Human placental mesenchymal stem cells regulate inflammation via the NF-κB signaling pathway

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Abstract. Emerging evidence has indicated that mesenchymal stem cells (MSCs) are involved in the modulation of inflammation. Human placenta-derived (HPL)-MSCs exist in sufficient quantities and play a role in immune regulation. However, the exact roles of HPL-MSCs in inflammation and the specific underlying mechanisms are not well defined. In the present study, HPL-MSCs were obtained from human fetal placentas, and further purified using a commercial kit. Using ELISA, reverse transcription-quantitative PCR, western blot, NO detection and other assays, the present study revealed that HPL-MSCs may improve lipopolysaccharide-induced macrophage inflammation by regulating macrophage polarization. Further mechanistic studies demonstrated that HPL-MSCs attenuated the NF-κB signaling pathway by regulating the expression of toll-like receptor 4 and the phosphorylation of IκBα and p65, which resulted in a reduction in the levels of inflammation. The present study indicated that HPL-MSCs may act as a novel target for the treatment of inflammation-related diseases.

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Introduction

Inflammation is an adaptive response to infection or tissue damage, and is considered to be a mechanism of immune defense and repair. However, when inflammation becomes chronic or lasts for a prolonged period of time, it contributes to a wide range of diseases, including cancers, as well as cardiovascular and autoimmune diseases (1). Understanding the underlying mechanism involved in the occurrence and development of inflammation may help the development of novel therapeutic targets for these diseases.

A large number of studies have previously focused on the association between macrophages and the progression and maintenance of inflammation. Macrophages, which are present in almost all tissues, play important roles in the maintenance of tissue homeostasis (2). They are considered the key drivers of innate immunity, and are crucial in host defense and inflammation (3). Macrophages can be divided into two types; namely, classically activated M1 phenotype and activated M2 phenotype, based on their distinct activation status and function. In different environments, there is a transformation between M1 and M2 phenotypes (4). M1 macrophages, also known as inflammatory macrophages, are formed in response to infection and/or injury by the human body. They are characterized by a strong bactericidal capacity and a high expression of inducible nitric oxide synthase (iNOS). In addition, these cells also secrete high levels of inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-12 (5). Moreover, studies have shown that the M1 phenotype is involved in lipopolysaccharide (LPS)-induced inflammation through the toll-like receptor 4 (TLR4)/nuclear factor κB (NF-κB) signaling pathway (6,7). M2 macrophages, a type of anti-inflammatory macrophage, are formed during stimulation by IL-4, produce low levels of inflammatory cytokines, and are characterized by high expression of arginase (Arg)-1, mannose receptor [cluster of differentiation (CD)206] and anti-inflammatory factor IL-10, which are closely associated with infection clearance and tissue repair (8). Thus,

it is reasonable to speculate that the timely transformation between M1 and M2 macrophages may be beneficial for tissue repair and regeneration. However, the therapeutic roles of macrophages in inflammatory diseases, as well as the detailed mechanisms, remain to be fully elucidated.

Previous research has focused on the use of mesenchymal stem cell (MSC) therapy for the treatment of inflammation-related diseases. Distributed in almost all parts of the human body, MSCs are multipotent cells with a capacity to differentiate into numerous mesenchymal lineages, such as osteoblasts, chondrocytes and adipocytes (9). A large number of clinical trials and *in vivo* experiments have revealed that MSCs are a promising candidate for the treatment of a variety of inflammation-related diseases, including inflammation caused by severe acute respiratory syndrome coronavirus 2 infection (10), Crohn's disease (11) and lupus erythematosus (12). MSCs were, initially, mainly isolated from bone marrow (BM). However, the surgical procedure of obtaining BM-MSCs may trigger injuries in the donor and the harvested cells are inadequate in number, limiting the clinical application of BM-MSCs to a certain extent (13). Therefore, further studies have focused on extracting MSCs from alternative sources (14-16). For example, human adipose-derived MSCs were found to treat rheumatoid arthritis via modulating T cell immunity and the production of inflammatory mediators (17). Treatment with human umbilical cord-derived MSCs has been found to reduce inflammatory cytokine levels in early diabetic nephropathy and inhibit fibrosis progression via the transforming growth factor (TGF)-β pathway (18).

As one of the readily accessible sources of MSCs, placentas have been largely used to study MSCs. Notably, human placenta-derived (HPL)-MSCs are more easily propagated and possess improved immunoregulatory properties, making them a suitable option for the future treatment of inflammation-related diseases (19). Although previous studies (10-12) have reported the therapeutic effects of MSCs derived from different sources on inflammation, relatively little is known regarding the use and mechanisms underlying HPL-MSCs in the inhibition of inflammation. Moreover, the specific roles of macrophage polarization in the regulation of HPL-MSCs are yet to be fully elucidated.

In the present study, HPL-MSCs were successfully isolated and purified from human fetal placentas. The functional role and underlying mechanisms of HPL-MSCs in LPS-induced macrophage inflammation were explored via a series of *in vitro* experiments. The current study provides a basis for exploring the therapeutic application of HPL-MSCs in inflammatory diseases.

