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ORIGINAL ARTICLE

Immunomodulatory effects of umbilical cord-derived mesenchymal stem cells

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ABSTRACT

Umbilical cord blood (UCB) is of great interest as a source of stem cells for use in cellular therapies. The immunomodulatory effect of mesenchymal stem cells (MSCs) originating from bone marrow, adipose tissue and amniotic membrane has previously been reported. In this study, MSCs were isolated from UCB with the aim of evaluating their immunomodulatory effects on proliferation of PB lymphocytes by two different techniques; namely, 5-bromo-2-deoxyuridine ELISA and a carboxy fluorescein diacetate succinimidyl ester flow cytometric technique. MSCs were isolated from UCB, propagated until Passage four, and then characterized for cell surface markers by flow cytometry and ability to differentiate towards osteocytes and adipocytes. Immunosuppressive effects on PB lymphocytes were examined by co-culturing mitomycin C-treated UCB MSCs with mitogen-stimulated lymphocytes for 72 hr. Thereafter, proliferation of lymphocytes was detected by CFSE flow cytometry and colorimetric ELISA. The titers of cytokines in cell culture supernatant were also assayed to clarify possible mechanisms of immunomodulation. UCB MSCs suppressed mitogen-stimulated lymphocyte proliferation, which occurs via both cell-cell contact and cytokine secretion. Titers of transforming growth factor beta and IL 10 increased, whereas that of IFN-γ decreased in the supernatants of co-cultures. Thus, UCB MSCs suppress the proliferation of mitogen-stimulated lymphocytes. However further *in vivo* studies are required to fully evaluate the immunomodulatory effects of UCB MSCs.

Key words cord blood, immunomodulation, mesenchymal stem cells, proliferation.

Mesenchymal stem cells are clonogenic cells with self-renewal and multilineage potential that can differentiate into cells of both mesodermal and non-mesodermal origin (1, 2). Additionally, MSCs can migrate to sites of inflammation and exert potent immunosuppressive and anti-inflammatory effects through interactions between lymphocytes associated with both the innate and adaptive immune systems. Along with their stemness and immune-suppressive properties, their easy accessibility and ability to proliferate suggest that MSCs may be useful therapeutically for various disorders (3).

Mesenchymal stem cells are of great interest because of their immune-modulatory characteristics; they have been shown to inhibit T-cell proliferation both *in vitro* and *in vivo*, confirming they have immune-suppressive effects (4). Thus they have potential roles in the prevention and treatment of GVHD after adult stem

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List of Abbreviations: 7 AAD, 7-aminoactinomycin D; BM, bone marrow; Brdu, 5-bromo-2-deoxyuridine; CFSE, carboxy fluorescein diacetate succinimidyl ester; DMEM, Dulbecco's modified Eagles medium; GVHD, graft-versus-host disease; IDO, indoleamine 2, 3-dioxygenase; LIF, lymphocyte inhibitory factor; MSC, mesenchymal stem cell; MNC, mononuclear cell; PB, peripheral blood; PE, phycoerythrin; PHA, phytohemagglutinin; SI, stimulation index value; TGF-β, transforming growth factor beta; UCB, umbilical cord blood.

cells transplantation, prevention of rejection after organ transplantation and treatment of autoimmune disorders (5).

The differentiation properties of MSCs seem to depend on micro-environmental factors *in vivo*, whereas the immunomodulatory effects appear to be intrinsic, thus providing an attractive basis for the therapy of autoimmune and inflammatory diseases by systemic infusion. Moreover, these intrinsic properties of MSCs include secretion of various factors, modulation of the local environment and activation of endogenous progenitor cells. Hence, MSC therapy offers therapeutic promise for management of GVHD, systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, diabetes, myocardial infarction, thyroiditis, various neurological disorders and other disorders (6).

Although MSCs have mainly been isolated from BM, similar populations have been isolated from other tissues, including adipose tissue, placenta, amniotic fluid and UCB (7).UCB-derived MSCs have been evaluated for use in cellular therapies because they are abundantly available and can be obtained without harming the donor (both mother and infant). Moreover, they are ontogenically primitive, relatively invulnerable to immunologic challenges, have a low risk of viral contamination and there are no ethical barriers to their use (8). Additionally, UCB MSCs can be cryopreserved for future use when needed as (off the shelf) therapy (9).

In this study, we aimed to isolate of MSCs from UCB and evaluate their immunomodulatory effects on proliferation of PB lymphocytes by using two different techniques; namely, Brdu ELISA and a CFSE flow cytometric technique.

