Alteration of the immune status of umbilical cord mesenchymal stem cells stimulated by TLR1/2 agonist, Pam3Csk

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Abstract. Mesenchymal stem cells (MSCs) have been widely used in clinical trials due to their multiple differentiation ability, low immunogenicity and immunosuppressant effects on immune response. However, accumulating evidence has indicated that MSCs may stimulate *in vivo* immune responses and result in the disappearance of MSCs following engrafting. Toll-like receptors (TLRs) are important in immune response induction against invaded pathogens, however, the function of TLRs in regulating the immune status of MSCs has been seldom reported. The present stimulated umbilical cord (UC) MSCs by treatment with the TLR1/2 agonist, Pam3Csk, the to determine whether activation of TLR1/2 signaling alters the immune status of UCMSCs. The results indicated that activation of TLR1/2 increased the proliferation of peripheral blood mononuclear cells (PBMCs) and the production of lactate dehydrogenase in a PBMC-MSC co-culture system. The study also demonstrated that Pam3Csk induced the secretion of pro-inflammatory molecules, and increased the expression levels of cytokine and chemokines in UCMSCs. Flow cytometry analysis indicated that the levels of surface co-stimulators, CD80 and CD86, were increased on UCMSCs in the presence of Pam3Csk, whereas activation of TLR1/2 exerted no observable effect on the differentiation abilities of UCMSCs. The results of the current study indicated that activation of TLR1/2 signaling may alter the immune status of UCMSCs, however, further mechanistic research is required in future studies.

Introduction

Mesenchymal stem cell (MSC)-based therapy has been widely used in clinical trials and led to exciting developments in cell therapy, including the prevention of graft versus host disease (1), reducing liver fibrosis (2), remodeling of broken bone (3) and treatment of cardiovascular diseases (4). An important characteristic of MSCs is the ability to differentiate into various cell types (5). Another feature of MSCs is the lack of co-stimulatory molecules including CD80, CD86 and HLA-II, which result in MSCs failing to induce immune responses (6). Additionally, MSCs also suppress the immune reactions mediated by T cells, B cells, natural killer (NK) cells, dendritic cells and complements (7-9). However, recent studies have indicated that although MSCs exhibit low-immunogenicity and immunosuppressant abilities, MSCs used as a therapeutic may be rejected or injured by the host immune system and eventually disappear *in vivo* (10-12).

Toll-like receptors (TLRs) are a family of pathogen-associated molecular patterns (PAMPs) involved in mediating immune responses induced by invading pathogens (13). There are 10 members in the human TLRs family, which recognize distinct microbial products from bacteria, viruses and fungi (14). The TLRs also important for in MSC functions, including increasing osteogenic differentiation (TLR3) (15), promoting MSCs migration (TLR5) (16) and inhibiting MSCs mediating immunosuppression (TLR4) (17). However, the importance of TLRs in regulating the immune status of MSC has not been widely investigated. A previous report demonstrated that TLR7 stimulates the immunogenicity of MSCs (18), whereas TLR3 and 4 did not alter the immune status of MSCs. However, TLR3 and TLR4 agonists enhanced the expression of various immune-associated molecules (19). To the best of our knowledge, no previous report has investigated the importance of TLR1/2 on the immune status of MSCs. The current study used MSCs isolated from umbilical cord (UC) and activated the TLR1/2 pathway using a specific agonist, aiming to determine whether the activation of TLR1/2 changes the immune status of MSCs.

Materials and methods

Culture and stimulation of MSCs. The MSCs from UC were provided by Sichuan Umbilical Cord Blood Stem Cell Bank (Chengdu, China). The UCMSCs were maintained at 37˚C with 5% CO_2 in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at $1x10^5$ cells/well in a 6-well plate.

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Table I. Primers used for reverse transcription-polymerase chain reaction.