Materials and methods

Materials. Fetal bovine serum (FBS) and cell culture medium (H-DMEM) were all purchased from Gibco (Thermo Fisher Scientific, Inc.). LPS extracted from *Escherichia coli* 0111: B4 was obtained from Sigma-Aldrich (Merck KGaA). IL-4 was purchased from PeproTech, Inc. An Enhanced BCA Protein Assay kit was obtained from Beyotime Institute of Biotechnology. OriCellP MSCs NCR Protein-Free Cryopreservation medium was purchased from Cyagen Biosciences, and CD11b monoclonal antibody (M1/70)

(cat. No. MA1-10082) was purchased from Thermo Fisher Scientific, Inc.

Isolation and culture of HPL-MSCs. Human fetal placenta was obtained from the Department of Obstetrics and Gynecology, Affiliated Hospital of Jiangnan University (Wuxi, China). A total of 30 placentae from healthy pregnant females aged 25-35 years (median age, 32 years) were collected. Inclusion criteria were as follows: Maternal blood tests for HBV, HIV, CV, EBV, CMV and syphilis were negative, and there were no other infectious diseases or congenital disease; full term cesarean section or natural delivery and newborn had no congenital disease. Samples were collected after obtaining written informed consent from each patient, and ethics approval was obtained from the Medical Ethics Committee of the Affiliated Hospital of Jiangnan University (approval no. LS2021046; Wuxi, China). Placental tissues were cut into small pieces, washed repeatedly with phosphate buffered saline (PBS) and digested with collagenase I (100 U/ml) at 37°C for 30 min. Following centrifugation at 937 x g for 5 min at room temperature, the pellet was resuspended in PBS, followed by filtration and centrifugation at 937 x g for 5 min at room temperature. Upon removal of the supernatant, the cells were re-suspended with MSC culture medium (ScienCell Research Laboratories, Inc.). Cells ($5x10^4$ cells/cm^2) were placed in cell culture dishes at 37°C with 5% CO_2. Unattached cells and debris were removed on day 2 after culture. Cells were harvested when a confluence of 80% was reached, and only cells between passage 3 and passage 9 were used for subsequent experiments.

Flow cytometry. Phenotypic analysis of the cultured HPL-MSCs was performed using flow cytometry. HPL-MSCs (passage 3; $1x10^6$ cells/ml) were collected in a flow tube in flow cytometry staining buffer (cat. No. 00-4222-26; Invitrogen; Thermo Fisher Scientific, Inc.), and subsequently stained with FITC-conjugated antibodies against human CD29 (cat. No. 11-0299-42; 1 μg/test), CD73 (cat. No. 11-0739-42; 0.25 μg/test), CD105 (cat.No.MA1-19594; 20μl/1x10^{6}cells), CD45 (cat.No.11-0459-42; 0.25 μg/test), CD34 (cat. No. 11-0349-42; 0.5 μg/test), CD14 (cat. No. 11-0149-42; 1 μg/test), human leukocyte antigen-DR isotype (cat. No. 11-9956-42; 0.125 μg/test) and CD11b (cat. No. MA5-16528; dilute to 80 times volume) from Invitrogen (Thermo Fisher Scientific, Inc.). The isotype controls (cat. No. 11-4714-42; 1 μg/test) were from Invitrogen (Thermo Fisher Scientific, Inc.). The isotype controls and the target antibodies were placed in the dark for 30 min at 4°C. After washing twice with PBS, the cells were resuspended in 200 μl PBS, followed by flow cytometry (B53037, Beckman Coulter, Inc.). Data were analyzed using FlowJo v10 software (BD Biosciences).

Adipogenic and osteogenic differentiation. Cell confluence at 100 and 80% was used for adipogenic and osteogenic differentiation, respectively, based on the results of the pre-experiments performed in accordance with the standard protocol of the OriCell Human related Stem Cell Adipogenic Differentiation Kit (cat. No. HUXXC-90031; Cyagen Biosciences) and OriCell Human related Stem Cell Osteogenic Differentiation kit (cat. No. HUXXC-90021; Cyagen Biosciences). HPL-MSCs (passage 3; $1.5x10^5$ cells/well) were seeded in a 6-well plate. Upon reaching a 100% confluence, cells were cultured

using OriCell™ human MSC adipogenic differentiation medium (cat. No. HUXXC-90031; Cyagen Biosciences). Oil red O staining (10 min staining at room temperature) was performed to analyze the differentiation potential of adipocytes on day 24 after induction. For osteogenic differentiation, OriCell™ human MSC osteogenic differentiation medium (cat. No. HUXXC-90021; Cyagen Biosciences) was added to the wells after reaching a confluence of 80%. On day 23 after induction, the potential for osteogenesis was assessed using Alizarin Red staining (30 min staining at 37°C).

In vitro co-culture experiment. Macrophage-like cells, RAW264.7, purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences, were cultured in H-DMEM with 10% FBS at 37°C in a humidified incubator supplied with 5% CO_2. Cells in the exponential growth phase were divided into blank control, LPS and LPS + HPL-MSCs. In the blank control group, cells ($5x10^5$) were incubated with 2 ml H-DMEM containing 10% FBS. For the LPS group, cells ($5x10^5$) were stimulated with LPS (1 μg/ml) for 4 h at 37°C to create an M1 inflammatory model. For the IL4 group, cells ($5x10^5$) were stimulated with IL-4 (20 ng/ml) for 4 h at 37°C to create an M2 inflammatory model. In the LPS + HPL-MSCs group, cells ($5x10^5$) were stimulated with LPS (1 μg/ml) for 4 h before co-cultivation with HPL-MSCs.

