Effects of bone marrow mesenchymal stem cells on the cardiac function and immune system of mice with endotoxemia

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Received March 25, 2015; Accepted December 22, 2015

DOI: 10.3892/mmr.2016.5151

Abstract. The present study aimed to investigate the effects of bone marrow mesenchymal stem cells (MSCs) on the cardiac function and immune system of mice with endotoxemia. The mice were divided into the following groups: Control group, endotoxemia group, lipopolysaccharide (LPS) treatment group, LPS and MSC treatment group (LPS + MSC group) and MSC group. Following treatment with LPS, the cardiac function of the mice was examined at after 2, 6 and 24 h, and on day 7. An enzyme-linked immunofluorescent assay was used to analyze the serum and the levels of cytokines in the myocardium, and western blotting was used to investigate any changes in the levels of signaling proteins associated with the myocardium. A 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay was used to investigate the growth rate of the splenic cells at after 24 h and on day 7, and the humoral immune function and phagocytosis of the macrophages in the mice were also examined. The cardiac function of the mice with endotoxemia declined, although this impairment was circumvented following treatment with MSCs. The levels of interleukin (IL)-1β, IL-6, tumor necrosis factor-α and IL-10 in the serum and the myocardium increased following stimulation by LPS, although these declined as a result of MSC treatment. The expression levels of Toll-like receptor 4, p65-nuclear factor-κB and phosphorylated p38 in the mouse myocardium were enhanced following stimulation by LPS, which subsequently decreased as a result of MSC treatment. Compared with the control group, the growth rate of the splenic cells, humoral immune function and the level of phagocytosis of macrophages were all increased, although these parameters declined following treatment with MSCs. Taken together, the present study revealed that the MSCs inhibited the inflammatory reaction in the mice with endotoxemia, and improved cardiac function. By contrast, the cellular and humoral immunity were depressed, and phagocytosis of the macrophages, which were enhanced following simulation with LPS, were decreased following treatment with MSCs. However, no overexpression of the anti-inflammatory factor, IL-10, was observed. The present study hypothesized that MSCs exert a bifunctional role in endotoxemia, by inhibiting inflammatory factors, including IL-1 and IL-6, and inhibiting the compensatory expression of IL-10 following LPS stimulation. This avoids the possibility of excessive inhibition of immunological function, as this results in immunosuppression, and a higher ratio of IL-10 to TNF-α is indicative of a poor prognosis in patients with sepsis.

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Key words: bone marrow mesenchymal stem cells, endotoxemia, cardiac function, immune function

Introduction

Sepsis poses a serious threat to the life of patients, resulting in multiple organ failure syndrome. Treatment options at present include surgery, antibiotics and other supportive treatments, however, the therapeutic options available remain unsatisfactory in terms of efficacy, with a mortality rate of ~30% (1). In the United States, ~250,000 patients succumb to sepsis-associated mortality every year (2). Sepsis predominantly results in organic dysfunction due to the LPS, which is produced by bacteria and interacts with the human inherent immune system, resulting in its dysfunction (3,4) and leading to activation of the inherent immune system, the inhibition of adaptive immunity, mitochondrial dysfunction and hypermetabolism accompanied by insulin resistance. Furthermore, the enhancement in capillary permeability leads to tissue edema, which eventually results in the dysfunction of tissue organs (4). One of the clinical manifestation of sepsis is the impairment and insufficiency of cardiac function. A previous study indicated that, among 235 patients who succumbed to sepsis or septic shock-associated mortality, those exhibiting cardiovascular system failure accounted for 35.3% (5). As the inflammatory reaction is important in sepsis, previous studies have attempted to use monoclonal antibodies raised against specific inflammatory factor receptors to inhibit the inflammatory reaction (2,6). However, sepsis results from interactions among various inflammatory factors and, as a result, treatments based on monoclonal antibodies lose

their therapeutic potential due to the targeting of a single receptor (2).

As the inflammatory reaction is closely associated with immunological abnormalities in sepsis, effective treatments, which are able inhibit excessive inflammatory reactions and regulate immunological abnormality are of interest. Previous studies have demonstrated that mesenchymal stem cells (MSCs) are able to regulate the immune system, and MSCs have been used to treat a range of conditions, including acute lung injury, inflammatory bowel disease, graft versus-host reactions and ischemic heart disease (7,8). Previous attempts have been made to treat sepsis using MSCs, and MSCs have been successfully used in animals to inhibit the systemic inflammation reaction through interleukin (IL)-10, and thereby relieve the functional impairment, which is associated with sepsis and endotoxemia (7-11).

MSCs have also elicited improved therapeutic effects on the cardiac insufficiency associated with sepsis, accompanied by a decreased expression of inflammatory factors, including tumor necrosis factor-α (TNF-α) and IL-1 (11). However, at present, the treatment for sepsis using MSCs is focused more on the inhibition of the excessive inflammatory reaction caused by sepsis, and the effect of MSCs on the immune system remain to be fully elucidated. As lipopolysaccharide (LPS) is the predominant compound promoting the involvement of Gram-negative bacteria in sepsis, and the severity of sepsis is correlated with the concentration of LPS in the circulatory system (12,13), LPS is commonly injected into an animal model in order to simulate sepsis.

In the present study, MSCs were used to treat mice with endotoxemia, with the aim to observe the effects of the MSCs on cardiac function, and on the levels of cytokines in the serum and myocardium of mice with endotoxemia. In addition, the effects of MSCs on the immune system were investigated, in order to assist in the development of novel therapies using MSCs for treating sepsis.

