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Immunological properties of umbilical cord blood-derived mesenchymal stromal cells

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ABSTRACT

Mesenchymal stromal cells (MSCs) are promising candidates for developing cell therapies for intractable diseases. To assess the feasibility of transplantation with human umbilical cord blood (hUCB)-derived MSCs, we analyzed the ability of these cells to function as alloantigen-presenting cells (APC) *in vitro*. hUCB-MSCs were strongly positive for MSC-related antigens and stained positively for human leukocyte antigen (HLA)-AB and negatively for HLA-DR. When treated with interferon (IFN)-γ, the expression of HLA-AB and HLA-DR, but not the co-stimulatory molecules CD80 and CD86, was increased. hUCB-MSCs did not provoke allogeneic PBMC (peripheral blood mononuclear cell) proliferation, even when their HLA-molecule expression was up-regulated by IFN-γ pretreatment. When added to a mixed lymphocyte reaction (MLR), hUCB-MSCs actively suppressed the allogeneic proliferation of the responder lymphocytes. This suppressive effect was mediated by soluble factors. We conclude that hUCB-MSCs can suppress the allogeneic response of lymphocytes and may thus be useful in allogeneic cell therapies.

1. Introduction

MSCs are multipotent cells found in fetal liver, bone marrow (BM), and cord blood [1,2], and have the capacity to differentiate *in vitro* into several mesodermal (bone, cartilage, tendon, muscle, and adipose), endodermal (hepatocyte), and ectodermal (neurons) tissues [3]. Classically, MSCs are defined as being able to adhere to plastic, expressing CD29, CD73, CD44, CD90, CD105, and major histocompatibility complex type [MHC] class I antigens, and not expressing the hematopoietic cell markers CD34, CD45, and MHC class II antigen. MSCs constitutively secrete a large number of cytokines and promote the expansion and differentiation of hematopoietic stem cells (HSCs) [4]. Furthermore, MSCs are not immunogenic, namely, they do not induce allogeneic lymphocytes to proliferate *in vitro* [5]. Indeed, MSCs appear to suppress these allogeneic proliferative responses. For example, in *in vivo* trials, co-infusion of MSCs as a third party delayed donor cell rejection, even when immunosuppressant drugs were not used [6]. These characteristics make MSCs potent candidates for the development of allogeneic cell-based therapeutic strategies. The therapeutic efficacy of MSCs for bone, joint, and neuronal diseases has recently been reported [7,8].

BM is the main source of MSCs and BM-MSCs have already been used in various clinical studies. However, the number of MSCs in the BM and their multi-lineage differentiation capacity decline with age [9]. Therefore, human umbilical cord blood is often used as an alternative source of stem or progenitor cells, namely, HSCs and MSCs [10]. hUCBs also have a practical advantage over BM-derived MSCs in that they are obtained by non-invasive methods that do not harm either the mother or the infant [11]. Furthermore, cord blood stem cells are more immature than adult MSCs and expand readily *in vitro* [12]. Given these features along with their potent differentiation potential [3], hUCB-MSCs are an attractive source for cellular or gene transfer therapy. Moreover, a female patient with chronic spinal cord injury who was given hUCB-MSCs showed functional and morphological improvement [13]. The transfer of hUCB-MSCs was also useful in treating Buerger's disease [14].

The aim of the present study was to investigate the immunological properties of hUCB-MSCs to assess their potential usefulness in allogeneic transplantation. We examined the effect of IFN-γ pretreatment on the expression of MHC molecules on the hUCB-MSC cell surface and assessed whether hUCB-MSCs can provoke an *in vitro* allogeneic reaction. We also asked whether hUCB-MSCs, like other MSCs [15,16], can inhibit allogeneic MLR and mitogen-induced lymphocyte proliferation. In addition, we examined whether these immunological properties of hUCB-MSCs change after they differentiate into various lineages.

2. Materials and methods

2.1. Isolation and culture of hUCB-MSCs

This study was approved by the Institutional Review Board of Medipost Inc., Seoul, Korea. The hUCB-MSCs were separated and

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maintained as described previously [3]. Briefly, hUCB samples were collected from the umbilical vein of deliveries with informed maternal consent. Mononuclear cells were isolated from the hUCBs by centrifugation through a Ficoll-Hypaque gradient (density 1.077 g/cm^3, Sigma, St. Louis, MO, USA). The separated mononuclear cells were washed, suspended in α-minimum essential medium (α-MEM, Gibco BRL, Carlsbad, CA, USA) supplemented with 10% FBS (HyClone, Logan, UT, USA), and seeded at a concentration of 5×10^6cells/cm^2. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 with a twice weekly change of culture medium. One to 3 weeks later, when the monolayer of fibroblast-like adherent cells colonies had reached 80% confluence, the cells were trypsinized (0.25% trypsin, HyClone), washed, resuspended in culture medium (α-MEM supplemented with 10% FBS) and sub-cultured.

2.2. Neurogenic differentiation

Poly-D-lysine- and laminin (Sigma)-coated plates were used to induce neuronal differentiation. Initially, hUCB-MSCs were pretreated for 2 days in DMEM/10% FBS with 20 ng/ml basic fibroblast growth factor (R&D System, Inc., Minneapolis, MN, USA). Neuronal differentiation was then induced by replacing this medium with neurogenic medium composed of DMEM/F12 medium supplemented with 200 μM butylated hydroxyanisole, 25 μg/ml insulin (Sigma), 25 mM KCl, 2 μM valproic acid (Sigma), 10 μM forskolin (Sigma), and 1 μM hydrocortisone (Sigma). After 72 h of differentiation, neurogenesis was evaluated by measuring the expression of MAP-2 and NeuN by Western blot analysis.