A Transwell assay was carried out to assess the effects of HPL-MSCs on inflammation. Briefly, a co-culture Transwell chamber (diameter, 2.4 cm; 0.4-μm pore size; Wuxi NEST Biotechnology Co., Ltd.) was utilized for the assay. RAW264.7 cells ($5x10^5$) were seeded into the lower chamber, containing 1 ml H-DMEM containing 10% FBS. Subsequently, HPL-MSCs ($5x10^5$) were seeded into the upper compartment, containing 0.5 ml H-DMEM containing 10% FBS. Following ~24 h in culture, the cells were harvested for subsequent analysis.

Enzyme-linked immunosorbent assay (ELISA) and nitric oxide (NO) detection assay. ELISA assays were performed to determine the concentration of mature IL-10 (IL-10 Mouse Uncoated ELISA Kit, cat. No. 88-7105-88) with, IL-6 (IL-6 Mouse Uncoated ELISA Kit, cat. No. 88-7064-88) and TNF-α (TNF alpha Mouse Uncoated ELISA Kit, cat. No. 88-7324-77) in RAW264.7 cell culture supernatant, all assays were performed in at least triplicate using a commercial ELISA kit purchased from Invitrogen (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The NO detection assay was performed using the nitric oxide assay kit (cat. No. S0021S; Beyotime Institute of Biotechnology). For ELISA of HPL-MSCs, for the stimulation group, cells ($5x10^5$) were stimulated with LPS (1 μg/ml), for the blank control group, cells ($5x10^5$) were not treated.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from each RAW.264.7 cell sample using TRIzol® reagent (Thermo Fisher Scientific, Inc.). RNA was reverse transcribed into cDNA using a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. qPCR was carried out to quantify gene expression using an UltraSYBR mixture (cat. No. CW0957M; CoWin Biosciences) in a LightCycler 96

Table I. Reverse transcription-quantitative PCR primers for detection of gene transcripts.

Gene	Sequence (5'-3')
GAPDH	Forward: GAGCCAAAAGGGTCATCATCT
	Reverse: GAGGGGCCATCCACAGTCTT
IL-6	Forward: ACAACCACGGCCTTCCCTACTT
	Reverse: CACGATTTCCCAGAGAACATGTG
IL-1β	Forward: GCAACTGTTCCTGAACTCAACT
	Reverse: ATCTTTTGGGGTCCGTCAACT
iNOS	Forward: GAGCTCGGGTTGAAGTGGTATG
	Reverse: GAAACTATGGAGCACAGCCACAT
IL-10	Forward: CCCTTTGCTATGGTGTCCTT
	Reverse: TGGTTTCTCTTCCCAAGACC
TNF-α	Forward: AAGCCTGTAGCCCACGTCGTA
	Reverse: GGCACCACTAGTTGGTTGTCTTTG

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; iNOS, inducible nitric oxide synthase; TNF-α, tumor necrosis factor α.

Real-Time System (LightCycler 96 Roche Diagnostics GmbH), the thermocycling conditions were as follows: 95°C for 10 min; 95°C for 15s followed by 60°C for 1min with 35 cycles. The sequences of specific primers are listed in Table I. The relative expression of each transcript was normalized to the expression of GAPDH. Amplification data were analyzed according to the $2^{-\Delta\Delta Cq}$ method (20).