MATERIALS AND METHODS

Collection of UCB

Six UCB samples were collected in heparinized syringes from normal full-term vaginal deliveries at Kasr AL Ainy hospital with the volunteers' informed consent. All samples were processed within 12 hr of their collection. This study was approved by the Ethics Committee of the Faculty of Medicine, Cairo University.

Isolation of MSCs from UCB

Umbilical cord blood was diluted 1:1 with PBS (Lonza, Verviers, Belgium), the diluted blood layered on Bicoll separating solution 1.077 g/mL (Biochrome, Berlin, Germany) and centrifuged at 400 *g* for 30 minutes. The mononuclear cell layer was then collected and washed twice with PBS. The cell pellets were resuspended in low glucose DMEM (Hyclone, Thermoscientific, Rockford, IL, USA) supplemented with 10% FCS (Euroclone, Pero, Italy), 1% penicillin/streptomycin (Lonza) and 1% L glutamine (Euroclone).

Mononuclear cells were cultured in 25 cm^2 tissue culture flasks at a density of 1×10^6 cells/cm^2. Cells were allowed to adhere over night, after which non-adherent cells were washed and fresh media added. Cells were kept in culture until they reached 90% confluence, then trypsinized with trypsin EDTA0.25% (Hyclone) to the next passage.

Immunophenotyping of cells using flow cytometry:

Passage four cells ($n = 4$) were trypsinized, washed and resuspended in PBS at a concentration of 1×10^6 cells/mL. They were then stained for 20 minutes in the dark with monoclonal antibodies conjugated with FITC to CD13, CD29, CD90, HLA ABC, CD73, CD34, HLA DR and CD45 and to PE CD105,CD166, CD146, CD44, CD133, CD14, CD144 and CD31. Monoclonal antibodies were purchased from Beckman Coulter (Marseille, France) except CD73, CD144 and CD31, which were obtained from BD Pharmingen (San Diego, CA, USA). Mouse isotypic antibodies served as controls. Viability stain 7 AAD (Sigma, St Louis, MO, USA) was added for 20 minutes to all tubes to ensure gating on viable cells. Cells were analyzed with a CYTOMICS FC 500 Flow Cytometer (Beckman Coulter, Miami, FL, USA) using CXP Software version 2.2.

Multi-lineage differentiation assays

A mesenchymal stem cells functional identification kit (#SC006, R&D Systems, Minneapolis, MN, USA) was used to evaluate the differentiation potential of UCB MSCs. Differentiation experiments were all repeated four times.

For osteogenic differentiation, Passage four MSCs were cultured at a density of 4.2×10^3 cells/cm^2 in 24-well tissue culture plates (Corning, Cambridge, MA, USA) precoated with fibronectin (Sigma) at a concentration of 1 $\mu g/cm^2$until they reached 70% confluence. Osteogenic differentiation media was added for 3 weeks, the media being changed twice per week. Osteocytes were fixed and stained with Alizarin red stain (Sigma).

For adipogenic differentiation, Passage four MSCs were cultured at a density of 2.19×10^4 cells/cm^2 in 24-well plates in alpha Minimum Essential Medium until they reached confluence. Adipogenic differentiation media was added for 3 weeks, fresh media being added twice per week. Adipocytes were fixed and stained with Oil red stain (Sigma).

Lymphocyte proliferation assay

Human PB lymphocytes were isolated from healthy donors by Bicoll density separation and the cell concentration adjusted to 1×10^6/mL in RPMI-1640 medium (Lonza) supplemented with 10% FCS.

Umbilical cord blood MSCs at Passage four were trypsinized, the concentration adjusted to 5×10^4/mL, and then plated in 96-well plates. The plates were incubated at 37°C with 5% CO_2 for 72 hr. Upon reaching 70% confluence, the medium was removed and 5 μL of mitomycin C (Serva Electrophoresis GmbH, Heidelberg, Germany) (1 μg/μL) added in 100 μL DMEM for 30 minutes at 37°C to stop the mitotic activity of the MSCs. Inactivated MSCs were then washed twice with PBS, then co-cultured for 72 hr with 1×10^5 human lymphocytes either unstimulated or stimulated with 5 μL PHA (Biochrome).

There were then three test groups: lymphocytes + PHA (positive control), unstimulated lymphocytes (negative control) and MSCs + stimulated lymphocytes. Proliferation was evaluated by using a Brd U cell proliferation kit (Roche, Mannheim, Germany) ELISA according to the manufacturer's instructions. The SI, defined as the mean optical density values of mitogen-stimulated culture/mean optical density value of unstimulated lymphocytes, was calculated for each treatment.

