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Human placental mesenchymal stem cell derived exosomes exhibit anti-inflammatory effects via TLR4-mediated NF-κB/MAPK and PI3K signaling pathways

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Exosomes are a type of nanoparticles in 40–200 nm extracellular vesicles secreted from living cells, containing a plurality of biologically active substances, which can be used as carriers of intercellular delivery signals. Among them, mesenchymal stem cell (MSC)-derived exosomes have been reported to play important roles in injury repair, alleviating inflammation; thus, MSC-derived exosomes have become hot spot in noncellular therapies. The role of human placental MSC-derived exosomes (hplMSC-Exos) in inflammation and their potential mechanisms are unclear. Therefore, we investigated the anti-inflammatory effects of hplMSC-Exos in lipopolysaccharide (LPS)-induced RAW264.7 cells and their intrinsic mechanisms. Our data demonstrated that hplMSCs-Exos can adjust inflammation by regulating TLR4-mediated NF-κB/MAPK and PI3K signaling pathways, indicating that hplMSCs-Exos can act as a new strategy for inflammatory treatment.

1. Introduction

Inflammation is an adaptive response when the body is exposed to noxious stimuli, such as infection and tissue damage (Medzhitov 2008; Newton and Dixit 2012); it is a double-edged sword. Moderate inflammation can help repair body damage, but excessive inflammation can lead to many degenerative diseases, such as cardiovascular disease, arthritis, type 2 diabetes, and cancer (Chai et al. 2015; Libby 2007). Therefore, maintaining a balance of inflammation in the body is particularly important. Pattern recognition receptors (PRRs), which were expressed intracellular or on the surface of innate immune cells (macrophages, mast cells, fibroblasts, dendritic cells, and circulating leukocytes), recognize pathogen invasion or cell damage signals and generate immune response (Segonzac and Zipfel 2011; Zelenay and Reis e Sousa 2013). These signals include pathogen-associated molecular patterns (PAMPs), such as microbial nucleic acids, lipoproteins and carbohydrates, and danger-associated molecular patterns (DAMPs) released by damaged cells.

Macrophages, as important immune cells, play a significant role in the process of inflammation, repair, and metabolism (Gordon 2007). Macrophages have great plasticity. According to changes in microenvironment, inflammatory mediators can promote macrophages into different phenotypes, namely, classic activated macrophages (CAMS or M1) and non-classic activation macrophages cells (alternatively activated macrophages, AAMS or M2) (Mosser and Edwards 2008). The M1 macrophages secrete proinflammatory cytokines, such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) to accelerate inflammation. On the contrary, M2 macrophages release anti-inflammatory cytokines interleukin-10 (IL-10), transforming Growth Factor β (TGF-β) or the like to limit inflammation and promote tissue repair (Fong et al. 2008; Martinez et al. 2008; Mosser and Edwards 2008). Therefore, inhibition of macrophages to M1 polarization or promotion of macrophages to M2-type polarization is a new strategy for treating inflammation.

Toll-like receptor 4 (TLR4), a member of the toll-like receptor family, is a pattern recognition receptor capable of recognizing PAMPs. Lipopolysaccharide (LPS), the main component of the outer membrane of gram-negative bacteria, can be recognized by TLR4 and trigger inflammation response (Kumar et al. 2011; Savva and Roger 2013). Once TLR4 is activated, TLR4 transmits signals by the adaptor protein, and the downstream effector nuclear factor kappa-B (NF-κB) is activated, resulting in the phosphorylation of NF-kappa-B inhibitor α (IκBα)and p65, which ultimately leads to the production and release of pro-inflammatory cytokines (Peng et al. 2010). Mitogen-activated protein kinase (MAPK) is another effector of the inflammatory signal caused by TLR4 (Peroval et al. 2013). Activated TLR4 causes the activation of MAPK and then phosphorylates c-Jun N-terminal kinase (JNK), extracellular-regulated kinase (ERK) and p38, causing inflammatory responses (Cargnello and Roux 2011).

Phosphoinositide 3-kinase (PI3K) is an important signaling molecule that regulates cellular function and activities (Vanhaesebroeck et al. 2016). PI3K signaling pathway is closely related to the development of inflammation and is also involved in cell apoptosis (Hawkins and Stephens 2015; Sun et al. 2010). After PI3K is activated by various types of cell stimuli or toxic injury, serine/threonine kinase Akt (Akt) participates in cell survival and apoptosis by phosphorylation.

Exosomes are nanoscale extracellular vesicles with a diameter between 40 and 200 nm containing bioactive substances such as protein, mRNA and DNA fragment, which play a major role in intercellular communication during physiology and pathology (Kalluri and LeBleu 2020). Mesenchymal stem cells (MSCs) are adolescent stem cells with self-renewal and multi-differentiation potential; they are capable of anti-inflammatory, immunosuppressive, and tissue repair (Deans and Moseley 2000; Pittenger et al. 1999). Exosomes derived from MSCs have a biological function similar to their maternal cells; they particularly have the ability of high security and easy transportation (Lou et al. 2017), which has become a hot spot in noncellular therapies. Studies have reported that exosomes derived from MSCs have shown to reduce the level of proinflammatory cytokines and repair tissue damage in various diseases (Chan et al. 2019; Li et al. 2013; Sun et al. 2017). We aim to explore whether human placental MSC-derived exosomes (hplMSC-Exos) can regulate the polarization of LPS-induced macrophages, improve the expression of related inflammatory cytokines, and investigate the mechanisms behind them.