Western blot analysis. RAW.264.7 cells were lysed with RIPA lysis buffer (cat. No. P0013B; Beyotime Institute of Biotechnology) containing proteinase inhibitors, after rinsing twice with PBS on ice. The protein content was evaluated using a BCA assay. Denatured proteins were separated using 10% SDS-PAGE (20 μg/lane) subsequently transferred to PVDF membranes and blocked with QuickBlock™ Blocking Buffer (cat. No. P0231; Beyotime Institute of Biotechnology) for 1 h at room temperature the specific steps of blocking operation were according to the manufacturer's instructions. Following blocking, membranes were incubated with rabbit anti-phosphorylated (p)-IκBα (1:1,000; cat. No. ab133462; Abcam), anti-IκBα (1:1,000 dilution; cat. No. ab76429; Abcam), anti-p65 (1:1,000 dilution; cat. No. ab32536; Abcam), anti-p-p65 (1:1,000 dilution; cat. No. ab76302; Abcam), anti-GAPDH (1:1,000 dilution; cat. No. AF1186; Beyotime Institute of Biotechnology), anti-IL-1β (1:1,000 dilution; cat. No. ab254360; Abcam), anti-interferon regulatory factor 5 (IRF5) (1:1,000 dilution; cat. No. AF2488; Beyotime Institute of Biotechnology), anti-Bcl-2 (1:1,000 dilution; cat. No. ab32124; Abcam), anti-Bax (1:1,000 dilution; cat. No. ab32503; Abcam) and anti-TLR-4 (1:1,000 dilution; cat. No. ab13556; Abcam) overnight at 4°C. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit (1:6,000; cat. No. CW0103; CoWin Biosciences) for 1 h at room temperature. Signals were visualized using an ECL detection kit (cat. No. P0018S; Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Bands were
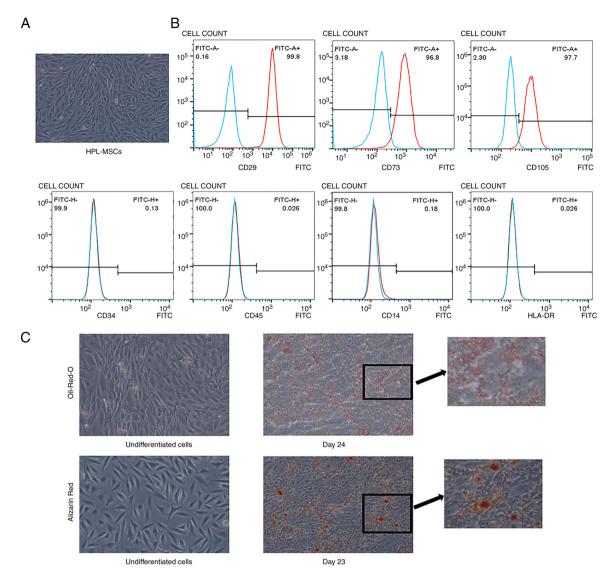
Figure 1. Identification of HPL-MSCs. (A) Representative photomicrograph of adherent cells on plastic cell culture dish. (B) Flow cytometry determined the expression of CD29, CD73, CD105, CD34, CD45, HLA-DR and CD14 in HPL-MSCs. The blue line and the red line represent the isotype control and the level of surface markers, respectively. The x-axis represents the fluorescence signal value and the y-axis represents counts. (C) HPL-MSCs showed multiple differentiation potentials. The experiments were representative of at least three independent trials, each with three technical replicates. Magnification, x10. HPL-MSC, human placenta-derived mesenchymal stem cell; CD, cluster of differentiation; HLA-DR, human leukocyte antigen-DR isotype.

visualized and imaged using a Bio-Rad Laboratories, Inc. image analysis system. GAPDH was used as the loading control. Semi-quantitative protein quantification was performed using ImageJ 1.8.0_172 software (National Institutes of Health).

Statistical analysis. Each experiment included three technical replicates. Data are presented as the mean ± standard deviation. Data analysis was conducted using SPSS 23.0 software (IBM Corp.). Unpaired Student's t-test was used to compare data between two groups. For multiple comparisons, one-way ANOVA with Tukey's post hoc test was used. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Identification and characterization of HPL-MSCs. The results of the present study demonstrated that both primary and passaged (Fig. 1A) cells formed a single layer of adherent cells,

which presented a spindle-shaped, fibroblast-like morphology. CD29, CD73 and CD105 were expressed in these cells, while no hematopoietic lineage markers (CD14, CD34 and CD45) were determined using flow cytometry (Fig. 1B). In addition, no HLA-DR expression was detected, which indicated that cells isolated from human placenta possessed low immunogenicity (Fig. 1B). After the induction of differentiation, cells demonstrated the potential to differentiate into adipocytes and osteocytes *in vitro*. After 24 days of induction, the presence of lipid droplets was confirmed using Oil red O staining (Fig. 1C). Cells were stained with Alizarin red 23 days after adding the differentiation medium, which confirmed the characteristics of osteocytes (Fig. 1C).

LPS does not affect the secretion of inflammatory factors by HPL-MSCs, apart from nitric oxide content. To evaluate the effects of LPS on HPL-MSCs, the secretion of inflammation-related factors was analyzed in HPL-MSCs in the presence
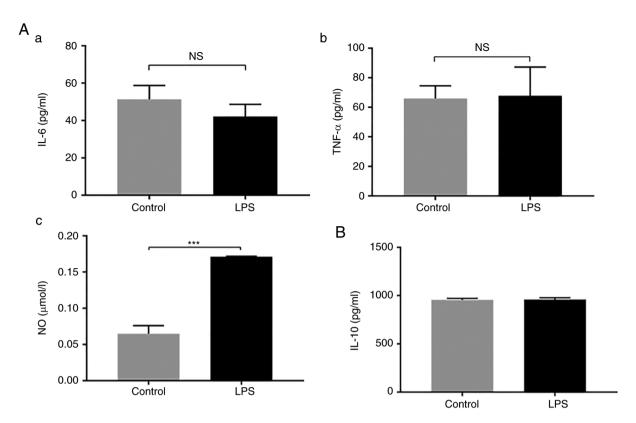
Figure 2. Secretion of inflammatory factors by human placenta-derived mesenchymal stem cells in the presence of LPS. (A) The levels of (Aa) IL-6 and (Ab) TNF-α were measured using ELISA. (Ac) Commercial NO kit was utilized to measure the release of NO. (B) The levels of IL-10 were measured using ELISA. The experiments were representative of at least three independent trials, each with three technical replicates. The error bars represent the standard deviations. ***P<0.001. LPS, lipopolysaccharide; IL, interleukin; TNF, tumor necrosis factor; NO, nitric oxide; NS, not significant.

of LPS. Following stimulation with LPS (1 µg/ml) for 24 h, the levels of IL-6, TNF-α and IL-10 in HPL-MSCs were measured using ELISA. There was no significant difference in the expression of IL-6 and TNF-α between the stimulation group and the blank control group (Fig. 2A). The nitric oxide (NO) content was significantly higher than that of the blank control group (Fig. 2A). In addition, no significant difference was observed in the expression level of IL-10 between the two groups (Fig. 2B). These results implied that LPS exerted no effects on the expression levels of IL-6, TNF-α or IL-10 secreted by HPL-MSCs.

