Original Article

Inhibition of M1 macrophage specific gene expression and promotion of M2 macrophage specific gene expression by bone mesenchymal stem cells

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Abstract: Objective: Considering that the bone mesenchymal stem cells (BMSCs) can be used for the therapy in an animal sepsis model and the macrophages play a key role in the pathological process of the sepsis, this study aims to investigate the influence of BMSCs on the macrophages. Methods: LPS and IFN-γ were used to irritate and induce J774.1 macrophages for 24 hours into M1 macrophages, while IL-4 was applied to irritate and induce J774.1 macrophages for 24 hours into M2 macrophages; BMSCs, together with M1 and M2 macrophages, were cultivated for 24 hours; and the macrophages and supernatant were collected to analyze the changes of cytokines. Results: (1) BMSCs significantly reduced the TNF-α, IL-6, CCL-2, iNOS and CD86 expressed by M1 macrophages; in addition, there was an obvious increase in IL-10, CD206 and Arg-1 expression; while in the supernatant, CD206 was significantly increased, and the content of TNF-α and IL-6 significantly declined. (2) BMSCs significantly increased the expression of Arg-1, CD206, FIZZ-1, Ym1 and IL-10 in M2 macrophages, on the contrary, the expression of TNF-α, IL-6, iNOS and CD86 were significantly reduced; while in the supernatant, the content of IL-6 and TNF-α were significantly reduced, and the content of CD206 was significantly increased. Conclusions: BMSCs can inhibit the specific gene expression of M1 macrophage, but promote the specific gene expression of M2 macrophage in contrast.

Keywords: Sepsis, bone mesenchymal stem cells, macrophages, M1/M2 macrophages, co-culture cytokines

Introduction

Sepsis refers to the life-threatening organ dysfunction caused by reaction disorders of the host to infections [1]. It is a kind of critical disease that presents with multiple organ dysfunction caused by injuries to cells and tissues due to systemic inflammatory response, immune imbalance and endothelial injury.

Macrophages play a key role in the inflammatory reaction and immune imbalance process of the sepsis. Macrophages can be categorized into two types: Type M1 (classically activated macrophages) and M2 (alternatively activated macrophages) [2]. Type M1 was induced by the interferon-γ (IFN-γ) or lipopolysaccharides, in which the interleukin-10 (IL-10) was expressed with the low content or was not expressed, and secretes a large amount of proinflammatory factors that participate in acute proinflammatory reactions, eliminate invading pathogens. And as a result, the Th1 immune response [3, 4] can be regulated and promoted. Type M2 was induced by IL-4 or IL-13, in which IL-10 was highly expressed and secretes a large amount of anti-inflammatory cytokines that participate in killing extracellular pathogens, repairing tissues and healing wounds. And as a result, Th2 immune response [4, 5] was regulated and promoted.

Mesenchymal stem cells (MSCs) are of potential multi-directional differentiation [6]. Some studies have indicated that MSCs can improve animal sepsis damage caused by endotoxin injection and the perforation of cecum ligation [7, 8]. However, a large amount of studies have focused on interactions between MSCs and T cells, and the immune mechanism [9, 10]. However, few studies have investigated the effect of MSCs on macrophages [11], and no study has investigated the influence of polarized M1 and M2 macrophages. Bone mesenchymal stem cells (BMSCs) can be used for the therapy in an animal sepsis model and the macrophages play a key role in the pathological process of the sepsis, this study aims to investigate the influence of BMSCs on the macrophages.
chymal stem cells (BMSCs) are more easily available and quickly proliferated, compared with other types of stem cells [12]. Therefore, the current study aims to investigate the influence of BMSCs on M1 and M2 macrophages.

