, we confirmed that MSCs could protect aged sepsis model rats through the regulation of the JAK-STAT signaling pathway, but we also acknowledge that this is only the tip of the iceberg of the mechanism of action of MSCs, and that more complex mechanisms remain to be investigated in depth.

Although our study confirmed the protective effect of MSCs in aged sepsis model rats, there are still some limitations; our understanding of the molecular mechanism of Th cell regulation by MSCs is incomplete. We also have not clarified whether the immune function of aged sepsis model rats is different or similar to that of adult sepsis model rats. We will continue to investigate related issues in depth in our subsequent studies.

Conclusion

In conclusion, this study confirmed that treatment with MSCs could improve survival, protect vital organ functions, regulate sepsis-induced inflammatory factor storms and ameliorate immune dysfunction in aged sepsis model rats. We propose that inhibition of the JAK-STAT signaling pathway may be one of the mechanisms of MSC-mediated protection.

Declarations

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of the Chinese PLA General Hospital. Animal experiments were approved by the Experimental Animal Welfare Ethics Committee of Chinese PLA General Hospital. Umbilical cords were obtained from the Department of Obstetrics and Gynecology, the First Medical Center, Chinese PLA General Hospital, China, and the mothers signed an informed consent.

Consent for Publication

Not applicable

Availability of data and materials

The authors declare that there are no primary datasets and computer codes associated with this study. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

All authors declare no competing interests.

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Author Contributions

L.W. performed experiments, analyzed data, and wrote the manuscript. Z.D. and Y.S. performed experiments and analyzed data. Y.L.(Yun Li), Y.Z. and Y.L.(Yuyan Liu) performed experiments. M.Y. and R.Y. analyzed data. F.Z. revised the manuscript. Z.Q. guided the experimental design. H.K. designed and guided the experiments and revised the manuscript.

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Not applicable

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Figures

Figure 1

Survival rate and biochemical markers of rats in each group. (A) Mean body weight of rats in each group; (B) Hemorrhagic necrosis of cecum and abdominal bulk of rats after CLP; (C) 24h mortality rate of rats in each group; (D) 72h mortality rate of rats in each group; (E) survival analysis of rats in each group (Log-rank (Mantel-Cox) Test); (F-K) levels of biochemical markers in serum of rats in each group (** $p < 0.01$, *** $p < 0.001$).

Figure 2

HE staining and tissue damage scores of lung, liver, kidney and ileum of rats in each group. (A) HE staining of lungs at 6h, 24h and 72h in each group of rats; (B) lung tissue damage scores in each group of rats; (C) liver HE staining at 6h, 24h and 72h in each group of rats; (D) liver tissue damage scores in each group of rats; (E) kidney HE staining at 6h, 24h and 72h in each group of rats; (F) kidney tissue damage scores in each group of rats; (G) ileum HE staining at 6h, 24h and 72h in each group of rats; (G)

ileum HE staining at 6h, 24h and 72h in each group of rats; (H) kidney tissue damage scores in each group of rats. (G) HE staining of ileum at 6h, 24h and 72h; (H) ileal tissue damage scores of rats in each group (n=5, scale bar=100μm, ***p<0.001).

Figure 3

Inflammatory cytokine levels and proportions of Th cell subpopulations in peripheral blood of rats in each group. (A-I) Inflammatory cytokine levels in serum of rats in each group (*compared with the model

group); (J) circle gating strategy for flow cytometry analysis of peripheral blood Th cell proportions; (K, L, N, O) flow analysis of the proportions of Th1, Th2, Th17, and Treg cells in peripheral blood as a percentage of CD4+ T cells; (M) ratio of Th1/Th2 in peripheral blood of rats in each group; (P) ratio of Th17/Treg in peripheral blood of rats in each group (*p<0.05, **p<0.01, ***p<0.001).

Figure 4

Levels of inflammatory cytokines and proportions of Th cell subpopulations in the spleen suspensions of rats in each group. (A-I) Levels of inflammatory cytokines in the spleen suspensions of rats in each group (compared with the model group); (J) circle-gating strategy for flow cytometry analysis of the proportion of splenic Th cells; (K, L, N, O) flow analysis of the proportion of Th1, Th2, Th17, and Treg cells in the spleen as a percentage of CD4+ T cells; (M) the ratio of Th1/Th2 in the spleen of rats in each group; (P) the ratio of The ratio of Th17/Treg in spleen of each group (*p<0.05, **p<0.01, ***p<0.001).

Figure 5

Tunel staining and STAT1 and STAT3 expression levels in the spleen of rats in each group. (A) Apoptosis of spleen lymphocytes at 6h, 24h and 72h in each group of rats (scale bar=100μm); (2) number of apoptotic cells per field of view in each group of rats; (C-D) expression levels and semi-quantitative analysis of STAT1 and p-STAT1 in spleen tissue of each group of rats; (E-F) expression levels and semi-quantitative analysis of STAT3 and p-STAT3 in spleen tissue of each group of rats. Results (**p<0.01, ***p<0.001).

Figure 6

Levels of inflammatory cytokines in cell supernatants and the proportion of Th cell subpopulations in lymphocytes in each group of in vitro experiments. (A-I) Levels of inflammatory cytokines in cell supernatants of each group (*compared with the model group); (J) circle gating strategy for flow cytometry analysis of the proportion of Th cells in lymphocytes of each group; (K, L, N, O) flow analysis of the proportion of Th1, Th2, Th17, and Treg cells in lymphocytes of each group as a percentage of CD4+ T cells; (M) ratio of Th1/ Th2 in lymphocytes of each experimental group; (P) ratio of Th17/Treg in lymphocytes of each experimental group (*p<0.05, **p<0.01, ***p<0.001).

Figure 7

Levels of inflammatory factors in cell supernatants and the proportion of Th cells in lymphocytes and the expression levels of STAT1 and STAT3 in each group after the use of inhibitors. (A-F) Proportions of Th1, Th2, Th17 and Treg cells in CD4+ T cells and the ratios of Th1/Th2 and Th17/Treg in each experimental group after the use of JAK inhibitors; (G) levels of IFN- γ in the supernatant of each experimental group after the use of JAK inhibitors; (H) levels of IL-17A in the supernatant of each experimental group after the use of JAK inhibitors; (I-K) Proportions of Th1 and Th2 cells in CD4+ T cells and Th1/Th2 ratio in each experimental group after using STAT1 inhibitor; (L) levels of IFN- γ in cell supernatants in each experimental group after using STAT1 inhibitor; (M-N) expression levels of STAT1 and p-STAT1 in lymphocytes in each experimental group and the results of semi-quantitative analysis; (O-Q) Proportions of Th17 and Treg cells to CD4+ T cells and Th17/Treg ratio in each experimental group after using STAT3 inhibitor; (R) Levels of IL-17A in cell supernatants of each experimental group after using STAT3 inhibitor; (S-T) Expression levels of STAT3 and p-STAT3 in lymphocytes of each experimental group and the results of semi-quantitative analysis (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; C: control group, M: model group, T: treatment group, I: inhibitor group, T+I: inhibitor+MSCs group). Supplying supplementary1 Identification results of umbilical cord-derived MSCs, including results of induced differentiation staining and flow cytometry identification of cell surface markers. Supplying supplementary1 Identification of umbilical cord-derived mesenchymal stem cells.

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