HPL-MSC co-culture attenuates LPS-induced M1 macrophage polarization at the protein expression level. In order to investigate the effects of HPL-MSCs on inflammation, HPL-MSCs and macrophages were co-cultured in a Transwell system (Fig. 3A). The levels of IL-6 and TNF-α were measured using ELISA. Compared with the LPS group, a significant reduction was observed in the expression of IL-6, TNF-α and NO in the LPS + HPL-MSCs group (Fig. 3B). Correspondingly, we detected the levels of IL-10, a marker of M2 macrophages. The blank control group was negative control, the IL4 group was positive control, ELISA assays showed that the level of IL10 in LPS + HPL-MSCs group was not significantly different from that in blank control group, and the level of IL10 in LPS + HPL-MSCs group was significantly lower than that in IL4 group (Fig. 3C). The results of the western blot analysis indicated that the level of M1 macrophage markers (IRF5 and IL-1β) were decreased in the LPS + HPL-MSCs group compared with the LPS group (Fig. 3D and E). Results of flow

cytometry demonstrated that the level of CD11b, a protein marker of M1 macrophages, in LPS + HPL-MSCs group was significantly decreased compared with the LPS group (Fig. 3F). Collectively, these results suggested that HPL-MSCs contributed to the reduction of M1 macrophage markers at the protein level, which implied that HPL-MSCs may exert certain anti-inflammatory effects.

HPL-MSC co-culture inhibits LPS-induced M1 macrophage polarization at the gene level. In order to further investigate the effects of HPL-MSCs on the mRNA expression levels of macrophage-related proteins, HPL-MSCs and macrophages were co-cultured in a Transwell system. The results of the RT-qPCR analysis demonstrated that the mRNA levels of pro-inflammatory factors (iNOS, TNF-α, IL-6 and IL-β) in the LPS+HPL-MSCs group were significantly lower than those in the LPS group (Fig. 4A). To verify whether HPL-MSCs could further promote the M2 polarization of macrophages by reducing the M1 polarization of macrophages, M2 macrophage-related markers were investigated. The mRNA levels of IL-10 in the LPS + HPL-MSCs group were significantly lower than the IL-4 group, and there was no significant difference compared with the blank control group (Fig. 4B). These results indicated that HPL-MSCs promoted the reduction of M1 macrophage polarization, at the mRNA level, which was consistent with the results at the protein expression level. Additionally, this confirmed that HPL-MSCs exerted certain anti-inflammatory effects. However, HPL-MSCs did not promote the M2 polarization of macrophages.
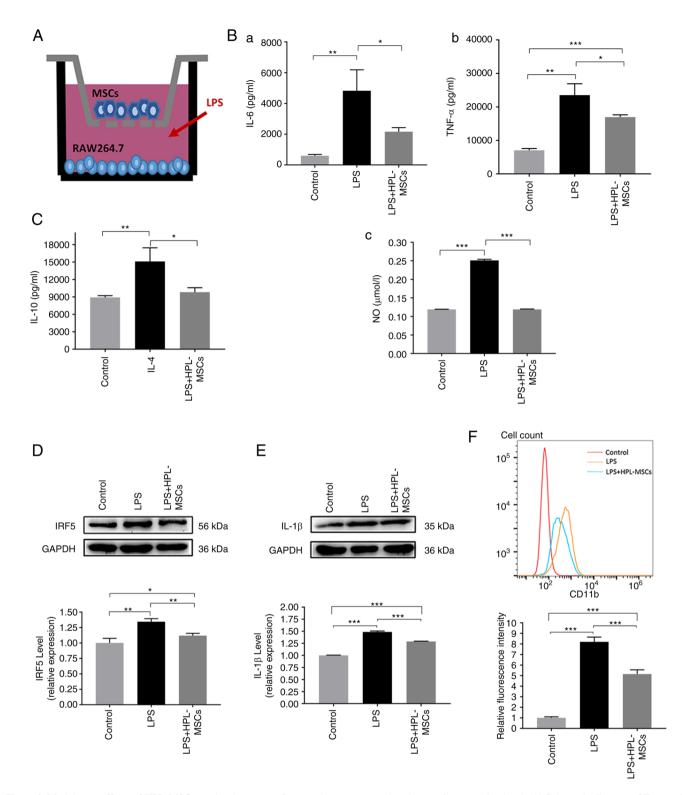
Figure 3. Modulatory effects of HPL-MSCs on the phenotype of macrophages, measured at the protein expression level. (A) Schematic diagram of Transwell co-cultivation. (B) The levels of (Ba) IL-6 and (Bb) TNF-α were measured with ELISA. (Bc) NO kit was utilized to measure the release of NO. (C) IL-10 concentration was measured with ELISA. (D) IRF5 protein concentration was measured by Western blot analysis. (E) Western blot analysis was also used to detect the expression of IL-1β. (F) The expression of CD11b in the cell surface was detected by flow cytometry. The x-axis represents the fluorescence signal value and the y-axis represents Counts. The experiments were representative of at least three independent trials, each with three technical replicates. The error bars represent the standard deviations. *P<0.05, **P<0.01, ***P<0.001. MSC, mesenchymal stem cell; HPL, human placenta-derived; LPS, lipopolysaccharide; IL, interleukin; TNF, tumor necrosis factor; NO, nitric oxide; IRF5, interferon regulatory factor 5; CD, cluster of differentiation.

