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*Original Article*

# The synergetic effects of interferon-γ and lipopolysaccharide on the phenotype, proliferation, and cytokine production of mouse bone marrow mesenchymal stem cells

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**Abstract:** The characteristics of mesenchymal stem cells (MSC) are regulated by the microenvironment. Also, interferon-γ (IFN-γ) and lipopolysaccharides (LPS) regulate the MSC. However, the synergetic effects of IFN-γ and LPS on MSC are yet to be elucidated. In this study, IFN-γ (20 ng/mL) and LPS (1 μg/mL) were used to stimulate mouse bone marrow (mBM)-MSC. The phenotype of mBM-MSC was analyzed using flow cytometry, and the proliferation of mBM-MSC was measured using an MTT assay. The secretion of interleukin-6 (IL-6) and the chemokine (C-X-C motif) ligand 1 (CXCL1) was analyzed using ELISA. The production of nitric oxide (NO) was measured by the kit, and the expression of iNOS (inducible nitric oxide synthase) was analyzed by qRT-PCR. After treatment with IFN-γ and LPS, the expressions of CD106 and CD54 were increased. However, IFN-γ reduced the proliferation of LPS-induced mBM-MSC. Furthermore, IFN-γ and LPS played a synergistic role in enhancing the IL-6, CXCL1, and NO secretions of mBM-MSC. IFN-γ induced the phosphorylation of STAT1 in mBM-MSC, and LPS enhanced the phosphorylation of p65. The phosphorylation of STAT1 and p65 exert the regulatory effects of IFN-γ + LPS on mBM-MSC. In conclusion, the current study shows that IFN-γ and LPS together regulate the phenotype, proliferation, and cytokine production of MSC. These finding provide an in-depth insight into the changes of MSC in infectious diseases.

**Keywords:** Interferon-γ, lipopolysaccharide, mesenchymal stem cells

## Introduction

Mesenchymal stem cells (MSC) proliferate in culture flasks in vitro and can differentiate into the cells of the mesoderm, such as osteoblasts, adipocytes, and chondrocytes, under specific conditions [1]. Thus, MSC are promising tools in regeneration medicine. The characteristics of MSC can be regulated by the microenvironment, and several cytokines and stimulants can also affect the MSC.

Interferon-γ (IFN-γ) is one of the major cytokines for Th1, regulating the Th1-related immune responses. IFN-γ binding to the receptor activates the JAK-STAT pathway [2] and regulates the expression of several immune-related genes. The stimulation of IFN-γ-mediated proliferation of MSC is inhibited [3]. Long-term IFN-γ treatment induces the premature senescence of MSC [3]. However, the IFN-γ treatment of MSC does not alter the basic phenotype or the expression of CD73, CD90, or CD105, but the expression of CD106 and CD54 is increased [4]. MSC possess the function of immune regulation and inhibit the secretion of the IFN-γ of T cells and NK cells. The immunoregulatory activity of MSC requires the activation of IFN-γ [5, 6]. In mice MSC, IFN-γ cooperates with TNF-α/IL-1β and induces the release of nitric oxide (NO) to regulate the immune response [7].

Lipopolysaccharide (LPS) is the main component of the surface of gram-negative bacteria, which is identified by the toll-like receptor 4 (TLR4); consequently, the downstream NF-κB pathway is activated. LPS increases the proliferation ability of mouse bone marrow MSC

(mBM-MSC) and promotes their osteogenic differentiation ability [8, 9]. Also, LPS increases the ability of IL-6 secretion by MSC [8], and LPS-pretreated MSC enhance the immune regulation ability of MSC [10].

After infection, IFN-γ together with LPS can regulate the MSC; however, the changes in MSC are yet to be elucidated. In this study, we treated MSC with IFN-γ and LPS in vitro and studied the changes in the phenotype, proliferation, and cytokine secretions of MSC.