Materials and methods

Materials

6-8 week-old C57BL/6J male mice graded as Specific Pathogen Free (SPF) were purchased from the Animal Laboratory of Inner Mongolia University of Science and Technology. Treatments for the animals during the experiment were in compliance with the medical ethical standards of the First Affiliated Hospital of Baotou Medical College. J774.1 cells were purchased from Shanghai Xuanyan Biotechnology Co., Ltd. Mouse anti-CD86/B7-2 antibodies, mice anti-mannose receptor/CD206 antibodies, isotype control of goat anti-mouse IgG and IgG2a marked with Alexa Fluor® 488, and a centrifuge were purchased from Thermo Fisher Scientific. Rat anti-mice CD90 antibodies marked with phycoerythrin (PE) (CD90-PE), CD45 and CD11b antibodies marked with allophycocyanin (APC) (CD45-APC, CD11b-APC), CD34 and CD44 antibodies marked with fluorescein isothiocyanate (FITC) (CD34-FITC, CD44-FITC), IgG1 isotype control marked with PE (IgG1-PE), IgG1 isotype control marked with APC (IgG1-APC), IgG2b isotype control marked with FITC (IgG2b-FITC), and the flow cytometry equipment were purchased from BD. Reverse transcription kits, TRIzol reagents and fluorescence quantitative kits were purchased from TaKaRa. IL-4, IFN-γ and LPS were purchased from Sino Biological Inc. Mice tumor necrosis factor-α (TNF-α), IL-6 and CD206 ELISA kits were purchased from Xinqidi Biotechnology. Transwell chamber and 6-well plate were purchased from Nunc. The cell incubator was purchased from SANYO. The fluorescence quantitative PCR instrument was purchased from BIOER. The phase contrast microscope was purchased from Olympus, and the ELISA instrument was purchased from Rayto.

Method

Separation and extraction of BMSCs: C57BL/6J mice aged 6-8 weeks were used and sacrificed by decapitation. Then, 75% ethyl alcohol was used for the sterilization for 10 minutes. The femur were removed. Next, a 10-ml DMEM/F12 culture solution was used to rinse the bone marrow in the centrifuge tube, and was gently blown to prepare the cell suspension at 1,600 rpm for 7 minutes. Then, these were rinsed with phosphate-buffered saline (PBS) (+) three times, and centrifuged at 1,000 rpm for five minutes. Afterwards, the solution was transferred into DMEM/F12 culture medium containing 5% fetal bovine serum and 1% double antibodies (meycin), and cultivated in 37°C in an incubator. After 24 hours, the medium was changed, and the floating cells were removed. The culture lasted for seven days until the third generation which will be cryopreserved.

Identification of BMSC surface markers

The third generation of BMSC single cell suspension at a concentration of 1×10^9 L^-1 was packed in five Eppend of tubes, respectively. Tube 1 was recorded as the negative control, and 5 μL of IgG1-FITC and IgG1-PE were added into it, respectively, while 25 μL of CD44-FITC, 20 μL of CD34-PE, 1 μL of CD45-FITC and 1 μL of CD90-PE were separately added into the remaining four tubes, and cultivated for 30 minutes at 4°C in the dark. Afterwards, these were rinsed with the buffer twice, and 25 μL of 40 g/L of paraformaldehyde and 250 μL of buffer was added for the fixation. The flow cytometry was used for the detection.

Co-culture of BMSCs with M1 and M2 macrophages

J774.1 macrophages were chosen and cultivated in the DMEM/F12+10% FBS system until 90% confluence was achieved, and cells were collected and counted. For Group C, J774.1 macrophages were diluted into 1×10^5/ml through the DMEM/F12+10% FBS system and inoculated into the chamber below the Transwell plate. For Group M1, J774.1 macrophages were diluted into 1×10^5/ml using the 100-ng/ml LPS+30-ng/ml IFN-γ M1 inductive system, and inoculated into the chamber below the Transwell plate. For Group M2, J774.1 macrophages were diluted into 1×10^5/ml in the 100-ng/ml IL-4 M2 inductive system, and inoculated into the chamber below the Transwell plate. Cells were placed in an incubator with 5% CO₂ at a constant temperature of 37°C for 24 hours, and the partially polarized macrophages were used for the flow and RT-PCR detection, respec-

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Table 1. Primer sequences for real-time PCR analysis