2.3. Chondrogenic differentiation

hUCB-MSCs were induced to differentiate into chondrogenic cells as described previously [3]. After 3 weeks of differentiation in chondrogenic medium [high-glucose DMEM (Gibco BRL) supplemented with 100 nm dexamethasone (Sigma), 50 mg/mL L-ascorbic acid (Sigma), 100 mg/mL sodium pyruvate (Sigma), 40 mg/mL L-proline (Sigma), 10 ng/mL transforming growth factor-β3 (TGF-β3, Sigma), 500 ng/mL bone morphogenic protein 6 (BMP-6, R&D Systems, Minneapolis, MN, USA) and 50 mg/mL ITS$^+$ premix (6.25 mg/mL insulin, 6.25 mg/mL transferrin, 6.25 ng/mL selenious acid, 1.25 mg/mL BSA, and 5.35 mg/mL linoleic acid, Becton Dickinson)], chondrogenesis was evaluated by safranin O staining. To dissect the cells into single cell suspensions, chondrogenic pellets were incubated for 1 h in dissection solution (DMEM medium containing 0.2% collagenase type I) and then pipetted vigorously until single cell suspensions were achieved.

2.4. MLRs

To assess T-cell reactivity against allogeneic cell populations, human responder PBMCs (1×10^5/well) were cocultured with inactivated allogeneic PBMCs (1×10^5/well) or hUCB-MSCs (1×10^2, 1×10^3 or 1×10^4/well) in 96-well tissue culture plates. The PBMCs were purchased from AllCells, LLC (CA, USA). The stimulator PBMCs and hUCB-MSCs were inactivated by treatment with 10 μg/ml mitomycin-C (Sigma) for 1 h at 37 °C. In some experiments, responder PBMCs were cocultured with allogeneic stimulator PBMCs in 96-well tissue culture plates at 1×10^5/well in the presence or absence of 1×10^2, 1×10^3 or 1×10^4/well preplated hUCB-MSCs. Due to the large size of hUCB-MSCs, these cells were not cultured at the same ratio with the PBMC responder cell (1×10^4 hUCB-MSCs in a 96-well plate well are 80% confluent). For experiments using transwell chambers (BD Bioscience), PBMCs from two HLA-type-mismatched individuals were seeded at 1×10^5/well in the presence or absence of 1×10^3 or 1×10^4 hUCB-MSCs. The hUCB-MSCs were separated from the PBMCs by a high density pore membrane (transwell chamber, BD Bioscience) by being added to the upper compartment. T-cell proliferation to alloantigens was determined by adding 20 μl of bromodioxyuridine (BrdU) after 6 days of MLR culture (the final BrdU concentration was 10 μM). The tissue culture plates were then incubated at 37 °C in 5% CO_2 for an additional 18 h. Thereafter, the tissue culture plates were centrifuged at 300*g* for 10 min, the substrate was added as described by the manufacturer's instructions and colorimetric immunoassays were immediately used to measure the BrdU that had been incorporated during DNA synthesis, which estimates cell proliferation (Roche, Boerhringer Mannheim). Thus, the absorbance of the samples was measured in an UV max kinetic microplate reader (Molecular Device) at 370 nm (reference wavelength: 492 nm). The data of three replicates are shown as means and standard deviation (SD).

2.5. Mitogen-induced proliferation assay

PBMCs were seeded in triplicate at a concentration of 1×10^5/50 μl/well in 96-well plates with 5 or 10 μg/ml phytohaemagglutinin (PHA, Sigma). RPMI 1640 medium (50 μl/well) or hUCB-MSCs (1×10^2, 1×10^3 or 1×10^4/well) were added. After 3 days of incubation, 20 μl BrdU was added for 18 h. In the experiments using transwell chambers, 1×10^5 PBMCs per well were added to the lower compartment and then 1×10^3 or 1×10^4 MSCs were added to the upper compartment of the transwell chamber. All studies were performed using HLA-unmatched donor populations of hUCB-MSCs and hPBMCs.

2.6. Phenotypic analysis

hUCB-MSCs were stained with various combinations of saturating amounts of monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE): CD45-FITC, CD34-FITC, CD31-FITC, CD14-FITC, HLA-DR-FITC (Becton Dickinson, San Jose, CA, USA), CD29-PE, CD44-PE, CD73-PE, CD90-PE, CD166-PE, HLA-AB-PE, CD80-FITC, CD86-PE, isotype-matched control (Pharmingen, Los Angeles, CA, USA), and CD105-PE (Serotec, NC, USA). At least 10^4 events were analysed by flow cytometry (FACScan, BD Biosciences) with the cellquest software.

2.7. Cytokine ELISA

The IFN-γ, transforming growth factor-beta (TGF-β), interleukin (IL)-2, IL-10, IL-1α, IL-1β, and tumor necrosis factor (TNF)-α levels in the conditioned supernatants obtained from MLR and proliferation assays were measured by enzyme-linked immunoassays (R&D system) according to the manufacturers' instructions.

2.8. Statistics

Statistical analyses of data were performed by the Student's *t*-test to determine statistical significance. Values are given as means ± SD (standard deviation).

3. Results

3.1. Characterization of the hUCB-MSCs

MSCs were successfully isolated from three hUCBs and could be separately cultured until passage 4. As reported previously [3], the hUCB-MSCs had a fibroblast-like morphology (Fig. 1A). Three different hUCB-MSC clones were uniformly positive for MSC-related antigens, namely, CD73 (96.1 ± 2.3%), CD105 (91.3 ± 4.1%), CD90 (96.8 ± 1.5%), CD166 (93.1 ± 3.5%), CD29 (91.1 ± 6.7%), and CD44 (92.1 ± 5.1%) (Fig. 1D). In addition, these cells were positive for HLA-class I (HLA-AB;

98.1 ± 3.3) but expressed neither HLA-class II (HLA-DR) nor the hematopoietic markers such as CD14, CD31, CD34, and CD45.

To evaluate the multi-lineage differentiation capacity of the hUCB-MSCs, we cultured them with chondrogenic- or neurogenic-differentiation media. As shown in Fig. 1, chondrogenic differentiation was observed, as shown by the accumulation of Safranin-O-staining sulfated proteoglycans (Fig. 1B). Neurogenic differentiation was also observed, as demonstrated by morphological changes (Fig. 1C) and the expression of the mature neuron markers MAP-2 and NeuN (data not shown).