2. Investigation and results

2.1. Characterization of hplMSC-Exos

Under TEM, the vesicles presented a round-like morphology with a lipid bilayer membrane structure and showed a typical cup-like shape. The diameter of the vesicles was approximately 40–200 nm (Fig. 1A). Consistent with the TEM results, NTA detection found that the diameter of exosomes was distributed between 40 and 200 nm, and the average vesicle size was 139.8±35.5 nm (Fig. 1B). Exosomal surface markers CD9, CD63, and TSG101 were highly expressed on the surface of vesicles (Fig. 1C). This finding proved that the vesicles extracted by differential ultracentrifugation were hplMSC-Exos.

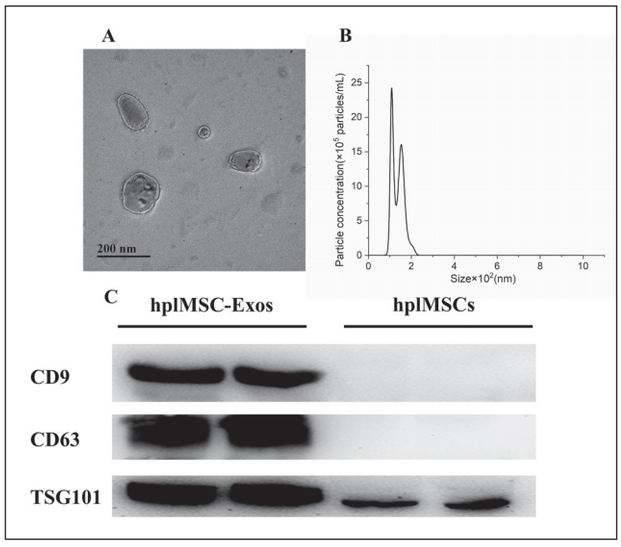


Fig. 1: Characterization of hplMSC-Exos. (A) Morphology of exosomes observed by TEM. Scale bar: 200 nm. (B) Particle size distribution of exosomes detected by NTA. (C) Expression of exosomal markers (CD9, CD63 and TSG101) evaluated by western blotting.

2.2. Effects of hplMSC-Exos on RAW264.7 cells proliferation

To evaluate the effect of hplMSC-Exos on RAW264.7 cells proliferation, exosomes in a concentration range from 10 to 90 μg/mL was exposed to RAW264.7 cells. As shown in Fig. 2, after 24 h stimulus, there was no significant differences (P > 0.05) in cell viability between blank control group and different concentrations of exosomes groups, indicating that hplMSC-Exos almost had no effects on the proliferation of RAW264.7 cells, that is, hplMSC-Exos had no cytotoxicity to RAW264.7 cells.

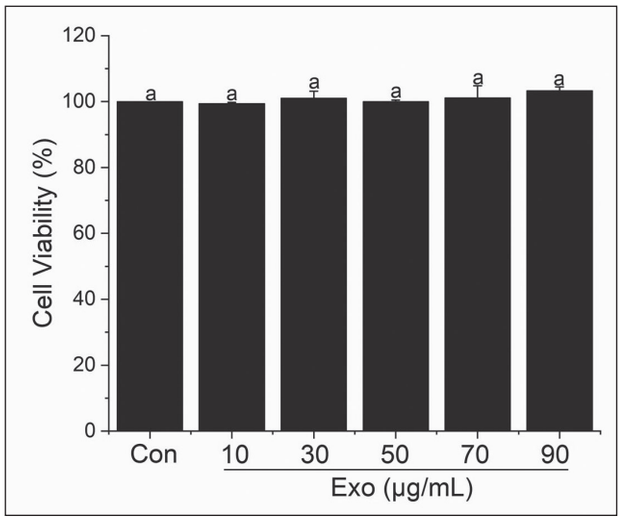


Fig. 2: Effects of hplMSC-Exos on RAW264.7 cells proliferation. Difference lowercases represent statistical significance between different groups (P<0.05).

2.3. HplMSC-Exos ameliorated inflammation by regulating the expression of inflammatory factors

ELISA showed a significant increase in the expression of the pro-inflammatory cytokines (IL-6, TNF-α), and the decrease in the expression of anti-inflammatory cytokine (IL-10) in LPS group (Figs. 3A–3C). On the contrary, the pro-inflammatory cytokines (IL-6, TNF-α) decreased and the anti-inflammatory cytokine (IL-10) increased after hplMSCs-Exos treatment. In accordance with ELISA, these inflammatory cytokines in mRNA level also exhibited similar trends (Figs. 3D–3F).

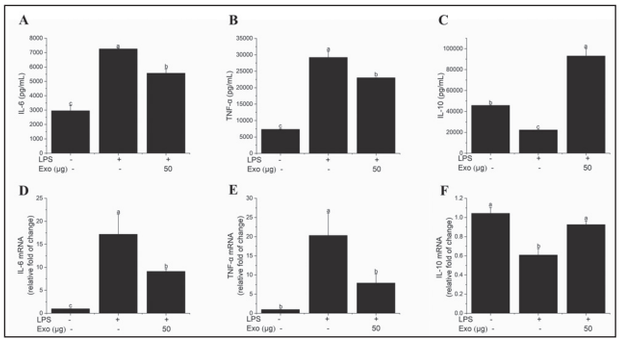


Fig. 3: HplMSC-Exos lowered the expression of pro-inflammatory cytokines and raised the expression of anti-inflammation cytokines. (A-C) Protein concentration of IL-6, TNF-α and IL-10 in culture supernatant measured by ELISA. (D-F) Gene expression of IL-6, TNF-α and IL-10 measured by qRT-PCR. Difference lowercases represent statistical significance between different groups (P<0.05).