HPL-MSCs regulate inflammation through the NF-κB signaling pathway. To determine whether HPL-MSCs inhibited LPS-induced cellular inflammation *in vitro*, the TLR4/NF-κB pathway in macrophages, the classical signaling pathway of LPS-induced inflammation (7), was examined in co-culture experiments following stimulation by LPS. The results of the western blot analysis demonstrated that the protein expression levels of TLR4, p-IκBα and p-p65 in the
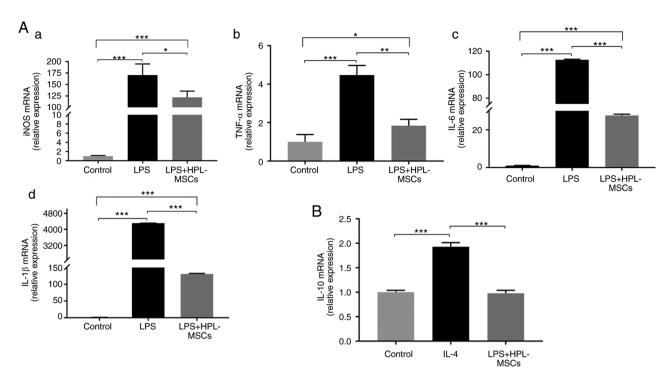
Figure 4. Modulatory effects of HPL-MSCs on the phenotype of macrophages at the gene level. (A) Transcription levels of (Aa) iNOS, (Ab) TNF-α, (Ac) IL-6 and (Ad) IL-1β in macrophages after co-culture with HPL-MSCs, as revealed by RT-qPCR. (B) RT-qPCR analysis of IL-10 transcription levels in macrophages after co-culture with HPL-MSCs. The experiments were representative of at least three independent trials, each with three technical replicates. The error bars represent the standard deviations. *P<0.05, **P<0.01, ***P<0.001. HPL-MSC, human placenta-derived mesenchymal stem cell; LPS, lipopolysaccharide; IL, interleukin; TNF, tumor necrosis factor; iNOS, inducible nitric oxide synthase; RT-qPCR, reverse transcription-quantitative PCR.

LPS group were significantly increased, compared with those of the blank control group (Fig. 5A and B). However, data obtained following co-cultivation revealed that HPL-MSCs may decrease the expression levels of TLR4 and p-IκBα (Fig. 5A and B), as well as inhibiting the phosphorylation of p65 (Fig. 5B). The results of the western blot analysis demonstrated that HPL-MSCs induced an increase in Bcl-2 protein expression and a decrease in Bax protein expression compared with the LPS group (Fig. 5C). These results indicated that HPL-MSCs were involved in the reversal of macrophage apoptosis. Collectively, these results highlighted that HPL-MSCs may regulate inflammation by modulating the NF-κB signaling pathway.

Discussion

Stem cells exhibit potential in both biotherapy and tissue engineering (19). MSCs derived from various sources have been demonstrated to be effective in the treatment of inflammatory diseases (21). However, obtaining a large number of MSCs remains a key problem in clinical practice. Placentas contain a large number of pluripotent stem cells with characteristics similar to that of BM-MSCs (22). Thus, the placenta has recently been considered as a promising source for the isolation of MSCs. The results of the present study demonstrated that HPL-MSCs are easily isolated from the donor placenta, and they exhibit a fibroblast-like morphology. The isolated cells expressed a large number of markers, such as MSC-specific surface markers (CD29, CD73 and CD105), but these cells did not express hematopoietic lineage markers (CD34, CD45 and CD14) or HLA-DR. Moreover, results of Oil

red O and Alizarin red staining indicated that these cells had the ability to differentiate into adipocytes and osteoblasts. The results of the present study suggested that the cells isolated from the placenta were MSCs, which was consistent with the results of previous research (23).

Macrophages play a key role in inflammation, defense, repair, metabolism and other physiological processes. They are crucial for the body in maintaining homeostasis. To regulate inflammation in different microenvironments or diseases, macrophages exhibit different morphologies, phenotypes and functions, as well as undergoing polarization (24). Unpolarized macrophages are categorized as the M0 phenotype. Following polarization, macrophages are divided into M1 and M2 phenotypes. In the presence of external stimuli, transformation occurs between M1 and M2 phenotypes (25).