Previous reports have demonstrated that pretreatment of MSCs with IFN-γ increases their cell surface expression of HLA-AB and HLA-DR [17]. Therefore, we treated with hUCB-MSCs with IFN-γ (100 U/ml) for 72 h and examined their expression of HLA-AB, HLA-DR, and the co-stimulatory molecules CD80 and CD86. The expression levels of HLA-AB and HLA-DR but not the co-stimulatory molecules were elevated by IFN-γ pretreatment (Fig. 1E).

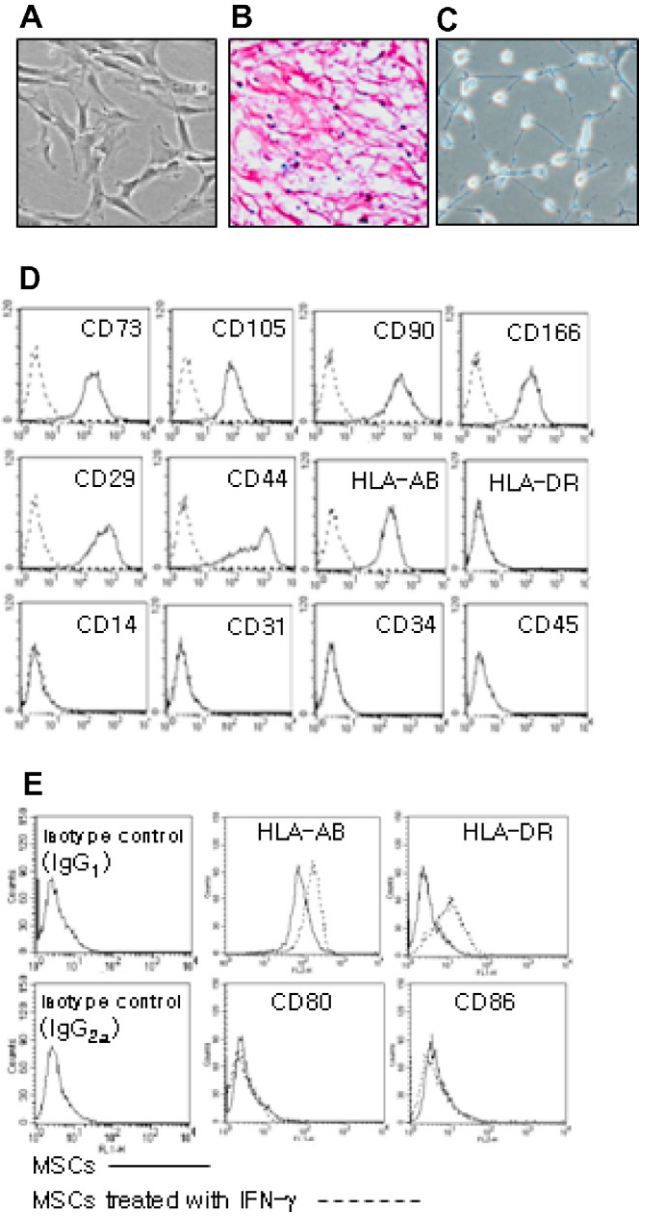

Fig. 1. Basic characteristics of hUCB-MSCs. (A–C) Inverted phase contrast microscope images of hUCB-MSCs in the 4th passage (A), 4 weeks after chondrogenic differentiation and Safranin-O staining (B), and 72 h after neurogenic differentiation (C). Original magnification, 200×. (D) Immunophenotype of the hUCB-MSCs. MSC-clone of human cord blood at passage 4 was labeled with antibodies against the indicated antigens and they were then analyzed by flow cytometry. The dotted lines indicate the isotype-matched IgG antibody control labeling. (E) Expression of immunologically important cell surface molecules on hUCB-MSCs. Data represents one of three experiments with similar results.

3.2. Allogeneic response of hUCB-MSCs

To determine whether hUCB-MSCs can function as APCs, various concentrations of inactivated hUCB-MSCs were cocultured with responder allogeneic PBMCs and PBMC proliferation was assessed 7 days later. As a control, inactivated allogeneic PBMCs served as the APCs. The allogeneic PBMCs were highly efficient APCs but in contrast, hUCB-MSCs did not stimulate lymphocyte proliferation (Fig. 2A). Significantly, coculture with hUCB-MSCs even suppressed the baseline proliferation capacity of the PBMCs (Fig. 2A). IFN-γ pretreatment did not improve the capacity of hUCB-MSCs to function as APCs (Fig. 2B).

To determine whether the differentiation of hUCB-MSCs increases their immunogenicity, inactivated hUCB-MSC-derived chondrocytes and neuron-like cells were cocultured with allogeneic responder PBMCs in 96-well tissue culture plates. The inactivated hUCB-MSC-derived chondrocytes were cocultured with allogeneic PBMCs as a pellet or as a single cell suspension (1×10^4/well). Neither the chondrocytes (suspension or pellet) nor the neuron-like cells induced allogeneic PBMC proliferation (Fig. 3), similar to the effect of undifferentiated hUCB-MSCs.

3.3. Immunosuppressive effect of hUCB-MSCs on MLR

It was interesting that in the MLR using inactivated hUCB-MSCs, the proliferation of the responder PBMCs was lower than when these cells were cultured on their own (Fig. 2). To investigate this effect further, we added inactivated hUCB-MSCs to an MLR culture composed of responder PBMCs and inactivated stimulator PBMCs. The hUCB-MSCs were added in three different concentrations at the initiation of the culture experiment. The HLA types of the hUCB-MSCs did not match either the responder or stimulator PBMCs. The hUCB-MSCs inhibited the MLR in a hUCB-MSC concentration-dependent manner (Fig. 4A). In experiments with three different hUCBs, maximal suppression (average 39%, range 33 ± 7.5–42 ± 12.6%) was observed when there was 1×10^4 hUCB-MSCs/well (a 1:10 MSC/effector cell ratio). In Fig. 2, the biggest suppressive effect on baseline PBMC proliferation is seen with the lowest hUCB-MSC concentration, which is not consistent with these observations. Pre-treatment of the hUCB-MSCs with IFN-γ (100 U/ml) for 3 days prior to co-culture with the PBMCs did not affect their MLR-suppressive abilities.