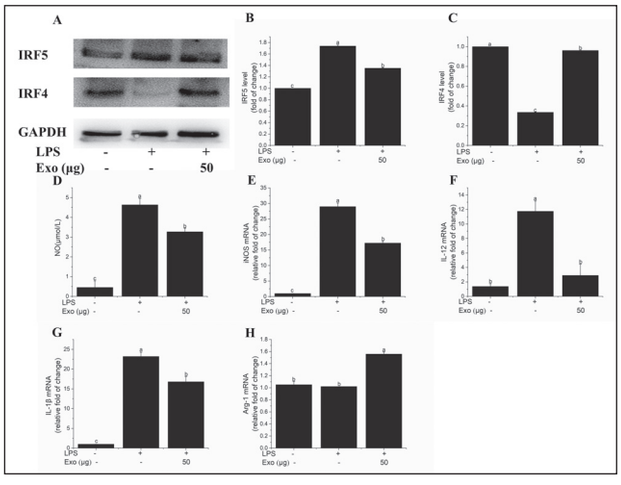


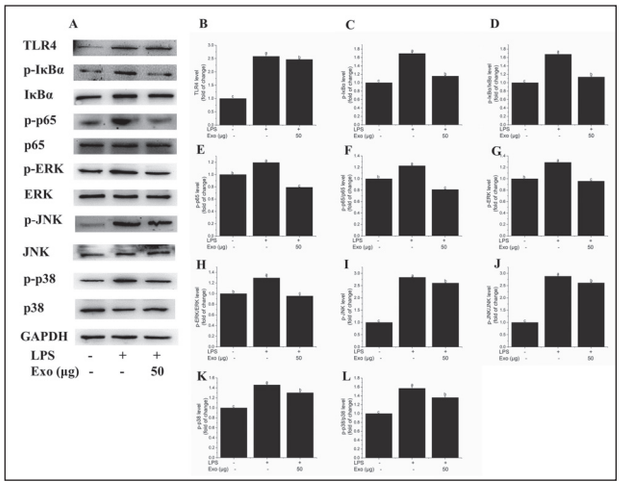
Fig. 4: HplMSC-Exos inhibited M1-type polarization in RAW264.7 cells. (A) Western blot analysis of IRF5 and IRF4. (B, C) Protein semi-quantitative analysis of IRF4 and IRF5. (D) Secretion of NO. (E-H) Gene expression of iNOS, IL-12, IL-1β and Arg-1 detected by qRT-PCR. Difference lowercases represent statistical significance between different groups (P<0.05).

2.4. HplMSCs-Exos reduced M1 polarization in LPS-induced RAW264.7 cells

After hplMSCs-Exos treatment, the expression of Interferon regulatory factor 5 (IRF5) significantly decreased whereas Interferon regulatory factor 4 (IRF4) significantly increased (Figs. 4A–4C). The secretion of NO prominently decreased (Fig. 4D). In addition, the transcription levels of iNOS, IL-12, and IL-1β decreased remarkably (Figs. 4E–4G). On the contrary, the transcription levels of Arg-1 increased (Fig. 4H). This finding indicated that hplMSCs-Exos can reduce the polarization of macrophages to M1 type.

2.5. HplMSC-Exos inhibited the activation of TLR4-mediated NF-κB/ MAPK signaling pathway

TLR4/ NF-κB/ MAPK signaling pathway is relatively closely related to M1 polarization and inflammation; thus, we investigated the potential role of hplMSC-Exos in this pathway. As shown in Fig. 5, TLR4 was activated by LPS, which subsequently resulted in the phosphorylation of IκBα and p65. The expression of p-ERK1/2, p-JNK, and p-p38 was also enhanced. However, after dealing with hplMSC-Exos, the expression of these proteins decreased and was statistically significant (P<0.05). In addition, the activation of NF-κB can lead to the nuclear translocation of its subunit. As shown in Fig. 5M, after LPS stimulation, NF-κB p65 was transferred to the nucleus, but hplMSC-Exos treatment inhibited this process.



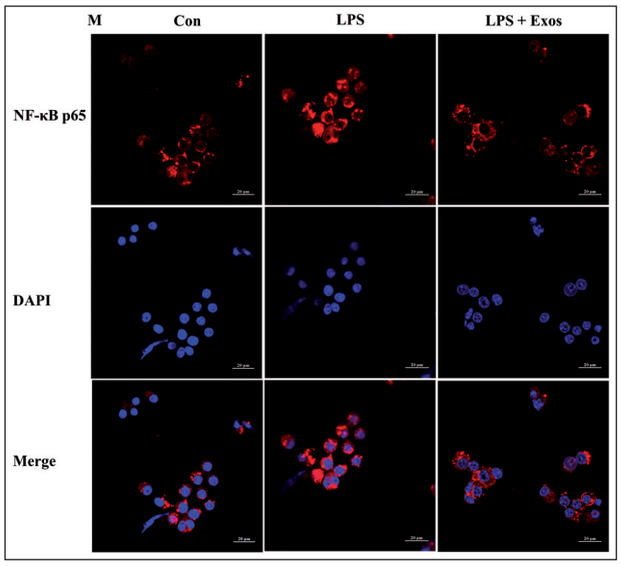


Fig. 5: Effects of hplMSC-Exos on the expression of TLR4/ NF-κB/ MAPK signaling pathway related proteins in RAW264.7 cells. (A) Western blot analysis of TLR4/ NF-κB/ MAPK signaling pathway related proteins. (B-L) Protein semi-quantitative analysis of TLR4/ NF-κB/ MAPK signaling pathway related proteins. Difference lowercases represent statistical significance between different groups (P<0.05). (M) Effects of hplMSC-Exos on the nucleus translocation of transcription factor NF-κB. Confocal immunofluorescence images of NF-κB nucleus translocation of the subunit p65 in RAW264.7 cells. Red: NF-κB p65; Blue: DAPI, nucleus.

2.6. HplMSC-Exos involved in PI3K/ Akt signaling pathway

PI3K/ Akt signaling pathway also plays an important role in inflammatory response and regulation. We explored whether exosomes regulate inflammation through this pathway. Compared with the blank control group, the expression of phosphorylated PI3K and Akt in LPS group increased prominently, and the addition of hplMSC-Exos reduced the expression of phosphorylated PI3K and Akt (P<0.05, Fig. 6).

