



Umbilical cord mesenchymal stem cells enhance the therapeutic effect of imipenem by regulating myeloid-derived suppressor cells in septic mice

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Background: Umbilical cord mesenchymal stem cells (UC-MSCs), which possess potent immunomodulatory effects and low immunogenicity, are considered to be a promising stem cell-based therapy for sepsis. In the current study, we aimed to investigate whether the combined use of UC-MSCs and imipenem has a better effect than imipenem alone in treating *Escherichia coli* (*E. coli*)-induced sepsis and to explore the mechanism by which UC-MSCs exert their therapeutic effect in septic mice.

Methods: We randomly divided mice into five groups with 10 mice in each group: the normal control group (control group), the sepsis group (vehicle group), the MSCs treatment group (MSCs group), the imipenem treatment group (imipenem group), and the imipenem plus MSCs treatment group (imipenem + MSCs group). We monitored the survival rate in each group every 12 h for 3 days. After observing the survival rate, another 50 mice were also randomly divided into five groups, and the mice were sacrificed after 24 h. Bacterial colonies from the blood and peritoneal lavage fluid were counted in a blinded manner. Organ injury was analyzed by hematoxylin and eosin (HE) staining. Frequencies of myeloid-derived suppressor cells (MDSCs) in the blood, spleen, and bone marrow (BM) were determined by flow cytometry. Plasma levels of tumor necrosis factor- α (TNF- α), interleukin (IL)-6, IL-1 β , and IL-10 were detected by enzyme-linked immunosorbent assay (ELISA).

Results: Compared with imipenem treatment, the co-administration of UC-MSCs and imipenem dramatically improved the survival rate, decreased the bacterial load, and ameliorated organ injury. Furthermore, UC-MSCs treatment, either alone or in combination with imipenem, significantly increased plasma levels of IL-10 and the percentage of MDSCs by inducing arginase-1 in septic mice.

Conclusions: Our results indicated that UC-MSCs protect mice against sepsis by acting on MDSCs. Combination therapy of UC-MSCs and imipenem may be a new approach for the future clinical treatment of sepsis.

Keywords: Sepsis; umbilical cord mesenchymal stem cells (UC-MSCs); imipenem; myeloid-derived suppressor cells (MDSCs)

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Introduction

Sepsis is a complicated inflammatory syndrome caused by dysregulated host response to severe infection, which leads to life-threatening organ dysfunction (1). Acute lung injury (ALI) and multiple organ dysfunction syndrome, complications that occur in most septic patients, are important causes of mortality in critical patients (2). Despite the improvement in intensive care and antibiotic therapy, the mortality rate remains at 25–30% (3). Therefore, novel strategies are necessary to advance the treatment of sepsis.

Mesenchymal stem cells (MSCs) are fibroblast-like multipotent cells that can be isolated from various tissues or organs (4,5). Our previous studies revealed that MSCs exert immunomodulatory effects on various immune cells, including B cells, T cells, macrophages, and dendritic cells (6–9). Because of their regenerative and immunomodulatory properties, MSCs are extensively used to treat a variety of human diseases (10). Recently, studies suggested that combination therapy of antibiotics and menstrual-derived MSCs (MenSCs) or adipose-derived MSCs can reduce organ damage and improve survival in the animal model of sepsis (11,12). However, whether the co-administration of UC-MSCs and antibiotics shows the same effect during sepsis development remains to be elucidated.

Myeloid-derived suppressor cells (MDSCs), which are defined as a heterogeneous population of myeloid progenitors, can potently suppress T cell responses (13). The MDSCs phenotype differs between mice and humans. Mouse MDSCs are characterized as CD11b⁺Gr-1⁺ cells and can be further divided into CD11b⁺Ly6G^{low}Ly6C^{high} monocytic MDSCs (M-MDSCs) and CD11b⁺Ly6G^{high}Ly6C^{low} granulocytic MDSCs (G-MDSCs) (14). Human MDSCs are CD11b⁺CD33⁺HLA-DR⁻ cells (15). Previous studies have shown that MDSCs dramatically expand in both septic patients and mice (16,17). It has been found that the numbers of CD11b⁺Gr-1⁺ cells dramatically increase in the spleen, lymph nodes, and bone marrow (BM) during polymicrobial sepsis, which can contribute to sepsis-induced T cell suppression and preferential Th2 polarization (18). Hence, exploring the mechanism of MDSCs generation and regulation may shed new light on the treatment of sepsis.

