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Priming of Mesenchymal Stem Cells for Enhanced Interleukin-10 Secretion via Conditioned Medium from Lipopolysaccharide-Activated Peripheral Blood Mononuclear Cells

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ABSTRACT

Background: Mesenchymal stem cells (MSCs) are known for their immunomodulatory properties, particularly their ability to secrete anti-inflammatory cytokines such as interleukin-10 (IL-10). Enhancing the secretion of IL-10 by MSCs could have significant therapeutic potential for treating inflammatory diseases. **Objective:** This study aimed to prime the secretion of IL-10 by MSCs through the use of conditioned medium (CM) derived from lipopolysaccharide (LPS)-induced peripheral blood mononuclear cells (PBMCs). **Methods:** MSCs were isolated from Wharton's Jelly and characterized using flow cytometry and differentiation assays. PBMCs were isolated from human blood samples and stimulated with LPS to produce a pro-inflammatory environment. The conditioned medium from these LPS-induced PBMCs was collected and add to MSCs culture medium in 5% and 7.5%. After 24h and 48h incubation, IL-10 secretion by MSCs was measured using an enzyme-linked immunosorbent assay (ELISA). **Results:** The results demonstrated that MSCs cultured in the conditioned medium from LPS-induced PBMCs showed a significant increase in IL-10 secretion compared to control conditions in 24h exposure, but not significantly different in 48h. **Conclusion:** The exposure of conditioned medium from LPS-induced PBMCs may effectively enhances the secretion of IL-10 by MSCs.

Keywords : MSCs, IL-10, PBMC, LPS, Cell priming

INTRODUCTION

A major issue in inflammatory illnesses is dysregulated wound healing, which is typified by an excessive build-up of macrophages and an abnormal release of pro-inflammatory cytokines[1–3]. The term "secretome" describes the entire set of chemicals that stem cells secrete or release onto the surface, including bioactive peptides like growth factors, chemokines, and cytokines[4–6]. However, the secretome can be influenced by the stem cell's place of origin. Many researchers have concentrated on the utilization of stem cells over the past few decades and have attempted to develop plans for extending very effective stem cell treatment[7–9]. Notably, research has shown that the complicated paracrine mechanisms of stem cell-free conditioned medium (CM) contribute to these positive outcomes[10–12]. Therefore, secretome-derived stem cell has gained scholarly attention because of these discoveries.

The primary source of pro- and anti-inflammatory ligands involved in controlling inflammatory responses is mononuclear cells, including monocytes, macrophages, neutrophil and dendritic [13–15]. When the infection is present, the cells can recognize lipopolysaccharides (LPS) pattern through their receptor and release most of the pro-inflammatory cytokines, such as TNF-α, interferon-γ (IFN-γ) and interleukin 1β (IL1β)[16–19]. These molecules induce nuclear factor kappa B (NF-κB) activation which are linked to many signalling pathways, leading to robust proinflammatory milieu [20]. One potent anti-inflammatory cytokine with distinct characteristics, interleukin-10 (IL-10) can restore the inflammatory response to its typical state and could be a helpful strategy inhibiting inflammatory niche [21,22].

In regenerative medicine, mesenchymal stem cells (MSC) therapy may prove to be an effective treatment for a range of inflammatory disorders[2]. MSCs are a type of multipotent cells characterized by their capacity to adhere to plastic surfaces. Several surface markers have been used to distinguish MSCs, such as CD90, CD105, CD44, CD73, and CD29, which are considered positive, while CD34, CD45, CD11b, CD14, CD19, and human leukocyte antigen (HLA) are considered negative [23,24]. Furthermore, MSCs have the capacity to differentiate into different mature cell types, including adipocytes, osteocytes, nerve cells, and osteocytes[25]. Multiple studies have demonstrated that MSCs can have a role in immunomodulatory and anti-inflammatory properties[26]. Numerous paracrine signalling factors, including anti-inflammatory molecules, specifically IL-10 have been shown to be expressed in response to stem cell transplantation into injured tissues[9]. Consequently, MSC secretory components may influence inflammatory immune cells through immunomodulatory capacity.

