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The Mechanisms Involved in Mesenchymal Stem Cell Alleviation of Sepsis-Induced Acute Lung Injury in Mice: A Pilot Study

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ABSTRACT

Background: Acute lung injury is a common complication of sepsis in intensive care unit patients. Inflammation is among the main mechanisms of sepsis. Therefore, suppression of inflammation is an important mechanism for sepsis treatment. Mesenchymal stem cells (MSCs) have been reported to exhibit antimicrobial properties.

Objective: The present study investigated the effects of MSCs on sepsis-induced acute lung injury.

Methods: Male C57BL/6 mice underwent a cecal ligation and puncture (CLP) operation to induce sepsis and then received either normal saline or MSCs (1×10^6 cells intravenously) at 3 hours after surgery. Survival after surgery was assessed. Lung injury was assessed by histology score, the presence of lung edema, vascular permeability, inflammatory cell infiltration, and cytokine levels in bronchoalveolar lavage fluid. Finally, we tested nuclear factor kappa-light-chain-enhancer of activated B cells activation in lung tissue.

Results: As expected, CLP caused lung injury as indicated by significant increases in the histopathology score, lung wet to dry weight ratio, and total protein concentration. However, mice treated with MSCs had amelioration of the lung histopathologic changes, lung wet to dry weight ratio, and total protein concentration. The levels of cytokines tumor necrosis factor alpha, interleukin 6, interleukin 1β, and interleukin 17 in bronchoalveolar lavage fluid were dramatically decreased after MSCs treatment. In contrast, expression of interleukin 10 was increased after MSCs treatment. Moreover, mice treated with MSCs had a higher survival rate than the CLP group. Neutrophil infiltration into bronchoalveolar lavage fluid was attenuated after MSCs injection, but the amounts of macrophages observed in the MSC group showed no significant differences compared with the CLP group. In addition, MSCs treatment significantly reduced nuclear factor kappa-light-chain-enhancer of activated B cells activation in lung tissue.

Conclusions: Based on the above findings, treatment with MSCs dampened the inflammatory response and inhibited nuclear factor kappa-light-chain-enhancer of activated B cells activation in the mouse CLP model. Thus, MSCs may be a potential new agent for the treatment of sepsis-induced acute lung injury. (Curr Ther Res Clin Exp. 2020; 81:XXX–XXX)

Background

Sepsis, a fatal syndrome of disordered inflammation, is the leading cause of death in intensive care units,[1] which incurs a staggering $16.7 billion cost to the US health economy with more than 750,000 annual cases and >200,000 deaths each year.[2] Lungs are the first organ affected by sepsis, and sepsis-induced acute lung injury (ALI) is the major cause of death by sepsis.[3] Despite the latest advances in this field, the mortality of patients with ALI is still almost 50%.[4] Hence, it is critical to investigate novel interventions for sepsis-induced ALI.

Septic ALI is characterized by increasing microvascular permeability that leads to leaking of protein-rich edematous fluid and circulating inflammatory cells into the pulmonary interstitium and air spaces.[5] However, the pathogenesis is complex and unclear. In recent years, it has been increasingly recognized that inflammatory cytokines play important roles during sepsis-induced ALI. Accumulating evidence suggests that septic lungs contain significant amounts of proinflammatory cytokines such as tumor necrosis fac-

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tor alpha (TNF-α), interleukin (IL) 6, and IL-1β.[6–8] IL-17 plays a uniquely powerful role in the inflammatory system, which mediates inflammation and induces neutrophil recruitment to inflammatory sites. IL-17 has been reported to increase in sepsis and in turn leads to parenchymal cell dysfunction and tissue damage.[9] The release of anti-inflammatory cytokines, particularly IL-10, is related to downregulation of proinflammatory cytokines and prevention of lung injury. Although mechanisms underlying the development of ALI are not clearly understood, the number of inflammatory cells and activation of inducible transcription factors in the lungs, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), is believed to lead to the development of septic ALI. Activation of NF-κB leads to the activation of various proinflammatory markers for the progression of lung inflammation.[10,11]

Thus, there have been many attempts to control the inflammatory response using specific anticytokine or antimediator therapies. Recently, bone marrow-derived mesenchymal stromal cells (MSCs) have become a hot topic of research, studied in several clinical settings of inflammatory diseases, including sepsis,[12] ischemic kidney injury,[13] and Crohn's disease.[14] The prominent features of MSCs include their ability to home to injured tissues, versatile paracrine signaling effects, an immunomodulatory capacity, and potential for direct antimicrobial effects.[15–18] The complexity of the mechanism of the inflammatory response in sepsis-induced ALI, which is not only involved in preventing infection or inflammation, but also in limiting tissue damage, underlines the critical nature of anti-inflammatory activity. MSCs may have a therapeutic effect in sepsis-induced ALI.

