

Original Paper

# Lipopolysaccharides Shapes the Human Wharton's Jelly-Derived Mesenchymal Stem Cells *in Vitro*

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## Key Words

Lipopolysaccharides • Mesenchymal stem cells • Toll-like receptor 4 • Wharton's jelly

## Abstract

**Background:** Although the expression of toll-like receptors (TLRs) on different types of human mesenchymal stem cells (hMSCs) has recently been reported, controversy remains regarding the presence of TLR4 as well as its engagement and impact on human Wharton's jelly-derived MSCs (hWJ-MSCs). **Methods:** In the present study, the expression and role of TLR4 in hWJ-MSCs was investigated using a model of lipopolysaccharide (LPS). Proliferation, apoptosis, and the expression of paracrine factors in hWJ-MSCs primed with LPS were analysed. **Results:** The expression of TLR4 was high at the RNA level but very low at the protein level. hWJ-MSCs responded to LPS stimulation and initiated a marked up-regulation of inflammatory cytokine (IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8) production. Moreover, hWJ-MSCs LPS stimulation resulted in the up-regulation of indoleamine 2,3-dioxygenase [IDO]-1, Cox2, interferon [IFN]- $\beta$ , and matrix metalloproteinase (MMP)-2 but a down-regulation of MMP-9, which affect the immunosuppressive potential of hWJ-MSCs. **Conclusions:** These data suggest that LPS engagement shapes hWJ-MSCs and results in the production of pro-inflammatory cytokines and inhibitory immune mediators, showing TLR4 agonist induces the hWJ-MSCs polarization to a pro-inflammatory and immunosuppressive state, which may be beneficial for the exploration of the clinical potential of hWJ-MSCs.

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## Introduction

The differentiation and immunomodulatory properties of mesenchymal stem cells (MSCs) suggest that they are useful cell therapeutic tools for regenerative medicine [1]. Human Wharton's jelly derived-MSCs (hWJ-MSCs), which are isolated from umbilical cord after the removal of blood vessels [2], have biological properties that are similar to human bone marrow-derived MSCs (hBM-MSCs) and are considered an alternative to MSCs derived from adult tissues because they are more primitive, easily obtained, rapidly expanded, immunoprivileged, have strong immunosuppressive potential, and have no related ethical issues [3-7]. Several disease models [8-10] and human clinical trials [11, 12] have further confirmed the therapeutic potential of hWJ-MSCs in recent years. MSCs are attracted to and migrate toward injured tissues in host [13]. However, MSCs are not spontaneously immunosuppressive [14], and the presence of inflammatory mediators may be essential for MSC-mediated immunosuppression and modulation of the functional properties, proliferation, and survival of implanted MSCs [14-18]. An understanding of the mechanism of the interaction between MSCs and local injured tissues would benefit the future clinical applications of MSCs.

Toll-like receptors (TLRs), a type of biological pattern recognition receptor (PRR), are transmembrane proteins that play critical roles in the immune system by mediating inflammatory responses, primarily through the binding of ligands [19, 20]. Specifically, TLRs recognise pathogen-associated molecular patterns (PAMPs) derived from microorganisms and also endogenous molecules released by injured tissues, such as heat shock protein (HSP) 60, HSP70, and fibrinogen, molecules that are considered "danger signals" [21]. To date, 10 TLRs have been identified in humans, and all TLRs (except TLR3) induce the production of TNF- $\alpha$ , IL-1, IL-6, and other cytokines [21, 22]. Recently, the TLR profiles in hMSCs derived from various tissues (including hWJ-MSCs) have been explored, and their regulatory effects on MSC differentiation, proliferation, and immunomodulation have been described [23-27]. However, MSCs derived from various sources display different TLRs profiles, functional properties, and responses to stimulation by TLRs' agonists [23-29].

LPS is a common model system that mimics the inflammatory process by acting as a TLR4 agonist [21, 22]. It has been used to investigate the response of human adipose tissue-derived MSCs (hAD-MSCs), hBW-MSCs, and human cord blood-derived MSCs (hCB-MSCs) to TLR4 triggering *ex vivo*, and it was found that TLR4 expressed at a high level and is a functional receptor in these cells [23, 25, 26]. In contrast, the presence and functional properties of TLR4 in hWJ-MSCs remains controversial [27, 29]. Therefore, this study aimed to investigate the expression profile of TLR4 in hWJ-MSCs and to evaluate the inductive effect of TLR4 agonist on the proliferation, apoptosis, paracrine profiles, and immunosuppressive properties of hWJ-MSCs.