According to numerous studies, MSCs actively engage in communication and interaction with both innate and adaptive immune cells to improve immunological diseases, such as multiple sclerosis, inflammatory bowel disease, rheumatoid arthritis, graft versus host disease, and systemic lupus erythematosus (SLE)[26–28]. Additional clinical studies have also demonstrated that MSC-based treatment is a successful means of controlling immunity in infection disease, including sepsis [29]. These studies were supported by various findings showing that MSCs can produce various anti-inflammatory cytokines when exposed to inflammatory milieu, including IL-10[29]. IL-10 serves as potent anti-inflammatory cytokines in controlling excessive inflammatory responses. Specifically, IL-10 attenuates pro-inflammatory cytokines release through inhibiting inflammatory signals, such as NF-kB[19]. In line with these findings, several studies have focused on strategies to augment the anti-inflammatory properties of MSCs, such pharmacological priming and hypoxia[30]. Lipopolysaccharide (LPS) preconditioning of MSCs has also shown to be promising strategy to promote anti-inflammatory features of MSCs[31], however there is insufficient evidence regarding paracrine effects of LPS preconditioned MSCs.

Regarding these facts, we speculate that molecules secreted by LPS-preconditioned mononuclear cells may optimize anti-inflammatory molecules produced by MSCs. In this study, we firstly pretreated mononuclear cells with LPS. We then incubated the MSCs with CM of LPS-preconditioned mononuclear cells and assessed their anti-inflammatory properties compared to untreated MSC.

MATERIAL AND METHODS

MSC Isolation and characterization

Umbilical cords were collected from full-term deliveries with informed consent. The cords were stored in sterile PBS with antibiotics and processed within 24 hours. The umbilical cords were washed with PBS, and the blood vessels were carefully removed. The remaining Wharton's Jelly was cut into small pieces. The umbilical cords were washed with PBS, and the blood vessels were carefully removed. The remaining Wharton's Jelly was cut into small pieces. The chopped Wharton's Jelly was digested using

0.1% collagenase type I and 0.25% trypsin-EDTA at 37°C for 1 hour. The digested mixture was filtered to remove any large pieces, and the cells were collected by centrifugation. The pellet was resuspended in low-glucose Dulbecco's Modified Eagle's Medium (DMEM-LG) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cell suspension was plated in T75 flasks and incubated at 37°C in a humidified atmosphere containing 5% CO2. Non-adherent cells were removed after 24 hours, and the medium was replaced every 3 days until 80% confluence was achieved.

To confirm the identity of the MSCs, flow cytometry was performed to assess the expression of surface markers. The cells were trypsinized, washed, and incubated with positive marker antibodies against CD73, CD90, CD105, and Lineage cocktail negative markers (CD14, CD34, CD45, and HLA-DR). The stained cells were analyzed using a flow cytometer. Additionally, MSCs were differentiated into osteoblasts and adipocytes using specific differentiation media to confirm their multipotent potential.

PBMC Isolation and culture

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples collected from healthy donors. Blood was diluted 1:1 with PBS and layered over a density gradient medium (Ficoll-Paque) in a 50 mL conical tube. After centrifugation at 2000 rpm for 30 minutes at room temperature, the PBMC layer was carefully extracted and washed twice with PBS. The final cell pellet was resuspended in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin.

Isolated PBMCs were seeded in T75 flask at a density of 1 × 10^6 cells/mL and incubated at 37°C in a 5% CO2 atmosphere. Cells were allowed to equilibrate for 24 hours before further experimental procedures.

PBMC induction with Lipopolysaccharides (LPS)

To activate PBMCs, cells were treated with 30ng/1.5x10^6 cells of Lipopolysaccharides (LPS) derived from *Escherichia coli* (O55) for 4 hours. After incubation, the supernatants were collected and stored at -80°C for next steps.