Animal models play an important role in sepsis research. The cecal ligation and puncture (CLP) model is the gold standard.[19] Therefore, in our study, we used the CLP model to elucidate the effect of MSCs treatment on sepsis-induced ALI.

Methods

Animal model

Male C57Bl/6 mice weighing 25 to 30 g were purchased from the animal center at Qingdao University (Qingdao, China). Mice were housed in a constant temperature environment a 12-hour light–dark cycle. The mice were allowed to access to food and water freely. They were acclimated for 1 week before conducting experiments. All experimental procedures were approved by the Animal Care and Use Committee of Qingdao University.

Culture of MSCs

C57Bl/6 mouse bone marrow-derived MSCs were purchased from Cyagen Biosciences (Sunnyvale, California) and cultured according to the supplier's instructions. Sixth, eighth, and 10th passage MSCs were used for experiments.

Mouse model of CLP

CLP-induced sepsis was generated as described previously.[19] Mice were anesthetized with 2% pentobarbital and a 1- to 2-cm midline incision was made along the linea alba of the abdominal muscle to isolate and exteriorize the cecum. Seventy-five percent of the cecum was ligated with a 4-0 silk suture, and the cecum was punctured twice with a 21 G needle. A small amount (droplet) of feces was gently extruded from the penetration holes to ensure patency. The cecum was then returned to the peritoneal cavity gently, and the abdominal incision was closed carefully with 4-0 silk sutures. In the sham group, mice underwent the same procedure but were neither ligated nor punctured.

Three hours after CLP, mice were injected with 1×10^6 MSCs in 0.2 mL normal saline via the tail vein.[22] As a control, 0.2 mL normal saline was injected to CLP and sham mice.

Survival study

Survival study was carried out as a separate experiment with 20 animals in each group. After grouping, the mice in each group were observed for their survival period until 120 hours from the time of surgery.

Animal death and tissue harvest

The mice were put to death by cervical dislocation at 24 hours after sham or CLP surgery. The lung tissues and bronchoalveolar lavage fluid (BALF) were obtained for the different purposes, respectively.

Histology to assess lung injury

Right lung tissue in each group (eg, sham, CLP, and CLP + MSCs) were used in this analysis. The lung tissue was perfused with 10% formalin, and embedded in paraffin. Five-millimeter sections were prepared for hematoxylin and eosin staining. All procedures were performed according to previously published methods.[20] A pathologist who was blinded to the experimental assignments analyzed all sections. In brief, lung histology alterations were assessed on the following parameters: a scale of 0 to 3 (0 = absent and appeared normal, 1 = light, 2 = moderate, and 3 = severe) according to the histologic features: edema, hyperemia and congestion, neutrophil margination and tissue infiltration, intra-alveolar hemorrhaging and debris, and cellular hyperplasia. Each parameter was scored from 0 to 3 based on severity. Then lung injury was assessed by the total score, which was calculated as the sum of all scores for each parameter (0–3 = normal to minimal injury, 4–6 = mild injury, 7–9 = moderate injury, and 10–12 = severe injury). The maximum score per animal was 12.

Bacterial load determination in lungs

To determine the pulmonary bacterial load, the lungs were harvested and equal amounts of wet tissue were homogenized and briefly centrifuged to remove gross particulate matter. Serial dilutions of tissue homogenates were prepared. A portion of each dilution was then plated on blood agar plates and incubated at 37°C for 24 hours under aerobic conditions. According to the dilution factor, the number of bacterial colonies was expressed as colony-forming units (CFUs) per gram of wet tissue.

Lung wet to dry weight ratio

Lung edema was assessed by calculating the ratio of lung wet to dry (W/D) weight ratio. Freshly harvested lung was washed 3 times with phosphate buffered saline at 4°C to remove residual blood, dried, and weighed to obtain the wet lung weight (W). For measurement of the dry weight (D), the tissue was then dried in an oven at 80°C for at least 24 hours. The W/D ratio was calculated as follows: W/D ratio (%) = [(wet weight – dry weigh) / dry weight] $\times$ 100

Total protein concentration and cell count in BALF

BALF was obtained by washing the airways 3 times with 1.5 mL saline. The BALF was centrifuged at 1500 rpm for 10 minutes at 4°C. The supernatant was collected for total protein analysis using Tripure Isolation Reagent (Roche Diagnostics, Basel, Switzerland)

according to the manufacturer's instructions. The cell pellet was resuspended in phosphate buffered saline for the total cell count using a hemacytometer, and differential cell counts were performed with cytospins by the Wright-Giemsa staining method.

Cytokine analysis by ELISA

Mouse TNF-α, IL-6, IL-1β, IL-17, and IL-10 were measured in BALF using commercially available enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, Minnesota), according to the manufacturer's instructions.