Materials and methods

Bone marrow MSCs. All the animal experiments in the present study were performed in accordance with the Experimental Animal Management Regulations in Nanjing Medical University (Nanjing, China). The present study was approved by the ethics committee of the First Affiliated Hospital of Nanjing Medical University (Nanjing, China). A total of 80 C57 BL/6 male mice (aged 8-10 weeks, weighing ~25 g, 12 h light/dark cycle), maintained at 24˚C and 50% humidity were purchased from the Animal Core Facility in Nanjing Medical University. The mice were sacrificed by cervical vertebra dislocation, and soaked in 75% alcohol for sterilization purposes for 15 min. The bilateral shafts of the femurs were extracted under sterile conditions. The *cavitas medullaris* was washed with 10 ml phosphate-buffered saline (PBS) using a sterile 5 ml syringe, prior to the disposal of the femoral shafts. The washed bone marrow was mixed by lashing and stirring, following which the marrow was centrifuged at 750 x g for 5 min at 20˚C, and the supernatant was discarded. The remainder was resuspended in high-glucose Dulbecco's modified Eagle's medium (DMEM; GE Healthcare Life Sciences, Logan, UT, USA) containing 10% Gibco fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and was subsequently inoculated in a Petri-dish (10 cm diameter) at 37˚C in an incubator containing 15% CO_2. Following incubation for 4 days, the medium was replaced for the first time with high-glucose DMEM containing 10% FBS, following which the medium was replaced every day until 80% of the cells were entering cell fusion at a 1:2 passage. The third generation cells were used.

Treatment of mice with endotoxemia using MSCs. A total of 80 C57 BL/6 male mice (aged 8-10 weeks, weighing ~25 g) were selected for the present study. The mice were provided with free access to food (grain feed) and water, and were housed at a temperature of 24˚C and 50% humidity. The following four groups of mice were established: Control; lipopolysaccharide (LPS) treatment; LPS and MSC treatment (LPS + MSC group) and MSC treatment group, with 20 mice in each group. LPS (Sigma-Aldrich, St. Louis, MO, USA) was used at a concentration of 0.5 mg/ml, and the concentration of MSCs used was $2x10^6$/ml. In the control group, 0.5 ml phosphate-buffered saline (PBS) was administered through peritoneal injection and, after 1 h, 0.5 ml PBS was administered via *vena caudalis* injection. In the LPS group, 10 mg/kg LPS was administered via peritoneal injection and, 1 h later, 0.5 ml phosphate-buffered saline (PBS) was administered via *vena caudalis* injection. In the LPS + MSC group, 10 mg/kg LPS was administered via peritoneal injection and, 1 h later, 10^6 bone marrow MSCs were administered via *vena caudalis* injection. In the MSC group, 0.5 ml PBS was administered via peritoneal injection and, 1 h later, 10^6 bone marrow MSCs were administered via *vena caudalis* injection. No mice died throughout the course of the experiment all were sacrificed by cervical vertebra dislocation. The cardiac functions of the mice were investigated using a two-dimensional echocardiogram (Vevo 2100; VisualSonics, Inc., Toronto, Canada) 2, 6 and 24 h, and 7 days following LPS injection. Subsequently, five mice in each group were sacrificed in order to examine the associated tissues.

Determination of the serum and the myocardial inflammatory factor. A total of 500 µl of blood was collected from each mouse and maintained at physiological temperature. Following incubation for 1 h, the blood was centrifuged at 2,000 x g for 10 min to obtain the serum. Myocardial tissues were homogenized in 10 weight/volume of sodium chloride on ice and then centrifuged at 10,000 x g for 15 min at 4˚C. The supernatants were collected for IL-1β, IL-6, TNF-α and IL-10 assays with enzyme-linked immunosorbent assay (ELISA) kits. An ELISA assay kit was purchased from R&D Systems, Inc. (Minneapolis, USA). Aliquots (50 µl) of the standard, control or sample solution mixed with 50 µl assay dilutant were added to each well, and the mixtures were incubated at room temperature for 2 h. Following the removal of unbound material by washing five times, 100 µl conjugate was added to each well and incubated at room temperature for 2 h. Following rinsing, 100 µl substrate solution was added and incubated at room temperature in the dark for 30 min, the reaction was terminated by adding 100 µl stop solution to each well, and the optical density was read at 450 nm within 30 min using an SH-1000 microplate reader (Corona Electric Co., Ltd, Hitachinaka, Japan).

Determination of myocardial signal pathway proteins. A total of 0.1 g of myocardial tissue was obtained, to which 1 ml lysate and 10 μl phenylmethyl sulfonylfluoride solution (Sigma-Aldrich) were added prior to homogenization. The homogenate was centrifuged at 12,000 x g for 20 min (4 μl), and the supernatant was collected to measure the concentration of protein using the Bradford protein assay method (Jiancheng Biotech, Nanjing, China), with bovine serum albumin as a standard (diluted to a concentration of 5 mg/ml). The proteins were separated using sodium dodecyl sulfate (SDS)-PAGE (5 μg/μl per well; 12%; Bio-Rad Laboratories, Inc., Hercules, CA, USA), and were electrotransferred (300 mA) onto a polyvinylidene fluoride (PVDF) membrane (EMD Millipore, Billerica, MA, USA). The myocardial proteins were sealed off using 5% defatted milk powder as the blocking agent for 2 h, and were subsequently separately incubated overnight at 4˚C with the corresponding primary antibody (1:5,000 dilution). The following primary antibodies were used: Rabbit anti-Toll-like receptor (TLR)-4 (cat. no. bs-1021R; Bioss Biotech, Woburn, MA, USA), rabbit anti-myeloid differentiation primary response gene 88 (MyD88; cat. no. bs-1047R; Bioss Biotech), rabbit anti-c-Jun N-terminal kinase (JNK; cat. no. ab179461; Abcam, Cambridge, UK), rabbit anti-phosphorylated (p)-JNK (cat. no. ab124956; Abcam) and rabbit anti-β-actin (cat. no. 4967; Cell Signaling Biotechnology, Inc., Danvers, MA, USA). Additionally, the NF-κB and the p38 MAPK Pathway Sampler kits were used (cat. nos. 9936 and 9913, respectively, Cell Signaling Biotechnology, Inc.). The PVDF membrane was washed with 0.5% TBS-T solution (Beyotime Institute of Biotechnology, Nantong, China) three times (5 min each). Subsequently, goat anti-rabbit secondary antibody (1:6,000 dilution) conjugated to horseradish peroxidase (cat. no. 3056-1; Epitomics, Inc., Hangzhou, China) was applied to the PVDF membrane, prior to agitation on a rocking bed for 4 h at room temperature. The membrane was subsequently washed with 0.5% TBS-T solution three times (5 min each). TMB Membrane Peroxidase Substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MA, USA) was added onto the membrane. The levels of target proteins were determined using a gel imaging system (ChemiScope 2850, Clinx Science Instruments Co., Ltd., Shanghai, China).