MSC induction with CM of LPS-induced PBMC

MSCs were exposed to the conditioned medium (CM) obtained from LPS-stimulated PBMCs to investigate the immunomodulatory effects of MSCs. Briefly, the CM was filtered through a 0.22 µm filter to remove any cellular debris and then added to the MSC culture at 5% and 7.5% with fresh DMEM-LG. The MSCs were incubated with CM for 48 hours at 37°C in a 5% CO2 atmosphere. After incubation, the supernatants were collected for cytokine analysis, and the cells were harvested for further assays.

Interleukin-10 level analysis by ELISA

The levels of Interleukin-10 (IL-10) in the culture supernatants were quantified using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Human ELISA Kit, Cat. No. 532925, Elabscience). Briefly, 100 µL of standards or samples were added to the pre-coated wells of a 96-well plate and incubated for 2 hours at room temperature. After washing, 100 µL of biotin-conjugated anti-IL-10 antibody was added and incubated for 1 hour. Following another wash, 100 uL streptavidin-HRP was added, and the plate was incubated for 30 minutes. The reaction was developed by adding the substrate solution and stop solution, and the absorbance was measured at 450 nm using a microplate reader. The IL-10 concentrations were calculated based on the standard curve generated with known concentrations of recombinant IL-10.

Statistical analysis

Statistical analyses were performed using SPSS 22.0 (SPSS Inc., Chicago, IL, USA). All data are presented as mean ± standard deviation (SD). Data analysis involved one-way ANOVA and followed by the Least Significant Difference (LSD) test, with a significant level sel at $p<0.05$.

RESULTS

MSCs characteristics

In compliance with ISCT recommendations, we assessed the cell shape, expression of membrane markers, and differentiation potential of UC-MSCs at the fifth passage in order to identify their properties. The cells had a spindle-shaped characteristic and resembled fibroblasts (Figure 1a). The capacity of UC-MSCs to differentiate was verified by adipogenic (Figure 1b) and osteogenic (Figure 1c) differentiation tests. The UC-MSCs underwent 21 days of incubation before differentiating into osteocytes and adipocytes, as shown by the red deposits of calcium and lipid, respectively. The cells' immunophenotyping profile showed that they expressed CD90, CD73, and CD105 positively whereas Lin was expressed negatively (Figure 1d).