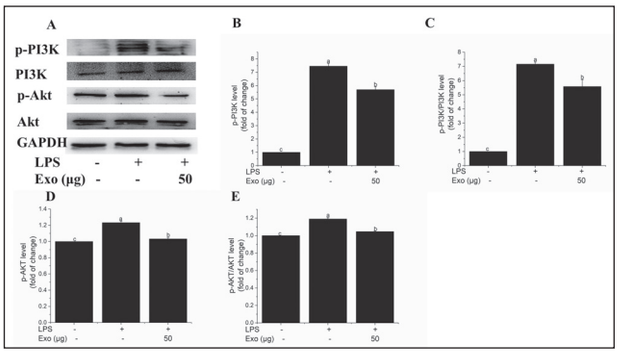


Fig. 6: Effects of hplMSC-Exos on the expression of PI3K/ Akt signaling pathway related proteins in RAW264.7 cells. (A) Western blot analysis of PI3K/ Akt signaling pathway related proteins. (B-E) Protein semi-quantitative analysis of PI3K/ Akt signaling pathway related proteins. Difference lowercases represent statistical significance between different groups (P<0.05).

2.7. HplMSC-Exos alleviates LPS-induced cell apoptosis

Western blotting was performed to study the effect of exosomes on cell apoptosis. Compared with the control group, the expression of Bax and Cleaved Caspase3 was significantly increased, whereas Bcl-2 expression notably decreased in the LPS-induced group (P<0.05). On the contrary, hplMSC-Exos significantly decreased the expression of Bax and cleaved caspase 3 and increased the expression of Bcl-2 (P<0.05, Fig. 7). This finding indicated that hplMSC-Exos could inhibit the apoptosis in LPS-induced RAW264.7 cells.

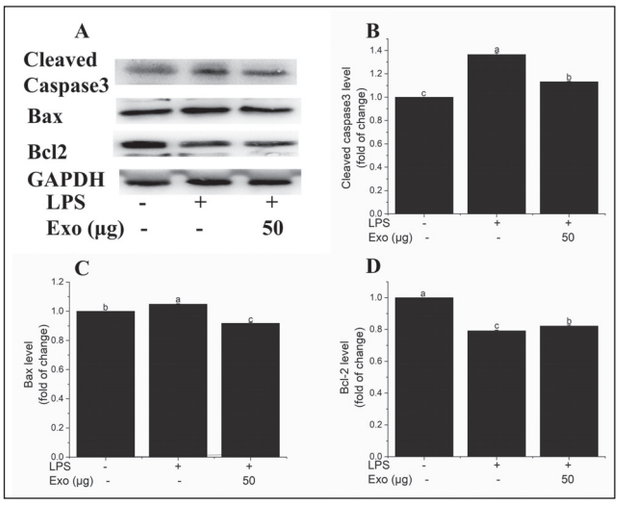


Fig. 7: Effects of hplMSC-Exos on the expression of apoptosis-related protein in RAW264.7 cells. (A) Western blot analysis of apoptosis-related proteins. (B-E) Protein semi-quantitative analysis of apoptosis-related proteins. Difference lowercases represent statistical significance between different groups (P<0.05).

3. Discussion

MSCs are a type of progenitor cells with the ability to update themselves, with a number of differentiation potential, low immunogenicity, and easy to obtain; they are widely used in organ transplantation, tissue engineering, and gene therapy (Nagamura-Inoue and He 2014; Payne et al. 2013). Studies have shown that MSCs are mainly involved in inflammatory response by affecting the secretion of immune cell proliferation, differentiation, and inflammatory cytokines (Shi et al. 2018). In addition,

MSCs have targeted homing capabilities, which can be directed to the damaged portion and colonized to repair tissues (Sordi et al. 2005). However, the low survival rate and abnormal differentiation of MSCs are the main disorders of their clinical application. MSC-derived extracellular vesicles in a diameter of 40–200 nm, namely exosomes, have a biological function similar to their source cells. In addition, exosomes do not cause abnormal differentiation and are easy to store; they have higher therapeutic values (Fais et al. 2016). Here, we collected MSCs of human placenta and extracted exosomes with differential ultracentrifugation. Vesicles were cup-shaped structures under TEM, and NTA displayed that the particle size was between 40 and 200 nm. Western blot showed that these vesicles specifically expressed CD9, CD63, and TSG101 which are exosomal surface markers, indicating that we successfully extracted hplMSCs-Exos (Fig. 1). In addition, we found that endotoxin in hplMSC-Exos were very low with a concentration between 10μg/mL and 90μg/mL (Fig. 2).

Inflammation is a type of defense response to infection and injury. Macrophages, which occupy a prominent position in immune system, serve an important role during inflammation. Macrophages mainly distinguish benign and harmful microbes through pathogen-related molecular patterns (Mogensen 2009). Macrophages can adapt to changes in microenvironment by changing their phenotypes and physiological properties, namely, the plasticity and heterogeneity of macrophages (Mosser and Edwards 2008). Bacterial, LPS, IFN-γ, and TNF can promote the formation of M1. Conversely, parasites, fungi (Noel et al. 2004), IL-4, and IL-13 (Martinez et al. 2008) can promote macrophages to M2-type polarization. M1 macrophages can induce the production of active oxygen, promote the synthesis of nitric oxide, and release various proinflammatory cytokines, such as IL-6 and TNF-α, thereby promoting inflammation and exacerbate tissue damage. M2 macrophages release anti-inflammatory cytokines IL-10, TGF-β, and the like to promote inflammation subsided and tissue repair (Mantovani et al. 2004). We used LPS to stimulate RAW264.7 cells to establish an inflammatory model *in vitro*. After hplMSC-Exos treatment, the expression of pro-inflammatory cytokines IL-6 and TNF-α significantly decreased, and the expression of anti-inflammatory factor IL-10 significantly increased (Fig. 3). Moreover, due to hplMSCs-Exos treatment, the concentration of NO and the transcriptional levels of M1 macrophages markers (IL-12, IL-1β, and iNOS) declined, whereas the expression of M2 macrophages marker (Arg-1) significantly improved (Figs. 4D–4G), demonstrating that hplMSC-derived exosomes can inhibit the polarization of macrophages to M1 type.

