

# Overexpression of COX-2 but not indoleamine 2,3-dioxygenase-1 enhances the immunosuppressive ability of human umbilical cord-derived mesenchymal stem cells

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**Abstract.** Owing to their immunosuppressive properties mesenchymal stem cells (MSCs) are widely applicable in the treatment of autoimmune disease. The aim of this study was to investigate whether the indoleamine 2,3-dioxygenase-1 (*IDO-1*) and cyclooxygenase-2 (*COX-2*) genes enhanced the immunosuppressive functional ability of MSCs following stable transfection. To strengthen the immunomodulatory ability of MSCs, *IDO-1* and *COX-2* were overexpressed in umbilical cord progenitor cell-derived MSCs using recombinant plasmids and electroporation. RT-qPCR analysis and western blotting confirmed the expression of *IDO-1* and *COX-2* in transfected MSCs. Further functional assays in co-culture experiments, including lymphocyte proliferation and cytotoxicity assays showed that *COX-2*-transfected MSCs possessed more potent immunomodulatory cells than the untreated MSCs, or MSCs transfected with *IDO-1*. Additionally, synthesis of interferon- $\gamma$  and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) was significantly inhibited in lymphocytes co-cultured with *COX-2*-transfected MSCs, which was consistent with changes in immune-related genes in MSCs. An enhanced expression of *IDO-1*, *COX-2*, heme-oxygenase-1, inducible nitric-oxide synthase, TNF- $\alpha$ -stimulated gene/protein-6, transforming growth factor- $\beta$  (TGF- $\beta$ ), human leukocyte antigen molecule 5 (HLA-G5) and interleukin-10 (IL-10) was identified following *COX-2* transfection. We showed that the overexpression of *COX-2* enhanced the immunosuppressive function of MSCs. *COX-2*-modified MSCs more potently inhibited the activation and proliferation of peripheral blood mononuclear cells.

## Introduction

Mesenchymal stem cells (MSCs) are of stromal origin and can be isolated from various human tissues, including bone

marrow, adipose tissue, skeletal muscle, synovium, gingiva, amniotic fluid, cord blood, and the umbilical cord. MSCs are an extremely promising source of adult stem cells that may be used for cell-based therapeutics, due in part to their substantial ability to differentiate into multilineages and broad secretory activities (1-4). Preclinical studies have suggested the potential for using MSCs in the settings of tissue repair in type 1 diabetes (5), acute lung injury (6,7), radiation protection (8) and nephropathy (9).

However, it was suggested that the immunomodulatory activity of MSCs is an important consideration in cell therapy (10). Additionally, *in vitro* evidence suggests that MSCs directly modulate T-cell function. MSCs inhibit the maturation and migration of various antigen-presenting cells, suppress B-cell activation, induce suppressor T-cell formation, and alter the expression of several receptors necessary for antigen capture and processing (11,12). This immunosuppressive activity of MSCs may also play an important role in the therapy of autoimmune diseases (13), and the prevention of acute graft-versus-host disease (aGVHD) (14).

Previous findings have shown that the recommended infusion of adoptively transferred MSCs ranges from 10<sup>5</sup>/kg to 10<sup>7</sup>/kg body weight, and that an association exists between the anticipated therapeutic effect and the increasing quantity of MSCs (15-19). However, infused MSCs are, not only found in the bone marrow and injured tissues, but are also located in the lungs (20,21). The dangers of pulmonary embolism and cardiac dysfunction increase with the number of adoptively transferred input cells (22). Therefore, this study aimed to obtain a new population of MSCs exhibiting more potent immunosuppressive functions with subsequent improvement in their clinical application without the need for an increasing cell dose.

Numerous molecules participate in the immunomodulation process of MSCs, including prostaglandin E2 (PGE2) (23), indoleamine 2,3-dioxygenase (IDO) (24), nitric oxide (25), transforming growth factor- $\beta$  (TGF- $\beta$ ) (26), and interleukin-6 (IL-6) (27). Of these molecules, *IDO-1* and cyclooxygenase-2 (*COX-2*) are key factors in tryptophan catabolism and PGE2 synthesis, respectively. When PGE2 production is inhibited, immunomodulation is weakened (23,24,28). Tryptophan is an essential amino acid in T-cell proliferation, and its depletion or accumulation of its degradation product kynurenine, adversely affect T-cell amplification (24).

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In view of the critical roles of IDO-1 and COX-2 in immunomodulation, we attempted to upregulate the expression levels of IDO-1 and COX-2 to enhance the synthesis of PGE2 and kynurenine, and to determine whether the immunosuppressive ability of MSCs can be improved.