In the present study, we aimed to investigate the hypothesis that umbilical cord MSCs (UC-MSCs) combined with imipenem is superior to imipenem monotherapy in reducing bacterial sepsis-induced mortality and organ damage. Moreover, we explored the mechanism by which UC-MSCs exert their therapeutic effect in septic mice. Our results showed

that the co-administration of UC-MSCs and imipenem significantly improved the survival rates of *Escherichia coli* (*E. coli*)-induced septic mice, decreased the bacterial load, and increased the percentage of MDSCs. These findings indicate that combination therapy of UC-MSCs and imipenem may be a new approach for the future clinical treatment of sepsis.

We present the following article in accordance with the ARRIVE reporting checklist (available at <http://dx.doi.org/10.21037/atm-20-6371>).

Methods

Bacterial strains

E. coli strains were kindly provided by the Medical Laboratory Center of Zhong da Hospital (Nanjing, Jiangsu, China). They were isolated from human clinical specimens and prepared in Luria-Bertani medium.

Isolation and culture of UC-MSCs

UC-MSCs were obtained and isolated as previously reported (19,20). Briefly, umbilical cords were collected from term infants immediately after birth. The cords were rinsed twice in phosphate-buffered saline (PBS) with 100 units/mL penicillin and streptomycin, and the cord blood vessels were carefully removed. Wharton's jelly was cut into 1–2 mm³ pieces and resuspended in Dulbecco's modified Eagle's medium (DMEM)/F-12 containing 10% fetal bovine serum (FBS) (all from Gibco, Life Technologies). After culture for 2 days, the medium containing non-adherent cells was discarded, and the medium was replaced twice a week thereafter. MSCs between passages 4 to 6 were used for subsequent experiments.

Mice

One hundred male ICR mice (30±2 g, 6 weeks old) were purchased from SPF (Beijing) Biotechnology Co., Ltd. (Beijing, China) and kept in specific pathogen-free conditions at the animal center of the Affiliated Drum Tower Hospital of Nanjing University Medical School. To observe the survival rate, 50 mice were divided into five groups: the normal control group, the vehicle group, the MSCs group, the imipenem group, and the imipenem + MSCs group; *E. coli*-induced sepsis was induced as previously described (21). *E. coli* were harvested, washed, and resuspended in PBS in a total volume of 200 µL (1×10⁸ CFU/mouse). Then, 0.2 mL bacterial suspension was intraperitoneally injected into the mice. Four hours after *E. coli*

injection, UC-MSCs (1×10^6 cells in a volume of 300 μL) were intravenously infused via the tail vein (22), and imipenem was intraperitoneally injected in a volume of 200 μL (25 mg/kg) (23,24). Mice in the control group received the same volume of PBS. The survival rate was monitored every 12 h for 3 days. In a separate experiment, another 50 mice were also divided into those five groups ($n=10$), and the mice were sacrificed after 24 h. Whole blood and peritoneal lavage fluid were collected, and the number of colonies was calculated and is expressed as the \log_{10} (CFU/mL). Experiments were performed under a project license (No. 2020AE01033) granted by the ethics board of the Affiliated Drum Tower Hospital of Nanjing University Medical School, in compliance with the institutional guidelines for the care and use of animals.

MDSCs isolation and coculture with BM-derived macrophages (BMDMs)

Splenic MDSCs were isolated by using Myeloid-Derived Suppressor Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions. To generate BMDMs, BM cells from normal control ICR mice were cultured in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin and 50 ng/mL M-CSF (PEPROTECH, Rocky Hill, USA). The cell medium was refreshed on day 3, and BMDMs were harvested on day 5. BMDMs were replated in 12-well plates (5×10^5 cells/well) and cocultured with the same number of MDSCs in the presence of 100 ng/mL lipopolysaccharide (LPS) for 24 h. The supernatants were collected for enzyme-linked immunosorbent assay (ELISA).

ELISA

Concentrations of plasma and supernatant tumor necrosis factor- α (TNF- α), IL-6, IL-1 β , and IL-10 were determined by ELISA according to the manufacturer's instructions (Bio-Legend, San Diego, CA, USA).

Flow cytometry

Before staining, spleens were mashed on ice to obtain single cell suspensions. BM cells were flushed from femurs and tibias. Then, BM cells and splenocytes were separately passed through a 200-mesh sieve. Single-cell suspensions were stained at 4 $^{\circ}\text{C}$ with a Fc receptor block (clone 93) for 20 min. Total MDSCs were labeled with APC anti-mouse CD11b (clone M1/70) and PE anti-mouse Gr-1 (clone RB6-8C5) antibodies. M-MDSCs and G-MDSCs were detected

with APC anti-mouse CD11b (clone M1/70), FITC anti-mouse Ly6-G (clone RB6-8C5) and PE anti-mouse Ly6C (clone HK1.4) antibodies. All antibodies were purchased from eBioscience. Data were collected with a fluorescence activated cell sorting (FACS) Calibur flow cytometer (BD Biosciences) and analyzed with FlowJo software.