## Materials and methods

### *Generation of mBM-MSC*

The experimental procedures used in this study were approved by the Ethics Committee of Zhejiang Hospital. The mBM-MSC were isolated and expanded as described previously [3]. The bone marrow cells were collected from 6-10-week-old C57BL/6 mice by flushing their femurs and tibias and seeding them at a density of $10^6/cm^2$. The MSC were isolated using a MesenCult™ Proliferation Kit (Stemcell Technologies, Vancouver, Canada). After 3 days, the non-adherent cells cultured at 37°C in an atmosphere maintaining 5% $CO_2$ at 80% confluency were harvested from passages 7-12.

### *Flow cytometric analysis*

The phenotypes of mouse BM-MSC were analyzed using the following antibodies: PE-conjugated CD29, CD34, CD45, CD54, CD106 and CD117. Non-specific isotype-matched antibodies served as controls. All the antibodies were purchased from eBioscience (San Diego, CA, USA). The cells were analyzed by flow cytometry in a Beckman Coulter FC 500, and the data were analyzed using FlowJo software (FlowJo LLC, Ashland, OR, USA).

### *Proliferation assay*

For the cell proliferation assay, mouse BM-MSC were seeded at a density of $10^3$ cells/well in 0.1 mL DMEM with 10% FBS in 96-well plates. IFN-γ (Peprotech, Rocky Hill, NJ, USA) and LPS (Sigma, St. Louis, MO, USA) were used at a final concentration of 20 ng/mL and 1 μg/mL, respectively. After 3 days, the cells were analyzed by MTT assay (Sigma).

### *Enzyme-linked immunosorbent assay*

mBM-MSC were isolated and seeded at a density of $3 \times 10^4$ cells/well in 0.5 mL DMEM with 10% FBS in 24-well plates. IFN-γ and LPS were used at final concentrations of 20 ng/mL and 1 μg/mL, respectively. The cell-free supernatants were collected after 24 hours and stored at -80°C. IL-6 ELISA kits were purchased from eBioscience, and CXCL1 ELISA kits were purchased from Peprotech (Rocky Hill, NJ, USA). ELISA was performed according to the manufacturer's instructions. Briefly, the plate was coated with capture antibody overnight and incubated with a blocking buffer for 1 h. Then, the cell culture supernatants were added to the plate for 2 h, followed by detection antibody and incubation for 1 h. Avidin-HRP was added, and the plate was incubated for 30 min. 1X TMB solution was added for 15 min, and the reaction was stopped by 1 M $H_2SO_4$. The absorbance was quantified at 450 nm using a microplate reader.

### *NO detection*

Cell-free supernatants were used for the detection of NO using the kit (Beyotime Biotechnology, Shanghai, China), following the supplier's instructions. Briefly, cell culture supernatants were mixed with 50 μL Griess Reagent I in each well in a 96-well plate. Then, 50 μL of Griess Reagent II was added, and the absorbance was measured at 540 nm by a microplate reader.

### *Real-time reverse transcription polymerase chain reaction*

The mBM-MSC were isolated and seeded at a density of $1 \times 10^5$ cells/well in a 6-well plate. IFN-γ and LPS were used at a final concertation of 20 ng/mL and 1 μg/mL, respectively. Total RNA was extracted from isolated MSC using a miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription was carried out using a TOYOBO ReverTra Ace qPCR RT Kit for 60 min at 37°C (Toyobo Co., Ltd, Osaka, Japan) in the presence of oligo-dT primer. Real-time polymerase chain reaction analyses were carried out using TOYOBO SYBR® Green Real-time PCR Master Mix. The expression of the target genes was normalized to that of *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase). The primers used were listed as follows: *GAPDH*: for-