Lymphocyte proliferation was also assessed by using a Cell Trace CFSE cell proliferation Kit (Invitrogen, Carlsbad, CA, USA); 50 μL of CFSE (10 Mm) was added to cells adjusted to 2×10^6/50 μL with RPMI 1640, then incubated for 7 mins in a CO_2 incubator at 37°C. Thereafter 1 mL RPMI supplemented with 10% FCS was added. The cells were washed twice and suspended at a concentration of 1×10^6/mL, after which 100 μL of CFSE-labeled cell suspension was incubated either alone or with 10 μg PHA in 96-well plates for 72 hr.

Thereafter, the cells were subjected to flow cytometric analysis. SI was calculated as the ratio of total proliferated cells (after subtracting proliferation of unstimulated cells) to unproliferated cells.

Assessment of proliferation by both techniques (Brdu and CFSE) was repeated four times ($n = 4$).

Cytokines ELISA assay

Passage four MSCs were adjusted to 5×10^3/mL and plated in 24-well plates. On reaching 70% confluence, the cells were mitotically inactivated with mitomycin C and then washed twice with PBS.

Three groups were established as follows; MSCs alone, MSCs co cultured with 2×10^5 PB lymphocytes and stimulated with 10 μL PHA and finally 2×10^5 lymphocytes stimulated with 10 μL PHA. The three groups were incubated for 72 hr in DMEM with 10% FCS. The supernatants of each group were collected and evaluated with an ELISA kit for human TGF-β1 (DRG Instruments GmbH, Marburg, Germany) ($n = 9$), human IFN-γ (Assay Pro, Cambridge, UK) ($n = 9$) and human IL 10 (AviBion, Helsinki, Finland) ($n = 7$) according to the manufacturers' instructions.

Statistical analysis

Data are presented as means ± SD. The results were analyzed by SPSS 13.0 (Chicago, IL, USA). Numerical variables were compared between the study groups using Student's *t*-test for independent samples when comparing two groups and ANOVA with *post hoc* multiple two-group comparisons when comparing more than two groups. P values <0.05 were considered statistically significant.

RESULTS

Isolation of UCB MSCs

Umbilical cord blood MNCs isolated and cultured at a density of 1×10^6/cm^{2}adhered to tissue culture flasks. A few colonies of elongated fibroblast-like cells appeared after 1 week. The cells gradually reached confluence with a whirlpool-like array at about 28 days of culture (Fig. 1a).

Immunophenotyping of cells by flow cytometry

Cell surface markers of the Passage four UCB MSCs were examined by flow cytometry. As shown in Figure 2, these cells showed a typical MSCs pattern; being negative to CD34, CD45 (hematopoietic markers), CD14 (monocytic marker), CD 144 and CD 31 (endothelial marker) and HLA DR, whereas they were positive for CD13, CD29, CD 90, HLA ABC, CD73(SH3), CD105 (SH2), CD166 and CD44.

Multi-lineage differentiation assay

The differentiation potential of UCB MSCs was examined. When the cells were cultured under conditions favorable for osteogenic differentiation, they began to flatten and form mineralized matrix as evidenced by Alizarin red stain thus confirming the osteogenic potential of the cells (Fig. 1b).

Under adipogenic inductive media, lipid vacuoles were seen by inverted microscopy and confirmed by red staining of the vacuoles with Oil Red O stain, confirming the adipogenic potential of the cells (Fig. 1c,d).

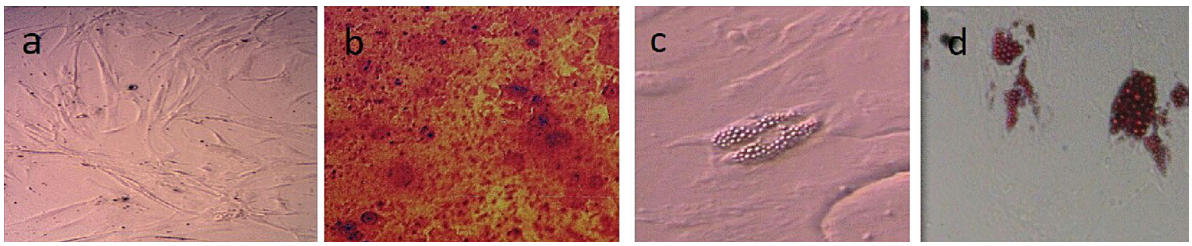

Fig. 1. Characterization of UCB MSCs by morphology and differentiation potential. (a) Morphology of UCB MSCs by phase contrast microscopy: the cells are fibroblast-like, elongated and spindle shaped, reaching about 70% confluence (magnification 10×). (b) Osteogenic differentiation of UCB MSCs as shown by calcium deposition in the extracellular matrix after staining with Alizarin red stain (magnification 10×). (c) Adipogenic differentiation of UCB MSCs as shown by appearance of lipid vacuoles that are visible by inverted microscopy (magnification 40×). (d) Oil Red O staining of MSCs induced by adipogenic supplement; cells show large cytoplasmic, red, lipid-rich vacuoles (magnification 40×).