<table>
<thead>
<tr>
<th>Name of primer</th>
<th>Primer sequences</th>
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<tbody>
<tr>
<td>IL-6</td>
<td>Forward: 5'-TGATGGATGCTACCAAACCTGG-3' reversed: 5'-TGCTTGGCTTATGATGCCA-3'</td>
</tr>
<tr>
<td>TNFα</td>
<td>Forward: 5'-TGGAAGTACAAAGTACCTGGACACCC-3' reversed: 5'-GCTGAGATGAGTCTGACCA-3'</td>
</tr>
<tr>
<td>CCL2</td>
<td>Forward: 5'-GGAACTCTGAACCTGACTC-3' reversed: 5'-GGCTGCTGATTGACACCC-3'</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Forward: 5'-AGCAGCTCACCTGTTTAT-3' reversed: 5'-GGAACTCTGAACCTGACTC-3'</td>
</tr>
<tr>
<td>CD86</td>
<td>Forward: 5'-ACGGACTTGAACAAACCAAGC-3' reversed: 5'-TGCTGAGATGAGTCTGACCA-3'</td>
</tr>
<tr>
<td>Arg-1</td>
<td>Forward: 5'-CAGAAAGAATTGGAAGTCTGAGC-3' reversed: 5'-CAGATATGCAGGGAGTACAC-3'</td>
</tr>
<tr>
<td>CD206</td>
<td>Forward: 5'-GGAACCGGAGAAACCCGATC-3' reversed: 5'-GCCGAGCATAAGATACAAAG-3'</td>
</tr>
<tr>
<td>FIZZ-1</td>
<td>Forward: 5'-TGAATGCTCCAGTGAATAC-3' reversed: 5'-GGCCCATCTGTTATGACACCC-3'</td>
</tr>
<tr>
<td>Ym-1</td>
<td>Forward: 5'-AGCAGCTCTGAACATGACCC-3' reversed: 5'-CCCCTCTATGGCCCTGAC-3'</td>
</tr>
<tr>
<td>IL-10</td>
<td>Forward: 5'-AGCCGGGAAGCATAACT-3' reversed: 5'-AGGCCAATAAATGCTC-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward: 5'-ACCCTGAGATTTCATGACACC-3' reversed: 5'-AGCTGAGATGAGTCTGACCA-3'</td>
</tr>
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</table>

Flow identification of surface markers of M1 and M2 macrophages

BMSCs were co-cultured with M1 and M2 macrophages Transwell™ for 24 hours and the macrophages were collected and counted. The macrophages were diluted into 1×10⁵/ml of cell concentration using the DMEM/F12+10% FBS system, and were marked, respectively. Then, cells were co-cultured in an incubator with 5% CO₂ at 37°C for 24 hours, and sterile forces were used to take and collect macrophages and the supernatant in upper and lower chambers.

Flow cytometry was used for detection.

Total RNA extraction and cDNA inverse transcription after BMSCs were co-cultured together with M1 and M2 macrophages

After the RNA was extracted from co-cultured macrophages, macrophages were polarized for 24 hours, and the polarized culture solution was removed. PBS (-) was used to gently rinse cells once, and 260 g was taken and centrifuged for 5 minutes. The PBS (-) was removed, 1 ml RNAiso Plus was added into each dish, placed for 1-2 minutes, gently blown, and transferred into sterile EP tubes. Then, 0.2 mL chloroform was added into the EP tube and was tightly covered, and gently mixed up and down until the solution presented the milk-white color. Then, the solution was placed at the ambient temperature for 5 min, and centrifuged at 12,000 g for 15 minutes at 4°C. Then, the supernatant was taken and placed into sterile EP tubes treated with DEPC water, 0.5 mL of isopropyl alcohol was added, and was gently mixed and placed on ice for 30 minutes. Next, 1 ml of 90% ethyl alcohol was added (pre-cooled at 4°C), gently mixed, and placed on an ice box for 30 minutes. A 260 g solution was selected and centrifuged for 5 minutes, cells were collected, and the supernatant was removed. Then, 300 μl of the first antibodies were separately added (CD86 and CD206), gently mixed, and incubated at the ambient temperature for 1 hour in the dark. Subsequently, a 260 g solution was selected and centrifuged for 5 minutes, cells were collected, and the supernatant was removed. Then, 300 μl of the second antibodies were separately added, and incubated at ambient temperature for 30 minutes in the dark. Flow cytometry was used for detection.
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Reverse transcription solution was prepared using 2 μL of 5× PrimeScript® Buffer, 0.5 μL of PrimeScript® reverse transcriptase mixture, 0.5 μL of OligodT Primer (50 μmol/L), 0.5 μL of randomized primer hexamer (100 μmol/L), and 6.5 μL of total RNA. Primers were synthesized by Shanghai Sangon Biotech, as shown in Table 1.