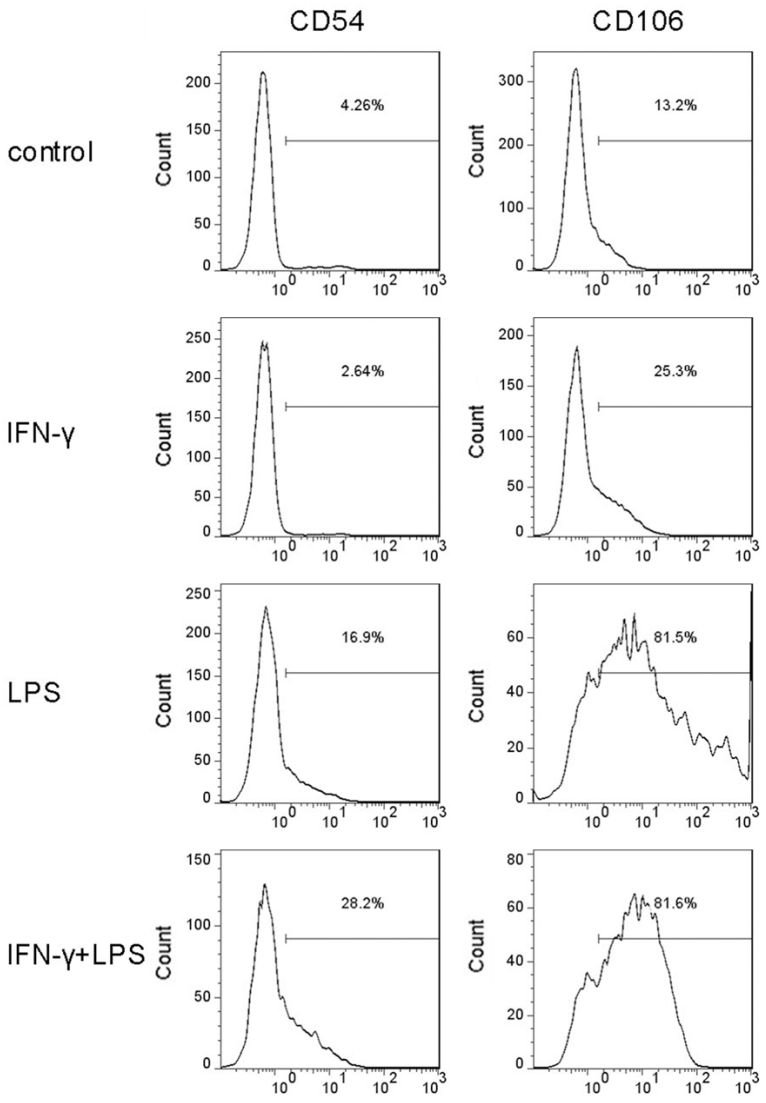


**Figure 1.** IFN-γ and LPS regulate the phenotype of mBM-MSC. mBM-MSC were cultured with IFN-γ (20 ng/mL) and/or LPS (1 μg/mL) for 24 h. The cells were analyzed by flow cytometry in a Beckman Coulter FC 500, and the data were analyzed using FlowJo software.

ward primer: 5'-ATCAACGACCCCTTCATTGACC-3', reverse primer: 5'-CCAGTAGACTCCACGAC-ATACTCAGC-3'; *iNOS* (inducible nitric oxide synthase) forward primer: 5'-CAGCTGGGCTGTAC-AAACCTT-3', reverse primer: 5'-CATTGGAAGT-GAAGCGTTTCG-3'.

*Western blot*

The mBM-MSC were isolated and seeded at a density of $1 \times 10^5$ cells/well in a 6-well plate. IFN-γ and LPS were used at final concertation of 20 ng/mL and 1 μg/mL, respectively. The cells were lysed in a lysis buffer (20 mM Tris (pH 8.0), 1% Triton X-100, 0.1% $NaDodSO_4$, 150 mM NaCl, 1 mM EDTA, 2.5 mM sodium pyrophosphate, 1 mM PMSF, and protease inhibitor mixture). The proteins were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Roche), followed by overnight blocking with 5% nonfat milk at 4°C. Then, the blots were probed with rabbit anti-p-STAT1, anti-STAT1, anti-p-p65, anti-p65 or GAPDH primary antibodies and HRP-conjugated goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The immunoreactive bands were visualized by ECL according to the manufacturer's protocol.