Western blots

Lung tissues were homogenized and total proteins were extracted using a tissue protein extraction reagent according to the manufacturer's instructions. The protein concentration was measured using a bicinchoninic acid protein assay kit (Beyotime Biotechnology, Haimen, China). A total of 100 μg protein was separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Anti-p-NF-κB p65 and -inhibitor kappa B-alpha (IκB-α) antibodies (Cell Signaling Technology, Danvers, Massachusetts) were applied as primary antibodies at 4°C overnight, and horseradish peroxidase-conjugated anti-rabbit/mouse immunoglobulin G (Santa Cruz Biotechnology, Dallas, Texas) were used as secondary antibodies. Densitometry was performed using Quantity One software (Bio-Rad Laboratories, Hercules, California).

Statistical analyses

Data are expressed as means (SEM). Multiple comparisons of parametric data were performed using one-way analysis of variance followed by the Student-Newman-Keuls post hoc test. Survival was estimated by Kaplan-Meier analysis. Analyses were conducted using SPSS for Windows version 10.1 (SPSS, Inc, Chicago, Illinois). Statistical significance was defined as P values < 0.05.

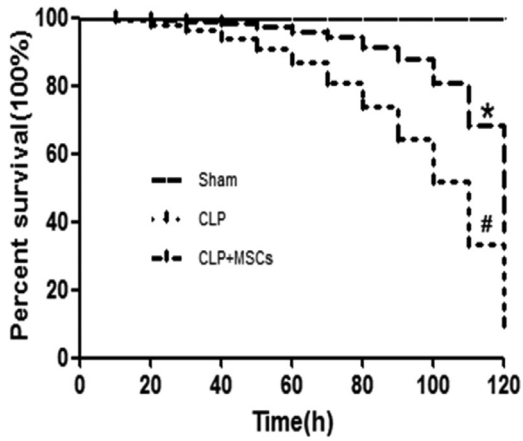

Figure 1. Mesenchymal stem cells (MSCs) improve the survival rate of cecal ligation and puncture (CLP) model mice. Each group included 20 animals. Kaplan-Meier curves showed the survival rate in each group. The survival rate at 120 hours was significantly higher in the CLP group than in the sham group. $^{*}P < 0.05$. The survival rate at 120 hours was significantly higher in the MSC group than in the CLP group. $^{\#}P < 0.05$.

Results

Effect of treatment with MSCs on survival

In the absence of antibiotic therapy, survival was observed for 120 hours after the CLP operation to investigate the improvement by treatment with MSCs. All sham-operated mice without the CLP operation survived, but the survival rate of the CLP group was only

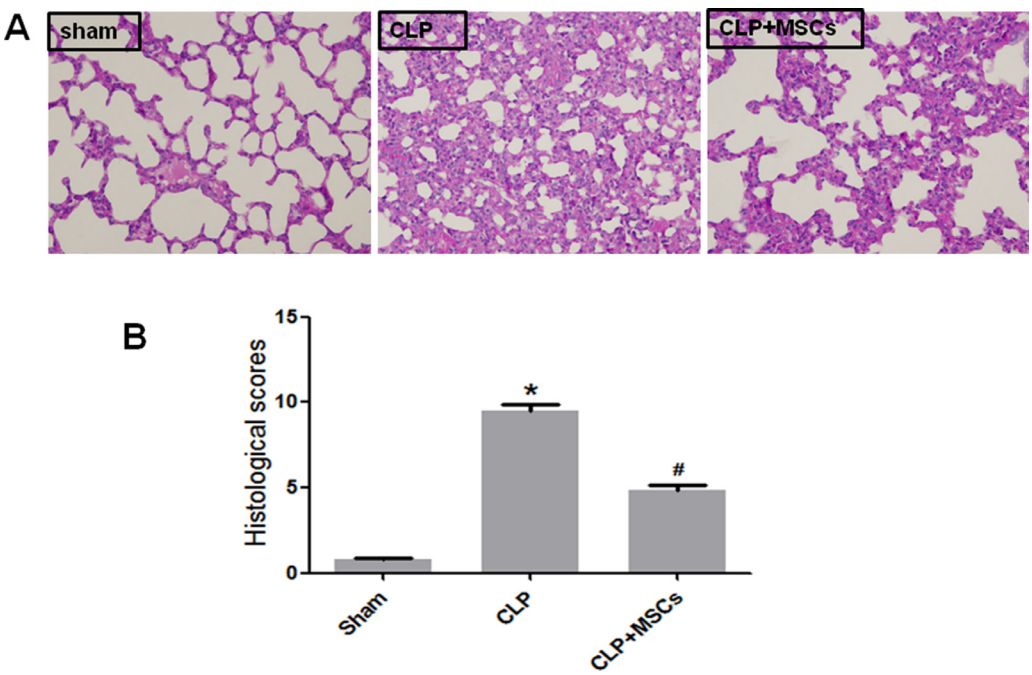

Figure 2. Mesenchymal stem cells (MSCs) alleviate cecal ligation and puncture (CLP) -induced histologic changes in the lungs. Lung tissue from each experimental group were processed for histological evaluation at 24 hours after CLP injury. (A) Representative hematoxylin and eosin staining of lung sections. Original magnification: × 200. (B) Histology scores of pulmonary damage in the various groups. All data are presented as means ± SD.$^{*}P < 0.05$ compared with the sham group. $^{\#}P < 0.05$ compared with the CLP group.

