CHAPTER 8

Generation of mesenchymal stromal cells in the presence of platelet lysate: a phenotypical and functional comparison between umbilical cord blood- and bone marrow-derived progenitors


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Summary

Mesenchymal stromal cells (MSCs) are employed in diverse clinical settings in order to modulate immune response. However, relatively little is known about the mechanisms responsible for their immunomodulatory effects, which could be influenced by both cell source and culture conditions.

We tested the ability of a 5% platelet lysate (PL)-supplemented medium to support isolation and *ex-vivo* expansion of MSCs from full-term umbilical-cord blood (UCB). We also investigated the biological/functional properties of UCB-MSCs, in comparison with PL-expanded bone marrow (BM)-MSCs.

Success rate of MSC isolation from UCB was in the order of 20%. UCB-MSCs exhibited the typical morphology, immunophenotype and differentiation capacity. Although they have a low clonogenic efficiency, UCB-MSCs may possess high proliferative potential. The genetic stability of UCB-MSCs was demonstrated by a normal molecular karyotype; in addition, these cells do not express hTERT and telomerase activity, express p16<sup>ink4a</sup> protein and do not show anchorage-independent cell growth. Concerning alloantigen-specific immune response, UCB-MSCs were able to: i) suppress T- and NK-lymphocyte proliferation, ii) decrease cytotoxic activity and iii) only slightly increase IL-10, while decreasing IFN<sub>γ</sub> secretion, in mixed lymphocyte culture (MLC) supernatants. While an IDO-specific inhibitor did not reverse MSC-induced suppressive effects, a PGE2-specific inhibitor hindered the suppressive effect of both UCB- and BM-MSCs on alloantigen-induced cytotoxic activity. Both UCB- and BM-MSCs expressed HLA-G.

UCB- and BM-MSCs may differ in terms of clonogenic efficiency, proliferative capacity and immunomodulatory properties; these differences may be relevant for clinical application.
Introduction

In addition to hematopoietic stem cells (HSCs), the bone marrow (BM) also contains mesenchymal stromal cells (MSCs). These latter cells exhibit multilineage differentiation potential and are endowed with immunomodulatory properties that have been demonstrated both in vitro and in vivo. In the setting of hematopoietic stem cell transplantation (HSCT), ex vivo expanded MSCs have been employed in view of both their immunomodulatory activity and their ability to support hematopoiesis. In particular, in a phase I/II multicenter study, MSCs proved safe and effective when administered to 55 patients with severe, steroid-refractory acute graft-versus-host disease (GvHD). Moreover, in another phase I/II study, co-transplantation of MSCs, together with T-cell depleted peripheral blood stem cells (PBSCs), overcame the problem of graft failure in 14 children given HSCT from an HLA-haploidentical family donor. Similarly, co-transplantation of MSCs and umbilical cord blood (UCB) stem cells is under investigation. Recently, in view of their immunosuppressive properties, as well as their role in tissue repair and trophism, MSC infusion has also been proposed as a novel approach for reparative/regenerative medicine in the treatment of autoimmune disorders and chronic inflammatory diseases.

Although BM represents the most commonly employed source of MSCs for both experimental and clinical use, MSCs have been isolated from other sources, including adipose tissue, placenta, amniotic fluid, fetal tissues and UCB. Notably, it has not always been possible to grow MSCs from UCB and in many cases the yield was low. In particular, the presence of mesenchymal progenitors in full-term UCB has been questioned in recent years by many groups, whose attempts to obtain MSC have either failed or yielded low numbers. In fact, the frequency of MSCs in UCB is very low and, in fetal blood, it has been reported to decline with gestational age from about 1/10^6
mononuclear cells in first trimester fetal blood to 0.3/10^6 mononuclear cells in term cord blood.\cite{20} Despite this limitation, Bieback et al. have demonstrated that, when critical parameters for the selection of ‘good quality’ term UCB units are employed, MSCs can be successfully isolated in more than 60% of the processed cord blood units.\cite{22}

MSCs have been mainly expanded in vitro in the presence of fetal calf serum (FCS). Cells thus obtained, when infused into patients, may potentially carry the risks for both transmitting zoonoses and causing immune reactions directed against residual animal proteins. For these reasons, culture supplements devoid of animal components, such as platelet lysate (PL), have been tested in recent years for the isolation and expansion of MSCs.\cite{26-28} In particular, our group previously demonstrated that a 5% PL-supplemented medium can support large-scale, ex-vivo expansion of BM-derived MSCs (BM-MSCs) and that this medium is superior to 10% FCS in terms of both clonogenic efficiency and proliferative capacity.\cite{27} Conversely, BM-MSCs expanded in PL seem to be endowed with relatively low immunosuppressive activity, as compared with BM-MSCs grown using FCS as culture supplement.\cite{27}

The aim of this study was to test the ability of a PL-supplemented medium to support the generation and ex vivo expansion of MSCs from full-term UCB (UCB-MSCs), as well as to characterize these latter cells for their biological and functional properties, in comparison with PL-expanded BM-MSCs. In particular, we focused on the investigation of both the genetic stability and the immunoregulatory function, exerted on alloantigen-specific immune response, by UCB-MSCs. Moreover, we have evaluated the possible mechanisms at the basis of UCB-MSC immunosuppressive effect.
Design and methods
UCB unit collection and selection
UCB units were collected after full-term delivery and stored at the Cord Blood Bank of our Hospital after obtaining signed written informed consent. The Institutional Review Board of the Fondazione IRCCS Policlinico San Matteo approved the study. Citrate Phosphate Dextrose-A, 20 ml, was employed as anticoagulant in the collection bags. Whole UCB units were employed for MSC generation. Ten fresh UCB units (median volume 45 ml, range 40-60) were selected according to the following criteria: 1) total nucleated cell (TNC) count ranging from 500 to 750 x10⁶; 2) manipulation performed within 24 hours from delivery; 3) overall cell viability greater than 75%, investigated by 7-amino-actinomycin D (7-AAD) and Aldefluor (ALDH). Samples obtained from UCB units before and after mononuclear cell (MNC) separation were analyzed by a Becton Dickinson FACSCanto instrument (BD BioSciences, San Jose, CA, USA), FACSDiva software 5.0, according to the EWGCCA guidelines (European Working Group on Clinical Cell Analysis). Cell viability was determined using the 7-AAD dye test (Molecular Probes, Eugene, OR, USA) within the context of expression of surface markers identified by fluorochrome-labeled antibodies. The following monoclonal antibodies were used: anti-CD34 phycoerythrine (PE), anti-CD45 peridinin chlorophyll protein (PerCP), anti-CD133 allophycocyanin (APC) (all from BD BioSciences) and ALDH (StemCell Technologies, Vancouver, Canada). ALDH was detected using the green fluorescence channel following manufacturer’s instructions.

