**Human umbilical cord-derived mesenchymal stem cells suppress proliferation of
PHA-activated lymphocytes *in vitro* by inducing CD4⁺CD25^{high}CD45RA⁺
regulatory T cell production and modulating cytokine secretion**

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Running title: Immunomodulation of T lymphocytes by UC-MSCs

Abstract

Bone marrow-derived mesenchymal stem cells (MSCs) are promising candidate cells for therapeutic application in autoimmune diseases due to their immunomodulatory properties. Unused human umbilical cords (UC) offer an abundant and noninvasive source of MSCs without ethical issues and are emerging as a valuable alternative to bone marrow tissue for producing MSCs. We thus investigated the immunomodulation effect of umbilical cord-derived MSCs (UC-MSCs) on human peripheral blood mononuclear cells (PBMCs), T cells in particular, in a co-culture system. We found that UC-MSCs efficiently suppressed the proliferation of phytohematoagglutinin (PHA)-stimulated PBMCs ($p < 0.01$). Kinetic analysis revealed that UC-MSCs primarily inhibited the division of generation 3 (G3) and 4 (G4) of PBMCs. In addition, UC-MSCs augmented the expression of CD127⁺ and CD45RA⁺ but reduced the expression of CD25⁺ in PBMCs stimulated by PHA ($p < 0.05$). Furthermore, UC-MSCs inhibited PHA-resulted increase in the frequency of CD4⁺CD25⁺CD127^{low/-} Tregs significantly ($p < 0.01$) but augmented PHA-resulted increase in the frequency of CD4⁺CD25^{high}CD45RA⁺ Tregs to about three times in PBMCs. The levels of anti-inflammatory cytokines, PEG₂, TGF- β , and IL-10 were greatly up-regulated, accompanied by a significant down-regulation of pro-inflammatory IFN- γ in the co-culture ($p < 0.01$). Our results showed that UC-MSCs are able to suppress mitogen-induced PBMC activation and proliferation *in vitro* by altering T lymphocyte phenotypes, increasing the frequency of CD4⁺CD25^{high}CD45RA⁺ Tregs, and modulating the associated cytokine production.

Further studies are warranted to investigate the therapeutic potential of UC-MSCs in immunologically-diseased conditions.

Key Words: UC-MSCs, Tregs, immunosuppressive function, soluble cytokines

Introduction

Bone marrow-derived mesenchymal stem cells (BM-MSCs) have been extensively investigated in recent years as a promising therapeutic candidate in regenerative medicine as well as in immunological disorders and graft-versus-host-disease (GvHD) [1-3]. Human umbilical cords as an alternative for BM tissue to isolate MSCs are becoming attractive because MSCs derived from umbilical cords (UC-MSCs) are more primitive, have lower immunogenicity, and maintain a high dividing potential compared to BM-MSCs [4]. In addition, there are no ethical issues and no harm to donors in isolating MSCs from umbilical cords. It has been well accepted that UC-MSCs are bio-equivalent to BM-MSCs in their differentiation and marker expression [5]. In addition, it has been proposed that UC-MSCs are able to inhibit the mitogen-activated lymphocytic proliferation and exert immunomodulatory functions *in vitro* as BM-MSCs and MSCs isolated from other tissues by similar mechanisms, including the secretion of soluble cell factors, cell-to-cell contact, and/or the induction of regulatory T cell (Treg) generation [6-8]. *In vivo*, UC-MSCs were able to prevent the progress of severe and refractory systemic lupus erythematosus by increasing the frequency of peripheral Treg cells (Tregs) and re-establishing the balance between Th1- and Th2-related cytokines [9].

It is well known that Tregs play a crucial role in maintaining immunological self-tolerance and immune homeostasis [10, 11]. *In vitro*, Tregs are able to suppress

the proliferation of T cells triggered by specific antigens or polyclonal TCR stimulators (such as anti-CD3 mAb) in the presence of antigen-presenting cells (APCs) by a granzyme-dependent or perforin-dependent mechanism or to deliver a negative signal to responder T cells [12]. However, whether the immunosuppressive effect of UC-MSCs on the proliferation of activated T cells is due to the induced conversion of effector T cells into Tregs is controversial [6, 13]. For example, S. Selleri et al suggest that it may not be the FoxP3⁺ Tregs but the IL-10 producing Th1 cells induced by UC-MSCs that have contributed to the suppression of activated PBMCs.[13].