3.4. Effect of hUCB-MSCs on mitogen-induced lymphocyte proliferation

We then examined whether hUCB-MSCs can also inhibit mitogen-induced lymphocyte proliferation. Thus, three different concentrations of inactivated hUCB-MSCs were added to PBMCs in the presence of PHA. After 4 days of culture, proliferation rate of PBMC were confirmed. The HLA types of the hUCB-MSCs and the PBMCs did not match. Two concentrations of PHA, namely, 5 and 10 μg/ml, were used since the higher concentration yielded maximal stimulation while the lower concentration was the lowest concentration that still generated a response close to the maximal response (lymphocyte responses declined dramatically when PHA was present at concentrations below 5 μg/ml). Significantly, hUCB-MSCs did not inhibit the lymphocyte proliferative response to PHA (Fig. 4B). Indeed, co-culture with hUCB-MSCs tended to stimulate the lymphocyte proliferative response to 5 μg/ml PHA. Moreover hUCB-MSCs did not inhibit the lymphocyte proliferation regardless of changing the incubation period (Fig. 4C).

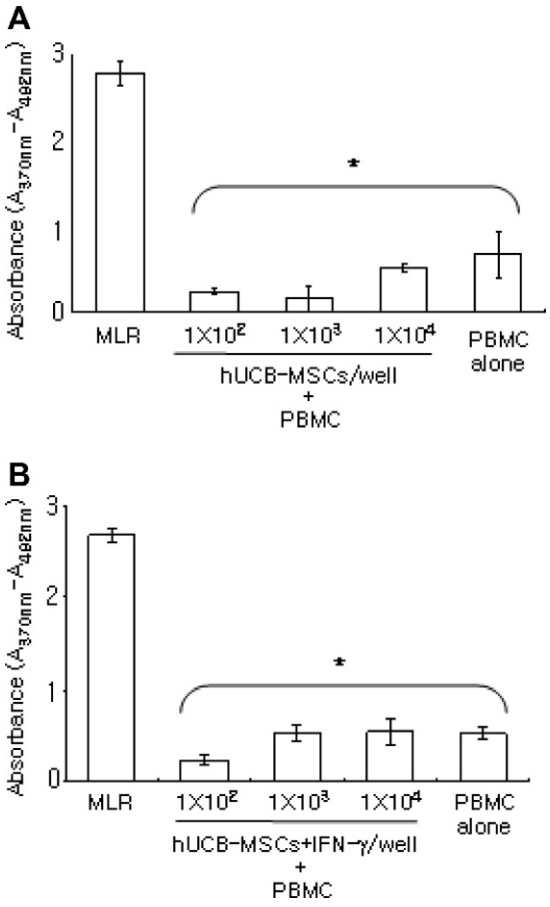

Fig. 2. Proliferative responses by PBMCs to allogeneic hUCB-MSCs. (A) Human responder PBMCs (1×10^5 cells/well) were cultured in 96-well tissue culture plates with inactivated allogeneic PBMCs (1×10^5 cells/well) (positive control; MLR) or three different concentrations of inactivated hUCB-MSCs. In (B), the hUCB-MSCs had been pre-treated with IFN-γ for 3 days. The cells were pulsed with BrdU for the final 18 h of culture and PBMC proliferation was measured by determining the absorbance at 370 nm (reference wavelength 492 nm). The data of four independent experiments, each of which was performed in triplicate, are expressed as means ± SD. *Statistical significant ($P < 0.05$, Student's *t*-test) as compared with MLR culture.

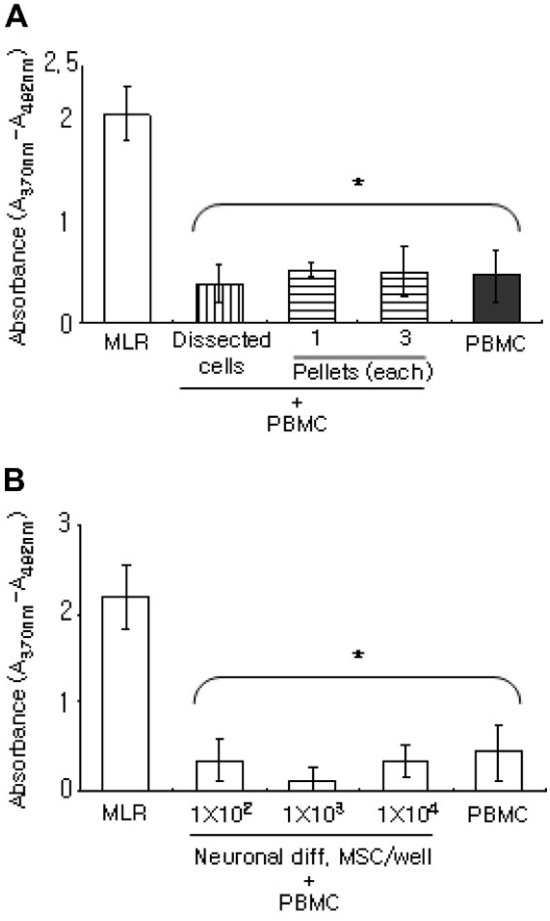

Fig. 3. Proliferative response of PBMCs to differentiated allogeneic hUCB-MSCs. (A) hUCB-MSCs were induced to differentiate into chondrocytes for 3 weeks in chondrogenic medium, after which the inactivated chondrocyte pellets or single cell suspensions (generated by collagenase type I digestion of the pellets) were cocultured with allogeneic PBMCs. (B) Neuronal differentiation was induced by culture in neurogenic media for 72 h, after which the neuron-like hUCB-MSCs were harvested by trypsinization, inactivated and plated for 6 h before allogeneic PBMCs were added. Inactivated PBMCs served as a positive control (MLR). PBMC proliferation was determined by measuring the absorbance at 370 nm (reference wavelength 492 nm). The data of three independent experiments, each of which was performed in triplicate, are expressed as means ± SD. *Statistical significant ($P < 0.05$, Student's *t*-test) as compared with MLR culture.

