

## Original Article

# Characterization of mesenchymal stem cells under the stimulation of Toll-like receptor agonists

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Infective factors cause the perpetuation of inflammation as a result of the permanent exposure of the immune system to exogenous or endogenous products of virus or bacteria. Mesenchymal stem cells (MSCs) can be exposed to this infective environment, which may change the characteristics and therapeutic potency of these MSCs. MSCs have the ability to repair damaged and inflamed tissues and regulate immune responses. In this study, we demonstrated that MSCs express functional Toll-like receptors (TLR) 3 and 4, the Toll-like receptor families that recognize the signals of viral and bacterial mimics, respectively. The specific stimulations did not affect the self-renewal and apoptosis capabilities of MSCs but instead promoted their differentiation into the adipocytes and osteoblasts with the TLR3 ligand. The reverse of these results were obtained with the TLR4 ligand. The migration of the MSCs to stimulate either of the two specific ligands was inhibited at different times, whereas the immunogenicity and immunosuppressive properties of the MSCs were not weakened unlike in the MSCs group. These results suggest that TLR3 and TLR4 stimulation affect the characterization of MSCs.

**Key words:** agonists, mesenchymal stem cell, Toll-like receptor.

## Introduction

Mesenchymal stem cells (MSCs) are multipotent nonhematopoietic progenitor cells that possess the ability of self-renewal and can differentiate into cell lineages of mesenchymal origin (i.e., adipocytes, osteoblasts, and chondrocytes) and nonmesenchymal cell lineages (i.e., pancreatic-like cells) (Chang *et al.* 2006; Sun *et al.* 2007). MSCs also have the ability to repair damaged and inflamed tissues and regulate immune responses through direct contact or by secreting soluble cytokines (Shi *et al.* 2010; Bassi *et al.* 2012). These features make MSCs an interesting resource of regeneration and cellular therapy (Si *et al.* 2011). Recently, MSCs have been reported to express functional Toll-like receptors (TLRs) that may play a

role in self-renewal and differentiation of MSCs (Hwa Cho *et al.* 2006; Pevsner-Fischer *et al.* 2007). TLRs are pattern recognition receptors that can recognize a wide variety of pathogen-associated molecular patterns, including polyinosinic-polycytidylic acid (Poly [I:C]) (TLR3) and lipopolysaccharide (LPS) (TLR4) (Akira *et al.* 2001; Iwasaki & Medzhitov 2004). The binding of TLRs and their specific stimulus activate MyD88-dependent or independent downstream signaling pathways, leading to the production of inflammatory cytokines or type I interferon (Akira *et al.* 2001).

In this study, we explored the expression of TLR3 and TLR4 in MSCs and the response of MSCs to Poly (I:C) and LPS stimulation in terms of self-renewal, multiple differentiation, apoptosis, immunogenicity, migration, immunosuppression, and secretion of cytokines.

## Materials and methods

### Mice

C57BL/6 mice (4–8 weeks old, male) were purchased from Shanghai Laboratory Animal Center, China Academy of Sciences, and housed in laminar flow cabinets under specific pathogen-free conditions. The mice

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were cared for and handled according to the recommendations of the National Institute of Health Guidelines for Care and Use of Laboratory Animals. The protocol was approved by the Committee on the Ethics of Animal Experiments of Shanghai Jiao Tong University. Surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. The work conformed to the provisions of the Declaration of Helsinki.

#### *Reagents and antibodies*

The basic medium used for MSCs culturing was low-glucose Dulbecco's modified Eagle medium (Hyclone) with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco). The medium used for spleen mononuclear cell culture was RPMI 1640 (Gibco) supplemented with 10% FBS, 1% penicillin, and 1% streptomycin. Poly (I:C) and LPS were purchased from Sigma. Anti-mouse CD34, CD44, CD45, CD11b, major histocompatibility complex (MHC)-I, MHC-II, Sca-1, CD80, CD86, CD3, CD28, and rat IgG2a and IgG2b isotype control monoclonal antibodies were purchased from eBioscience. Adipogenic and osteogenic differentiation media were purchased from Cyagen. Cell counting kit-8 (CCK-8) was purchased from Dojindo. A polymerase chain reaction (PCR) kit was purchased from TaKaRa. Carboxyfluorescein diacetate succinimidyl ester (CFSE) was purchased from Invitrogen. Primers were supplied by BioTNT.

#### *Isolation and expansion of MSCs*

Bone marrow cells were harvested from the femurs and tibiae of the C57BL/6 mice. After the erythrocytes were split, the remaining cells were plated in a basic medium. The cultures were kept at 37°C in a humidified atmosphere containing 95% air and 5% CO<sub>2</sub>. Non-adherent cells were removed after 8 h, and adherent cells were maintained with medium replacement every 3 days. When the clones of the primary cultures became 70% to 90% confluent, the culture was treated with 0.25% Trypsin containing 0.02% ethylenediaminetetraacetic acid (EDTA) for 2 min at room temperature. The lifted cells were harvested and reseeded. The cells were used for the experiments when they exhibited a homogeneous pattern.

#### *Flow cytometry measurement*

In the immunophenotype assay, flow cytometry analysis was performed using the following antibodies: CD34-FITC (clone RAM34), CD44-PE (clone IM7),

CD45-PE (clone 30-F11), CD11b-PE (clone M1/70), Sca-1-APC (clone 17-5981), MHC I-PE (clone 34-1-2S), MHC II-PE (clone NIMR-4), CD80-FITC (clone 16-10A1), and CD86-PE (clone GL1). The cells stained with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-labeled rat anti-mouse IgG were used as negative controls. MSCs were either untreated or treated with 50 µg/mL Poly (I:C) or 1 µg/mL LPS for 24 h. The MSCs were harvested and incubated with specific antibodies at 4°C for 30 min. The cells were washed with phosphate-buffered saline (PBS) plus 0.5% bovine serum albumin and analyzed using flow cytometry (LSR II, BD FACSAria). At least 10 000 events were collected and further analyzed using FlowJo software.