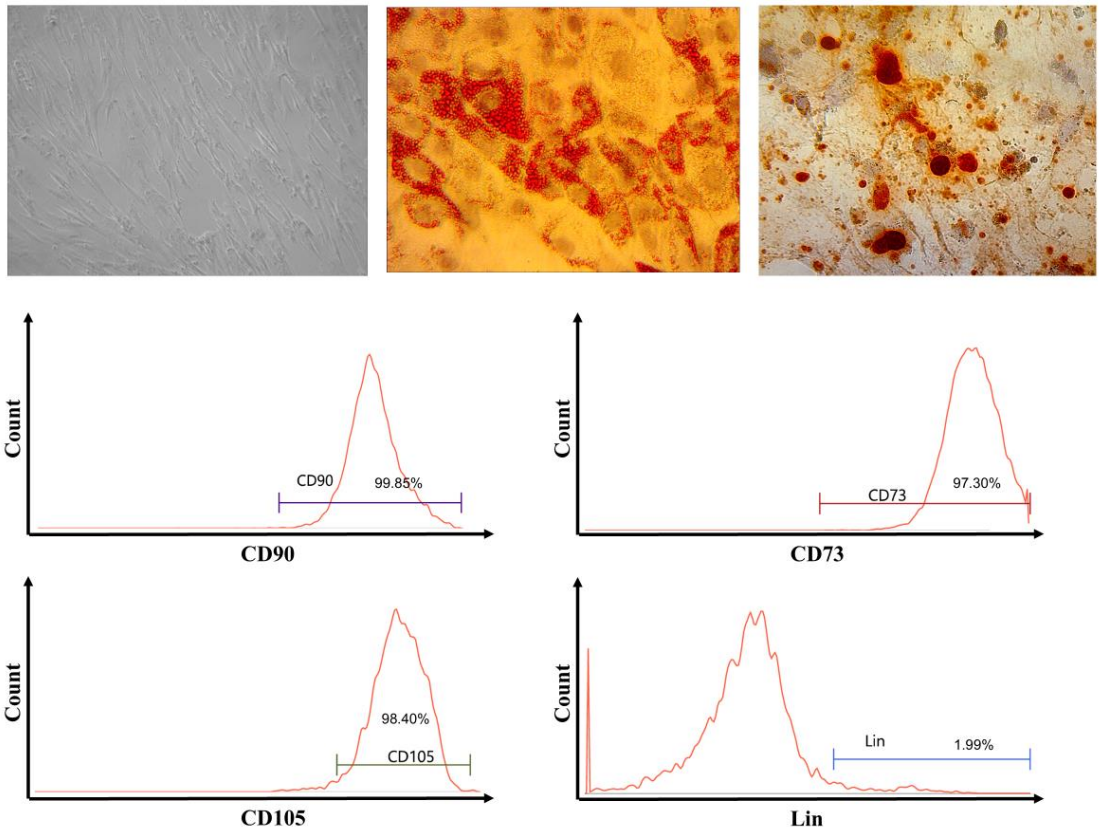

Figure 1. At the fifth passage, the cells had a 200× magnification and looked spindle-shaped and fibroblast-like. After staining with oil red O and alizarin red, the UC-MSCs showed that they could differentiate into osteocytes and adipocytes, which were distinguished by the deposition of lipid and calcium, respectively, and by being colored red. The phenotyping research further revealed that the UC-MSCs expressed CD90 (99.85%) and CD73 (97.30%) positively, while CD105 (98.40%) and Lin (1.99%) were expressed negatively.

LPS-preconditioned mononuclear cells CM enhance IL-10 secretion of MSCs

To promote anti-inflammatory molecules secretion of MSCs, we incubate MSCs with LPS-preconditioned mononuclear cells CM. The CM was produced by preconditioning mononuclear cells with LPS for 4 hours. Then, we incubated MSCs with preconditioned mononuclear cells CM for 24

hours and 48 hours. After incubation, Human IL-10 ELISA Kit (Cat. No. 532925, Elabscience) was used to analyse the level of IL-10 anti-inflammatory cytokines produced by MSCs. ELISA assay showed that treating MSCs with 7,5% LPS-preconditioned mononuclear cells CM for 24h significantly enhanced IL-10 secretion as anti-inflammatory ligand (IL-10 = 4,5 pg/mL) compared to 5% group (IL-10=3,5 pg/mL) and untreated MSCs (IL-10 =3,7 pg/mL) (Figure 2). In other hand, IL-10 secretion by MSCs after 48h incubation with LPS-induced PBMC CM did not significantly increase, either 5% or 7,5% group (3,6 pg/mL and 3,9 pg/mL respectively).

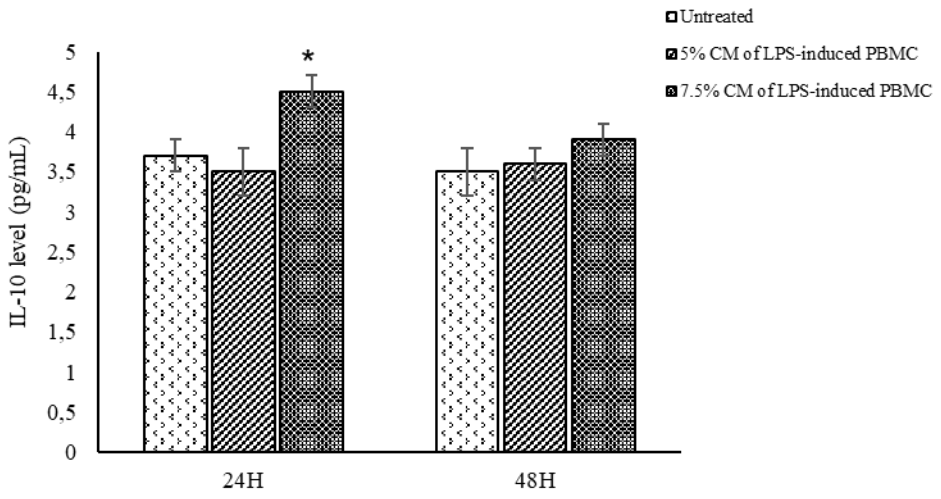

Figure 2. IL-10 levels secreted by MSC after 24h and 48h incubation with CM of LPS-induced PBMC.

