Exosomal miR-146a Contributes to the Enhanced Therapeutic Efficacy of IL-1β-Primed Mesenchymal Stem Cells Against Sepsis

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ABSTRACT

Improving the immunomodulatory efficacy of mesenchymal stem cells (MSCs) through pretreatment with pro-inflammatory cytokines is an evolving field of investigation. However, the underlying mechanisms have not been fully clarified. Here, we pretreated human umbilical cord-derived MSCs with interleukin-1β (IL-1β) and evaluated their therapeutic effects in a cecal ligation and puncture (CLP)-induced sepsis model. We found that systemic administration of IL-1β-pretreated MSCs (βMSCs) ameliorated the symptoms of murine sepsis more effectively and increased the survival rate compared with naïve MSCs. Furthermore, βMSCs could more effectively induce macrophage polarization toward an anti-inflammatory M2 phenotype through the paracrine activity. Mechanistically, we demonstrated that βMSC-derived exosomes contributed to the enhanced immunomodulatory properties of βMSCs both in vitro and in vivo. Importantly, we found that miR-146a, a well-known anti-inflammatory miRNA, was strongly up-regulated by IL-1β stimulation and selectively packaged into exosomes. This exosomal miR-146a was transferred to macrophages, resulted in M2 polarization, and finally led to increased survival in septic mice. In contrast, inhibition of miR-146a through transfection with miR-146a inhibitors partially negated the immunomodulatory properties of βMSC-derived exosomes. Taken together, IL-1β pretreatment effectively enhanced the immunomodulatory properties of MSCs partially through exosome-mediated transfer of miR-146a. Therefore, we believe that IL-1β pretreatment may provide a new modality for better therapeutic application of MSCs in inflammatory disorders. Stem Cells 2016; 00:000–000

SIGNIFICANCE STATEMENT

Mesenchymal stem cells (MSCs) are attractive candidates for treating many immune and inflammatory disorders. However, the immunosuppressive capacities of naïve MSCs are benign, and their therapeutic efficacies are limited. Here, we provide a new strategy to effectively enhance the therapeutic effect of MSCs against sepsis by pre-stimulating MSCs with IL-1β. We also elucidate the involved mechanism that the up-regulated miR-146a by IL-1β stimulation is packaged into exosomes, transferred to recipient macrophages, where regulates M1-M2 transition, and finally contributes to the reduced inflammation and increased survival in septic mice. Our data add new insights into elevating MSC functions by pre-treating inflammatory factors.

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INTRODUCTION

Sepsis, which is a clinical syndrome that occurs in patients following an infection or injury, is characterized by overwhelming systemic inflammation and multiple organ dysfunctions. Despite recent advances in antibiotic therapies and intensive care, the mortality rate as a result of sepsis remains high in intensive care units [1]. Therefore, a new treatment regimen is desperately needed to improve clinical outcomes. Encouragingly, many studies have indicated that because of their immunomodulatory properties, mesenchymal stem cells (MSCs) could be a novel therapeutic candidate for the treatment of sepsis by modulating the deregulated inflammatory response [2-4].

Accumulating evidence suggests that naive MSCs have tenuous immunomodulatory effects, or even cannot suppress immune reactions. But the cells are usually activated by inflammatory cytokines in the inflammatory microenvironment to exert their powerful immunomodulatory effects [5-7]. Therefore, finding strategies to modify MSCs using cytokines or drugs to improve their immunomodulatory activities can improve the development of MSC-based therapies for inflammatory diseases, including sepsis. In the past few years, we and others have reported that pre-stimulation of MSCs with toll-like receptor 3 ligand poly(I:C) [8] or cytokines such as interferon-γ (IFN-γ) [9] or interleukin-17 (IL-17) [10] could enhance the immunomodulatory function of MSCs. Interleukin-1β (IL-1β), which is an important pro-inflammatory cytokine, is enriched in the early stage of sepsis and is associated with the severity and evolution of organ dysfunction [11]. Although it has emerged as a therapeutic target for inflammatory diseases [12], growing evidence has indicated that the presence of IL-1β is beneficial for MSC-based therapies. For example, IL-1β combined with other cytokines can enhance paracrine activity and improve the immunomodulatory effects of MSCs [13, 14]. In addition, IL-1β alone can induce the migration of MSCs to injury sites [15, 16]. Our previous study also demonstrated that IL-1β pretreatment enhanced the therapeutic efficacy of MSCs on dextran sodium sulfate (DSS)-induced colitis, partially by promoting the migration of MSCs to the inflammation sites [17]. However, the underlying mechanism is still fragmentary and incomplete. And whether IL-1β pretreatment could also benefit the therapeutic efficacy of MSCs on sepsis is still unknown.

Exosomes are membrane vesicles with diameters of 30–150 nm that originate in the late endosomes of cells [18]. Growing evidence has suggested that exosomes can mediate local and systemic cell-to-cell communications through the transfer of proteins, mRNAs and microRNAs [19-21]. Recently, exosomes have been described as important paracrine products of MSCs, which suggests a new mechanism for the function of MSCs [22, 23]. Numerous studies have proved that MSC-derived exosomes can improve recovery in animal models of tissue injury [24-27] and immunological diseases, including experimental autoimmune encephalomyelitis (EAE) and graft versus host disease (GvHD) [28, 29]. Intriguingly, recent data also demonstrated that MSC-derived exosomes are protective against sepsis-associated cardiac injury [30]. However, it remains unknown whether IL-1β pretreatment could change the components of MSC-derived exosomes in order to influence their protective effects against sepsis.

