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IL-6 Production Stimulated by CD14+ Monocytes-paracrined IL-1β does not Contribute to the Immunosuppressive Activity of Human Umbilical Cord Mesenchymal Stem Cells

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Key Words

Umbilical cord mesenchymal stem cells (UC-MSCs) • IL-6 • PGE2 • IL-1β

Abstract

Background/Aims: Human umbilical cord mesenchymal stem cells (hUC-MSCs) possess immunosuppressive activities but the mechanisms of such activities are not fully understood. Here, we investigated the role of IL-6, one of the characteristic factors of MSCs, in the immunoregulating effect of hUC-MSCs on $CD4^+$ T lymphocytes. Methods: The condition media from human peripheral blood mononuclear cells (hPBMCs) or CD14+/- cell were tested if stimulating IL-6 production by hUC-MSCs. The related signaling pathway of IL-6, and the immunosuppressive activity of IL-6 on $CD4^+$ T lymphocytes were studied. Result: IL-6 production was dramatically increased by hUC-MSCs when co-culturing with resting or activated hPBMCs. $CD14^+$ monocytes-paracrined IL-1β promoted the secretion of IL-6 by hUC-MSCs via JNK and NF-κB signaling pathway. Blocking of PGE2 synthesis did not affect the secretion of IL-6, anti-IL-6 antibody was not able to reverse hUC-MSCs-mediated inhibition on $CD4^+$ T lymphocytes. IL-6 did not mediate the suppressive activity of IL-1β-hUC-MSCs- PGE2 on $CD4^+$ T cell. Conclusion: $CD14^+$ monocytes-paracrined IL-1β promotes IL-6 secretion by hUC-MSCs through activating JNK and NF-κB signaling pathway. However, increased IL-6 production does not contribute to immunosuppressive activity of IL-1β-hUC-MSCs-PGE2 on $CD4^+$ T cells.

Introduction

Mesenchymal stem cells (MSCs) have been initially identified in the bone marrow (BM) as multipotent nonhematopoietic progenitor cells that differentiate into cells of the mesoderm lineage, including bone, fat and cartilage [1]. Although BM-MSCs were widely studied in the basic and clinical fields, human umbilical cord derived MSCs (hUC-MSCs) seem more competitive as

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hUC-MSC can readily be isolated with no harm to donors and expanded rapidly in large quantities [2-6].

hUC-MSCs are not only poorly immunogenic but also have potent immunosuppressive activities. hUC-MSCs, like BM-MSCs, express human leukocyte antigen (HLA) major histocompatibility complex (MHC) class I, but not HLA MHC class II and co-stimulatory molecules such as CD40, CD80 and CD86 [7, 8]. MSCs can inhibit the activation and proliferation of T lymphocyte in a major histocompatibility complex (MHC)-independent manner [9] and also regulate the immune response indirectly through modulation of professional antigen presenting cells such as dendritic cells [10]. Some clinical trials have shown that MSCs contribute to the reduction of the incidence of graft-versus-host disease following hematopoietic stem cell transplantation [11], and to the improvement of multiple sclerosis and type 2 diabetes [12].

The mechanisms of the immunosuppression capability of MSCs are not fully understood, both cell-to-cell contact [13, 14] and soluble mediators are likely involved. A batch of soluble factors secreted by MSCs have been suggested to contribute to the immunosuppression including transforming growth factor-β1 (TGF- β1), hepatocyte growth factor (HGF) [15], indoleamine 2,3-dioxygenase (IDO) [16], nitric oxide (NO [17], IL-6, vascular endothelial growth factor (VEGF) [18] and prostaglandin E2 (PGE2) [2]. Previously, we have demonstrated that PGE2 secreted by hUC-MSCs plays an important role in inhibiting the proliferation of human peripheral blood mononuclear cells (hPBMCs) in response to mitogenic or allogeneic stimulus but not TGF-β, IDO or NO [19].

IL-6 plays an important role in immunomodulation [20] ,angiogenesis [21], normal wound healing [22], and drug resistance of cancer [23]. And IL-6 has been demonstrated to be one of the characteristic factors of MSCs to contribute to the immunoregulation activities of MSCs and relates to the immunosuppression of PGE2 [18, 20]. However, the exact role of IL-6 in the immunoregulation of hUC-MSCs remains to be further illustrated. In this study, we have carefully investigated the mechanism of IL-6 production by hUC-MSCs and found that $CD14^+$ monocytes secreted IL-1β play a key role in the induction of IL-6 production through activating JNK and NF-κB signaling pathway. Our previous study has shown that IL-1β promotes PGE2 production which mediates the immumosuppresion of hUC-MSCs on $CD4^+$ T cells, and IL-6 does not affect PGE2 production of MSC [19]. In this article, we further demonstrate that PGE2 does not affect the secretion of IL-6 and IL-6 does not directly affect the suppressive activity of IL-1β-hUC-MSCs- PGE2 on $CD4^+$ T cell. Our data indicate that IL-6 secreted by hUC-MSCs following IL-1β stimulation does not contribute to the immunosuppressive activity of hUC-MSCs on $CD4^+$ T lymphocytes.