In this study, we co-cultured UC-MSCs with human peripheral blood mononuclear cells (PBMCs) collected from healthy donors and investigated the potential regulatory effect of UC-MSCs on the proliferation of PHA-stimulated PBMCs, alteration of lymphocytic phenotype, Treg frequency, and soluble cytokine secretion profile in the co-cultures.

Materials and Methods

Isolation and culture of UC-MSCs

Human umbilical cords (n=3) were purchased from StemExpress (Placerville, CA).

The protocol for isolation of UC-MSCs was previously described [14]. In brief, the vessels of UC were removed to retain Wharton's jelly. Wharton's jelly was cut into 1mm³ pieces, put into the bottom of the tissue culture dish (BD, Franklin Lakes, New

Jersey), and incubated at 37°C in a 5% carbon dioxide incubator without culture medium for two hours to allow tissue attachment. Following a two hour incubation, 15ml complete culture medium containing DMEM (low glucose) supplemented with 10% FBS (Fetal bovine serum), 1% L-glutamine, and 1% penicillin-streptomycin (all from Invitrogen, Grand Island, NY, USA) were added to the tissue culture dishes and incubated at 37°C in 5% carbon dioxide incubator for an additional 7 days. After 7 days, the tissue pieces were removed and the primary cells, which had migrated out from the tissues and attached to the tissue culture dishes, were passaged by 1 min treatment with 0.25% trypsin and 0.02% EDTA at 37°C. The cells were cultured in a medium of DMEM (low glucose) supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin and maintained at 37°C in 5% carbon dioxide incubator. The medium was changed every 3 days. UC-MSCs were further passaged when they reached 90% confluency. All UC-MSCs used in experiments were controlled within passages 3-5.

Co-culture of UC-MSCs and PBMCs

PBMCs of healthy donors (n=10) were purchased from AllCells Inc (Emeryville, CA, USA) and StemExpress (Placerville, CA, USA). Co-culture experiments were performed in a 12-well plate (BD, Franklin Lakes, New Jersey, USA). 1×10^5 UC-MSCs were plated into each well, cultured in 1ml of advanced RPMI 1640 culture medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% FBS, two mM glutamine, 100U/ml penicillin and streptomycin, and allowed to adhere for 2

h at 37°C in 5% carbon dioxide incubator. 5×10^5 PBMCs were then seeded onto the cultured UC-MSCs at a ratio of 1:5 for UC-MSCs:PBMCs in the presence of 10 µg/ml phytohaemagglutinin (PHA, Sigma, St Louis, MO, USA). PBMCs cultured alone (without UC-MSCs) with or without PHA served as controls. After 3 days, the suspended PBMCs were collected for cell count, cell proliferation assay, and flow cytometry analysis. The supernatant of cell cultures was collected and kept at -80°C for later examination of ELISA.

Cell proliferation

The proliferation of PBMCs was assessed by Carboxy Fluorescein Diacetate Succinimidyl Ester (CFSE, Invitrogen, Grand Island, NY, USA) assay. The protocol for cell labeling was conducted by an established protocol in our group [14]. Briefly, PBMCs were labeled with 10 µM CFSE in pre-warmed phosphate-buffered saline (PBS) containing 2% FBS at a final concentration of 10^6 cells/ml. After incubation at 37 °C for 15 min in the dark, cells were washed twice with fresh co-culture medium. CFSE-labeled PBMCs were stimulated by PHA in the presence or absence of UC-MSCs or cultured alone without PHA stimulation. Three days later, PBMCs were suspended, washed by cold PBS, and collected for cell count and analysis of flow cytometry.

