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ORIGINAL ARTICLE

Umbilical Cord Blood Mesenchymal Stem Cells Enhance Lipopolysaccharide-Induced IL-10 and IL-37 Production in THP-1 Cells

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Abstract—Umbilical cord blood mesenchymal stem cells (UCB-MSCs) have been shown to be a source of stem cells for use in cellular therapies and have immunomodulatory effects on several immune cells in an inflammatory environment. However, whether UCB-MSCs have immunomodulatory effects against lipopolysaccharide (LPS)-induced inflammatory cytokine secretion in macrophages and whether it is involved in phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) signaling pathway remain unclear. After co-culture of UCB-MSCs and phorbol 12-myristate 13-acetate (PMA)-activated human THP-1 cells using a transwell system, it showed that LPS significantly induced increases in the expression levels of interleukin 10 (IL-10), interleukin 37 (IL-37), phospho-PI3K (p-PI3K), and phospho-Akt (p-Akt) in macrophages. UCB-MSCs upregulated the expression of IL-10, IL-37, p-PI3K, and p-Akt, while it had no obvious effect on PI3K and Akt levels. Inhibitors of PI3K (LY294002) significantly suppressed the expression of IL-10, IL-37, p-PI3K, and p-Akt; however, it had no effect on the expression levels of PI3K and Akt. The present study demonstrated that UCB-MSCs increased the LPS-stimulated expression of IL-10 and IL-37 in macrophages through the PI3K/Akt signaling pathway.

KEY WORDS: umbilical cord blood mesenchymal stem cells; THP-1 cells; IL-10; IL-37; immunomodulation.

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Abbreviations: *UCB-MSCs*, Umbilical cord blood mesenchymal stem cells; *PI3K/Akt*, Hosphoinositide 3-kinase/protein kinase B; *LPS*, Lipopolysaccharide; *IL-10*, Interleukin 10; *IL-37*, Interleukin 37; *PMA*, Phorbol 12-myristate 13-acetate

INTRODUCTION

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection [1]. Gram-negative bacteria are common pathogenic bacteria causing sepsis in clinical practice, whose main pathogenic component is lipopolysaccharide (LPS). A large number of studies have reported that LPS stimulation as a model of endotoxemia is frequently used to understand the pathophysiology of sepsis and test new therapies [2–5]. In addition, *in vitro* studies have shown that LPS can increase the expression of inflammatory cytokines in macrophages [6, 7], which have crucial roles in the cytokine storm and sepsis [8]. Despite recent advances in antibiotic therapies and intensive care, there are still no effective therapies for the disease and sepsis is still one of the main causes of

death in intensive care units [9]. Therefore, new treatments are urgently needed to improve clinical outcomes. Recent studies have demonstrated that mesenchymal stem cells (MSCs) may become new therapeutic candidates for the treatment of sepsis due to their immunomodulatory and immunosuppressive activities [10, 11].

Umbilical cord blood mesenchymal stem cells (UCB-MSCs) are isolated from human umbilical cord blood, so they are easily accessible and could not be harmful to the human body (both mother and infant) [12]. Additionally, they are antigenically primitive, relatively invulnerable to immunologic challenges, a low risk of viral contamination and there are no ethical barriers to their use [13]. Recent studies have suggested that UCB-MSCs also had immunomodulatory functions, such as effects on inhibiting the proliferation of PB lymphocytes, immunosuppressive properties against lymphoid cell populations, and improving the survival of the human neuronal cells [14–16]. However, whether UCB-MSCs could affect the expression of anti-inflammatory mediators interleukin 10 (IL-10) and interleukin 37 (IL-37) in macrophages remains less known.

Macrophage dysfunction is a major manifestation of sepsis-induced immunosuppression. After endotoxin stimulates macrophages, they secrete a large number of cytokines and other inflammatory mediators to cause inflammatory cascade amplification [17, 18]. After treated with PMA, a human monocytic leukemia cell line (THP-1) can be induced to differentiate to macrophages [19], which can be stimulated by LPS as cell model to explore the inflammatory response of sepsis [20, 21]. It was reported that LPS elicited the expression of cytokines IL-10 and IL-37 in THP-1 cells, partly through the phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) signaling pathway [22–24].