PL preparation
PL was prepared as previously described. In brief, aliquots of 50 ml platelet-rich plasma, collected by apheresis, were obtained from ten healthy volunteers at the Transfusion Service of our Hospital. All apheresis
products contained a minimum of $5 \times 10^{11}$ platelets (PLTs). Written informed consent from donors was always obtained and all apheresis products were screened for infectious agents according to National regulations. Immediately after collection, PLT apheresis products were frozen at -80°C and subsequently thawed at 37°C to obtain the release of PLT-derived growth factors. Heparin (5000 UI) was added to PLT bags to avoid gel formation. Apheresis products were centrifuged three times at 900 g for 30 minutes to eliminate PLT bodies. Finally, PL preparations obtained through this procedure were pooled in a single culture supplement to be used for the generation and expansion of UCB-MSCs.

Isolation and culture of UCB- MSCs
MNCs were isolated from the 10 UCB units by density gradient centrifugation (Ficoll 1.077 g/ml; Lymphoprep, Nycomed Pharma, Zurich, Switzerland) after 1:1 dilution with Dulbecco’s phosphate buffered saline (D-PBS; Euroclone, Celbio, Milan, Italy) and plated in non-coated 75-175 cm² polystyrene culture flasks (Corning Costar, Celbio) at a density of 160,000/cm² in complete culture medium: Mesencult (StemCell Technologies) supplemented with 2 mM L-glutamine, 50 µg/ml gentamycin (Gibco-BRL, Life Technologies, Paisely, UK) and 5% PL. This concentration of PL was chosen on the basis of results previously obtained with BM-MSCs. Cultures were maintained at 37°C, in a 5% CO₂ humidified atmosphere. After 48 hours, non-adherent cells were discarded; culture medium was replaced twice a week. Upon the appearance of MSC-like clones, cells were harvested using Trypsin (Sigma-Aldrich, Milan, Italy), re-plated for expansion at a density of 4,000 cells/cm² and propagated in culture until reaching a senescence phase. Senescent cells were monitored for up to eight weeks, in order to reveal any change in morphology and/or proliferation rate. Cell growth was
analyzed by direct cell counts and cumulative population doublings (PD) were determined. Number of PD were calculated using the formula \( \log_{10}(N)/\log_{10}(2) \) where \( N= \text{cells harvested/cells seeded} \) and results were expressed as cumulative PD. \(^{31}\)

UCB-MSC multilineage differentiation potential
The adipogenic and osteogenic differentiation capacity of UCB-MSCs was determined at passage (P) 2, as previously described. \(^{27}\) To detect osteogenic differentiation, cells were stained for alkaline phosphatase (AP) activity using Fast Blue (Sigma-Aldrich) and for calcium deposition with Alzarin Red (Sigma-Aldrich). Adipogenic differentiation was evaluated through the morphological appearance of lipid droplets stained with Oil Red O (Sigma-Aldrich).

Immunophenotypic characterization of UCB-MSCs
FITC, PE, PerCP-Cy5.5 monoclonal antibodies specific for the following antigens were employed: 1) CD45 (clone HI30), CD14 (clone MΦP9), CD34 (clone 581), CD13 (clone L138), CD80 (clone L307.4), CD31 (clone L133.1), HLA A-B-C (clone G46-2.6), HLA-DR (clone G46-6[L243]), CD90 (clone 5E10), CD73 (clone AD2) (all from BD Biosciences), CD105 (clone SN6; Serotec, Kidlington, Oxford, UK), HLA-G (clone MEM-G/9; Exbio, Praha, CZ) for the assessment of MSC surface phenotype; 2) CD3 (clone SK7), CD4 (clone SK3), CD8 (clone SK1), CD56 (clone NCAM16.2), CD25 (clone 2A3), CD152 (CTLA4; clone BNI3), Foxp3 (clone PCH101; eBioscience, San Diego, CA, USA) for evaluation of lymphocyte subsets. Appropriate isotype-matched controls (BD Bioscience, eBioscience) were included. Intracellular staining for CD152 (CTLA4), Foxp3 and HLA-G was performed as previously described. \(^{27,32}\) Two-color or three-color direct immune fluorescence cytometry was
performed with FACScalibur flow cytometer (BD Biosciences) and data calculated using CellQuest software (BD Biosciences).

Telomerase activity detection assay and reverse transcription (RT)-PCR analysis of the human telomerase reverse transcriptase (hTERT)
Telomerase activity was measured by the polymerase chain reaction (PCR)-based telomeric-repeat amplification protocol (TRAP) using the TRAPEze kit (Intergen Company, FLA, USA) on samples containing 0.6 and 6.0 µg of protein. Protein extract from a telomerase-positive human cell line (JR8) was used as a positive control. A sample was scored as telomerase activity-positive when positive TRAP results were obtained from at least one protein concentration.