## Materials and methods

**Culture of MSCs.** Umbilical cords were obtained from clinically normal pregnancies after participants provided informed consent. The study was approved by the local ethics committee of Qilu Hospital. Umbilical cords were excised and washed in 0.1 mol/l phosphate buffer (pH 7.4) to remove residual blood. The cords were dissected, and the blood vessels were removed. The remaining tissues were cut into small sections (1–2 mm<sup>3</sup>) and placed in tissue culture plates with low-glucose Dulbecco's modified Eagle's medium (DMEM), which was supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL, Grand Island, NY, USA), 2 ng/ml vascular endothelial growth factor (VEGF), 2 ng/ml epidermal growth factor (EGF), 2 ng/ml fibroblast growth factor (all from R&D Systems, Minneapolis, MN, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco-BRL). Cultures were maintained at 37°C in a fully-humidified atmosphere with 5% CO<sub>2</sub> in air. The media were changed every 3–4 days. Adherent cells proliferated from individual explanted tissues 7–12 days after the culture was initiated. Subsequently, small tissue specimens were removed from the culture, and the adherent fibroblast-like cells were cultured to confluence for 2–3 weeks. The cells were trypsinized using 0.25% trypsin (Gibco-BRL) and then passaged at a density of 1×10<sup>4</sup> cells/ml in the culture medium exactly as described above. The cells were used after 5 or more passages.

**Cell surface antigen phenotyping.** Fifth- to seventh-passage cells were collected and treated with 0.25% trypsin. The cells were then stained with either fluorescein isothiocyanate (FITC)-conjugated or phycoerythrin (PE)-conjugated monoclonal antibodies in 100 µl phosphate buffer for 15 min at room temperature, as per the manufacturer's instructions. The antibodies used in this assay were targeted against human cell-surface expressed antigens including CD29, CD34, CD31, CD44, CD45, CD73, CD90 and CD105 (SeroTec, Raleigh, NC, USA). The cells were analyzed by flow cytometry (Guava easyCyte 6HT-2L; Merck Millipore, Billerica, MA, USA). Positive cells were counted and compared with the signal of the corresponding immunoglobulin isotypic controls.

**Recombinant plasmid construction and transfection.** For construction of the expression plasmid, the IDO-1 and the COX-2 inserts were isolated by polymerase chain reaction (PCR) amplification from a complementary DNA (cDNA) library (GeneChem, Shanghai, China) and digested with the restriction endonucleases *Eco*RI and *Bgl*II (MBI Fermentas, Burlington, ON, Canada). The inserts were subsequently linked to the pEGFP-N3 expression plasmids (Bio-Asia Co., Shanghai, China) with T4 DNA ligase (TransGen, Beijing, China). The ligation products were transformed into competent *Escherichia coli* DH5α (TransGen) and then selected using the kanamycin resistance method. Recombinant plasmids were sequenced (ABI Prism 3100 DNA Sequencer; Applied

Biosystems, Foster City, CA, USA) and confirmed to contain the entire coding sequence of IDO-1 or COX-2. Clones with the correct sequence were amplified for further transfection. The new recombinant plasmids were designated as pEGFP-IDO-1 and pEGFP-COX-2.

The recombinant plasmids were transfected into MSCs with a gene transfection instrument (SCIEN TZ-2C; Scientz Co., Ningbo, China) and electroporation cuvettes (Bio-Rad, Hercules, CA, USA). A successful transduction was confirmed by visualizing the expression of enhanced green fluorescent protein (EGFP; included in the pEGFP-N3 vector) after 4 days of culture. The cells were maintained and allowed to grow for another 3–5 days, and the expression level of IDO-1 and COX-2 was confirmed by western immunoblotting as described below.

The experiments were divided into four groups: i) untreated MSCs were the control group, ii) cells transfected with only the *IDO-1* gene were the MSCs (IDO-1) group, iii) cells transfected with only the *COX-2* gene were the MSCs (COX-2) group, and iv) cells described as the MSCs (IDO-1/COX-2) group, were simultaneously transfected with the IDO-1 and COX-2 plasmids. The expression levels of the transferred plasmids were the same in each group.

**Protein extraction and western immunoblotting.** To verify the expression levels of IDO-1 and COX-2, the transfected cells were collected and resuspended in protein lysis buffer (RIPA: PMSF at a ratio of 100:1; Solarbio, Beijing, China) according to the manufacturer's instructions. Lysates were incubated on ice for 30 min and then centrifuged at 12,000 rpm and 4°C for 20 min. Equal amounts of proteins (15 µg for each sample) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with antibodies against IDO-1, COX-2 or β-actin (Abcam Inc., Cambridge, MA, USA) at 4°C overnight. Antibody binding was assessed by incubation with horseradish peroxidase-conjugated secondary antibodies (Beyotime, Shanghai, China). Chemiluminescence was detected using an ECL Plus immunoblotting detection system (Beyotime).