MicroRNAs (miRNAs) have been reported to be important components of exosomes that are transferred to recipient cells to mediate intercellular communication and regulate cell functions [20, 31, 32]. The discovery of miRNAs in MSC-derived microparticles, including exosomes, has garnered increased attention [33]. Among the miRNAs, some have important immunoregulatory properties, such as miR-21, miR-143, miR-146a, miR-147 and miR-149. Intriguingly, these miRNAs can be up-regulated or down-regulated by inflammation in other immune cells [8, 34-36]. These results lead us to speculate that IL-1β stimulation may alter MSC miRNAs, which would, in turn, contribute to the enhanced therapeutic effects of IL-1β-primed MSCs.

In the present study, we investigated the therapeutic effect of IL-1β-primed MSCs (BMSCs) in a cecal ligation and puncture (CLP)-induced mouse model of sepsis and the underlying mechanism involving exosomes. We found that IL-1β promoted the therapeutic efficacy of MSCs against sepsis by inducing macrophage polarization to the M2 phenotype. Moreover, we found that BMSC-derived exosomes contained high levels of miR-146a and had similar enhanced immunomodulatory effects both in vitro and in vivo. Importantly, we demonstrated that the up-regulation of miR-146a in exosomes contributed to the enhanced immunomodulatory properties of BMSCs. These findings indicate that IL-1β pretreatment further enhanced the therapeutic efficacy of MSCs against sepsis and that the exosome-mediated transfer of miR-146a was necessary for the protective effects of the BMSCs.

MATERIALS AND METHODS

Cell cultures

MSCs were isolated from human umbilical cords according to previously described methods [37]. Human umbilical cords were obtained from full-term caesarian section births from the Department of Gynecology and Obstetrics at the Affiliated Drum Tower Hospital of Nanjing University Medical School. MSCs were cultured in complete Dulbecco’s Modified Eagle’s Medium (DMEM)/F12 (Life Technologies) with antibiotics (penicillin 100 U/ml, streptomycin 10 µg/ml; Invitrogen) and 10% fetal bovine serum (FBS; Gibco, Australia). MSCs between passages 3 and 7 were used for the subsequent experiments. The immunophenotype of the culture-expanded MSCs was characterized using flow cy-
tometry based on previously described methods [37]. All MSCs showed positive expression for CD29, CD44, CD73, CD90 and CD105 and negative expression for CD11b, CD14, CD19, CD31 and HLA-DR.

Bone marrow-derived macrophages (BMDMs) were isolated from the bone marrow of 6- to 8-weeks-old male C57BL/6 mice by culturing red blood cell-depleted BM in complete RPMI 1640 (10% FBS, penicillin 100 μg/ml and streptomycin 10 μg/ml) with 25 ng/ml macrophage colony-stimulating factor (PeproTech) for 5 days at 37 °C with 5% CO₂.

MSC treatment and co-culture with BMDMs

MSCs were seeded in standard 12-well plates or in the upper compartment of Transwells (0.4-μm pore size, Costar) and then treated with 10 ng/ml IL-1β or vehicle for 12 h, followed by washing twice with PBS. In some experiments, we treated MSCs with 10 μM GW4869 (Sigma) or vehicle for 1 h before administering the IL-1β treatment. BMDMs (2.5×10⁵ cells/well) were collected and co-cultured with MSCs directly or in the lower compartment of Transwells (MSCs:BMDMs = 1:5). BMDMs and MSCs were then co-incubated in the presence of LPS (100 ng/ml) for 24 h. The supernatants were collected for ELISA assay, and cells were collected for RNA or protein extraction.

Reverse transcription and real-time quantitative PCR

Total RNA was extracted from cells or exosomes using TRIzol reagent (Invitrogen) following the manufacturer’s instructions. The concentration and purity of the RNA were determined by measuring the absorbance at 260 nm and 280 nm using a spectrophotometer (Smart Spec Plus, Bio-Rad). Total RNA (1 μg) was reverse transcribed in a 20-μl system using the Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas). Subsequently, real-time quantitative PCR was performed using the SybrGreen PCR Master Mix (with Rox) (Invitrogen) and Step-one plus Real-Time PCR System (Applied Biosystems, Foster City, CA). The sequences of the primers are shown in supplemental Table 1.

ELISA

The protein levels of TNF-α, IL-10 and IL-6 in the cell culture supernatants or mouse serum were detected using the corresponding mouse enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer’s instructions (Biolegend).

Flow cytometric analysis

MSCs were stained for MSC markers according to previously described methods [37]. Single-cell suspensions of lung or liver cells were obtained based on methods described by Nemeth et al. [2]. In brief, freshly removed lung or liver tissues were minced into small pieces and incubated in RPMI 1640 medium with 300 U/ml collagenase type IV (Sigma) and 50 U/ml DNase I (Sigma). After the incubation, the cell suspensions were filtered through a 70-μm cell strainer and then washed with complete RPMI medium. The single-cell suspensions were incubated with a Fc receptor block (CD16/32, eBioscience) to reduce nonspecific antibody binding. The panel of antibodies used in these experiments included CD45-PerCP, F4/80- PE, TNF-α-APC and iNOS-APC (all from eBioscience), and CD68-FITC and CD206-APC, which were obtained from Biolegend. Among these antibodies, CD45 and F4/80 received cell surface staining, CD68, iNOS and TNF-α received intracellular staining, and CD206 received both cell surface staining and intracellular staining. Flow cytometry was performed using FACS Calibur flow cytometer (BD Bioscience), and data were analyzed with FlowJo software (TreeStar, Ashland, OR).