3.5. Importance of soluble factors in the immunosuppressive effect of hUCB-MSCs

To determine whether the immunosuppressive effect of hUCB-MSCs is mediated by soluble factors or cell-to-cell contact, MLR and mitogen-induced proliferation assays were performed in transwell chambers that prevent cell-to-cell contact between the responder PBMCs and the inactivated hUCB-MSCs. Experiments with three hUCBs showed that the hUCB-MSCs continued to inhibit MLR by 37 ± 11.1–45 ± 9.8% (Fig. 5A). Moreover, the hUCB-MSCs again failed to suppress the mitogen-induced proliferation of lymphocytes (Fig. 5B). Thus, it appears that soluble factors secreted from hUCB-MSCs suppress allogeneic immune responses but not mitogen-induced responses.

We also directly demonstrated that soluble factors are involved in immunosuppressive effect. Responder PBMCs were cocultured with allogeneic stimulator PBMCs in α-MEM (10% FBS) supplemented with 50% hUCB-MSC culture supernatants or conditioned hUCB-MSC/MLR medium. As shown in Fig. 5C, proliferation of responder PBMCs was suppressed by conditioned hUCB-MSC/MLR medium, but not by hUCB-MSC culture supernatants.

3.6. hUCB-MSCs suppress the production of cytokines

We then sought to further characterize the immunosuppressive effect of hUCB-MSCs by subjecting the conditioned supernatants obtained from MLR and mitogen-induced proliferation assays to cytokine ELISAs for IL-1α, IL-1β, IL-2, IL-10, TGF-β, IFN-γ, and TNF-α. In MLR, the hUCB-MSCs reduced the responder cell production of IFN-γ and IL-2 (Fig. 6A), which are well known for their ability to promote lymphocyte proliferation. Interestingly, however, the hUCB-MSCs also decreased the production of these cytokines by PHA-stimulated PBMCs (Fig. 6B). Indeed, hUCB-MSCs inhibited the IL-2 production by PHA-stimulated PBMCs more severely than the IL-2 production by MLR.

When the pro-inflammatory cytokines TNF-α, IL-1α and IL-1β, and the immunosuppressive cytokine IL-10 and TGF-β were examined, the hUCB-MSCs were found to significantly suppress TNF-α levels in the MLR-conditioned medium (Fig. 6A). This effect was not observed in the mitogen-induced proliferation assay (Fig. 6B). The concentrations of IL-1α, IL-1β, and IL-10 were below the level of detection (10 pg/ml) in all culture conditions. Furthermore, we found similar amounts of TGF-β (0.7 ± 0.045 ng/ml) in MLR supernatants that had been performed with or without hUCB-MSCs.

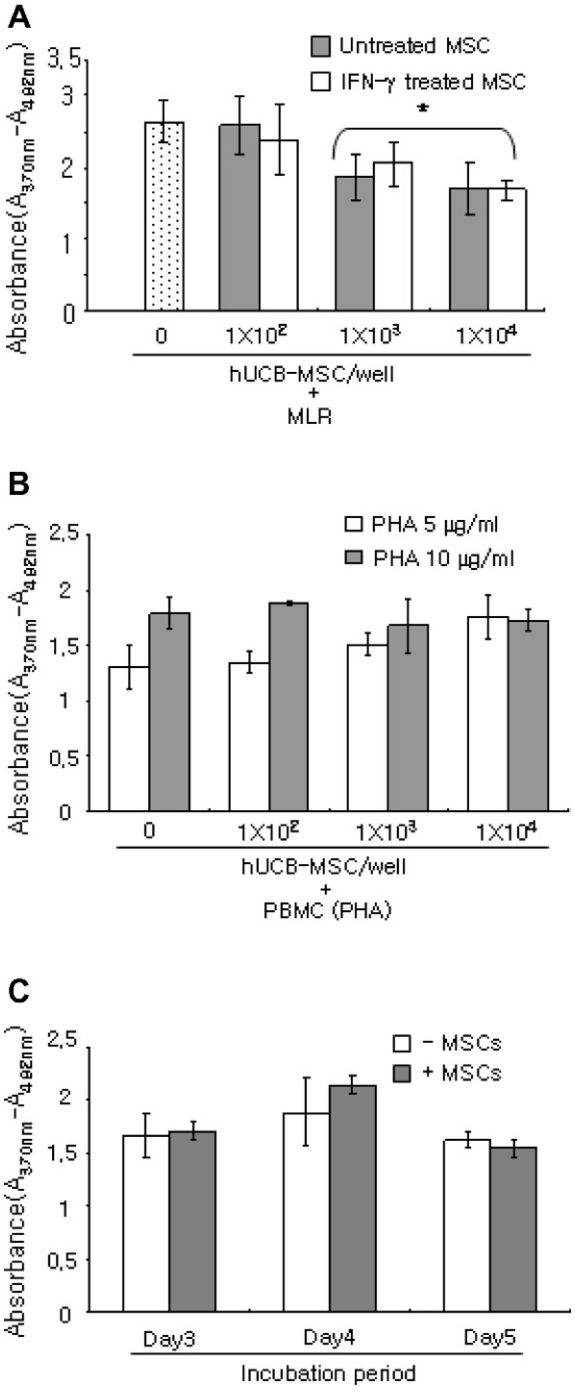

Fig. 4. Inhibitory effect of hUCB-MSCs on MLR and PHA-induced PBMC proliferation. (A) hUCB-MSCs were cultured with or without IFN-γ for 3 days and then added to MLR cultures (effector PBMC: responder PBMC = 1:1), respectively. (B) PBMCs (1×10^5) were stimulated with 5 or 10 μg/ml PHA and inactivated hUCB-MSCs at the indicated dose per well were added to the cultures on day 0. (C) PBMCs (1×10^5) were stimulated with 10 μg/ml PHA and inactivated hUCB-MSCs (1×10^4) were added to the cultures on day 0. Responder PBMC proliferation was determined by measuring absorbance at 370 nm (reference wavelength 492 nm).The data of three independent experiments, each of which was performed in triplicate, are expressed as means ± SD. *Statistical significant ($P < 0.05$, Student's t-test) as compared with MLR culture (without hUCB-MSCs).