Materials and Methods

Isolation and culture of hUC-MSCs

hUC-MSCs were isolated from umbilical cords obtained from local maternity hospitals with donors' informed consent. Human tissue collection for research was approved by the Institutional Review Board of the Chinese Academy of Medical Science and Peking Union Medical College. Briefly human umbilical cords were collected from normal, full-term infants delivered by cesarean section. The cord was cut into pieces of 2 mm diameter after flushing the residual blood off, and digested with type II collagenase (Sigma) at 37°C for 30 minutes. The digested fragments were treated with 0.125% trypsin (Gibco) for another 30 min with gentle agitation at 37°C. The digested mixture was then passed through 100 μm and 200 μm filters. The collected cells were resuspended in DMEM/F-12 (Gibco) containing 10% fetal bovine serum (FBS;Hyclone), 10ng/mL epidermal growth factor (EGF; Sigma), 100U penicillin/streptomycin (Sigma), 2 mM L-glutamine (Gibco) and 0.1 mM nonessential amino acids. The cells were plated at a density of 1.0×10^6cells/cm^2 in non-coated T-25 or T-75 flasks (Beckon Dickinson) and maintained in a humidified atmosphere at 37°C under 5% CO2 in air. The medium was changed in 24 hours, while adherent cells kept growing. Medium was changed twice a week, until 80% confluence was reached. Cells were collected by digesting with 0.125 % trypsin. The viability was examined by trypan blue exclusion and regularly found greater than 95%. Passages were carried out at 1x10^{4}cells/cm^2 in complete DMEM/F-12 medium for cells expansion. Periodic control of cells markers by FACS as well as the differentiation ability were performed and showed stable up to, at least, the 10th passage. hUC-MSCs at P4 to P8 were used in this study.

Immunophenotype analysis of MSCs by flow cytometry

hUC-MSCs (n=5) were stained with PE conjugated antibodies specific for the following surface markers: CD3, CD13,CD14,CD19, CD29,CD31, CD44, CD49e, CD73, CD80, CD86,CD105, CD106, CD133,CD166, or with FITC-conjugated antibodies specific for the following surface markers: CD34, CD45, CD90, HLA-ABC and HLA-DR. Non-specific isotype matched antibodies served as controls. All the antibodies were purchased from BD Pharmingen, and the flow cytometry analysis was performed on a FACSCalibur using the CellQuest software (Becton Dickinson).

Multilineage differentiation

hUC-MSCs were plated in 24-well plate at a density of 3000 cells/well. Specific induction medium was added 24 hours

later. For adipogenic induction, the cells were treated in a defined Medium DMEM-LG(Gibco) supplemented with 10% FBS, 100U penicillin/ streptomycin (Sigma), 2 mML-glutamine (Gibco), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate,1 μM dexamethasone (Sigma), 0.5 mM indomethacin (Sigma), 10μM insulin (Sigma) and 0.5 mM isobutylmethylxanthine(Sigma). Three weeks after the induction, the cells were stained with oil-red. For osteogenic induction, the induced medium was DMEM-LG supplemented with 10% FBS, 100U penicillin/streptomycin, 2 mML-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 0.1μM dexamethasone, 50 μg/ml ascorbate phosphate (Sigma) and 10mM glycerophosphate (Sigma). Osteogenic differentiation was evaluated by Von Kossa assay three weeks afterward. For chondrogenic induction, the cells were cultured in DMEM-HG supplemented with 10% FBS, 100U penicillin/streptomycin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, pyridoxine hydrochloride (Gibco), 50 ug/ml L-ascorbic acid-2-phosphate (Sigma), 0.4 mM L-proline (Sigma), 10^7M dexamethasone, 1% ITSt1 (Sigma) and 10 ng/ml recombinant human transforming growth factor-beta (TGF-beta; Peprotech). Chondrogenic differentiation was assessed by safranin O staining three weeks later.

Isolation of human peripheral blood mononuclear cells (hPBMCs), $CD4^+$ T cells and $CD14^+$ monocytes

hPBMCs were isolated by Ficoll-Paque (Axis-Shield) density gradient centrifugation (density 1.077 ± 0.002 at 2200rpm×20min) from the venous blood of health volunteers after informed consent. $CD4^+$ T cells and $CD14^+$ monocytes were purified using relevant magnetic MicroBead kits (Miltenyi Biotec). Briefly, the hPBMCs were magnetically labeled with CD4 or CD14 microbeads (Miltenyi Biotech) respectively, and then loaded onto the column in a magnetic field according to the manufacturer's instructions. The magnetically labeled cells were retained on the column and were eluted as the positively selected cell fraction. The purity of isolated cells was more than 90%.

Coculture of hUC-MSCs and the immune cells

Cells were maintained in culture in DMEM/F-12 medium or RPMI-1640 medium with 10% FBS, 100U penicillin/ streptomycin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate. hUC-MSCs (20 Gy irradiated) were plated in 96-well flat-bottom plate 2-4 hours before the addition of hPBMCs or $CD4^+$ T cells. For dose dependent experiment, various numbers of hUC-MSCs were incubated with hPBMCs (10^5/well) in the presence or absence of 10 ug/ml Phytohaemagglutinin (PHA; sigma) for 3 days. Additionally, anti-CD3/CD28 Dynabeads (Invitrogen)-activated $CD4^+$ T cells (10^5/well, Dynabeads/T=1/1) were cocultured with hUC-MSCs (2×10^4/well) for 3 days. In some experiments, exogenous PGE2 (100ng/ml, Sigma), exogenous IL-6 (20ng/ml, Peprotech), NS-398 (PGE2 synthesis inhibitor, purchase from Cayman Chemicals, 50uM) or anti-IL-6 antibody (5ug/ml, Biolegend) was added to the culture system. In some experiment, signal pathway inhibitors were used. Wortmannin (Calbiochem) was used at 50 nM, and Go6983 and Go 6976 (Sigma) at 100 nM and 10 nM, respectively. PD98059 (Sigma) at 10 μM, SB203580 (Sigma) at 20 μM, SP600125 (Sigma) at 20 μM, IKK-NBD peptide (Biomol) at 10 μM, PDTC (Sigma) at 50 μM were used in the experiments. Cell-free supernatants were collected and kept frozen at -80 °C, and the cytokine expression was examined by ELISA kit. IFN-γ, IL-1β, and IL-6 ELISA kits were purchased from Jingmei Biotech Co, Ltd, PR China. PGE2 ELISA kit was purchased from Cayman Chemicals. All assays were carried out according to the protocols of the suppliers.