**Reverse transcription-quantitative PCR (RT-qPCR) assay of immune-regulated gene mRNA expression.** In order to detect changes in immune-related genes in transfected MSCs, total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Total RNA was reverse transcribed to cDNA with the Omniscript cDNA Synthesis kit (Qiagen, Hamburg, Germany) according to the manufacturer's instructions. In a typical procedure, 0.2 µg of total RNA was reverse transcribed in a final volume of 20 µl containing the following: 1X RT buffer, deoxynucleotide triphosphate mix (5 mM each), RNase inhibitor (10 U/µl RNase out; Invitrogen), oligo(dT) primers and 4 units Omniscript RT. The samples were incubated at 37°C for 60 min, and the resulting cDNA was stored at -80°C until RT-qPCR analysis was initiated using an ABI 7500 PCR system and SYBR-Green I dye (Toyobo, Osaka, Japan). The primers used are provided in Table I. The reagents and primers were obtained from Bioasi Co., Ltd., Shanghai, China. β-actin was used as an internal control. The expression of each gene

Table I. Primer sequences used in PCR.

Genes	Primer sequences	Size (bp)
<i>IDO-1</i> <sup>a</sup>	5'-GGAAGATCTTCCATGGCACACGCTATGGAAAAC 5'-CCGGAATTCGGACCTTCCTTCAAAAGGGATTTC	1,209
<i>COX-2</i> <sup>a</sup>	5'-GGAAGATCTTCCATGCTCGCCCGCGCCCTGCTGC 5'-CCGGAATTCGGCAGTTCAGTCGAACGTTCTTTTAG	1,812
<i>IDO-1</i>	5'-GCCCTTCAAGTGTTTCACCAA 5'-GCCTTTCCAGCCAGACAAATAT	90
<i>COX-2</i>	5'-GGTCTGGTGCCTGGTCTGAT 5'-TCCTGTTTAAGCACATCGCATACT	80
<i>HMOX-1</i>	5'-AGGGAAGCCCCCACTCAAC 5'-ACTGTCGCCACCAGAAAGCT	81
<i>IL-2</i>	5'-CCAGGATGCTCACATTAAAGTTTTAC 5'-GAGGTTTGAGTTCTTCTTCTAGACACTGA	87
<i>IL-10</i>	5'-GCCTTGCTGAGATGATCCAGTT 5'-TCACATGCGCCTTGATGTCT	85
<i>iNOS</i>	5'-GGTGAAGCGGTAACAAAGG 5'-TGCTTGGTGGCGAAGATGA	81
<i>TGF-β1</i>	5'-GGGAAATTGAGGGCTTTTCG 5'-GAACCCGTTGATGTCCACTTG	83
<i>IL-1α</i>	5'-GACGCCCTCAATCAAAGTATAATTC 5'-TCAAATTTCACTGCTTCATCCAGAT	89
<i>IL-1β</i>	5'-GCGGCATCCAGCTACGAAT 5'-GTCCATGGCCACAACAACCTG	80
<i>IFN-γ</i>	5'-CCAACGCAAAGCAATACATGA 5'-TTTTCGCTTCCCTGTTTTAGCT	72
<i>β-actin</i>	5'-GGACATCCGCAAAGACCTGTA 5'-GCATCCTGTTCGGCAATGC	80

<sup>a</sup>Primers of IDO-1 and COX-2 used to obtain complete CDS of genes by polymerase chain reaction. PCR, polymerase chain reaction; IDO-1, indoleamine 2,3-dioxygenase-1; COX-2, cyclooxygenase-2; IL-2, interleukin-2; iNOS, inducible nitric-oxide synthase; TGF-β1, transforming growth factor-β; IFN-γ, interferon-γ.

was determined using the 2<sup>-ΔΔCT</sup> method. The RT-qPCR conditions used were: 1 cycle at 95°C for 4 min followed by 94°C for 15 sec at 60°C for 1 min, and for a total of 40 cycles. Data were analyzed using Sequence Detection software version 1.4 (Applied Biosystems). Data were reported as mean ± standard deviation (SD) of at least three independent experiments. mRNA expression was presented as fold-change compared with the untreated control groups. Control group values were set at a fold-change equal to unity.

