Original Research

Mouse bone marrow-derived mesenchymal stem cells induce macrophage M2 polarization through the nuclear factor-κB and signal transducer and activator of transcription 3 pathways

Shuo Gao[1], Fei Mao[1], Bin Zhang[1], Ling Zhang[1], Xu Zhang[1], Mei Wang[1], Yongmin Yan[1], Tingting Yang[1], Jie Zhang[1], Wei Zhu[1], Hui Qian[1] and Wenrong Xu[1,2]

[1]School of Medical Science and Laboratory Medicine, Jiangsu University, Zhenjiang, Jiangsu 212013, P. R. China; [2]The Affiliated Hospital, Jiangsu University, Zhenjiang, Jiangsu 212001, P. R. China

Corresponding author: Wenrong Xu. Email: icls@ujs.edu.cn; Hui Qian. Email: lstmmmlst@163.com

The first two authors contributed equally to this work.

Abstract

Increasing evidence has demonstrated that mesenchymal stem cells (MSCs)-mediated regulation of macrophages is critical for inflammation response and tissue injury repair. However, the underlying mechanism is not well understood. In this study, we investigated the effect of mouse bone marrow-derived MSCs on macrophages under normal and inflammatory conditions. Co-culture with MSCs or treatment with MSC-conditioned medium (MSC-CM) reduced the expression of tumor necrosis factor-α while inducing the expression of interleukin 10 (IL-10) and arginase 1 in lipopolysaccharide (LPS)-stimulated mouse RAW264.7 cells and splenic $CD11b^+$ cells. MSC-CM treatment increased the expression of CD206, a marker of alternatively activated M2 macrophages, in RAW264.7 cells. In addition, MSC-CM promoted the proliferation and migration of RAW264.7 cells. MSC-CM treatment activated signal transducer and activator of transcription 3 (STAT3) but inhibited nuclear factor-κB (NF–κB) pathways in LPS-stimulated RAW264.7 cells. Moreover, STAT3 inhibitor S3I-201 antagonized the induction of IL-10, arginase 1, and CD206 by MSC-CM in RAW264.7 cells. Conclusively, our findings suggest that mouse MSCs induce macrophage M2 activation through the NF-κB and STAT3 pathways.

Keywords: Mesenchymal stem cells, macrophage, inflammation, polarization, signaling pathway

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Introduction

Mesenchymal stem cells (MSCs) are pluripotent adult stromal cells which have self-renewal and multi-differentiation abilities.[1] Under injury and inflammation conditions, MSCs can be mobilized from bone marrow to the site of damage, respond to the local microenvironment, and exert anti-inflammatory and immunosuppressive functions.[2] Our previous studies have demonstrated that MSCs have therapeutic effects for multiple diseases, including liver injury,[3,4] ischemia/reperfusion-induced acute kidney injury,[5] and collagen-induced arthritis.[6]

The secretion of extensive bioactive molecules is now considered to be the main mechanism by which MSCs achieve their therapeutic effects.[7] Increasing evidence has shown close interactions between MSC derived soluble factors and immunocytes such as lymphocytes,[8,9] dendritic cells,[10] natural killer cells,[11] and macrophages.[12] Macrophages have remarkable plasticity in response to environmental cues.[13] Depending on the stimulated signals, macrophages undergo classical M1 activation or alternative M2 activation. M1 macrophages, stimulated by TLR ligands and interferon-γ, are characterized by the release of pro-inflammation cytokines and high production of reactive nitrogen and oxygen intermediates. M2 macrophages, stimulated by interleukin 4 (IL-4) or IL-13, are characterized by the release of IL-10, high expression of arginase 1 (Arg-1) and mannose receptors.[14] Macrophages of the M2 phenotype are considered to be anti-inflammatory cells and play critical roles in tissue remodeling.[15]

Studies have demonstrated that MSCs may affect the activation and function of macrophages *in vitro* and *in vivo* in both cell contact-dependent and -independent manners.[16–18] We have previously observed that human umbilical cord-derived MSCs promote the M2 polarization of macrophages at tissue injury sites, contributing to the protection from ischemia-reperfusion injury.[19] Although the MSCs–macrophages interaction has been suggested, the

underlying mechanism responsible for this interaction remains largely unknown.

In the present study, we determined the effects of mouse bone marrow-derived MSCs on mouse RAW264.7 cells and mouse splenic CD11b$^+$ cells and explored the signaling pathways responsible for these effects. We demonstrated that macrophages co-cultured with MSCs or treated with MSC-conditioned medium (MSC-CM) skewed towards an M2 phenotype under inflammatory condition. MSC-educated macrophages were characterized by decreased expression of tumor necrosis factor (TNF)-α and IL-6, and increased expression of Arg-1 and IL-10. In addition, MSC-educated macrophages displayed enhanced proliferation and migration abilities. Moreover, MSCs-induced M2 polarization of macrophages involved the regulation of nuclear factor-κB (NF-κB) and signal transducer and activator of transcription 3 (STAT3) pathways.

Materials and methods

Cell culture

Mouse MSCs were isolated and cultured using standard protocols.[3] Bone marrow cells from BALB/c mice were collected by flushing the femurs from six- to eight-week-old mice with phosphate-buffered saline (PBS) and then cultured in low glucose Dulbecco's modified Eagle's medium (LG-DMEM) containing 15% fetal bovine serum (FBS, Invitrogen, CA) at 37°C with 5% CO_2. All protocols of mice were approved by the Institutional Animal Care Committee of Jiangsu University. Culture medium was changed at day 4 to remove non-adherent cells and subsequently at three-day intervals. When cells reached 80–90% confluence, the adherent cells were trypsinized with 0.25% trypsin-EDTA (Invitrogen) and passaged into new flasks for further expansion. MSCs at passage 3–5 were used for the detection of immunophenotype and multipotent differentiation potentials. The mouse macrophage-like cell line RAW264.7 was purchased from Cells Resource Center of Shanghai Institutes for Biological Sciences, The Chinese Academy of Sciences and cultured in high glucose DMEM (HG-DMEM) containing 10% heat-inactivated FBS (Invitrogen).