DISCUSSION

The purpose of the current investigation was to optimize anti-inflammatory molecules secretion by MSCs using LPS-preconditioned mononuclear cells CM. As mentioned before, mononuclear cells were firstly incubated with 10 ng/mL LPS for 4 hours. The LPS-preconditioned mononuclear cells CM then collected and incubated the MSCs with 5% and 7.5% CM concentration for 24 and 48 hours. We finally collected the CM after incubation and analyze the IL-10 concentration compared to untreated MSCs.

This study showed that 5% LPS-preconditioned mononuclear cells CM incubation for 24 hours could enhance the secretion of IL-10 level in MSCs. Mononuclear cells can recognize and respond gram-negative bacteria-produced LPS through LPS-binding protein (LBP)[31]. LBP is a part of LPS that facilitates its transfer to membrane-bound CD14 (mCD14), a 55-kDa glycoprotein produced on the surface of monocytes monoclonal cells. In the lipid bilayer, CD14 releases LPS, which is then intercalated and binds to a complex of receptors, including the heat shock proteins 70 and 90[32]. Toll-like receptor 4 (TLR4) and MD-2 complex is further recruited and promotes many signalling pathways, including NF-κB[31]. This pathway then enhances the secretion of proinflammatory molecules, such as TNF-α, IFN-γ and IL1β which can promote the expression of anti-inflammatory cytokines in MSCs.

MSCs increase TLR-3 expression in response to a sufficient dosage of TNF-α, which releases IL10 and other anti-inflammatory molecules[31]. TLR-4 expression was upregulated, and cyclooxygenase-2 (COX2) was produced as a result of TNF-α-TNFR-1 binding complex on MSCs, NF-κβ and ERK signaling[33]. The TLR4-primed MSC, sometimes referred to as MSC type-1, has a proinflammatory profile that includes increased PGE2 production due to COX2 activation [34]. PGE2 bind to EP2 and EP4 receptors in MSCs, causing a P110δ isoform of PI3k kinase and switch the pathway from MyD88-dependent proinflammatory (MyD88-independent) to TRIF-TRAM mediated anti-

inflammatory leading to the secretion of IL-10[22]. According to these findings, MSCs may polarize into MSC type-1 when they first stimulate TNF-α, as shown by the production of COX2, and then repolarize into MSC type-2 when an inflammatory signal accumulates inside the MSCs[35].

The present study showed that CM of LPS-preconditioned mononuclear cells can optimize anti-inflammatory capacity of MSCs. Proinflammatory cytokines contained in CM provides a stimulation to MSCs to release IL-10 level. Nevertheless, these effects may vary in experimental in vivo settings and may be adjusted by a variety of parameters, including MSC supplies, LPS concentration and time of incubation, and TLR4 expression levels. Therefore, further studies are needed to overcome these limitations.

CONCLUSIONS

The paracrine actions of MSCs can be enhanced with proinflammatory molecules secreted by inflammatory mononuclear cells. This study indicated that LPS preconditioning can provide inflammatory condition in mononuclear cells and improve the improve anti-inflammatory secretion of MSCs, as evidenced by the considerable increase of IL-10.

CONFLICT OF INTEREST

Competing interests: No relevant disclosures.

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CONTRIBUTORS

AP and IA were involved in concepting and designing the study. LMS and AP was involved in the experiment. LMS provided the data analysis. IA and AN performed the statistical analysis. AP and IF wrote and edited the manuscript. Meanwhile AP helped in reviewing the manuscript.

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