In the differentiation study, the cells were measured using the 5, 6-CFSE staining method. C57BL/6 spleen mononuclear cells were prepared by standard centrifugation on a Ficoll Hypaque density gradient and resuspended at a concentration of  $1 \times 10^7$  cells/mL in PBS. CFSE was added to a final concentration of 5 µM and incubated for 15 min at 37°C. Labeling was stopped by washing with RPMI 1640 plus 10% FBS. The labeled cells were stimulated with anti-CD3 and CD28 antibodies at a final concentration of 1 µg/mL. One day prior to activation, the MSCs were plated in a 96-well plate at different ratios to adhere for 6 h. Afterwards, 50 µg/mL Poly (I:C) or 1 µg/mL LPS was added to the appropriate well for the next 24 h. The control MSCs were left untreated. After stimulating for 24 h, MSCs were washed twice with PBS, and stimulated spleen mononuclear cells ( $2 \times 10^4$  cells per well) were added to the wells. The control cultures were spleen mononuclear cells stimulated by antibodies without MSCs. The ratios of MSCs to spleen mononuclear cells were 1:200, 1:100, 1:50, and 1:10. Five days later, the spleen mononuclear cells were harvested, and cell proliferation was determined by flow cytometry.

#### *Evaluation of MSC differentiation*

In the adipogenesis assay, the cells were incubated in basic medium with or without Poly (I:C) or LPS at a density of  $2 \times 10^4$  cells/cm<sup>2</sup> in a six-well plate. One day later, the medium was replaced with an adipogenic induction medium. The cells were then cultured for an additional 7 days with maintenance medium after four cycles of induction/maintenance. At the end of the cultivation period, the cells were fixed with 4% formaldehyde solution and stained with Oil Red O for adipocyte detection.

In the osteogenesis assay, the cells were incubated in basic medium with or without Poly (I:C) or LPS at a density of  $2 \times 10^4$  cells/cm<sup>2</sup> in a 6-well plate. After

24 h, the growth medium was replaced with osteogenic differentiation medium, and the cells were fed every 3 days for 3 weeks. Finally, the cells were fixed and stained with Alizarin Red for calcium deposition.

#### *Reverse transcriptase-polymerase chain reaction*

To determine the levels of TLR3 and TLR4 mRNA expressions, MSCs untreated or treated with Poly (I:C) or LPS for 24 h were harvested. Total RNA was isolated from the MSCs using RNAsimple Total RNA Kit (Tiangen Bio Tech, Beijing) according to the manufacturer's protocols. A total of 1  $\mu$ g of RNA was reverse-transcribed into cDNA, and the cDNA was processed with AMV-Optimized Taq using the One Step RNA PCR Kit (AMV) (TaKaRa). The primer sequences (5'-3' forward and reverse, respectively) specific to mouse mRNA were glyceraldehyde 3-phosphate dehydrogenase (GAPDH), AACCTTGGCATTGTGGAAGG, and ACACATTGGGGGTAGGAACA (280 bp); TLR3, ACATACCTGATGATCTTCCCTC, and TATTTGGCACAGTTCTG GCTC (228 bp); and TLR4, AATCTGGTGGCTGTG GAG, and CCCTGAAAGGCTTGGTCT (228 bp). The amplification conditions were as follows: 50°C (30 min), 94°C (2 min) followed by 35 cycles at 94°C (30 s), 60°C (30 s), and 72°C (2 min) for GAPDH; 94°C (30 s), 61°C (30 s), and 72°C (2 min) for TLR3; and 94°C (30 s), 65°C (30 s), and 72°C (2 min) for TLR4. The PCR products were separated on a 3% agarose gel electrophoresis, stained with ethidium bromide, and analyzed under ultraviolet light. The relative gene expression level was quantified as the ratio of band density (TRL/GAPDH).

#### *Cytokines detection*

Mesenchymal stem cells at  $3 \times 10^5$  cells per well were seeded in a 6-well plate to adhere for 6 h. The medium was then replaced with MSC growth medium with or without Poly (I:C) or LPS. After 24 h, the supernatants were collected. Cytokine concentration was tested by enzyme-linked immunosorbent assay (Neobioscience) for interleukin (IL)-1 $\beta$ , IL-6, IL-10, interferon (IFN)- $\beta$ , transforming growth factor (TGF- $\beta$ ), and IFN- $\gamma$  according to the manufacturer's instructions.

#### *Cell proliferation assays*

Mesenchymal stem cells were seeded in 96-well plates at  $4 \times 10^3$  per well in basic medium. After 6 h, the medium was replaced with fresh medium containing Poly (I:C) or LPS, respectively. At 6, 12, and 24 h, the medium was removed and fresh medium containing CCK-8 (V/V = 10%) was added to each well. After 2 h at 37°C, spectrophotometric measurement at 450 nm was performed using a 96-well plate reader.

#### *Scratch test*

The MSCs were seeded in a 6-well plate at  $3 \times 10^5$  cells per well. After starvation for 24 h in low-FBS medium (1% FBS), cells were removed by connecting 200  $\mu$ L tips while confluent with a monolayer. The cells were washed with PBS and given growth medium (1% FBS) with or without Poly (I:C) or LPS. After 6, 12, and 24 h, cell growth was observed using a Nikon TS100 microscope.

#### *Transwell migration assay*

Migration assay was performed using transwell inserts with 8  $\mu$ m pore member filters (Corning). MSCs were harvested through trypsinization and loaded into the upper chamber at  $2 \times 10^4$  cells per well. MSC growth medium (1% FBS) with or without TLR ligands was loaded into the bottom chamber. At 6, 12, and 24 h, the non-migrating cells on the upper side of the filters were removed using a cotton-tipped applicator. The migrating cells were fixed with 4% formaldehyde solution and stained with crystal violet. Crystal violet was decolorized using 33% acetic acid; the destaining solution was collected. Fluorescence was taken at 570 nm. The number of migrated cells was reflected indirectly by calculating their mobility. Mobility (%) = the value of OD (number of migrating cells)/the value of OD (the number of total cells)  $\times$  100%.