Histopathological examination

After mice were sacrificed, lungs and livers were collected and fixed in 4% paraformaldehyde immediately. Paraffin-embedded lungs and livers were cut into 4- μm -thick sections and subsequently stained with hematoxylin and eosin (HE) for morphologic analysis according to previous studies (25,26) by a blinded observer.

Real-time PCR

Total RNA was extracted from isolated splenic MDSCs using TRIzol Reagent (Invitrogen). Real-time PCR assays were then performed with SYBR Green Master Mix (Invitrogen) on a Step One sequence detection system (Applied Biosystems Waltham, MA, USA). The relative abundance of genes was calculated by using the $2^{-\Delta\Delta\text{CT}}$ method with GAPDH as an internal control. The primers used for real-time PCR are listed in [Table S1](#).

Statistical analysis

Data are expressed as the mean \pm SEM, and data analysis was performed using GraphPad Prism 8 software. The survival rate was analyzed with Kaplan-Meier survival curves. One-way analysis of variance (ANOVA) followed by Tukey's post-test was used to analyze multiple groups. P values less than 0.05 were considered statistically significant.

Results

UC-MSCs improve the survival rate of imipenem monotherapy and ameliorate organ injury in E. coli-induced septic mice

To observe the effect of combined treatment on acute bacterial sepsis, we established a mouse sepsis model by intraperitoneal injection of 1×10^8 CFU *E. coli* per mouse. At 72 h after different treatments, the survival rate reached by the imipenem+ UC-MSCs group was 90%, while the survival rates of the vehicle, UC-MSCs, and imipenem groups were 10%, 40%, and 50%, respectively (*Figure 1A*).

