Immunology
The Journal of cells, molecules, systems and technologies

British Society for immunology

IMMUNOLOGY ORIGINAL ARTICLE

The immunomodulatory activity of human umbilical cord blood-derived mesenchymal stem cells *in vitro*

Meng Wang,[1] Yuan Yang,[2] Dongming Yang,[1] Fei Luo,[1] Wenjie Liang,[1] Shuquan Guo[1] and Jianzhong Xu[1]

[1]*Department of Orthopaedics, Southwest Hospital, and* [2]*State Key Laboratory of Trauma, Burns and Combined Injury, Institute of Combined Injury of PLA, Third Military Medical University, Chongqing, China*

doi:10.1111/j.1365-2567.2008.02891.x
Received 31 October 2007; revised 21 May 2008; accepted 22 May 2008.
Correspondence: M. Wang, Department of Orthopaedics, Southwest Hospital, Third Military Medical University, 30 Gaotanyan Road, Shapingba District, Chongqing 400038, China. Email: wangmen99@yahoo.com
Senior author: Jianzhong Xu, email: xu_jianzhong@yahoo.cn

Summary

Bone marrow-derived mesenchymal stem cells (BM-MSC) are currently being investigated in preclinical and clinical settings because of their self-renewal and multipotent differentiative capacity or their immunosuppressive function. However, BM may be detrimental because of the highly invasive donation procedure and BM-MSC decline with age. Therefore, MSC derived from other sources have been considered as an alternative. However, there is only limited knowledge on their immunomodulatory properties. Human umbilical cord blood (UCB) cells are good substitutes for BM-MSC because of the immaturity of newborn cells. In this study, we successfully isolated MSC from UCB. The morphological phenotypes, cell cycle status, surface markers and differentiation potential of these clonally expanded cells are consistent with BM-MSC. Furthermore, UCB-MSC expanded *in vitro* retain low immunogenicity and an immunomodulatory effect. Flow cytometry analysis showed that UCB-MSC did not express CD40, CD40 ligand, CD80, CD86 and major histocompatibility complex class II molecules. We have demonstrated that UCB-MSC are incapable of inducing allogeneic peripheral blood mononuclear cell (PBMC) proliferation and have a dose-dependent inhibition of PBMC immune responses in mixed lymphocyte reactions (MLR) and phytohaemagglutinin activation assays, even after interferon-γ treatment. Additionally, we have found that UCB-MSC can suppress the function of mature dendritic cells. Using transwell systems, we have demonstrated an inhibition mechanism that depends on both cell contact and soluble factors. Based on the findings we conclude that banked UCB could serve as a potential alternative source of MSC for allogeneic application in the future.

Keywords: immunogenicity; immunoregulatory; mesenchymal stem cells; umbilical cord blood

Introduction

Mesenchymal stem cells (MSC) are characterized as undifferentiated cells, able to self-renew with a high proliferative capacity, and have the potential to differentiate into several types of cells, including osteocytes, chondrocytes, adipocytes, cardiomyocytes and neurons.[1–3] For these reasons, MSC have drawn considerable interest as useful materials for tissue engineering and cell-based therapy. To date, human bone marrow (BM) represents the major source of MSC. Autologous MSC derived from BM have been applied for cell-based therapies, including the treatment of osteogenesis imperfecta, intracoronary transplantation in patients with acute myocardial infarction, and support of haematopoiesis.[4–8] However, the harvest of BM is a highly invasive procedure, and the possibility of donor morbidity as well as the number, differentiation potential and maximum life span of human BM-derived autologous MSC significantly decline with the age of the donor.[9–11] Therefore, alternative sources from which to isolate MSC are subject to intensive investigation.

The MSC isolated from umbilical cord blood (UCB) have the potential to be expanded and cryopreserved for future use as an 'off-the-shelf' therapy. An attractive alternative source of MSC, UCB can be obtained by a less invasive method, without posing harm to the mother or infant. Cells

from UCB have many advantages because of the immature nature of newborn cells compared to adult cells. Moreover, UCB cells provide no ethical barriers for basic studies and clinical applications.[12,13] Recently, UCB banking for transplantation of haematopoietic stem cells has become increasingly popular.[14] As a result of their availability and the easy-to-access system, we can utilize the UCB bank to establish a UCB-MSC bank network system immediately.