UCB MSCs suppress mitogen-induced proliferation of PB MNCs:

In order to determine the ability of UCB MSCs to suppress mitogen-induced proliferation of allogenic lymphocytes; both non-stimulated and PHA-stimulated MNCs were cultured for 72 hr in the presence or absence of UCB MSCs.

ELISA quantitated the amount of thymidine analog BrdU incorporated into the newly synthesized DNA of replicating cells. The proliferation of stimulated human PB lymphocytes was reduced to 56% of the control ($n = 4$); the mean SI of the PHA-stimulated lymphocytes was 3.7 ± 1.6 and the mean SI of MSCs co-cultured with PHA-stimulated lymphocytes was 2.16 ± 1.1 (Fig. 3a).

In addition, proliferation of PHA-stimulated lymphocytes was assessed by flow cytometric CFSE assay, which allowed the percentage of proliferated precursors distributed across two peaks to be calculated (mean $82.3\% \pm 5$ SD) and compared with the percentage of proliferating precursors in co-culture with UCB MSCs (mean $67.3\% \pm 5$ SD) (Fig. 4). The mean SI of PHA-stimulated lymphocytes was 5.025 ± 3 and of MSCs co-cultured PHA-stimulated lymphocytes 1.9 ± 0.49 (lymphocyte proliferation was reduced to 37% in the co-culture, $n = 4$) (Fig. 3b).

Cytokine ELISA assay

To examine the effect of UCB MSCs on the activity of the lymphocytes, production of IFN-γ, TGF-β1 and IL-10

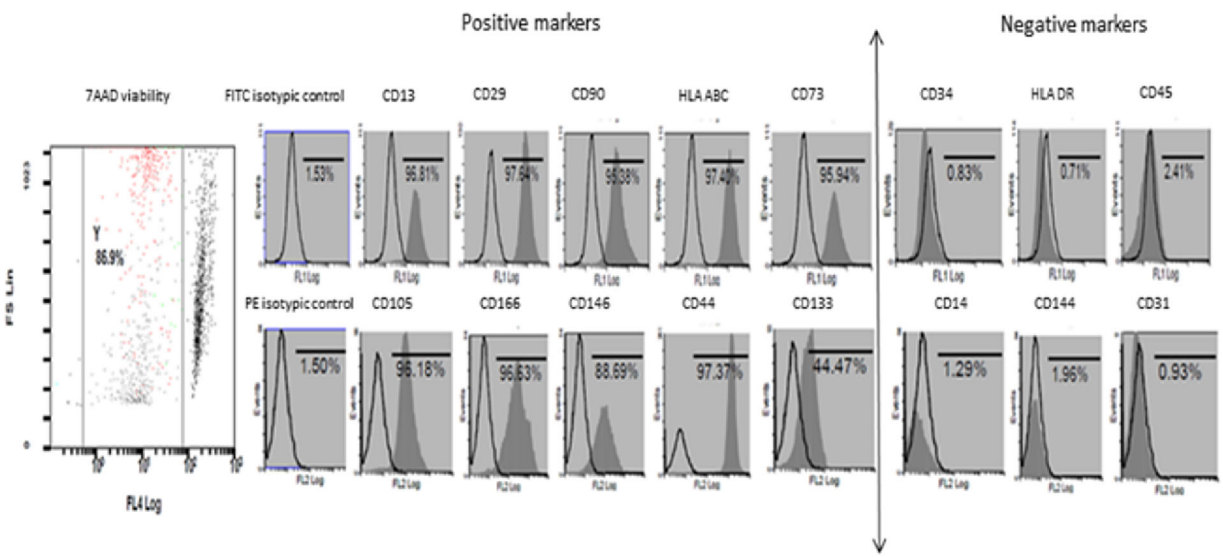

Fig. 2. Flow cytometric study of Passage four UCB MSCs surface markers. Y is the gated viable cells from which the viability dye 7 AAD was excluded, cells showed positive surface expression of CD13, CD29, CD90, HLA ABC, CD73, CD105, CD166, CD146, CD44 and CD133 and negative expression for CD34, HLADR, CD45, CD14, CD144 and CD31. The unshaded peaks represent the isotype-matched antibody controls.