Using transwell co-culture of UCB-MSCs and macrophages in a cell model of endotoxemia induced by LPS, we investigated the effects of UCB-MSCs on the production of IL-10 and IL-37 and explored whether the PI3K/Akt signaling pathway was involved in LPS- induced IL-10 and IL-37 expression in macrophages.

MATERIALS AND METHODS

Cell Culture and Treatment

UCB-MSCs were donated by Boya Life Stem Cell Technology and have been identified by flow cytometry [25] and cultured in DMEM medium (NanJing KeyGen Biotech Co., Ltd., Nanjing, China) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. MSCs between passages four and seven were used for the subsequent experiments. Human THP-1 cells were donated by the Key Laboratory of Systematic Bioengineering, Ministry of Education, Tianjin University and cultured in RPMI1640 medium (NanJing KeyGen Biotech Co., Ltd., Nanjing, China) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. UCB-MSCs were seeded in the upper compartment of transwells (0.4 μM pore size, Corning, Lowell, MA, USA) 12 h. THP-1 cells were cultured in 6-well plate and pretreated for 48 h with 100 ng/mL PMA (Beyotime Institute of Biotechnology, Shanghai, China) to induce differentiation to macrophages [26], followed by washing three times with phosphate buffer saline. In some experiments, THP-1 macrophages were treated 10 μM LY294002 (Sigma-Aldrich, St. Louis, MO, USA) for 1 h before administering the LPS treatment. Macrophages (5.0×10^5 cells per cell) were co-cultured with UCB-MSCs directly or in the lower compartment of transwells (UCB-MSCs: macrophages = 1:5). Macrophages and UCB-MSCs were then co-incubated in the presence of LPS (1 μg/mL) for 24 h. The supernatants were collected for enzyme-linked immunosorbent assay (ELISA) assay, and cells were collected for RNA or protein extraction.

ELISA Assay

For co-culture experiment, the supernatants were collected after co-culture for 24 h. For single-culture experiment, the supernatants were collected after UCB-MSCs supernatant was added into the wells of LPS-stimulated THP-1 macrophages for 24 h. The protein levels of IL-10 and IL-37 in the cell culture supernatants were detected using human IL-10 ELISA kit and human IL-37 ELISA kit (ABclonal Biotech Co., Ltd., Cambridge, USA) according to the manufacturer's instructions.

Real-Time Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Tiangen Biotech Co. Ltd., Beijing China) following the manufacturer's instructions. One microgram of RNA was reverse-transcribed with specific antisense primer by using the one-step RT-PCR kit in accordance with the manufacturer's instructions (Vazyme Biotech Co., Ltd., Nanjing, China). RT-PCR was conducted using LightCycler® 480 type II system (Roche, Basel, Switzerland). The primers used for the PCR amplifications are as follows: GAPDH forward, AGA AGG CTG GGG CTC

ATT TG; GAPDH reverse, AGG GGC CAT CCA CAG TCT TC; IL-10 forward, TCT CCG AGA TGC CTT CAG CAG A; IL-10 reverse, TCA GAC AAG GCT TGG CAA CCC A; IL-37 forward, AGT GCT GCT TAG AAG ACC CGG; IL-37 reverse, AGA GTC CAG GAC CAG TAC TTT GTG A. The cycling conditions were as follows: 95 °C for 30 s, 40 cycles of 95 °C for 5 s, and 60 °C for 30 s. All samples were measured in triplicate. Differences in gene expression were calculated using the $2^{-\Delta\Delta ct}$ method.

Western Blotting

Total cell lysates were prepared using RIPA lysis buffer plus protease inhibitors and phosphatase inhibitors. Protein concentration was measured using the NanoDrop ND-2000 spectrophotometer (Thermo Forma, USA). Samples of cell lysates were separated by 10% SDS/PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Following blocking with 5% bovine serum albumin for 2 h at room temperature, the membranes were incubated at 4 °C overnight with a primary antibody recognizing PI3K (Proteintech, Wuhan, China); Akt (Proteintech); phospho-PI3K (p-PI3K) (Abcam, Cambridge, UK); phospho-Akt (p-Akt) (Cell Signaling Technology, MA, USA); and GAPDH (Proteintech). Then, peroxidase-conjugated secondary antibodies and electrochemiluminescence (ECL) detection systems were used according to routine methods.