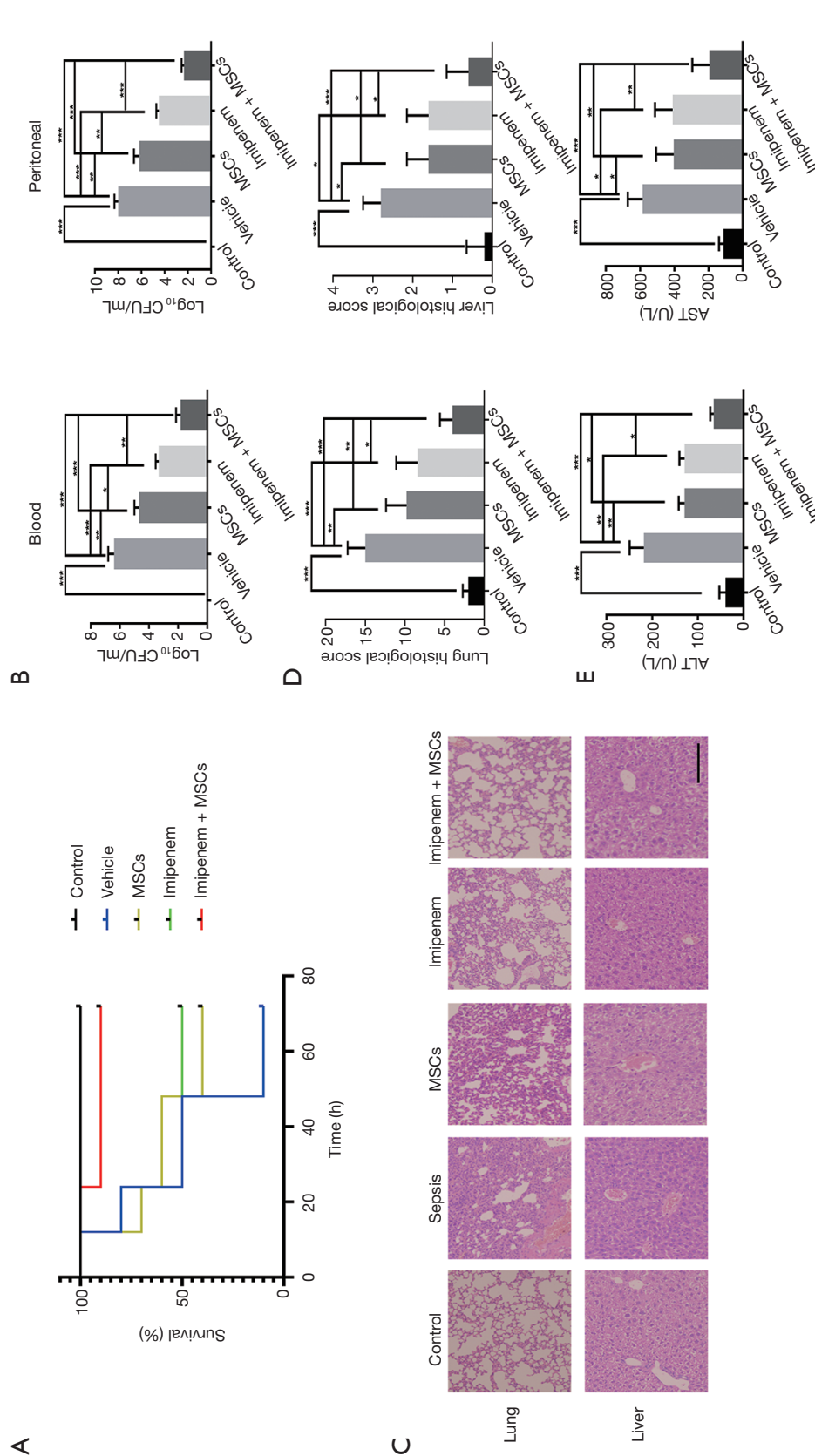


Figure 1 UC-MSCs improve the survival rate of imipenem monotherapy and ameliorate organ injury in *E. coli*-induced septic mice. Septic mice were treated with UC-MSCs and imipenem together or separately 4 h after *E. coli* infection (1×10^8 CFU/mouse). Mice in the control group were treated with PBS. The survival rate of each group was monitored every 12 h for 3 days. (A) Kaplan-Meier curves were used to analyze survival rates ($n=10$). (B) In a separate experiment, mice were treated as in (A) except they were anesthetized and killed 24 h after *E. coli* infection. Blood and peritoneal fluid were collected and plated for 16 h. Statistical analysis of the CFU number is shown ($n=5$). (C) Lung and liver tissues were analyzed using HE staining (original magnification, $\times 20$); the scale bar represents 150 μm . (D) Injury scores of lungs and livers in different experimental groups ($n=5$). (E) Serum concentration of ALT and AST were measured ($n=5$). The data are presented as the mean \pm SEM. The experimental data shown in this figure are representative of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. UC-MSC, umbilical cord mesenchymal stem cell; *E. coli*, *Escherichia coli*; PBS, phosphate-buffered saline; HE, hematoxylin and eosin; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

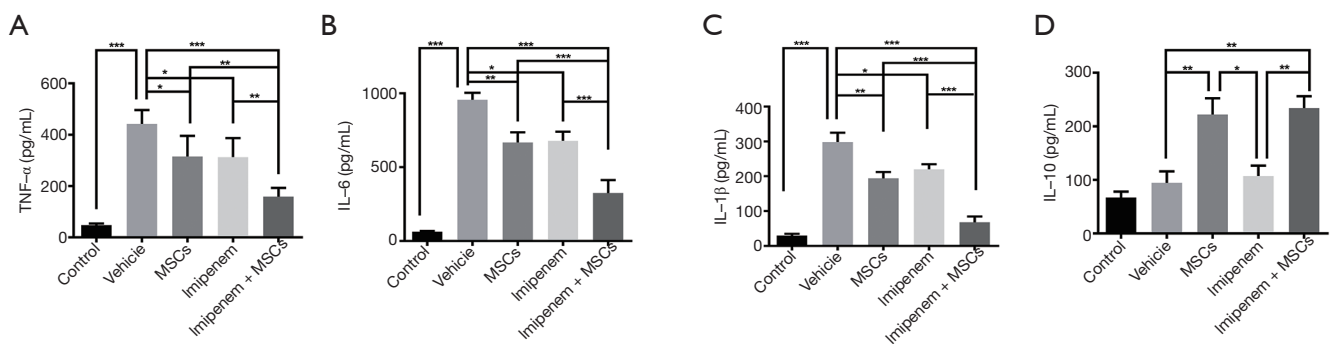


Figure 2 Effect of UC-MSCs treatment on the inflammatory cytokine response. (A,B,C,D) Plasma levels of TNF- α , IL-6, IL-1 β , and IL-10 in each group were determined by ELISA. The data are presented as the mean \pm SEM (n=5). The experimental data shown in this figure are representative of three independent experiments. *, P<0.05; **, P<0.01; ***, P<0.001. UC-MSC, umbilical cord mesenchymal stem cell; TNF- α , tumor necrosis factor- α ; IL, interleukin; ELISA, enzyme-linked immunosorbent assay.