Gene	Forward primer	Reverse primer	Gen bank no.
IFN-β	CAGCAATTTTCAGTGTCAGAAGCT	TCATCCTGTCCTTGAGGCAGT	M28622
IL-6	GACCCAACCACAAATGCCA	GTCATGTCCTGCAGCCACTG	M14584
IL-8	CTGGCCGTGGCTCTCTTG	CCTTGGCAAAACTGCACCTT	NM_000584
IL-10	GGTGATGCCCCAAGCTGA	TCCCCCAGGGAGTTCACA	U16720
TNF-α	GGTGCTTGTTCCTCAGCCTC	CAGGCAGAAGAGCGTGGTG	M10988
CCL5	GACACCACACCCTGCTGCT	TACTCCTTGATGTGGGCACG	NM_002985
MCP-1	AGCAGAGGCTGGAGAGCTACA	GGGTCAGCACAGATCTCCTTGT	NM_006273
MCP-3	CCTCTCCTGCCTCATGCTTATT	CTCTGTCTCTGCATCATTTGTGAA	U58914
IP10	TGAAATTATTCCTGCAAGCCAA	CAGACATCTCTTCTCACCCTTCTTT	NM_001565
MIP-1	GACACCACACCCTGCTGCT	TACTCCTTGATGTGGGCACG	NM_002985
Nanog	CCAAAGGCAAACAACCCACTT	CGGGACCTTGTCTTCCTTTTT	NM_00129769
Sox2	CCCCTTTATTTTCCGTAGTTGTATTT	GATTCTCGGCAGACTGATTCAA	NM_003106.3
Lin28	GTCATCAGCGTCAGCAAAGG	CCCTGCTGCTCAGCACTT	NM_004235.4
Otx2	GGTTTCCTCTCCCTCTCCAC	AATTTGAATTTTTACGTCTGCTG	NM_002448.3
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	J04038

IFN-β, interferon-β; IL, interleukin; TNF-α, tumor necrosis factor-α; CCL5, C-C motif chemokine ligand 5; MCP, monocyte chemoattractant protein; IP10, interferon γ-induced protein 10; MIP-1, macrophage inflammatory protein-1; Nanog, Nanog homeobox; SOX2, sex determining region Y-box 2; Lin28, Lin-28 homolog A; Otx2, orthodenticle homeobox 2.

TLR1/2 agonist, Pam3Csk, was purchased from Novus Biologicals, Ltd. (Cambridge, UK) and dissolved in sterile water to 0.5 mg/ml as the stock concentration. The final concentration of Pam3Csk used to stimulate UCMSCs was 100 ng/ml.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA of treated and untreated UCMSCs was extracted using the RNeasy kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. ReverTra Ace kit (Toyobo Co., Ltd., Osaka, Japan) was used to perform the synthesis of cDNA with the following RT conditions: 65˚C (5 min), 37˚C (15 min) and 98˚C (5 min). qPCR was performed using RealMaster Mix SYBR Green (cat. no. FP202; Tiangen Biotech Co., Ltd., Beijing, China) in an iCycler iQ (Bio-Rad Laboratories, Inc., Hercules, CA, USA) under the following conditions: 95˚C (30 sec), 58˚C (30 sec) and 72˚C for (30 sec), followed by a melt curve from 55-95˚C in 0.5˚C increments and 10 sec intervals for 40 cycles. For quantification, GAPDH was used as the internal control while untreated UCMSC was negative control. The by $2^{-\Delta\Delta Cq}$ method was used for relative quantification (20). The primers used in detection were listed in Table I. All detections of qPCR were performed three times.

Antibody array. Supernatants from treated and untreated groups were collected at 4 h post-stimulation. Supernatants were centrifuged (800 x g, 10 min, 10˚C) to remove the residual cells and then stored at -80˚C. All samples, including TLR1 agonist treated and untreated, were screened for secreted protein using RayBio Human Antibody Array C Series 1000 (RayBiotech, Inc., Norcross, GA, USA) according to the manufacturer's protocol. Blots were analyzed using ImageJ software, version 1.50 (National Institutes of Health, Bethesda, MD, USA).

Leukocyte proliferation and leukocyte-mediated cytotoxicity detection. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteers and labeled by with carboxyfluorescein diacetate succinimidyl ester (CFSE) at 37˚C for 10 min with a final concentration 10 μM. The current study was approved by the ethics committee of The Central Hospital of Dazhou (Dazhou, China), and written informed consent was obtained. The labeling reaction was stopped by adding 5 ml pre-cooled complete medium. The remaining CFSE was removed by three washes with cold phosphate-buffered saline (800 x g, 5 min, 4˚C). PBMCs were then co-cultured with UCMSCs with or without TLR1/2 agonist Pam3Csk. The ratio of PBMCs and UCMSCs in co-culture system was 5:1. PBMCs were collected for proliferation detection by fluorescence-activated cell sorting (FACS) following 72-h stimulation.