#### *Immunosuppression assay*

The ability of MSCs to modulate the proliferation of spleen mononuclear cells was evaluated in a co-culture system. MSCs with different ratios were seeded in a flat-bottom 96-well plate in RPMI 1640 for 6 h. Afterwards, 50  $\mu$ g/mL Poly (I:C) or 1  $\mu$ g/mL LPS was added to the appropriate well. The control MSCs were left untreated. After 24 h, the MSCs were washed twice with PBS, treated with mitomycin C at a final concentration of 25  $\mu$ g/mL for 30 min, and washed three times with PBS. The freshly isolated spleen mononuclear cells ( $2 \times 10^5$  cells per well) were stimulated with anti-CD3 and CD28 antibodies (1  $\mu$ g/mL), and immediately added to the wells for 5 days. The control cultures were spleen mononuclear cells stimulated by antibodies without MSCs. The ratios of MSCs to spleen mononuclear cells were 1:200, 1:100, 1:50, and 1:10. Before the end of the co-culture, the cultures were pulsed with 1  $\mu$ Ci/well [ $^3$ H] thymidine (5 Ci/mmol) for the last 18 h. The cells were harvested, and the radionuclide uptake was measured through scintillation counting. All cultures were done in triplicate.

### Statistical analysis

All values are given as mean  $\pm$  standard deviation (SD). Independent groups were compared using the two-tailed standard Student's *t*-test. Cell proliferation and migration rates were compared at different periods using repeated measures analysis of variance. The SPSS 16.0 program was used for statistical analysis (SPSS, Chicago, IL, USA). The value  $P < 0.05$  was considered statistically significant.

## Results

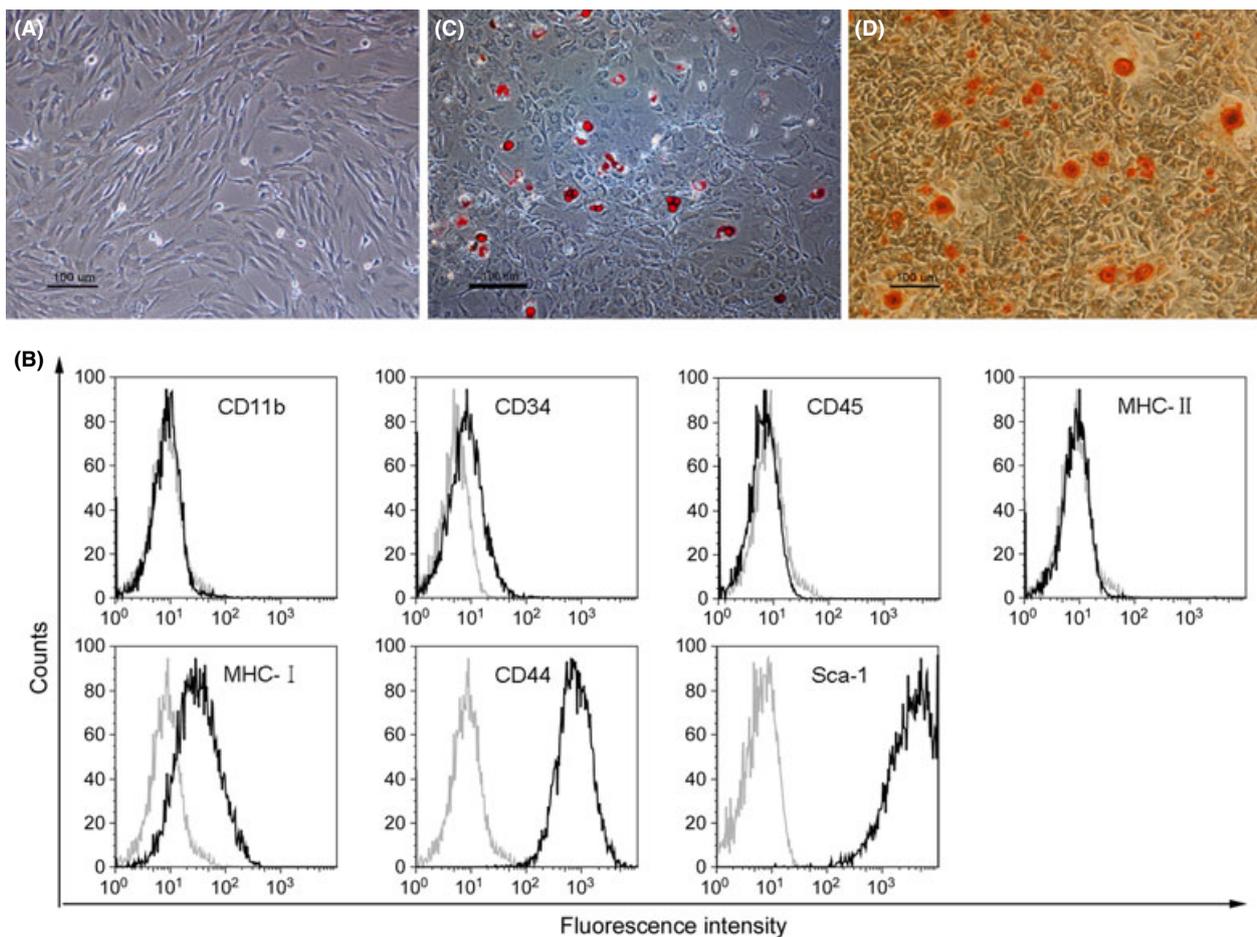
### Characteristics of MSCs

Plastic adherent cells from mouse bone marrow were isolated and propagated, and approximately 40% of these cells showed a typical fibroblast-like morphology and appeared as swirls (Fig. 1A). A lack of

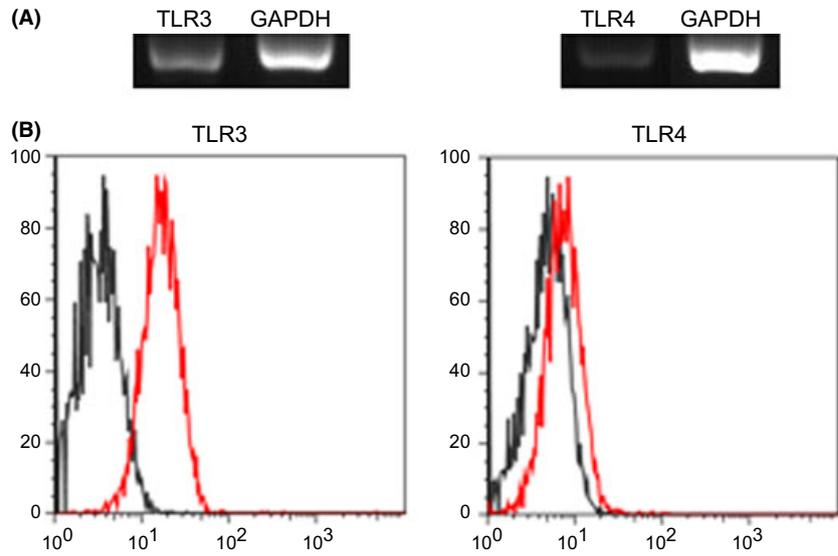
CD45, CD11b, and MHC-II expression was observed, and a weak, positive CD34 expression, as well as strong positive CD44 and stem cell antigen-1 (Sca-1) expressions, occurred (Fig. 1B). The cells then differentiated into adipocytes (Fig. 1C) and osteoblasts (Fig. 1D).