## Materials and Methods

### *Isolation and expansion of hWJ-MSCs*

hWJ-MSCs were isolated from human umbilical cords obtained in our hospital with informed consent from the parents. Human tissue collection for research was approved by the medical ethics committee of the People's Liberation Army General Hospital in the Beijing Military Region. Collagenase II combined with trypsin digestion for isolating hWJ-MSCs was used as previously described [30]. Briefly, umbilical cords isolated from full-term newborn infants were immediately immersed in PBS under sterile conditions and processed within 2-6 h. The blood vessels were carefully removed, and the cords were washed repeatedly. The cords were cut into 1-2-mm<sup>2</sup> segments and digested with 0.075% collagenase II (Sigma, St Louis, MO, USA) for 30 min with gentle agitation at 37°C and then filtered through a 150- $\mu$ m mesh cell sieve. Next, the samples were trypsinised with 0.125% trypsin (Gibco, Grand Island, NY, USA) for another 30 min with gentle agitation at 37°C. Trypsin activity was terminated with pre-warmed fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), and the digested mixture was passed through a 75- $\mu$ m mesh cell

sieve to obtain single-cell suspensions. The cells were washed with phosphate-buffered saline (PBS) three times and stained with trypan blue to determine their viability and number. The cells were then cultured in non-coated plastic flasks in DF12 medium (Gibco, Grand Island, NY, USA) supplemented with 10% FBS, 100 U/mL penicillin-streptomycin (Sigma), and 2 mM glutamine (Sigma); non-adherent cells were removed by completely changing the medium after 24 h. The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> and passaged at 80-90% confluency. The hWJ-MSC cultures were characterised by the analysis of cell surface markers using flow cytometry and *in vitro* differentiation assays with the Mesenchymal Stem Cell Adipogenesis Kit (Cyagen Biosciences Inc., USA) and Mesenchymal Stem Cell Chondrogenic Kit (Cyagen Biosciences Inc., USA). The cell number and viability were determined by trypan blue staining; a cell viability percentage of at least 95% was required for all of the tests. Cells after two to seven passages in the logarithmic growth phase (80-90% confluency) were used in the experiments described in this article.

## *RT reaction and quantitative real-time PCR (qRT-PCR)*

hWJ-MSCs were exposed to LPS (final concentration of 1.0 µg/mL) for 24 h, 48 h, and 72 h for hWJ-MSC activation; the same volume of PBS was used to treat the cells as a control. The supernatants were harvested by centrifugation at 3000 rpm for 20 min and stored at -80°C for protein detection after LPS exposing respectively. The adherent hWJ-MSCs were trypsinised with 0.25% trypsin, washed twice with PBS, and collected by centrifugation at 1500 rpm for 10 min. The pellets were lysed with 1 mL Trizol Reagent, and the total RNA was subsequently isolated following the manufacturer's protocol (Invitrogen, Carlsbad, CA). The extracted RNA was quantified using a Nanodrop2000 ND-2000 (Nanodrop, USA), and cDNA was synthesised using 2 µg RNA and the Revertaid H minus first strand cDNA synthesis kit (Fermentas Life Sciences, EU). qRT-PCR analysis for TLR4, CD14, IL-1β, IL-1α, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, indoleamine 2,3-dioxygenase [IDO]-1, IDO2, TNF-α, Cox-2, interferon [IFN]-β, IFN-γ, MMP-2, and MMP-9 transcripts was performed in triplicate using an Applied Biosystems 7500 Fast Real-Time PCR system thermal cycler. A DyNAmo™ Flash SYBR® Green qPCR Kit (Thermo, USA) and primers (listed in Table 1) at a final concentration of 0.2 µM were used with the following programme: hot start at 95°C for 7 min, 40 cycles of annealing at various temperatures for 30 s and extension at 72°C for 32 s, followed by the final single-peak melting curve programme. The qRT-PCR analysis was further confirmed by analysis of the PCR products using 1.8% agarose gel electrophoresis. The comparative threshold cycle method (2-ΔΔCt) was used for data analysis. The relative expression of the target gene mRNA was calculated and normalised based on the GAPDH levels using SDS software version 1.4.

## *Flow cytometry*

hWJ-MSCs were trypsinised, harvested and aliquoted at 1×10<sup>5</sup> cells per tube, and labelled with FITC-conjugated mouse anti-human CD90, CD105, CD73, CD31, CD14, HLA-ABC, HLA-DR, isotype antibody (Biolegend, USA), or Phycoerythrin conjugated to mouse anti-human TLR4 and isotype antibody (ebioscience, USA) for 45 min at 4°C. To detect the intracellular expression of TLR4, cells were fixed in ice-cold 4% paraformaldehyde for 10 minutes and were permeabilized in ice-cold acetone for another 10 minutes prior to stain with monoclonal anti-TLR4 antibody. The cells were rinsed in PBS before, between and after the fixation and permeabilization. After incubation, the cells were washed once again with PBS and resuspended in 400 µl PBS. The cells were analysed using a FACScan flow cytometer (Beckman FC500, USA) within 1 h after filtering through a 150-µm mesh screen.

## *Proliferation: MTT assay*

Cells were seeded in a 96-well plate at 1000 cells per well in 100 µl complete medium and incubated for six hours to allow attachment. The cells were then exposed to LPS (*E. coli* serotype O55:B5; Sigma, USA) (final concentration of 1.0 µg/mL) for up to 24 h, 48 h, 72 h, and 120 h; the same volume of PBS was used as a control. When cell culture time is over, the medium was removed, and 200 µl of fresh medium and 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (10%, final concentration of 0.5 mg/ml) was added per well. The plates were incubated for 4 h in an incubator, and 150 µl DMSO per well was added to formazan produced. The colourimetric determination of MTT reduction was measured using a plate reader at 490 nm (SynergyMx Multi-Mode Microplate Reader, Biotek, USA).