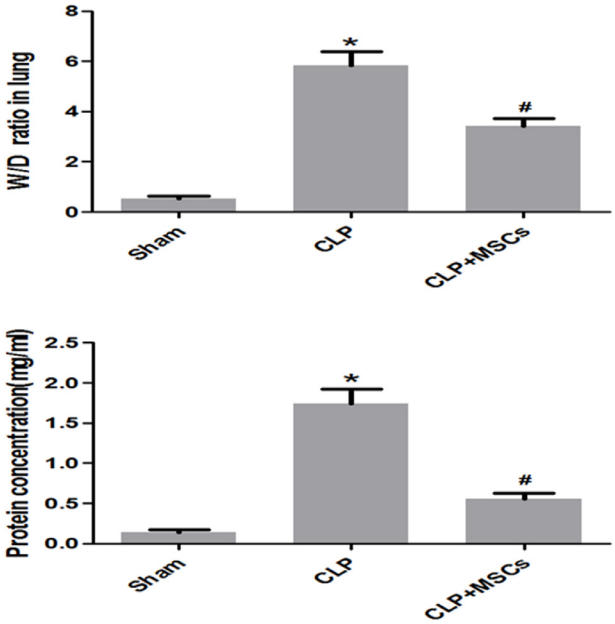

Figure 3. Mesenchymal stem cells (MSCs) reduce the lung wet to dry (W/D) weight ratio and protein concentration in the lungs. Sepsis significantly increased the lung W/D weight ratio and protein concentration in BALF compared with the sham group. Treatment with MSCs significantly reduced the W/D weight ratio and protein concentration in the lungs compared with cecal ligation and puncture (CLP) mice. Data are presented as means ± standardization. $^{*}P < 0.05$ compared with the sham group. $^{\#}P < 0.05$ compared with the CLP group.

30% at 120 hours (Figure 1). Treatment with MSCs significantly improved the survival rate compared with the septic mice ($P < 0.05$).

Effects of treatment with MSCs on lung histology

To evaluate histologic changes after MSC treatment on CLP-induced ALI, lung tissues were harvested at 24 hours after the CLP operation. No histologic alteration was observed in lung specimens of the sham group (Figure 2A). Conversely, in the CLP group, there was a severe inflammatory response characterized by hemorrhaging, alveolar congestion, thickening of the alveolar wall/hyaline membrane formations, and infiltration and aggregation of neutrophils in airspaces or vessel walls (Figure 2B). However, these inflammatory alterations were markedly attenuated in the MSCs group (Figure 2C). Consistent with these findings, histological scores of lung tissue were significantly higher after the CLP operation (Figure 2D), and the MSCs treatment group had a significantly lower lung injury score than the CLP group.

Effects of treatment with MSCs on the lung W/D ratio and protein content

To evaluate changes in pulmonary vascular permeability, the lung W/D weight ratio was analyzed. As shown in Figure 3A, the lung W/D weight ratio was significantly higher at 24 hours after the CLP operation compared with the sham group ($P < 0.05$). MSCs treatment significantly decreased the lung W/D weight ratio ($P < 0.05$). As expected, mice in the CLP group had significantly higher levels of total protein in BALF than those in the sham group as shown in Figure 3B. Accordingly, the protein content after MSCs treatment was significantly reduced compared with the CLP group ($P < 0.05$).

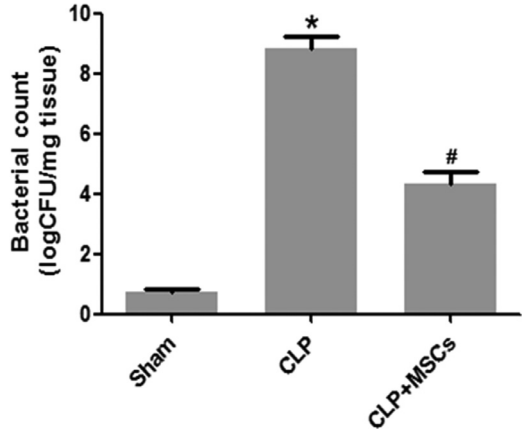

Figure 4. Mesenchymal stem cells (MSCs) inhibit the cecal ligation and puncture (CLP)-induced bacterial load in lung tissue. Lung tissues were collected at 24 hours after the CLP operation and cultured on 5% sheep blood agar plates. The numbers of bacterial colonies were counted after incubation. Data are presented as means ± SD. $^{*}P < 0.05$ compared with the sham group. $^{\#}P < 0.05$ compared with the CLP group.