Compared to the vehicle group, the number of bacterial colonies from the blood and peritoneal cavity were decreased in both the MSCs group and imipenem group, while a greater inhibition of bacterial growth was observed in response to combination treatment than MSCs or imipenem treatment alone at 24 h (Figure 1B). Both morphological damage and injury scores were ameliorated in the cotreatment group (Figure 1C,D). Additionally, the serum concentration of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which are biochemical indicators of liver function, were markedly decreased in the combined treatment group 24 h after *E. coli* injection (Figure 1E). These results suggest that UC-MSCs therapy in combination with imipenem provides a more protective effect against *E. coli*-induced sepsis than imipenem or UC-MSCs treatment alone.

Effect of UC-MSCs treatment on the inflammatory cytokine response

To elucidate the anti-inflammatory effect of UC-MSCs treatment, we evaluated plasma levels of several inflammatory cytokines at 24 h post-infection. We found that TNF- α , IL-6, and IL-1 β levels in the plasma collected from septic mice significantly decreased after UC-MSCs plus imipenem treatment (Figure 2A,B,C). Additionally, the level of IL-10 significantly increased in the UC-MSCs group and the combined treatment group (Figure 2D). All these data indicated that co-administration of UC-MSCs and imipenem may bring immune responses back into balance.

UC-MSCs treatment increased the percentage of MDSCs in septic mice

It has been reported that the percentage of MDSCs increases in different tissues of septic mice, including the blood, spleen, and BM (18,27). In the present study, we found that the proportion of MDSCs significantly increased in the blood, spleen, and BM of *E. coli*-induced septic mice at 24 h. Compared with the other treatments, UC-MSCs treatment and cotreatment further increased the frequency of MDSCs in the spleen but not the blood and BM (Figure 3A,B). In addition, UC-MSCs treatment and cotreatment preferentially increased G-MDSCs in the spleen of septic mice (Figure 3C,D). These data revealed that the therapeutic effect of UC-MSCs treatment may be associated with the elevation of MDSCs in septic mice.

UC-MSCs significantly increased arginase-1 expression in MDSCs

MDSCs suppress the activity of immune cells through various functional molecules, including arginase-1, transforming growth factor- β (TGF- β), reactive nitrogen species (ROS), and inducible nitric oxide synthase (iNOS) (28). Thus, we explored whether UC-MSCs treatment exerted its function through these molecules. We found that UC-MSCs treatment and combination treatment significantly enhanced the mRNA level of arginase-1 but did not affect iNOS, TGF- β , gp91-phox or p47-phox expression in splenic MDSCs (Figure 4A,B,C,D,E). The protein level of arginase-1 also significantly increased in splenic MDSCs in the UC-MSCs and combination treatment groups compared with the other groups (Figure 4F).

