MALP-2, an agonist of TLR6, promotes the immune status without affecting the differentiation capacity of umbilical cord mesenchymal stem cells

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Abstract. Mesenchymal stem cells (MSCs) are increasingly used in cell-based therapy due to their multiple differentiation capacity, low expression of co-stimulatory factors and immunosuppressive effect. However, accumulating studies reported the recognition and rejection of engrafted MSCs, which eventually led to the fail of clinical trials. Toll-like receptors (TLRs) are important in mediating the immune response. In the present study, macrophage-activated lipopeptide-2 (MALP-2) was introduced to activate the TLR6 pathway in umbilical cord MSCs (UCMSCs). PBLs isolated from healthy volunteers were co-cultured with UCMSCs to measure whether activation of TLR6 of UCMSCs could stimulate immune responses. Reverse transcription-quantitative polymerase chain reaction and immunohistochemistry were performed to detect pro-inflammatory molecules and differentiation status of UCMSCs, respectively. The results indicated that activation of TLR6 in UCMSCs increased the proliferation of peripheral blood leukocytes (PBLs) and enhanced the release of lactate dehydrogenase in damaged UCMSCs, which confirmed the role of TLR6 in promoting the immunogenicity of UCMSCs. Furthermore, quantitative polymerase chain reaction demonstrated that the expression of proinflammatory molecules (including IL-1β, IL-6, IL-8, IL-10, CCL1 and CCL4) was induced, whereas the expression of stem cell markers (Klf4 and Nanog) was inhibited. The differentiation results indicated

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that activation of TLR6 had no effect on the differentiation capacity of UCMSCs. All these findings suggest that stimulation of TLR6 pathway may increase the immunogenicity of UCMSCs in *in vitro* detections. In conclusion, the results of the current study indicated a new role of TLR6 in regulating the biological function of UCMSCs.

Introduction

Mesenchymal stem cells (MSCs) are currently widely used in stem cell therapy for chronic inflammatory disorders, autoimmune diseases, graft-vs.-host disease and allograft rejection (1-3). MSCs are widely distributed *in vivo* and are found in the bone marrow, adipose tissue, brain, heart, skin, umbilical cord and peripheral blood (4). These cells can be differentiated into numerous tissue phenotypes, including neural, pancreatic and hepatocytic phenotypes, in specific microenvironments (5). The lack or low expression of co-stimulatory factors, such as CD80, CD86, CD40, CD34 and human leukocyte antigen-II, largely benefit the MSC-based therapy (6). Another advantage of MSCs in clinical therapy is their immunosuppressive effect. MSCs are able to inhibit the immune responses by blocking the functions of T-, B-, natural killer (NK) and dendritic cells (7-9).

The mechanism underlying the effect of MSCs on the *in vivo* biological functions remains unknown (10). In spite of the known immunosuppressive effect of MSCs, several studies have reported that MSCs induced immune responses, resulting in the injury and rejection of MSCs, and eventually leading to the failure of clinical therapy (11-15). Implanted MSCs are recognized by the immune system of recipients and are eliminated (12). Previous studies have confirmed this notion, since MSCs activated NK cell-mediated immune rejection and induced memory T cell response (13,14). Evidence also indicated the injury of MSCs was mediated by the complement system (15).

Toll-like receptors (TLRs) are a family of pathogen-associated molecular patterns, which serve important roles in the activation of immune responses. There are 11 members in the human TLRs family, which bind to distinct components from

Key words: umbilical cord mesenchymal stem cells, Toll-like receptor 6, macrophage-activated lipopeptide-2, immunogenicity, biological function

bacteria, viruses, fungi, as well as circulating endogenous ligands (16). TLR6 is located on the surface of cell membrane and recognizes diacylated lipoprotein of bacteria and conserved regions of dengue virus (17). However, to the best of our knowledge, no previous study has elucidated the role of TLR6 in regulating the immune status of MSCs isolated from the umbilical cord. In the present study, the TLR6 pathway was stimulated by a TLR6 agonist, namely macrophage-activated lipopeptide-2 (MALP-2), and the effects of TLR6 activation on the immune status of umbilical cord MSCs (UCMSCs) was investigated.