The prepared reverse transcription system was bathed in water at 37°C for 15 minutes, inactivated at 85°C for 5 seconds, and then the reverse transcription completed. The real-time quantitative PCR reaction solution was prepared according to the following methods: 10 μL of SYBR® Premix (2×), 1.0 μL PCR forward primer (10 μmol/L), 1.0 μL reverse primer (10 μmol/L), 1.0 μL cDNA and 7.0 μL deionized water were used. The prepared PCR reaction solution was placed in the PCR instrument, and the setting procedures and conditions were as follows: the initial denaturation at 95°C for 30 seconds, at 95°C for 5 seconds, 60°C for 30 seconds, which lasted for 40 cycles; the reaction for amplification at 95°C for 15 seconds, at 60°C for 1 minute, at 95°C for 15 seconds, and at 60°C for 15 seconds. The fluorescence quantitative PCR instrument automatically collects the Ct values of the target and reference genes, and the multiple of the relative expression level of the target genes in the experimental group to the control group= 2-ΔΔCT, where ΔCT, the relative expression level of target genes=Ct of target genes of the experimental group or control group -Ct of β-actin, ΔΔCT=ΔCT of experimental group -ΔCT of control group.

Detection of changes in IL-6, TNF-α and CD206 content in macrophage supernatant by ELISA after BMSCs were co-cultured with M1 and M2 macrophages

The supernatant in which M1 and M2 macrophages were cultured for 24 hours, and the supernatant, in which M1 and M2 macrophages were co-cultured with BMSCs, were added into the corresponding wells with the standard solution. According to the instructions of the ELISA kit, measurement was conducted at an absorbance value of 450 nm (A450).

Statistical analysis

SPSS 18.0 statistical software was adopted to make the statistical analysis. The measurement data were expressed as the mean ± SD. The independent sample t-test was used for the comparisons of these two groups, and the
post-hoc test analysis was conducted for the comparisons of various groups. P<0.05 indicates statistical significance.

Results

Observations on BMSC morphology

BMSCs were conducted with the diffused distribution after the inoculation for 3-4 days and adhered to the wall. The cell body grew in round and centered forms. The cellular morphology tended to be unified after 6-7 days in a long spindle shape with the strong proliferation capacity and the convergence degree of 90%. P2 and P3 generation are described in Figure 1.

Adipogenesis–induced and osteogenesis–induced differentiation of BMSC

After the third generation of BMSC adipogenesis was induced for three days, significantly increased lipid drops were observed (Figure 2A), a large amount of lipid drops was observed on the 7th day, and oil red O was stained in bright red color (Figure 2B). After the third generation of BMSC osteogenesis was induced, the aggregation phenomenon was partially observed in cells, and calcium deposits were found around the cells after induction for 21 days. After staining by alizarin red, red calcium nodules were observed (Figure 2C, 2D). This indicates that the cultured BMSCs had strong differentiation ability.

BMSC identification

For the third generation of BMSCs, the flow identification was carried out for the markers on the surface of cells, and the results demonstrated the positive CD44 and CD90 and the negative CD45 and CD34 results, which conforms to the international identification of markers on the surface of BMSCs (Figure 3).

Morphology of macrophages after BMSCs were co-cultured with M1 and M2 macrophages

Macrophages diffusely grown with a round shape after the incubation. The cell membrane was intact and the cytoplasm was rich in many particles. The nucleus was large and deviated
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Figure 4. Morph of macrophages (20×). A: M1 macrophages; B: M1+BMSCs; C: M2 macrophage; D: M2+BMSCs; E: Macrophage.