Preparation of condition medium

$1x10^6$ resting hPBMCs, $1x10^6$ $CD14^+$ monocyte, $1x10^6$ $CD14^-$ cells or $5*10^5$ hUC-MSCs were cultured in 24 well plate using 1ml PRMI 1640 medium with 10% FBS, 100U penicillin/ streptomycin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate. After 18 hours, supernatants were collected and filtered by 0.4 um filter as condition medium (CM), aliquoted and stored at -80°C.

Immunoblotting

Cell extracts were prepared using cell lysis buffer (Cell Signaling Technology), were centrifuged at 12,000 rpm×5 min after heating up at 95°C for 10 minutes. Proteins were then separated by SDS PAGE and transferred to PVDF membrane (0.2 μm, Amersham) using a wet tank transfer system (Bio-Rad). Membranes were blocked with 20 mM Tris, pH 7.4, 150 mM NaCl (TBS) containing 0.1% (v/v) Tween-20, 5% (v/v) calf serum for 2 hours at room temperature and incubated with either anti-IκB monoclonal antibody (2 μg/ml, Santa Cruze) or anti-β-actin monoclonal antibody (1 μg/ml, Santa Cruze) for 2 hours at room temperature. Membranes were washed three times (10 minutes each) with TBS, 0.1% (v/v) Tween-20, and the bounded antibodies were detected with goat anti-mouse HRP-conjugated secondary antibody (1:1000, Santa Cruze) and a chemiluminescence detection kit (Amersham) according to the manufacturer's instructions. Protein bands were quantified by densitometric analysis using ImageJ software.

Statistical analysis

All experimental protocols were done with at least triplicate points and the results were analyzed using the GraphPad Prism software package (version 4, GraphPad Software). Data are presented as mean ± standard deviation (SD). Means between groups are compared with the unpaired student t test. Differences between groups are considered statistically significant when $p< 0.05$ (*) and highly significant when $p< 0.01$ (**).

Results

Characterization of hUC-MSCs

The immunophenotype of hUC-MSCs was analyzed by flow cytometry. hUC-MSCs were negative for CD3, CD14, CD19, CD31, CD34, CD45, CD80, CD86, CD133 and HLA-DR, but positive for CD13, CD29, CD44, CD49e, CD73, CD90, CD105, CD106, CD166 and HLA-

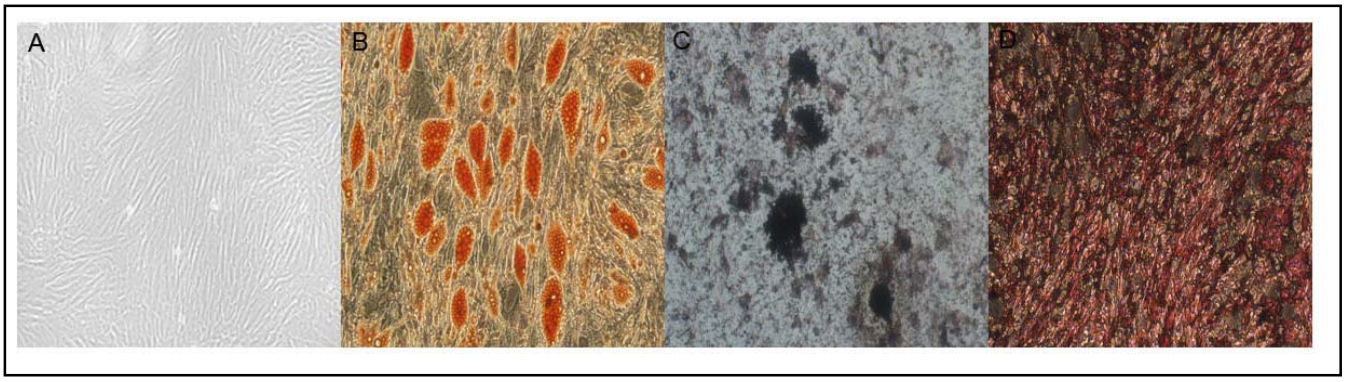

Fig. 1. The multipotent abilities of hUC-MSCs. (A) hUC-MSCs display a fibroblastic morphology. (B) Adipogenic differentiation was assayed by oil red O staining. (C) Osteogenic differentiation was assayed by von kossa staining. (D) chondrogenic differention was assessed by safranin O staining. Images were visualized using an Olympus IX71microscope and captured with a Nikon Coolpix 995 camera.

ABC [19]. In addition, hUC-MSCs were able to differentiate into adipogenic, osteogenic and chondrogenic cells in lineage-specific induction conditions (Fig. 1).

IL-6 is upregulated by hUC-MSCs upon coculture with hPBMCs

The IL-6 level was very low in the culture supernatant of either hUC-MSCs or hPBMCs. The expression of IL-6 was significantly upregulated to 70 ng/ml and 100 ng/ml when hUC-MSCs were co-cultured with resting or activated hPBMCs at ratio 1:1. The IL-6 production was enhanced when the ratio of hUC-MSCs increased (Fig. 2A). Moreover, a dose and time-dependent upregulation of IL-6 was strongly evident in this coculture system, IL-6 expression was largely elevated after culturing for 24 hrs (Fig. 2B).