Flow cytometry

Flow cytometry was performed according to our previous protocol [14]. In brief,

PBMCs were washed twice with washing buffer (PBS containing 2% FBS), centrifuged, resuspended in 100 μ l washing buffer, and incubated with various fluorescent dye-conjugated specific antibodies (APC-conjugated mouse anti-human CD4, PE-conjugated mouse anti-human CD25, PE-Cy7-conjugated mouse anti-human CD127, and eFluor®450-conjugated mouse anti-human CD45RA, all from eBioscience, San Diego, CA, USA) or appropriate isotypic controls on ice in the dark. After incubation for 30 min, cells were washed with washing buffer and resuspended in 200 μ l washing buffer for analysis of flow cytometry with the LSRFortessa™ cell analyzer (BD, Franklin Lakes, New Jersey, USA).

Cytokine examination

ELISA kits for assessing TGF- β 1, IL-10, and IFN- γ were purchased from eBioscience (San Diego, CA, USA). The ELISA kit for assessing PGE2 was purchased from R&D Systems (Minneapolis, CA, USA). ELISA was performed according to the manufacturer recommended protocols. In brief, the collected cell-free supernatant was put into 96-well specific antibody-coated plate. Absorbance was recorded at 450 nm using a microplate reader (Bio-Rad, Philadelphia, PA, USA). Each cytokine standard and sample was run in duplicate.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 5 (GraphPad). Data were expressed as the means $\pm$ SEM. Two-sample *t* tests were used to analyze cell kinetics.

One-way ANOVAs were used to analyze other data, including cell counting, the proliferation index of lymphocytes, and the frequency of Tregs in CD4⁺ cells.

Significance was set at $p < 0.05$.

Results

UC-MSCs significantly inhibited the proliferation of PHA-activated PBMCs in co-culture.

The suppressive effect of UC-MSCs on the proliferation of PHA-activated PBMCs was assessed by cell counting and flow cytometry. Our data showed that UC-MSCs significantly decreased the proliferative number of PBMCs stimulated by PHA with cell counting (Fig. 1A, $p < 0.01$). Flow cytometry was used to further confirm the effect of UC-MSCs on the proliferation of PHA-activated PBMCs, which were analyzed by using the proliferation index of CFSE-labeled PBMCs by means of the software ModFit. Our data revealed that UC-MSCs significantly reduced the proliferation index of PHA-activated PBMCs (Fig. 1B, $p < 0.01$). However, UC-MSCs did not affect the proliferation of PBMCs in co-cultures without PHA stimulation (data not shown). Further analysis of the kinetics of PHA-activated PBMCs revealed that UC-MSCs primarily suppressed the cell division of generation 3 (G3) and G4 of PBMCs (Fig. 1C, $p < 0.05$).

UC-MSCs induced the change of lymphocyte marker expression and increased the frequency of CD4⁺CD25^{high}CD45RA⁺ Tregs in PHA-activated PBMCs.

To assess the immunoregulatory effect of UC-MSCs on lymphocyte marker expression in PHA-activated PBMCs in co-culture, we separately analyzed the percentage of CD25⁺, CD127⁺, and CD45RA⁺ cells in PBMCs from different groups using flow cytometry. Compared to resting PBMCs (without PHA stimulation), PHA greatly increased the expression of CD25 but decreased the expression of CD127 and CD45RA in PBMCs (Fig. 2A-C). UC-MSCs augmented the expression of CD127 and CD45RA but reduced the expression of CD25 in PHA-activated PBMCs (Fig. 2A-C, $p<0.05$). To investigate the effect of UC-MSCs on the frequency of Tregs in PHA-activated PBMCs, multicolor flow cytometry was performed to measure the frequency of Tregs in different groups. Our data showed that PHA stimulation increased the frequency of CD4⁺CD25⁺CD127^{low/-} cells in CD4⁺ T cells while UC-MSCs significantly reduced this increased frequency of CD4⁺CD25⁺CD127^{low/-} cells induced by PHA in PBMCs (Fig. 2D, $p<0.01$). In contrast, PHA increased the frequency of CD4⁺CD25^{high}CD45RA⁺ cells in CD4⁺ T cells compared to resting PBMCs (Fig. 2E). Interestingly, UC-MSCs further augmented this increased frequency of CD4⁺CD25^{high}CD45RA⁺ cells induced by PHA in PBMCs (Fig. 2E, $p<0.01$). These data suggest that UC-MSCs are able to differentially modulate phenotypes of T cells and Tregs in PHA-activated PBMCs.