### MSCs expressed functional TLR3 and TLR4

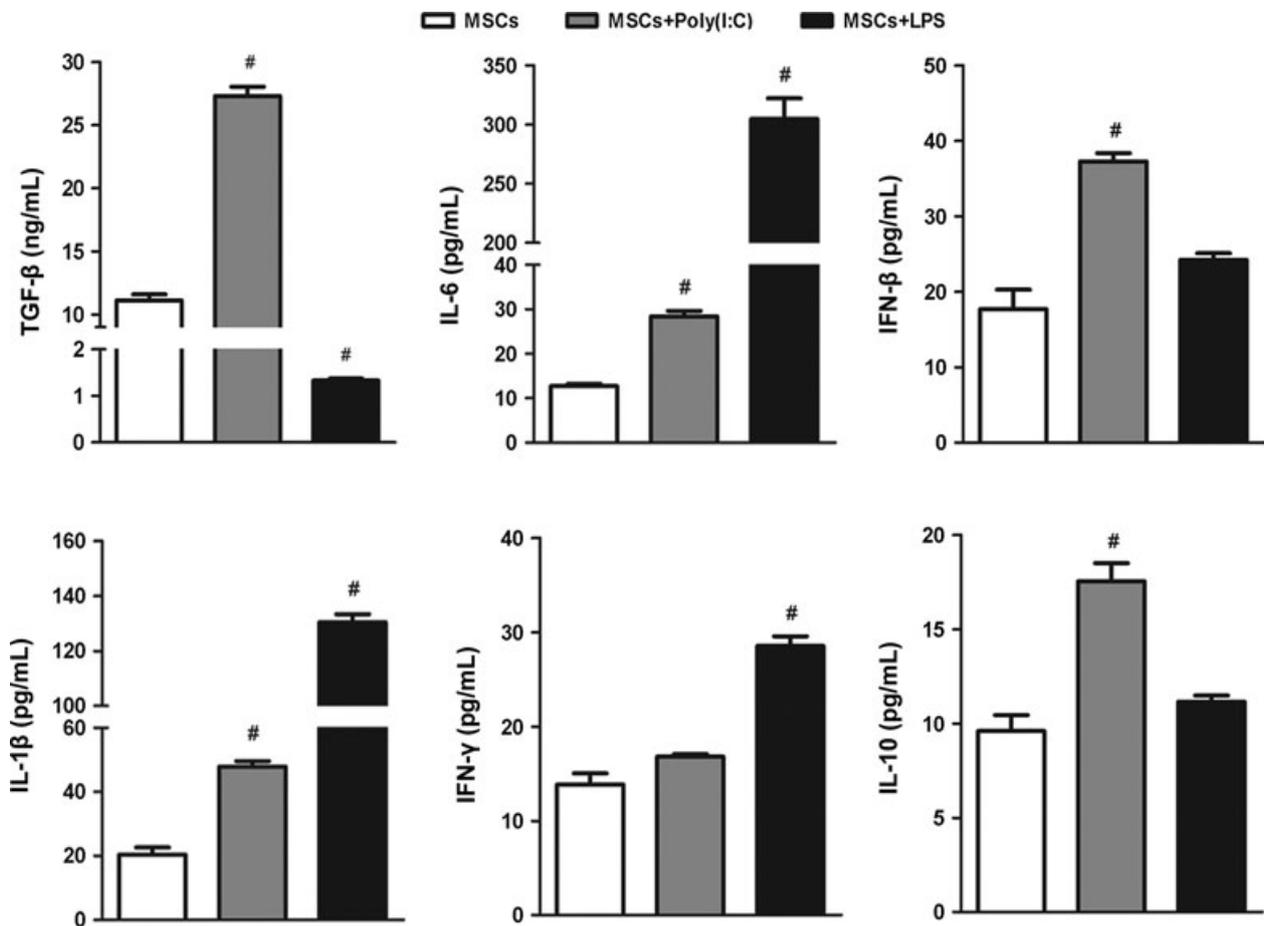
Mesenchymal stem cells expressed detectable TLR3 and TLR4 (Fig. 2). The relative gene expression level was 0.18 for TLR3 and 0.05 for TLR4. The cytokines secreted by MSCs changed under different stimuli after 24 h incubation (Fig. 3). With Poly (I:C) stimulation, the levels of TGF- $\beta$ , IL-10, and IFN- $\beta$  increased significantly and those of IL-6 and IL-1 $\beta$  increased mildly. By contrast, LPS-stimulated MSCs secreted high-level IFN- $\gamma$ , IL-6, and IL-1 $\beta$  compared with MSCs alone and MSCs with Poly (I:C).



**Fig. 1.** The characteristics of mouse bone marrow-derived mesenchymal stem cells (MSCs). (A) Expanded MSCs were fibroblast-like shapes and arrange in swirl. (B) Phenotypic properties of MSCs by flow cytometry. (C) Adipogenic differentiation potential of MSCs by detecting intercellular lipid vacuoles by Oil Red O staining. (D) Osteogenic differentiation potential of MSCs evaluated by detecting the formation of calcium nodule staining by Alizarin S.



**Fig. 2.** The expression of TLR3 and TLR4 on mesenchymal stem cells (MSCs). (A) mRNA expression of TLR3 and TLR4. (B) Representative flow cytometry analysis of TLR3, TLR4 (red line) and corresponding isotype antibodies controls (black line).