$CD14^+$ monocytes-paracrined IL-1β trigger IL-6 production in hUC-MSCs

To determine the resource of IL-6, we cultured hUC-MSCs in cell-free condition medium (CM) of hPBMCs, which had been cultured for 18 hrs. The IL-6 production was largely enhanced after 24 hrs culture when 100ul of hPBMCs CM added. On the other hand, we cultured hPBMCs in cell-free condition medium (CM) of hUC-MSCs to see if hPBMCs could produce IL-6. However IL-6 level didn't change even when 100 µl of hUC-MSCs CM added (Fig. 3A). We cultured hUC-MSCs in cell-free condition medium (CM) of $CD14^+$ monocytes, $CD14^-$ cells or hPBMCs, respectively. As shown in Fig. 3B, IL-6 expression of hUC-MSCs increased in a volume-dependent manner only in the CM from $CD14^+$ monocytes but not in the CM from $CD14^-$ cells. The level of IL-6 of hUC-MSCs cultured in $CD14^+$ monocytes CM is similar to that in hPBMCs CM.These

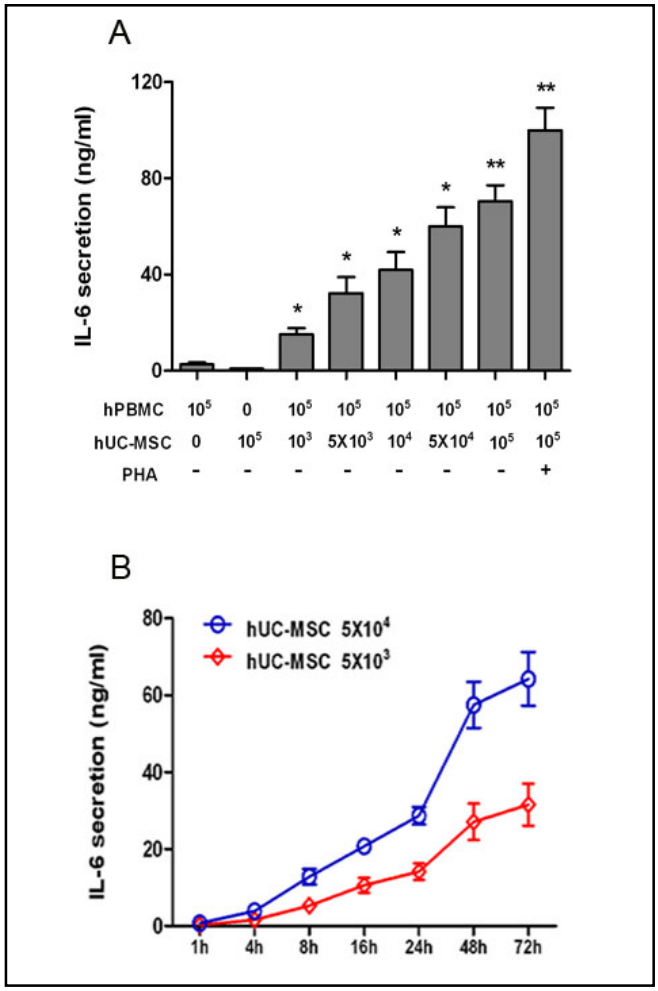

Fig. 2. IL-6 is upregulated by hUC-MSCs upon coculture with hPBMCs. IL-6 expression was determined using ELISA in the supernatant of coculture of hUC-MSCs with hPBMCs at different ratios in the presence or absence of PHA for 3 days (A), and for different time points (B). Data represent the mean ± SD of experiments performed in three independent experiments (triplicate of each experiment), each performed with hUC-MSCs from different donors.*, $p < 0.05$; **, $p < 0.01$.