Interferon regulatory factors (IRF) are involved in the regulation of polar transformation of macrophages, where IRF5 and IRF4 are key factors of macrophages to M1- and M2-type polarization. IRF5 can combine the promoter sequence of the M1 type marker IL-12 and activate IL-12, which induces cells to M1 polarization (Takaoka et al. 2005). IRF4 is related to M2 type polarization by activating Jmjd3-mediated H3k27 demethylation (Satoh et al. 2010). We found that hplMSC-Exos can reduce the expression of IRF5 and upgrade the expression of IRF4 in LPS-induced RAW264.7 cells (Figs. 4A–4C), further confirming that hplMSC-derived exosomes can restrain macrophages to M1-type polarization.

TLR4 is an important member of TLR family and is a significant pattern recognition receptor (PRRs) in innate immune system, which is a key figure in antibacterial and antiinfection. TLR4 is the main receptor of bacterial infection; it activates various intracellular signaling transductions through identifying pathogen-associated molecular patterns (PAMPs), prominently the activation of transcription factor NF-κB (Kumar et al. 2011). TLR4 is activated by LPS at the aid of LPS-binding protein (LBP) and CD14 (Ciesielska et al. 2021), and the downstream IκBα is phosphorylated and degraded. Subsequently, the NF-κB p65 subunit is activated after phosphorylation; it enters into the nucleus, starting the expression of a series downstream targets, such as inflammatory cytokines (IL-6, TNF-α) and protease (iNOS). The latter further activates NF-κB by positive feedback, resulting in a cascading reaction, which further expands and extends inflammation (Lai et al. 2017). Mitogen-activated protein kinases (MAPKs), containing c-Jun N-terminal kinase (JNK), extracellular-regulated kinase (ERK), and p38, are also activated by LPS-combined TLR4 (An et al. 2002). Activated MAPKs perform pro-inflammation effects by promoting the release of inflammation cytokines (TNF-α, IL-1β, IL-6, and IL-12). Therefore, we explored whether hplMSC-Exos can slow down the inflammation process. Our results showed that hplMSC-Exos can inhibit the expression of TLR4 and the activation and signal transduction of NF-κB signaling pathway by suppressing phosphorylation of NF-κB p65 subunit and IκBα and the nucleus translocation of NF-κB in LPS-induced RAW264.7 cells. Similarly, we found that exosomes reduced the phosphorylation of ERK, JNK, and p38; thus, the activation of p38/MAPK signaling pathway was repressed (Fig. 5). Accordingly, we speculated that hplMSC-Exos can play a protective role in inflammation via TLR4/ NF-κB/ MAPK signaling pathway.

Phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway is another important intracellular signaling pathway involving inflammation and participates in regulating cell survival and apoptosis. PI3Ks are signal downstream molecules of TLR4, which can be activated by phosphorylation after LPS treatment. Subsequently, Akt is activated, leading to the initiation of downstream signaling pathways and releasing inflammatory cytokines and pro-apoptotic proteins; thus, apoptosis and tissue damage are promoted (Cianciulli et al. 2020; Song et al. 2005). We discovered that compared with the LPS group, hplMSC-Exos treatment reduced the phosphorylation level of PI3K and Akt, indicating that the activation of Akt was inhibited (Fig. 6). After hplMSCs-Exos treatment, the expression of apoptotic protein Bcl2-associated X (Bax) and Cleaved Caspase3 was significantly down-regulated, and the expression of anti-apoptotic protein B-cell lymphoma-2 (Bcl2) was significantly up-regulated (Fig. 7). In summary, these results indicate that hplMSC-Exos inhibits the activation of PI3K/ Akt and the apoptosis of RAW264.7 cells.

In conclusion, hplMSC-Exos can inhibit M1 polarization of RAW264.7 cells, reducing the production of pro-inflammatory cytokines and their effects related to TLR4-mediated NF-κB/ MAPK and PI3K/ Akt signaling pathways. The anti-inflammatory roles of hplMSC-Exos occurred in inhibiting the phosphorylation of IκBα, p65, ERK, JNK, p38, PI3K, and Akt, the nucleus translocation of NF-κB and the expression of apoptosis-related proteins, thereby relieving inflammation. These findings may identify a novel noncellular therapy for inflammation-related diseases.

4. Experimental

4.1. Chemicals and reagents

LPS (L6529) extracted from Escherichia coli O55:B5 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), penicillin G, streptomycin, and Dulbecco's modified eagle medium (DMEM) were purchased from Gibco (Carlsbad, CA, USA). The rabbit anti-IRF5, anti-IRF4, anti-TLR4 and anti-GAPDH were purchased from Beyotime (Shanghai, China). The rabbit anti-phospho-p65 and anti-p65 were purchased from Sangon Biotech (Shanghai, China). The rabbit anti- phospho-IκBα and anti-IκBα were purchased from Abcam (Cambridge, UK). The rabbit anti-phospho-ERK, anti-ERK, anti-phospho-JNK, anti-JNK, anti-phospho-p38, anti-p38, anti-phospho-PI3K, anti-PI3K, anti-phospho-Akt, anti-Akt, anti-Cleaved caspase3, anti-Bax and anti-Bcl2 were purchased from Cell Signaling Technology (Mass, USA).