**Inhibition of peripheral blood mononuclear cell (PBMC) proliferation by IDO-1- and COX-2-modified MSCs.** Allogeneic PBMCs were isolated by Ficoll/Hypaque gradient centrifugation of peripheral venous blood (20-30 ml, that was collected from healthy volunteers). The preliminary tests showed that phytohemagglutinin (PHA; Sigma-Aldrich, St. Louis, MO, USA) stimulated lymphocyte proliferation at a final concentration of 10 μg/ml. Additionally, the inhibitory

effect of MSCs on lymphocyte proliferation was notable at a ratio of MSCs to lymphocytes of 1:10 to 1:20 (data not shown).

MSCs of the four groups were adapted to the co-culture medium (RPMI-1640 medium without phenol red, and supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) by gradient reduction of DMEM and plated in triplicate in 96-well microtiter plates. When MSCs achieved a 100% confluence, the cells were co-cultured with PHA-activated PBMCs. After three days, the suspended cells (mainly lymphocytes) from each well were transferred to a new 96-well plate, and then incubated with 10 μl of Cell Counting kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Absorbance at 450 nm was measured with a model 450 microplate reader (Bio-Rad Laboratories, Richmond, CA, USA). Experiments were performed in triplicate and were repeated at least twice.

**Cytokine measurement in culture supernatant.** After three days of co-culture of lymphocytes and MSCs, culture supernatants

were collected to measure the levels of interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D Systems).

**Cytotoxicity analysis.** HeLa cells at a density of  $3 \times 10^4$ , were resuspended in RPMI-1640 medium (without phenol red), supplemented with 10% FBS, and seeded in 96-well plates and used as target cells for cytotoxicity analysis. PHA-activated PBMCs were respectively co-cultured with MSCs from the four groups at a 1:10 ratio of MSCs to lymphocytes, respectively. After three days, suspended cells (mainly lymphocytes) from each group were transferred to HeLa cell pre-cultured 96-well plates with lymphocytes seeded at a density of  $3 \times 10^5$  lymphocytes per well. The cells were incubated at 37°C in 5% CO<sub>2</sub> in air for 6 h. After this time period, 10  $\mu$ l of CCK-8 was added to the cultures and incubations were continued for an additional 1.5 h. Relative absorbance values were measured at 450 nm as described above. Cytotoxic activity was calculated as:  $[\text{OD}_{(\text{PBMC}+\text{HeLa})} - \text{OD}_{(\text{PBMC})}]/\text{OD}_{(\text{HeLa})}$ .

**Statistical analysis.** Data are presented as the means  $\pm$  standard deviation. Data sets were compared via analysis of variance (ANOVA) followed by the Student's t-test for paired analysis of data sets between and within groups. Data were analyzed using SPSS software version 14.0 (SPSS Inc., Chicago, IL, USA). Differences between values were considered statistically significant at an  $\alpha$ -value of  $P < 0.05$ .

## Results

**Biological characteristics of UC-derived MSCs.** After several passages, adherent cells from UC formed a monolayer of typical fibroblastic cells (Fig. 1A). Flow cytometric results showed that UC-derived cells shared most of their immunophenotype with MSCs, including a positive expression for stromal markers (CD29, CD44, CD73, CD90 and CD105), but a negative expression for the endothelial marker CD31, and the hematopoietic markers (CD34 and CD45) (Fig. 1B).

**Construction of recombinant eukaryotic expression plasmids and electro-transfection.** The amplification products of the *IDO-1* and *COX-2* genes (with relative size of 1,209 and 1,812 bp, respectively) were purified and digested with the restriction endonucleases *Bgl*/II and *Eco*RI to construct a recombinant plasmid by insertion into the pEGFP-N3 (4729 bp) vector. The result of rDNA sequence analysis confirmed the correct sequence and reading frame of *IDO-1* (GenBank, NM\_002164.5) and *COX-2* (GenBank, NM\_000963). MSCs were transfected with recombinant vectors with a transfection efficiency of  $\sim 30\%$  (Fig. 2A and B, as evaluated by observation of GFP fluorescence). The results of the western immunoblot analysis confirmed the existence of a thicker band for the *IDO-1* and *COX-2* proteins in the transfected MSCs as compared to MSCs without transfection, which demonstrated that the *IDO-1* and *COX-2* cDNA was expressed in MSCs (Fig. 2C).