Statistical Analysis

The data were presented as the means ± standard deviation. Statistical analysis involved Graph Pad Prism 7.0 (La Jolla, CA, USA) and was performed using analysis of variance (ANOVA). A P value of < 0.05 was considered statistically significant. All experiments were performed at least three times.

RESULTS

Production of IL-10 and IL-37 Is Increased by UCB-MSCs Co-culture in LPS-Stimulated THP-1 Macrophages

To examine the anti-inflammatory effect of UCB-MSCs on LPS-stimulated THP-1 macrophages, we measured the levels of IL-10 and IL-37 by ELISA. As shown in Fig. 1, compared with the negative control group, IL-10 production in THP-1 macrophages (a) was increased after LPS stimulation ($P < 0.01$) and IL-37 production in THP-1 macrophages (b) was increased after LPS stimulation $P < 0.05$). After co-culture with UCB-MSCs for 24 h, IL-10 and IL-37 levels in LPS-stimulated THP-1 macrophages were significantly increased ($P < 0.05$) and inhibited by co-treatment with LY294002 ($P < 0.01$).

IL-10 and IL-37 mRNA Levels Were Increased by UCB-MSCs Co-culture in LPS-Stimulated THP-1 Macrophages

To further detect IL-10 and IL-37 expression at mRNA level in LPS-stimulated THP-1 macrophages, real-time PCR was used to measure cytokine mRNA level. As shown in Fig. 2, compared with the negative control group, IL-10 (a) and IL-37 mRNA production in THP-1 macrophages (b) was increased after LPS stimulation ($P < 0.05$). After co-culture with UCB-MSCs for 24 h, IL-10 and IL-37mRNA levels in LPS-stimulated THP-1 macrophages was significantly increased ($P < 0.01$) and was significantly inhibited by co-treatment with LY294002 ($P < 0.01$).

Expression of Phosphorylated PI3K and Akt Is Enhanced by UCB-MSCs Co-culture and Reduced by LY294002

To evaluate whether PI3K/Akt signaling pathway was involved in the regulatory effects of UCB-MSCs on THP-1 macrophages, we investigated the phosphorylation of two PI3K/Akt signaling molecules, PI3K and Akt. As shown in Fig. 3, the total levels of PI3K and Akt did not significantly change among these groups. LPS increased expression levels of p-PI3K and p-Akt compared with THP-1 macrophages and THP-1 macrophages after co-culture with UCB-MSCs. UCB-MSCs markedly enhanced the levels of p-PI3K and p-Akt. However, p-PI3K and p-Akt were reduced by LY294002, a specific pharmacological inhibitor of PI3K.

DISCUSSION

MSCs have potential hopes for the treatment of sepsis [27, 28]. As a kind of mesenchymal stem cells, UCB-MSCs also have immunoregulatory properties. A few groups have investigated the anti-inflammatory effect of UCB-MSCs; however, the mechanism of action