Western blot

Western blotting was performed according to previously described methods [38]. Protein concentrations were determined using a Bradford assay (Pierce). The total protein content (30 μg) of each sample was subjected to SDS-PAGE and immunoblotting with the desired antibodies against iNOS (Abcam, ab15323), Arg-1 (Bioworld, BS6971), IRAK1 (Cell Signaling Technology, 4504), TRAF6 (Abcam, ab33915), IRF5 (Abcam, ab181553), β-Tubulin (Bioworld, AP0064) and GAPDH (Bioworld, AP0063).

Transfection of miR-146a inhibitors

MSCs were cultured in the standard 12-well plates, upper compartment of Transwells or 10-cm dishes and then transfected with miRNA-146a mimics (30 nM), miRNA-146a inhibitors (50 nM) or negative controls using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. Four hours later, the cells were stimulated with or without IL-1β (10 ng/ml) for 12 h, washed twice with PBS and then either collected and administered to mice, changed with fresh DMED/F12 containing normal FBS for an additional 24 h before collected for miR-146a level detection, co-cultured with BMDMs for an additional 24 h or changed with fresh DMED/F12 containing exosome-depleted FBS for exosome isolation.

Exosome isolation

MSCs or IL-1β-primed MSCs were cultured in DMED/F12 containing exosome-depleted FBS (by 18 h centrifugation at 100,000 g) for 48 h. In some experiments, MSCs were pretreated with 10 μM GW4869 (Sigma) for 12 h. Exosomes from the culture supernatants were isolated using differential centrifugation based on previously described methods [39] with some modifications. In brief, the supernatants were centrifuged at 2,000 g for 10 min and then 10,000 g for 30 min to remove the debris and apoptotic bodies (Beckman Avanti Centrifuge J-26XP). Subsequently, the su-
pernatast were centrifuged at 110,000 g for 70 min (Beckman Optima L-80 XP Ultracentrifuge with 70Ti rotor), followed by washing with PBS and purification by centrifugation at 110,000 g for 70 min. All the centrifugations were performed at 4°C. The pellet was resuspended in PBS and sterilized by filtration through a 0.22-µm filter (Millipore). In each exosome preparation, the concentration of total proteins was quantified using a Bradford Protein Assay (Pierce) and then stored at -80°C until further use.

Characterization of exosomes

Protein markers of purified exosomes were determined using western blotting with anti-CD63 (Santa Cruz, sc-15363) and anti-Alix antibodies (Santa Cruz, sc-49268). Exosome morphology was characterized using transmission electron microscopy (JEM-200CX, Japan). Briefly, put a drop of 10 µl of exosome suspension on clean parafilm. A 200-mesh Formvar-carbon-coated electron microscopy (EM) grid was floated onto the drop with the coated side facing the suspension and left to adsorb for 3 min at room temperature. The grid (membrane side down) was transferred to drops of 2% uranyl acetate and left to negative stain for 5 min. After air-drying for 5 min, the grid was observed under a transmission electron microscopy (TEM) at 100kV. The size distribution of the particles was measured using qNano (Izon Science, Christchurch, New Zealand).

Labeling of exosomes

Exosomes were pre-labeled with PKH-67 (Sigma) or CM-Dil (Invitrogen) according to the manufacturer’s instructions and then washed with PBS and centrifuged at 110,000 g for 70 min to remove the excess dye. BMDMs were grown on 12-well plates and then incubated with PKH-67-labeled exosomes (10 µg/mL) for 4 h. The cells were then washed three times with PBS, fixed with 4% paraformaldehyde (PFA) for 10 minutes and permeabilized with ice cold methanol for 15 min. The nuclei were stained with DAPI, and images were taken under a confocal microscope (Olympus FV10C).

Induction and treatment of sepsis

Cecal ligation and puncture (CLP) surgery was performed in male C57BL/6 mice (25-30 g) according to previously described methods [40]. In order to induce mid-grade sepsis resulting in survival rates of approximately 40%, we ligated the cecum at half the distance between the distal pole and the base of the cecum. In order to induce high-grade sepsis (100% lethality), we ligated the cecum at approximately 75% of the distance between the distal pole and the base of the cecum. In the sham-operated mice, we exteriorized the cecum but neither ligated nor punctured it. After surgery, we re-suscitated the mice by injecting pre-warmed normal saline (37 °C; 1 ml per mouse, subcutaneously). CLP-operated mice were randomly assigned to injections of PBS (150 µl), the different treated MSCs (1×10⁶ cells per mouse in 150 µl of PBS) or exosomes (30 µg per mouse in 150 µl of PBS) into the tail vein 4 h after the operation. Blood, peritoneal lavage fluid, liver and lung tissues were collected for subsequent determination 48 h after the CLP operation. Otherwise, the survival rate was monitored every 12 h for 7 days.

H&E staining

Freshly collected liver, lung and kidney tissues were fixed with 4% PFA (pH 7.4) and then gradually dehydrated, embedded in paraffin, cut into 3-µm sections and stained with hematoxylin and eosin for light microscopy.

Immunofluorescent staining

Six hours after the CM Dil-labeled exosomes were injected, liver and lung tissues were collected, and immunofluorescence staining was employed to determine the distribution of exosomes and their localization with macrophages. Briefly, 8-µm-thick frozen liver or lung slices were fixed with cold methanol/acetone (1:1) for 10 min at -20 °C. Following three extensive washings with PBS, the samples were blocked with 3% bovine serum albumin (BSA) in PBS for 60 min at room temperature and then incubated with anti-F4/80 primary antibody (Abcam, ab6640) at a 1:200 dilution for 2 h. After rinsing three times in PBS, the samples were incubated with Alexa Fluor 488- conjugated secondary antibody at a 1:400 dilution for 1.5 h at room temperature in the dark, and then the nuclei were stained with DAPI (Bioword, China). The slides were visualized using a Nikon Eclipse Ti-U fluorescence microscope equipped with a digital camera (DS-Ri1, Nikon).