Magnetic cell sorting for mouse splenic CD11b$^+$ cells

Mouse splenic mononuclear cells were obtained by Ficoll density gradient centrifugation (Chuan Ye Biochemical Products, Tianjin, China) from mice at 6–8 weeks. The isolated cells were incubated with phycoerythrin (PE) labeling reagent for 10 min, PE selection cocktail for 10 min and magnetic nanoparticles for 15 min, and the magnetic field was subsequently applied using Magnet (EasySep, Stem cell, USA) for the positive selection of CD11b cells. The purity of the CD11b$^+$ cells, as assessed by FACS analysis, was greater than 91%.

Conditioned medium from MSCs

For MSC-CM, MSCs were cultured in complete medium for 48 h. The supernatants were collected and spun down to remove possible cell debris (500 *g* for 10 min).

Co-culture of MSCs with macrophages

Two different co-culture methods were used: (1) RAW264.7 cells and MSCs were seeded into the lower and upper chambers of transwell plates in a 10:1 ratio (0.4 μm, pore size; Costar, USA). Cells were co-cultured for 24 h and then incubated with lipopolysaccharide (LPS; 1 μg/mL) for 6 or 18 h. (2) RAW264.7 cells were plated in the lower chambers of transwell plates in the presence or absence of LPS (1 μg/mL) for 4 h. The cells were then washed with PBS for three times. MSCs were added into the upper chambers or MSCs-CM were added into the cells. At 24 h after incubation, RAW264.7 cells were collected for further experiments. The LG-DMEM with 15% FBS diluted 1:1 with fresh medium to the macrophages was used as control groups. For enzyme-linked immunosorbent assay (ELISA), RAW264.7 cells were incubated with MSC-CM (1:1 in fresh medium). After 24 h, LPS was added to reach a final concentration of 1 μg/mL. The isolated CD11b$^+$ cells were co-cultured with MSCs or incubated with MSC-CM for 24 h, followed by incubation with or without LPS for 6 or 18 h. The cells and supernatants were collected and stored at −70°C until use.

Quantitative RT-PCR

Total RNA was extracted from RAW 264.7 cells and splenic CD11b$^+$ cells and reversely transcribed to cDNA by using a reverse transcription kit according to the manufacturer's instruction (Invitrogen). Quantitative RT-PCR was performed by using mouse-specific primers for *TNF-α*, *IL-10*, and *Arg-1* genes (Table 1). β-actin was used as the internal control. Reactions were performed by using SYBR Green PCR mix (Bioer Technology Company Limited, Hangzhou, China) in the CFX96^{TM} Real-Time System (Bio-Rad, CA).

ELISA

The levels of mouse IL-6 and IL-10 in the supernatants were evaluated by ELISA following the manufacturer's instructions (Excell Biology, Shanghai, China).

Table 1 Primer sequences of target genes

Genes	Primer sequence (5′–3′)	Amplicon size (bp)	Annealing temp. (°C)
TNF-α	For: AACTCCAGGCGGTGCCTATG	242	65
	Rev: TCCAGCTGCTCCTCCACTTG		
IL-10	For: ACTCTTCACCTGCTCCACTG	415	63
	Rev: GCTATGCTGCCTGCTCTTAC		
Arg-1	For: CCAGATGTACCAGGATTCTC	191	56
	Rev: AGCAGGTAGCTGAAGGTCTC		
IL-6	For: AAGTCCGGAGAGGAGACTTC	487	58
	Rev: TGGATGGTCTTGGTCCTTAG		
IL-1β	For: AGCTTCAGGCAGGCAGTATC	215	60
	Rev: TCATCTCGGAGCCTGTAGTG		
β-Actin	For: CACGAAACTACCTTCAACTCC	265	56
	Rev: CATACTCCTGCTTGCTGATC		

Flow cytometry

RAW264.7 cells were plated in six-well plates and incubated with MSC-CM or control medium for 48 h. Cells were stained with anti-CD206 antibody (1:200, Santa Cruz, Minneapolis, USA) at 4°C for 30 min, followed by incubation with FITC-labeled secondary antibody (Boster Bioengineering Company Limited, Wuhan, China) for another 30 min. The percentage of CD206-positive cells was determined by using flow cytometry.

Immunofluorescence staining

RAW264.7 cells were grown on glass cover lips with MSC-CM or control medium for 48 h. For immunofluorescence staining, the sections were incubated with anti-CD206 monoclonal antibody (1:50, Santa Cruz) at 4°C overnight and incubated with FITC-labeled secondary antibody at 37°C for 45 min. Cy3-labeled secondary antibody was used in this section (1:100, Jackson, USA). The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Beyotime Institute of Biotechnology, Shanghai, China). All images were obtained using a fluorescent microscope (ECLIPSE Ti-S, Nikon, Japan).

Cell proliferation assay

RAW264.7 cells were plated in 96-well plates (2000 cells in 100 μL medium) and incubated with 100 μL MSC-CM in the presence or absence of LPS (1 μg/mL). Control cells were treated with complete medium. MTT reagent (Aeresco, USA) was added into the cells at different time points (24, 48, and 72 h) and the cells were cultured for another 4 h. The optical density (OD) values were measured at 450 nm. For the cell counting assay, cells were seeded at a density of 10,000/well in 24-well plates and incubated with 250 μL MSC-CM in the presence or absence of LPS (1 μg/mL). The number of cells was counted at 24, 48, and 72 h after treatment, respectively.

Cell migration assay

A cell transwell migration assay was performed as previously described with slight modifications.[20] MSCs were plated at a density of 20,000/well in 24-well plates for 24 h. Then RAW264.7 cells (200,000/well) were added into the top chambers (8.0 μm, pore size; Costar, USA). LPS (1 μg/mL) was added into the system. At 8 h after incubation, cells in the top chambers were fixed with 4% paraformaldehyde and stained with crystal violet and then counted.