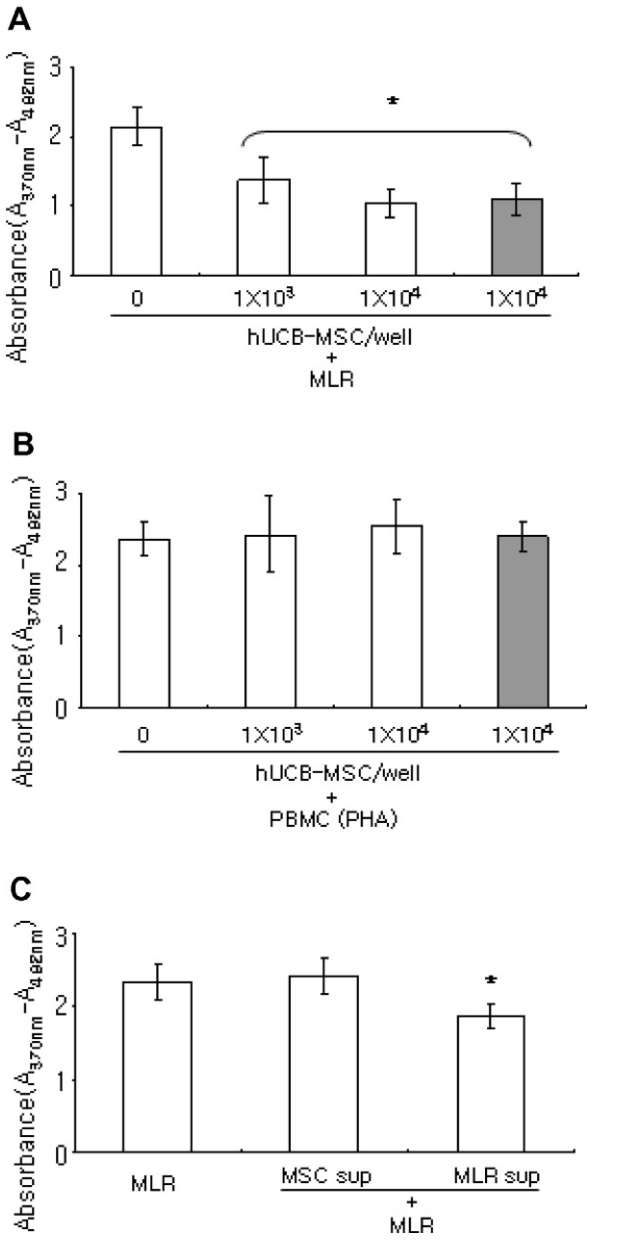

Fig. 5. The immunosuppressive effect of hUCB-MSCs is mediated by a soluble factor. MLR (A) and mitogen-induced proliferation (B) assays were performed with allogeneic hUCB-MSCs separated by a transwell membrane (white bar) or without transwell separation (grey bar). In (C), MLR assay was performed in the presence of hUCB-MSCs supernatants or conditioned hUCB-MSC/MLR medium. The data of four independent experiments, each of which was performed in triplicate, are expressed as means ± SD. *Statistical significant ($P < 0.05$, Student's t-test) as compared with MLR culture (without hUCB-MSCs).

4. Discussion

hUCB is currently used as an alternative to BM as a source of stem cells for management of hematological malignancies. The possible usefulness of hUCB-MSCs in many preclinical models ranging from myocardial ischemia and stroke to cartilage regeneration is also under intense experimental investigation [7,18]. The main theoretical limitation hampering the clinical transplantation of hUCB cells would be graft rejection due to the immunological barrier imposed by HLA mismatching. However, in this study, we showed that hUCB-MSCs actually suppress the allogeneic responsiveness of human lymphocytes, as they suppress their proliferation and reduce their production of immunostimulatory cytokines. Significantly, previous studies have shown that MSC-like cells from other tissues, namely, BM [15,19,20], adipose tissue [16], and fetal lung tissue [21], also inhibit lymphocyte proliferation and inflammatory cytokine production.