Statistical analysis

All data are expressed as the mean ± S.E.M unless otherwise indicated. Statistical significance between groups were analyzed using a t-test or one-way ANOVA followed by Tukey’s post hoc test to correct for multiple comparisons in GraphPad Prism 5 (San Diego, USA). A P value of < 0.05 was considered statistically significant.

RESULTS

IL-1β pretreatment enhances the therapeutic efficacy of MSCs in a mouse model of sepsis

Before addressing the function of MSCs, we first determined that human umbilical cord-derived MSCs continued to fulfill the minimal criteria for defining MSCs [41] after human recombinant IL-1β exposure. First, the IL-1β-pretreated MSCs (βMSCs) retained their morphology and capacity to adhere to plastic (supplemental Fig. S1a). Second, they maintained their surface markers, including the positive expression of CD29, CD44, CD73, CD90 and CD105 and negative expression of CD11b, CD14, CD19, CD31 and HLA-DR (supplemental Fig. S1b). Third, the βMSCs still possessed the capacity to differentiate into osteoblasts and adipocytes in vitro (sup-
The therapeutic effects of BMSCs in vivo were examined using a CLP-induced sepsis mouse model. Mice were randomly grouped and injected with BMSCs, MSCs, or PBS through the tail vein 4 h after a mid-grade CLP operation (Fig. 1A). First, we examined survival rates after CLP in the treated mice. As previously reported, the mid-grade CLP resulted in a 40% survival rate at the end of day 7 [40]. We found that the survival rate in the BMSC-treated mice (approximately 75%) was higher than in the naïve MSC-treated mice (approximately 58.3%) (Fig. 1B), which indicated that IL-1β pretreatment increased the protective effects of MSCs for sepsis. We also confirmed that BMSCs could effectively increase the survival rate in the high-grade CLP-induced sepsis (supplemental Fig. S3).

To determine whether a change in bacterial burden could contribute to the BMSC-related protection, we measured the bacterial colony forming unit (CFU) counts in the peritoneal lavage fluid and blood, which were collected 48 h after the CLP operation. The results showed that the bacterial CFU counts in the peritoneal lavage fluid of the PBS-treated septic mice were significantly increased (P < 0.0001) compared with the sham-operated mice. In contrast, the bacterial CFU counts were remarkably reduced following the administration of MSCs, and further reduced with the administration of BMSCs (Fig. 1C).

Because death as a result of sepsis is tightly associated with the systemic inflammatory response, we measured the concentrations of proinflammatory cytokines TNF-α and IL-6 and anti-inflammatory cytokine IL-10 in the serum. Eighty-four hours after the CLP operation, the levels of these cytokines were all increased in the serum compared with the sham-operated mice. There was a more significant reduction in the serum concentrations of IL-6 and TNF-α in the BMSC-treated mice versus the MSC-treated mice. However, the serum concentration of IL-10 was significantly elevated in the BMSC-treated mice but not in the MSC-treated mice (Fig. 1D).

We also examined the pathology and function of major organs, including the livers, lungs, and kidneys, which are often injured in sepsis. The serum levels of the liver enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), were remarkably decreased in the BMSC-treated mice (P < 0.001 versus the CLP + PBS mice) but only slightly decreased in the MSC-treated mice (Fig. 1E). The similar results were also shown in the serum levels of creatinine (Fig. 1E). Histological assessment of liver, lung, and kidney sections from mice with CLP-induced sepsis revealed evidence of edema, inflammatory cell infiltration, and severe hemorrhage. These damages were more significantly ameliorated in the BMSC-treated mice (Fig. 1F). These findings indicate that IL-1β pretreatment can effectively enhance the therapeutic activity of MSCs in sepsis.

IL-1β-primed MSCs promote macrophage polarization to the M2 phenotype in vivo

Because macrophages are reported to play critical roles in the therapeutic effects of MSCs in sepsis [2], we investigated the influence of IL-1β-primed MSCs on macrophages in injured lung and liver tissues. The populations of macrophages and their classical (M1) or alternative activation (M2) phenotypes were measured using flow cytometric analysis. First, we observed that the proportion of CD45+ CD68+ F4/80+ macrophages was significantly increased in the lung tissue of the PBS-treated septic mice compared with the sham-operated mice, whereas the administration of BMSCs reduced the macrophage population significantly more than MSC administration (Fig. 2A). Furthermore, the M1 markers of gated macrophages, inducible NO synthase (iNOS) (Fig. 2B) and TNF-α (Fig. 2C), were remarkably reduced in the BMSC-treated (but not MSC-treated) mice compared with the PBS-treated mice. Meanwhile, the M2 marker CD206 was up-regulated by BMSC treatment (Fig. 2D). In the liver tissue of the septic mice, BMSC treatment was also observed to decrease the macrophage population and M1 markers and increase the M2 marker (supplemental Fig. S4) as compared with MSC treatment. These data indicate that IL-1β pretreatment enhances the regulatory effect of MSCs on macrophage polarization to the M2 phenotype in vivo.