**Table 1.** The primers for qRT-PCR analysis. Footnote: \*the annealing temperature varied from 55°C to 65°C, but no detectable result was obtained by the qRT-PCR and PCR analysis.

Gene	Sequence	Annealing temperature (°C)
IL-1 $\beta$	Forward: 5' AAACCTCTTCGAGGCACAAG 3'	60
	Reverse: 5' GTTTAGGGCCATCAGCTTCA 3'	
MMP-2	Forward: 5' AGTCTGAAGAGCGTGAAG 3'	58
	Reverse: 5' CCAGGTAGGAGTGAGAATG 3'	
IL1- $\alpha$	Forward: 5' GAATGACGCCCTCAATCAAAGT 3'	58
	Reverse: 5' TCATCTTGGCAGTCACATACA 3'	
MMP-9	Forward: 5' GGCAGGACCGTCTCTACTGGCGCGT 3'	57
	Reverse: 5' CAGAACAGAATACCAGTTTGTATC 3'	
IDO1	Forward: 5' GCCTGATCTCATAGAGTCTGG 3'	62
	Reverse: 5' TTACTGCAGTCTCCATCAGC 3'	
IL-6	Forward: 5' GTGTGAAAGCAGCAAAGAGGC 3'	59
	Reverse: 5' CTGGAGGTACTCTAGGTATAC 3'	
TNF- $\alpha$	Forward: 5' CAAAGTAGACCTGCCAGAC 3'	*
	Reverse: 5' GACCTCTCTAATCAGCCC 3'	
CD14	Forward: 5' ACAGGGCGTTCTTGCTCGC 3'	62
	Reverse: 5' CGGGAAGGCGCGAACCTGTT 3'	
TLR4	Forward: 5' TACAAAATCCCCGACAACCTC 3'	56
	Reverse: 5' AGCCACCAGCTTCTGTAAACT 3'	
IL-8	Forward: 5' TCTTGGCAGCCTTCCTGATT 3'	60
	Reverse: 5' AACTTCTCCACAACCCTCTG 3'	
IL-12	Forward: 5' TCACAAAGGAGGCGAGGTTCTAAGC 3'	64
	Reverse: 5' CCTCTGCTGCTTTTGACACTGAATG 3'	
COX-2	Forward: 5' ACTCTGGCTAGACAGCGTAA 3'	60
	Reverse: 5' ACCGTAGATGCTCAGGGAC 3'	
IDO2	Forward: 5' AAATGCACTGCCAGTTGAAA 3'	60
	Reverse: 5' CTGGTGGGTGAAGTGTCAG 3'	
IFN- $\gamma$	Forward: 5' TCGGTAAGTGAATGTCCA 3'	60
	Reverse: 5' TCCTTTTCGCTTCCCTGTTTT 3'	
IFN- $\beta$	Forward: 5' ACTGCCTCAAGGACAGGATG 3'	60
	Reverse: 5' AGCCAGGAGGTTCTCAACAA 3'	
GVPDH	Forward: 5' GACCACTTTGTCAAGCTCATTTC 3'	61
	Reverse: 5' GTGAGGGTCTCTCTCTCTCTTGT 3'	

## Apoptosis and cell cycle: PI staining

hWJ-MSCs were seeded in a 6-well plate as described above. Apoptosis and the cell cycle were assessed by PI staining using a commercial Coulter DNA PREP Reagent kit according to the manufacturer's protocol (Beckman Coulter, Inc., USA) after exposure to LPS (final concentration of 1.0  $\mu$ g/mL) for up to 24 h, 48 h, and 72 h.

## ELISA

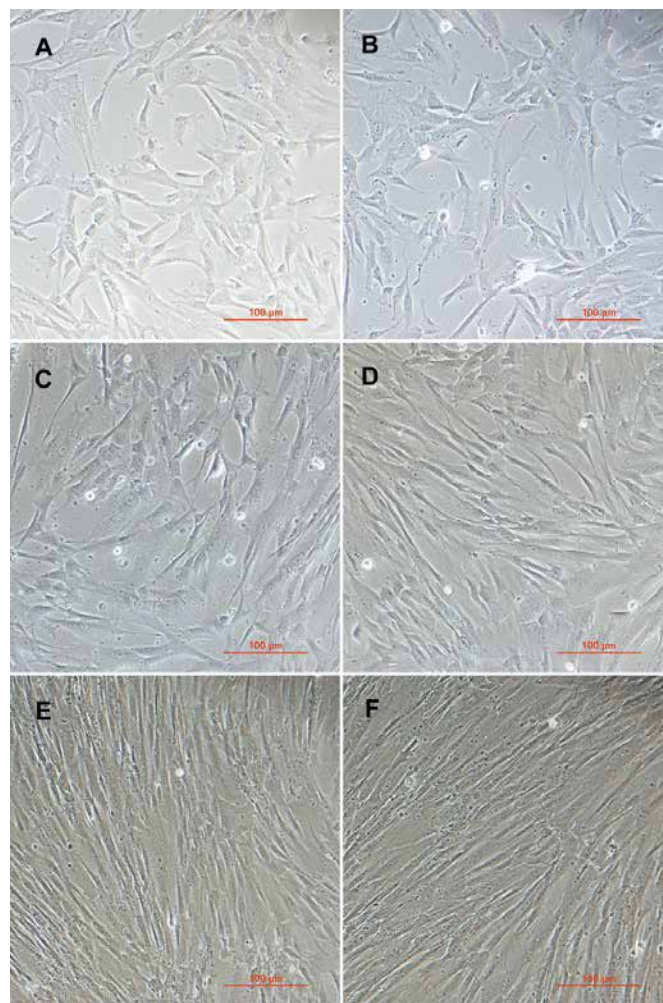
The quantitative determination of IL-1 $\beta$ , IL-1 $\alpha$ , IL-6, IL-8, IL-12, and TNF- $\alpha$  in the supernatants of hWJ-MSC cultures at 24 h, 48 h, and 72 h after LPS treatment was performed using commercial ELISA kits (Neobioscience, China) according to the manufacturer's protocols.