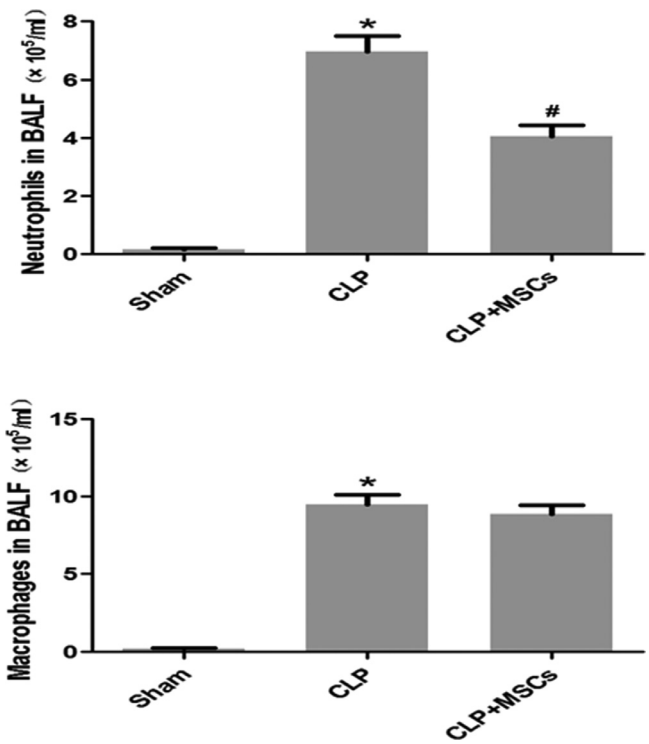

Figure 5. Neutrophil and macrophage numbers in bronchoalveolar lavage fluid (BALF). BALF was collected at 24 hours after cecal ligation and puncture (CLP) injury to measure the numbers of neutrophils and macrophages in BALF. Mesenchymal stem cells (MSCs) reduced the number of neutrophils compared with the CLP group. $^{*}P < 0.05$. However, the change in the number of macrophages in BALF compared with the CLP group showed no statistically significant difference.

Effect of treatment with MSCs on bacterial load

The CLP model is known to be associated with the development of bacteremia. To understand the mechanism of MSCs in sepsis-induced ALI mice, we examined the bacterial load in pulmonary samples at 24 hours after CLP injury. The CLP group had high CFU values, whereas the MSCs group had significantly reduced CFU values (Figure 4).