IL-1β-primed MSCs induce M2 polarization through their paracrine activity

To better understand how MSCs interact with macrophages, we co-cultured MSCs or BMSCs with bone marrow-derived macrophages (BMDMs) directly in a cell-cell contact manner or in a Transwell system as shown in figure 3A. Lipopolysaccharide (LPS) was added to these co-culture systems to induce an inflammatory microenvironment. The results showed that 24 h of LPS stimulation remarkably up-regulated the protein levels of the M1 markers, TNF-α (Fig. 3B) and iNOS (Fig. 3D). In contrast, MSCs significantly inhibited LPS-induced up-regulation of TNF-α (Fig. 3B) and iNOS (Fig. 3D), and notably, the inhibitory effect of BMSCs was much greater. Meanwhile, BMSCs were observed to more effectively increase the LPS-induced up-regulation of the M2 markers IL-10 (Fig. 3C) and arginase-1 (Arg-1) (Fig. 3D) compared with MSCs. The effects on the mRNA levels of the M1 (Fig. 3E) and M2 markers (Fig. 3F) were also similar. Of note, there was no significant difference between the cell-cell contact co-culture or Transwell coculture in the BMSC-induced macrophage polarization. These data indicate that IL-1β pretreatment promotes MSC-induced M2 polarization in vitro and that the mechanism is through alterations in paracrine activity and not direct cell contact.
Exosomes derived from βMCSs are sufficient to induce M2 polarization and protect mice from sepsis

In recent years, exosomes have been shown to be important paracrine products of MSCs [22, 23]. To investigate whether exosomes contribute to the enhanced immunomodulatory effects of βMCSs, we blocked exosome secretion using GW4869, an inhibitor of neutral sphingomyelinase 2 (nSMase2), which controls exosome secretion [42]. In this study, 10 µM GW4869 successfully decreased the release of exosomes by MSCs (Fig. 4A) without affecting cell growth (Fig. 4B). GW4869 pretreated or untreated MSCs or βMCSs were co-cultured with BMDMs in a Transwell system in the presence of LPS for 24 h. We found that the down-regulation of TNF-α and iNOS by MSCs or βMCSs was partially inhibited by GW4869 pretreatment (Fig. 4C). Meanwhile, the up-regulation of IL-10 and Arg-1 by MSCs or βMCSs was also attenuated by GW4869 pretreatment (Fig. 4D). These results indicate that exosomes are involved in MSC-induced macrophage polarization.

To further validate the direct effects of exosomes on BMDMs, we purified exosomes from the culture supernatants of MSCs and βMCSs using differential centrifugation. Western blot analysis confirmed that exosomes derived from MSCs (MSC-exo) and βMCSs (βMSC-exo) both express CD63 and Alix (Fig. 5A), which are two widely recognized molecular markers for exosomes [39]. Particle tracking analysis showed that the purified extracellular vesicles from the MSCs and βMCSs were 92 ± 34.1 nm (mean ± s.d.) and 106 ± 45 nm in diameter, respectively(Fig. 5B). Furthermore, TEM showed that the isolated MSC vesicles had a cup-shaped canonical exosome morphology [43, 44][Fig. 5C]. When added to the cultured BMDMs in the presence of LPS, the PKH67 (green)-labeled exosomes (Fig. 5D) were internalized by the BMDMs within four hours. Twenty-four hours later, we detected the concentrations of TNF-α and IL-10 in the culture supernatants and the mRNA expression levels of iNOS and Arg-1 in the BMDMs. We found that the βMSC-exo (10 µg/ml) more efficiently inhibited the LPS-induced up-regulation of TNF-α and iNOS and that is promoted the up-regulation of IL-10 and Arg-1(Fig. 5E) compared with MSC-exo.

We further explored whether βMSC-derived exosomes could, similar to βMCSs, improve the therapeutic efficacy against sepsis. To this end, the CM-Dil-labeled MSC-exo or βMSC-exo (30 µg per mouse) or an equal volume of PBS were intravenously injected into mice 4 h after a high-grade CLP operation. After 6 h, we found that 83.2 ± 3.8% of the labeled exosomes (red) were colocalized with F4/80+ macrophages (green) in the liver (Fig. 5F), which indicated that exosomes were mainly internalized by macrophages in vivo. The survival rates of the mice were monitored over 7 days. Similar to previous reports, we found that no mice survived in the PBS-treated group 4 days after the CLP operation in this high-grade sepsis model [40] (Fig. 5G). Encouragingly, administration of βMSC-exo significantly improved the survival rate of mice to 69.2% over 7 days compared with 33.3% of mice that survived in the MSC-exo-treated group (Fig. 5G).

The above data confirmed that the immunomodulatory effects of βMSC-exo were greater than that of MSCs-exo at the same dose, which was consistent with their cellular counterparts. Therefore, we suggest that more functional molecules may be embedded in the exosomes derived from βMCSs than from MSCs.

MiR-146a is critical for the immunomodulatory effects of exosomes derived from βMCSs

Studies suggest that miRNAs are selectively packaged into exosomes, which largely dictates the effects of exosomes on recipient cells [20]. Therefore, we determined whether the exosomes contained any pivotal miRNAs that contributed to the βMCSs-induced immunomodulatory effects. We screened exosomes derived from MSCs or βMCSs for several known immunomodulatory miRNAs including miR-21, miR-143, miR-146a, miR-147a and miR-149-5p, some of which have been reported to be found in both MSCs and MSC-derived exosomes [33]. Intriguingly, compared with other miRNAs, miR-146a was the most abundant in MSC-exo (approximately 4-fold higher relative to U6) and was remarkably increased in βMSC-exo (approximately 156-fold higher relative to U6) (Fig. 6A and B). When BMDMs were incubated with these exosomes for 12 h in the presence of LPS, the intracellular level of miR-146a was up-regulated by MSC-exo, which was further increased by βMSC-exo (Fig. 6C). After an additional 12 h, we found that the protein levels of well-known miR-146a targets IRAK1, TRAF6 and IRF5 in BMDMs were down-regulated by βMSC-exo (Fig. 6D); however, the mRNA levels of these targets were not changed (supplemental Fig. 5S). These results suggest that the increased miR-146a in exosomes is transferred to macrophages and regulates their functions by targeting the IRAK1, TRAF6 and IRF5 signaling cascades.