Determination of the growth rate of splenic cells using a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Concanavalin A (ConA), at a final concentration of 5 μg/ml, was added to RPMI-1640 culture medium to a 0.2 μm filter membrane and stored at 4˚C. MTT (Sigma-Aldrich) was dissolved in PBS to a final concentration of 5 mg/ml and maintained at 4˚C. The spleen was removed from the mice under the sterile conditions and placed on a flat plate, followed by crushing or cutting. The spleen was subsequently washed with Hank's solution (Sigma-Aldrich) and filtered through four layers of sterile gauze to obtain a suspension of single cells, which were further purified through two successive centrifugation steps at 250 x g for 5 min at 4˚C. The precipitated cells were resuspended in RPMI-1640 culture medium, and the splenic cells obtained were cultured on a 96-well culture plate, with 100 μl/well. The final concentration of splenic cells was $1.5x10^6$ /ml, to which 100 μl ConA solution (5 μg/ml) was added, and the cells were cultured in a 5% CO_2 incubator at 37˚C for 16 h. An aliquot of 10 μl MTT was added into each well prior to the completion of the incubation period. Finally, the cell suspension was centrifuged at 250 x g for 10 min at 4˚C, the supernatant was discarded, and 200 μl 10% SDS containing 0.04 mol/l HCl was added. The culture plate was agitated on a decolorizing rocking bed for 10 min. The photoabsorption of each well was measured at a wavelength of 490 nm using a microplate reader (SH 1000; Corona Electric Co., Ltd, Hitachinaka, Japan), the results were recorded and a cell growth curve was constructed, with time on the x-axis and light absorption on the y-axis. The incremental increases in the growth rate of the splenic cells were calculated 24 h and 7 days following treatment with LPS.

Determination of the humoral immune function. A total of 4 mice were used for this part of the current study, all mice were sacrificed by cervical vertebra dislocation. The mice were immunized with sheep red blood cells (SRBCs; Cell-Bio Biotechnology Co., Ltd., Shanghai, China) and 50 mg of splenic tissues were extracted after 4 days, which were then ground using a 100 mesh copper screen. Subsequently, the extracts were washed twice in Hanks solution and made into a cell suspension with PBS ($5x10^6$/ml). For the experimental groups, 1 ml of 0.2% SRBCs and 1 ml of complement were added to 1 ml of the splenic cell suspension, and the mixture was incubated at 37˚C for 1 h. Centrifugation was performed at 250 x g for 5 min at 4˚C, and the supernatant was obtained. The photoabsorption was measured using a SH-100 microplate reader at 413 nm (antibody optical density value). In the control group, 1 ml 0.2% SRBCs was added to 1 ml splenic cell suspension in a blank tube without complement, and the other procedures were identical with those detailed above.

Assessment of macrophage phagocytosis in mice. Venous blood was extracted from a chicken wing, to which was added physiological saline, and the mixture was centrifuged twice at 250 x g for 5 min at 4˚C. Subsequently, an appropriate volume of physiological saline was added, which was determined by the volume of erythrocytes obtained, in order for the concentration of chicken red blood cells (CRBCs; Cell-Bio Biotechnology Co., Ltd.) to be 1%. At 3 days prior to the experiment, 1 ml soluble amylum (Sigma-Aldrich) was administered to the mice via peritoneal injection every day. At 30 min prior to the experiment, 1 ml 1% CRBCs was administered to the mice via intraperitoneal injection. After 3 days, the mice were sacrificed and 200 μl of peritoneal fluid was extracted. A blob of physiological saline solution was dropped onto the slide, to which was added a blob of peritoneal fluid. The liquid was allowed to stand for 10 min to enable cellular adherence of the ascites to occur, following which the physiological saline solution was discarded, and Wright's staining (Sigma-Aldrich) was performed when the slide had been allowed to dry. A total of 100 mononuclear macrophages were counted on each slide using a CX41 routine microscope (Olympus Corporation, Tokyo, Japan), and the percentage of cells phagocytosing CRBCs were calculated. The percentages of phagocytosing cells were compared across the groups.

Statistical analysis. Data is presented as the mean ± standard error of the mean, and statistically significant differences

were assessed by one-way analysis of variance followed by post-hoc analysis. All statistical analyses were processed with Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Changes in the levels of cytokines in the serum. The level of IL-1β in the LPS treatment group was increased at the 2 h time point following LPS stimulation, with no statistically significant difference following treatment with MSCs, although more marked increases in the levels of IL-1β were observed at the 6 h, 24 h and day 7 time points, with a marked decline following treatment with MSCs. The levels of IL-6 and TNF-α in the LPS treatment group were increased at the 2 h time point, although without significant difference, compared with the control group. The increase was more clearly discernible at 6 h, 24 h and day 7 time points, with a significant decline following treatment with MSCs. The level of IL-10 in the LPS group exhibited no marked change at the 2 h time point, and a marginal increase was observed following treatment with MSCs, but without statistical significance, compared with the control and LPS groups. However, a significant increase ($P<0.05$) in the levels of IL-10 were observed after 6 h, 24 h and 7 days, with a marked decrease observed following treatment with MSCs. The level of IL-10 in the MSC group declined after 24 h and 7 days, with a statistically significant difference ($P<0.05$), compared with the LPS group. Compared with the control group, no marked changes in cytokines were observed in the MSC group (Fig. 1).