Supernatants from the PBMC-UCSMSCs were harvested at 24, 48 and 72 h post stimulation. Three centrifugation steps were performed to remove the remaining cells that may influence the detection. Release of lactate dehydrogenase (LDH) from injured cells was detected by a cytotoxicity kit according to the manufacturer's protocol. Cytotoxicity (% lysis) was calculated using the following formula: (E - M)/(T - M) x 100; E is the experimental release, M is the spontaneous release in the presence of media alone, and T is maximum release in the presence of 5% Triton X-100.

Detection of surface markers and co-stimulators detection by FACS. Pam3Csk treated and untreated UCMSCs were collected for FACS detection by following 72-h stimulation. The UCMSCs were fixed with 10% formaldehyde for 10 min, then were stained

with different antibodies to detect surface stem cells markers and co-stimulatory molecules. The assay was performed using CXP flow cytometry software, version 2.0 (Beckman Coulter, Inc. Brea, CA, USA). The positive and negative standard was gated according to the control groups. The antibodies used in detection are listed in Table II. The dilution of all antibodies in FACS assay was 1:100 and incubated 30 min at room temperature. All assays were conducted three times.

Differentiation detection of UCMSCs. Conditioned medium of chondrocytes (cat. no. A10071-01), osteocytes (cat. no. A10072-01) and adipocytes (cat. no. A10070-01) were obtained from Gibco (Thermo Fisher Scientific, Inc.) and added to UCMSCs ($1.5x10^5$ per well) in 6-well plates in the presence of 100 ng/ml Pam3Csk. Oil-red O for adipocytes, alizarin red for osteocytes and safranine staining for chondrocytes was conducted on day 5, 14 and 20. Prior to staining [staining solutions diluted at 1:3 with double distilled water (ddH_2O)], cells were fixed with 10% formaldehyde soulution for 10 min at room temperature, then washed three times with ddH_2O. Subsequently, Mayer's hematoxylin staining was conducted for 5 min, followed by three further washes with ddH_2O.

Statistical analysis. The analysis of RT-qPCR results was performed using Bio-Rad iQ5 software (Bio-Rad Laboratories, Inc.). Results are expressed as the mean ± standard error and were analyzed with Student's t-test using SPSS software (version 16.0; SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference. All figures were created using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

Table II. Monoclonal antibodies used for fluorescence-activated cell sorting analysis.

Name	Company	Cat no.
CD40	eBioscience, Inc. (San Diego, CA, USA)	17-9953
CD80	eBioscience, Inc.	11-0809
CD86	eBioscience, Inc.	12-0869
CD59	eBioscience, Inc.	11-0596
CD74	eBioscience, Inc.	11-0748
CD90	eBioscience, Inc.	45-0909

CD, cluster of differentiation.

Table III. Lactate dehydrogenase levels in the supernatant of umbilical cord MSC-PBMC co-culture system.

Time	MSC + PBMC	MSC + PBMC + Pam3Csk	P-value
24 h	10.3±2.2%	11.7±2.8%	0.265
48 h	13.0±1.6%	23.9±5.1%	<0.05[a]
72 h	22.3±3.3%	32.7±4.5%	<0.05[a]

[a]$P<0.05$ vs. control group. Results are expressed as mean values ± standard error of the mean. MSC, mesenchymal stem cell; PBMC, pheripheral blood mononuclear cell.

Results

TLR1/2 activation of UCMSCs increases the proliferation of PBMC and cytotoxicity effect. PBMCs from healthy volunteers were co-cultured with UCMSCs in the presence of 100 ng/ml Pam3Csk. The results indicated that the proliferation of PBMCs was higher following Pam3Csk stimulation (20.7%) in PBMC-UCMSCs co-culture system compared with the untreated group (10.2%; Fig. 1). The results also demonstrated that Pam3Csk only led to 10.2% proliferation rate in PBMC. The results suggested that activation of TLR1/2 pathway in UCMSCs increases the immune response.

The immune attack was measured by detecting the LDH levels in culture supernatants from injured cells, which is a classical method for measuring leukocyte-mediated cytotoxicity. In the current study, PBMCs were co-cultured with UCMSCs and Pam3Csk was added to activate TLR1/2 signaling. The negative was PBMCs-UCMSCs without Pam3Csk. The results indicated no difference significant difference between the two groups at 24 h post-co-culture (10.3% vs. 11.7%: P=0.265), whereas LDH levels were significantly increased in the Pam3Csk treatment group compared with the untreated group at 48 h (12.9% vs. 23.9%; P=0.01) and 72 h (22.3% vs. 32.7%; P=0.037) post-co-culture (Table III).