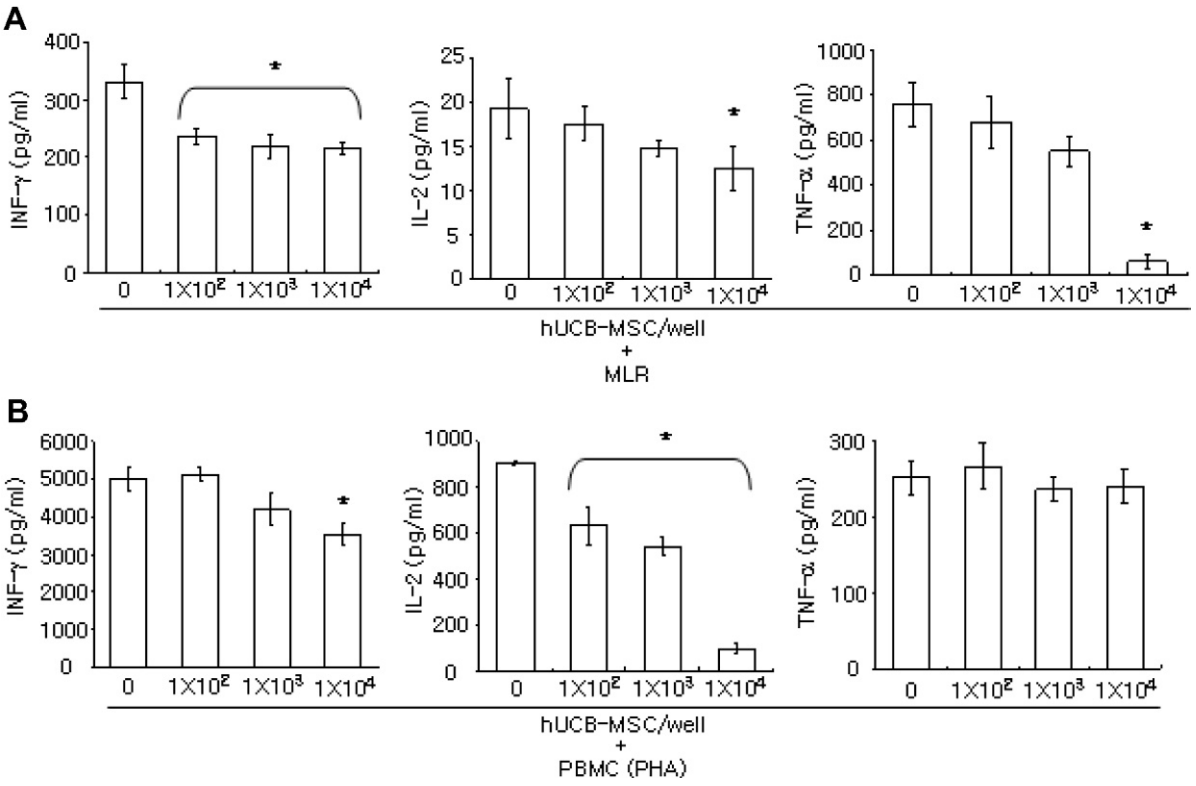

Fig. 6. hUCB-MSCs suppress IFN-γ, IL-2, and TNF-α production in MLR and mitogen-induced proliferation assays. Supernatants of MLR (A) and PHA-stimulated lymphocyte (B) assays performed in the presence and absence of hUCB-MSCs were subjected to ELISAs to measure IFN-γ, IL-2, and TNF-α production. The responder PBMC cells alone, the inactivated PBMCs alone, and the hUCB-MSCs alone produced less than 10 pg/ml IFN-γ, IL-2, and TNF-α. The data from three independent experiments, each of which was performed in triplicate, are expressed as means ± SD. *Statistical significant ($P < 0.05$, Student's t-test) as compared with hUCB-MSCs untreated cultures.

We obtained three different hUCB-MSC populations for this study and checked that they had the same phenotype and multilineage differentiation capacity that have been reported for hBM-MSCs [16]. In line with previous observations, our cell populations were positive for the MSC-related antigens CD73, CD105, CD90, CD166, CD29, and CD44, and negative for HLA-class II and T-cell co-stimulatory molecule B7 [3]. HLA-class II and B7 are well known to play important roles in antigen presentation and the allogeneic response. IFN-γ pre-treatment elevated HLA-class I and II expression on our hUCB-MSCs but these cells nevertheless continued to be unable to provoke a proliferative response from alloreactive lymphocytes. Similarly, we found that when hUCB-MSCs were pre-treated with the pro-inflammatory cytokine IL-1β or TNF-α, they were still unable to provoke an allogeneic response (our unpublished data). Thus, hUCB-MSCs fail to act as allogeneic response-inducing APCs despite carrying HLA molecules. This suggests that hUCB-MSCs can be implanted into inflammation sites without having to use long-term immunosuppressive drug therapies. Our results are in agreement with previous studies concerning hBM-MSCs [17], which reported that hBM-MSCs do not enhance the proliferation of allogeneic T lymphocyte, even when their alloantigens are up-regulated by IFN-γ or B7 gene transduction. Ryan et al. also reported that the pro-inflammatory cytokine IFN-γ did not block MSC inhibition of alloantigen-driven proliferation, while IFN-γ induced the production of indoleamine 2,3-dioxygenase (IDO), which enhanced immunosuppressive activity of MSCs [22]. Taken together, although we failed to reveal the reason why IFN-γ did not augment or suppress of immunosuppressive activities of hUCB-MSCs, we carefully suppose that the pro-inflammatory activity of IFN-γ on MSCs should be compensated by immunosuppressive molecules which are secreted by IFN-γ.

When our hUCB-MSCs were induced to differentiate into chondrocytes or neuron-like cells, they did not elicit an immunologic response when added to allogeneic PBMCs. Thus, the immune evasion properties of the hUCB-MSCs were not lost upon their differentiation. A previous study has also shown that MSCs that had differentiated into osteocytes continued to be unable to induce allogeneic T-cell proliferation, regardless of whether they were treated with IFN-γ or not [17]. Moreover, MSCs that had differentiated into osteocytes or adipocytes were as effective immunosuppressors as undifferentiated MSCs when added to a two-way MLR at the initiation point [21]. When allogeneic MSCs were transplanted and differentiated in a site-specific manner into chondrocytes, adipocytes, myocytes and cardiomyocytes, they have persisted for 13 months [23]. Notably, it has been shown that when chondrocytes and myoblasts are transplanted into HLA-mismatched recipients to treat model articular and muscular diseases, these cells are acutely rejected [24,25]. Moreover, allogenic transplanted cartilage was surrounded by infiltrations composed mainly of lymphocyte and showed signs of acute rejection [26]. Neural allografts are usually rejected in the brain as well [27,28]. Consequently, our results suggest that the differentiated MSCs can be successfully transplanted even when they are MHC-mismatched, which is a unique property of hUCB-MSCs.

While the immunosuppressive properties of MSCs have been extensively studied, the mechanism of suppression remains to be clarified. Our experiments showed that hUCB-MSCs suppressed the alloreactive proliferation of T lymphocytes depending on the concentration of hUCB-MSCs, with low concentrations (100 cells/well) failing to suppress the alloreactivity of the lymphocytes. Low hUCB-MSC concentrations also did not promote T-cell alloreactivity. However, a previous study has reported that low MSC concentrations (under 1000 cells/well) are profoundly immunosuppres-

sive [16], while yet another recent study has found that low MSC concentrations suppress the lymphocyte alloproliferative response less well than higher concentrations and may even be stimulatory [5]. Thus, the effect of low MSC concentrations on alloreactivity remains unclear at this point. We also sought to inhibit the lymphocyte proliferation induced by two different concentrations of PHA. Interestingly, our MSCs did not inhibit mitogen-induced lymphocyte proliferation. This was unexpected since several studies have suggested that BM- and adipose tissue-derived MSCs not only suppress alloreactive T lymphocytes, they also greatly reduce the lymphocyte proliferation induced by the potent T-lymphocyte mitogens PHA, Con A and SpA [5,16,29], moreover, inhibition by MSCs occurred when both autologous and allogeneic PBMC were stimulated. Our observations suggest that the immunological properties of hUCB-MSCs may be very different from those of BM- and adipose tissue-derived MSCs (although it should be noted that we did not directly compare hUCB-MSCs with other MSCs in this study). In addition, it appears that alloreactive lymphocyte proliferation is stimulated by a different pathway from the one used for mitogen-induced lymphocyte proliferation. In the past several years, T-cell activation by mitogens like ConA or PHA has been considered as mimicry of T-cell activation by APC. According to this theory, although the immediate result of a comparative study has not been reported until now, it appears that both T-cell activation pathways use similar cell signaling pathway and activation mechanism [30–32]. Further work will be required to clarify how hUCB-MSCs reduce T-cell proliferation and what are differences between allogeneic and PHA-stimulated T-cell activation.