Changes in levels of cytokines in the myocardium. The level of IL-1β in the LPS group was marginally increased at the 2 h time point following LPS stimulation, however, the increase was not statistically significant, compared with the control group. However, the levels of IL-1β were markedly increased at the 6 h, 24 h and day 7 time points, with a decrease observed following treatment with MSCs, although this was not statistically significant. The levels of IL-6 and TNF-α in the LPS group exhibited no marked changes at the 2 h time point, although a marked increase was observed at the 6 h, 24 h and day 7 points, with a significant ($P<0.05$) decline observed following MSC treatment. The level of IL-10 in the LPS group did not exhibit a marked change at the 2 h time point, however, the level was significantly increased at the 6 h time point ($P<0.05$) following MSC treatment. Marked increases in the levels of IL-10 were also observed at the 24 h and 7 day times points, although the decrease in levels following MSC treatment at these time points were not statistically significant (Fig. 2).

Changes in cardiac function. The level of the ejection fraction (EF) in the LPS group markedly declined at the 2 h, 24 h and 7 day time points following LPS stimulation, which recovered to a differing extent following treatment with the MSCs, which was statistically significant ($P<0.05$) for all the time points, with the exception of the 6 h time point (Fig. 3). On monitoring the changes in the fractional shortening, the results obtained were similar to those obtained for the EF (Fig. 3).

Changes in the protein expression level of myocardial signaling proteins. The protein expression levels of TLR-4 and p65-nuclear factor-κB (NF-κB) in the LPS group exhibited no marked changes at the 2 h time point, however. the observed levels were markedly increased at the 6 h, 24 h and 7 day time points, with lower levels of expression observed following treatment with the MSCs. The protein expression of MyD88 in the LPS group was increased at the 24 h time point, with a decrease observed following treatment with MSCs. The levels of p-p38 in the LPS group were markedly increased at the 2 h, 6 h, 24 h and 7 day time points, with a decrease observed for all the time points following treatment with MSCs (Fig. 4).

Changes in immunological function. The growth rate of the splenic cells in the LPS group were markedly increased at the 24 h and 7 day time points, with a marked decease observed following MSC treatment (Fig. 5). No significant differences were identified between the MSC and control group. The humoral immune function in the LPS group was markedly increased at the 24 h and 7 day time points, with marked decreases observed following treatment with MSCs (Fig. 6). The humoral immune function in the MSCs group declined, compared with the control group. Phagocytosis of the macrophages in the LPS group occurred to a greater extent at the 24 h and 7 day time points, with marked decreases following treatment with MSCs, although the level of phagocytosis remained higher following MSC treatment, compared with the control group at the 24 h time point (Fig. 7). Phagocytosis in the MSCs group occurred to a lesser extent than that in the control group after 7 days (Fig. 7).

Discussion

In the late 1980s, it was hypothesized that inflammatory factors are important in sepsis, and aimed to identify a biomarker to improve diagnostic and prognostic purposes and interfere with inflammatory factors, in order to improve the treatment of sepsis (14). Subsequently, the modes of seizure of sepsis were identified and delineated, with the liberation of several inflammatory factors upon stimulation by LPS at an early stage of disease, and patients with sepsis acquiring immunosuppression at more advanced stages of the disease (15). Therefore, attempts have been made to improve the effectiveness of strategies to treat sepsis clinically, by inhibiting the excessive inflammatory reaction. As LPS is important in sepsis, investigations of the mechanism underlying its action have revealed that LPS activates the inherent (or native) immune system, predominantly through TLR-4 (16), and, following the formation of a complex between TLR-4 and myeloid differentiation factor (2MD-2), LPS activates NF-κB via MyD88 to elicit the production of various types of inflammatory factors. The results in the present study revealed that, following stimulation of the mice with LPS, the protein expression levels of TLR-4, MyD88 and NF-κB in the myocardium increased, with corresponding increases in the levels of inflammatory factors in the serum and myocardium. Therefore, the present study hypothesized that an antagonist of TLR-4 may be used to treat sepsis through the interruption of the stimulatory role exerted by LPS on the immune system (2). Although, in early investigations, the antagonist of TLR-4,