For hTERT assessment, total cellular RNA was extracted from frozen samples with the RNeasy micro kit (Qiagen GmbH). A 0.5 µg aliquot from each sample was reverse-transcribed by using the RT-PCR Core kit (Applied Biosystems, Foster City, CA, USA) with random hexamers, and the resultant cDNA was then amplified with the same kit. Amplification of hTERT cDNA was obtained as previously described.

Western immunoblotting
MSCs were lysed on ice in lysis buffer. Total cellular lysates were separated on 15% SDS-polyacrylamide gel and were transferred onto Hybond ECL nitrocellulose membranes (GE Healthcare Europe GmbH, Cologno Monzese, Italy). Nitrocellulose membranes were blocked in PBS-Tween 20 with 5% skim milk, first incubated overnight with the primary antibody specific for p16ink4a (Abcam Inc., Cambridge, MA, USA) and then with the secondary peroxidase-linked whole antibody (GE Healthcare Europe). Bound antibody was detected using the enhanced chemiluminescence Western blotting detection system (GE Healthcare Europe).
DU145 human prostate cancer and U2OS human osteogenic sarcoma cell lines were used as positive and negative controls for p16<sup>ink4a</sup> expression, respectively.

**Clonogenic assay**

Single-cell suspensions of 100,000-1,000 cells/ml in complete medium and 0.3%(w/v) agarose were plated in triplicate in 35 mm culture dishes, over chilled 0.6% agarose feeder layers. Cultures were incubated at 37°C in 5% CO<sub>2</sub> humidified atmosphere and examined at 14 days after plating under an inverted microscope.

**Molecular karyotyping**

Molecular karyotyping of UCB-MSCs at early (P3) and late (P8-9) passages was performed through array-comparative genomic hybridization (array-CGH) with the Agilent kit 44B (Human Genome CGH Microarray, Agilent Technologies, Santa Clara, CA, USA), as previously described. A pool of characterized genomic DNA (Human Genomic DNA Male, Promega, Madison, WI, USA) was used as control DNA for all experiments. Quality control parameters for every experiment were evaluated using the CGH Analytics Agilent software-QC tool.

**Mixed lymphocyte cultures (MLCs) and cytotoxicity assay**

Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque density gradient from heparinized PB samples from healthy volunteers. Primary MLCs were set up according to previously described methods. Briefly, non-irradiated, “third-party” UCB-MSCs, allogeneic to both responder (R) and irradiated stimulator (S) PBMCs, were added at a R to MSC ratio of 10:1. Control MLC (ctrl-MLC) was set-
up in the absence of UCB-MSCs. UCB-MSCs employed in MLC experiments had been harvested and cryopreserved at P3. The total number of PBMCs recovered after 10-day MLC was counted in vitality with Trypan Blue (Sigma-Aldrich). Then, recovered cells were analyzed by flow cytometry and percentage of lymphocyte subsets (CD3^+CD4^- and CD3^+CD8^- T-cells, as well as CD3^-CD56^- NK-cells) was determined. Number of lymphocyte subsets per ml culture was, therefore, calculated and compared with the initial number of cells (day 0). Differentiation of regulatory T cells (Treg) was evaluated by measuring the percentage of CD4^-CD25^- and CD4^-CD25^bright T lymphocytes, together with the expression of Foxp3 and CTLA4 on CD4^-CD25^- lymphocytes. Alloantigen-induced cell-mediated cytotoxic activity was tested in a 5-hour 51Cr-release assay, as previously described. Results are expressed as percent specific lysis of target cells. 51Cr-labeled target cells included PHA-activated S-PBMCs (S-PHA) and the same lots of UCB-MSCs that had been added to MLCs.

To evaluate the possible involvement of indoleamine 2,3-dioxygenase (IDO) and/or prostaglandin E2 (PGE2) in the immunosuppressive effect of PL-expanded UCB-MSCs, we set up MLCs in which, in some wells, specific inhibitors of IDO activity (1-Methyl-Tryptophan, 1-M-Trp, Sigma-Aldrich), or of PGE2 (NS-398, Cayman Chemicals, Irvine, CA) were added to MSCs/MLC co-culture at a concentration of 1 mM and 5 µM, respectively. In this set of experiments, BM-MSCs, expanded in 5% PL-supplemented medium, were employed as a control for UCB-MSCs. Cell counts per ml of culture recovered after 10-day MLC as compared to day 0, and alloantigen-induced cell-mediated cytotoxic activity (using 51Cr-labeled S-PHA as target cells) were evaluated for each culture condition. After 72-hour culture, supernatants were collected for the evaluation of IDO activity and PGE2 quantification.
Detection of IDO activity

IDO activity was evaluated by quantifying tryptophan (tryp) and kynurenine (kyn) concentrations in 72-hour culture supernatants with high-performance liquid chromatography (HPLC), by using the HPLC pump, model SCL-10 VP (Shimadzu, Kyoto, Japan). For separation, pre-columns (cartridge holder and guard cartridge) from Phenomenex (Torrance, CA, USA) and reverse-phase C18 (octyl) columns (250 mm length, 460 mm internal diameter, 5 micron grain size) from Beckman-Coulter (Milan, Italy), were used. The incorporated UV/VIS detector model UV-SPD-M10 VP (Shimadzu) was employed for detection of both kyn and nitrotyrosine at a wavelength of 360 nm. Tryp was detected by a fluorescence detector (Shimadzu, Model RF-535) at 285-nm excitation wavelength and an emission wavelength of 365-nm. Samples were prepared as previously reported.37 L-tryp, L-kyn, 3-nitro-L-tyrosine, trichloroacetic acid, potassium phosphate and acetonitrile for the HPLC elution buffer were obtained from Sigma-Aldrich. All chemicals used were of analytical grade. Peak area counts were used to calculate concentrations (EZStart software, version 7.3). Tryp and kyn were referred to nitrotyrosine. The reproducibility of the system was controlled by nitrotyrosine counts and variations less than 5% were tolerated.