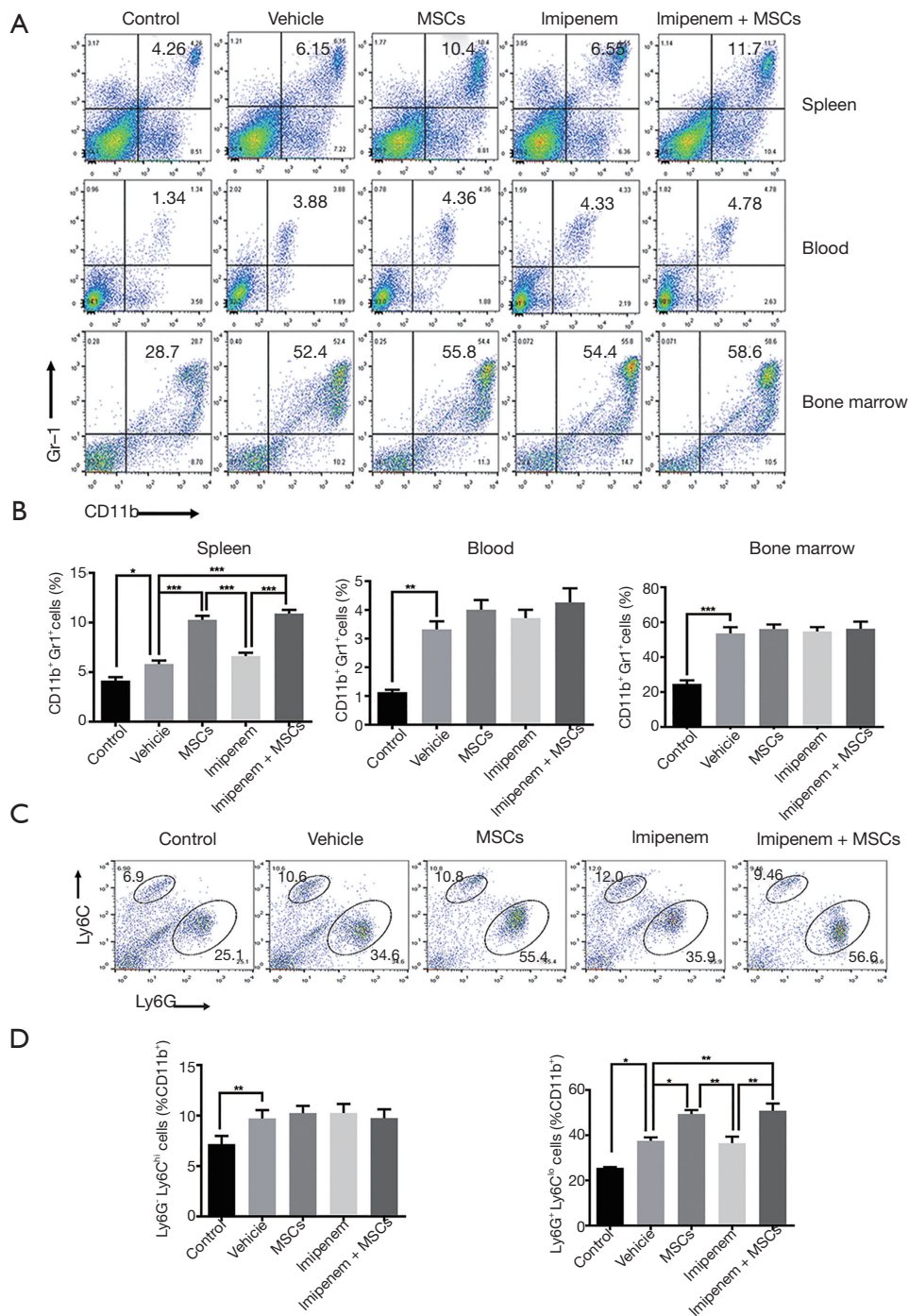


Figure 3 UC-MSCs treatment increased the percentage of MDSCs in septic mice. (A) Flow cytometry was used to determine the percentage of MDSCs in the blood, BM, and spleens of mice treated with or without MSCs and imipenem 24 h after *E. coli* infection. (B) Statistical analysis of the percentage of MDSCs in different tissues. (C) The percentage of G-MDSCs and M-MDSCs in the spleens of mice. (D) Statistical analysis of the percentage of G-MDSCs and M-MDSCs in the spleens of mice. The data are presented as the mean \pm SEM (n=5). The data shown in this figure are representative of three independent experiments. *, P<0.05; **, P<0.01; ***, P<0.001. UC-MSC, umbilical cord mesenchymal stem cell; MDSC, myeloid-derived suppressor cell; BM, bone marrow; *E. coli*, *Escherichia coli*; G-MDSC, granulocytic MDSC; M-MDSC, monocytic MDSC.