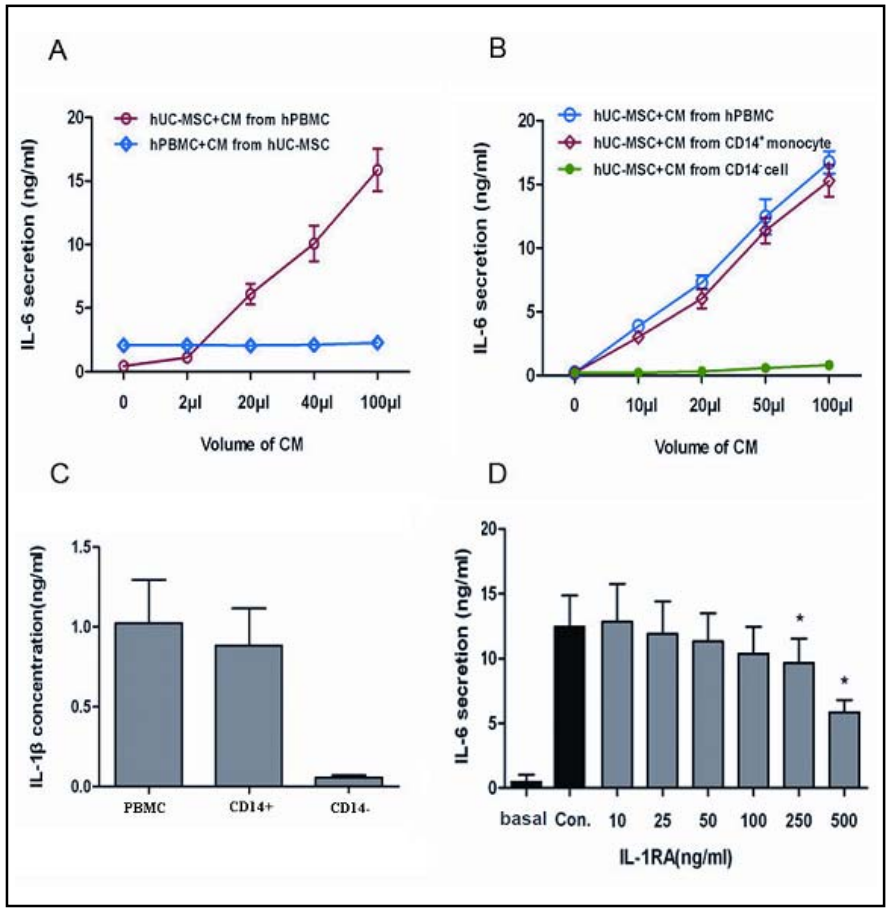

Fig. 3. CD14+ monocytes-paracrined IL-1β triggers IL-6 production in hUC-MSCs. (A) IL-6 expression in the supernatant was determined when hUC-MSCs were stimulated by hPBMCs CM or hPBMCs were stimulated by hUC-MSCs CM for 8 hours, the hPBMCs CM were collected after culturing $1*10^6$ hPBMCs with 1ml PRMI 1640 medium with 10% FBS 100U penicillin/streptomycin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate (1640)and $5*10^5$ hUC-MSCs with 1ml 1640 in 24 well plate for 18 hrs, respectively. (B) IL-6 expression in the supernatant was determined when hUC-MSCs were stimulated by the hPBMCs CM, CD14+ cells CM and CD14- cells CM for 8 hrs. CM were collected after culturing $1*10^6$ hPBMCs , $1*10^6$ CD14+ cells and $1*10^6$ CD14- in 24 well plate using 1ml 1640 for 18 hrs. (C) The level of IL-1β was quantified in the CM of hPBMCs, CD14+ monocytes or CD14- cells. (D) IL-6 expression in the supernatant was determined when hUC-MSCs were stimulated by hPBMCs CM in addition of IL-1RA, and when Data represent the mean ± SD of experiments performed in three independent experiments (triplicate of each experiment), each performed with hUC-MSCs from different donors. * $p < 0.05$.

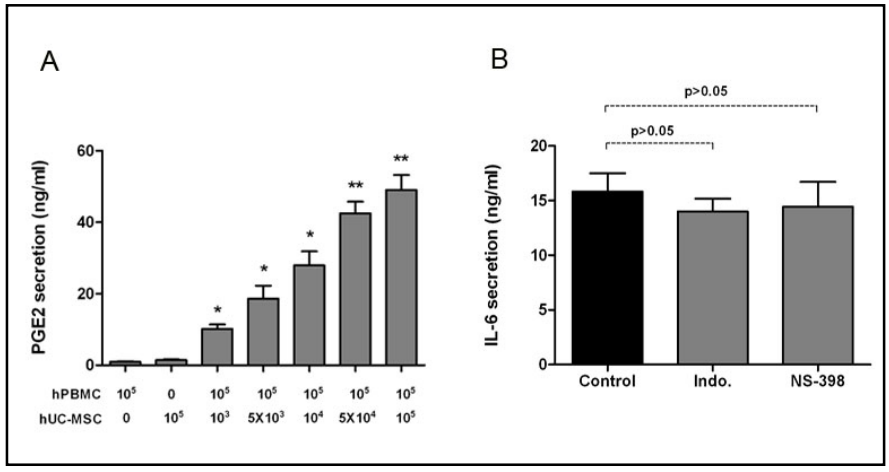

Fig. 4. PGE2 does not affect the production of IL-6. (A) the level of PGE2 were determined in the supernatant of coculture of hUC-MSCs with hPBMCs at different ratios for 3 days. (B) hUC-MSCs were stimulated by hPBMCs CM(100ul) in the presence of indomethacin (10μM) and NS-398(50μM) for 8 hours, and IL-6 concentration in the supernatant was measured. Data represent the mean ± SD of experiments performed in three independent experiments (triplicate of each experiment), each performed with hUC-MSCs from different donors.*, $p < 0.05$; **, $p < 0.01$.

results showed that hUC-MSCs were the main supplier of IL-6 in the coculture system, and the upregulation of IL-6 was in a volume-dependent manner. It also indicated that soluble factors in the CM of hPBMCs were the major driving force for IL-6 production by hUC-MSCs.

IL-1β was one of the characteristic factors of monocyte/macrophage [24, 25], and may induce the production of IL-6 [26, 27].To confirm if IL-1β is one of the potential inducers of IL-6, we tested the concentrations of IL-1β in the hPBMCs , CD14+ monocytes CM , CD14- monocytes CM. We found that concentrations of IL-1β in the hPBMCs and CD14+ monocytes CM were up to 10-fold higher than that in CD14- cells CM (Fig. 3C). To investigate the contribution of IL-1β in the upregulation of IL-6 by hUC-MSCs, we added IL-1RA (IL-1 receptor antagonist) in the culture of hUC-MSCs activated by