It has been proposed that inflammation is regulated and controlled by macrophages of both the M1 and M2 phenotypes (26). M1 macrophages release chemokines, such as NO, reactive oxygen species and pro-inflammatory cytokines (TNF-α and IL-6) (4,5), while M2 macrophages express chemotactic factors and anti-inflammatory cytokines, such as IL-10, TGF-β, Arg-1 and prostaglandin E2 (8). M1 macrophages are mainly involved in the initiation and maintenance of inflammation, while M2 macrophages are mainly involved in inflammation control (8). To the best of our knowledge, the number of M2 macrophages is increased to eliminate inflammation. The phenotype of macrophages determines the ultimate outcomes of inflammation. Therefore, how to effectively regulate the balance of M1 and M2 macrophages is particularly important to manage an uncontrolled inflammatory response. In a previous study, Liu et al (27) demonstrated that
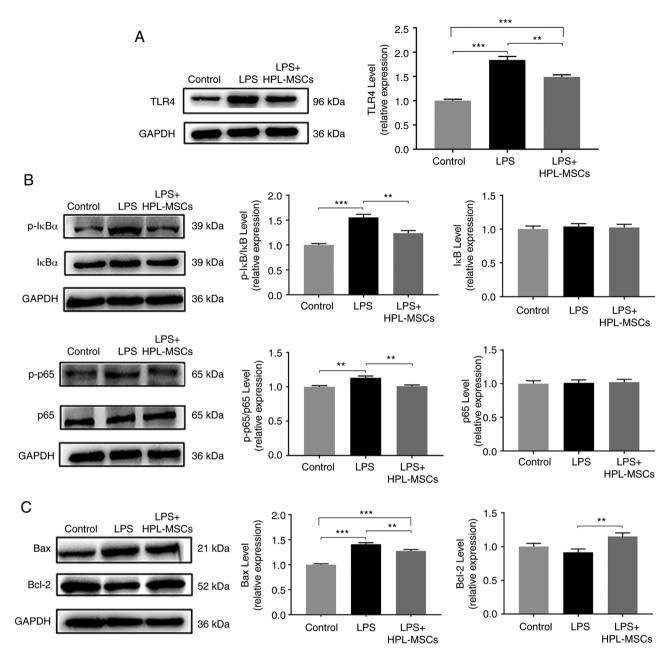
Figure 5. HPL-MSCs inhibit inflammation through the NF-κB signaling pathway. Western blot analysis was used to detect (A) expression of TLR4, (B) the expression of p-IκBα, IκBα, p-p65, p65, p-IκBα/IκBα and p-p65/p65 (C) the expression of Bax and Bcl-2 proteins in macrophages. The experiments were representative of at least three independent trials, each with three technical replicates. **P<0.01 and ***P<0.001; columns without asterisks have no significant difference. The error bars represent standard deviations. HPL-MSC, human placenta-derived mesenchymal stem cell; LPS, lipopolysaccharide; TLR4, toll-like receptor-4; p, phosphorylated.

that Fasudil mediated the conversion of M1 macrophages to M2 macrophages, which contributed to the anti-inflammatory effects on encephalomyelitis. These results confirmed that the phenotype of macrophages play an important role in the inflammatory immune response.

In previous studies, LPS (1 μg/ml) and IFN-γ (20 ng/ml) have been utilized to induce the generation of M1 macrophages (28,29), and IL-4 (20 ng/ml) and IL-13 (20 ng/ml) have been used to induce macrophage polarization to the M2 phenotype (30,31). A variety of polarization systems were designed for the present study. Notably, RT-qPCR, ELISA and flow cytometry were utilized, and the results indicated that the M1 phenotype may be induced by 1 μg/ml LPS, and the

M2 phenotype may be induced by IL-4 (20 ng/ml). Results of the present study demonstrated that expression levels of IL-6, TNF-α, iNOS and IL-1β were significantly increased in the LPS group compared with the control (6,7), which verified that the model establishment was successful.

To rule out the effects of inflammatory factors produced by HPL-MSCs in the presence of LPS, HPL-MSCs were stimulated with LPS alone for 24 h, and the results indicated no significant changes in the expression of inflammatory factors, except NO. The results of the present study indicated that within the time range, HPL-MSCs were not sensitive to the majority of cytokines in the presence of LPS; however, the generation of NO may have been affected. Following co-cultivation, the

content of NO was not decreased compared with the control group, which further illustrated that the anti-inflammatory effects of HPL-MSCs were significant.

As previously described, the co-culture of MSCs with non-polarized macrophages *in vitro* contributed to the development of M2 macrophages. In addition, a significant increase in the proportion of non-polarized M2 macrophages was observed, along with an increase in CD206 expression and IL-10 synthesis. There was a notable decrease in the expression levels of TNF-α. These results implied that MSCs mediated the conversion of M1 macrophages to M2 macrophages, leading from a tissue pro-inflammatory response to an anti-inflammatory response, which thereby improved the uncontrolled inflammatory response (32). The results of the Transwell assay in the present study demonstrated that compared with the LPS group, regardless of the transcriptional or translational levels, the expression of M1 phenotype markers like IL-6, TNF-α, iNOS or NO and IL-1β were decreased in the LPS + HPL-MSCs group, which was consistent with previous literature (33). However, inconsistent with the findings of previous studies (34,35), the results of the present study demonstrated that there was no significant increase in the expression of M2 phenotype markers. We hypothesized that the HPL-MSC stimulation contributed to the transformation of the macrophage phenotypes, and that the M1 phenotype originally induced by LPS would alternate to the M0 or M2 phenotype. It is possible that within a short period of time, macrophages will transform into the M0 phenotype.