Materials and methods

Culture and stimulation of UCMSCs. Human UCMSCs were provided by the Sichuan Umbilical Cord Blood Stem Cell Bank (Chengdu, China). The UCMSCs were isolated by the cell bank according to a previously described method (18). Briefly, following disinfection in 75% ethanol for 10-20 min, the umbilical cord was diced and then placed into 25 mm² plates. The culture medium was added when tissues were firmly attached to the plates (usually 15-20 h). Next, the mesenchymal tissues were removed 5-8 days later, and then dissociated MSCs were collected. The UCMSCs were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.) and then stored in liquid nitrogen (-160°C) for future use. The present study was approved by the Ethics Committee of the West China Second Hospital, Sichuan University (Chengdu, China). The TLR6 agonist MALP-2 was purchased from Novus Biologicals LLC (Littleton, CO, USA) and prepared as recommended by the instruction manual.

Leukocyte proliferation and cell injury assay. Peripheral blood leukocytes (PBLs) were isolated from blood samples of healthy volunteers (subsequent to obtaining written informed consent) by centrifugation gradient (1,200 x g for 5 min and 10°C). PBLs were then labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) at a final concentration of 10 μ M, and cold complete DMEM was added for 10 min to stop the reaction. CFSE-labeled PBLs were then washed three times by cold phosphate buffered saline and centrifuged at 1,200 x g for 5 min at 10°C to remove the unlabeled CFSE. Subsequently, the cells were co-cultured with UCMSCs (co-culture ratio, 10:1) in the presence of MALP-2 (100 μ g/ml) and the MALP-2 untreated co-culture group was used as control. UCMSCs treated with 5% Triton X-100 was used as the positive control, which acted as the maximum release group. Cells were collected at 72 h after stimulation for detection of the PBL proliferation by fluorescence-activated cell sorting, (increased PBL proliferation dilutes the labeled fluorescence). The supernatants from the two PBL-UCMSC co-culture groups (untreated control and MALP-2-treated group) were collected by centrifugation at 3,000 x g for 20 min at 4°C, and the lactate dehydrogenase (LDH) levels were measured by a cytotoxicity detection kit (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions. The lysis percentage was calculated using the following formula: Lysis $(\%) = (E-M)/(T-M) \times 100$, where E is the experimental release, M is the spontaneous release in the presence of media alone, and T is the maximum release in the presence of 5% Triton X-100.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for quantification detection. The mRNA expression levels of interleukin (IL)-1β, IL-6, IL-8, IL-9, IL-10, chemokine (C-C motif) ligand 1 (CCL1), CCL2, CCL4, CCL26, Kruppel-like factor 4 (Klf4), Lin28, Nanog, SRY-box 17 (Sox 17) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were determined by RT-qPCR. Briefly, total RNA was extracted from the treated and untreated UCMSCs, and reverse transcription was performed using a ReverTra Ace qPCR RT kit (FSQ-101; Toyobo Co., Ltd., Kagoshima, Japan) to synthesize cDNA, using the following conditions: 65°C for 5 min, 37°C for 15 min and 98°C for 5 min. Next, qPCR was conducted with the SYBR Green RealMaster Mix (FP202; Tiangen Biotech Co., Ltd., Beijing, China) under the following thermal cycling conditions: One cycle at 95°C for 30 sec, 40 cycles at 95°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec, followed by a melt curve from 55 to 95°C in 0.5°C increments and 10 sec intervals. The primers used for qPCR assay are listed in Table I. GAPDH was used as an internal control. Quantitative data were analyzed using Bio-Rad iQ5 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The figures were completed using GraphPad Prism5 (GraphPad Software, Inc., La Jolla, CA, USA).

Detection of surface markers. MALP-2-treated and untreated UCMSCs were harvested at 72 h post-stimulation. The UCMSCs were stained with different antibodies to detect the expression levels of surface molecules, including co-stimulators and stem cell markers. The antibodies used in this assay were the following: CD40 (cat. no. 11-0809), CD59 (cat. no. 11-0596), CD86 (cat. no. 12-0869) and CD90 (cat. no. 45-0909; all from eBioscience, Thermo Fisher Scientific, Inc.), all were diluted to 1:200 with serum-free DMEM. Flow cytometry was then conducted and analyzed using CXP flow cytometry software (Beckman Coulter, Brea, CA, USA). Positive expression was gated according to the fluorescence intensity of the negative control group.