Figure 5. CD86 Flow cytometry identification of macrophages. A: Isotype control, B: M1 macrophages, C: M1 macrophages+BMSCs, D: M2 macrophage, E: M2 macrophage+BMSCs.

to one side with the strong proliferation capacity, general adherence and high cell refraction (Figure 4E). In the Group M1, the morphology of macrophages varied and the cell refraction was weakened. The increased particles were found in the abundant cytoplasm. Round cells were reduced, and partial cells extended pseudopods in branch, rod and radiating shapess (Figure 4A). In the co-culture group of M1 macrophages and BMSCs, compared with the Group M1, the rod-shaped, star-shaped and branch-shaped macrophages were increased; the cells became round. The cell adhesion was increased with the abundant cytoplasm (Figure 4B). In the Group M2, the volume of most cells was slightly enlarged as the oval shape with the increased cell adhesion. The cells grew in aggregation or cluster with the abundant cytoplasm (Figure 4C). In the co-culture group of M1 macrophages and BMSCs, compared with the Group M2, the cells became round; and the rod-shaped, star-shaped and branch-shaped macrophages was increased with the increased cell adhesion; in addition, the cytoplasm was abundant, and the particles was increased (Figure 4D).
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Results of flow cytometry identification of markers on the surface of M1 and M2 macrophages

Flow cytometry results for CD86: After M1 macrophages were co-cultured with BMSCs, the expression of CD86 presented as the negative. In the M1 group, the expression of CD86 presented as the positive, and the CD86 was negatively expressed in Group M1+BMSCs, Group M2 and Group M2+BMSCs (Figure 5).

Flow cytometry results for CD206 (the mannose receptor): After M1 and M2 macrophages were respectively co-cultured with BMSCs, the expression of CD206 presented as the positive. CD206 was positively expressed in Group M1+BMSCs, M2 and M2+BMSCs; but was negatively expressed in Group M1 (Figure 6).

mRNA content of M1 and M2 macrophage cytokines: Compared with Group M2, the TNF-α, IL-6, chemokine ligand 2 (CCL-2), inducible nitric oxide synthase (iNOS) and CD86 mRNA content was significantly increased in the Group M1. Furthermore, the arginase-1 (Arg-1), CD206 and IL-10 mRNA content significantly increased in Group M2 compared with the low mRNA content in Group M1. In addition, the chitinase 3-like-ponit 3 (Ym-1) and inflammatory molecular 1 (FIZZ-1) were almostnot detected in Group M1, but these werehighly expressed in Group M2 (Figure 7).

Co-culture of BMSCs and M1 macrophages to promote M1 macrophages to be transferred into M2 macrophages, and the co-culture of BMSCs and M2 macrophages to further activate M2 macrophages.

After M1 macrophages were co-cultured with BMSCs, the IL-6, TNF-α, iNOS, CD86 and CCL2 were detected in Group M1+BMSCs, but not in Group M2+BMSCs.
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mRNA content were significantly decreased, while the Arg1, CD206 and IL-10 mRNA content were significantly increased (P<0.05, P<0.01). Furthermore, theFizz1 and Ym-1 were also expressed. After M2 macrophages were co-cultured with BMSCs, the IL-6, TNF-α, iNOS, CD86 and CCL2 mRNA content was significantly decreased, while the Arg1, CD206, IL-10, Fizz1 and Ym-1 mRNA content was significantly increased (P<0.05, P<0.01; Figure 7).

Changes in cytokines in the supernatant detected by ELISA assay after M1 and M2 macrophages were respectively co-cultured with BMSCs

After M1 and M2 macrophages were respectively co-cultured with BMSCs, the TNF-α and IL-6 content in the supernatant were significantly decreased, and the CD206 content was significantly increased (P<0.01, Figure 8).