## Statistical analysis

The results are presented as the mean  $\pm$  SD. The statistical analysis was performed using a Student's t test or a one-way ANOVA, followed by Tukey's post hoc test.



**Fig. 1.** hWJ-MSCs viewed under a Nikon ECLIPSE TE2000-S microscope at 350× magnification. No difference in morphology was observed between the control groups and groups exposed to LPS for lengths of time. A: Unstimulated MSCs at 24 h. B: LPS-stimulated MSCs at 24 h. C: Unstimulated MSCs at 48 h. D: LPS-stimulated MSCs at 48 h. E: Unstimulated MSCs at 72 h. F: LPS-stimulated MSCs at 72 h.



## Results

### *Characterisation of hWJ-MSCs*

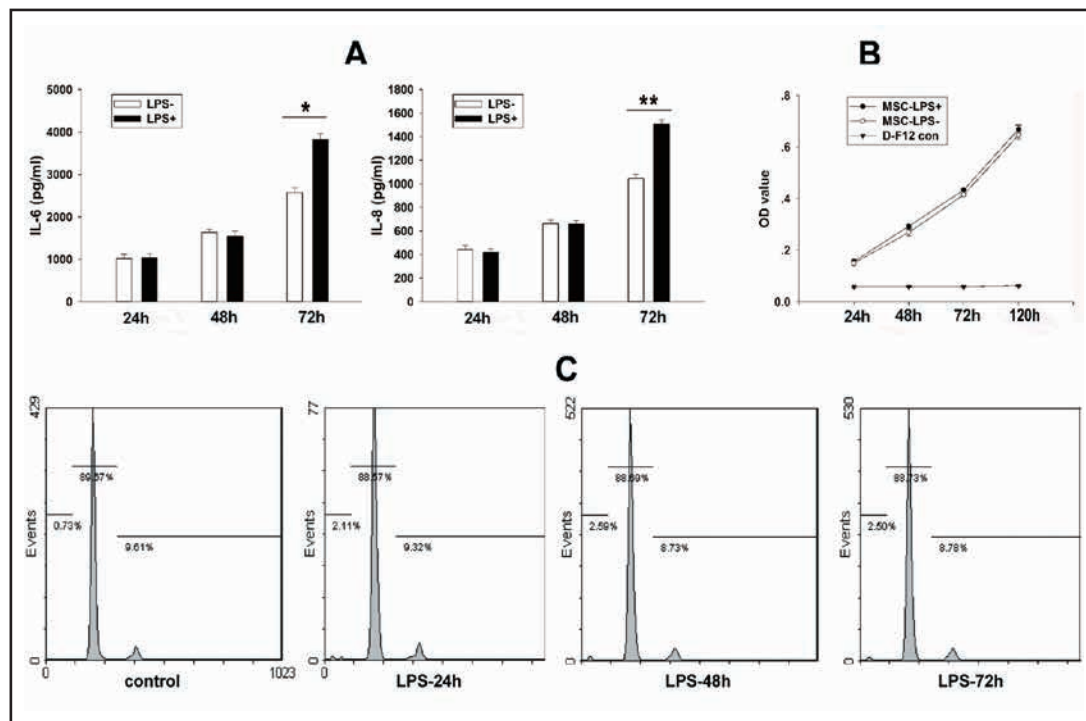
hWJ-MSCs were isolated from three samples and analysed for the positive expression of CD105, CD73, CD90, and HLA-ABC and negative expression of CD31, CD14, and HLA-DR surface markers using flow cytometry analysis, respectively. Stem cell differentiation tests *in vitro* showed that chondrogenic and adipogenic differentiation were successfully induced in hWJ-MSCs (data not shown).

### *LPS does not affect hWJ-MSC morphology, proliferation, and apoptosis*

The morphology of hWJ-MSCs was not affected by LPS exposure, as determined by microscopy using a Nikon eclipse TE2000-S Microscope at 350× magnification. In addition, the proliferation and apoptosis of hWJ-MSCs, as analysed by MTT assay and PI staining, respectively, showed no differences between the control and groups exposed to LPS at different time points (Fig. 1, Fig. 2B and C).