Measurement of cytokines and HLA-G by ELISA

The concentrations of interferon-gamma (IFN-γ), interleukins (IL)10, IL6, IL12, IL7, IL2, IL15 and transforming growth factor beta (TGFβ) in MLC supernatants after 12, 24, and 48 hours, were quantified by ELISA using monoclonal antibody pairs (Pierce Endogen, Rockford, IL), as previously described.27 PGE2 levels were evaluated using a commercially available ELISA (R&D System, Minneapolis, MN), according to manufacturer’s
instructions. The concentration of soluble HLA-G (sHLA-G) in culture supernatants was quantified by ELISA (sHLA-G ELISA, Exbio); clone MEM-G/9 was employed as anti-HLA-G capture antibody.

Statistical analysis
The non-parametric Kolmogorov-Smirnov test for independent samples was performed for the comparison of cumulative cell counts at P0 and P5 of UCB- and BM-MSCs. Due to the small size of the groups, the maximum significance value obtained was P = 0.1.

Results
Characterization of UCB-MSCs
Ten UCB units obtained at full-term delivery were selected according to the ‘quality’ criteria described in the Design and Methods section. MNCs were separated, plated as P0 and cultured in medium supplemented with 5% PL. The cultures at P0 were monitored for up to four weeks to allow identification of MSC clones in the flasks. Two out of the ten UCB units (20%; UCB3 and UCB6) gave rise to three and four MSC-like clones after 15- and 14-day culture, respectively. In the remaining 8 UCB units, we did not obtain MSCs, despite the long observation time in culture. MSC clones from UCB3 (UCB3-MSCs) and UCB6 (UCB6-MSCs) were expanded ex vivo and characterized for their morphology, differentiation potential, immunophenotype, proliferative capacity, biosafety profile and immunoregulatory properties.

UCB-MSCs displayed the typical spindle-shaped morphology, similar to that of BM-MSCs expanded in the same culture medium (see Supplementary data, Figure 1s A). As already observed for BM-MSCs expanded in the presence of PL, UCB-MSCs required only 2-3 minutes incubation with trypsin at room temperature to obtain the complete
detachment of cells from the flasks, whereas 5–8 min at 37°C are usually necessary to harvest MSCs grown in the presence of 10% FCS. UCB-MSCs were induced to differentiate into osteoblasts and adipocytes and examined for this capacity by histological staining. The cells were able to differentiate into osteoblasts, as demonstrated by the histological detection of AP activity (purple reaction) and calcium deposition stained with Alizarin Red (Figure 1A), and into adipocytes, as revealed by the formation of lipid droplets, stained with Oil Red O (Figure 1B).

The surface phenotype of UCB-MSCs was analyzed by flow cytometry every two passages (at P1, P3, P5 and so on) and showed the typical panel of MSC markers, in agreement with previous reports. In particular, by the second passage, contamination with hematopoietic cells was no longer detectable and more than 98% UCB-MSCs were positive for CD90, CD73, CD105 and CD13 surface antigens and negative for CD34, CD45, CD14, CD80, CD31 molecules. The expression of HLA-DR was always less than 2%, whereas HLA-class I was uniformly present on UCB-MSCs (more than 98% of positive cells). See Supplementary data, Figure 1s B.

Calculated cumulative cell counts from P0 to P5 for UCB3- and UCB6-MSCs, together with counts for BM-MSCs cultured in the presence of 5% PL, for comparison are shown in Figure 1C. UCB-MSCs yielded similar numbers at P5 (1.62 x 10^9 MSCs for UCB3 and 2.02 x 10^9 MSCs for UCB6), as compared with BM-MSCs (3.2 ± 1.02 x 10^9 MSCs as mean ± SD of 8 BM donors; P>0.1), even when starting with low cell numbers. In fact, the number of UCB-MSCs collected after trypsinization at P0 was 0.2 x 10^6 for both units, whereas the mean of MSCs from eight BM donors at the same passage was 2.6 ± 0.58 x 10^6 cells (P<0.1). UCB-MSC growth was also evaluated in terms of PD; cumulative PD from P1 to P5 were as follows: 12.9 for UCB3-MSCs, 13.3 for UCB6-MSCs, 10.9 for BM-MSCs (mean of 8 BM donors). Moreover, because of the extremely low
frequency of clones in case of UCB-MSCs, a comparison of the colony-
forming unit-fibroblast (CFU-F) assay with BM-MSCs could not be
performed. The median time to reach 80% confluence for all passages from
P1 to P5 was 6.5 days for both UCB3- and UCB6-MSCs, as compared with
5.5 days in case of BM-MSCs expanded in the presence of 5% PL.27 Taken
together, these results suggest that UCB-MSCs, although displaying a
rather low clonogenic efficiency, possess high proliferative potential.
UCB-MSCs were cultured continuously in vitro until reaching senescence
and monitored daily for up to eight weeks, in order to investigate their
propensity to undergo spontaneous transformation in vitro. UCB3-MSCs
and UCB6-MSCs displayed a progressive decrease in their proliferative
capacity until they reached a senescence phase after 73- and 81-days
culture at P9 and P10, respectively. The cells maintained their typical
spindle-shaped morphology, differentiation capacity and surface markers
throughout the culture period.
Figure 1. A) Osteogenic differentiation capacity of UCB-MSCs, as compared with BM-MSCs (right panel). Shown are representative photographs from UCB3-MSCs at P3 and BM-MSCs from donor 2. Magnification x 20. B) Adipogenic differentiation capacity of UCB-MSCs, as compared with BM-MSCs (right panel). Shown are representative photos from UCB3-MSCs at P3 and BM-MSCs from donor 2. Magnification x 20. C) Calculated cumulative cell counts from P0 to P5 of UCB3- and UCB6-MSCs, as compared with BM-MSCs cultured in the presence of 5% PL-additioned medium (mean of 8 BM-donors) and already reported.