Activation of TLR1/2 signaling increases the surface expression of co-stimulators of UCMSCs. The data of the current study indicated that TLR1/2 agonist induces immune attack and causes injury to UCMSCs. Thus, the study subsequently examined the effect of activation of TLR1/2 signaling on the UCMSCs surface expression of co-stimulators, which are important for mediating immune responses. The results indicated that CD80 (11.5 vs. 1.1%) and CD86 (12.4 vs. 2.0%) were significantly upregulated (P=0.036 and P=0.043, respectively) in UCMSCs treated with Pam3Csk compared with the control group (Fig. 2A). The expression variation of specific markers of UCMSCs were also detected and the results indicated that CD59 (98.2 vs. 96.6%) and CD74 (98.6 vs. 98.8%) and CD90 (89.8 vs. 98.6%) levels were marginally inhibited following Pam3Csk stimulation compared with untreated cells (Fig. 2B). The FACS results indicated that activation of TLR1/2 altered the surface expression of co-stimulators of UCMSCs, however the effect was not marked.

Immune-modulation molecules were upregulated in the presence of Pam3Csk. The UCMSCs were stimulated with TLR1/2 agonist, Pam3Csk, and the expression of pro-inflammatory cytokines (IFN-β, IL-6, IL-8 and TNF-α), chemokines (CCL-5, MCP-1, IP-10 and MIP-1α) and stem cell markers (Nanog, Sox2, Lin28 and Otx2) were examined at 4, 12, 24, 72 and 120 h following agonist treatment. It was demonstrated that IL-6, CCL-5, IP-10 and MIP-1α were significantly induced to high expression levels upon Pam3Csk stimulation compared with the control (Fig. 3A and B). Additionally, it was observed that although IFN-β, IL-8, TNF-α and MCP-1 expression levels were significantly induced in the presence

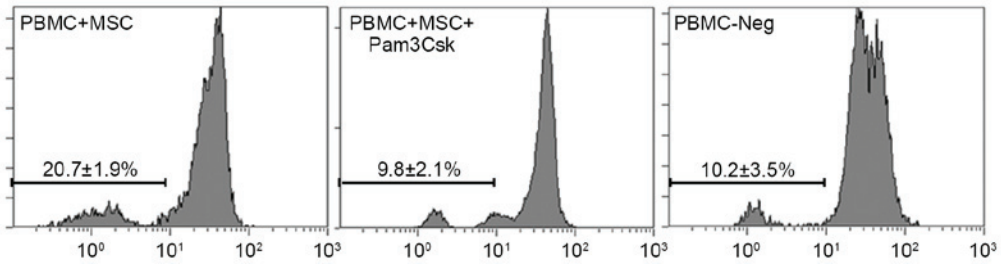

Figure 1. Toll-like receptor 1/2 agonist increase the proliferation of allogeneic PBMCs in a PBMC-umbilical cord MSC co-culture system. PBMC, peripheral blood mononuclear cell; MSC, mesenchymal stem cell.