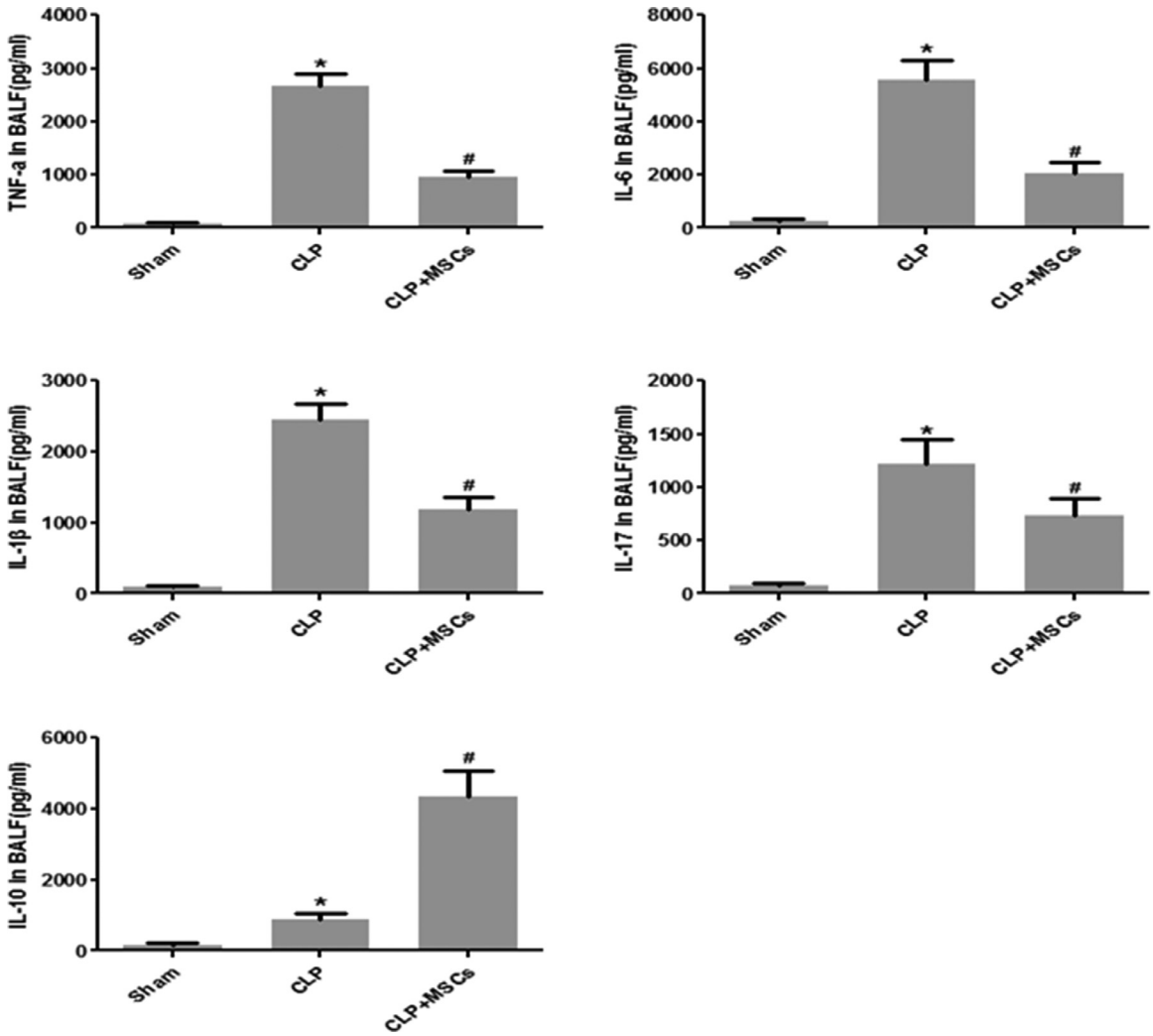

Figure 6. Mesenchymal stem cells (MSCs) reduce the level of proinflammatory cytokines and increase the level of interleukin (IL) 10 in bronchoalveolar lavage fluid (BALF). Enzyme-linked immunosorbent assays were used to quantify cytokines in the BALF of each group at 24 hours after cecal ligation and puncture (CLP) injury. MSCs significantly reduced the levels of tumor necrosis factor alpha (TNF-α), IL-1β, IL-6, and IL-17, and increased the level of IL-10. Data are presented as means ± SD. $^{*}P < 0.05$ compared with the sham group. $^{\#}P < 0.05$ compared with the CLP group.

Effects of treatment with MSCs on lung inflammatory cells in BALF

Accumulation of activated inflammatory cells in the lungs correlates directly with the severity of septic ALI. To further assess the inflammatory responses in septic lungs, we examined the number of neutrophils and macrophages in BALF at 24 hours after CLP injury. As expected, the CLP operation significantly increased the number of neutrophils and macrophages in BALF compared with sham group ($P < 0.05$). In contrast, MSCs administration decreased the number of neutrophils in BALF, but there was no significant change in the number of macrophages between MSCs and CLP groups ($P > 0.05$) (Figure 5).

Effect of treatment with MSCs on inflammatory cytokines in BALF

Next, we evaluated the effects of MSCs on the production of inflammatory cytokines related to septic ALI. As illustrated in Figure 6, the concentrations of TNF-α, IL-6, IL-1β, IL-10, and IL-17 in BALF were significantly increased in the CLP group. In contrast, MSCs treatment decreased the levels of TNF-α, IL-6, IL-1β and IL-17 in BALF compared with the CLP group. However, in contrast to inflammatory mediators, MSCs treatment increased the levels of the anti-inflammatory cytokine IL-10 in BALF compared with CLP mice (Figure 6).

Effects of treatment with MSCs on NF-κB activation

To determine whether MSCs treatment has any effect on the NF-κB pathway in the lungs, western blotting was performed to analyze the phosphorylated NF-κB p65 and IκB-α that reflect activation of NF-κB. As shown in Figure 7, the CLP operation significantly increased IκB-α and phosphorylated NF-κB p65 compared with the sham group. However, MSCs treatment markedly decreased IκB-α and phosphorylated NF-κB p65 induced by sepsis ($P < 0.05$) (Figure 7). These results showed that MSCs inhibited NF-κB activation in sepsis-induced ALI mice.