**Expression of immune-related genes in *IDO-1*- and *COX-2*-modified MSCs.** The results showed that the MSCs transfected

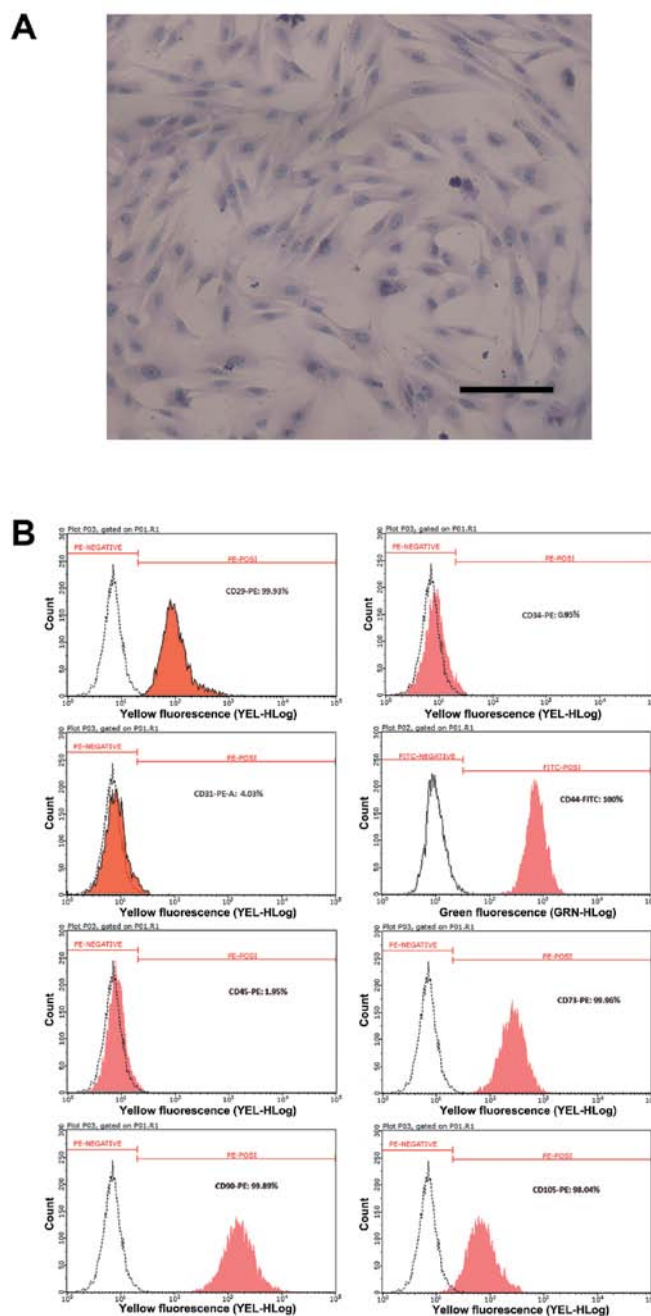


Figure 1. Morphological and immunophenotypic assessment of umbilical cord-derived mesenchymal stem cells. (A) Hematoxylin and eosin (H&E) staining of umbilical cord-derived cells. These cells had a bipolar spindle shape and a monolayer pattern of growth. Scale bar, 100  $\mu$ m. (B) Surface markers were detected by flow cytometric analysis. The cells expressed CD29, CD44, CD73, CD90 and CD105, but not CD31, CD34 or CD45.

with recombinant plasmids markedly upregulated the expression of the inserted genes (Fig. 3). Compared with the control group, the MSCs (*IDO-1*) group expressed very high levels of *IDO-1*, and downregulated other immunosuppressive factors, such as *COX-2*, heme-oxygenase-1 (HO-1), inducible nitric-oxide synthase (iNOS), TGF- $\beta$ , IL-10, TNF- $\alpha$ -stimulated gene/protein-6 (TSG-6) and human leukocyte antigen molecule 5 (HLA-G5). For the MSCs (*COX-2*) group, the expression levels of *COX-2* and other immune regulatory genes were all elevated. However, apart from the expression of *IDO-1* and