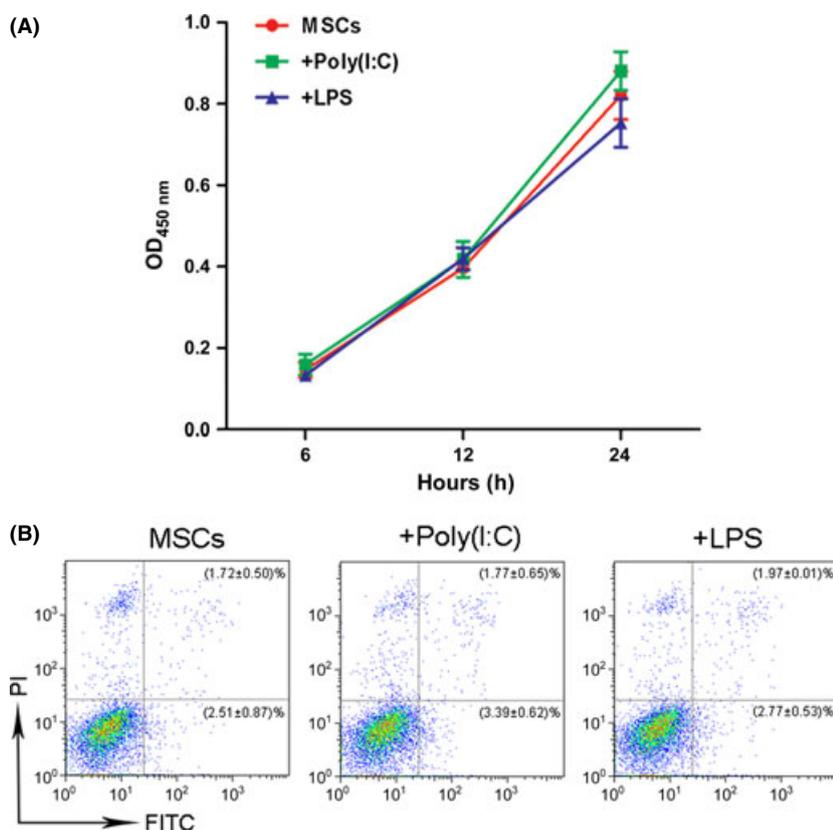
**Fig. 3.** The style of cytokines secreted by mesenchymal stem cells (MSCs) stimulated with or without Poly (I:C) and LPS. Data shown was representative of three different experiments (<sup>#</sup>*P* < 0.01).

### Effects of TLR ligands on MSCs proliferation, apoptosis, differentiation, and migration

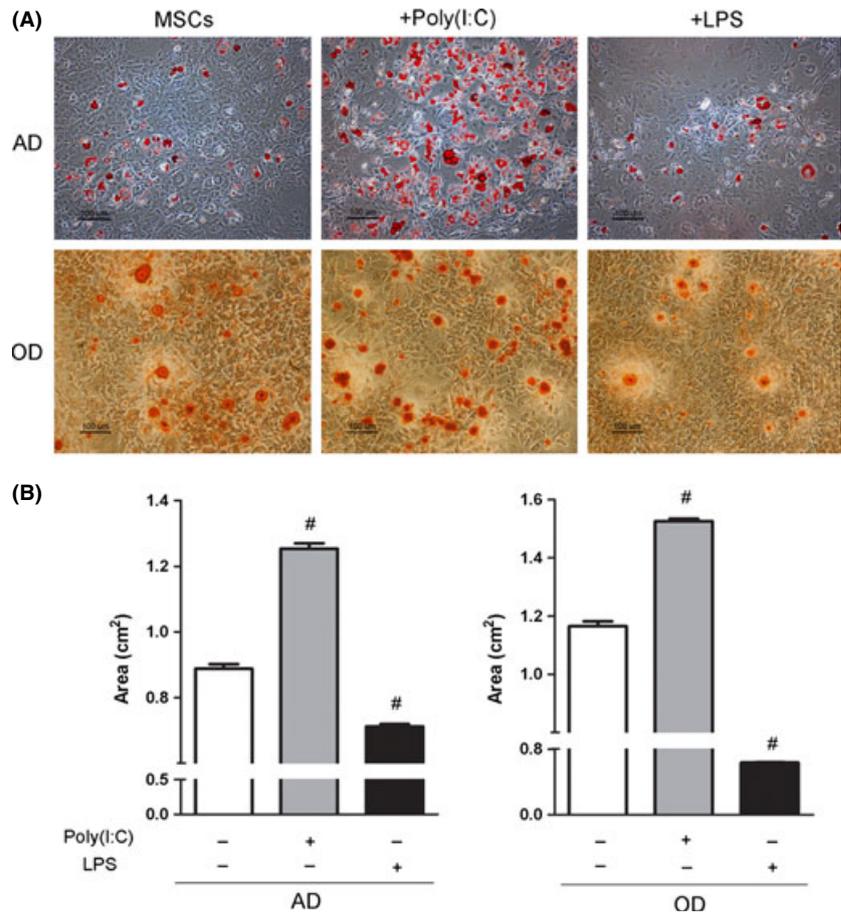
Regardless of the duration of exposure to either Poly (I:C) or LPS stimulation, i.e., for 6, 12, or 24 h, neither Poly (I:C) nor LPS affected the proliferation of MSCs (Fig. 4A) as well as the apoptosis of MSCs (Fig. 4B). Unlike in the control group, the differentiation of both adipocytes and osteoblasts of MSCs was enhanced in the presence of Poly (I:C) but was inhibited by LPS (Fig. 5A). NIS-Elements BR 3.2 software was used to quantify the area of coloration, and the difference was significant ( $P < 0.01$ ) (Fig. 5B). MSCs migrated into the injured region 6 h after the scratch, and the healing process was time-dependent. At 24 h, the injured region was covered by MSCs. However, the migration of MSCs treated with Poly (I:C) or LPS was delayed compared with that in the control group (Fig. 6A). Similar results were confirmed using the Transwell migration assay. The migrated MSCs were stained with crystal violet in different groups (Fig. 6B). The number of migrated MSCs was reflected indirectly by mobility, and the difference was significant ( $P < 0.05$ ) (Fig. 6C). Moreover, Poly (I:C)-stimulated MSCs migrated more than those stimulated with LPS (Fig. 6C).