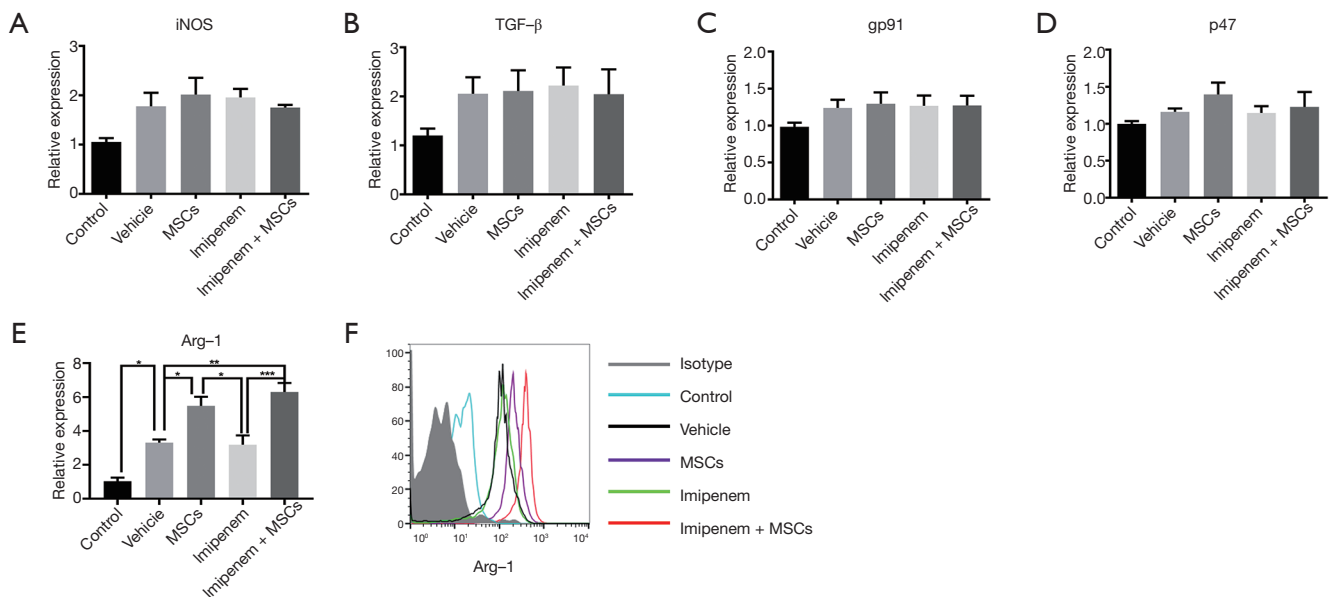


Figure 4 UC-MSCs significantly increase arginase-1 expression in MDSCs. (A,B,C,D,E) The mRNA levels of iNOS, TGF- β , gp91-phox, p47-phox, and Arg-1 were determined by real-time PCR. (F) The protein level of arginase-1 was detected by flow cytometry. The data are presented as the mean \pm SEM (n=5). The data shown in this figure are representative of three independent experiments. *, P<0.05; **, P<0.01; ***, P<0.001. UC-MSC, umbilical cord mesenchymal stem cell; MDSC, myeloid-derived suppressor cell; iNOS, inducible nitric oxide synthase; TGF- β , transforming growth factor- β ; gp91, gp91-phox; p47, p47-phox; Arg-1, arginase-1.

UC-MSCs enhance the immunosuppressive function of splenic MDSCs through arginase-1

Considering that MDSCs may interact with macrophages and influence inflammatory cytokine secretion, splenic MDSCs were cocultured with LPS-stimulated BMDMs. Cytokine levels in the supernatant were assessed. As shown in *Figure 5A,B,C,D*, MDSCs isolated from the spleens of mice in the UC-MSCs treatment and the combination treatment groups exhibited suppressed proinflammatory TNF- α , IL-6, and IL-1 β production but significantly increased production of the anti-inflammatory cytokine IL-10. The presence of nor-NOHA (500 μ M, an arginase inhibitor) blocked these effects. These results suggest that UC-MSCs treatment enhances the immunosuppressive activity of splenic MDSCs mainly through arginase-1, which may contribute to re-balanced cytokine production in septic mice.

Discussion

In the present study, we found that combination therapy of UC-MSCs and imipenem significantly reduced the bacterial load in both peritoneal lavage fluid and peripheral

blood in septic mice and improved the survival rate in mice with lethal *E. coli* infection. Additionally, levels of TNF- α , IL-6, and IL-1 β in the plasma collected from septic mice significantly decreased after combination treatment.

Sepsis initiates an overwhelming proinflammatory response in the first few hours of onset (29), and if not treated early, the patient's condition will deteriorate rapidly. Studies have revealed that MSCs might be favourable in sepsis therapy when infused at the early stage (11,30). As UC-MSCs can be easily isolated from the umbilical cord and expanded *in vitro*, they are considered a promising tool for treating sepsis. He *et al.* showed that intravenous infusion of UC-MSCs was safe and well-tolerated in patients with severe sepsis (31). The mechanism, however, is still unknown.

Previous studies revealed that MDSCs play an important role in the pathogenesis of infectious diseases along with sepsis (18,32,33). Despite MDSCs have been considered to be deleterious in patients with cancer (34), the role of MDSCs in sepsis is complex. In the early stages, MDSCs may be beneficial by limiting excessive inflammation, thus protecting against organ dysfunction. Derive *et al.* demonstrated that adoptive transfer of day 10 MDSCs into septic mice decreased