To confirm the role of miR-146a in exosomes, we inhibited miR-146a in βMSC-exo by transfecting βMCSs with miR-146a inhibitors and subsequently isolating the exosomes (called in-146a-βMSC-exo) from the culture supernatants. Quantitative RT-PCR analysis demonstrated that expression levels of miR-146a were decreased in in-146a-βMSC-exo compared with in-NC-βMSC-exo (Fig. 6E). We then treated BMDMs with in-NC-βMSC-exo or in-146a-βMSC-exo in the presence of LPS for 24 h and then collected the cells for RNA isolation and qRT-PCR analysis. The results showed that the down-regulation of M1 markers (TNF-α and iNOS) as well as the up-regulation of M2 markers (IL-10 and Arg-1) induced by in-NC-βMSC-exo were partially negated by in-146a-βMSC-exo (Fig. 6F). These data indicate that miR-146a is involved in the βMSC-exo-mediated macrophage M2 polarization in vitro.
To further confirm the effect of exosomal miR-146a in vivo, we administered in-NC-βMSC-exo or in-146a-βMSC-exo (30 μg per mouse) to septic mice and monitored the survival rates over 7 days. The results showed that the survival rate of the in-146a-βMSC-exo-treated mice was 41.7%, which was lower than the in-NC-βMSC-exo-treated mice (66.7%). These observations suggest that exosomal miR-146a plays an essential role in the protective effects of βMSC-derived exosomes against sepsis.

Given that exosomal miR-146a was derived from MSCs, we then investigated the direct effects of miR-146a in MSCs. We first observed that miR-146a was strongly up-regulated in MSCs after IL-1β stimulation (Fig. 7A). Subsequently, we increased the expression of miR-146a in MSCs using transfection of miR-146a mimics or decreased the expression of miR-146a in βMSCs using transfection of miR-146a inhibitors (Fig. 7B). When these cells were co-cultured with LPS-induced BMDMs, miR-146a overexpression improved the MSC-induced down-regulation of M1 markers (TNF-α and iNOS), whereas miR-146a inhibition impaired the βMSC-induced down-regulation of M1 markers (Fig. 7C). Meanwhile, there was an opposite effect on M2 markers (IL-10 and Arg-1) (Fig. 7D). In the septic mice, miR-146a overexpressing MSCs (MSC-mi-146a) improved the survival rate (53.8%) compared with the negative control transfected MSCs (MSC-mi-NC) (33.3%), whereas inhibition of miR-146a partially reversed the protective effects of βMSCs (Fig. 7E). These results indicate that miR-146a is involved in the IL-1β-induced immunomodulatory effects of MSCs.

**DISCUSSION**

In this study, we confirmed that IL-1β pretreatment could increase the immunomodulatory effects of MSCs. We proved that when compared with naive MSCs, βMSCs could significantly ameliorate the symptoms of sepsis, including increasing the survival rates and bacterial clearance, reducing the excessive inflammatory response and alleviating organ injuries. Studies have proved that the transition from a proinflammatory M1 into an alternative M2 state is beneficial for sepsis recovery [2, 45]. Our results are consistent with these early studies and show that βMSCs effectively promote macrophage polarization to the M2 phenotype not only in vitro but also in lung and liver tissues in septic mice. These results also suggest that IL-1β pretreatment can be a useful strategy to enhance the immunomodulatory ability of MSCs.

It is generally believed that the functions of MSCs are mediated by soluble factors and/or in a cell contact-dependent pattern in response to immune cells. However, many studies have shown that MSCs are only transiently recruited to the injured organs, and only a few MSCs are observed afterwards [46-48]. In addition, several animal models of organ injury highlight the efficacy of conditioned media from MSC cultures [14, 49-51]. An important observation of our work is that the immunomodulatory effects of MSCs are not limited to cell contact. We find that similar to cell-to-cell contact co-culture, MSCs and βMSCs co-cultured with BMDMs in a Transwell system can also promote macrophage polarization to a M2 phenotype. This indicates that MSCs secrete bioactive factors that mediate their beneficial therapeutic effects through a paracrine mechanism.

Most previous studies have suggested that the pre-treatment of MSCs with inflammatory factors enhances their functions through increased release of soluble factors, such as chemokines and nitric oxide [6], insulin-like growth factor 1 (IGF-1) [14], IL-6 [10], vascular endothelial growth factor (VEGF) [52], or prostaglandin E2 (PGE2) [53]. In fact, apart from these trophic factors, the amount or content of the MSC-released exosomes may change following pretreatment with inflammatory factors in order to improve the function of MSCs. Recently, Ti and colleagues found that LPS pre-activated MSCs release more exosomes and contained higher levels of let-7b, which contributed to the improved effects of MSCs on wound healing [54]. Our results also focus on the effects of exosomes. We found that inhibition of exosome release by GW4869 decreased the enhanced immunomodulatory effects of βMSCs. Furthermore, the purified exosomes from βMSCs are sufficient for their immunomodulatory activity both in vitro and in vivo. We also demonstrated that IL-1β treatment significantly up-regulated miR-146a expression and packaging within exosomes.