*Statistical analysis*

The data were analyzed using the GraphPad Prism software (San Diego, CA, USA). Data are presented as the mean ± SD. ANOVA with Tukey's multiple comparisons test were used to determine the statistical significance at $P < 0.05$.

## Results

*Regulation of the BM-MSC phenotype by IFN-γ and LPS*

The regulatory effects of IFN-γ and LPS on the surface markers of mBM-MSC were examined. As shown in **Figure 1**, IFN-γ (20 ng/mL) and LPS (1 μg/mL) did not alter the expression of the classical markers, CD29, CD34, and CD45. Both IFN-γ or LPS increased the expression of CD106, while only the LPS-treated group increased the level of CD54 slightly. After treatment with IFN-γ and LPS, the expressions of CD106 and CD54 increased. Thus, IFN-γ and LPS regulated the MSC adhesion molecules.

*Regulation of proliferation of BM-MSC by IFN-γ and LPS*

Consistent with other studies, the proliferation ability of MSC in the IFN-γ group decreased ($P <$

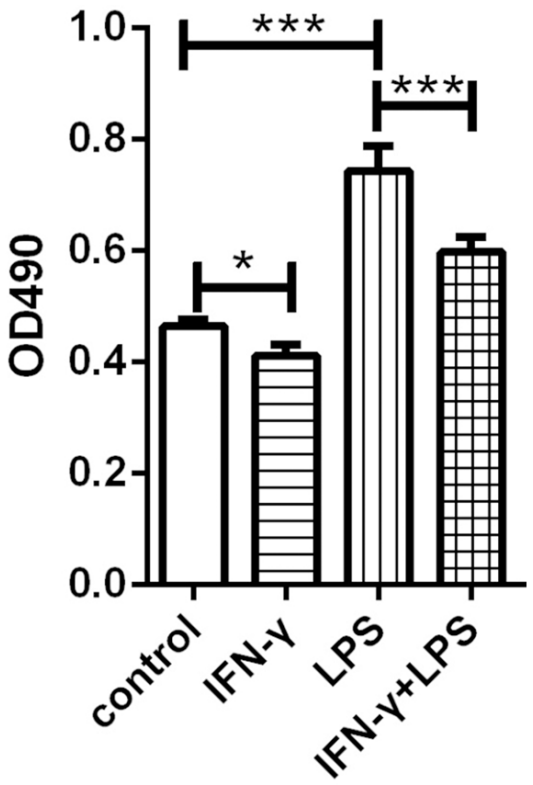


**Figure 2.** IFN-γ and LPS regulate the proliferation of mBM-MSC. mBM-MSC were cultured with IFN-γ (20 ng/mL) and/or LPS (1 μg/mL) for 72 h. The cell viability was assessed by MTT. ($^{*}P < 0.05$, $^{***}P < 0.001$).

0.05), but the proliferation ability of the LPS group increased ($P < 0.001$). The proliferation ability of the IFN-γ and LPS groups was lower than the proliferation ability of LPS ($P < 0.001$), indicating that IFN-γ reduces the proliferation of LPS-induced MSC (**Figure 2**).

*Regulation of IL-6 and CXCL1 secretion by IFN-γ and LPS in BM-MSC*

IL-6 and CXCL1 are critical cytokines secreted by MSC, and the changes in these two cytokines after IFN-γ and LPS treatment were measured. LPS significantly increased the secretion of IL-6 and CXCL1 in MSC. IFN-γ could not markedly increase the expression of IL-6 and CXCL1. However, the addition of IFN-γ in the presence of LPS increased the level of IL-6 ($P < 0.001$) and CXCL1 ($P < 0.001$) (**Figure 3**).