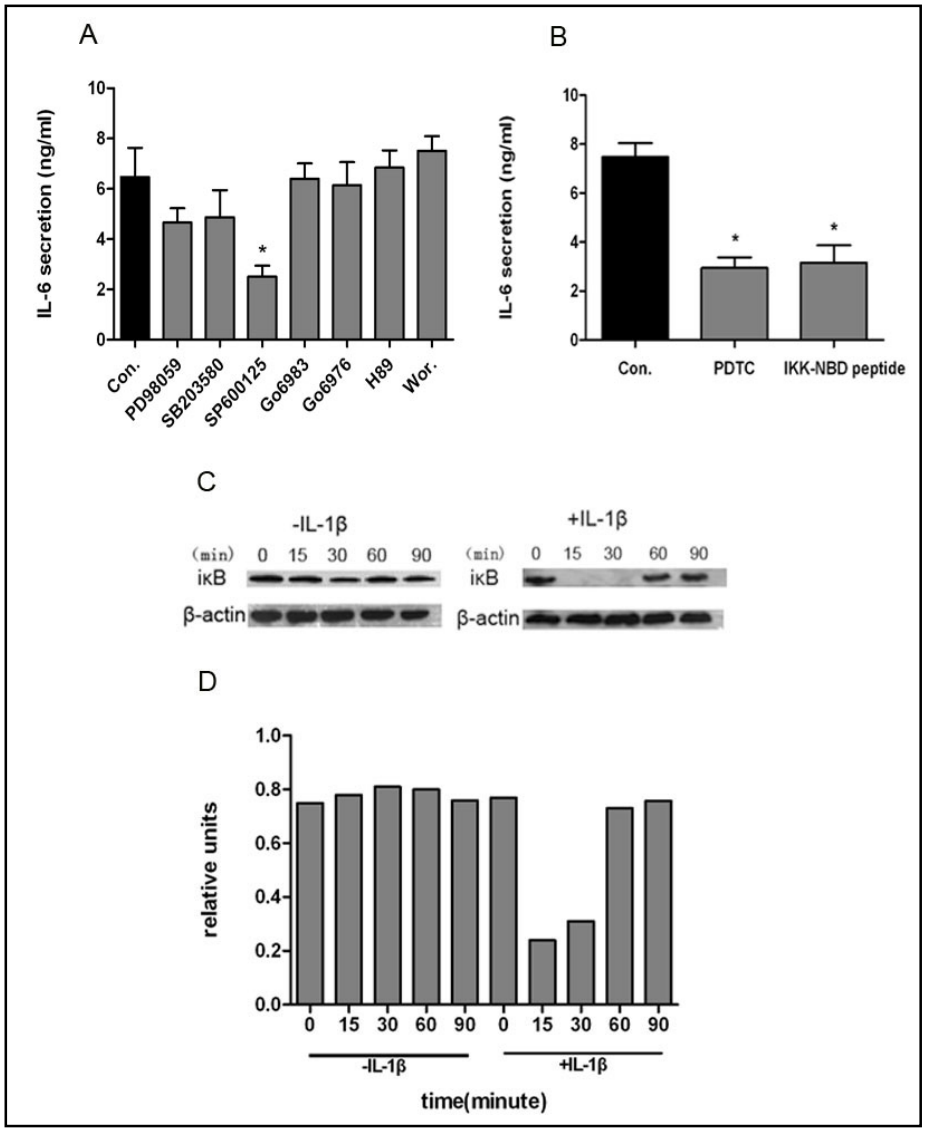

Fig. 5. JNK and NF-κB signaling pathway were activated in IL-1β-induced IL-6 production by hUC-MSCs. (A) and (B) hUC-MSCs were stimulated with IL-1β (2ng/ml) for 8 hours after 1 hours pretreated with the specific inhibitors of different signaling proteins. Then the IL-6 level in each supernatant was detected by ELISA. Data represent the mean ± SD of experiments performed in three independent experiments (triplicate of each experiment), each performed with hUC-MSCs from different donors. *, $p < 0.05$. (C) hUC-MSCs were incubated without or with IL-1β for the indicated times. Total cellular proteins were extracted and subjected to immunoblot analysis using anti-IκB monoclonal antibody. Samples were also immunoblotted with anti-β-actin monoclonal antibodies in parallel as a loading control (triplicate of each experiment). (D) densitometric quantitation of IκB was measured for the relative units.

hPBMCs CM. IL-6 production was significantly blocked by IL-1RA in a dose-dependent way (Fig. 3D). We also tested the mRNA expression of IL-6 in UC-MSCs incubated IL-1β by real time PCR, and we found that addition of IL-1β into the hUC-MSCs culture induced a notable increase (30 fold) in the IL-6 mRNA expression (data not shown). These data suggested that IL-1β may promote the production of IL-6 by hUC-MSCs. However the IL-6 did not decrease to the basal level even when 500 ng/ml of IL-1RA added. IL-1β as one of the characteristic factors of monocyte/macrophage [24, 25] may induce the production of IL-6 [26, 27].

PGE2 does not affect IL-6 production

PGE2 has been suggested to be a candidate to promote the IL-6 expression by MSC [18]. The results shown in Fig. 4A indicate that the expression of PGE2 was significantly upregulated when coculturing of hUC-MSCs with resting hPBMCs in a dose dependent manner, and the tendency of IL-6 and PGE2 production were similar in coculture system. To identify the effect of PGE2 on IL-6 production, hUC-MSCs were cultured in hPBMCs CM containing indomethacin or NS-398 (PGE2 synthesis inhibitors) for 24 hrs and IL-6 level were determined. The results showed that blocking of PGE2 synthesis didn't affect the secretion of IL-6 (Fig.4B). Collectively, the PGE2 and IL-6 secreted by hUC-MSCs were increased by allogeneic hPBMCs, but they were relatively independent.

JNK and NF-κB signaling pathways were activated in IL-1β-induced IL-6 production by hUC-MSCs.

To define the potential signaling pathway involved in IL-1β-induced IL-6 production, hUC-MSCs were pretreated with specific inhibitors of some signaling