### TLR ligands did not change the phenotype and immunosuppression of MSCs

With MSCs already expressing functional TLR3 and TLR4, we investigated whether their specific ligands could affect the phenotype of MSCs. We found that the addition of Poly (I:C) or LPS in the culture did not change the expression of stemness markers, such as CD34, CD11b, CD44, and Sca-1, and the expression of MHC-I, MHC-II, CD80 and CD86 were not induced or upregulated (Fig. 7). MSCs have a powerful immunosuppressive function. We examined the role of TLR3 and TLR4 in the immunosuppression of MSCs. The addition of MSCs significantly inhibited the proliferation of activated splenocytes, and this efficacy was dependent on the number of MSCs. When the ratio of MSCs and splenocytes was 1/10, the depressive effect was strongest ( $P < 0.05$ ) (Figs. 8A,B). We then tested the role of TLR3 and TLR4. As shown in Figure 8C, a lack of significant difference between TLR3 and TLR4 ligands in terms of MSCs-mediated suppression was observed using [ $^3$ H] thymidine assay given any ratio of MSCs to splenocytes.



**Fig. 4.** The proliferation (A) or apoptosis (B) of mesenchymal stem cells (MSCs) was not affected by Poly (I:C) and LPS. The data represented the results from three experiments.



**Fig. 5.** The differentiation potential of mesenchymal stem cells (MSCs) affected by Poly (I:C) and LPS. (A) Both adipocytes and osteoblasts differentiation of MSCs were enhanced in the presence of Poly (I:C), but it was inhibited with lipopolysaccharide (LPS) compared with control group. (B) NIS-Elements BR 3.2 software was used to quantify the area of coloration, respectively, and the difference was significant. <sup>#</sup> $P < 0.01$ ; AD, adipogenic differentiation; OD, osteogenic differentiation.

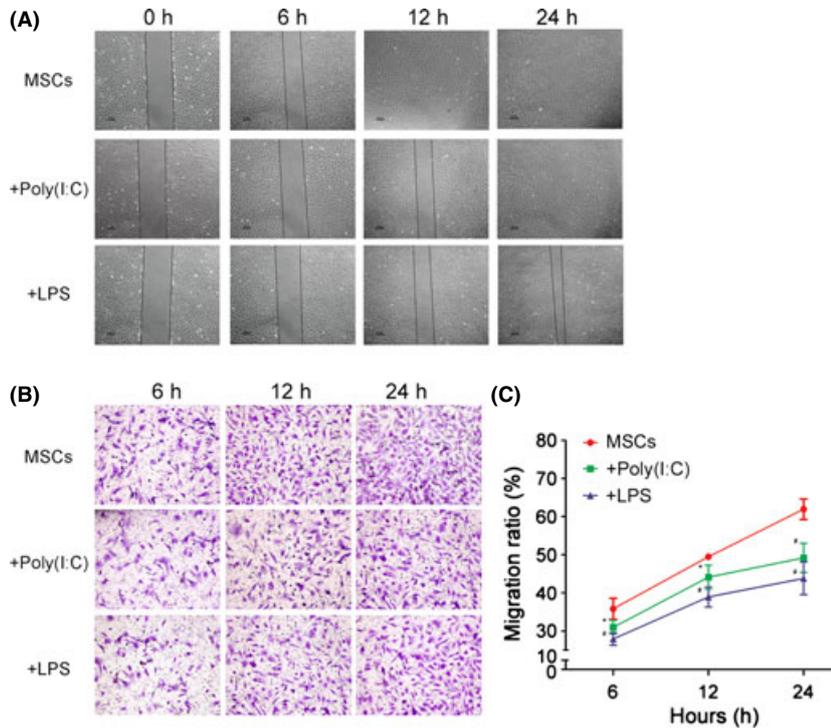
## Discussion

In this study, we confirmed that murine bone marrow-derived MSCs expressed TLR3 and TLR4 at the level of mRNA and protein. By detecting the level of cytokines secreted by MSCs with TLR3 and TLR4 agonists, we identified the functional status of TLR3 and TLR4 in MSCs. With Poly (I:C) stimulation and TLR3 agonist, MSCs were prone to secrete anti-inflammatory factors TGF- $\beta$  and IL-10. A number of pro-inflammatory cytokines such as IL-1 $\beta$ , IFN- $\gamma$ , and IL-6 were produced mainly with LPS stimulation.

We revealed that Poly (I:C) promoted the differentiation potency of MSCs to adipocytes and osteoblasts, and that LPS inhibited this process. The transcription factor nuclear factor (NF)- $\kappa$ B, which is triggered by the TLR4-MyD88-dependent pathway and inhibits the induced differentiation of MSCs into adipocytes, osteoblasts, and chondrocytes (Wehling *et al.* 2009; Yarmo *et al.* 2010; Yamaguchi & Weitzmann 2012), was activated. This result confirms that a molecular mechanism leads to the different results of MSC differentiation with TLR 3 and TLR4 ligands. In fact,

MSCs derived from patients with multiple myeloma showed impaired osteogenic differentiation potential compared with the normal group (Xu *et al.* 2012). This finding suggests the attenuation of MSC differentiation potential in immune disorder diseases. Moreover, our data illustrated that both Poly (I:C) and LPS did not damage the activity of MSCs given their lack of effect on proliferation and apoptosis. However, the results showed indirectly that a single signal from TLR3 and TLR4 to the MSCs might not affect the control of cell life and death *in vitro*.