### *The constitutive expression of TLR4 in hWJ-MSCs*

Both the expression profiles of TLR4 and CD14, were analysed at the mRNA and protein levels by qRT-PCR analysis and flow cytometry. Flow cytometry analysis showed that hWJ-MSCs were marginally positive for TLR4 expression (approximately 3%) but did not express CD14 in comparison to the isotype control; no obvious change was observed in response to LPS stimuli. Few intracellular expression of TLR4 was observed in hWJ-MSCs after cells



**Fig. 2.** A: IL-6 and IL-8 concentrations, as detected by ELISA at 24 h, 48 h, and 72 h, with or without LPS treatment. The data represent the mean of three independent experiments, each performed in triplicate (\* $P < .05$  compared with control; \*\* $P < .01$  compared with control). B: No effect on cell proliferation was observed with the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)) at 24 h, 48 h, 72 h, and 120 h after exposure to LPS in hWJ-MSCs. C: PI staining analysis of hWJ-MSC cell cycle.

exposing to LPS for up to 72 h compared to the expression on the surface of hWJ-MSCs (data not shown). However, qRT-PCR analysis revealed that TLR4 was expressed at relatively higher levels compared to GAPDH expression, and both TLR4 and CD14 were markedly up-regulated when the cells were activated with LPS for up to 72 h ( $p=0.000$  and  $0.000$ ) (Fig. 3).

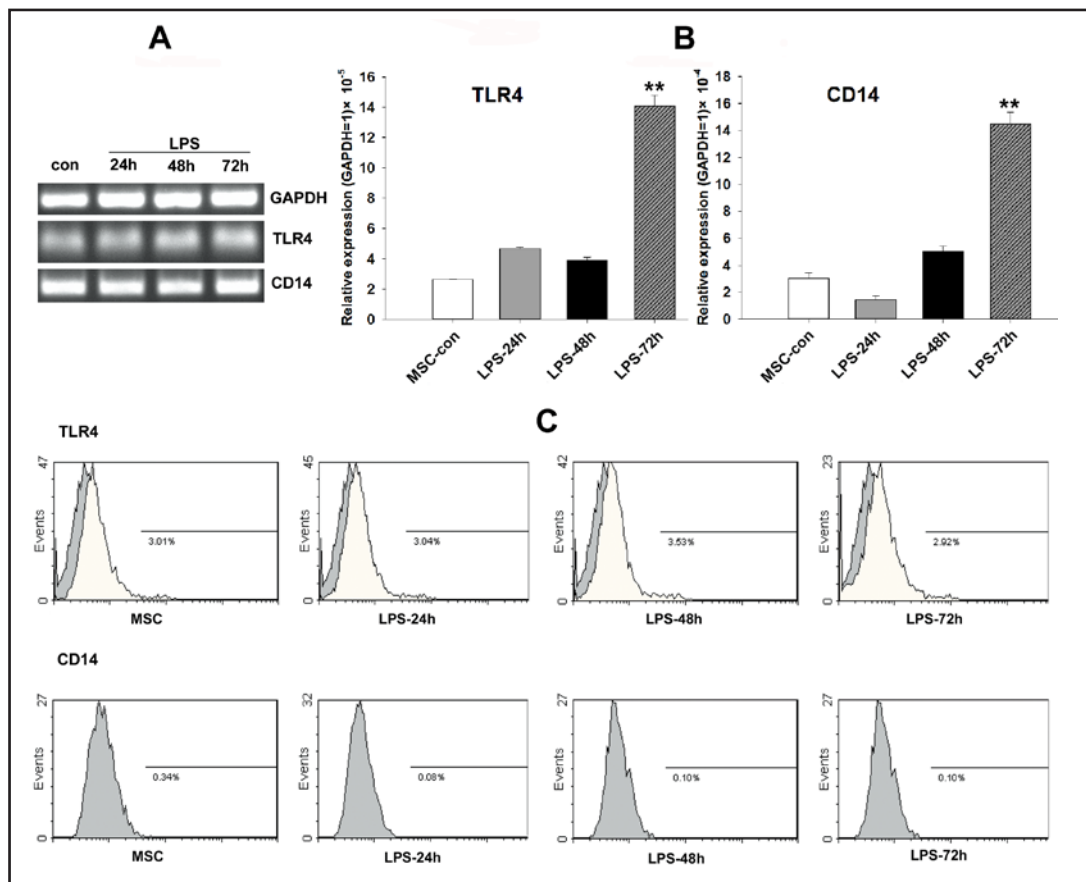
#### *LPS stimulation resulted in increased inflammatory cytokines and chemokine expression in hWJ-MSCs*

qRT-PCR was utilised to analyse the Th1 (IL-2, IFN- $\gamma$ , and TNF- $\alpha$ ) and Th2 cytokines (IL-4, IL-10, and IL-13), inflammatory cytokines (IL-1 $\beta$ , IL-1 $\alpha$ , IL-6, and IL-12), and chemokine (IL-8) expression profiles in hWJ-MSCs. The results demonstrated the constitutive expression of IL-1 $\beta$ , IL-1 $\alpha$ , IL-6, and IL-8 at very high levels, whereas the expression levels of IL-2, IL-4, IL-10, IL-13, and TNF- $\alpha$  were undetectable (data not shown). LPS had no/slight effect on the expression of IL-1 $\beta$ , IL-1 $\alpha$ , IL-6, and IL-8 in hWJ-MSCs at 48 h though a marked increase was observed after 72 hours (all  $p=0.000$ ). Very low IL-12 and IFN- $\gamma$  mRNA levels were detected by qRT-PCR; overall, IL-12 was inhibited significantly (all  $p=0.000$ ) (Fig. 4) and IFN- $\gamma$  was not induced by LPS after the entire treatment (data not shown).