As previously described, the LPS/TLR4 mediated signaling pathway is the main macrophage endotoxin pathway (34). Results of a previous study demonstrated that LPS produced by bacteria induced the activation of macrophages through the myeloid differentiation factor 88-dependent and –independent pathways, after activation of TLR4 (35). Consequently, the inflammation cascade became imbalanced, and both monocytes and macrophages were jointly regulated by the diacylglycerol-protein kinase C (PKC) signaling pathway and the PKC-NF-κB pathway. Therefore, in macrophages, NF-κB is closely associated with the inflammatory response signaling pathway.

Under normal circumstances, NF-κB is localized in the cytoplasm and is composed of two functional subunits, namely p65 and p50, while it is bound to its endogenous inhibitors (IκB-α and IκB-β). IκB-β blocks the entry of NF-κB to the nucleus and regulates the expression of target genes. In response to certain stimuli, IκB-specific serine residues are phosphorylated by IκB kinase, causing the polyubiquitination of IκB. Subsequently, NF-κB enters into the nucleus following activation, which contributes to the generation of inflammatory mediators upon binding to the target gene(s). Moreover, the gene products further activate NF-κB, triggering an expanded cascade of uncontrolled inflammation (36).

Results of the present study highlighted the protective roles of HPL-MSCs in LPS-induced inflammation. HPL-MSCs inhibited the release of pro-inflammatory factors at the transcriptional and translational levels. Thus, we hypothesized that MSCs mediated the immune inflammatory response by regulating macrophage polarization and the NF-κB signaling pathway. On this basis, HPL-MSCs and macrophages were
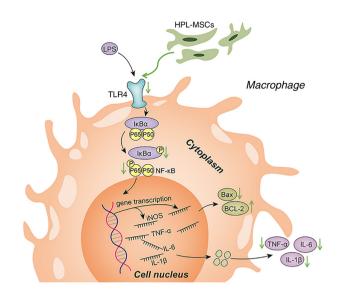

Figure 6. Roles of HPL-MSCs in LPS-induced macrophage inflammation. HPL-MSC, human placenta-derived mesenchymal stem cell; LPS, lipopoly-saccharide; TLR4, toll-like receptor-4; p, phosphorylated; IL, interleukin; TNF, tumor necrosis factor; iNOS, inducible nitric oxide synthase.

co-cultured to explore the roles of the NF-κB signaling pathway in inhibiting the polarization of macrophages by HPL-MSCs. Results of the present study confirmed that HPL-MSCs resulted in the decreased expression of TLR4 in macrophages induced by LPS, and in the decreased phosphorylation of IκBα and NF-κB p65. Thus, HPL-MSCs may deactivate the NF-κB signaling pathway, to a certain extent. In addition, the secretion of IL-6, a pro-inflammatory biomarker regulated by the NF-κB signaling pathway, was also inhibited.

A previous study has highlighted that inflammatory mediators (NO and TNF-α) produced by LPS-stimulated macrophages contribute to apoptosis (37). Thus, the levels of macrophage apoptosis may reflect the levels of inflammation, to a certain extent. The results also demonstrated that the expression of anti-apoptotic protein Bcl-2 increased, while the expression of pro-apoptotic protein Bax decreased in the LPS + HPL-MSCs group. These results suggest that HPL-MSCs may also inhibit macrophage apoptosis. Cytokines and genes associated with apoptosis are regulated by the transcription factor NF-κB (38). Therefore, these results further verified that HPL-MSCs may regulate inflammation through the NF-κB signaling pathway (Fig. 6).

Inflammation and undesirable immune responses may cause a variety of diseases or complications, including inflammation-related cancers (39,40) and postoperative lymphedema (41). Thus, effective anti-inflammatory therapy will play an important role in the early prevention and treatment of several diseases, leading to improvement in patient prognosis. The present study explored the anti-inflammatory mechanisms and therapeutic potential of HPL-MSCs. It was shown that HPL-MSCs attenuated the NF-κB signaling pathway by regulating the expression of TLR4, as well as the phosphorylation of IκBα and p65. HPL-MSCs may attenuate inflammation and reduce the release of inflammatory factors. However, the present study is not without limitations. For example, numerous mechanisms are involved in MSC-mediated inflammatory regulation, but only one signaling pathway remained

the focus of the present study. Moreover, the present study was only performed *in vitro*, and further *in vivo* studies are required.

In conclusion, based on the results of the present study, as well as those of previous studies, HPL-MSCs may exhibit potential in the future treatment of inflammatory diseases.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YL and XZ designed and performed experiments. YL was responsible for the funding acquisition. YH processed placental samples and isolated HPL-MSCs. MK and YWu performed patient examinations, were responsible for the delivery of labor, the initial handling and preservation of the placentas and they did all the preparatory work and pre-experiments of our study. YWa conceived the study. CD designed the study. YL and XZ confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Human fetal placenta was obtained from the Department of Obstetrics and Gynecology, Affiliated Hospital of Jiangnan University (Wuxi, China) with written informed consent from the patients. This study was approved by the Medical Ethics committee of The Affiliated Hospital of Jiangnan University (Wuxi, China; approval no. LS2021046).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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