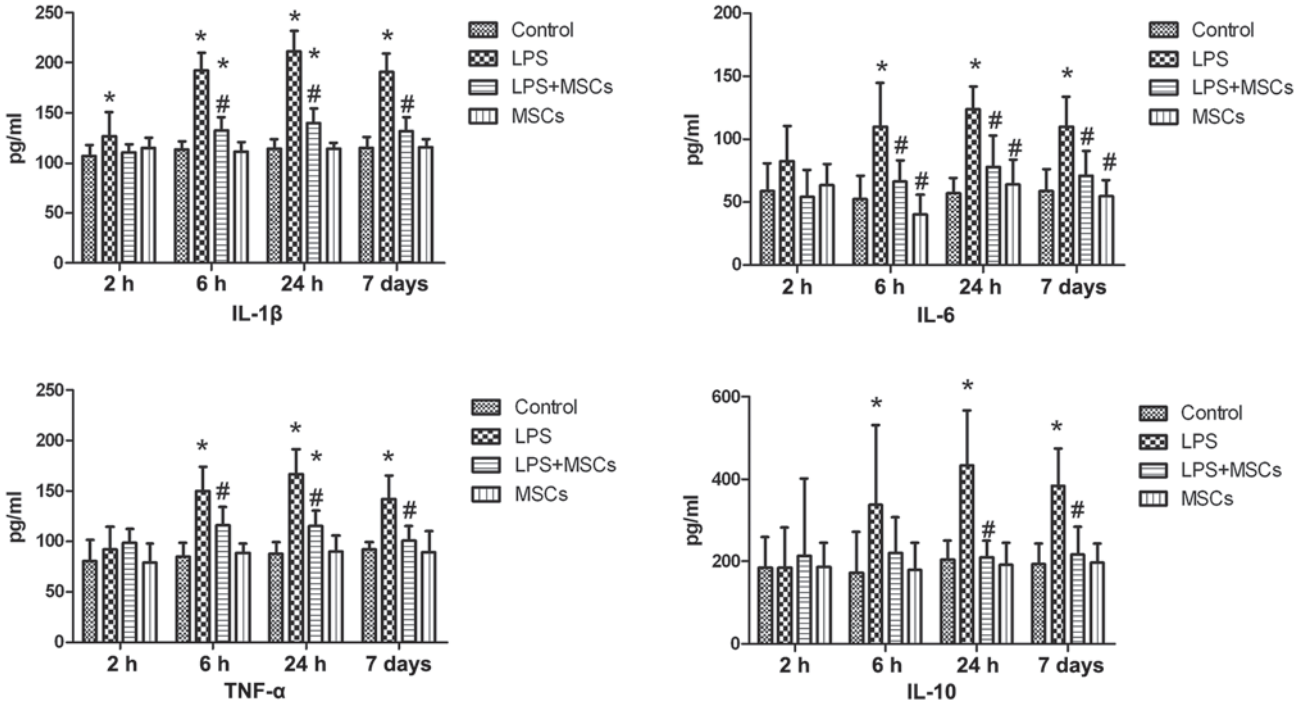

Figure 1. Changes in serum levels of cytokines. The level of IL-1β in the LPS group increased at the 2 h time point, and more marked increases in the levels of IL-1β were observed at the 6 h, 24 h and 7 day time points, with a marked decline following treatment with MSCs. The levels of IL-6 and TNF-α in the LPS group were increased at the 6 h, 24 h and 7 day time points, with a marked decline following treatment with MSCs. Compared with the control, the level of IL-10 in the LPS group was not significantly different at the 2 h time point, but levels were significantly increased at the 6 h, 24 h and 7 day time points, with a significant decline following treatment with MSCs. *P<0.05, compared with the control group; #P<0.05, compared with the LPS group. LPS, lipopolysaccharide; MSC, mesenchymal stem cell; IL, interleukin; TNF-α, tumor necrosis factor-α.

eritoran, exhibited an apparent tendency to increase the survival rate of patients with sepsis (17), a subsequent large three-stage clinical trial indicated that the mortality rates of patients with sepsis were not lowered as a consequence of treatment with eritoran (6). The possible explanation for this is that the signaling pathway mediated by TLR-4 is not the only route by which LPS acts on the human body. Caspase-11, independently of TLR-4, is also involved in apoptosis induced by LPS (18,19). LPS acts in association with caspase-11, producing an inflammasome following entry to macrophages, and active IL-1 is liberated by the inflammasome under the stimulation of caspase-11 (20,21). In the present study, the level of p-p38 was markedly increased in the LPS group, which was decreased following treatment with MSCs. These results suggested that, as the complex pathogenesis of sepsis remains to be fully elucidated, it is difficult for a treatment regimen aimed at a single factor to exert a marked therapeutical effect on sepsis.

In subsequent studies on sepsis, it has been revealed that the hypothesis suggesting patients with sepsis suffer an excessive inflammatory reaction in the early stages of the disease, and immunosuppression in the advanced stage, was not supported by genomic analyses (22). Thereafter, it has been suggested that the onset of sepsis is accompanied by the activation and inhibition of the immune system (23), with characteristics of abnormal activation predominantly in the early stage, and immunosuppression predominantly in the advanced stage. In addition, the timing and intensity of early immune activation of the body was associated with a range of factors, including the patients' physical status, virulence of pathogenic bacteria and other complicating factors (24). Among patients with refractory sepsis, due to the immunosuppression resulting from a deficiency in the inflammatory factors to activate the adaptive immune system, it is difficult for persistent infections to be controlled (25). The immunosuppression of patients with sepsis is predominantly characterized by the apoptosis of lymphocytes and dendritic cells, decreased expression levels of the cell-surface antigen-presenting complex and human leucocyte antigen-death receptor, and an increase in the expression level of inhibitory immune regulatory molecules, including programmed death 1 and cytotoxic T-lymphocyte-associated antigen 4. In targeting the immunosuppressive condition, immunostimulatory therapies have been suggested for the treatment of sepsis (26), however, this remains a preliminary stage of investigation, with concerns that they may aggravate the inflammatory reaction and provoke autoimmunity.

There has been increased interest in the ability of MSCs to adjust the function of the immune system extensively, the action of which is twofold and differs from previous treatment regimens. A wealth of evidence has established that MSCs exert a suppressive effect on the innate and adaptive immune systems (27-29). MSCs have been revealed to stimulate the immune system (30). γ-Interferon stimulates MSCs, generating their antigen-presenting capability, which leads to the stimulation of the adaptive immune system (31). MSCs also stimulate natural B cells and their differentiation and proliferation during transit times, and promote B cells to differentiate into plasma cells following antigenic stimulation (32). As sepsis is associated with an excessive