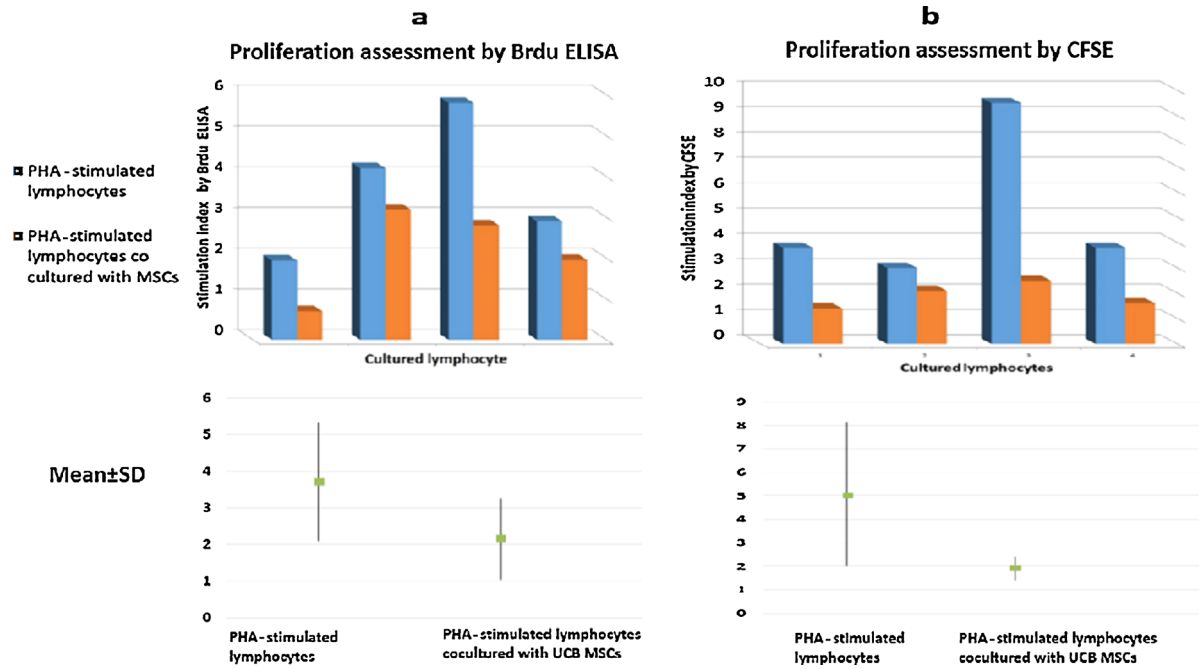

Fig. 3. Immunomodulatory properties of UCB MSCs on lymphocyte proliferation. Stimulation indexes of PHA-stimulated lymphocytes are shown as blue bars and of PHA-stimulated lymphocytes co-cultured with UCB MSCs as red bars ($n = 4$). (a) Brdu proliferative assay: the mean SI of PHA-stimulated lymphocytes is 3.7 ± 1.6 and of MSCs co-cultured PHA-stimulated lymphocytes 2.16 ± 1.1. (b) Flow cytometric CFSE assay: the mean SI of PHA-stimulated lymphocytes is 5.025 ± 3 and of MSCs co-cultured PHA-stimulated lymphocytes 1.9 ± 0.49.