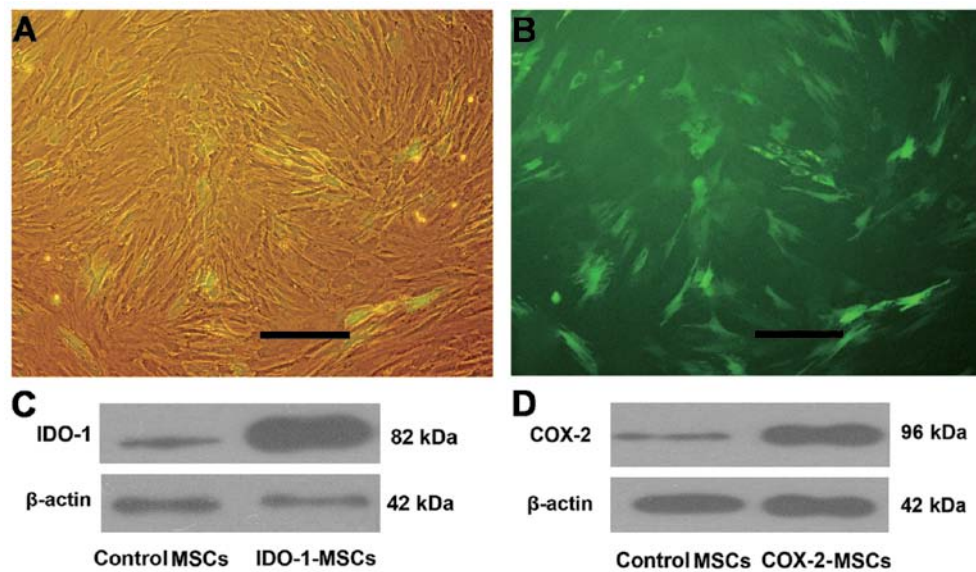


Figure 2. Electrical transfection of umbilical cord in mesenchymal stem cells (MSCs) shown as imaging and western blot analysis. (A and B) The expression of enhanced green fluorescent protein was observed by fluorescence microscopy after 48 h, and the transfection efficiency was ~30%. Scale bar, 100  $\mu$ m. (C) The expression of indoleamine 2,3-dioxygenase-1 (IDO-1) protein in the IDO-1 group of MSCs was higher than that in the untreated control MSCs. (D) The expression of cyclooxygenase-2 (COX-2) protein was more highly expressed in the COX-2 group of MSCs than the untransfected MSCs.

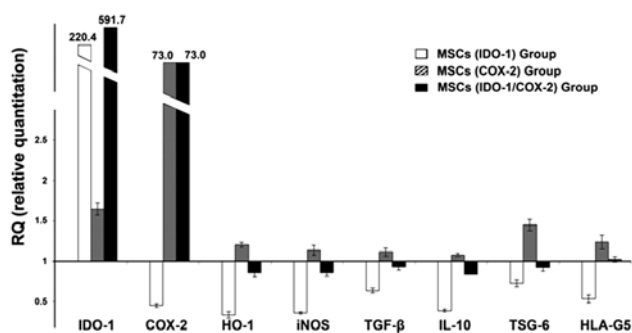


Figure 3. Immune-related gene expression in indoleamine 2,3-dioxygenase-1 (IDO-1)- and cyclooxygenase-2 (COX-2)-modified mesenchymal stem cells (MSCs). Immunomodulation-related gene expression was quantified by the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay (the control group values were set at fold-change, 1). Compared with the untreated control group, the expression of IDO-1, COX-2, heme-oxygenase-1 (HO-1), inducible nitric-oxide synthase (iNOS), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) stimulated gene/protein-6 (TSG-6), transforming growth factor- $\beta$  (TGF- $\beta$ ), human leukocyte antigen molecule 5 (HLA-G5) and interleukin-10 (IL-10) were enhanced in the COX-2 MSC group. However, apart from IDO-1 and COX-2, almost all the detected genes were dampened in the IDO-1 MSC group and the IDO-1/COX-2 MSC group.

COX-2, almost all the detected genes were dampened in the MSCs (IDO-1) and (IDO-1/COX-2) groups.

#### Potent immunosuppressive ability of COX-2-modified MSCs.

In the preliminary experiments, PHA-stimulated lymphocytes showed significant proliferation from day 3 onwards compared to the untreated group (Fig. 4A). Thus, we collected the data from day 3 in subsequent co-culture assays. The results (Fig. 4B) showed that the inhibitory effect of MSCs on lymphocyte proliferation was statistically significant when transfected with IDO-1 or COX-2 compared with the control

MSCs. However, the inhibitory effect of MSCs (IDO-1/COX-2) group was lower than that of the single-transfected MSCs (IDO-1) group or MSCs (COX-2) group.

Inflammatory cytokines expressed by PBMC, such as IFN- $\gamma$  and TNF- $\alpha$ , were activated by PHA stimulation. However, following co-culture with MSCs, the expression levels of IFN- $\gamma$  and TNF- $\alpha$  were significantly reduced in PBMC. By contrast, the MSCs (COX-2) group more effectively inhibited the protein expression levels of IFN- $\gamma$  and TNF- $\alpha$  than their control group counterparts ( $P < 0.05$ ) (Fig. 5A and B).

In the cytotoxicity analysis, the results showed that the 10:1 ratio of lymphocytes relative to HeLa cells exerted a highly acceptable killing effect. By contrast, PHA-activated PBMC exhibited markedly increased cytotoxicity against HeLa cells. After co-culture with MSCs, the cytotoxic activity of PBMC was significantly lower than the non-transfected MSCs group ( $P < 0.05$ ). In addition, PBMC incubated with the MSCs (COX-2) group, had the lowest killing activity as compared with the non-transfected control group. However, the observations were not statistically different from each other (Fig. 5C).