UCMSC differentiation. UCMSCs were seeded into 6-well plate with a density of 1.5×10^5 cells/well. Conditioned mediums for chondrocyte induction (A10071-01; Gibco; Thermo Fisher Scientific, Inc.), adipocyte induction (A10070-01; Gibco; Thermo Fisher Scientific, Inc.) and osteocyte induction (A10072-01; Gibco; Thermo Fisher Scientific, Inc.) were added in each well with or without MALP-2 treatment. The results of differentiation were collected at 10- and 15-days post-induction, and analyzed using oil-red staining for adipocytes, alizarin red staining for osteocytes and safranin staining for chondrocytes (eBioscience; Thermo Fisher Scientific, Inc.).

Statistical analysis. Values are expressed as the mean ± standard error of the mean using SPSS version 16.0 software (SPSS, Inc., Chicago, IL, USA). Significant differences of cytotoxicity percentage between MALP-2 treated and untreated groups were analyzed using a Student's t-test. Significant differences



Gene	Forward primer (5'-3')	Reverse primer (5'-3')	GenBank no. M15330	
IL-1β	ACGAATCTCCGACCACCACT	CCATGGCCACAACAACTGAC		
IL-6	GACCCAACCACAAATGCCA	GTCATGTCCTGCAGCCACTG	M14584	
IL-8	CTGGCCGTGGCTCTCTTG	CCTTGGCAAAACTGCACCTT	NM_000584	
IL-9	CTCTGTTTGGGCATTCCCTCT	GGGTATCTTGTTTGCATGGTGG	M30134	
IL-10	GGTGATGCCCCAAGCTGA	TCCCCCAGGGAGTTCACA	U16720	
CCL1	GCAGATCATCACCACAGCC	GTCCACATCTTCCGGCCA	NM_002981	
CCL2	CTGCTCTCCAGCGCTCTCA	GTAAGAAAAGCAGCAGGCGG	NM_002984	
CCL4	CAGTGCTTCTGTGCCTGCTG	TGCATCTGGCTGAGCAAGTC	NM_005408	
CCL26	CCAAGACCTGCTGCTTCCAA	GAATTCATAGCTTCGCACCCA	NM_006072	
Klf4	GTCATCAGCGTCAGCAAAGG	CCCTGCTGCTCAGCACTT	NM_004235	
Lin28	TGCTGTCGAGGGATGGATA	CCACCCAATGCGTTCTATTG	NM_024674	
Nanog	CCAAAGGCAAACAACCCACTT	CGGGACCTTGTCTTCCTTTTT	NM_001297	
Sox 17	TGGCGCAGCAGAATCCA	CGACTTGCCCAGCATCTTG	NM_022454	
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	J04038	

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MALP-2, macrophage-activated lipopeptide-2; IL, interleukin; CCL, chemokine (C-C motif) ligand; Klf4, Kruppel-like factor 4; Sox 17, SRY-box 17; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

between untreated UCMSCs and different time points of MALP-2 treated UCMSCs were analyzed by analysis of variance and post hoc test (Tukey's) after homogeneity test of variances. Values of P<0.05 and P<0.01 were considered to indicate statistically significant differences compared with the control group.

Results

Activation of TLR6 in UCMSCs enhances the proliferation of PBLs. The proliferation of CFSE-labeled PBLs is widely used to detect an increased immune attack. In the present study, CFSE-labeled PBLs were co-cultured with UCMSCs in the presence of MALP-2. PBLs were harvested at 72-h after the co-culture, and the proliferation of PBLs was then detected by flow cytometry. The results indicated that the proliferation of PBLs in the group treated with MALP-2 was increased (18.4%) as compared with that in the untreated co-culture group (9.2%), which suggested that TLR6 activation was able to increase the immune response of PBLs against UCMSCs (Fig. 1A).