To confirm the qRT-PCR results, we assessed the cytokines' levels in the conditioned medium from hWJ-MSCs treated with LPS. Of all the tested cytokines, only IL-6 and IL-8 were detectable, showing a significant increase after cells exposing to LPS for up to 72 h compared to controls ( $p=0.01$  and  $0.007$ ) (Fig. 2A).

#### *LPS promotes MMP-2 expression but inhibits MMP-9 expression in hWJ-MSCs*

As shown by qRT-PCR analysis, MMP-2 and MMP-9 were constitutively expressed in hWJ-MSCs, and MMP-2 was expressed at high levels; in contrast, MMP-9 was expressed at a relatively low level. LPS stimulation resulted in the significant up-regulation expression of



**Fig. 3.** TLR4 and CD14 expression, as analyzed by qRT-PCR and normalised to GAPDH (A and B) (\*\* $p < .01$  compared with control). MSCs (3<sup>rd</sup> passage) not treated or treated with LPS were negative for CD14, as shown by flow cytometry, but were only constitutively and marginally positive for TLR4 (C).

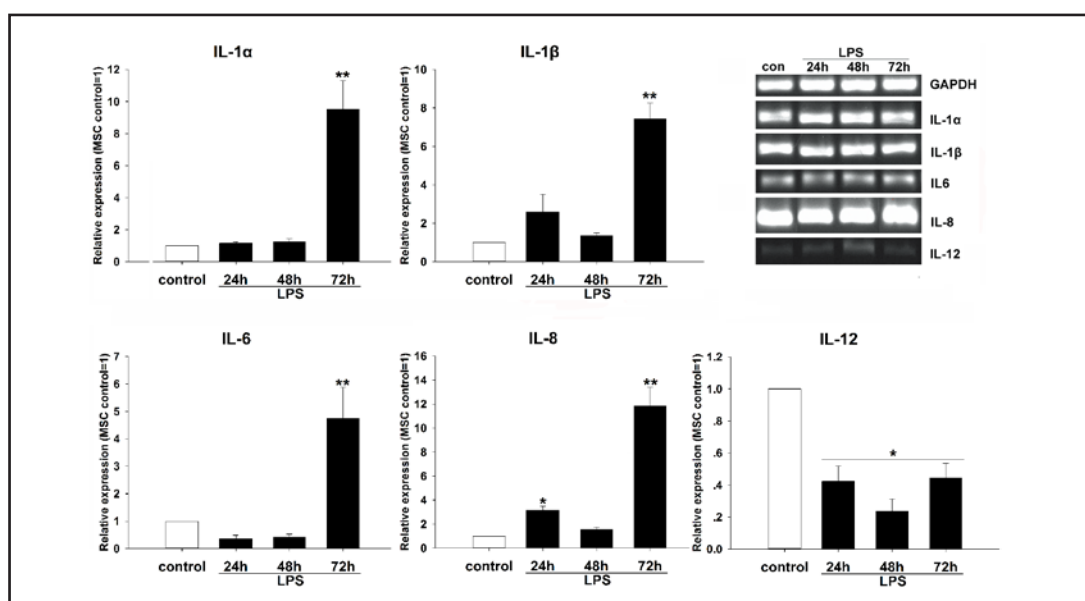
MMP-2 and transiently down-regulated expression of MMP-9 in hWJ-MSCs. The expression of MMP-2 was not altered in the first 48 h ( $p=0.997$  and  $0.828$ ) but increased in the next 24 h ( $p=0.000$ ). This finding is in sharp contrast to MMP-9, which was inhibited by LPS in the first 48 h ( $p=0.000$  and  $0.000$ ) and increased to a relatively normal level after 72 h ( $p=0.236$ ) (Fig. 5).