4.2. Cell culture and treatment

The placenta of full-term health woman was obtained from Affiliated Hospital of Jiangnan University (Wuxi, China). The informed consent of the volunteer maternity was acquired and the experiment was approved by ethics committee of Affiliated Hospital of Jiangnan University (LS2021046).

HplMSCs were extracted with tissue blocks and sorted to purify by EasySep™ Human CD271 positive selection kit II and EasySep™ magnet (StemCell Tech, Vancouver, Canada). Typical MSCs' morphology under microscope, flow cytometry and Adipogenic and osteogenic differentiation verified the successful extraction of HplMSCs (Data not shown). These hplMSCs were cultured in complete MSCM (Sciencell, USA) containing 5% fetal bovine serum, 1% MSC growth supplement (MSCGS), and 1% antibiotics (100 U/mL penicillin and 100 g/mL streptomycin) at 37 °C in a humidified atmosphere containing 5% CO_2. Cells at passage 3 to 9 were used for experiments.

Macrophage-like cell line RAW264.7 cells were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). They were cultured in DMEM containing high glucose (4.5 g/L) and 10% fetal calf serum at 37 °C in a humidified

atmosphere containing 5% CO_2. Cells were used for experiment when grown to exponential phase.

To investigate the roles of hplMSC-derived exosomes in regulating inflammation and the underlying mechanism, RAW264.7 cells were divided into the following groups: (I) blank control: cells cultured in DMEM medium; (II) LPS group: cells cultured in DMEM containing 1 μg/mL LPS; (III) LPS+Exo group: cells subjected to 1 μg/mL LPS for 4 h prior to addition of 50 μg/mL exosomes for 24 h.

4.3. Extraction of exosomes

For extracting exosomes, when the cell growth density reached 70%–80%, the complete medium was changed to serum-free medium and then cultured for 48 h. The conditioned medium was collected, and differential ultracentrifugation was used to purify exosomes, as previously reported (Thery et al. 2006). In summary, the conditioned medium was centrifuged at 300, 2,000, and 10,000 g for 10 min, 10 min, and 60 min, respectively, to pellet, and cells, dead cells and cell debris were removed. All supernatants were stored for the next process. Subsequently, the supernatant was centrifuged at 100,000 g for 70 min twice using a Hitachi CP80NX ultracentrifuge (Hitachi High-Technologies, Tokyo, Japan), and the precipitation was obtained each time. Finally, the precipitation was resuspended by modest PBS to obtain the exosomes and stored at −80 °C for subsequent experiments. All the centrifugation steps were performed at 4 °C. The exosomal protein concentration was detected by a BCA Protein Assay Kit (Beyotime, Shanghai, China).

4.4. Characterization of exosomes

The morphology of exosomes was detected by transmission electron microscopy (TEM). In summary, the exosomes were resuspended in PBS and pipetted 10 μL to the Formvar/Carbon film-coated transmission electron microscope grid. After storing for 3 min, the excess liquid was removed from the grid using Whatman paper, and the grid was dried overnight. Finally, the exosomes were observed under JEOL-2100F TEM (JEOL, Japan) at 120 kV. The size of exosomes was detected by Malvern's Nanosight NS300 (Britain). The particle size distribution of the exosomes in the suspension was obtained using the scattering and Brownian motion. The protein expression of exosomes was detected by western blotting analysis. The specific antibodies for TSG101 (Abcam, Cambridge, UK), CD9 (Abcam, Cambridge, UK) and CD63 (Abcam, Cambridge, UK) were used to identify exosomes. The dilution ratio of these antibodies was 1:1,000.

4.5. Cell proliferation assay

RAW264.7 cells were placed in a 96-well plate at a density of 10^4 cells/wells containing exosome-depleted complete medium. Different concentration of hplMSC-Exos (10 μg/mL, 30 μg/mL, 50 μg/mL, 70 μg/mL, and 90 μg/mL) were added to incubate for 24 h at 37 °C, 5% CO_2. MTT assay was used to evaluate the cell viability. The absorbance was measured at 570 nm by a microplate reader. Cell activity was calculated according to the following formula:

Cell vialibity (%) = $OD_{\text{absorbance of exosomal group}}$ / $OD_{\text{absorbance of control group}}$ × 100

4.6. Total RNA extraction and real-time quantitative polymerase chain reaction (qRT-PCR)

The total RNA was extracted from each group using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. The extracted RNA was adjusted to the same concentration and preformed reverse transcription using RevertAid First Strand cDNA Synthesis Kit (Thermo, USA). The gene expression was quantified through a qRT-PCR by using UltraSYBR mixture in a Roche light cycler 96 real time system. The amount of GAPDH in the same cDNA was used to normalize the relative amount of each transcript, and $2^{-\Delta\Delta Ct}$ method was used for data analysis. The primer sequences of each gene are shown in the Table.

4.7. Detection of nitric oxide (NO) generation

NO generation was detected by using a commercial NO assay kit (Beyotime, Shanghai, China) under the manufacturer's protocol. Each result was tested at least three times.

4.8. Enzyme-linked immunosorbent assay (ELISA)

Concentration of cytokines (IL-6, TNF-α) secreted by RAW264.7 cells was determined by a commercial ELISA kit (Invitrogen, USA) under the manufacturer's guidance.