Discussion

In this study, we evaluated the protective effects of MSCs in the CLP-induced ALI mouse model. Consistent with a previous study,[21] MSC administration attenuated lung inflammatory injury after the CLP operation, as revealed by the decreased lung W/D weight ratio, bacterial load, protein content, and inflammatory cells in BALF, which was associated with reduced lung histologic damage. In ad-

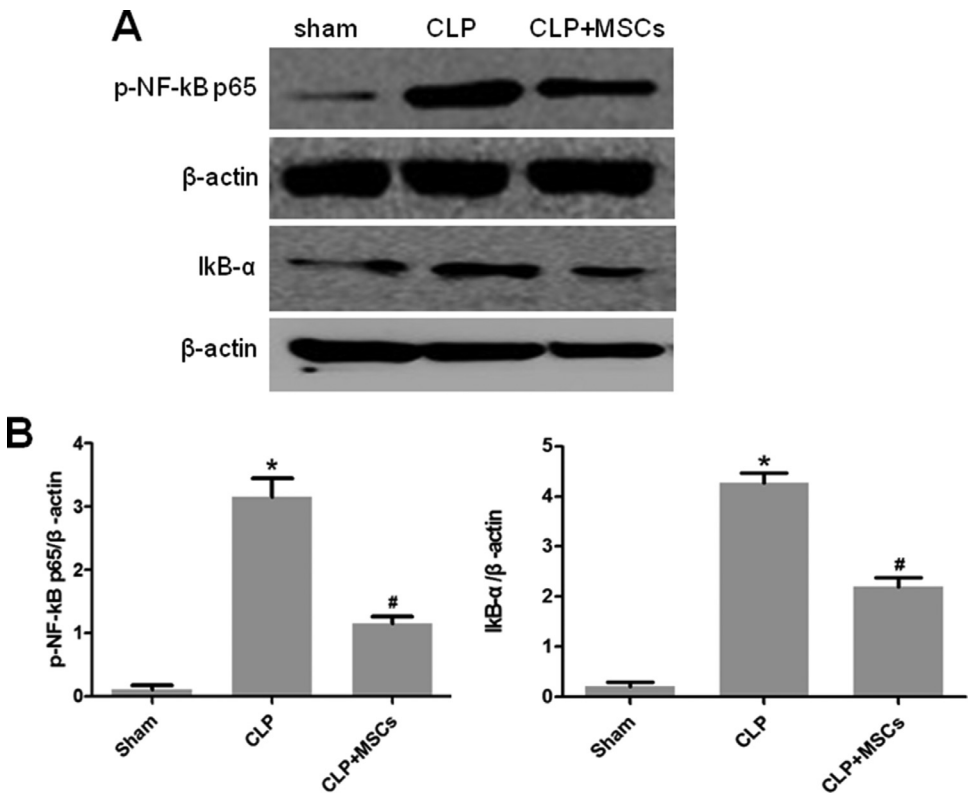

Figure 7. Mesenchymal stem cells (MSCs) reduce the expression of phosphorylated nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p65 and inhibitor kappa B-alpha (IκB-α) in lung tissues. (A) Western blotting of phosphorylated NF-κB p65 and IκB-α proteins in lung tissues. (B) Quantitative analysis of phosphorylated NF-κB p65 and IκB-α in lung tissues. The protein levels are presented as means ± SD. $^{*}P < 0.05$ compared with the sham group; $^{\#}P < 0.05$ compared with the cecal ligation and puncture (CLP) group.

dition, we found that MSCs treatment markedly inhibited the release of proinflammatory cytokines in BALF. Furthermore, MSCs significantly inhibited CLP-induced NF-κB activation in lung tissues and increased the expression of anti-inflammatory cytokine IL-10. Moreover, our data support previous studies showing that MSCs improved the septic mouse survival rate, even in the presence of antibiotic therapy.[21]

Although many different pathogeneses are involved in the development of sepsis, bacteria are the most recognized pathogenesis. Previous studies have shown that MSCs inhibit the inflammatory responses in endotoxin-induced sepsis.[22–24] However, the mechanism needed to be investigated further. Few studies have indicated that MSCs secrete antibacterial proteins/peptides such as LL-37[17] and lipocalin-2,[25] leading to improved bacterial clearance. Pati and colleagues[26] reported that treatment with MSCs increases bacterial clearance because of enhanced phagocytic activity in host immune cells. The antimicrobial effect was explained in part by an increase in monocyte phagocytosis of MSC-treated mice. However, there is considerable debate regarding the antimicrobial effects of MSCs. Another study showed that isolated MSCs lacked the ability to directly kill *Escherichia coli* in vitro.[27] And Li and colleagues[28] reported that blocking NF-κB activity can effectively reduce acute lung injury in mice, but without compromising bacterial host defense after CLP. Many factors may account for the preservation of bacterial host defense. Fortunately, in our study, MSCs therapy significantly reduced the bacterial load in BALF, which may have contributed to the survival benefit and reduction of inflammation in sepsis-induced ALI.