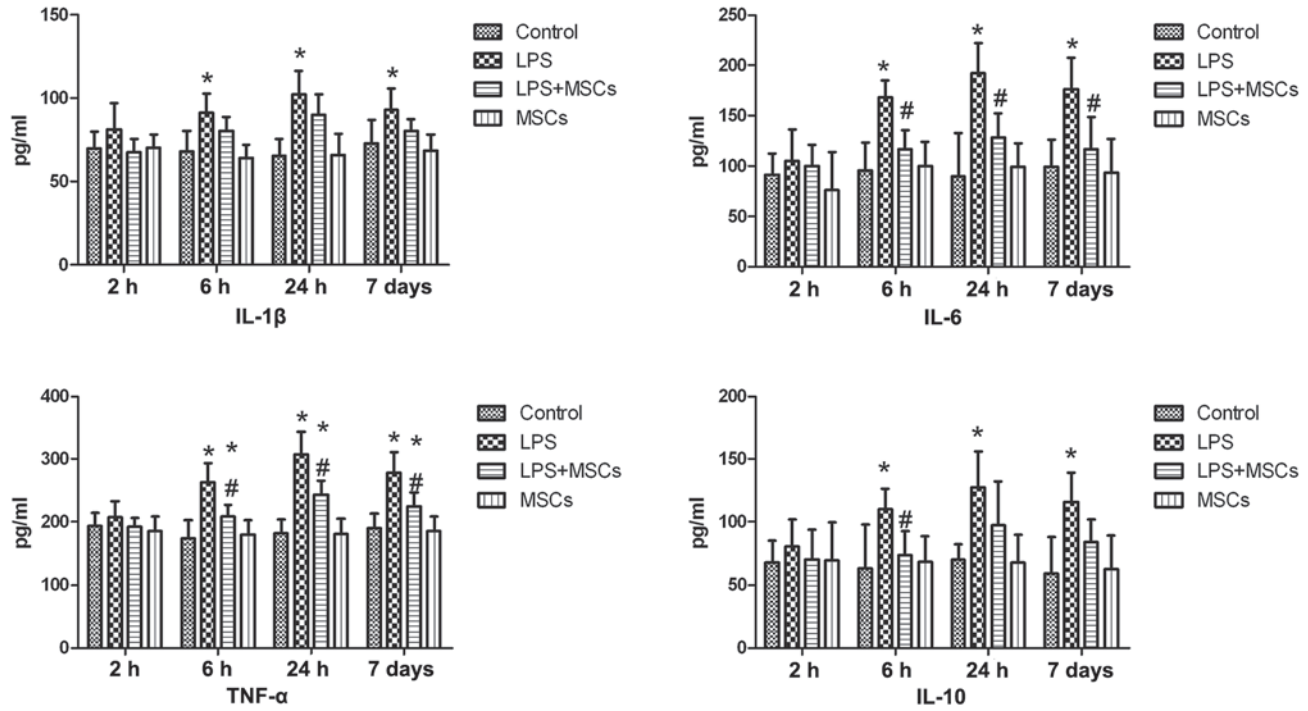

Figure 2. Changes in the levels of cytokines in the myocardium. Compared with the control, the levels of IL-1β increased at the 6 h, 24 h and 7 day time points, with a decline following treatment with MSCs, which was not statistically significant. The levels of IL-6 and TNF-α in the LPS group exhibited no marked changes at the 2 h time point, although significant increases in their levels were observed at the 6 h, 24 h and 7 day time points, with a significant decline following MSC treatment. The level of IL-10 in the LPS group exhibited no marked change at the 2 h time point, although a significant increase was observed at the 6 h time point, which declined following MSC treatment. A significant increase in the level of IL-10 was also observed at the 24 h and 7 day time points, although the decline in levels following MSC treatment was not statistically significant. *P<0.05, compared with the control group; #P<0.05, compared with the LPS group. LPS, lipopolysaccharide; MSC, mesenchymal stem cell; IL, interleukin; TNF-α, tumor necrosis factor-α.

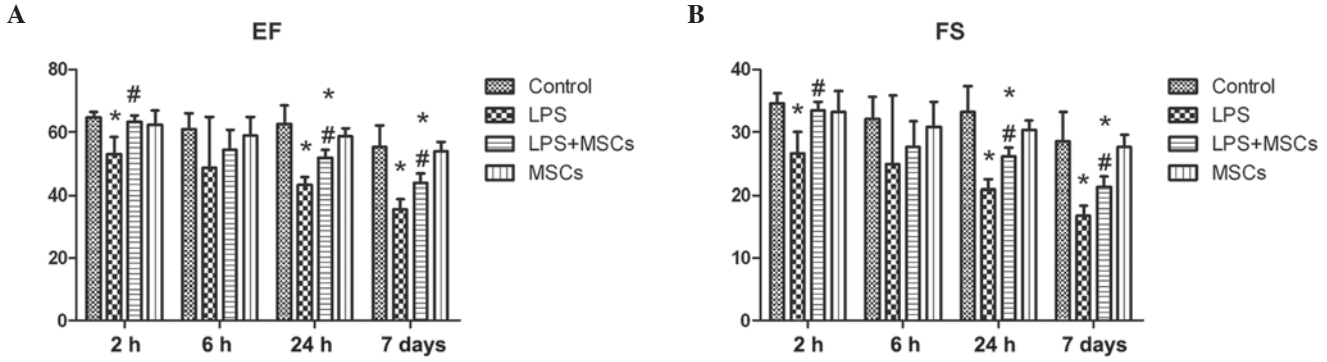

Figure 3. Changes in cardiac function in the different treatment groups. (A) Level of the EF in the LPS group decreased at the 2 h time point, and also at the 24 h and 7 day time points, and recovered to a differing extent following MSC treatment. The changes were statistically significant, with the exception of the 6 h time point. (B) Changes in FS were similar to those for EF. *P<0.05, compared with the control group #P<0.05, compared with the LPS group. EF, ejection fraction; FS, fractional shortening; LPS, lipopolysaccharide; MSC, mesenchymal stem cell.

inflammatory reaction, as well as immunosuppression, the appropriateness of using MSCs in treatment strategies for sepsis remains to be fully elucidated.