Biosafety profile of UCB-MSCs - Lack of telomerase expression in UCB-MSCs during long-term in vitro culture

MSC cultures from both UCB3 and UCB6 were tested at two different in vitro passages (P4 and P8) for the expression of telomerase catalytic activity by the TRAP assay. TRAP results failed to evidence the presence
of enzyme catalytic activity in all tested samples (Figure 2A). To gain insights into the molecular mechanisms responsible for the repression of telomerase activity in UCB-MSCs, we assessed the expression of the hTERT gene, which codes for the catalytic component of human telomerase, in the same cultures screened for telomerase activity. RT-PCR failed to evidence the expression of hTERT mRNA (see Supplementary data, Figure 2s), thus indicating that the absence of telomerase activity in cultured UCB-MSCs was ascribable to lack of hTERT gene transcription.

MSC cultures from both UCB3 and UCB6 were found to express p16ink4a protein, as detected by Western immunoblotting, at all tested in vitro passages (Figure 2B). In addition, UCB3-MSCs (P3 and P6) and UCB6-MSCs (P4 and P11) did not show anchorage-independent cell growth, since they failed to generate colonies when plated in double-layer agarose (data not shown).

UCB-MSCs were also tested for their genomic assets; in particular UCB3- and UCB6-MSCs were investigated at early passages (P3) and at later passages in culture (P8 and P9, respectively) by means of array-CGH (see Figure 2C for UCB6-MSCs at P3 and P9). The results of array-CGH experiments revealed that UCB-MSCs expanded in vitro do not show unbalanced chromosomal rearrangements; in fact, deletions or duplications of genomic material, excluding Copy Number Variations (CNVs) constitutionally present, were absent in the UCB-MSCs studied.
Figure 2. (A) Telomerase activity of UCB3- and UCB6-MSC cultures at P4 and P8. Telomerase activity was detected by the TRAP assay using two protein concentrations. The telomerase-positive cell line JR8 was used as a positive control. The blank represents a negative control to which no protein extract was added. The lane labelled +RNAse represents an additional negative control containing 0.6 μg cell extract of JR8 pretreated with RNAse. The location of the internal amplification standard (ITAS) is reported. (B) P16^{ink4a} expression in UCB3- and UCB6-MSC cultures at different in vitro passages as evaluated by Western immunoblotting. The p16^{ink4a}-positive DU145 cell line and the p16^{ink4a}-negative U2OS cell line were used as positive and negative controls, respectively. (C) Representative array-CGH profiles of chromosomes 1 of UCB6-MSCs at P3 (left, red profile) and P9 (middle, blue profile). The array-CGH profiles are linear and perfectly overlapping (right), thus demonstrating that in vitro expanded UCB- MSCs do not show unbalanced chromosomal rearrangements.
Immune regulatory properties of UCB-MSCs

In a first set of experiments, the immune regulatory capacity of UCB-MSCs was evaluated by assessing UCB-MSC interaction with alloantigen-specific immune response, elicited \textit{in vitro} in primary MLC. In agreement with previously reported studies, we observed that UCB-MSCs were able to strongly inhibit alloantigen-induced lymphocyte proliferation (Figure 3A). A strong inhibitory effect was evident on whole T lymphocytes and their subsets (CD3+, Figure 3B; CD3+CD4+, Figure 3C; CD3+CD8+, Figure 3D), as well as on NK lymphocytes (CD3\textsuperscript{neg}CD56+, Figure 3E). The percentage of CD4+CD25\textsuperscript{+} T cells considerably increased, as compared with day 0, after 10-day primary MLC, both in the presence and absence of UCB-MSCs, even though the percentage of this subset was higher in UCB-MSC/MLC as compared with ctrl-MLC (Figure 3F). In an attempt to discriminate CD4+CD25\textsuperscript{+} Tregs from conventional, recently activated CD4+CD25\textsuperscript{+} T lymphocytes, the degree of expression of CD25 (CD4+CD25\textsuperscript{bright} T cells, Figure 3G), as well as of CTLA4 and FoxP3 molecules was evaluated within the CD4+CD25\textsuperscript{T} cell subset (Figure 3H). We found a higher percentage of CD4+CD25\textsuperscript{bright} and CTLA4+ cells in UCB-MSC/MLCs as compared with ctrl-MLCs, while the percentage of FoxP3+ was lower in the presence than in the absence of UCB-MSCs (Figure 3H).
Figure 3. Immune modulatory effect of UCB-MSCs on the expansion of T and NK-lymphocyte subsets, induced by allogeneic stimulus. Recovery of total number of lymphocytes (A), CD3⁺ (B), CD3⁺CD4⁺ (C), CD3⁺CD8⁺ (D), CD3⁺negCD56⁺ NK cells (E), CD4⁺CD25⁺ (F), CD4⁺CD25bright (G) T-lymphocytes subsets and with respect to the initial number (white columns), was assessed after 10-days primary culture (gray columns). Percentages of CTLA4⁺ and Foxp3⁺ cells were calculated on gated CD4⁺CD25⁺ T cells (H). MLC was performed in the absence (Ctrl-MLC) or presence of third-party MSCs derived from UCB3 (MLC+UCB3-MSCs) or UCB6 (MLC+UCB6-MSCs). Results are expressed as the number of cells/ml in panels A-E and as percent of positive cells in panels F-H.
The cytokine production kinetics induced in vitro by allogeneic stimulus documented that addition of UCB-MSCs: (i) inhibits IFN\(\gamma\) secretion; (ii) strongly increases IL-6 secretion in MLC supernatants (see Supplementary data, Table 1s). Differently from what shown in a previous study where the addition of BM-MSCs grown in 5%PL to the MLC substantially increased IL-10 secretion,\(^{27}\) UCB-MSCs only slightly enhanced IL-10 secretion in 24-hour MLC supernatants; (iii) IL-2, IL-7, IL-12, IL-15 were undetectable in all experimental conditions.