UC-MSCs modulated cytokine secretion profiles in co-culture with PHA-

activated PBMCs.

To investigate the effect of UC-MSCs on the cytokine production of PHA-activated PBMCs, levels of various cytokines in the supernatant of different groups were examined by ELISA. The levels of PGE2 and TGF- β 1 were increased while the levels of IFN- γ were decreased in the supernatant of the co-culture of PHA-activated PBMCs and UC-MSCs compared to those in the culture of resting PBMCs alone and to those in the culture of PHA-activated PBMCs without UC-MSCs (Fig. 3A, B & C, $p < 0.01$). No significant difference was detected in the production levels of PGE2, IFN- γ , and TGF- β 1 between the cultures of resting PBMCs and PHA-activated PBMCs (Fig. 3A, B & C). There was an increased production of IL-10 in culture of PHA-activated PBMCs compared to that in the culture of resting PBMCs (Fig. 3D, $p < 0.01$). UC-MSCs further augmented the production of IL-10 in co-culture with PHA-activated PBMCs (Fig. 3D, $p < 0.05$). However, the functional significance of these differences in cytokine profiles remains to be elucidated.

Discussion

In the past few years, UC-MSCs attracted significant interest because of two distinctly different major biological properties, including the ability to repopulate the damaged host tissue for repair and regeneration as well as to exert immunomodulation and immunosuppression both *in vitro* and *in vivo* [15]. In the current study, we successfully isolated and expanded MSCs from human umbilical cords. In a co-culture system, we demonstrated that UC-MSCs significantly inhibited the

proliferation of PHA-stimulated PBMCs, modulated the phenotype of PBMCs, and changed the cytokine secretion profile. Consistent with previous studies [7, 8], the cell numbers and proliferation index of PHA-stimulated PBMCs were significantly decreased by co-cultured UC-MSCs (Fig. 1A & B, $p < 0.01$). In addition, we found that most PHA-stimulated PBMCs in the presence of UC-MSCs were in phases G1 and G2 while most PHA-stimulated PBMCs in the absence of UC-MSCs were in phases G3 and G4 (Fig. 1C). These data clarified that the decreased cell numbers and proliferation index of PHA-stimulated PBMCs was due to the inhibitory properties of UC-MSCs on the division of PBMCs.

There is increasing evidence showing that MSCs may alter lymphocyte markers or subtypes of T cells in PHA-stimulated PMBCs [16, 17]; for example, the expression of CD25 as an activation marker of T cells is significantly augmented due to the stimulation of PHA [16]. Consistent with published studies by others, our results demonstrated that the percentage of CD25⁺ cells was significantly increased in those PHA-stimulated PBMCs (Fig. 2A, $p < 0.01$). However, UC-MSCs were able to decrease the increased percentage of CD25⁺ cells in PBMCs stimulated by PHA (Fig. 2A, $p < 0.05$). CD45RA is considered to be a marker of naïve T cells [18]. CD127 as IL-7 receptor are able to become rapidly down-regulated in the presence of anti-CD3 stimulation [19]. We found that the percentages of CD127⁺ and CD45RA T⁺ cells were significantly decreased in PHA-stimulated PBMCs compared to resting PBMCs (Fig. 2B & C, $p < 0.01$), while UC-MSCs increased the expression of CD127 and

CD45RA in co-cultures with PHA-stimulated PBMCs (Fig. 2B & C, $p<0.05$). Thus, UC-MSCs were able to change the marker expression of PHA-stimulated PBMCs.