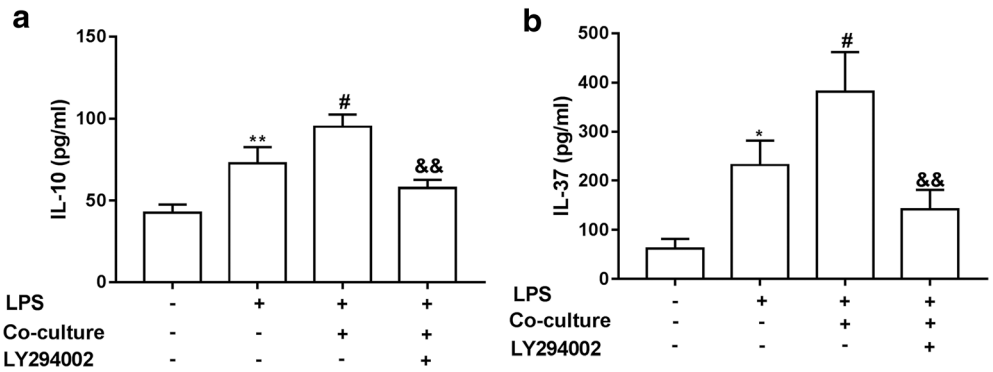

Fig. 1. UCB-MSCs co-culture increases LPS-induced IL-10 and IL-37 production in THP-1 macrophages. THP-1 cells were pre-treated with PMA to induce macrophage. Then, they were stimulated with 1 μg/mL LPS with or without UCB-MSCs and with or without LY294002 in a 6-well transwell plate for 24 h at 37 °C. Expression levels of IL-10 (**a**) and IL-37 (**b**) in the culture supernatants were detected by ELISA. Data represent the mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$ *vs.* THP-1(+) LPS (−) group; #$P < 0.05$ *vs.* THP-1(+) LPS (+) group; &&$P < 0.01$ *vs.* THP-1(+) LPS (+) LY294002 (+) group.

is still poorly understood. In this study, we demonstrated that UCB-MSCs increased LPS-induced production of IL-10 and IL-37 in THP-1 macrophages partly due to modulation of PI3K/Akt pathway.

When a pathogen breaches the body's natural barriers and enters into the body, it activates the innate immune system *via* specific conserved molecular patterns known as pathogen-associated molecular patterns which triggers an inflammatory response [26]. However, excessive inflammation can cause inflammatory diseases [29, 30], such as sepsis. It is well-known that Gram-negative bacteria are common pathogenic bacteria causing sepsis in clinical practice and the main pathogenic component is LPS [31]. According to a large number of reports, LPS stimulation can be used to establish a model of endotoxemia to understand the pathophysiology of sepsis *in vitro* and *in vivo* [3, 5, 32, 33]. Macrophages are the first line of defense against pathogenic microorganisms and they can phagocytose and destroy pathogens [17]. Human THP-1 cell is a mononuclear leukemia cell with all the characteristics of monocytes and it can be differentiated into macrophages after PMA stimulation, and this cell line is widely used in research [19, 34]. So, we chose LPS-stimulated macrophages as an endotoxemia model *in vitro*. LPS stimulates macrophages and secretes a large number of cytokines such as tumor necrosis factor-α (TNF-α), IL-6, IL-10, *etc.*, causing a cascade of inflammatory response [35, 36]. IL-37 is a recently discovered anti-inflammatory cytokine [37, 38] and can be secreted by monocytes, macrophages, epithelial cells, and so on [37]. It is reported that the UCB-MSCs had immunomodulatory functions [14–16]. However, the role of UCB-MSCs on THP-1 macrophages immune

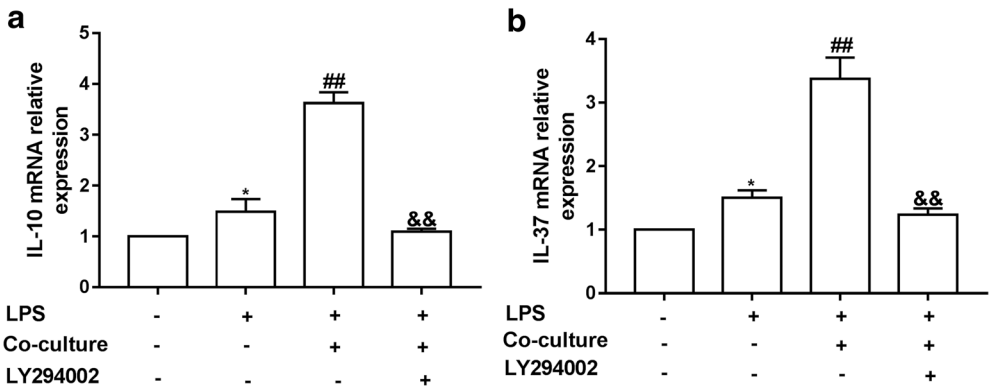

Fig. 2. UCB-MSCs co-culture increases LPS-induced IL-10 and IL-37 mRNA production in THP-1 macrophages. THP-1 cells were pre-treated with PMA to induce macrophage. Then, they were stimulated with 1 μg/mL LPS with or without UCB-MSCs and with or without LY294002 in a 6-well transwell plate for 24 h at 37 °C. Expression levels of IL-10 mRNA (**a**) and IL-37 mRNA (**b**) expressions were determined by RT-PCR. GAPDH was used as a quantitative control. All experiments were performed three times. *$P < 0.05$ *vs.* THP-1(+) LPS (−) group. ##$P < 0.01$ *vs.* THP-1(+) LPS (+) group. &&$P < 0.01$ *vs.* THP-1(+) LPS (+) LY294002 (+) group.