#### Discussion

The immunosuppressive properties of MSCs potentially endow them with properties ideally suited as cellular products in the treatment of autoimmune and other immune-mediated disorders (29,30). Improving the immune regulatory efficiency of MSCs inevitably leads to improved clinical outcomes. As previously mentioned, IDO-1 and COX-2 play critical roles in the immunomodulation of MSCs (23,24). In tryptophan catabolism, IDO-1 is a rate-limiting enzyme that inhibits antigen-specific T-cell proliferation and suppresses T-cell responses. In addition, COX-2 plays a key role in prostaglandin biosynthesis, which is helpful in PGE<sub>2</sub>-mediated immunosuppression (31-34). Therefore, in the current study,



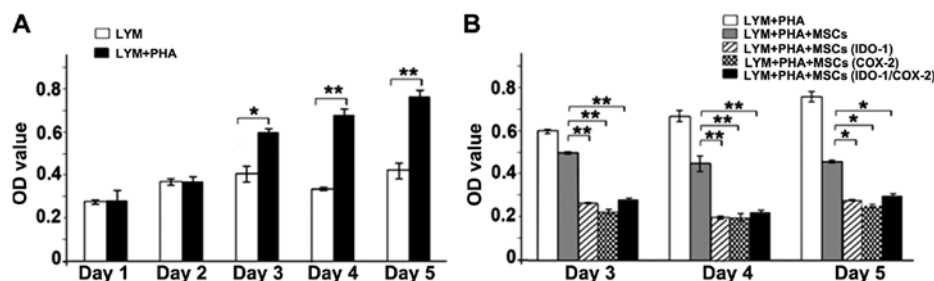


Figure 4. Lymphocyte proliferation assay of indoleamine 2,3-dioxygenase-1 (IDO-1)- and cyclooxygenase-2 (COX-2)-modified mesenchymal stem cells (MSCs). (A) Phytohemagglutinin (PHA)-primed lymphocytes showed significant proliferation from day 3 onwards as compared to the untreated group. (B) Proliferation of lymphocytes was suppressed by MSCs in the co-culture system. Overexpression of IDO-1 or COX-2 in MSCs promoted greater inhibitory functions as compared to their untreated MSC counterparts. However, no statistically significant differences were observed in the modified groups (\* $P < 0.05$  and \*\* $P < 0.01$ ).

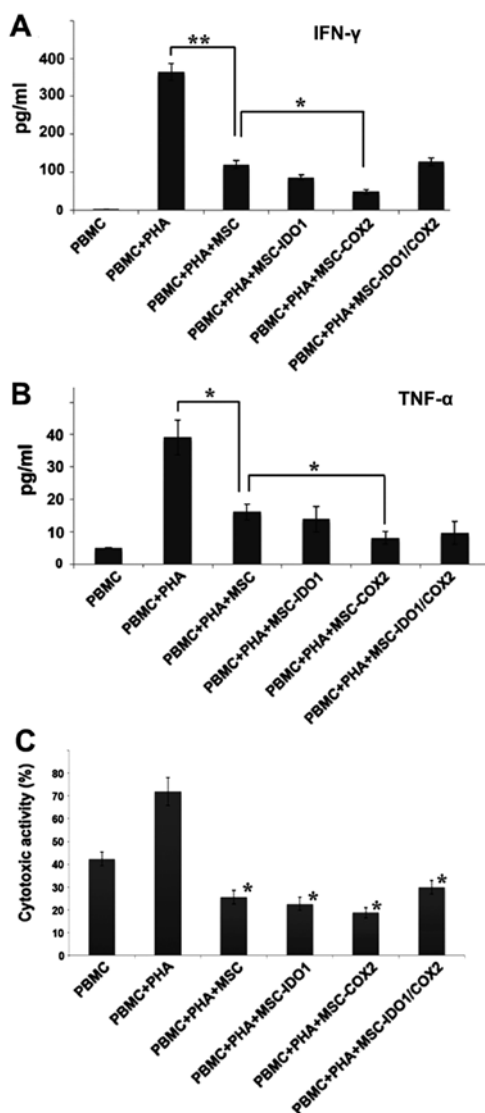


Figure 5. Reduction of lymphocyte activation by co-culture assay. (A and B) Enzyme-linked immunosorbent assay (ELISA) measurements show that the synthesis of interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) was significantly inhibited in peripheral blood mononuclear cell (PBMC) after co-culture with mesenchymal stem cells (MSCs), which was especially evident in the cyclooxygenase-2 (COX-2) MSC group (\* $P < 0.05$  and \*\* $P < 0.01$ ). Cytotoxicity assays showed that phytohemagglutinin (PHA)-activated PBMC exhibited markedly increased cytotoxicity against HeLa cells. After co-culturing with MSCs, the cytotoxicity of PBMC for their targets was significantly reduced ( $P < 0.05$ , as compared with PBMCs not cultured with MSCs). In addition, PBMC incubated with COX-2-transfected MSCs had the lowest killing activity as compared with the other groups.

we attempted to improve the expression levels of IDO-1 or COX-2 to expand the active functional expression of key immune regulatory molecules that are needed in adoptive cellular immune therapy, thereby increasing the potential immune regulatory efficiency of MSCs.