#### *LPS regulates the transcription of immunosuppressive mediators in hWJ-MSCs*

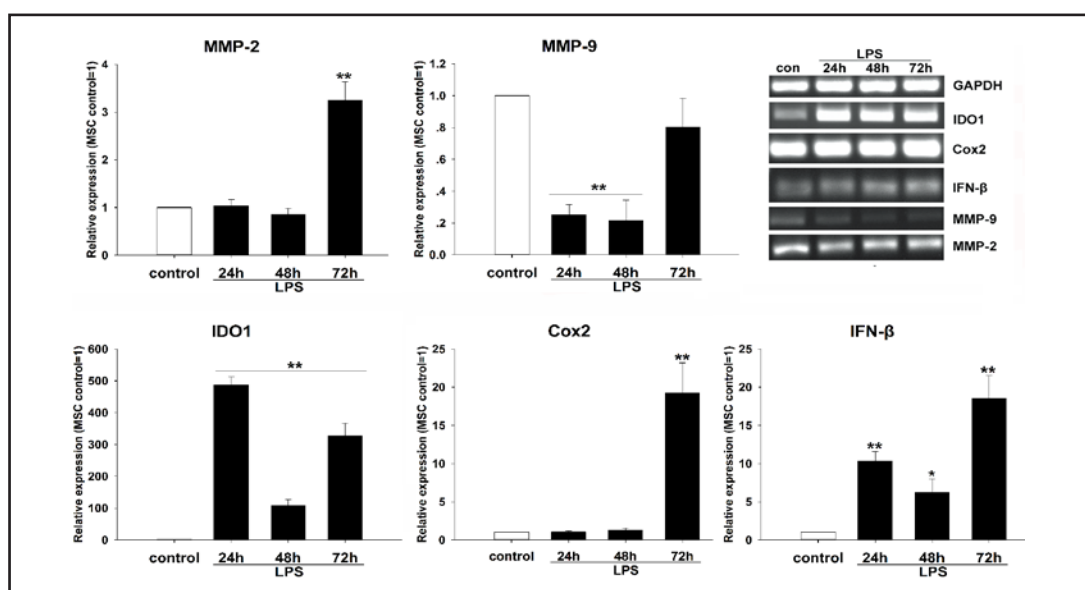
The expression of IDO-1, IDO2, Cox2, and IFN- $\beta$  was detected by qRT-PCR analysis. The expression of IDO-1 and IFN- $\beta$  at the mRNA level showed a significant increase after LPS exposure over the whole period of 72 h ( $p=0.000, 0.003, 0.000$  and  $p=0.001, 0.034, 0.000$ ), whereas the significant induction of Cox2 only appeared after a 72-h LPS exposure ( $p=0.000$ ) (Fig. 5). Regarding the expression of IDO2, no change was detected at different exposure times to LPS (data not shown).

## Discussion

The results of this study demonstrated the expression profile of TLR4 in hWJ-MSCs. An LPS model mimicked the inflammatory changes in the transcription and/or translation profiles of paracrine factors, such as cytokines and chemokine related to inflammation (IL-1 $\beta$ , IL-1 $\alpha$ , IL-6, IL-8, and IL-12), matrix metalloproteinases (MMP-2 and MMP-9),



**Fig. 4.** The inflammatory cytokine expression profiles of hWJ-MSCs exposed or not exposed to LPS for different time periods, as analysed by qRT-PCR (n=3, hWJ-MSCs not exposed to LPS as control; \*  $p < .05$  and \*\*  $p < .01$  compared with control).



**Fig. 5.** The expression profiles of metalloproteinases (MMP-2 and MMP-9) and immunosuppressive molecules (IDO1, Cox2, and IFN-β) of hWJ-MSCs exposed or not exposed to LPS for different lengths of time, as analysed by qRT-PCR (n=3, hWJ-MSCs not exposed to LPS as control; \*  $p < .05$  and \*\*  $p < .01$  compared with control).

and immunosuppressive molecules (IDO1, Cox2, and IFN-β). In contrast, no effect on the proliferation and apoptosis of hWJ-MSCs was found. The results illustrated that LPS engagement modulate the biological function and immune immunosuppressive properties of hWJ-MSCs, similar to hBW-MSCs, hAT-MSCs, and hCB-MSCs [23, 25, 26].

Specific PAMPs recognised by their specific TLRs trigger the production of different cytokines [15]. MSCs express Toll-like receptors (TLRs) that activate the TLR signalling