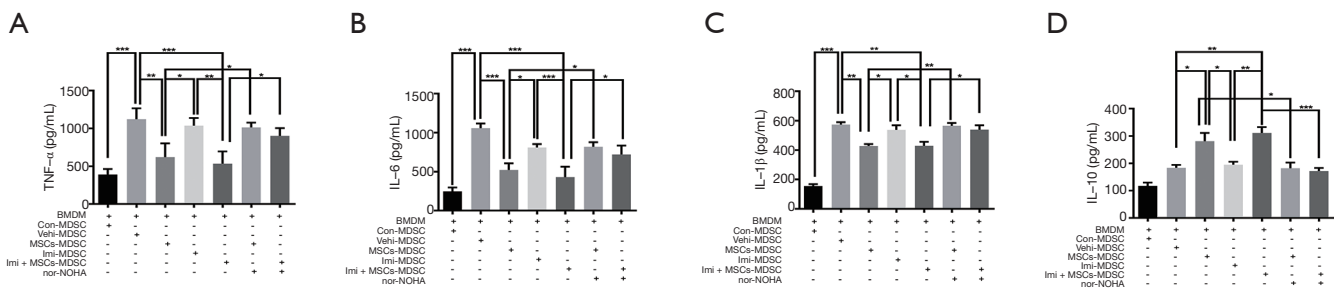


Figure 5 UC-MSCs enhance the function of MDSCs via arginase-1. MDSCs isolated from the spleens of control mice, septic mice, MSCs-treated septic mice, imipenem-treated septic mice, and MSCs + imipenem-treated septic mice were cocultured with equal numbers of LPS (10 ng/mL)-stimulated BMDMs (5×10^5 cells/well) for 24 h. (A,B,C,D) The levels of TNF- α , IL-6, IL-1 β , and IL-10 in the supernatant were determined by ELISA. The data are presented as the mean \pm SEM (n=5). The data shown in this figure are representative of three independent experiments. *, P<0.05; **, P<0.01; ***, P<0.001. UC-MSC, umbilical cord mesenchymal stem cell; MDSC, myeloid-derived suppressor cell; LPS, lipopolysaccharide; BMDM, bone marrow-derived macrophage; TNF- α , tumor necrosis factor- α ; IL, interleukin; ELISA, enzyme-linked immunosorbent assay.

peritoneal cytokine production and enhanced the survival rate (17). Sander *et al.* showed that septic mice lacking gp130 were unable to expand their MDSCs population and exhibited markedly higher mortality associated with increased inflammatory cytokine production (27). However, overzealous MDSCs proliferation may lead to persistent immunosuppression through inhibition of T cell proliferation and elaboration of anti-inflammatory cytokines in the late stage of sepsis. Mathias *et al.* suggested that persistently increased circulating MDSCs are associated with adverse long-term outcomes (35).

Until now, whether UC-MSCs can exert their immunomodulatory properties via MDSCs during early sepsis has not been reported. In this study, we focused on the amount and function of MDSCs in the acute phase, and the results revealed that UC-MSCs treatment, either alone or in combination with imipenem, significantly increased levels of the inhibitory cytokine IL-10 and the number of splenic MDSCs other than in the blood and BM. More importantly, we also demonstrated that the suppressive activity may be related to the upregulation of arginase-1 in MDSCs *in vivo* and *in vitro*. Therefore, we speculated that UC-MSCs inhibit excessive inflammation in the early stage of sepsis by regulating the number and function of MDSCs. The detailed mechanism, however, of how UC-MSCs regulate MDSCs needs further exploration. In addition, further studies are also needed to elucidate whether UC-MSCs can promote the differentiation and maturation of MDSCs, reduce the number of MDSCs in the late stage, and thus restore the immune response during sepsis.

More recently, Alcajaga and colleagues reported beneficial effects of MenSCs and antibiotics in sepsis and proposed a mechanism involving the antimicrobial and immunomodulatory properties of MenSCs (11). Although we observed comparable reductions in experimental sepsis-induced mortality, the antimicrobial effect of UC-MSCs *in vitro*, which will be our next research focus, was less involved in this study. Altogether, these data suggested that MSCs derived from different sources combined with antibiotics can improve the survival rate of septic mice, and the mechanism may be related to multiple targets. Collectively, our work could partly explain the noteworthy decrease in inflammation and mortality in animals with sepsis after receiving combination treatment. To our knowledge, this is the first report on the therapeutic effect of UC-MSCs combined with imipenem in mice with sepsis.

Conclusions

In summary, our data showed that the co-administration of UC-MSCs and imipenem significantly improved the survival rates of *E. coli*-induced septic mice, decreased the bacterial load, and increased the percentage of MDSCs. These findings highlighted the therapeutic potential of utilizing a combination of UC-MSCs and imipenem for the treatment of sepsis, especially in the early stage.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Experiments were performed under a project license (No. 2020AE01033) granted by the ethics board of the Affiliated Drum Tower Hospital of Nanjing University Medical School, in compliance with the institutional guidelines for the care and use of animals.

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