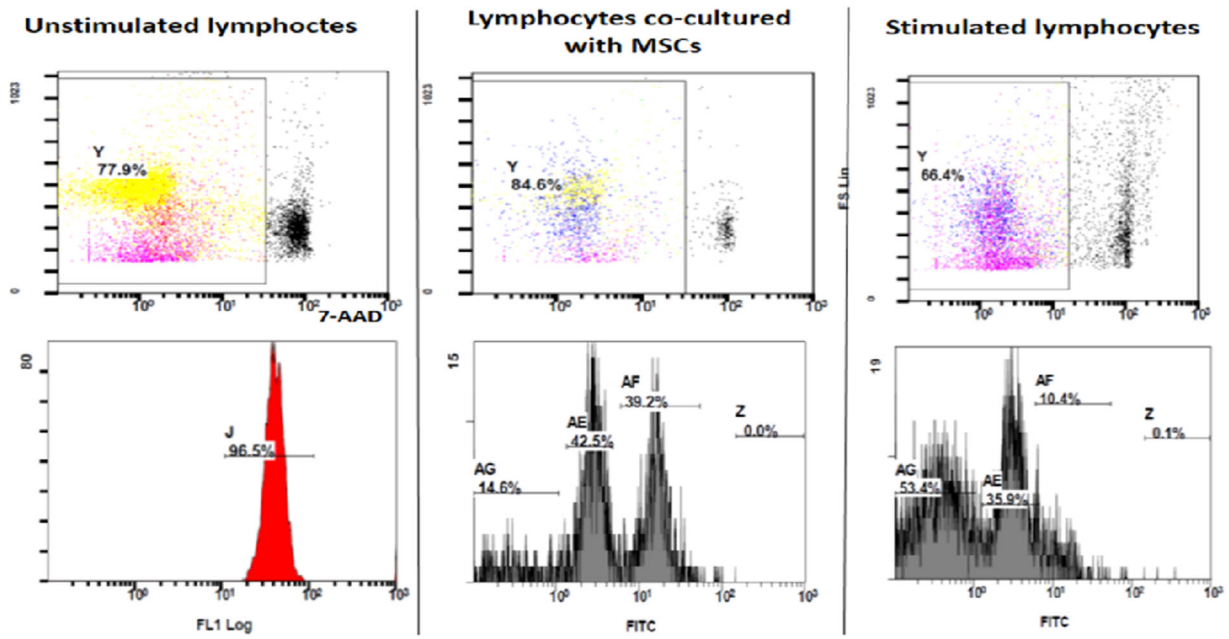

Fig. 4. Flow cytometric CFSE assay of lymphocyte proliferation. Result of one representative experiment; Y is the gated viable cells from which the viability dye 7 AAD was excluded (upper row). The lower row presents single parameter histograms for CFSE dilution; the one on the right shows the proliferation of PHA-stimulated lymphocytes (positive control) after 72 hr culture; it shows 10.4% of cells with no proliferation, 35.9% of cells with one division peak and 53.4% of cells with two division peaks. The left histogram presents the proliferation of unstimulated lymphocytes after 72 hr culture; it shows 96.5% of cells with no proliferation. The middle histogram presents the stimulated lymphocytes proliferation in co-culture with UCB MSCs; it shows 39.2% of cells with no divisions, 42.5% with one division and 14.6% with two divisions.