pathway *ex vivo*, impacting the proliferation, immunomodulation, and migration of these cells [23-27]. The functions of adult MSCs, such as hBW-MSCs and hAT-MSCs, were modulated by the TLR4-LPS signalling pathway *ex vivo*, and LPS stimulation resulted in the increased production of such inflammatory cytokines and chemokines as IL-1 $\beta$ , IL-6, and IL-8 [17, 23-25, 28]. However, for the role of TLR4 in hWJ-MSCs, two early reports came to the opposite conclusion [27, 29]. Raicevic et al. explored the details of the hWJ-MSCs TLRs expression profiles and found that TLR4 is not expressed in these cells at transcription level or protein level and consequently do not respond to LPS activation [29]. In contrast, report by Chen et al. demonstrated the presence of TLR4 on the surface of human umbilical cord-derived MSCs (hUC-MSCs) at a very low level (5%-6%) by flow cytometry analysis but further confirmed that LPS engagement induced NF- $\kappa$ B translocation and resulted in the up-regulation of IL-6, IL-8, and IFN- $\gamma$  mRNA expression in hUC-MSCs, which indicated TLR4 is a functional receptor [27]. Because of that LPS stimulation increased the TLR4 mRNA expression in MSCs at low concentration but decreased at very high concentration [31]. Moreover, the two groups used different isolating method respectively to obtain MSCs from human Wharton's jelly though no difference was found between the MSCs isolated by the two methods so far as we know. We speculated that the isolation method and the concentration and length of incubation with LPS might contribute to the confliction between the two reports. In this study, we isolated hWJ-MSCs like Chen et al. and exposed the cells to LPS at an appropriate concentration (1.0  $\mu$ g/ml) [32] for a longer (72 h) time period and wished make a comprehensive evaluation for the TLR4 expression profiles and the paracrine effect changes in hWJ-MSCs response to LPS engagement at different time. Our results showed that the constitutive expression of TLR4 was low at the protein level (approximately 3%) but high at the mRNA level in hWJ-MSCs, consistent with the report by Chen et al. [27]. The TLR4 profile on the surface of hWJ-MSCs was not altered during the course of LPS stimulation according to our flow cytometry analysis, but the continuous transcription of TLR4 was significantly up-regulated when hWJ-MSCs were stimulated for 72 h. This same increase was observed for CD14, a co-receptor of TLR4, though the CD14 protein is absent in hWJ-MSCs. The data showed LPS initiated a transcriptional regulation of TLR4 and CD14 expression in hWJ-MSCs. The discrepancy between transcription and translation could be explained by the translational regulation, which was also observed for TLR4 expression in hCB-MSCs [26]. The pro-inflammation cytokines (IL-1 $\beta$ , IL-1 $\alpha$ , and IL-6) and chemokine (IL-8) related to the TLR4-LPS signalling pathway was also up-regulated at the mRNA and/or protein levels in hWJ-MSCs treated with LPS. The pro-inflammatory state shift provoked by LPS in hWJ-MSCs in our study showed a "dull" response compared to the responses observed in hBM-MSCs and hAT-MSCs [23-25, 27] but showed similar characteristics to hCB-MSCs [26]. These findings suggested that the hMSCs derived from foetal tissue are not sensitive as the MSCs derived from adult tissues to the LPS engagement [2, 4, 7, 25, 26].

MMPs appear to play an essential role in angiogenesis, proliferation, apoptosis, and invasion. MMP-2 but not MMP-9 is secreted by hBM-MSCs, which promotes their migration through the extracellular matrix, and MMP-2 is a key factor involved in the transmigration and invasion of hMSCs [33-36]. Ding Y et al. [37] showed that hBW-MSCs prevent the rejection of fully allogenic islet grafts in a mouse model via the immunosuppressive activity of MMP-2 and MMP-9. In another report, TLR9 ligands increased hBW-MSC invasiveness, at least partially mediated by MMP-13 [38]. These reports showed that TLRs modulate MSCs through MMPs. In the present study, we also detected the transcriptional expression of MMP-2 and MMP-9 in hWJ-MSCs. The results showed the constitutive expression of MMP-2 at a relatively high level and MMP-9 expression at a relatively low level in hWJ-MSCs compared to the expression of GAPDH. MMP-2 was up-regulated in response to LPS stimuli, whereas MMP-9 expression was temporary inhibited by LPS in the first 48 h and increased to a relatively normal level at 72 h. Our results suggest that both MMP-2 and MMP-9 play roles in the TLR4-LPS signalling pathway in hWJ-MSCs [39], but show different responses.

hMSCs secrete some paracrine factors that mediate immunosuppression, such as PGE2, IDO, IFN- $\beta$ , and transforming growth factor- $\beta$  [27, 40, 41]. However, Cox2 but not

IDO or IFN- $\beta$  has been reported to respond to LPS stimulation in hWJ-MSCs [27]. In our study, we found that IDO1 and IFN- $\beta$  mRNA were constitutively expressed in hWJ-MSCs at low level and were both up-regulated in the presence of LPS. The expression of IDO1 and IFN- $\beta$  displayed a sharp increase in the whole period of LPS exposing and they showed the same increase trend at the three time points by qRT-PCR analysis. It might result from that an autocrine IFN- $\beta$  could up-regulate the expression profile of IDO1 by LPS engagement in MSCs [42]. The expression of Cox2 also increased, but only when the cells were exposed to LPS for up to 72 h, in contrast to the results in previous reports [27]. These results suggest that hWJ-MSCs have different immune properties in the inflammatory environment and that the immunomodulatory capability of hWJ-MSCs primed with LPS is enhanced as well as hBW-MSCs [27, 42].

In summary, TLR4 was constitutively and functionally expressed in hWJ-MSCs. Compared with hAT-MSCs and hBW-MSCs, a longer LPS exposure time was necessary to initiate a pro-inflammatory shift in hWJ-MSCs, which suggests that hWJ-MSCs are less sensitive in an inflammatory milieu. Moreover, the up-regulation of MMP-2, Cox2, IDO1 and IFN- $\beta$  in hWJ-MSCs in response to LPS stimuli suggested the greater immunosuppressive potential of hWJ-MSCs in the presence of LPS. These data strengthen the viewpoints previously reported that suggest that hWJ-MSCs are primitive stromal cells and have good clinical potential for regenerative therapy [2, 29, 43].

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## Conflict of Interest

The authors have no commercial or financial interest in the products or companies mentioned in this article.

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