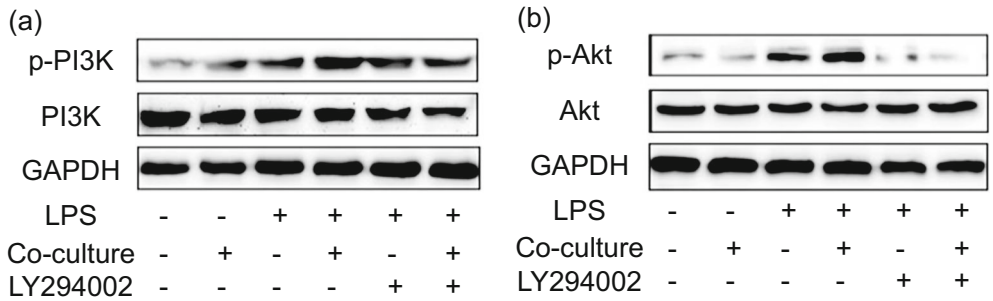

Fig. 3. UCB-MSCs enhance the phosphorylation of PI3K and Akt activation in LPS-stimulated THP-1 macrophages. THP-1 cells were pretreated with PMA to induce macrophage. Then, they were stimulated with 1 μg/mL LPS with or without co-culture UCB-MSCs and with or without LY294002 in a 6-well transwell plate for 24 h at 37 °C. The cells were then collected and extracted. **a** Western blot analysis was performed to detect p-PI3K and total PI3K. **b** Western blot analysis was performed to detect p-Akt and total Akt.

response is still incompletely defined. In this study, it showed that UCB-MSCs co-cultured with LPS-stimulated THP-1 macrophages effectively enhanced the production of anti-inflammatory cytokine IL-10 and IL-37, which further supported the anti-inflammatory activity of UCB-MSCs. Elevated macrophage-derived IL-10 helps to reduce tissue damage because it could prevent neutrophils from migrating into tissues and causing oxidative damage, thus mitigating multi-organ damage *in vivo* [39]. Several studies have demonstrated the ability of MSCs to increase the level of IL-10 during sepsis [21, 39–42], which could be benefit to the body during sepsis.

PI3Ks are heterodimeric complexes, which include a p110 catalytic and a p85 regulatory subunits [43]. Following phosphorylation by activating kinases, Akt affects various cellular targets that increase metabolism, growth, synthetic processes, and suppress apoptosis [44]. PI3K/Akt signaling pathway has been shown to play an important role in regulating LPS-induced acute inflammatory responses and is crucial for regulation of the production of inflammatory mediators [45, 46]. These findings show that PI3K/Akt signaling pathway has a modulatory role in cell fate, but the relationship of PI3K/Akt to immunomodulation of UCB-MSCs remains obscure. Our unpublished data indicate that PI3K/Akt pathway regulates IL-10 and Il-37 expression in human umbilical vein endothelial cells. The current study demonstrated that the levels of phosphorylation of PI3K and Akt were upregulated in THP-1 macrophages induced by LPS, and were significantly upregulated after co-culture with UCB-MSCs. However, this beneficial effect was blocked by LY294002, a PI3K specific inhibitor [47], which indicated that PI3K/Akt activation in THP-1macrophages was necessary for the immunomodulation function of UCB-MSCs. After the addition of PI3K/Akt inhibitor LY294002, the activation of MSC disappeared. This also implies that PI3K/Akt signaling pathway is necessary for UCB-MSCs to exert anti-inflammatory effect.

UCB-MSCs increased the levels of IL-10 and IL-37 in LPS-stimulated macrophages *in vitro*, and UCB-MSCs may be a new therapeutic strategy for sepsis for the anti-inflammatory effect, however, whether UCB-MSCs exert anti-inflammatory effect *in vivo* remains unclear. The present study showed that UCB-MSCs exert anti-inflammatory effect through PI3K/Akt signaling pathway; nevertheless, the downstream proteins of PI3K/Akt are unknown. More studies are needed to solve these problems.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

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