Western blot

RAW264.7 cells were incubated with MSC-CM for 48 h. After being stimulated with LPS for 1 h, fractionized proteins were extracted to detect NF-κB p65 protein expression. For STAT3 inhibition, RAW264.7 cells were incubated with MSC-CM for 2, 4, and 6 h in the presence or absence of 100 μmol/L S3I-201 (Sigma, USA). The total proteins lysates were then prepared. Equal amounts of proteins were electrophoresed on a 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, followed by immunoblotting as previously described.[21] The sources and dilution factors for primary antibodies were: NF-κB p65 (1:500, SAB, CA), NF-κB p-p65 (Ser529, 1:500, SAB), JAK1 (1:500, Bioworld), p-JAK1 (Y1022, 1:500, Bioworld), STAT3 (1:500, SAB) and p-STAT3 (Tyr705, 1:500, SAB). Histone H3.1 and β-actin were used as loading controls for nuclear and cytoplasmic proteins, respectively. The goat anti-rabbit secondary antibody was purchased from Bioworld (1:2000). The membranes were visualized by using Luminata^{TM} Crescendo Western HRP substrate (Millipore, USA).

Statistical analysis

All values were expressed as mean ± standard deviation (SD). Differences between groups were examined for statistical significance using Student's *t*-test and analysis of variance (ANOVA). A *P* value less than 0.05 was accepted as statistically significant. Statistical analysis was performed with Graph Pad Prism 5 (Graph Pad Software).

Results

MSCs induce the expression of M2 macrophage genes in RAW264.7 cells

The harvested mouse MSCs expressed CD29 and CD90, but negative for CD45 by FACS analysis, and they had ability to differentiate into osteocytes and adipocytes (Fig.S1A, B). To determine the effects of mouse MSCs on the expression of polarization-related genes in macrophages, RAW264.7 cells were co-cultured with MSCs for 24 h, followed by stimulation with LPS for different times (6 or 18 h). As shown in Figure 1A, the expression of *TNF-α* gene was reduced while the expression of *IL-10* and *Arg-1* genes was enhanced by co-culture with MSCs in a transwell system (MSC-TW + LPS groups). To confirm the ability of MSCs to modulate the gene profile in macrophages, RAW264.7 cells were stimulated with LPS for 4 h, followed by co-culture with MSCs in a transwell system or incubation with MSC-CM for additional 24 h. The results showed that MSC co-culture decreased the expression of TNF-α while increasing the expression of IL-10 and Arg-1 in LPS-stimulated macrophages (Figure 1B a, b, c). Similar effects were also observed in RAW264.7 cells that were incubated with MSC-CM (Figure 1B a, b, c). To further prove that MSCs induce the expression of M2 macrophage genes, we incubated LPS-stimulated RAW264.7 cells with MSC-CM and detected the levels of IL-6 and IL-10 by ELISA. As shown in Figure 1C a, the MSCs-CM reduced the production of IL-6, an important pro-inflammatory cytokine, in LPS-stimulated RAW264.7 cells. In contrast, MSC-CM increased the secretion of IL-10 in LPS-stimulated RAW264.7 cells (Figure 1C b).

MSCs induce M2 phenotype in mouse splenic CD11b$^+$ cells

To confirm that MSCs induce the change in phenotype in macrophages, we isolated macrophages from mouse spleen by positive selection with CD11b magnetic beads. The percentage of CD11b$^+$ cells was 11.3% in mouse splenic mononuclear cells and improved to 91.0% after sorting (Figure 2A). Consistent with observations in RAW264.7 cells, incubation with MSC-CM decreased the expression of TNF-α (Figure 2B a) but increased the expression of