In order to assess the effect of UCB-MSCs on alloantigen-induced cytotoxic activity, effector cells recovered after 10-day MLC were tested in a cytotoxicity assay, employing as targets either MLC-stimulator PHA– blasts (Figure 4A) or third-party UCB-MSCs from the same lots added to the MLC at day 0 (Figure 4B, Figure 4C). Results obtained in all experiments showed a striking inhibitory effect mediated by both lots of UCB-MSCs (UCB3- and UCB6-MSCs) on alloantigen-induced cytotoxic activity.
Figure 4. Immune modulatory effect of third-party UCB-MSCs on cell-mediated cytotoxic activity induced by an allogeneic stimulus (MLC). $^{51}$Cr-labeled target cells included PHA-activated S-PBMCs (S-PHA) (A) and the same UCB3-MSCs (B) and UCB6-MSCs (C) added to MLCs. Effector to target (E:T) ratios ranged between 20:1 and 0.6:1. Results are expressed as percent specific lysis of target cells.

With the aim of better understanding the biological mechanisms responsible for the immunosuppressive effect exerted by UCB-MSCs on alloantigen-induced immune response, in a second set of experiments, MLCs were carried out in the presence of IDO- or PGE2-specific inhibitors. As control, the same experimental conditions were also tested either in the presence or in the absence of BM-MSCs cultured in PL-
supplemented medium. Moreover, the constitutive expression of intracellular, membrane and soluble HLA-G (iHLA-G, mHLA-G, sHLA-G, respectively) was evaluated in UCB-MSCs, as well as in BM-MSCs harvested at P3.

In terms of alloantigen-induced lymphocyte proliferation, neither IDO-specific inhibitor, nor PGE2-specific inhibitor were able to reverse the MSC-induced suppressive effect (data not shown). By contrast, when alloantigen-induced cytotoxic activity was evaluated, a clear-cut effect of the PGE2-specific inhibitor was observed; indeed, addition of PGE2-specific inhibitor to MLC was able to reverse the suppressive effect exerted by both UCB-MSCs and BM-MSCs on alloantigen-specific cytotoxic activity, even though this reagent was apparently more effective when BM-MSCs were employed (see Figure 5). On the contrary, IDO-specific inhibitor was not able to reverse the suppressive effect exerted by both UCB-MSCs and BM-MSCs on alloantigen-specific cytotoxic activity (Figure 5).

Figure 5. Involvement of IDO and PGE2 in UCB- and BM-MSC mediated suppression of cell-mediated cytotoxic activity induced by an allogeneic stimulus (MLC). 51Cr-labeled target cells were PHA-activated S-PBMCs (S-PHA). Effector to target (E:T) ratio ranged between 10:1 and 1:1; results are expressed as % lysis.
specific lysis of target cells. BM-derived MSCs (BM1-MSCs and BM2-MSCs) expanded in PL-supplemented medium were employed as a control. IDO or PGE inhibitors (inhib) were added to the MLC as described in the “Design and Methods” section. Results obtained at the E:T of 1:1 for Exp. 1 (broken bar) and 10:1 for Exp. 2 (full bar) are shown, UCB3-MSCs and BM1-MSCs were employed in Exp. 1 whereas UCB6-MSCs and BM2-MSCs were tested in Exp 2.

As shown in Table 1, PGE2 concentrations were considerably higher in supernatants of MLCs carried out in the presence of BM-MSCs than in the presence of UCB-MSCs. These data might explain the striking effect of a PGE2-specific inhibitor observed in MLCs performed using BM-MSCs (Figure 5). The presence of PGE2-specific inhibitor considerably decreased PGE2 secretion in MLC experiments carried out with either BM-MSCs or UCB-MSCs (Table 1). Interestingly, a striking increase in PGE2 secretion was observed in the presence of the IDO-specific inhibitor (Table 1).

This observation may explain why IDO-specific inhibitor was unable to reverse the suppressive effect exerted in vitro by MSCs on alloantigen-specific cytotoxic activity (see Figure 5). In fact, the striking increase in PGE2 secretion, induced by the presence of IDO-specific inhibitor, could exert an effective suppressor function on alloantigen-specific cytotoxic activity, thus masking the effect of the IDO-specific inhibitor on IDO-mediated suppressive activity.
Table 1. Concentration of PGE2, Tryptophan (tryp) and Kynurenine (kyn) in MLC-supernatants

Concentrations of PGE2, tryptophan and kynurenine were quantified in MLC-supernatants collected after 72-hours culture in the absence (ctrl-MLC) or presence of BM-MSCs (MLC+BM-MSCs) or UCB-MSCs (MLC+UCB-MSCs) and in the absence or presence of IDO inhibitor (MLC+MSCs+IDO inhib) or PGE2 inhibitor (MLC+MSCs+PGE2 inhib). PGE2 levels are reported as pg/ml. Tryptophan and kynurenine levels are reported in µM as mean ±SD of three repeated runs for the same samples. Two independent experiments are presented, in which UCB3-MSCs and BM1-MSCs from donor 27 (Exp 1) and UCB6 MSCs and BM2-MSCs from donor 527 (Exp 2) were tested.*NA = not assessable. The tryptophan value could not be evaluated in the experimental condition as its fluorescence signal was superimposed by the fluorescence signal of the PGE2 inhibitor.