Mesenchymal stem cells can evade immune surveillance because of their low expression of MHC-class I molecules and their lack of expression of MHC-class II molecules and co-stimulatory molecules, such as CD40, CD80, and CD86 (Zhao *et al.* 2004). Allogeneic MSCs can be used as transplant donors because MSCs lack MHC restriction. However, in an inflammatory environment, MSCs can be activated and express the immune molecules, and in turn become the phenotype of antigen-presenting cells (APC) (Romieu-Mourez *et al.* 2009). Romieu-Mourez *et al.* (2009) classified MSCs into immature and mature cells according

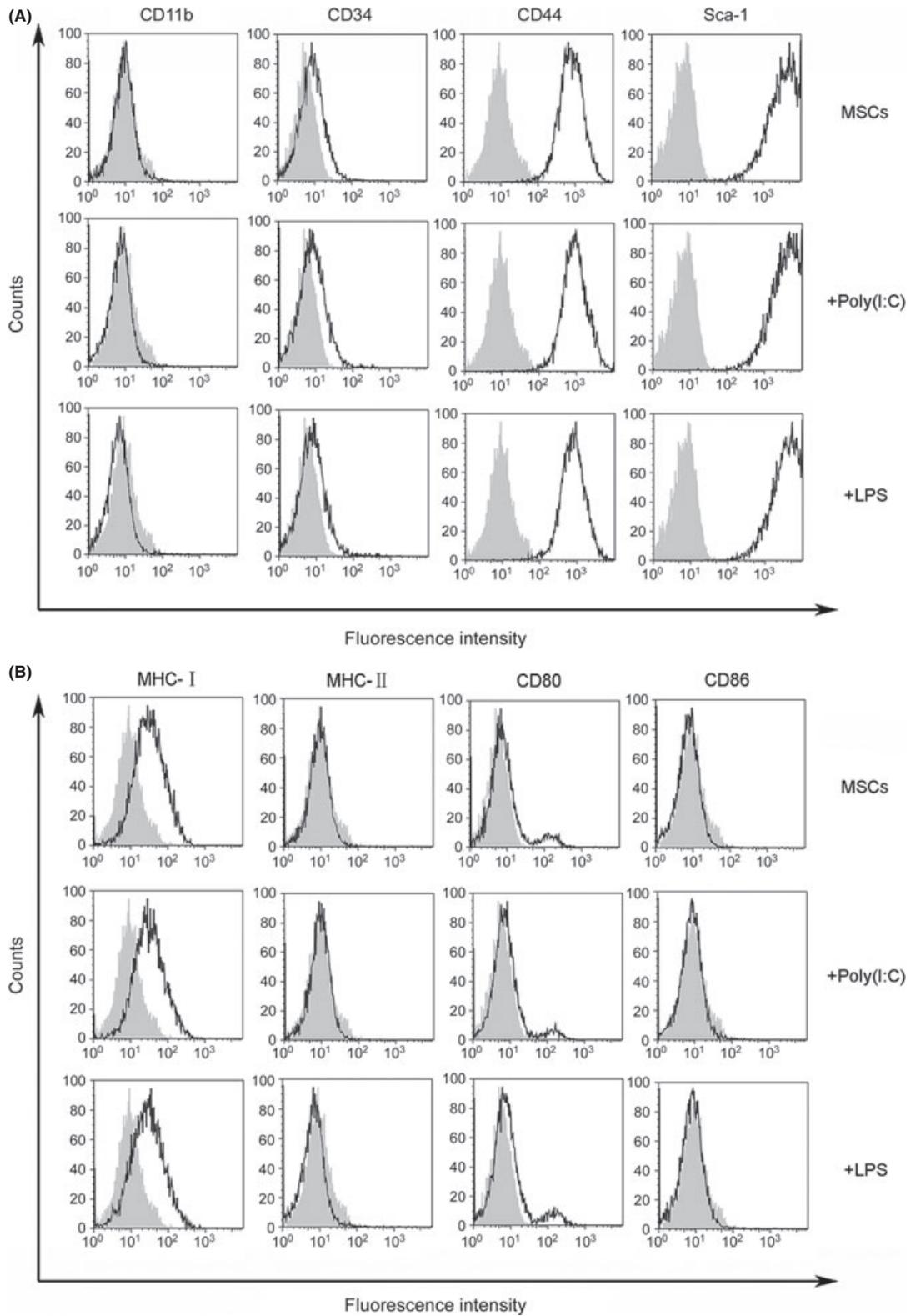


**Fig. 6.** The migration ability of mesenchymal stem cells (MSCs) affected by Poly (I:C) and LPS. (A) The migration of MSCs treated with Poly (I:C) or LPS was delayed compared with control group. (B) Similar results were confirmed by using Transwell migration assay. The migrated MSCs were stained by crystal violet in different group. (C) The difference of the number of migrated MSCs was significant.  $^{\#}P < 0.01$ ;  $^*P < 0.05$ .

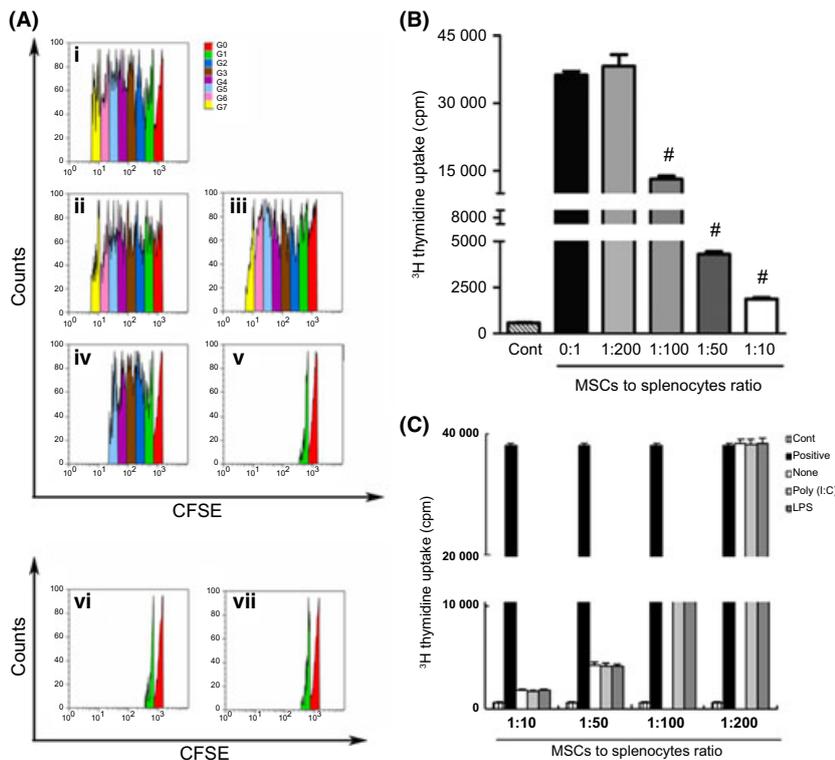
to the expression of immune molecules under the stimulation of inflammatory factors, similar to the classification of dendritic cells (DCs). However, this view has not been approved unanimously because even if the expression of MHC-II is induced, MSCs can still inhibit the proliferation of lymphocytes (Tse *et al.* 2003). Recent studies show that TLRs ligands can upregulate the expression of immunogenic molecules on DCs and induce DC maturation (Pasare & Medzhitov 2004). However, our results show that Poly (I:C) or LPS alone cannot contribute to the change in the phenotype of MSCs and in the expression of immunogenicity molecules.