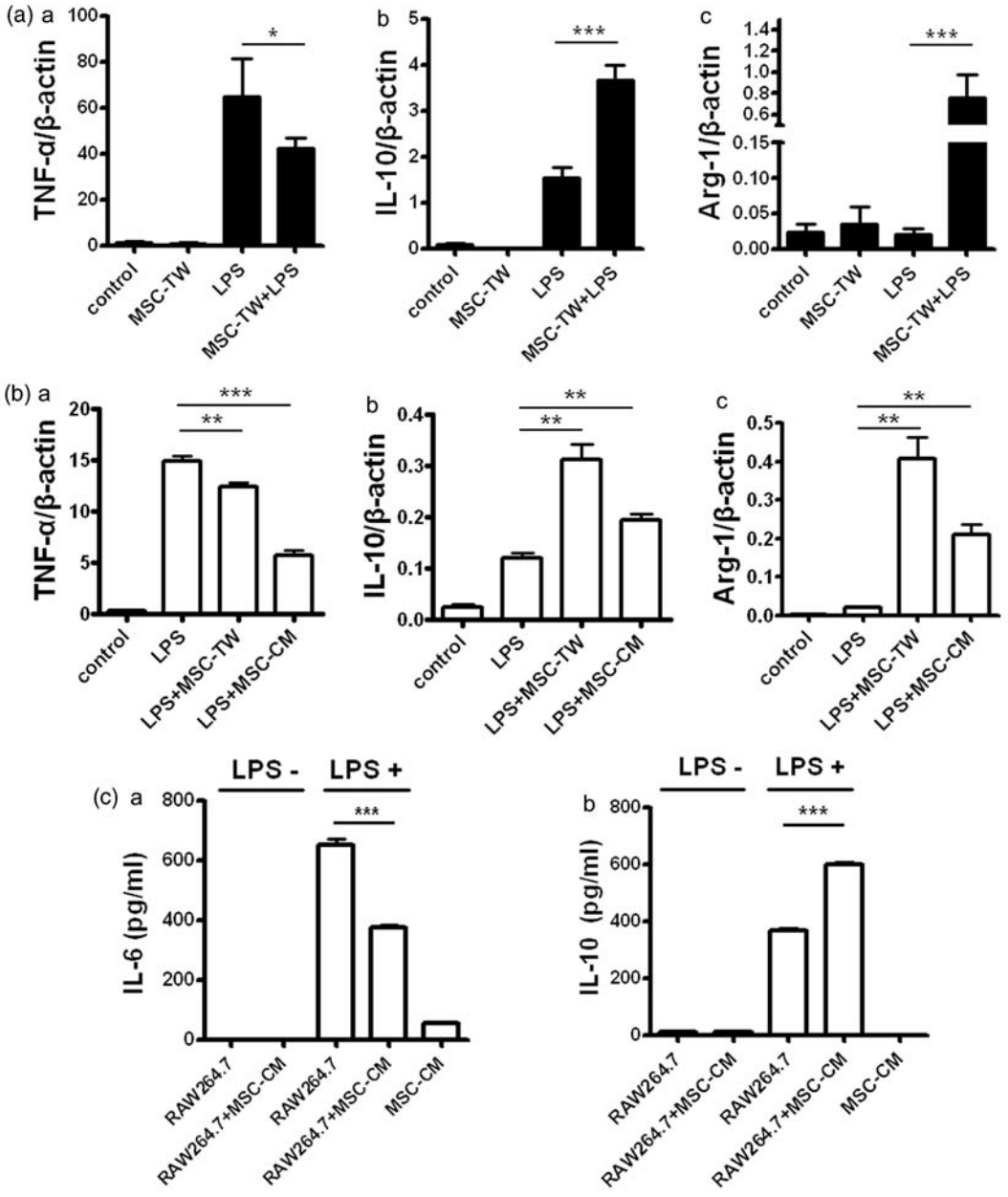

Figure 1 MSCs induce the expression of M2 macrophage genes in RAW264.7 cells. A. RAW264.7 cells were co-cultured with MSCs in a transwell system for 24 h, followed by incubation with LPS (1 μg/mL) for 6 h (TNF-α) or 18 h (Arg-1 and IL-10). The mRNA levels of TNF-α (a), IL-10 (b), and Arg-1 (c) were determined by qRT-PCR. B. RAW264.7 cells were stimulated with LPS (1 μg/mL) for 4 h. After washed with PBS, cells were co-cultured with MSCs in a transwell system or incubated with conditioned medium from MSCs (50% v/v). The mRNA levels of TNF-α (a), IL-10 (b), and Arg-1 (c) were examined by qRT-PCR. The results represent three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. C. RAW264.7 cells were incubated with MSC-CM for 24 h, followed by stimulation with LPS for 6 h (IL-6) or 18 h (IL-10). The levels of IL-6 (a) and IL-10 (b) in the supernatant from RAW264.7 cells were detected by ELISA. The results represent three independent experiments. $^{***}P < 0.001$. Arg-1: arginase 1; IL-10: interleukin 10; LPS: lipopolysaccharide; MSCs: mesenchymal stem cells; MSC-CM: MSC-conditioned medium; MSC-TW: MSC-transwell system; PBS: phosphate-buffered saline; TNF-α: tumor necrosis factor α

Arg-1 (Figure 2B b) in mouse splenic $CD11b^{+}$ cells (MΦ + MSC-CM + LPS groups). The production of IL-10 in mouse splenic $CD11b^{+}$ cells was also enhanced by co-culture with MSCs (Figure 2B c).

MSC-CM induces the expression of CD206 in RAW264.7 cells

To directly assess if MSCs induce an M2 phenotype in macrophages, we determined the expression of mannose receptors (CD206), a well-accepted marker for M2 macrophages,[22] in RAW264.7 cells by using flow cytometry. The results showed that incubation with MSC-CM greatly increased the percentage of CD206-positive macrophages (Figure 3A). Consistent with the results of flow cytometry, immunofluorescence staining also showed a significantly higher expression of CD206 in MSC-CM-treated RAW264.7 cells than in control cells (Figure 3B).