<table>
<thead>
<tr>
<th></th>
<th>PGE2</th>
<th>Tryp</th>
<th>kyn</th>
<th>PGE2</th>
<th>tryp</th>
<th>Kyn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-MLC</td>
<td>117</td>
<td>232±15</td>
<td>≤0.01</td>
<td>45</td>
<td>193±11</td>
<td>≤0.01</td>
</tr>
<tr>
<td>MLC+BM-MSCs</td>
<td>1440</td>
<td>164±10</td>
<td>1.54±0.4</td>
<td>700</td>
<td>95±4</td>
<td>1.6±0.21</td>
</tr>
<tr>
<td>MLC+BM-MSCs+IDO inhib</td>
<td>7500</td>
<td>215±15</td>
<td>≤0.01</td>
<td>1500</td>
<td>220±20</td>
<td>≤0.01</td>
</tr>
<tr>
<td>MLC+BM-MSCs+PGE2 inhib</td>
<td>234</td>
<td>*NA</td>
<td>≤0.01</td>
<td>35</td>
<td>*NA</td>
<td>≤0.01</td>
</tr>
<tr>
<td>MLC+UCB-MSCs</td>
<td>180</td>
<td>54±1</td>
<td>4.53±0.1</td>
<td>60</td>
<td>184±12</td>
<td>3.06±0.76</td>
</tr>
<tr>
<td>MLC+UCB-MSCs+IDO inhib</td>
<td>270</td>
<td>200±14</td>
<td>≤0.01</td>
<td>156</td>
<td>240±21</td>
<td>≤0.01</td>
</tr>
<tr>
<td>MLC+UCB-MSCs+PGE2 inhib</td>
<td>60</td>
<td>*NA</td>
<td>≤0.01</td>
<td>20</td>
<td>*NA</td>
<td>≤0.01</td>
</tr>
</tbody>
</table>
IDO activity was evaluated in MLCs supernatants, as indirect evidence of IDO-mediated tryp degradation. Results reported in Table 1 demonstrate the presence of detectable levels of kyn only in culture supernatants recovered from MLCs carried-out in the presence of either UCB- or BM-MSCs. This finding indicates that IDO-activity is dependent on the interaction between MSCs and cells active in MLC. Kyn was more abundant in the presence of UCB-MSCs, as compared with BM-MSCs. As expected, kyn was undetectable in culture supernatants collected from MSC/MLCs carried out in the presence of IDO-specific inhibitor; however, kyn was also undetectable in culture supernatants collected from MSC/MLCs carried-out in the presence of PGE2-inhibitor. The latter observation is in accordance with recently published data demonstrating that PGE2 is able to up-regulate IDO activity in dendritic cells. The inter-relationship among PGE2 secretion and IDO activation could also hypothetically explain why, even though PGE2 levels detected in MLC+BM-MSC+PGE2 inhibitor culture condition of Experiment 1 is comparable to that detected in Ctrl-MLC (see Table 1), alloantigen–induced cytotoxic activity is strikingly higher in the former than in the latter culture condition (see Figure 5).

Evaluation of constitutive HLA-G-expression in MSCs showed that UCB-MSCs displayed a higher percentage of mHLA-G+ cells as compared with BM-MSCs, while more than 95% of both UCB-MSCs and BM-MSCs were iHLA-G+. UCB-MSCs and BM-MSCs secreted similar amount of sHLA-G in culture supernatants (see Supplementary data, Table 2s).

Discussion
In this study, we have demonstrated that MSCs generated from full-term UCB in a culture medium containing 5% PL are similar to BM-MSCs in terms of morphology, differentiation potential, immunophenotype and
proliferative ability. On the contrary, they differ for clonogenic efficiency. In spite of their high proliferative potential, UCB-MSCs do not show any signs of *in vitro* transformation. Moreover, in view of our findings, although obtained in a limited number of UCB-MSC samples, UCB-MSCs are apparently as efficient as BM-MSCs when tested in terms of capacity of suppressing an alloantigen-specific immune response (see Figure 3s and Figure 4s).

Bieback *et al.* have demonstrated that MSCs can be isolated from full-term UCB with a success rate greater than 60%, when critical parameters for the selection of ‘good quality’ units are employed. These critical parameters included: a storage time shorter than 15 hours, a net UCB volume greater than 33 ml; a MNC count greater than $1 \times 10^8$ and absence of clots or signs of hemolysis. The quality criteria adopted in our study were less stringent in terms of storage time and UCB cellularity, but included a viability test on the manipulated cells. Despite this, the 5% PL-supplemented culture medium, which we had documented to be superior to 10% FCS in terms of clonogenic efficiency and proliferative capacity when employed for BM-MSCs, did not provide higher efficiency of isolation of UCB-MSCs, as compared to a FCS-based medium. In fact, only 20% of UCB units we tested gave rise to MSCs, whereas in the remaining UCB units MSCs could not be obtained, despite the extended culture time. We cannot exclude that either the relatively long storage time of the UCB units employed or their inferior cellularity might have influenced our inferior isolation rate, despite the use of a PL-supplemented medium.

Once obtained, UCB-MSCs expanded in the presence of 5% PL display the typical MSC morphology, immune phenotype and differentiation capacity (see Figure 1s and Figure 1A and 1B). UCB-MSCs possess high proliferative potential, yielding numbers comparable to BM-MSCs cultured
with the same medium\textsuperscript{27} at P5, although starting from lower cell counts at P0 (Figure 1C).

Given the high proliferative capacity and the published reports on \textit{in vitro} transformation of MSCs,\textsuperscript{41,42} we monitored UCB-MSCs during their whole culture period and, particularly, during their senescence phase, which occurred after 9 and 10 passages for UCB3- and UCB6-MSCs, respectively. Neither phenotypical, nor functional alterations of the cells were observed; the favorable bio-safety profile of UCB-MSCs was further demonstrated by the absence of telomerase activity and hTERT expression, the expression of p16\textsuperscript{ink4a} protein, the absence of anchorage-independent cell growth and by a normal molecular karyotype as proved by array-CGH analysis (Figure 2).