miR-146a is a well-known anti-inflammatory miRNA that plays a key role in inflammatory disorders [36, 55-57]. It can be induced by Toll-like receptor (TLR) agonists, TNF-α or IL-1β, and in turn, it inhibits inflammation by targeting IRAK1, TRAF6 and IRF5 in immune cells [58-60]. Interestingly, evidence has emerged that miR-146a is found in exosomes released by dendritic cells [61] or cardiomyocyte-derived cells [62] and is transferred between cells to regulate the inflammatory response. However, studies concerning the relationship between miR-146a and MSCs are limited, and the role of miR-146a in the immunomodulatory effects of MSCs is unclear. One study reported that miR-146a was up-regulated in neuronally differentiated bone marrow-derived MSCs (nBMSCs) and inhibited the immunomodulatory effects of nBMSCs by targeting PGE2 [63]. In contrast, another group found that increased miR-146a levels contributed to the therapeutic effects of MSCs in diabetic chronic inflammation, which indicates that miR-146a improves the immunomodulatory effects of MSCs [64]. Our results support the latter view. Here, we are the first to report that IL-1β stimulation can up-regulate miR-146a expression in MSCs. Furthermore, miR-146a is embedded in exosomes and transferred to macrophages, where it induces the down-regulation of M1 markers and up-regulation of M2 markers. Finally, exosomal miR-146a contributes to the increased therapeutic effects of βMSCs on CLP-induced sepsis.

The observation that miR-146a is critical for the elevated therapeutic effects of BMSCs is consistent with recent observations that delivery of lentivirus-expressing miR-146a protects from sepsis-induced cardiac dysfunction [65]. Moreover, high levels of miR-146a is reported to protect against acute lung injury [66] or atherosclerosis [59] by targeting TRAF6 and IRAK1. We found that inhibition of miR-146a expression partially reversed, but not completely abolished, the immunomodulatory effects of BMSC-derived exosomes. It is possible that other miRNAs or soluble factors may also be involved in the therapeutic effects of BMSCs, which needs further study. In this study, our results support that exosomal miR-146a from IL-1β-primed MSCs is necessary for their therapeutic effects in sepsis.

**CONCLUSION**

In summary, this study demonstrated that IL-1β pre-treatment enhanced MSC-induced alternative macrophage polarization. Moreover, IL-1β pretreatment improved the therapeutic efficacy of MSCs on sepsis. Mechanistically, IL-1β-mediated up-regulation of miR-146a in exosomes plays an essential role in the protective effects of BMSCs.

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**AUTHOR CONTRIBUTIONS**

Y.S.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, and final approval of the manuscript; H.D.: conception and design, collection and/or assembly of data, data analysis and interpretation; X.Z.: financial support, manuscript writing; Y.L.: provision of study material, collection and/or assembly of data, data analysis and interpretation; J.J.: collection and/or assembly of data; F.L.: collection and/or assembly of data; L.D.: manuscript writing; Y.N.: manuscript writing; Y.H.: conception and design, financial support, manuscript writing, and final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors declare no competing financial interests.