Tregs play an important role in maintaining immune homeostasis *in vivo* as well as suppressing the proliferation of activated T cells *in vitro* [11, 12, 20]. It is well known that cells with the $CD4^+CD25^+FoxP3^+$ phenotype have suppressive properties [21]. Many investigators suggested that $CD4^+CD25^+CD127^{low/-}$ could be an alternative for the classic biomarker of $CD4^+CD25^+FoxP3^+$ on Tregs, although CD127 expression inversely correlated with FoxP3 [19]. It is controversial whether $CD4^+CD25^+CD127^{low/-}$ cells represent the same population of $CD4^+CD25^+FoxP3^+$ Tregs since a high percentage ($34.0\pm15.1\%$) of $CD127^{low/-}$ cells did not express FoxP3 [22]. It has been reported that only $CD4^+CD25^{high}CD45RA^+$ T cells are able to produce homogeneous Treg lines on *in vitro* expansion and are able to suppress the proliferation of activated T cells [18]. Our results showed that there was an increased expansion of $CD4^+CD25^+CD127^{low/-}$ Tregs in PHA-stimulated PBMCs compared to naïve PBMCs (Fig. 2D, $p<0.01$). However, UC-MSCs significantly inhibited this expansion of $CD4^+CD25^+CD127^{low/-}$ Tregs in co-culture with PHA-stimulated PBMCs. In addition, there was an increased expansion of $CD4^+CD25^{high}CD45RA^+$ Tregs (Fig. 2E, $p<0.01$) in PHA-stimulated PBMCs. Interestingly, UC-MSCs further increased the frequency of $CD4^+CD25^{high}CD45RA^+$ Tregs in PHA-stimulated PBMCs (Fig. 2E, $p<0.01$). Thus, the inhibitory ability of UC-MSCs on T cell proliferation in PHA-stimulated PBMCs might be attributed, at least in part, to the induction and

augmented generation of $CD4^+CD25^{high}CD45RA^+$ Tregs in co-cultures.

Growing evidence showed that UC-MSCs secreted a number of soluble suppressive cytokines (such as PGE2, TGF- β 1, and IL-10) to produce immunoregulative functions [23]. MSC-mediated immunoregulation is the result of cumulative actions of several molecules because none of these molecules alone lead to a complete abrogation of T-cell proliferation [23]. Our results have shown that UC-MSCs increased the concentrations of PGE2, TGF- β 1, and IL-10 but decreased the concentration of IFN- γ in co-culture with PHA-stimulated PBMCs (Fig. 3A, B, & D, $p < 0.05$). However, no significant difference in the concentrations of PGE2, TGF- β 1, and IFN- γ was detected between naïve PBMCs and PHA-stimulated PBMCs without UC-MSCs (Fig. 3A, B, & C), suggesting that UC-MSCs contributed to these changes in cytokine production (PGE2, TGF- β 1 and IFN- γ). Our results showed that the levels of IL-10 in the supernatant of PHA-stimulated PBMC cultures and co-cultures with UC-MSCs and PHA-stimulated PBMCs were significantly increased (Fig. 3D, $p < 0.01$), accompanied by an increased frequency of $CD4^+CD25^{high}CD45RA^+$ Tregs, when compared to resting PBMCs without PHA stimulation. There are two major populations of Tregs, which include naturally occurring Tregs and IL-10 secreting Tregs (known as induced Tregs) [20]. The induced $CD4^+CD25^{high}CD45RA^+$ Tregs are able to secrete a certain level of IL-10 [18]. Thus, the increased IL-10 levels in the co-culture of UC-MSCs and PHA-stimulated PBMCs should be produced by UC-MSCs and the induced $CD4^+CD25^{high}CD45RA^+$ Tregs, which may have played an important part in the

inhibition of T cell proliferation in in our study.

In this study, we reported that UC-MSCs can produce potent immunosuppressive function on the proliferation of PHA-stimulated PBMCs by increasing the frequency of $CD4^+CD25^{high}CD45RA^+$ Tregs and the production of associated suppressive cytokines in co-culture.

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Legends:

Fig. 1. UC-MSCs significantly inhibited the proliferation of PHA-stimulated PBMCs in co-culture

1×10^5 UC-MSCs (n=3) were co-cultured with CFSE-labeled PBMCs of healthy adult donors (n=10) at a ratio of 1:5 in the presence of PHA (10 μ g/ml) for 3 days. After 3 day co-culture, the number of cells was quantified by cell counting and flow cytometry respectively. The proliferation index and suppression kinetics of PBMCs were analyzed by the software ModFit. Data were presented as the means $\pm$ SEM of three independent experiments.