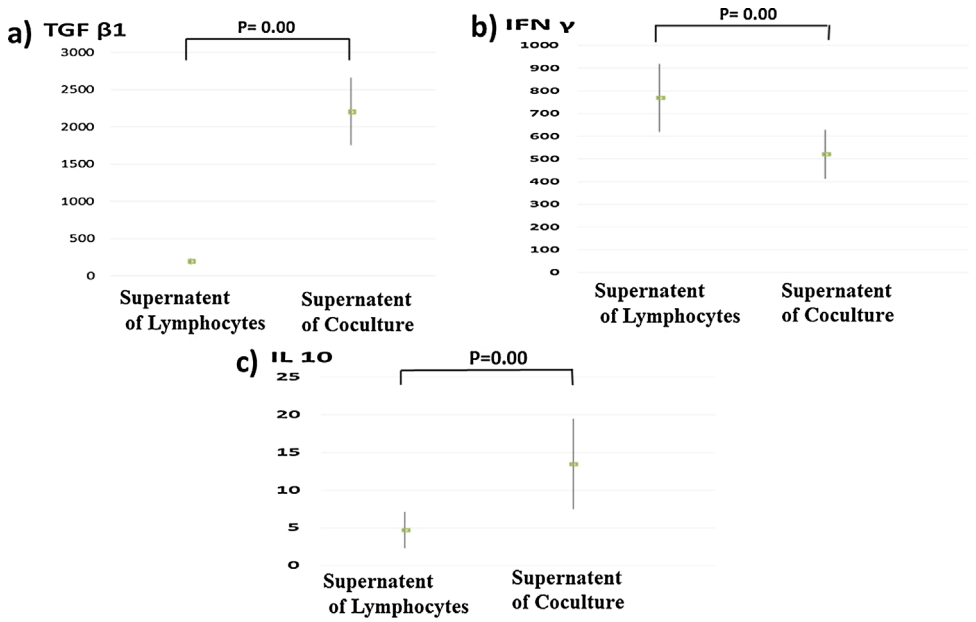

Fig. 5. Cytokine production in the supernatants of PHA-stimulated lymphocytes and in the co-cultures. (a) In stimulated lymphocyte wells, TGF-β1 was $195 \pm 35/10^5$cells versus $2210 \pm 450/10^5$ cells in the co-culture wells; this difference is highly significant ($P = 0.00$). (b) In stimulated lymphocyte wells, IFN-γ was $768 \pm 104/10^5$ cells versus $520 \pm 66/10^5$ cells in the co-culture wells; this difference is highly significant ($P = 0.00$). (c) In stimulated lymphocyte wells, IL-10 was $4.6 \pm 1.5/10^5$ cells versus $13.5 \pm 4.5/10^5$ cells in the co-culture wells; this difference is highly significant ($P = 0.00$).

was measured in culture supernatant obtained from cultured lymphocytes, cultured UCB MSCs and co-culture of UCB MSCs and lymphocytes.

The concentration of TGF-β1 was 2366.85 ± 891 pg/mL in the medium of UCB MSCs cultured alone, 4928.57 ± 1227 pg/mL in the medium of PB MNCs co-cultured with UCB MSCs and 595.7 ± 166 pg/mL in the medium of PB MNCs cultured alone ($P = 0.000$, $n = 9$).

The concentration of IFN-γ secreted by human PB lymphocytes was 2350 ± 470 pg/mL in the absence of MSCs. Its concentration decreased significantly (mean 1163.125 ± 239 pg/mL) in the supernatants of co-culture of lymphocytes with UCB MSCs. However, MSCs alone showed traces of IFN-γ secretion 14.725 ± 0.51 pg/mL ($P = 0.000$, $n = 9$). Thus the immune-modulatory effect of MSCs may be due to inhibition of IFN-γ secretion from activated lymphocytes.

The concentration of IL-10 secreted in the culture supernatant of UCB MSCs was 1.08 ± 0.42 pg/mL and 14.55 ± 7.5 pg/mL in PHA-stimulated lymphocyte culture, whereas in co-culture it was 30.25 ± 14 pg/mL ($P = 0.001$, $n = 7$).

Cytokine concentrations were correlated with the lymphocyte count in each well to exclude the effect of MSCs on proliferation of lymphocytes as a cause of changes in cytokine concentrations. In the stimulated lymphocyte wells, TGF-β1 was $195 \pm 35/10^5$ cells, IFN-γ $768 \pm 104/10^5$ cells and IL-10 $4.6 \pm 1.5/10^5$ cells, whereas in the co-culture wells TGF-β1 was $2210 \pm 450/10^5$ cells, IFN-γ $520 \pm 66/10^5$ cells and IL-10 $13.5 \pm 4.5/10^5$ cells ($P = 0.00$) (Fig. 5a–c).

DISCUSSION

Because of their property of immunomodulation, MSCs are being considered as a double-edged sword that can not only bring about tissue repair, but also attenuate adverse inflammatory reactions in an allogenic setting. MSCs appear to use a surprising array of mechanisms to avoid deletion by the host, including hypo-immunogenicity, modulation of dendritic cells and T cell function and creation of a suppressive microenvironment (10). MSCs can be expanded and cryopreserved until they are needed. However, use of allogenic MSCs may be associated with adverse reactions that can be as serious as rejection. UCBs are becoming increasingly important as an alternative source of MSCs; UCB cells can be used as universal donors that are not vulnerable to the HLA barrier (4).

In this study, we were able to isolate fibroblast-like cells from UCB that had adhered to a plastic substrate. They have the same surface markers as MSCs isolated from other sources. They were negative for

hematopoietic, monocytic and endothelial markers and HLA DR and positive for epitopes such as CD13, CD29, CD 90, HLA ABC, CD73 (SH3), CD105 (SH2), CD166 and CD44. However, we found slight positivity for CD 133, which is not in agreement with the findings of Lee *et al.*, who reported that UCB-MSCs are negative for CD133, indicating that these cells are not of hematopoietic origin (11). However, Liyang *et al.* reported that CD133-positive MSCs can be isolated from UCB (12). They had high proliferative potential and were passaged every 5–6 days. They were also able to differentiate *in vitro* into osteocytes and adipocytes.

For cell-based therapy, there is a great need for cells with low immunogenicity that do not cause problems upon allogenic use. Previous studies have shown that BM MSCs are not immunogenic and have immunomodulatory effects (13–15). We performed our study to assess whether UCB MSCs share the immunomodulatory effects of BM MSCs and found that UCB MSCs can suppress proliferation of allogenic mitogen-stimulated PB mononuclear cells. The mechanism of immunosuppression may be direct intercellular contact or via secretion of soluble factors.