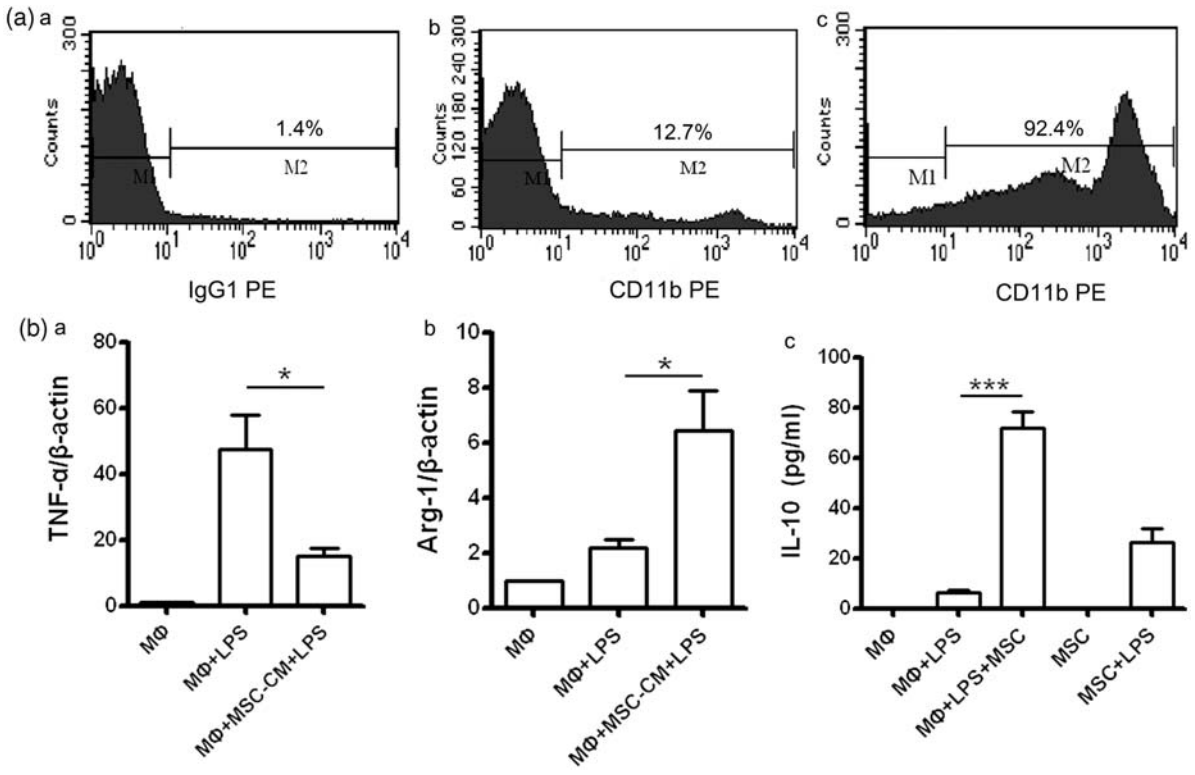

Figure 2 MSCs induce M2 phenotype in mouse splenic CD11b$^+$ cells. A. FACS analysis of the purity of mouse spleen splenic CD11b$^+$ cells. (a) PE isotype control; (b) PE-labeled cells before sorting; (c) PE-labeled cells after sorting. B. MSCs induce the expression of M2 macrophage genes in mouse splenic CD11b$^+$ cells. Mouse splenic CD11b$^+$ cells were incubated with MSC-CM (50% v/v) for 24 h, followed by stimulation with LPS (1 μg/mL) for 6 h. The mRNA levels of TNF-α (a) and Arg-1 (b) were examined by qRT-PCR. (c) The levels of IL-10 in co-culture supernatants were detected by ELISA. The results represent three independent experiments (mean ± SD). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Arg-1: arginase 1; FACS: fluorescence-activated cell sorting; LPS: lipopolysaccharide; MSC: mesenchymal stem cell; MSC-CM: MSC-conditioned medium; PE: phycoerythrin; TNF-α: tumor necrosis factor α

MSCs promote the proliferation and migration of RAW264.7 cells

To examine the biological effects of MSCs on macrophages, we determined the proliferation of RAW264.7 cells by using MTT and cell counting assays. RAW264.7 cells were incubated with control medium or MSC-CM (50% v/v) in the presence or absence of LPS for 24, 48, and 72 h. As shown in Figure 4A, the OD value in MSC-CM group was higher than that in control group at 48 and 72 h after treatment. To confirm that MSC-CM treatment affect cell proliferation, RAW264.7 cells were treated with control medium or MSC-CM in the presence or absence of LPS for 24, 48, and 72 h. The results of cell counting assay showed that at 48 and 72 h after treatment, there were more cells in MSC-CM group than that in control group (Figure 4A b). We next investigated the effect of MSCs on the migratory ability of macrophages by using a transwell migration assay. As shown in Figure 4B a, b, the number of migrated cells in the MSC group was markedly higher than that in the control group. In the presence of LPS stimulation, MSC co-culture also significantly induced the migration of RAW264.7 cells (Figure 4B c, d).

MSC-CM antagonizes LPS-induced activation of NF-κB p65 in macrophages

We next wanted to know the underlying mechanism responsible for the role of MSC-CM in the activation of M2 macrophages. RAW264.7 cells were treated with MSC-CM in the presence or absence of LPS and the nuclear and cytoplasmic proteins were extracted. The results of Western blot showed that at the resting state, NF-κB p65 proteins were mainly located in the cytoplasmic fraction. LPS stimulation for 1 h led to the nuclear translocation of NF-κB p65 and an increase of phosphorylated NF-κB p65. However, this effect was inhibited by MSC-CM treatment (Figure 5A). The induction of NF-κB p65 downstream targets IL-1β (Figure 5B a) and TNF-α (Figure 5B b) by LPS was also suppressed by MSC-CM.

MSC-CM activates STAT3 pathway to induce M2 phenotype

To further demonstrate the signaling pathways involved in the activation of M2 macrophage by MSCs, RAW264.7 cells were incubated with MSC-CM for different times. The results of Western blot showed that the expression of p-JAK1 and p-STAT3 was markedly increased at 2 h after treatment (Figure 6A). To determine whether the activation of STAT3 is responsible for the induction of polarization by MSCs, RAW264.7 cells were incubated with MSC-CM with or without STAT3 inhibitor under normal and LPS-stimulated conditions. As shown in Figure 6B, STAT3 inhibitor S3I-201 inhibited the induction of p-STAT3 by MSC-CM. In addition, S3I-201 also inhibited the upregulation of IL-10 and Arg-1 by MSC-CM (Figure 6C).