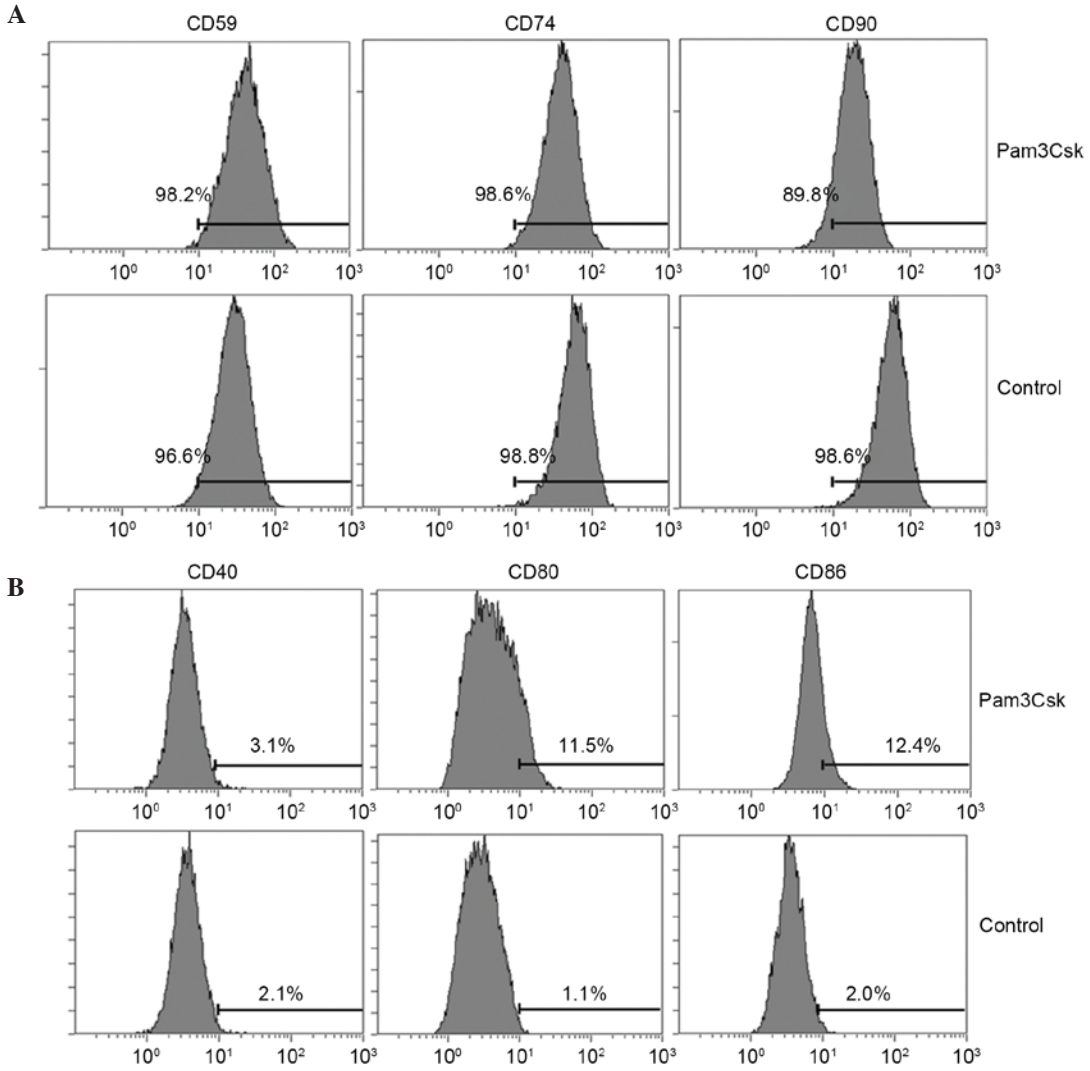

Figure 2. Pam3Csk induced the expression of co-stimulatory factors in umbilical cord mesenchymal stem cells, however had no influence on stem cell markers. (A) Stem cell markers, (B) co-stimulatory factors.

of Pam3Csk compared with the control, the expression levels decreased markedly at the later time points (Fig. 3A and B).

Additionally, the expression levels of stem cells markers were examined to determine whether activation of Pam3Csk affects the stemness of UCMSCs. The present study demonstrated that the expression level of Nanog was significantly inhibited following Pam3Csk stimulation at 12 h compared with control levels (P=0.021), whereas Sox2 levels were only inhibited compared with the control at 120 h treatment (P=0.028; Fig. 3C). It was also observed that the expression levels of Lin28 and Otx2 were not altered in the presence of Pam3Csk (Fig. 3C). Thus, the activation of TLR1/2 signaling upregulated the expression of pro-inflammatory molecules and may inhibit the stemness maintenance of UCMSCs.

Pam3Csk increases the secretion of pro-inflammatory cytokines in UCMSCs. The secretion of pro-inflammatory cytokines in the supernatants of the Pam3Csk-treated and