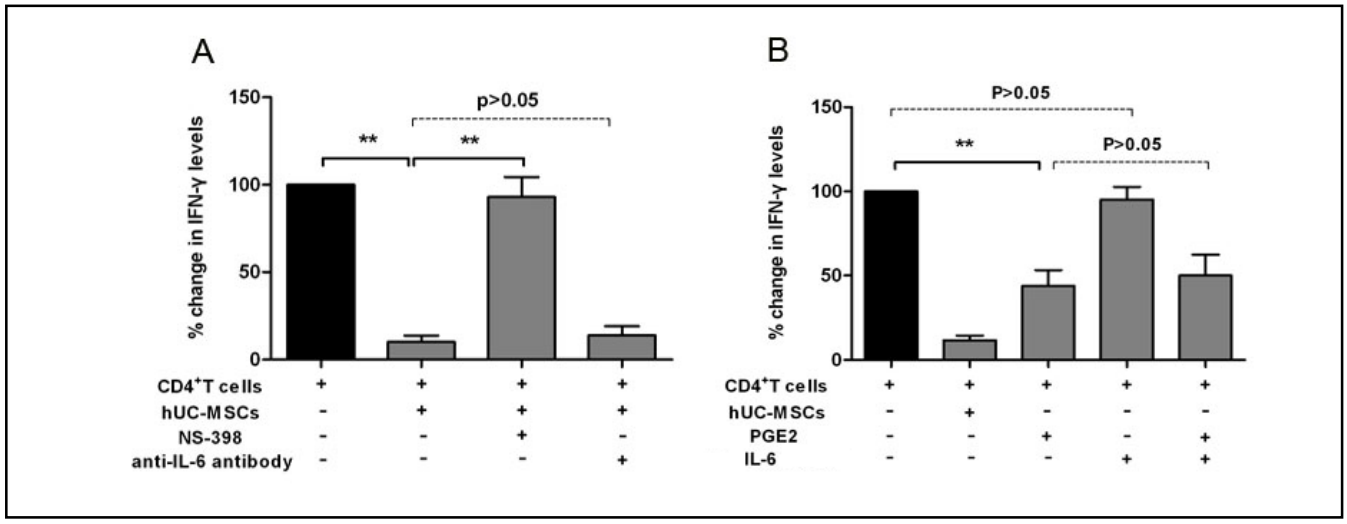

Fig. 6. IL-6 did not mediate the suppressive activity of IL-1β-hUC-MSCs- PGE2 on CD4+ T cell. Irradiated hUC-MSCs($2*10^4$/well) were cocultured with Dynabeads -stimulated CD4+ T cells ($1*10^5$/well) in a 96-well plate for 18 hrs in the presence (+) of indomethacin NS-398 (50 μM) or anti-IL-6 antibody (5 μg/ml) (A), or in the presence (+) of exogenous PGE2 (100 ng/ml) and /or IL-6 (20 ng/ml) (B). The level of IFN-γ was determined by ELISA. Percentage change of IFN-γ was obtained by direct comparison to the control of T cells alone with Dynabeads (=100%). Data represent the mean ± SD of experiments performed in three independent experiments (triplicate of each experiment), each performed with hUC-MSCs from different donors.*, $p < 0.05$; **, $p < 0.01$.

proteins for 1 hour before exogenous IL-1β was added. These specific inhibitors have been listed in Table 1, showing the possible signaling pathway involved. The IL-6 production was significantly reduced when the cells were pretreated with SP600125 (a specific inhibitor of JNK), PDTC and IKK-NBD peptide (two specific inhibitors of NF-κB) (Fig 5A, B). These results demonstrated that JNK and NF-κB signaling pathways were activated in IL-1β-induced IL-6 production by hUC-MSCs. To further confirm this, we detected the IκB expression in hUC-MSCs with or without IL-1β induction. IκB was rapidly degraded within 15 minutes after IL-1β added and reappeared after 60 minutes. There was no evidence of IκB degradation in hUC-MSCs without addition of IL-1β (Fig. 5C, D). However IκB was rapidly degraded within 15 minutes after IL-1β added and reappeared after 60 minutes (Fig.5C, D). The degradation of IκB was responsible for NF-κB activation and its translocation to the nucleus. The results confirmed that IL-1β-induced IL-6 was produced by hUC-MSCs through NF-κB signaling pathway.

Specific inhibitors	Signaling pathways
PD98059	extra-cellular signal-regulated kinase, ERK
SB20358	p38mitogen-activated protein kinase, p38 MAPK
SP600125	c-jun-N-terminal kinase, JNK
H89	protein kinase A, PKA
Wortmannin	phosphatidylnsitol 3-kinase, PI3K
IKK-NBD	IKK-NBD
Go6983	protein kinase C, PKC
Go6976	protein kinase C, PKC

Table 1. Specific inhibitors for signaling pathways

IL-6 didn't mediate the suppressive activity of IL-1β-hUC-MSCs- PGE2 on CD4+ T cell.

We have previously demonstrated that CD14+ monocytes promote hUC-MSCs-mediated immunosuppression probably via IL-1β-hUC-MSCs- PGE2 axis [28]. As IL-1β could induce IL-6 production by hUC-MSCs, we were wondering if IL-6 had effect on the immunosuppressive activity of hUC-MSCs. The cells were cocultured with anti-CD3/CD28 Dynabeads activated CD4+ T cells in the presence or absence of anti-IL-6 antibody or NS-398 (the PGE2 synthesis inhibitors), and IFN-γ level was determined. As we expected, IFN-γ secreted by activated CD4+ T cells was dramatically decreased, which indicated the immunosuppressive activity of hUC-MSCs. NS-398 almost completely abolished the inhibition of hUC-MSCs on IFN-γ secretion by activated CD4+ T cells and exogenous PGE2 partly mimicked the inhibitory effect of hUC-MSCs. These results demonstrated that PGE2 played an important role in the hUC-MSCs mediated immunosuppressive activity. However, the neutralizing anti-IL-6 antibody had little impact on the IFN-γ secreted by activated CD4+ T cells (Fig. 6A). Moreover, the exogenous IL-6 didn't affect the IFN-γ expression as the difference was not significant (Fig. 6B, P>0.05), and the addition of exogenous IL-6 had no impact on the

function of exogenous PGE2 to hUC-MSCs (Fig. 6B, P>0.05). The results demonstrated that IL-6 didn't mediate the suppressive activity of hUC-MSCs and had little effort on PGE2 for this suppression. This finding suggested that IL-6 was unlikely associated with immunosuppressive activity of IL-1β-hUC-MSCs- PGE2 on $CD4^+$ T cells.