These data suggest that UCB-MSCs, expanded in our culture system in the presence of 5\% PL, do not display a tendency for spontaneous transformation, in accordance with data published evaluating BM-MSCs cultured with both PL- and FCS-supplemented media.\textsuperscript{27,33} This is, to our knowledge, the first report to include a thorough investigation of the genetic stability of MSCs derived from UCB. This type of evaluation, also in view of the high proliferative potential of UCB-MSCs, is mandatory, in our opinion, for any clinical application of these cells.

When tested for their capacity to influence the alloantigen-specific immune response, in comparison to BM-MSCs grown in 5\% PL (see also Figure 3s and 4s for details on BM-MSCs),\textsuperscript{27} UCB-MSCs have similar suppressive effect on T- and NK-lymphocyte subset proliferation and on alloantigen-induced cytototoxic activity, while only slightly increase IL-10 in MLC supernatants. The results obtained in our study suggest that UCB-MSCs are able to exert an immunosuppressive effect on alloantigen-specific immune response by means of several mechanisms, including IDO activation and production of kyn, PGE2 secretion and HLA-G expression.\textsuperscript{4,11,36,43-49} All
these biological mechanisms have been previously described to be active in MSCs derived from other sources, such as BM-MSCs.\textsuperscript{4,11,35,43-49} In particular, it is noteworthy that our data confirm and extend previously reported results, underlying the inter-relationship among PGE2 secretion and IDO activation, two well-known mechanisms involved in anti-inflammatory immune response.\textsuperscript{39,40} Indeed, the presence of PGE2-specific inhibitor, besides reducing PGE2 levels in culture supernatants, was also able to inhibit IDO activity. Moreover, in the presence of IDO-specific inhibitor, we observed a striking increase in PGE2 secretion.

A distinctive feature of UCB-MSCs seems to be the constitutive surface expression of HLA-G on the majority of cells, while it has been reported that BM-MSCs mainly express only the soluble isoform of HLA-G.\textsuperscript{45} However, it is worth considering that MEM-G/9 monoclonal antibody, which is specific for both membrane-bound (HLA-G1) and soluble (HLA-G5) HLA-G isoforms was employed to evaluate HLA-G expression in flow cytometry. Therefore, we were unable to formally prove that soluble HLA-G5 isoform is the only one expressed by UCB-MSCs, as documented by Selmani et al.\textsuperscript{45} for BM-MSCs. Further experiments are warranted to clarify this point.

Membrane HLA-G expression, as well as sHLA-G isoforms have been demonstrated to exert a strong suppressive effect on proliferation and activation of effector functions of both T and NK lymphocytes. For instance, it is well known that HLA-G expression at the feto-maternal interface is one of the most potent mechanisms protecting the fetus from maternal immune attack.\textsuperscript{48} Moreover, surface HLA-G expression is one of the systems employed by tumor cells to evade the cytotoxic activity of both tumor-specific T lymphocytes and NK cells, and it has been recently suggested that transfer of membrane patches containing HLA-G molecules from mHLA-G\textsuperscript{pos} cells to activated T and NK lymphocytes (“trogocytosis”)
might be a mechanism of immune suppression protecting HLA-G<sup>-</sup> tumor cells. It may, thus, be speculated that UCB-MSCs expressing mHLA-G may be more protected than BM-MSCs from attack mediated by the host immune system. However, it has been recently demonstrated in a murine model that while local implantation of MSCs results in ectopic bone formation in syngeneic recipients, it leads to transplant rejection in allogeneic mice. This is in line with previously published data that MSCs can be lysed by cytotoxic T-lymphocytes, when infused into MHC-mismatched mice, resulting in their rejection. These observations supports the use of MSCs, in hard tissue repair strategies, preferably in an autologous or tolerant host. Further studies specifically addressing this issue are underway.

In conclusion, while the ability of BM-MNCs to generate MSCs reaches 100% under appropriate culture conditions, the success rate of isolating MSCs from UCB ranges, according to different reports, from 20 to 63%. In particular, Reinisch et al. have recently shown that MSCs can be obtained from full-term UCB in the presence of human PL, yielding cell numbers suitable for clinical application. The same authors report an isolation efficiency of 46%, considering both FCS-expanded and PL-expanded MSCs. However, their PL preparation procedure, percentage of PL employed in the culture medium (10%), as well as MSC plating density, differ from our approach. These differences might explain the different results obtained, in particular in terms of isolation efficiency, as compared with our data. Despite this, also in our experience, UCB-MSCs display a high proliferative capacity, which allow the expansion of sufficient cell numbers for clinical application, in a reasonable time-frame. Given their high proliferative capacity, immunosuppressive properties and potential for avoiding attack by immune cells, UCB-MSCs, also in view of their easy collection, could be considered to be used in clinical practice for
prevention and treatment of alloreactive-related immune complications, namely severe GvHD and graft rejection, following HSCT. However, as note of caution, recent data, obtained in a xenogenic model of NOD/SCID mice, showed that human UCB-MSCs, when administered in multiple doses, are effective in the prevention, but not in the treatment of GvHD. This discrepancy with the clinical efficacy displayed by MSCs on acute GvHD\textsuperscript{5} in human HSCT might be explained by the animal model employed and also by the unfavorable ratio between the number of UCB-MSCs and the huge number of effector cells mediating the tissue damage at time of acute GVHD onset.

UCB-MSCs could also serve as a tool in strategies of reparative/regenerative medicine, where the combination of the immunosuppressive and tissue repair properties could ameliorate the management of autoimmune and chronic inflammatory diseases.\textsuperscript{9-11}

Our results, although obtained in a limited number of MSC samples tested, suggest that the differences between BM- and UCB-derived MSCs and between cells expanded in the presence of PL and FCS may be relevant for the clinical application of MSCs.

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