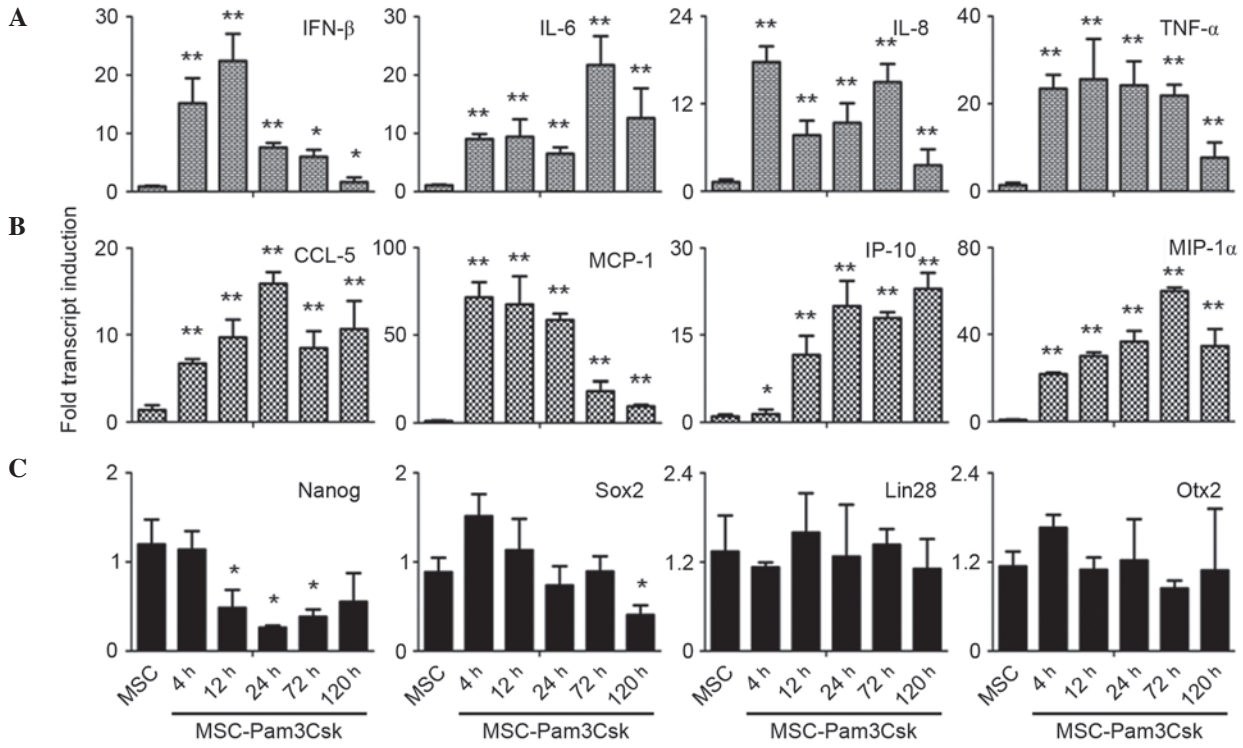

Figure 3. Secretion of pro-inflammatory molecules was significantly increased in UCMSCs treated with Pam3Csk. The mRNA levels of (A) cytokines, (B) chemokines and (C) stem cell markers were measured. *P<0.05, **P<0.001 vs. control group. Results are expressed as mean values ± standard error of the mean. IFN-β, interferon-β; IL, interleukin; TNF-α, tumor necrosis factor-α; CCL5, C-C motif chemokine ligand 5; MCP, monocyte chemoattractant protein; IP10, interferon γ-induced protein 10; MIP-1α, macrophage inflammatory protein-1α; Nanog, Nanog homeobox; SOX2, sex determining region Y-box 2; Lin28, Lin-28 homolog A; Otx2, orthodenticle homeobox 2.