4.9. Western Blot analysis

Western blot was performed to explore the mechanism of exosomes on inflammation in vitro. Cells were lysed with RIPA lysis buffer containing a protease inhibitor to extract total protein. Then, the lysed cells were centrifuged at 12,000g at 4 °C for 15 min, and the supernatant was collected. A BCA Protein Assay Kit (Beyotime, Shanghai, China) was used to detect the protein concentration. Denatured proteins were separated using 10% SDS-PAGE prior to transfer to polyvinylidene difluoride membranes (Millipore, MA, USA). The membranes were blocked with rapid block buffer (Beyotime, Shanghai, China) for 10 min at room temperature. After using TBST washing thrice, the membranes were incubated with primary antibodies overnight at 4 °C. Subsequently, the membranes were washed with TBST thrice and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG for 1 h. Finally, the membranes, which had been washed by TBST thrice, were photographed using the Tanon-2500B image analysis system (China). The semiquantitative protein quantification of each immunoreactive bands was performed by the Image J software, where GAPDH acted as a probed for loading control in the same membrane.

4.10. Effect of hplMSC-Exos on NF-κB nucleus translocation

Immunofluoresence was used to determine the effects of hplMSC-Exos on NF-κB nucleus translocation. RAW264.7 cells (2×10^5) were incubated in a confocal plate and cultured in 37 °C, 5% CO_2 for 24h according to the above treatment. PBS, 4 % paraformaldehyde solution and immunostaining blocking buffer (Beyotime, Shanghai, China) were used to wash, fix and block the cells, after which the cells were incubated with rabbit monoclonal antibody NF-κB p65 (Beyotime, Shanghai, China) at 4 °C overnight. Then the cells were washed thrice by PBS and incubated with Cy3-labeled goat anti-rabbit IgG (Beyotime, Shanghai, China) for 1 h at room temperature in dark. DAPI (Beyotime, Shanghai, China) was used to incubate for another 10 min, and followed by PBS washing thrice, the cells were observed under a CLMS (Nikon, Japan).

4.11. Statistical analysis

Statistical analysis was performed by using SPSS 26.0 statistical software. All data are presented as the mean±standard deviation (SD). Significant differences among groups were evaluated by one-way ANOVA. Statistical differences were indicated by different lowercase letters. $P < 0.05$ was considered statistically significant.

Conflicts of interest: The authors declare no conflict of interest.

Table: Primer sequences used for reverse transcription-quantitative polymerase chain rection analysis

Target gene	Primer sequence (5'-3')	
	Forward	Reverse
IL-6	ACAACCACGGCCTTCCCTACTT	CACGATTTCCCAGAGAACATGTG
TNF-α	AAGCCTGTAGCCCACGTCGTA	GGCACCACTAGTTGGTTGTCTTTG
IL-10	CCCTTTGCTATGGTGTCCTT	TGGTTTCTCTTCCCAAGACC
iNOS	GAGCTCGGGTTGAAGTGGTATG	GAAACTATGGAGCACAGCCACAT
IL-12	GGAAGCACGGCAGCAGAATA	AACTTGAGGGAGAAGTAGGAATGG
IL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
Arg-1	AGACAGCAGAGGAGGTGAAGAG	CGAAGCAAGCCAAGGTTAAAGC
GAPDH	GAGCCAAACGGGTCATCATCT	GAGGGGCCATCCACAGTCTT

References

An H, Yu Y, Zhang M, Xu H, Qi R, Yan X, Liu S, Wang W, Guo Z, Guo J, Qin Z, Cao X (2002) Involvement of ERK, p38 and NF-kappaB signal transduction in regulation of TLR2, TLR4 and TLR9 gene expression induced by lipopolysaccharide in mouse dendritic cells. Immunology 106, 38–45.

Cargnello M, Roux PP (2011) Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol Mol Biol Rev 75: 50–83.

Chai EZ, Siveen KS, Shanmugam MK, Arfuso F, Sethi G (2015) Analysis of the intricate relationship between chronic inflammation and cancer. Biochem J 468, 1–15.

Chan BD, Wong WY, Lee MM, Cho WC, Yee BK, Kwan YW, Tai WC (2019) Exosomes in inflammation and inflammatory disease. Proteomics 19: e1800149.

Cianciulli A, Porro C, Calvello R, Trotta T, Lofrumento DD, Panaro MA (2020) Microglia mediated neuroinflammation: focus on PI3K modulation. Biomolecules 10: 137.

Ciesielska A, Matyjek M, Kwiatkowska K (2021) TLR4 and CD14 trafficking and its influence on LPS-induced pro-inflammatory signaling. Cell Mol Life Sci 78: 1233–1261.

Deans RJ, Moseley AB (2000) Mesenchymal stem cells: biology and potential clinical uses. Exp Hematol 28: 875–884.

Fais S, O'Driscoll L, Borras FE, Buzas E, Camussi G, Cappello F, Carvalho J, Cordeiro da Silva A, Del Portillo H, El Andaloussi S, Ficko Trček T, Furlan R, Hendrix A, Gursel I, Kralj-Iglic V, Kaeffer B, Kosanovic M, Lekka ME, Lipps G, Logozzi M, Marcilla A, Sammar M, Llorente A, Nazarenko I, Oliveira C, Pocsfalvi G, Rajendran L, Raposo G, Rohde E, Siljander P, van Niel G, Vasconcelos MH, Yáñez-Mó M, Yliperttula ML, Zarovni N, Zavec AB, Giebel B (2016) Evidence-based clinical use of nanoscale extracellular vesicles in nanomedicine. ACS Nano 10: 3886–3899.

Fong CH, Bebien M, Didierlaurent A, Nebauer R, Hussell T, Broide D, Karin M, Lawrence T (2008) An antiinflammatory role for IKKbeta through the inhibition of "classical" macrophage activation. J Exp Med 205: 1269–1276.

Gordon S (2007) The macrophage: past, present and future. Eur J Immunol 37 Suppl 1: S9–17.

Hawkins PT, Stephens LR (2015) PI3K signalling in inflammation. Biochim Biophys Acta 1851: 882–897.

Kalluri R, LeBleu VS (2020) The biology, function, and biomedical applications of exosomes. Science 367(6478): eaau6977

Kumar H, Kawai T, Akira S (2011) Pathogen recognition by the innate immune system. Int Rev Immunol, 30: 16–34.