In the present study, the inflammatory reaction resulting from LPS stimulation led to a decrease in left cardiac function, similar to sepsis. MSCs have more marked therapeutic effects on cardiac insufficiency in rats with endotoxemia; and they also reduce the expression levels of host of inflammatory factors, including IL-1, TNF-α and IL-6 (7). However, in the present study, the expression of the inflammatory factor, IL-10, following stem cell therapy produced a different experimental result. Németh *et al* (9) demonstrated that, through stimulating the expression of IL-10, stem cells alleviate the organic damage caused by sepsis. Weil *et al* (11) demonstrated that, following stem cell therapy, the level of IL-10 in the serum increases. Notably, the present study revealed that, following LPS stimulation, no significant change in the serum level of IL-10 was observed in the LPS group, however, an increase was observed in the MSC group. At 6 h, 24 h and 7 days following treatment, the level of IL-10 in the LPS group increased significantly, and the level of IL-10 declined following treatment with MSCs. The level of IL-10 in the myocardium increased markedly after 6 h, 24 h and 7 days, and declined following treatment with MSCs. The above-mentioned experimental results are in agreement

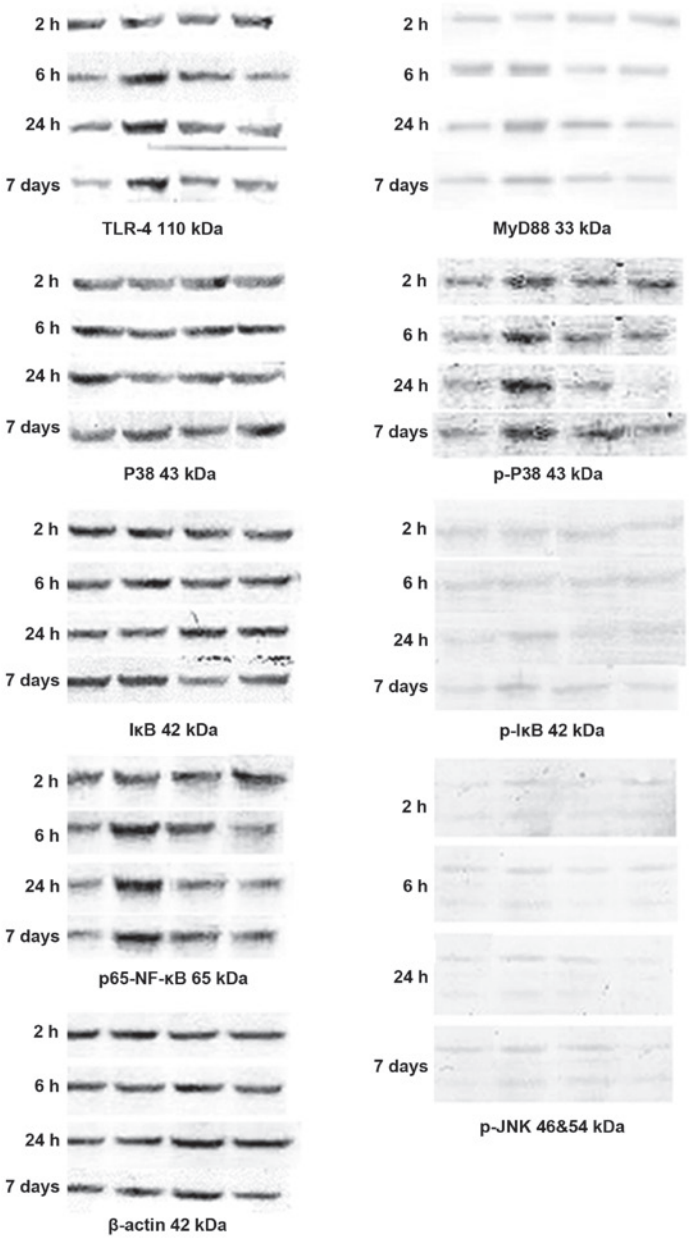

Figure 4. Changes in the levels of signaling proteins in the myocardium. The protein expression levels of TLR-4 and p65-NF-κB in the LPS group exhibited no marked changes at the 2 h time point, although the levels were markedly increased at the 6 h, 24 h and 7 day time points, and decreased following treatment with MSCs. The expression of MyD88 in the LPS group increased at the 24 h time point and declined following MSC treatment. The level of p-p38 in the LPS group was markedly increased at the 2 h, 6 h, 24 h and 7 day time points, and decreased following MSC treatment. β-actin was used as an experimental control. p-, phosphorylated; TLR-4, Toll-like receptor 4; NF-κB, nuclear factor-κB; MyD88, myeloid differentiation primary response gene 88; IκB, inhibitor of κB; JNK, c-Jun N-terminal kinase.

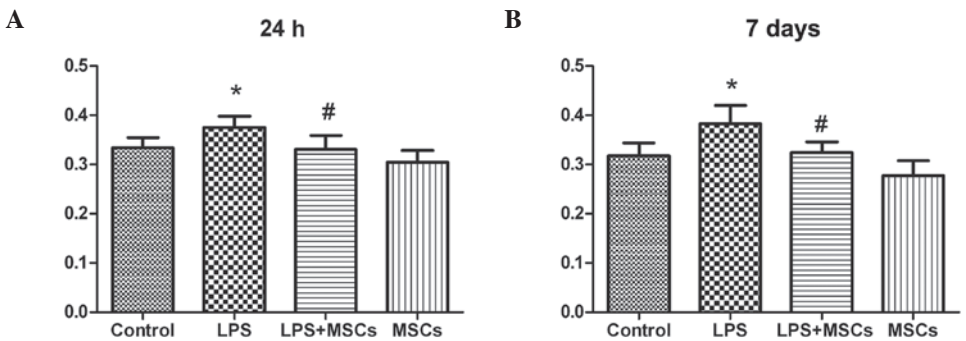

Figure 5. Changes in the splenic cell growth rate. The growth rate of splenic cells in the LPS group was increased markedly at the (A) 24 h and (B) 7 day time points, with marked decreases observed following MSC treatment. No significant differences were identified between the MSCs alone group and the control group. *P<0.05, compared with the control group; $^{\#}$P<0.05, compared with the LPS group. LPS, lipopolysaccharide; MSC, mesenchymal stem cell.