Considering the clinical safety of the cell product, viral vectors are not the optimal choice. The short lifespan of allogeneic MSCs in recipient subjects also does not require a stable transfection (35-37). Thus, we reconstructed two recombinant plasmids to enhance the expression levels of IDO-1 and COX-2. The results of RT-qPCR and western immunoblotting confirmed the expression of IDO-1 and COX-2 in transfected MSCs. Further functional tests in co-culture assays, including lymphocyte proliferation and cytotoxicity assays, showed that COX-2-transfected MSCs exhibit more potent immunomodulatory properties than other groups. As shown by ELISA, the synthesis of IFN- $\gamma$  and TNF- $\alpha$  was significantly inhibited in PBMC after co-culture with COX-2-transfected MSCs. IFN- $\gamma$  is a cytokine that is produced primarily by T-lymphocytes and natural killer cells. IFN- $\gamma$  is critical for innate and adaptive immunity against viral and intracellular bacterial infections and for tumor control (38). It also alters the transcription of up to 30 genes that produce a variety of physiological and cell responses, including driving NK cell activity, increasing antigen presentation and lysosomal activity of macrophages. TNF- $\alpha$  was produced by macrophages, lymphoid cells, and fibroblasts, among other cells. TNF- $\alpha$  was originally characterized by its ability to induce tumor cell apoptosis and cachexia, and is now considered a central mediator of inflammation (39). Reduction in the levels of IFN- $\gamma$  and TNF- $\alpha$  dampens the occurrence of inflammation and immune rejection.

To elucidate the changes caused by transfection, we examined the expression of several immune regulatory genes in MSCs. In addition to *IDO-1* and *COX-2*, the expression of *HO-1*, *iNOS*, *TSG-6*, *TGF- $\beta$* , *HLA-G5* and *IL-10* were all increased after COX-2 transfection in MSCs. Previous findings have demonstrated that secretion of TGF- $\beta$  and induction of IL-10 contribute to the immunological dampening functions of MSC (40). MSC-released TSG-6 was also identified and shown to improve immunosuppression by interacting with CD44 on resident macrophages, which decreased TLR2/NF- $\kappa$ B signaling and thereby decreased the secretion of proinflammatory mediators (41,42).

Another important molecule involved in MSC immune regulation involved the expression and secretion of HLA-G5.

The soluble isoform of HLA-G5 that is secreted by MSCs is responsible for the suppression of T-cell proliferation, NK cell-mediated cytotoxicity, and IFN- $\gamma$  secretion (43). It was shown that iNOS is important in MSC immune suppression by limiting T-cell proliferation (44). The stress-inducible enzyme HO-1, which catalyzes the rate-limiting step of the degradation of heme to biliverdin, also has a suppressive effect on T-cell proliferation in human and rat MSCs (45,46). Increased expression of these genes indicated that COX-2-enhanced MSCs were the most effective at enhancing immunosuppression, which is consistent with the results obtained from the functional tests.

The immunosuppressive effect of the MSCs (IDO-1) group was less than that of the MSCs (COX-2) group, suggesting the importance of PGE2 as compared to tryptophan/kynurenine among several immunomodulatory mechanisms. Notably, the immunosuppressive effect of MSCs with the two recombinant plasmids was weaker than MSCs transfected with only one recombinant plasmid. We hypothesize that the two plasmids may have competitive properties in the cell, resulting in inadequate translation. However, COX-2 transfection promoted positive outcomes, despite the instability of electroporation among different batches and cell membrane damage caused by the electrical pulses, which may have affected the efficacy of cell therapy. Additionally, the influence of COX-2 overexpression on cell viability remains to be investigated.

In conclusion, we obtained a novel population of MSCs that was derived following the overexpression of the immune-related COX-2 gene. The modified MSCs more potently inhibited the activation and proliferation of PBMCs. Thus, this gene-modified population of MSCs overexpressing COX-2, are expected to reduce the requirement of the number of infused cells, and improve the efficacy of treating autoimmune diseases and aGVHD.

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