Discussion

$CD14^+$ monocytes are capable of killing infected host cells via antibody and they are antigen presentation cells that lead to activation of T lymphocytes [29]. Monocytes play an important role in changing the level of characteristic factors and modulate the function of MSCs. It has been reported that the role of monocytes in the immunoinhibitory effects of BM-MSC was similar to that of human adipose-derived MSCs (hASCs) [30, 31]. Consistent with other studies, we have confirmed that $CD14^+$ monocytes promote PGE2 secretion which may be responsible for hUC-MSCs-mediated immunosuppressive activity [28]. In this study, we found that not only PGE2 but also IL-6 were significantly upregulated once hUC-MSCs were cocultured with resting or activated MHC-unmatched hPBMCs, indicated that hUC-MSCs shared similar characteristic with BM-MSCs which also upregulated IL-6 in addition of allogeneic hPBMCs [2, 18]. We found that IL-6 was basically low expressed by hUC-MSCs but it dramatically increased in the coculture system. Furthermore, we discovered that $CD14^+$ monocyte CM was sufficient for the induction of IL-6 by hUC-MSCs. IL-6 was another cytokine promoted by $CD14^+$ monocytes, which motivated us to investigate the role of IL-6 in the immunosuppressive activity of hUC-MSC.

In this study, we demonstrated that IL-1β was a major inducer for IL-6 production by hUC-MSCs. IL-6 secretion significantly decreased when the specific inhibitors of JNK and NF-κB were added, and IκB was rapidly degraded in hUC-MSCs following IL-1β treatment, suggesting that IL-1β upregulated hUC-MSCs derived IL-6 expression through JNK and NF-κB signaling pathway. IL-1β has also been demonstrated to promote the secretion of IL-6 in astrocytes [32], intestinal epithelial cells [33], synoviocytes [34] and mast cells [35], and this effect was through p38 MAPK [34, 35] and NF-κB signaling pathway [32, 33, 35]. Interestingly we found that three MAPK (ERK, p38 MAPK, and JNK) signaling pathways but not NF-κB was involved in IL-1β-induced PGE2 production in hUC-MSCs (unpublished data). This indicated that IL-1β induced IL-6 and PGE2 production by hUC-MSCs through different signaling pathways. PGE2 has been illustrated to stimulate IL-6 secretion in BM-MSCs [18]. However, our previous study has shown that IL-6 was not effective on the induction of PGE2 by hUC-MSCs [28]. Here we confirmed that PGE2, a crucial soluble factor mediated the suppressive effect of hUC-MSCs, had no effect on the secretion of IL-6. Thus, we postulate that PGE2 and IL-6 secreted by hUC-MSCs do not interfere with each other and play a relatively independent role in the biological effectiveness of hUC-MSCs. We were interesting to note a recent observation that IL-6-activated PGE2 secretion mainly participates in immunosuppressive potential of MSCs *in vivo* [20]. Although this was different from our finding, the divergence may be explained by the possibility that the lock-out of IL-6 gene may lead to change of the microenvironment of mice, which reduces PGE2 secretion from MSC in the mice deficient IL-6 but not in normal mice. Meanwhile, Djouad F et al found that IL-6 was involved in the immunoregulatory mechanism mediated by MSC through a partial inhibition of DC differentiation, they studied IL-6 secretion by mice BM-MSCs inhibited DC differentiation which was responsible for a decrease in T-cell proliferation. While we emphasized IL-β induced IL-6 secretion by hUC-MSCs and IL-6 was unlikely associated with immunosuppression activity of IL-1β-hUC-MSCs- PGE2 on $CD4^+$ T cells, the role of IL-6 on DC differentiation need further study.

We have illustrated that hUC-MSCs exert their immunomodulatory effects via IL-1β-PGE2 axi [19]. The PGE2 synthesis inhibitors almost completely abolished the inhibition of hUC-MSCs on IFN-γ secreted by activated $CD4^+$ T cells. Whereas here we showed that IL-6 had little effect on hUC-MSCs-induced inhibition on $CD4^+$ cells. However, IL-6 may also mediate immunomodulatory on other immune cell. Hegde et al. indicated that IL-6 was important in the differentiation of monocytes to dendritic cells [36, 37], and MSCs modulate dendritic cell maturation, migration and function through IL-6 at different stages [18].

IL-6 is a key molecule for neutrophil to rescue from programmed cell deat [38]. IL-6 also mediates the promotional effect of BM-MSCs and hUC-MSCs in megakaryocytopoiesis [39]. In addition, IL-6 is important in normal wound healing [22] and angiogenesis [21]. A few researchers have predicted that this proangiogenic effect of IL-6 may promote tumor growth [40]. It was also found that IL-6 mediated the promotional effect of BM-MSC on the proliferation of MCF-7 [41] and multiple

myeloma [37], the sphere formation and tumor initiation of colorectal tumor [42], the drug resistance, and overexpression of pluripotency markers of lung cancer [23]. As MSCs possess the ability to migrate to the tumor site with high selectivity following intravenous injection in experimental animals [43], further animal studies are required to evaluate the *in vivo* effect of hUC-MSCs on the potential risk of malignant transformation by the downregulation of antitumor immune and/or the direct impact of IL-6.

Several observations have indicated that allogeneic hPBMCs are required for the upregulation of IL-6 by BM-MSCs [2, 18]. In agreement with those reports, we found that IL-6 was basically low expressed by hUC-MSCs, and it increased dramatically when co-culturing with resting or activated MHC-unmatched hPBMCs. Furthermore we discovered that $CD14^+$ monocyte CM was sufficient for the induction of IL-6 production in hUC-MSCs. IL-1β promoted the secretion of IL-6 by hUC-MSC, which was mediated by c-jun- N- terminal kinase (JNK) as well as NF-κB. Finally, our results indicate that IL-6 derived from the activated hUC-MSCs is not associated with the direct immunosuppressive effect of hUC-MSCs on $CD4^+$ cells. And further studies on the role of IL-6 in the function of hUC-MSCs will be summarized in our next study.

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