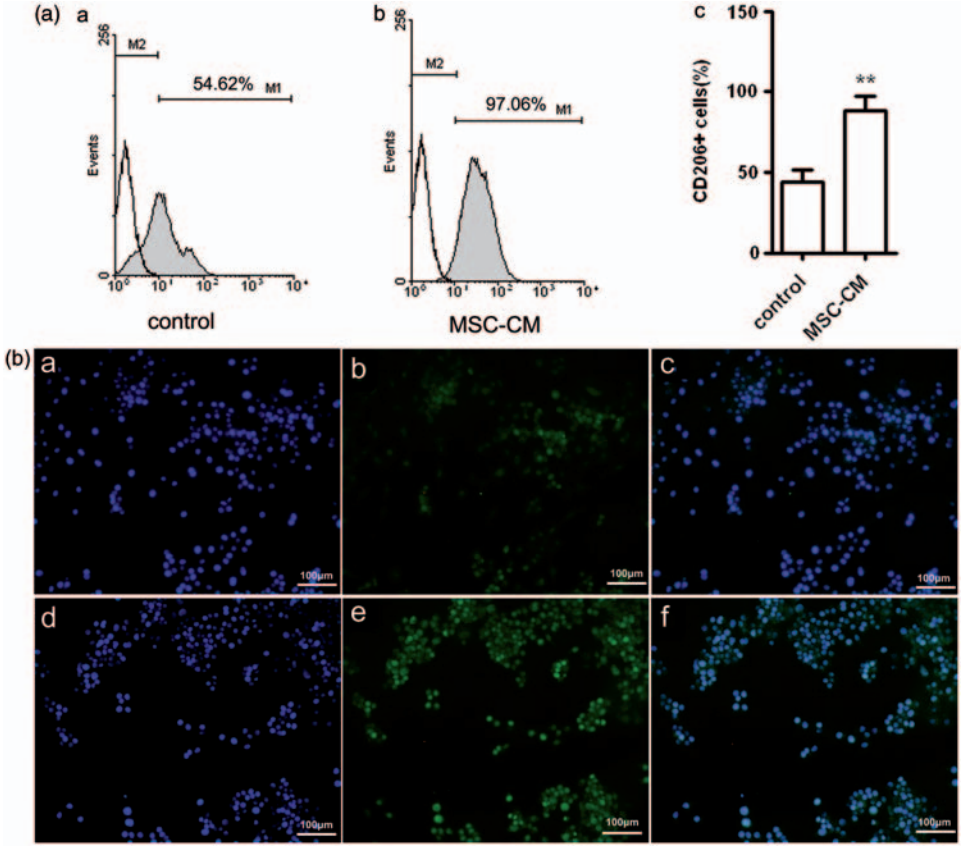

Figure 3 MSC-CM improves the expression of CD206 in RAW264.7 cells. A. RAW264.7 cells were untreated (a) or incubated with MSC-CM (b) for 48 h, then stained with CD206 primary antibody and FITC-labeled secondary antibody. The percentage of CD206-positive cells was analyzed by flow cytometry. The mean value from three independent experiments was shown in (c). $^{**}P < 0.01$. B. Immunofluorescent staining of CD206. RAW264.7 cells were untreated (a, b, c) or incubated with MSC-CM for 48 h (d, e, f). The expression of CD206 in RAW264.7 cells were determined by immunofluorescent staining. Cell nuclei were counterstained with DAPI. DAPI: 4',6-diamidino-2-phenylindole; FITC: fluorescein isothiocyanate; MSC-CM: mesenchymal stem cell-conditioned medium. (A color version of this figure is available in the online journal)

The increase of CD206-positive macrophages by MSC-CM was also suppressed by S3I-201 (Figure 6D). In summary, these data suggest that MSC-CM activates STAT3 pathway to induce M2 phenotype in RAW264.7 cells.

Discussion

In this study, we investigated the effects of MSCs on macrophage activation and explored the potential mechanism of action. We found that MSCs induced the expression of *M2* genes in macrophages in a cell contact-independent manner, which is consistent with the results from Maggini et al.[23] We also demonstrated that MSCs induced M2 activation in mouse splenic $CD11b^+$ cells. MSC-CM is strongly chemoattractive to monocytes *in vitro* and increases the number of macrophages during wound healing.[20] The results from Dayan et al. suggest that infusing MSCs into acute myocardial infarction mice significantly increase the number of macrophages in the circulation and heart, which may be associated with enhanced cardiac function.[17] In our study, we demonstrated that the MSC-CM promoted the proliferation and migration of RAW264.7 cells in the presence or absence of LPS stimulation, indicating that in response to an inflammation microenvironment, MSCs derived bioactive molecules could recruit macrophages, as well as induce their phenotype switch to favor the alleviation of inflammation.

We further explored the mechanism by which MSCs mediated macrophage polarization. A predominant activation of NF-κB and STAT1 promotes M1 macrophage polarization while STAT3 and STAT6 activation promotes M2 macrophage polarization, resulting in immune suppression.[24] Recent studies have provided a preliminary molecular basis for MSC–macrophage interaction. MSCs-derived prostaglandin E2 (PGE2) interacts with the EP4 receptor on macrophages, increasing the secretion of IL-10 in macrophages and reducing inflammation.[12,25] Activated MSCs produce anti-inflammatory protein TSG-6 and attenuate zymosan-induced mouse peritonitis by decreasing TLR2/NF-κB signaling in resident macrophages.[26] NF-κB activation is associated with the macrophage M1 phenotype and production of inflammatory mediators,[27] and the p65 member (RelA) is critical for this process.[24] Therefore, we explored whether MSC might affect the NF-κB signaling pathway in macrophages. Zhang et al. suggest that co-culture with MSCs inhibits LPS-induced upregulation of NF-κB p50 in THP-1 cells.[18] In our study, we found that MSC-CM inhibited NF-κB p65 nucleolar translocation in response to LPS stimulation, which may partially explain the down-regulation of M1 macrophage-related genes after