Poly (I:C) could promote the migration of human MSCs significantly. After the application of TLR3 blocking antibodies, the mobility of MSCs was inhibited by up to 55%, suggesting that TLR3 was necessary for MSC migration. LPS could also promote the migration of MSCs but the effect was relatively weak. The differences between the effects of TLR3 and those of TLR4 were explained by the different chemokine receptors expressed on MSCs stimulated by different TLR ligands (Tomchuck *et al.* 2008). However, our data contradicted these findings. This contradiction can be explained by two major mechanisms: (i) the migration of MSCs mediated by TLRs had species and cell type-specific requirements (Lundberg *et al.* 2007), and (ii) the type and level of chemokine receptors on MSCs differ among different species (Sordi *et al.* 2005; Ringe

*et al.* 2007) and microenvironments (Zhukareva *et al.* 2010). We assumed that at the early stage of infection, the chemokines and inflammatory factors released by damaged tissues were at a low level. Thus, the chemotaxis of MSCs was extremely weak. When the release of chemokines and inflammatory factors reached its peak, the migration of MSCs to the infected sites became strong to identify and remove pathogens quickly. Once MSCs arrived at the infective sites and encountered large amounts of bacteria or viruses, the migration weakened or even disappeared. At this time, MSCs repaired damaged tissues and inhibited the excessive inflammatory responses in the infected area. Our findings could also be supported by the phenomenon that the number of migrated MSCs to the infarct sites lessened shortly after an intravenous injection. One of the mechanisms was that the level of chemokines released by infarcted cardiocytes, such as stromal cell-derived factor 1 (SDF-1), reached its peak at 24 h after myocardial infarction (Ma *et al.* 2005). Aside from Poly (I:C) and LPS, Pam3Cys (TLR2 ligand) could also inhibit the migration of MSCs, enabling the MSCs to remain at the infected sites to suppress immune responses (Pevsner-Fischer *et al.* 2007). Moreover, in the present study, Poly (I:C)-stimulated MSCs were revealed to have migrated more compared with those stimulated with LPS. This finding is similar to that of Tomchuck's report that TLR3 and TLR4 had varying effectiveness in promoting the migration of



**Fig. 7.** The expression of stemness markers and immunogenic molecules on mesenchymal stem cells (MSCs) affected by Poly (I:C) and LPS. The stemness phenotype of MSCs (A) and co-stimulatory molecules (B) were determined by flow cytometry after being cultured in the absence or presence of Poly (I:C) or LPS for 24 h. Poly (I:C) and LPS did not change stemness markers and immunogenic molecules on MSCs. The gray line represented the isotype control and the black line represented the specific antibodies.



**Fig. 8.** The immunosuppression of mesenchymal stem cells (MSCs) affected by Poly (I:C) and LPS. (A, B) The addition of MSCs significantly inhibited the proliferation of activated splenocytes and this efficacy was dependent on the number of MSCs. When the ratio of MSCs and splenocytes was 1/10, the depressive effect was strongest ( $P < 0.05$ ). (C) There was no significant difference of TLR3 and TLR4 ligands on MSCs-mediated suppression at any ratio of MSCs to splenocytes  $^{\#}P < 0.01$ .

MSCs (Tomchuck *et al.* 2008). However, the mechanisms underlying the different abilities of TLR3 and TLR4 to inhibit the migration of MSCs are unclear at present.

Mesenchymal stem cells are generally accepted as immune privilege cells because of their immunomodulatory function. Studies show that the immunosuppression of MSCs is universal and non-specific through direct contact and soluble cytokines (Shi *et al.* 2010; Bassi *et al.* 2012). They not only inhibit the proliferation and differentiation of effector T lymphocytes, as well as inhibit the secretion of antibodies by B lymphocytes, but also regulate the function of DCs and natural killer cells (Bassi *et al.* 2012; Zhao *et al.* 2012). However, the role of TLRs in the immunosuppression of MSCs is not yet clear. The proliferation of CD4<sup>+</sup>T cells inhibited by human MSCs had been reversed after the activation of TLR3 and TLR4 by impairing Jagged-1/Notch signal pathway (Liotta *et al.* 2008). Inversely, Opitz *et al.* (2009) pointed out that the activation of TLR3 and TLR4 could strengthen the inhibition of human MSCs in the proliferation of peripheral blood mononuclear cells through the secretion of IFN- $\beta$  and the transcriptional activation of IDO-1. However, Waterman *et al.* (2010) confirmed through an experiment that the immunosuppression of human MSCs was enhanced when pretreated with TLR3 ligand but

reversed almost to 100% when pretreated with TLR4 ligand compared with that of MSCs alone. Although the tendency of cytokines secreted by MSCs after the stimulation of TLRs ligands was similar to their study, the immunosuppression of MSCs pretreated with Poly (I:C) or LPS was not affected in our experiment. We assumed that the various reported contrasting effects could be explained by the differences in terms of sources, expression level of TLR3 and TLR4, amount and time of incubation with TLR ligands, and kinds of lymphocytes in the co-culture system.

In summary, our results suggest that TLR3 and TLR4 stimulations affect the characterization of MSCs derived from mouse bone marrow.

### Acknowledgments

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### Conflicts of interest

The authors confirm that there are no conflicts of interest.

## Author contributions

Xi Chen and Zheng-Yun Zhang: study concept and design; performed the experiments; acquisition of data; analysis and interpretation of data; drafting of the manuscript. Hao Zhou: performed the experiments; acquisition of data. Guang-Wen Zhou: study concept and design; acquisition of data; critical revision of the manuscript for important intellectual content; study supervision.

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