Discussion

Macrophages are of plasticity, and M1 and M2 macrophages can be transformed between each other [13]. The cyclic adenosine phosphate can inhibit the secretion of the proinflammatory factors in the macrophages and elevate the proinflammatory factors to generate the phenotype transformation between M1 and M2 macrophages [14]. Liu et al. [15] demonstrated that Fasudil could induce M1 macrophages to be transferred into M2 in order to develop anti-inflammatory effects and address encephalomyelitis. Curcumin can promote M1 macrophages to be polarized into M2 to develop anti-inflammatory effects to fight against atherosclerosis [15]. Polarization to a beneficial direction through interfering and inducing the phenotype of macrophages is the study target in the future.

MSCs were used to treat inflammation and immune diseases due to low immunogenicity [16] and immunoregulation features [17-20], which has remained a hot topic in recent studies. Few studies have investigated the effects of macrophages in the treatment of sepsis by MSCs [11], indicating the features MSCs provided with M2 macrophages after MSCs were co-cultured with non-polarized macrophages, and the increase in the proportion of M2 macrophages. In addition, the expression of CD206 was upregulated, IL-10 expression increased, and TNF-α expression decreased; which describes that MSCs can regulate the phenotype of macrophages to be transferred into M2 macrophages, from proinflammatory response to anti-inflammatory reaction; thus, improving the uncontrolled inflammatory reaction [11]. There are no related studies on whether MSC has an influence on polarized macrophages (M1/M2 macrophages). Hence, this study adopted the Transwell™ system to separately co-culture BMSCs with M1 and M2 macrophages, in order to investigate the influence of BMSCs on M1/M2 macrophages. This study investigates the influence of BMSCs on M1 and M2 macrophages in terms of changes in the morphology of M1 and M2 macrophages, CD86 and CD206, as well as changes of proinflammatory factor and anti-inflammatory factor content.

We found that the following: (1) In the co-culture group of BMSCs and M1 macrophages, compared with M1 group, in terms of the morphology, the rod-shaped, star-shaped and branch-shaped macrophages decreased, and
cells became round. Cell adhesion increased and the cytoplasm was abundant. These were transformed into the morphology of M2 macrophages with negative CD86. CD86 [21] was the specific surface marker of M1 macrophages, indicating that BMSCs inhibited the activity of M1 macrophages. When sepsis occurs, related molecular patterns of LPS and other pathogens (PAMP) activate IKK compounds through TLR4 and IL-1 signals, and regulate the activity of M1 macrophages through NF-κB key signaling pathways [8, 22]. Furthermore, BMSCs influence the polarization of M1 macrophages by inhibiting the NF-κB signaling pathway [8, 23].

(2) BMSCs reduced the expression of IL-6, TNF-α, iNOS and other inflammatory factors expressed by M1 macrophages, and increased the expression of IL-10, Arg-1, Fizz-1 and CD206, with positive CD206. CD206 [21] is the specific surface marker of M2 macrophages, indicating that BMSCs could not only inhibit the activity of M1 macrophages, but also promote the polarization of M1 phenotype macrophages into M2 phenotype macrophages; which is the new idea from our studies. (3) With BMSCs, IL-10, CD206, Arg-1, Fizz-1 and Ym1 expressed by M2 macrophages were significantly increased, indicating that BMSCs may further enhance the functions of M2 macrophages; which are important findings from our study. The polarization process of M2 macrophages involves the activity of the JAK1 and STAT3 pathways [8, 24]. BMSCs can significantly increase the phosphorylation of JAK1 and STAT and activate STAT3 pathways [25], which may result in the further activation of M2 macrophages.

It can be concluded that one of the potential mechanisms for BMSCs in treating sepsis is that BMSCs not only inhibit the activity of M1 macrophages, but also induce the polarization of M1 phenotype macrophages into M2 phenotype macrophages, in order to further activate the functions of M2 macrophages. On this basis, the generation of anti-inflammatory factors increased and the generation of proinflammatory factors decreased, from proinflammatory reaction to anti-inflammatory reaction; thus, improving the uncontrolled inflammatory reactions.

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**Disclosure of conflict of interest**

None.
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