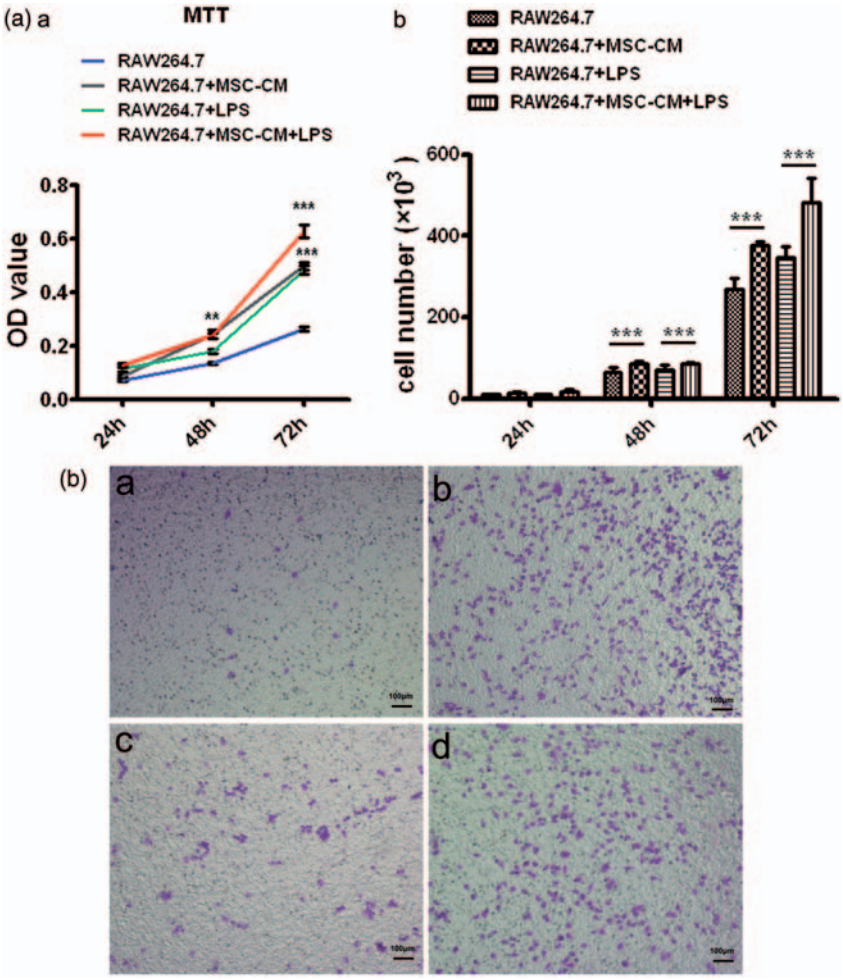

Figure 4 MSCs promote the proliferation and migration of RAW264.7 cells. A. MSC-CM promotes the proliferation of RAW264.7 cells. RAW264.7 cells were treated with MSC-CM for 24, 48, or 72 h. (a) and cell counting assays (b) were used to determine the proliferation of RAW264.7 cells. The results represent three independent experiments. $^{**}P < 0.01$; $^{***}P < 0.001$. B. MSCs enhance the migration of RAW264.7 cells. RAW264.7 cells were seeded into the top chamber and induced for migration by MSCs. Factors in the low chambers are control medium (a), MSCs (b), control medium with LPS (c), and MSCs stimulated with LPS (d), respectively. The migrated cells were photographed and counted. LPS: lipopolysaccharide; MSCs: mesenchymal stem cells; MSC-CM: MSC-conditioned medium. (A color version of this figure is available in the online journal)

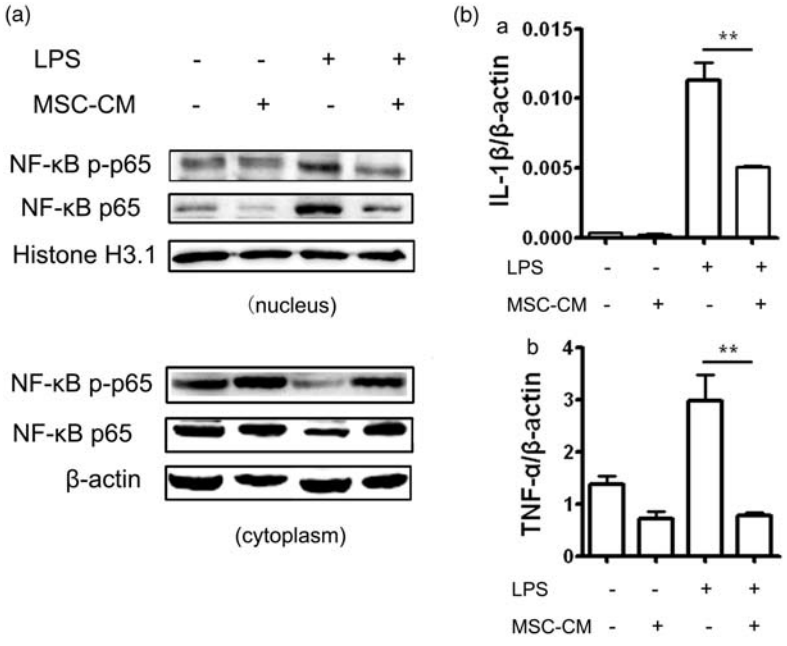

Figure 5 MSC-CM inhibits LPS-induced activation of NF-κB p65 in RAW264.7 cells. A. RAW264.7 cells were incubated with MSC-CM for 48 h, followed by stimulation with LPS (1 μg/mL) for 1 h. The levels of NF-κB p65 and p-NF-κB p65 (Ser 529) in the nucleus and cytoplasm of RAW264.7 cells were detected by using Western blot. Histone H3.1 and β-actin were used as loading control for cell nuclear and cytoplasmic proteins, respectively. B. RAW264.7 cells were incubated with MSC-CM for 48 h, followed by stimulation with LPS (1 μg/mL) for 6 h. The mRNA levels of IL-1β and TNF-α were determined by using qRT-PCR. IL-1β: interleukin 1β; LPS: lipopolysaccharide; MSC-CM: mesenchymal stem cell-conditioned medium; NF-κB: nuclear factor κB; TNF-α: tumor necrosis factor α