The damage of cells is frequently detected on the basis of LDH release from injured cells into the culture supernatant. The results of the current study indicated that cytotoxicity percentage were not significantly different between the MALP-2 treated and control groups following 24 and 48 h of co-culture (Fig. 1B). However, the cytotoxicity percentage was significantly increased in the TLR6-activated group compared with the untreated group after 72 h (P<0.05; Fig. 1B). These results suggested that the activation of TLR6 stimulated the immune attack of PBLs against UCMSCs.

TLR6 activation increases the expression of surface co-stimulatory molecules in UCMSCs. Given the aforementioned results stating that TLR6 activation increased the proliferation and immune attack in the co-culture of PBLs and UMCSCs, the present study also investigated whether MALP-2 treatment influences the expression of surface co-stimulatory factors and stem cell surface markers on UCMSCs. The UCMSCs were treated in the presence or absence of MALP-2, and the surface markers were measured after 72-h incubation by flow cytometry, including measurement of the stimulatory molecules CD40 and CD86, as well as of the stem cell specific markers CD59 and CD90. The results indicated that TLR6 activation enhanced the expression levels of CD40 (15 vs. 4.3%) and CD86 (13.5 vs. 2.0%) as compared with the untreated group (Fig. 2). By contrast, the surface stem cell marker CD90 presented no evident variation, while the expression of CD59 was slightly inhibited in MALP-2 treated UCMSCs (97.9 vs. 90.2%; Fig. 2).

Treatment with MALP-2 promotes the expression of cytokines and chemokines. RT-qPCR was conducted to detect the expression levels of important cytokines, chemokines and stem cell markers. UCMSCs were stimulated with MALP-2, and total RNA was extracted at 4, 12, 24, 72 and 120 h post stimulation. qPCR was performed to assay the expression of these important immune system-associated molecules. In cytokine detection, the expression level of IL-10 was significantly induced after treatment for 12-120 h (all P<0.01; Fig. 3A), while IL-1 β expression only increased after 24-120 h (24 h, P<0.01; 72-120 h, P<0.05). IL-6 (12 h, P<0.05; 24-120 h, P<0.01) and IL-8 (all P<0.01) expression increased 12-120 h post stimulation. In chemokine assessment, CCL4 was significantly induced from 24-120 h of treatment (all P<0.01; Fig. 3B), and CCL1 expression increased after 120 h of stimulation (P<0.05). However, the other two chemokines, CCL26 (72 h, P<0.05; 120 h, P<0.01) and CCL2 (12-24 h, P<0.05; 72 h, P<0.01; 120 h, P<0.05), presented inhibited expression, possibly due to the complex biological functions of chemokines in vivo.



Figure 1. (A) Flow cytometry results on PBL proliferation and (B) detection of LDH level as an indicator of cytotoxicity in the PBL-UCMSC co-culture system. *P<0.05 vs. control group. PBL, peripheral blood leukocyte; MSC, mesenchymal stem cell; UCMSC, umbilical cord MSC; MALP-2, macrophage-activated lipopeptide-2.



Figure 2. Analysis of co-stimulatory molecules and stem cell markers in UCMSCs upon MALP-2 stimulation. UCMSCs, umbilical cord mesenchymal stem cells; MALP-2, macrophage-activated lipopeptide-2.

To confirm whether activation of TLR6 influences the stemness of UCMSCs, expression levels of stem cell markers, including Klf4, Lin28, Nanog and Sox17, were detected in the present study. The Nanog expression significantly decreased from 4-120 h of treatment (all P<0.01; Fig. 3C), while Klf4 expression increased at 4 h (P<0.01), but was then significantly inhibited from 24-120 h of stimulation with MALP-2 (all P<0.01; Fig. 3C). Furthermore, the expression of Lin28 was significantly increased after 12 h of MALP-2 stimulation (12 h, P<0.05; 24-120 h, P<0.01), and Sox17 expression was only induced after 120 h of treatment with MALP-2 (P<0.05; Fig. 3C).