Pulmonary edema is a representative symptom of sepsis-induced ALI,[29] which is often caused by the loss of alveolar-capillary barrier integrity.[30] The magnitude of pulmonary edema was evaluated by examining the lung W/D weight ratio. Our present study showed that MSCs significantly decreased the W/D weight ratio and protein content in BALF, which is in line with the pulmonary histopathology results indicating that MSCs obviously alleviated alveolar epithelial cell edema and decreased the percolate in alveolar cavities. These results demonstrated that MSCs suppressed the filtration of protein-rich edema into the interstitial and alveolar spaces.

It is increasingly recognized that inflammatory cytokines play important roles during sepsis-induced ALI. Indeed, proinflammatory cytokines, such as TNF-α, IL-6, and IL-1β, are the major mediators of early inflammatory responses during sepsis-induced ALI.[31,32]

Indeed, MSCs have an anti-inflammatory activity. Several reports have demonstrated that MSCs modulate innate and adaptive immune cells by enhancing anti-inflammatory pathways in the injured organ milieu, which is mediated by cell contact-dependent and -independent mechanisms through the release of soluble factors such as TSG-6 and PGE_2.[33–35] In this study, CLP-induced neutrophil infiltration in the lungs was significantly reduced by treatment with MSCs. This reduction of leukocyte accumulation was accompanied by a reduction in the production of cytokines, including TNF-α, IL-1β, IL-6, and IL-17, in MSC-treated mice. A study has reported that MSCs promote the repolarization of monocytes and/or macrophages from the type 1 phenotype (proinflammatory) to the type 2 (anti-inflammatory) phenotype characterized by high levels of IL-10 secretion in sepsis.[23] However, in our study, although we found an increase of IL-10, there was no effect on the number of macrophages in BALF, which is inconsistent with previous reports. Our previous study showed that MSCs attenuate ischemic acute kidney injury by inducing regulatory T cells through splenocyte interactions.[36] We also found that MSCs attenuate sepsis-induced acute kidney injury by reducing IL-17.[37] MSCs

have been shown to inhibit the differentiation of naive T cells into Th17 cells,[38] inhibit secretion of proinflammatory cytokine IL-17, and promote expression of IL-10 by immunosuppressive FoxP31 T regulatory cells.[39,40] Therefore, we believe that treatment with MSCs for sepsis-induced ALI may be mediated through the balance of anti-inflammatory T regulatory cells and proinflammatory Th17 cells. MSCs ameliorate this potential injury through decreasing proinflammatory cytokines and increasing anti-inflammatory cytokines. In our previous study we found that MSCs mainly homed to lungs in the CLP model. These results suggested that the protective effects of MSCs on ALI may be related to the cell-to-cell contacts between MSCs and lung cells.

Additionally, NF-κB signaling plays a central role in regulation of the inflammatory response by participating in the transcription of many genes encoding cytokines and inflammatory mediators such as TNF-α, IL-6, and IL-1β.[41,42] Therefore, blocking the NF-κB signaling pathway may alleviate lung injury and the inflammatory response in CLP-induced ALI mice. In recent years, several studies have demonstrated that MSCs exert significant anti-inflammatory effects. A recent study demonstrated that MSCs effectively downregulate NF-κB signaling through secreting TSG-6 to decrease expression of proinflammatory cytokines.[43,44] In the present study, we found that MSCs markedly suppressed CLP-induced nuclear accumulation of p-NF-B p65 and IκB-α. Based on these results, MSCs may exert ALI treatment effects by blocking the release of inflammatory factors and inhibiting activation of the NF-κB pathway.

There are some limitations in our study. Our previous study reported that MSCs mainly homed to the lungs, although we observed a protective effect of MSCs on sepsis-induced ALI. However, the mechanism by which MSCs protected against sepsis-induced ALI was still unclear, which may be transdifferentiation or endocrine mechanisms. We have reported that MSCs protect against sepsis-induced AKI in which MSCs rarely homed to the kidney. Therefore, we need to research further to verify whether MSCs effects are mediated through different mechanisms for protection against sepsis-induced ALI and AKI. In this study, we found an increase of IL-10 and reduction of IL-17. However, the relationship between these changes needs further study.

Conclusions

Our study demonstrated that treatment with MSCs protects against sepsis-induced ALI. The mechanism may be mediated through the balance between sepsis-induced pulmonary proinflammatory cytokines and anti-inflammatory cytokines partially through blockade of the NF-κB signaling pathway. Overall, MSCs may have potential preventive and therapeutic effects to protect against inflammatory responses in sepsis-induced ALI.

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F. Luo and W. Jiang were the main implementers of the experiment. C. Luo was the designer of the experiment and the author of the manuscript. Y. Xu and X.-M. Liu were in charge of experiment implementation and data statistics W. Wang and W. Zhang were part implementers of the experiment.

Conflicts of Interest: The authors have indicated that they have no conflicts of interest regarding the content of this article.

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