*Regulation of NO production by IFN-γ and LPS in BM-MSC*

NO is a vital immunoregulatory molecule of MSC in mice. Herein, we studied the regulation of IFN-γ and LPS on NO. IFN-γ or LPS alone did not significantly increase the secretion of NO. Next, the expression of NO was detected when both IFN-γ and LPS were added (**Figure 4A**). Similarly, the expression of iNOS also required the existence of LPS and IFN-γ (**Figure 4B**).

*Effects of IFN-γ and LPS on the phosphorylation of STAT1 and p65 in BM-MSC*

STAT1 and NF-κB pathways are critical for cytokine production. Furthermore, p-STAT1, STAT1, p-p65, and p65 were detected by Western blot. As shown in **Figure 5**, IFN-γ induces the phosphorylation of STAT1, but LPS showed little effect on STAT1. Moreover, LPS enhanced the phosphorylation of p65, and the effects of IFN-γ on p-p65 were not distinct.

## Discussion

In the present study, the regulatory effects of IFN-γ and LPS on BM-MSC in mice were studied. IFN-γ and LPS regulate the expressions of CD106 and CD54, which exert synergistic effects on the induction of IL-6, CXCL1, and NO in MSC. IFN-γ and LPS antagonize each other while regulating the proliferation of MSC. Together with TNF-α/IL-1β, IFN-γ can regulate the expression of NO, cytokines, and chemotactic factors [7, 11], which exerts a critical role in the regulation of the immune function of mouse MSC. IFN-γ + LPS have similar effects, suggesting that it might activate the immunoregulatory function of MSC, without the synergistic effect of other inflammatory cytokines.

LPS and IFN-γ induce the polarization of M1 macrophages. The M1 macrophages secrete the inflammatory cytokines and chemokines, present the antigens, participate in pro-inflammatory responses, and play a central role in host defense against bacterial and viral infections [12, 13]. LPS and IFN-γ might lead to a strong inflammatory response after infection in the body, resulting in organ damage. LPS and IFN-γ can simultaneously activate the MSC and exert immune regulation, which in turn, controls the inflammatory reaction and maintains homeostasis.

The effects of LPS on the secretion of IL-6 and CXCL1 are stronger than the effect of IFN-γ, but the effect of LPS on these two cytokines can be enhanced by IFN-γ. The enhanced expression

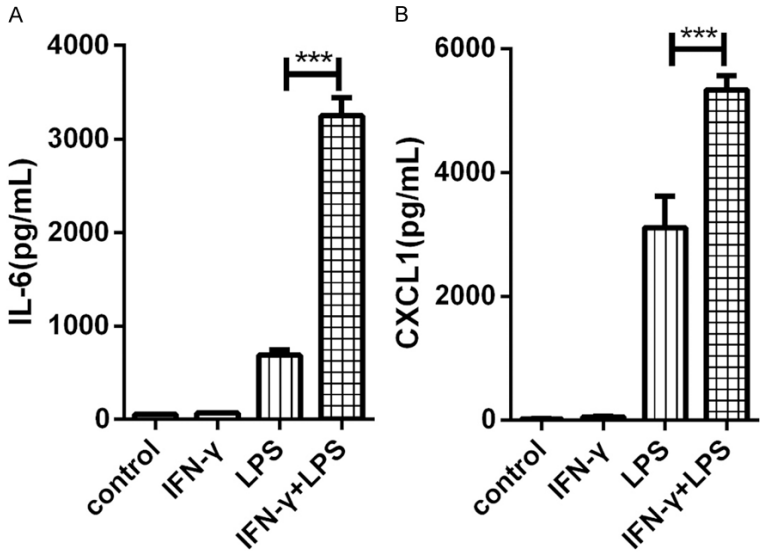


**Figure 3.** IFN-γ and LPS regulated the secretion of IL-6 and CXCL1 by mBM-MSC. mBM-MSC were cultured with IFN-γ (20 ng/mL) and/or LPS (1 μg/mL). The culture media were collected after 24 h, and the concentrations of IL-6 (A) and CXCL1 (B) were tested by ELISA (***P < 0.001).