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Figure 1. IL-1β pretreatment enhanced the therapeutic effects of MSCs in a mouse model of sepsis. (A): Experimental design for the in vivo study. Mid-grade sepsis was induced by CLP in male C57BL/6 mice. Sham-operated mice underwent the same procedure without ligation and puncture of the cecum. PBS or a suspension of MSCs or βMSCs (10^6 cells in 150 μl of PBS) was injected through the tail vein 4 h after the CLP operation. Mice were sacrificed, and samples were collected 48 h after the CLP procedure. The survival of the mice was monitored over 7 days. (B): The survival curves of the mice after CLP and MSC therapy. Sham, n = 5; the other groups, n = 10-12. (C): Bacterial counts in the peritoneal fluid and peripheral blood. n = 5 for each group. CFU, colony forming unit. (D): Levels of the cytokines TNF-α, IL-6 and IL-10 in the serum as measured by an ELISA. Sham, n = 5; the other groups, n = 8. (E): Biochemical indicators of liver and kidney functions including the serum concentration of AST, ALT and creatinine (SCr). Sham, n = 5; the other groups, n = 8. (F): H&E staining of livers, lungs and kidneys. Histologic injury scores are shown at right. n = 5 for each group. Bar = 100 μm. #p < 0.05, ###p < 0.0001 versus Sham; *p < 0.05, **p < 0.001, ***p < 0.0001 versus CLP + PBS group.
Figure 2. IL-1β-primed MSCs altered the macrophage population and M1/M2 polarization in the lungs. The flow cytometry plots and analyses of the percentages of CD68⁺ F4/80⁺ macrophages (A), the M1 markers iNOS (B) and TNF-α (C), and the M2 marker CD206 (D) in single-cell suspensions from whole lung tissues. Sham, n = 5; the other groups, n = 8. #p < 0.05, ###p < 0.0001 versus Sham; *p < 0.05, **p < 0.001, ***p < 0.0001 versus CLP + PBS group.
Figure 3. IL-1β-primed MSCs improved the modulation of activated macrophages to the M2 phenotype in vitro. (A): A diagram of the cell co-culture system. MSCs were seeded in the upper chamber of a 12-well insert, followed by IL-1β (10 ng/ml) treatment for 12 h. MSCs were then washed twice with PBS and co-cultured with BMDMs, which were seeded in the lower chamber, in the presence of LPS (100 ng/ml) for 24 h. The protein levels of TNF-α (B), IL-10 (C), iNOS and Arg-1 (D) were detected using an ELISA and western blotting, respectively. The mRNA levels of TNF-α and iNOS (E), and IL-10 and Arg-1 (F) were detected using qRT-PCR. Data are mean ± S.E.M of one representative experiment. Similar results were seen in at least three independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001; #p < 0.05, ###p < 0.0001. BMDMs, bone marrow-derived macrophages; βMSC, IL-1β pretreated MSCs; L + trans MSC, BMDMs co-cultured with MSCs in a Transwell system in the presence of LPS.
**Figure 4. Inhibition of exosome secretion impaired βMSC-induced M2 polarization.** (A-B): GW4869 inhibited exosome secretion without influencing cell growth. Four dishes of MSCs were treated for 12 h with GW4869 (10 μM) or DMSO, respectively, the cells were then washed and replaced with fresh complete medium. Two days later, exosomes were purified from the supernatants and the total protein in exosomes was detected using the Bradford protein assay (A). The number of cells was counted at the end of the exosome production period (B). (C-D): GW4869 attenuated the MSC-induced macrophage polarization. BMDMs were co-cultured in a Transwell system with GW4869-treated MSCs or βMSCs for 24 h in the presence of 100 ng/ml LPS, and M1 markers (C) and M2 markers (D) were measured using an ELISA or qRT-PCR. Data are mean ± S.E.M of one representative experiment. Similar results were seen in at least three independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001. GW, GW4869, inhibits exosome release.
Figure 5. Exosomes derived from IL-1β-primed MSCs enhanced the immunomodulatory effects *in vitro* and *in vivo*. (A): Western blot assay for CD63 and Alix expression in exosomes derived from MSCs or βMSCs. (B): Nanoparticle Tracking Analysis using qNano showed that the average size of MSC-exo was approximately 100 nm. (C): Transmission electron micrograph of MSC-derived exosomes with 2% uranyl acetate negative staining. (D): Representative images (150x) of BMDMs incubated with PKH67-labeled MSC-exo (10 μg/ml) at 37 °C for 4 h. (E): Exosomes from IL-1β-primed MSCs improved the modulation of activated macrophages to an M2 phenotype. BMDMs were incubated with MSC-exo or βMSC-exo for 24 h in the presence of LPS. M1 and M2 markers were measured using qRT-PCR or ELISA. Data are mean ± S.E.M of one representative experiment. Similar results were seen in at least three independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001; ###p < 0.001, ####p < 0.0001. (F): Immunofluorescent staining showing that exosomes (CM-Dil pre-labeled) were co-localized with macrophages in the liver. Bar = 50μm. (G): Survival curves of the mice after a high-grade CLP and MSC-exo or βMSC-exo therapy. Exosomes (30 μg in 150 μl PBS) derived from MSCs or βMSCs were injected through the tail vein 4 h after the CLP operation. n = 10-13.
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Figure 6. MiR-146a was transferred by exosomes and contributed to the immunomodulatory effects of βMSCs. (A): Real-time PCR analysis of miR-21, miR-143, miR-146a, miR-147a and miR-149-5p expressions in exosomes derived from MSCs and βMSCs. The expression levels of the miRNAs were normalized to U6. n = 4. (B): Representative real-time PCR melt-curves and amplification curves of miR-146a and U6 from exosomes. (C): MiR-146a levels of BMDMs treated with MSC-exo or βMSC-exo (10 μg/ml) for 12 h in the presence of LPS. (D): Western blot analysis of the miR-146a targets IRAK1, TRAF6 and IRF5 in BMDMs treated with or without βMSC-exo for 24 h in the presence of LPS. (E): Relative miR-146a levels in exosomes (30 μg) derived from miR-146a inhibitors (in-146a-βMSC-exo) or negative control oligonucleotide transfected βMSCs (in-2NC-βMSC-exo). (F): QPCR analysis of M1 and M2 markers in BMDMs treated with in-146a-βMSC-exo or in-NC-βMSC-exo (10 μg/ml) for 24 h. Data are mean ± S.E.M of one representative experiment in C, E and F. Similar results were seen in at least three independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001 and ###p < 0.0001. (G): Survival curves of the mice after a high-grade CLP and in-146a-βMSC-exo or in-NC-βMSC-exo (30 μg in 150 μl PBS) therapy. n = 12.
Figure 7. MiR-146a is involved in βMSC-mediated immunomoinhibition both in vitro and in vivo. (A): QPCR analysis of miR-21, miR-143, miR-146a, miR-147a and miR-149-5p expressions in MSCs or βMSCs. The expression levels of the miRNAs in MSCs were arbitrarily set to 1. (B): MiR-146a expression in MSCs transfected with miR-146a mimics (mi-146a) or negative controls (mi-NC), and βMSCs transfected with miR-146a inhibitors (in-146a) or negative controls (in-NC). MSCs were transfected for 4 h before treating with or without IL-1β for another 12 h. (C-D): BMDMs were cocultured with different treated MSCs (as in B) in a Transwell system for 24 h in the presence of LPS. M1 markers (C) and M2 (D) were measured by qRT-PCR or ELISA. (E): Survival curves of mice after a high-grade CLP and miR-146a-transfected MSCs therapy. Different treated MSCs were injected via the tail vein 4 h after CLP operation. Data are mean ± S.E.M of one representative experiment. Similar results were seen in at least three independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001 and ###p < 0.0001 in A-D.
IL-1β pretreatment enhances immunomodulatory function and therapeutic effect of MSCs on sepsis via exosome-mediated transfer of miR-146a to macrophages, leading to macrophage polarization to M2 phenotype, and finally reducing inflammation and increasing survival in septic mice.