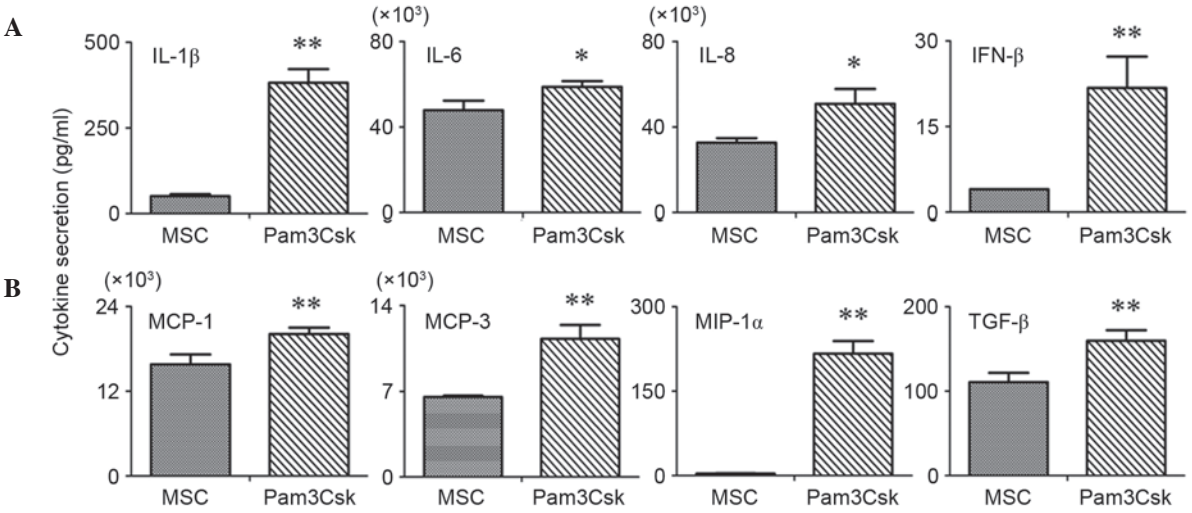

Figure 4. Toll-like receptor 1/2 agonist upregulates the expression levels of co-stimulatory molecules in umbilical cord MSCs. The levels of (A) co-stimulatory molecules and (B) surface markers were measured. *P<0.05, **P<0.001 vs. MSC. Results are expressed as mean values ± standard error of the mean. IL, interleukin, IFN-β, interferon-β; MSC, mesenchymal stem cell; MCP, monocyte chemoattractant protein; MIP-1α, macrophage inflammatory protein-1α; TGF-β, tumor growth factor-β.

untreated UCMSCs was measured using a RayBio antibody chip. The results indicated that IL-1β, IFN-β, MCP-1, MCP-3, MIP-1α and TGF-β were significantly upregulated in the supernatants of Pam3Csk treated UCMSCs compared with untreated UCMSCs (P<0.001; Fig. 4). Additionally, IL-6 and IL-8 levels were significantly induced upon TLR1/2 agonist stimulation compared with controls (P=0.032 and P=0.029, respectively), but not as markedly as MCP-1, MCP-3, etc (Fig. 4).

Pam3Csk stimulation had no effect on the differentiation ability of UCMSCs. A previous study indicated that differentiation of UCMSCs alters the immune status and increases immune responses (21). Thus, aimed to

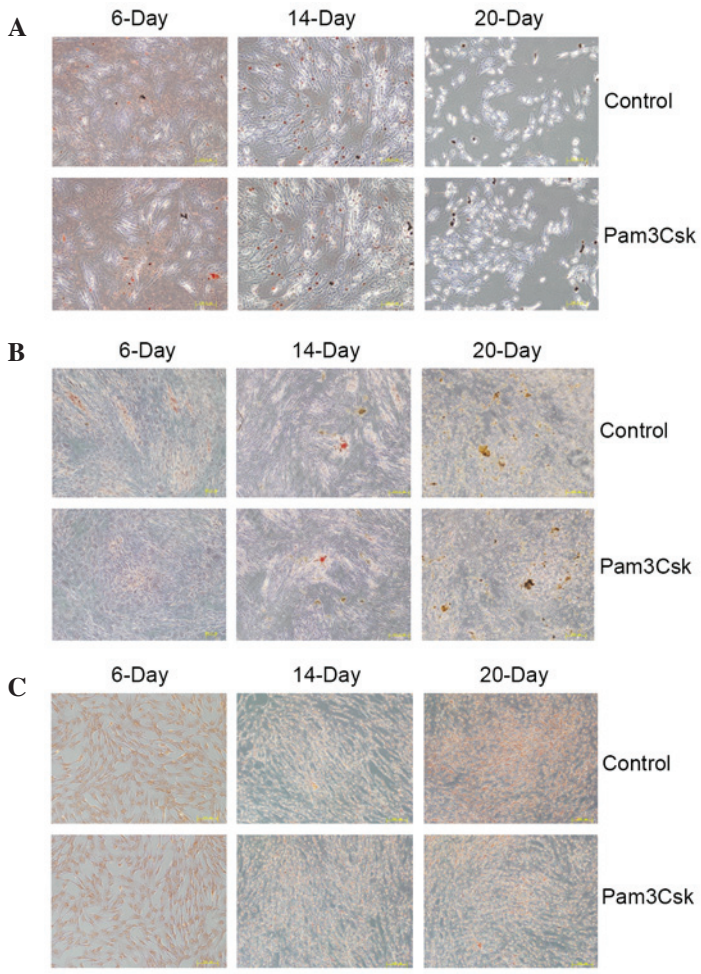

Figure 5. Pam3Csk treatment had no influence on the differentiation ability of UCMSCs. UCMSCs were stimulated to differenctate into (A) adipocytes (oil-red O staining, x400 magnification), (B) osteoblasts (alizarin red staining, x200 magnification) and (C) chondrocytes (safranine staining, x200 magnification). UCMSCs, umbilical cord mesenchymal stem cells.