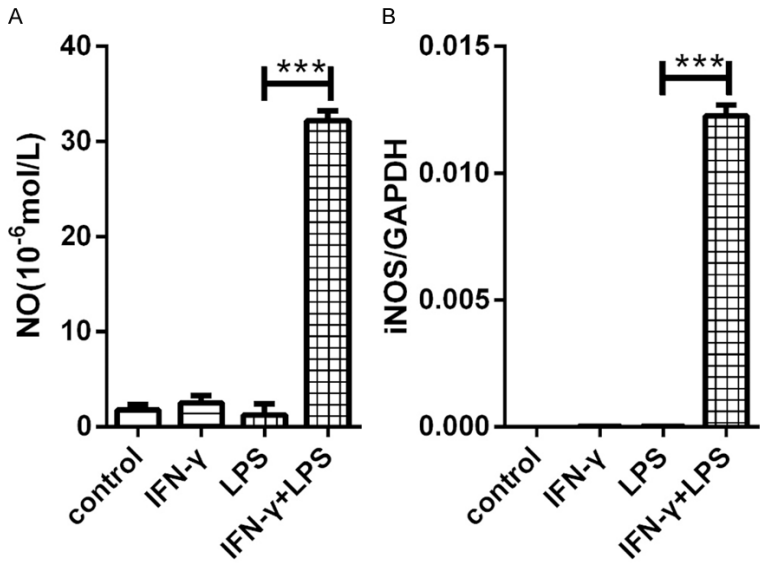


**Figure 4.** IFN-γ and LPS regulate the NO secretion of mBM-MSC. mBM-MSC were cultured with IFN-γ (20 ng/mL) and/or LPS (1 μg/mL). A. Culture media were collected after 24 h, and NO was detected. B. The cells were isolated after 24 h, and the relative expression of iNOS was measured by qPCR (***P < 0.001).

of NO requires the addition of IFN-γ and LPS, indicating their importance in inducing NO. The different regulatory modes of NO and IL-6 reflect the precise response of MSC to external stimuli. In BM-MSC, IFN-γ induces the phosphorylation of STAT1, and LPS enhances the phosphorylation of p65. Thus, both STAT1 and NF-κB have been shown to regulate CXCL1 and NO [14, 15]. The synergetic effects of IFN-γ and LPS are attributed to the activation of the STAT1 and NF-κB pathways.

MSC can be regulated by other cytokines such as TNF-α [16, 17], IL-1β [18], IL-10 [19], and IL-17 [20, 21]. In addition, these cytokines and LPS might exhibit synergetic effects in MSC after infection. Thus, further studies are required to investigate the synergistic effects of these cytokines and LPS.

In conclusion, the current study showed that IFN-γ and LPS regulate the phenotype, proliferation, and cytokine production of MSC. These findings provide an in-depth insight into the changes in MSC in infectious diseases, thereby providing a theoretical basis for the application of these cells in infectious diseases.

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**Disclosure of conflict of interest**

None.

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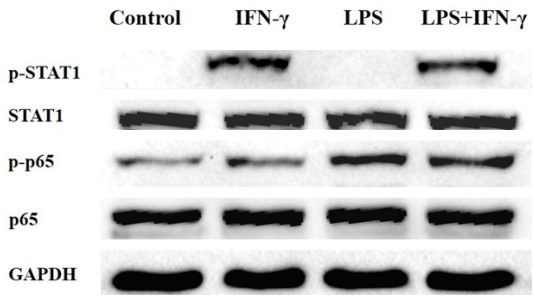


**Figure 5.** IFN-γ and LPS regulate the phosphorylation of STAT1 and p65 in BM-MSC. mBM-MSC were cultured with IFN-γ (20 ng/mL) and/or LPS (1 μg/mL). The expressions of p-STAT1, STAT1, p-p65, p65, and GAPDH was detected by Western blot.

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