We demonstrated that hUCB-MSCs suppress lymphocyte alloreactivity *via* a soluble factor(s) since the MSCs continued to suppress MLR across a transwell membrane. Similarly, it has been shown previously that BM-MSCs significantly inhibit alloreactivity even when they are separated from the responder lymphocytes by a transwell membrane [19,33,34]. Notably, these studies found that the degree of inhibition achieved in the transwell chamber experiments was less than that observed in the non-separated situation. We did not observe this. This disparity may be due to the different sources of MSCs used in our experiments. We also found that mitogen-induced lymphocyte proliferation was not suppressed by hUCB-MSCs regardless of whether there was cell-to-cell contact or not.

The soluble factors from hUCB-MSCs that inhibit lymphocyte alloreactivity may be produced spontaneously or only when the MSCs are exposed to allogeneic lymphocytes. We have found that when MLR is performed in the presence of hUCB-MSC culture supernatants, alloreactive T-lymphocyte proliferation is not suppressed; indeed, in some cases, the response is enhanced. However, when conditioned hUCB-MSC/MLR medium was used, it inhibited allogeneic lymphocyte proliferation, albeit slightly (18–20% reduction in proliferation). This low effect could be because the supernatants had been frozen and thawed previously. Moreover, the suppressive soluble factors would have been diluted by the fresh medium used in the MLR assay. Nevertheless, these results suggest hUCB-MSCs produce their suppressive soluble factor(s) only when allogeneic lymphocytes are in their vicinity.

Analysis of the IFN-γ and IL-2 levels in the supernatants of hUCB-MSC-suppressed MLR cultures revealed they were reduced by almost 30% relative to unsuppressed MLR cultures. This is significant because these cytokines are considered to play important roles in allogeneic immune responses and T-lymphocyte proliferation. IFN-γ is very important because it increases cell-surface HLA I and II class molecule expression and reinforces the T-lymphocyte activities, together with lymphotoxins-CD4 or CD8, produced by lymphocytes. IL-2 is also necessary for the growth, differentiation, and survival of antigen-selected cytotoxic T-cells and supports long-term T-cell proliferation [35–38]. Interestingly, hUCB-MSCs also reduced the production of IFN-γ and IL-2 by PHA-stimulated PBMCs, although this was not matched with the data of PBMC proliferation (Fig. 6). These results were unexpected since reduced IFN-γ and IL-2 production should limit lymphocyte proliferation. In contrast, a previous study has shown that MSCs reduce both the significant IFN-γ production of PHA-stimulated PBMCs and their proliferation [39]. This discrepancy may relate to the type of MSC being used in these experiments.

We found that IL-1α and IL-1β were not involved in the mechanism by which hUCB-MSCs suppress lymphocyte alloreactivity since they were not detectable in hUCB-MSC-suppressed MLR cultures (or PHA-stimulated PBMC cultures). The other side TNF-α levels in MLR supernatants were greatly reduced when the MLR had been performed in the presence of hUCB-MSCs. Interestingly hUCB-MSCs had failed to reduce the TNF-α levels in PHA-stimulated PBMC culture supernatants. TNF-α is produced by mitogens-stimulated monocyte or macrophages, and it has been shown that it can enhance the proliferation of mature T lymphocytes [40,41]. According to this mechanism, considerable amounts of TNF-α in the culture supernatants of PBMC only or PBMC plus hUCB-MSCs, stimulated with PHA, are correlated to the degree of T-cell proliferation. These data support the notion that the reduction of TNF-α level by hUCB-MSCs in the supernatants of MLR culture has caused suppression of lymphocyte alloreactivity. Our results are in agreement with previous studies [15,17,39], which reported that MSCs suppress T-lymphocyte proliferation by reducing their TNF-α production.

Anti-inflammatory cytokines such as TGF-β and IL-10 have been thought to mediate the immunosuppressive effects of MSCs [42]. However, hUCB-MSCs did not elevate the IL-10 levels in MLR culture supernatants, which were undetectable in both suppressed and unsuppressed conditions. These observations are consistent with previous studies concerning BM-MSCs, which reported undetectable levels of IL-10 in the supernatants of BM-MSC–suppressed MLRs. Furthermore, we found similar amounts of TGF-β in MLR supernatants that had been performed with or without hUCB-MSCs. These observations agree with those of Le Blanc et al. [5]. Consequently, we did not determine whether monoclonal antibodies against TGF-β or IL-10 would abrogate the immunosuppressive effect of hUCB-MSCs on MLR.

It has been also known that immunosuppressive molecules commonly produced by PBMC such as indoleamine 2,3-dioxygenase, prostaglandin E_2 and nitric oxide are involved in the inhibitory effect of hUCB-MSCs on T-lymphocyte proliferation [15,29,43,44]. Moreover, it has been recently reported that the soluble HLA-G secreted by BM-MSCs inhibited cytotoxic T-lymphocyte activity [45]. In our unpublished data, we have failed to block suppression of PBMC proliferation in the MLR cultures by adding specific blocker for indoleamine 2,3-dioxygenase (blocked with 1-methyl-tryptophan), prostaglandin E_2 (blocked with NS-398) and nitric oxide (blocked with N^{ω}-nitro-L-arginine). These results are considered as scientific evidences that the immunosuppressive effect of hUCB-MSCs is not related with these molecules.

In summary, although the mechanisms for the immunological effect of hUCB-MSCs were not addressed in the present study, our observation show that hUCB-MSCs are unable to act as APCs or antigens, moreover these cells are able to act as immune suppressor in allogenic lymphocyte responses. The ability of hUCB-MSCs to inhibit allogeneic responses appears to be mediated by soluble factors which are secreted by interaction between hUCB-MSCs and allogeneic PBMCs.

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