determine whether TLR1/2 activation alters the differentiation of UCMSCs. The conditioned media for adipocyte, osteoblast and chondrocyte differentiation were added to UCMSCs following stimulation with Pam3Csk to detect the importance of TLR1/2 on UCMSCs differentiation ability. At day 6, 14 and 20 post-stimulation, alizarin red for osteoblasts, safranine for chondrocytes and oil-red O staining for adipocytes was conducted to assess the differentiation of UCMSCs. The results indicated that activation of TLR1/2 by Pam3Csk stimulation exerted no observable effect on the differentiation UCMSCs to adipocytes, osteoblasts and chondrocytes (Fig. 5).

Discussion

Multiple differentiation and self-renewal properties of MSCs enable its usage in clinical cell-based therapies (21). MSC differentiate into cell types for various tissues, including bone, cartilage, adipocytes, connective stomal cells, hepatocytes and muscle (22). In addition to differentiating into specific cells types, MSCs are also involved in tissue regeneration due to their trophic effects (23). Thus, knowledge of molecules, and mechanisms, that regulate the properties and potential immunogenicity of MSCs is important for the therapeutic use of MSCs. Immunomodulatory properties enable MSCs to suppress the activation and proliferation of T and B cell responses, and to interfere with the maturation of dendritic and NK cells (7-9). However, previous studies indicated that the *in vivo* microenvironment alters the immune status, enhances immune responses and causes to failure of MSC-based therapy (10-12).

TLR is the most important (PAMP) family, with an important role in defending against invading pathogens (15). Among the 11 members of the human TLR family, TLR1/2, which is located on the cell surface and recognized by gram-positive bacteria, is involved in the recognition of a variety of microbial components, including lipoproteins. Previous research demonstrated that activation of TLR1/2 exhibited no effect on the immune status of MSC from bone marrow (17), while no studies focussing upon the role of TLR1/2 in regulating the immune status of MSCs from the umbilical cord have been conducted. As UCMSCs attract attention in cell-based therapy, it is important to analyze whether activation of TLR1/2 pathway may alter the immunogenicity of UCMSCs. (24). The current study demonstrated that activation of TLR1/2 signaling in UCMSCs promoted immunogenicity by increasing the proliferation of PBMC in co-culture with UCMSCs and enhancing the release of LDH into the supernatant of the PBMC-UCMSCs

co-culture system. In support of this observation, the treatment of Pam3Csk also upregulated the expression of surface co-stimulators, CD80 and CD86, to a certain extent, however Pam3Csk exhibited no obvious influence on the levels of stem cell markers, including CD59, CD74 and CD90. Antibody array chip and RT-qPCR analysis was also performed. The antibody chip array detecting secretion of pro-inflammatory molecules indicated the levels of IL-1β, INF-β, MCP-1, MCP-3, MIP-1α and TGF-β in the supernatants of Pam3Csk-treated UCMSCs were significantly increased. RT-qPCR for gene expression levels also indicated that the expression of various pro-inflammatory cytokines (INF-β, IL-6, IL-8 and TNF-α) and chemokines (CCL-5, MCP-1, IP-10 and MIP-1α) was increased by Pam3Csk. Huang *et al* (21) suggested that MSCs lost the immune privilege properties when differentiated into cardiac cells and finally resulted in the rejection of engrafted MSCs. Thus, the present study aimed to establish whether the enhanced immune status was associated with alteration of the differentiation abilities in UCMSCs. Conditioned media were introduced for adipocyte, osteoblast and chondrocyte differentiation of UCMSCs upon stimulation with Pam3Csk, however, no observable change in differentiation ability was detected following activation of TLR1/2 signaling with Pam3Csk.

In clinical trials, numerous endogenous ligands, including heparin sulfate, oxidized low-density lipoprotein, uric acid and heat shock proteins have been previously demonstrated to activate TLRs. These endogenous TLR agonists may regulate functions of UCMSCs by endogenous stimuli during tissue repair. Future studies are required to study the regulatory mechanisms of the biological functions of UCMSCs. The current study firstly confirmed that activation of the TLR1/2 pathway increased the immunogenicity of UCMSCs. In clinical cell-based therapy, the engrafted MSCs encountered numerous endogenous ligands which may activate TLR pathways. Thus, the present study identified the potential risks of the use of MSCs in clinical therapy

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