A & B. Bar graphs showing that the cell numbers (A) and the proliferation index (B) of PBMCs were significantly increased in PHA-stimulated PBMCs while UC-MSCs significantly reduced the increases in cell numbers and the proliferation index in PHA-stimulated PBMCs. **p<0.01, ANOVA.

C. Bar graphs showing that UC-MSCs increased the ratios of G1 (generation 1, parent cells) and G2 but decreased the ratios of G3 and G4 in PHA-stimulated PBMCs to nearly undetectable levels, suggesting that UC-MSCs primarily inhibited the division of G3 and G4 of PBMCs. **p<0.01, Two-sample t test.

D, E & F. Representative results showing the kinetics of PBMC proliferation without

PHA (D), with PHA (10µg/ml) (E), and with PHA (10µg/ml) and UC-MSCs (F).

Fig. 2. UC-MSCs induced the changes of lymphocyte marker expression and increased the frequency of CD4⁺CD25^{high}CD45RA⁺ Treg cells in PHA-activated PBMCs

After 3-day culturing, naïve PBMCs (without PHA), PHA-**activated** PBMCs, and PHA-activated PBMCs collected from co-cultures with UC-MSCs were used to perform flow cytometry analyses. PBMCs were labeled by specific antibodies for CD4, CD25, CD127, and CD45RA conjugated with APC, PE, PE-Cy7, and Pacific Blue. Data were presented as the means ± SEM of three independent experiments.

A, B, & C. Bar graphs showing that PHA stimulation significantly increased the percentage of CD25 (A) but decreased the percentages of CD127⁺ (B) and CD45RA (C) in PBMCs compared to those in naïve PBMCs. In contrast, UC-MSCs significantly decreased the percentage of CD25 but increased the percentages of CD127 (B) and CD45RA (C) in PHA-stimulated PBMCs, suggesting that UC-MSCs tend to reverse the alteration of lymphocyte marker expressions induced by PHA *p<0.05, **p<0.01.

D, & E. Bar graphs showing that the frequencies of CD4⁺CD25⁺CD127^{low/-} (D) and CD4⁺CD25^{high}CD45RA⁺ Tregs (E) were significantly increased in PHA-activated

PBMCs compared to those in naïve PBMCs ($p < 0.01$). However, the frequency of $CD4^+CD25^+CD127^{low/-}$ Tregs was greatly decreased (D) while $CD4^+CD25^{high}CD45RA^+$ Treg frequency was increased up to about 3-fold (E) in co-cultures of UC-MSCs and PHA-activated PBMCs. $**p < 0.01$.

F. Representative dot plot of homogeneity of the purified $CD4^+$ T cells in PBMCs.

G, H & I. Representative dot plots of $CD25^+CD127^{low/-}$ Tregs in the $CD4^+$ T cells without PHA (G), with PHA (10ug/ml), and (H) with PHA (10ug/ml) and UC-MSCs (I). M, N & K. Representative dot plots of $CD25^{high}CD45RA^+$ Tregs in the $CD4^+$ T cells without PHA (M), with PHA (10ug/ml) (N), and with PHA (10ug/ml) and UC-MSCs (K).

Fig. 3. UC-MSCs improved the cytokine secretion profile in co-cultures with PHA-activated PBMCs.

The levels of soluble cytokines in the supernatant of PBMC cultures without PHA , with PHA (10ug/ml), and with PHA (10ug/ml) and UC-MSCs were assessed by ELISA after culturing for 3 days. Data were presented as the means $\pm$ SEM of three experiments.

A, B, C, & D. Bar graphs showing that UC-MSCs significantly increased the levels of PGE2 (A, $p < 0.01$), TGF- β 1 (B, $p < 0.05$), and IL-10 (D, $p < 0.05$) but decreased the

levels of IFN- γ (C, $p < 0.01$) in co-cultures with PHA-activated PBMCs ($p < 0.01$). The levels of IL-10 production were significantly increased in culture of PHA-activated PBMC compared to that of naïve PBMCs ($p < 0.01$). However, no significant differences were detected in the production of PGE2, TGF- β 1, and IFN- γ between PHA-activated PBMCs and naïve PBMCs in culture. * $p < 0.05$, ** $p < 0.01$, ANOVA.