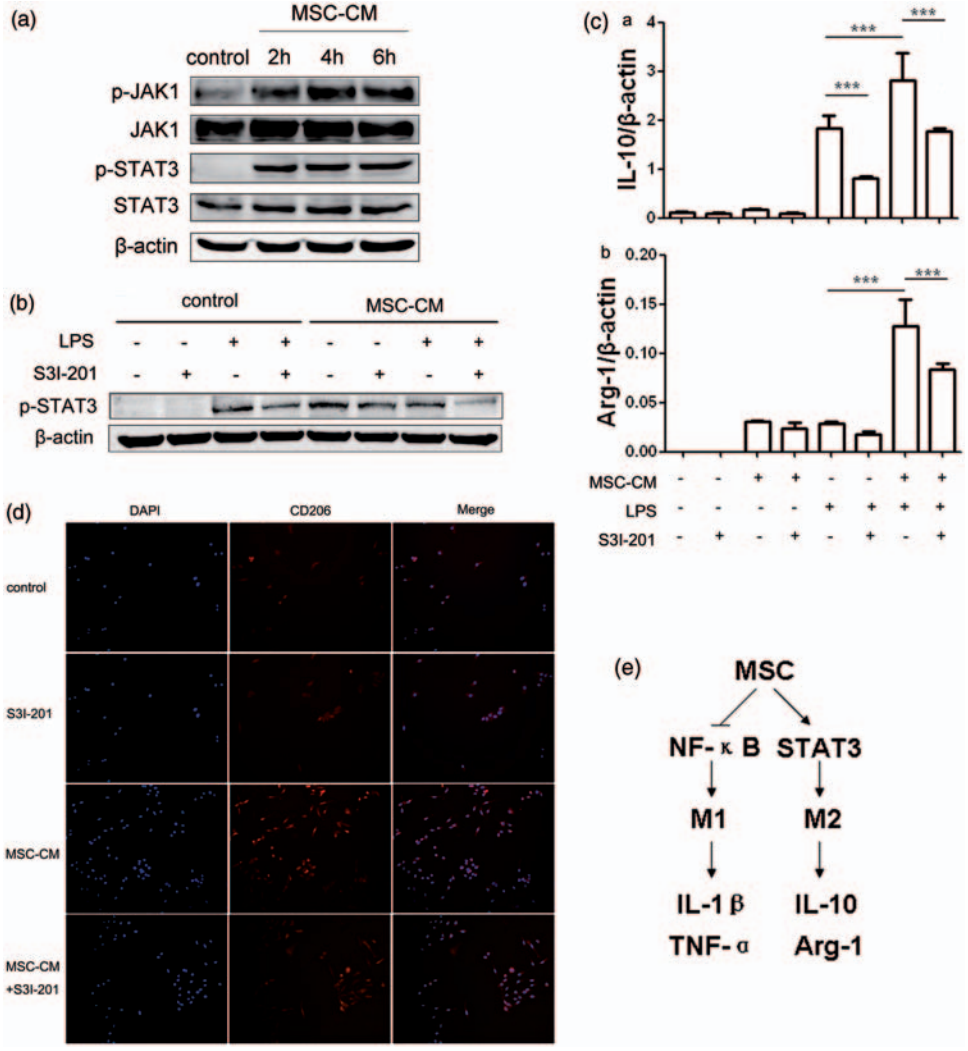

Figure 6 MSCs induce M2 polarization through the activation of STAT3 pathway. A. RAW264.7 cells were cultured with MSC-CM or control medium for 2, 4, and 6 h. The protein levels of JAK1, p-JAK1 (Y1022), STAT3, and p-STAT3 (Tyr 705) were examined by using Western blot. B. RAW264.7 cells were cultured with MSC-CM or control medium for 48 h in the presence or absence of S3I-201 (100 μmol/L). RAW264.7 cells were then incubated with LPS (0.1 μg/mL) for 4 h and the levels of p-STAT3 (Tyr 705) were examined by using Western blot. C. RAW264.7 cells were cultured with MSC-CM or control medium for 24 h in the presence or absence of S3I-201 (100 μmol/L). RAW264.7 cells were then incubated with LPS (0.1 μg/mL) for 18 h, and the mRNA levels of IL-10 and Arg-1 were determined by using qRT-PCR. D. RAW264.7 cells were cultured with MSC-CM or control medium for 48 h in the presence or absence of S3I-201 (100 μmol/L). The expression of CD206 in RAW264.7 cells was determined by using immunofluorescent staining. Cell nuclei were counterstained with DAPI. E. The proposed model for MSC in macrophage M2 polarization upon LPS-induced inflammation. MSC inhibits the translocation of NF-κB p65, inhibits macrophage M1 polarization, and decreases the expression of IL-1β and TNF-α. On the other hand, MSC induces the activation of STAT3, results in macrophage M2 polarization and increases the expression of IL-10 and Arg-1. Arg-1: arginase 1; DAPI: 4',6-diamidino-2-phenylindole; IL-10: interleukin 10; LPS: lipopolysaccharide; MSCs: mesenchymal stem cells; MSC-CM: MSC-conditioned medium; NF-κB: nuclear factor κB. (A color version of this figure is available in the online journal)

MSC-CM treatment. To further demonstrate whether MSCs modulate other signaling pathways for M2 polarization, we determined the effect of MSC-CM on STAT3, which is known to play essential roles in maintaining the homeostatic balance between pro- and anti-inflammatory signals in immune competent cells.[28] Lang et al. suggest that a predominance of STAT3 activation results in M2 macrophage polarization.[29] In our study, the level of phosphorylated JAK1 and STAT3 was markedly increased by MSC-CM treatment in RAW264.7 cells. We further confirmed that STAT3 inhibitor S3I-201 antagonized the activation of STAT3 and induction of IL-10, Arg-1, and CD206 expression by MSC-CM in RAW264.7 cells, suggesting the activation of STAT3 pathway is involved in MSC-CM-induced macrophage M2 polarization. However, the molecular mechanism for macrophage polarization is complicated, and further studies are needed to explore if the STAT3 pathway is of predominance in this setting. Overall, soluble factors released by MSCs induce the development of M2 phenotype in macrophages through the inhibition of NF-κB and activation of STAT3 pathways (Figure 6E).

Macrophages from different species, individual, and tissue are heterogeneous in morphology, phenotype, and function.[30] Rat MSCs ameliorate experimental peritoneal fibrosis by suppressing transforming growth factor (TGF)-β1 signaling in a paracrine manner.[31] Human bone

marrow MSCs suppress the secretion of TNF-α in THP-1 cells through CD200.[32] In this study, we used mouse-derived macrophages and bone marrow-derived MSCs to study the effect of MSCs on macrophage activation. It is interesting to investigate whether human MSCs regulate macrophage activation through the similar mechanisms in future studies. In addition, it is not clear whether MSCs affect the invasion of macrophages although we demonstrated that MSCs enhanced the migration of macrophages. Therefore, it will be better to model the tissue environment through both the migration and invasion assays.

In conclusion, we demonstrate that mouse bone marrow derived MSCs skewed macrophages towards an M2 phenotype in a cell contact-independent manner. MSCs exerted this effect through the inhibition of NF-κB p65 and the activation of STAT3 pathways. Our results not only suggest the regulation of macrophage polarization by MSCs, but also shed light on the mechanism for the anti-inflammatory ability of MSCs in tissue injury and infection.

Author contributions: SG and FM performed most of the experiments, analyzed the data, and wrote the paper; BZ, LZ, YY, TY, and JZ performed the experiments; XZ, MW, and WZ contributed to analyze the data and write the paper; WX and HQ designed the experiments, analyzed the data, revised the paper critically, and oversaw the research project.

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