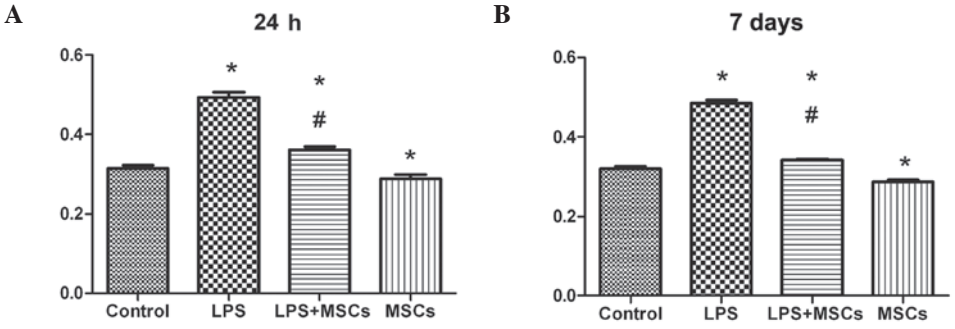

Figure 6. Changes in humoral immune function. Compared with the control, the humoral immune function in the LPS group was significantly increased at the (A) 24 h and (B) 7 day time points, with significantly decreases observed following MSC treatment. The humoral immune function in the MSCs group decreased, compared with the control group. *P<0.05, compared with the control group; #P<0.05, compared with the LPS group. LPS, lipopolysaccharide; MSC, mesenchymal stem cell.

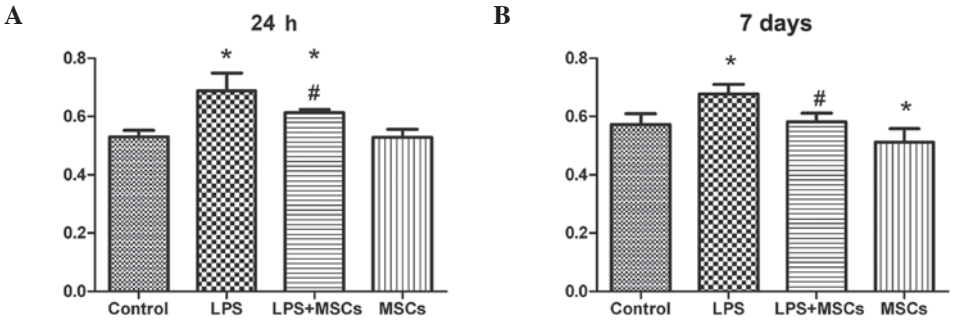

Figure 7. Changes in the levels of phagocytosis in the macrophages. Compared with the control, the extent of phagocytosis of macrophages in the LPS group increased at the (A) 24 h and (B) 7 day time points, with decreases observed following MSC treatment, although the extent of phagocytosis remained higher at the 24 h time point, compared with the control group. Levels of phagocytosis in the MSCs group were lower, compared with those in the control group at the 7 day time point. *P<0.05, compared with the control group; #P<0.05, compared with the LPS group. LPS, lipopolysaccharide; MSC, mesenchymal stem cell.

with the findings of previous studies (33,34). Differences in results among studies may be associated with the different experimental conditions used. Although the expression of IL-10 following treatment with MSCs requires further analysis, the present study hypothesized that MSCs exert a bifunctional role in endotoxemia, by inhibiting inflammatory factors, including IL-1 and IL-6, and inhibiting the compensatory expression of IL-10 following LPS stimulation. This avoids excessive inhibition of immunological function, as excessively inhibiting the inflammatory reaction results in immunosuppression, and a higher ratio of IL-10 to TNF-α is indicative of a poor prognosis in patients with sepsis (35).

In the assessment of the immunological function, the present study demonstrated that the mice in the LPS group manifested an abnormal reinforcement of cellular immunity, humoral immunity and phagocytosis of macrophages during the first week, which declined following treatment with MSCs. The humoral immune function in the MSC group was lower, compared with that in the control group. By contrast, no significant differences were identified in humoral or cellular immune function among the LPS, MSC or control groups, which indicated that the presence of MSCs in the mice with endotoxemia did not markedly suppress their adaptive immune response. In the present study, during the monitoring of the mice for 1 week, the immunological function remained in the stage of reinforcement, although immunosuppression did not manifest itself during sepsis, which may have been associated with the experimental approach used. Therefore, further investigations are required to examine the effects of MSCs on mice with sepsis predominantly accompanied by immunosuppression.

In conclusion, the present study indicated that MSCs exert regulatory roles in the immune system in several diverse ways, by inhibiting the excessive inflammatory reaction and reinforcing the immunological function, and by avoiding the abnormal increase in anti-inflammatory IL-10. Using MSCs to treat sepsis may circumvent the difficulties associated with therapies are aimed at single cytokines or other molecules. MSCs are readily obtained and easy to culture *in vitro*, with rapid and simple amplification. Previous studies have also shown that MSCs may exert antibiotic action by excreting antimicrobial peptide LL-37, thereby stimulating neutrophil granulocytes (36,37). Another advantage is that their immunogenicity is low, which makes it possible to treat sepsis using allogeneic stem cells. Taken together, the results of the present study confirmed that the therapeutic effects and underlying mechanism of MSCs in the treatment of sepsis require further investigation, in order to develop novel approaches for the treatment for sepsis.

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