Umbilical cord blood MSCs can act directly on T cells and affect the early step of immune response through inhibition of dendritic cell function *in vitro* (9). Hepatocyte growth factor, prostaglandin E2, TGF-β1, IDO, nitric oxide and IL-10 are among the soluble factors implicated in the immune-modulatory response (16). IDO is an enzyme that catabolizes L-tryptophan, thereby depleting the local environment of an essential amino acid. Although it is not a soluble factor, expression of IDO may contribute to a tolerogenic environment. The suppressive effects of IFN-γ and/or TNF-α produced by activated T-cells correlate with induction of IDO expression in MSCs, which in turn inhibits T-cell proliferation (17).

In our study, we found an increase in TGF-β1 and IL-10 and decrease in IFN-γ in culture supernatants of UCB MSCs co-cultured with mitogen-stimulated lymphocytes. IL-10 is a well-known cytokine involved in promoting activation and proliferation of regulatory or anti-inflammatory cells (18). Previous studies on co-culture of stimulated lymphocytes with BM MSCs or adipose tissue MSCs have shown increased IL-10 secretion in co-culture supernatants (13, 19, 20). However other groups have found no change in its secretion (21) or not even detected it (22).

Our data showed traces of IL-10 secretion from UCB MSCs and lymphocytes; however, the secretion increased upon co-culture. Tse *et al.* concluded that soluble factors from mouse BM MSCs induce IL-10 production by mouse splenocytes that induces apoptosis and suppresses proliferation of T cells(15). Nasef *et al.* reported two mechanisms of T cell tolerance due to MSCs; the first is inducing tolerogenic genes such as IDO, lymphocyte inhibitory factor, HLA-G, and the second via IL-10 and TGF-β1 gene expression (23).

Bommireddy *et al.* observed that adding TGF-β1 neutralizing antibodies can partially restore the proliferation of BM MSCs inhibited T lymphocytes (24), whereas recombinant TFG-β1 reportedly enhances the immunomodulatory properties of MSCs (25).

Regarding the role of IFN-γ similar results, Zhou *et al.* found that, although MSCs do not secrete IFN-γ, they significantly reduce its secretion by human lymphocytes, thus reducing the incidence of immune rejection (26). Zappia *et al.* demonstrated that MSCs can reduce serum concentrations of IFN-γ by activated T cells (27).Similar results were reported by Kang *et al.*: they found marked reduction in IFN-γ secretion and significant increase in production of anti-inflammatory cytokines IL-10 and TGF-β1 in the supernatants of co-cultures of human amniotic membrane MSCs with MNCs in the presence of mitogen compared with in supernatants obtained from MNCs alone(18).

Mesenchymal stem cells inhibit production of IFN-γ and increase production of IL-4 by T-helper 2 cells, thus shifting T cells from a pro-inflammatory (IFN-γ production) state to an anti-inflammatory (IL-4 production) state (28).

Chen et al. concluded that MSCs from fetal sources appear to be less immunogenic than BM MSCs and even more immunomodulatory than their adult counterparts. Human UCB MSCs increase the proportion of regulatory T cells, which contributes to the suppression of T cell proliferation (29). Human MSCs from different sources differ in their immunomodulatory mechanisms; these differences may be attributable to their different sources or to differences in the methods used to measure targeted lymphocyte proliferation (10).

The use of flow cytometry and CFSE dilution assay in this study allowed us to trace the kinetics of suppression of lymphocyte proliferation; thus, it provided superior and more informative results than Brdu ELISA assay. CFSE is an effective and popular means of monitoring lymphocyte division. CFSE covalently labels long-lived intracellular molecules with the fluorescent dye, carboxyfluorescein. When a CFSE-labeled cell divides, its progeny are endowed with half the number of carboxyfluorescein-tagged molecules; thus, the number of cell divisions can be assessed by measuring the corresponding decrease in cell fluorescence by flow cytometry. The capacity of CFSE to label lymphocyte populations with a high fluorescent intensity of exceptionally low variance, coupled with its low cell

toxicity, make it an ideal dye to measure cell division. Because it is a fluorescein-based dye, it is also compatible with a broad range of other fluorochromes, making it applicable to multi-color flow cytometry (30).

In conclusion, we found that UCB MSCs suppress the proliferation of mitogen-stimulated lymphocytes. However further *in vivo* studies are still required to fully evaluate the immunomodulatory effects of UCB MSCs.

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DISCLOSURE

All authors have no conflict of interest.

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