Stimulation with a TLR6 agonist has no effect on the differentiation ability of UCMSCs. The differentiation ability of UCMSCs was also assayed to confirm whether it was regulated by the activation of the TLR6 pathway. Conditional media for adipocytes, osteoblasts and chondrocytes were used to induce UCMSC differentiation. The specific cell phenotypes were identified by different staining methods, more specifically alizarin red for osteoblasts (Fig. 4A), safranin for chondrocytes (Fig. 4B) and oil red O for adipocytes (Fig. 4C), in order to detect the differentiation status of UCMSCs at 7 and 15 days post MALP-2 treatment. The results indicated that there





Figure 3. Effect of MALP-2 treatment on the expression levels of (A) proinflammatory cytokines, (B) chemokines and (C) stem cell markers. Data are presented as the mean ± standard derivation from three independent experiments. *P<0.05 and **P<0.01 vs. control group. MALP-2, macrophage-activated lipopeptide-2; IL, interleukin; CCL, chemokine (C-C motif) ligand; Klf4, Kruppel-like factor 4; Sox 17, SRY-box 17.



Figure 4. Differentiation of UCMSCs with or without MALP-2 stimulation. Differentiation of (A) osteoblasts, (B) chondrocytes, and (C) adipocytes. UCMSCs, umbilical cord mesenchymal stem cells; MALP-2, macrophage-activated lipopeptide-2.

were no differences between the control and TLR6-activated groups, which suggested that the TLR6 pathway had no role in regulating the differentiation of UCMSCs (Fig. 4A-C).

Discussion

The characteristics of MSCs that result in their important role in cell-based therapy include multipotent differentiation ability, lack of co-stimulatory molecule expression and an immunosuppressive effect on the immune system (19). However, recent clinical trials, including those using MSCs to treat graft versus host disease (GVHD), myocardial infarction and liver cirrhosis have not achieved encouraging results due to incomplete understanding of the engrafted MSCs (11,12). Human and animal studies have demonstrated the recognition and elimination of the MSCs within a few days of infusion by modifying the low immune status of MSCs in specific *in vivo* microenvironments, which eventually resulted in enhanced immune responses.

Proteins of the TLR family are important in bridging innate and adaptive immune response by recognizing conserved components of invasive pathogens, as well as circulating endogenous ligands (20). As UCMSCs is currently becoming more important in cell therapy, the role of TLRs in regulating the biological functions of UCMSCs is critical for improving the controlled and desirable clinical outcomes. Previously, it was confirmed that TLRs are important in regulating the differentiation capacity and immunosuppressive effect, as well as serving a role in MSC survival (21). However, the role of TLRs in the alteration of immunogenicity has seldom been reported. For instance, the study by Zhang *et al* reported that a TLR7 agonist increased the immunogenicity of UCMSCs (22), while the activation of TLR3, TLR4 and TLR9 had no effect on the immune status of UCMSCs (23,24).

In the present study, the role of TLR6 in the immune status of UCMSCs was assessed by stimulation with the TLR6 agonist, MALP-2. The results indicated that activation of TLR6 increased the proliferation of PBLs and enhanced the release of LDH in the co-culture system, which demonstrated the existence of immune response against UCMSCs. In addition, surface molecule detection revealed increased expression of CD40 and CD86 following stimulation, which are well known co-stimulatory factors in mediating immune response. Furthermore, the expression levels of proinflammatory cytokines (IL-1 β , IL-6, IL-8 and IL-10) were significantly induced upon MALP-2 stimulation, as well as the levels of two chemokines (CCL1 and CCL4). Huang et al has suggested that differentiation of MSCs into cardiomyocytes increased the immunogenicity of MSCs (25), while Zhang et al also confirmed that activation of TLR7 increased the immune status of UCMSCs by enhancing their differentiation ability (22). Thus, the differentiation capacity of UCMSCs was detected by adding conditional media in the present study, and the results revealed no marked difference between the control and TLR6 agonist-stimulated groups, which suggested that TLR6 had no effect on the differentiation of UCMSCs.

In conclusion, the present study demonstrated that activation of TLR6 resulted in alteration of the immune status of UCMSCs. In clinical trials, the infused UCMSCs will encounter a number of endogenous ligands that may function

as agonists to activate different TLR pathways. Thus, future studies are required to confirm the mechanisms underlying the effect of immune response-associated factors in regulating the biological functions of UCMSCs via the TLR6 pathway.

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