Lai JL, Liu YH, Liu C, Qi MP, Liu RN, Zhu XF, Zhou QG, Chen YY, Guo AZ, Hu CM (2017) Indirubin inhibits LPS-induced inflammation via TLR4 abrogation mediated by the NF-kB and MAPK signaling pathways. Inflammation 40: 1–12.

Li T, Yan Y, Wang B, Qian H, Zhang X, Shen L, Wang M, Zhou Y, Zhu W, Li W, Xu W (2013) Exosomes derived from human umbilical cord mesenchymal stem cells alleviate liver fibrosis. Stem Cells Dev 22: 845–854.

Libby P (2007) Inflammatory mechanisms: the molecular basis of inflammation and disease. Nutr Rev 65: S140–146.

Lou G, Chen Z, Zheng M, Liu Y (2017) Mesenchymal stem cell-derived exosomes as a new therapeutic strategy for liver diseases. Exp Mol Med 49: e346.

Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M (2004) The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol 25: 677–686.

Martinez FO, Sica A, Mantovani A Locati, M. (2008) Macrophage activation and polarization. Front Biosci 13, 453–461.

Medzhitov R (2008) Origin and physiological roles of inflammation. Nature 454(7203): 428–435.

Mogensen TH (2009) Pathogen recognition and inflammatory signaling in innate immune defenses. Clin Microbiol Rev 22: 240–273.

Mosser DM, Edwards JP (2008) Exploring the full spectrum of macrophage activation. Nat Rev Immunol 8: 958–969.

Nagamura-Inoue T, He H (2014) Umbilical cord-derived mesenchymal stem cells: Their advantages and potential clinical utility. World J Stem Cells 6: 195–202.

Newton K, Dixit VM (2012) Signaling in innate immunity and inflammation. Cold Spring Harb Perspect Biol 4: a006049

Noel W, Raes G, Hassanzadeh Ghassabeh G, De Baetselier P, Beschin A (2004) Alternatively activated macrophages during parasite infections. Trends Parasitol 20: 126–133.

Payne NL, Sun G, McDonald C, Layton D, Moussa L, Emerson-Webber A, Veron N, Siatskas C, Herszfeld D, Price J, Bernard CC (2013) Distinct immunomodulatory and migratory mechanisms underpin the therapeutic potential of human mesenchymal stem cells in autoimmune demyelination. Cell Transplant 22: 1409–1425.

Peng SC, Wong DS, Tung KC, Chen YY, Chao CC, Peng CH, Chuang YJ, Tang CY (2010) Computational modeling with forward and reverse engineering links signaling network and genomic regulatory responses: NF-kappaB signaling-induced gene expression responses in inflammation. BMC Bioinformatics 11: 308.

Peroval MY, Boyd AC, Young JR, Smith AL (2013) A critical role for MAPK signalling pathways in the transcriptional regulation of toll like receptors. PLoS One 8: e51243.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284: 143–147.

Satoh T, Takeuchi O, Vandenbon A, Yasuda K, Tanaka Y, Kumagai Y, Miyake T, Matsushita K, Okazaki T, Saitoh T, Honma K, Matsuyama T, Yui K, Tsujimura T, Standley DM, Nakanishi K, Nakai K, Akira S (2010) The Jmjd3-Irf4 axis regulates M2 macrophage polarization and host responses against helminth infection. Nat Immunol 11: 936–944.

Savva A, Roger T (2013) Targeting toll-like receptors: promising therapeutic strategies for the management of sepsis-associated pathology and infectious diseases. Front Immunol 4: 387.

Segonzac C, Zipfel C (2011) Activation of plant pattern-recognition receptors by bacteria. Curr Opin Microbiol 14: 54–61.

Shi Y, Wang Y, Li Q, Liu K, Hou J, Shao C, Wang Y (2018) Immunoregulatory mechanisms of mesenchymal stem and stromal cells in inflammatory diseases. Nat Rev Nephrol 14: 493–507.

Song G, Ouyang G, Bao S (2005) The activation of Akt/PKB signaling pathway and cell survival. J Cell Mol Med 9: 59–71.

Sordi V, Malosio ML, Marchesi F, Mercalli A, Melzi R, Giordano T, Belmonte N, Ferrari G, Leone BE, Bertuzzi F, Zerbini G, Allavena P, Bonifacio E, Piemonti L. (2005) Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets. Blood 106: 419–427.

Sun L, Li D, Song K, Wei J, Yao S, Li Z, Su X, Ju X, Chao L, Deng X, Kong B, Li L (2017) Exosomes derived from human umbilical cord mesenchymal stem cells protect against cisplatin-induced ovarian granulosa cell stress and apoptosis in vitro. Sci Rep 7: 2552.

Sun ZJ, Chen G, Hu X, Zhang W, Liu Y, Zhu LX, Zhou Q, Zhao YF (2010) Activation of PI3K/Akt/IKK-alpha/NF-kappaB signaling pathway is required for the apoptosis-evasion in human salivary adenoid cystic carcinoma: its inhibition by quercetin. Apoptosis 15: 850–863.

Takaoka A, Yanai H, Kondo S, Duncan G, Negishi H, Mizutani T, Kano S, Honda K, Ohba Y, Mak TW, Taniguchi T (2005) Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. Nature 434: 243–249.

Thery C, Amigorena S, Raposo G, Clayton A (2006) Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol, Chapter 3, Unit 3 22. doi:10.1002/0471143030.cb0322s30

Vanhaesebroeck B, Whitehead MA, Pineiro R (2016) Molecules in medicine mini-review: isoforms of PI3K in biology and disease. J Mol Med, 94: 5–11.

Zelenay S, Reis e Sousa C (2013) Adaptive immunity after cell death. Trends Immunol 34: 329–335.