Although some groups did not succeed in isolating MSC,[15,16] the number of groups able to isolate MSC from full-term UCB is growing. In the present study, UCB-MSC have been found to have a mesenchymal multipotency *in vitro*, and the immunophenotype of the clonally expanded cells is consistent with BM-MSC. Furthermore, several groups report that UCB-MSC can be cultured for longer and demonstrate a higher proliferation capacity compared with MSC derived from other sources.[17–20]

A major clinical challenge is finding a suitable cell source for the regeneration of damaged tissues. In addition to fulfilling the function of tissue reconstruction, the low immunogenicity of MSC is advantageous for clinical applications. Although the expansion and differentiation potentials of UCB-MSC have already been well characterized, immunogenic behaviour has been studied thoroughly only for MSC from BM. As an allogeneic cell source, the use of UCB-MSC may raise the issue of a possible adverse reaction or eventual rejection of the allogeneic MSC by the recipient patient. Previous studies have indicated that MSC derived from BM may be immune-privileged cells. It is believed that the surface characteristics of the MSC may enable it to avoid rejection. Previous analysis has demonstrated that BM-MSC do not express major histocompatibility complex (MHC) class II molecules or the costimulatory molecules (B7 and CD40) required for the T-cell activation responsible for transplant rejection. Furthermore, BM-MSC fail to elicit a proliferative response of allogeneic PBMC during coculture *in vitro*.[21–25] The BM-MSC are already extensively characterized so we asked whether MSC derived from UCB share the characteristics of BM-MSC. Therefore, the present study was designed to isolate the MSC from UCB and not only confirm that allogeneic MSC derived from UCB do not elicit an adverse immune response *in vitro* but also identify the possible mechanism responsible for the immune reaction. Furthermore, we investigated the influence of interferon-γ (IFN-γ) treatment on their immunomodulatory potential.

Materials and methods

Collection of UCB

The local ethical committee approved the collection of human UCB. Informed consent was obtained before the operation from all individuals included in the study. According to the institutional guidelines, UCB units were obtained from normal full-term and preterm deliveries, which had faced no complications throughout pregnancy, in a multiple bag system containing citrate phosphate dextrose anticoagulant and were processed within 12 hr of collection. The units were stored and transported at 18 ± 3°. No complications were encountered upon UCB collection, and none of the samples had signs of coagulation or haemolysis.

Isolation and culture of MSC

After cord blood was diluted 1 : 1 with 2 mM ethylenediaminetetraacetic acid–phosphate-buffered saline (EDTA-PBS; Gibco-BRL, Gaithersburg, MD), UCB-derived mononuclear cells (MNC) were isolated using Ficoll–Paque density gradient centrifugation (1·077 g/ml, Amersham Biosciences, Uppsala, Sweden) and resuspended in Dulbecco's modified Eagle's low glucose medium (DMEM; Gibco-BRL) that was supplemented with 10% fetal bovine serum (FBS; Gibco-BRL). The UCB-derived MNC were set in culture at a density of 2×10^6 cells/cm^2 in six-well culture plates (Falcon, Franklin Lakes, NJ). Cells were allowed to adhere overnight; non-adherent cells were removed and fresh medium was added to the wells. Half the medium was changed weekly thereafter. A few colonies were found in the wells 1 month after the collected cells were seeded. Culture wells were screened continuously to seek and obtain developing colonies of adherent fibroblastoid cells. The fibroblastoid colonies originating from single cell were trypsinized as passage 1 using a colony cylinder and then used for the experiment.

After clones derived from single cells were expanded in culture and had reached approximately 50–60% confluence, the cells were trypsinized (0·05% trypsin/EDTA solution; Gibco-BRL), washed twice with PBS, and replated at a density of 1×10^4 cells/cm^2 under the same culture conditions.

Cell proliferation assays

The population doubling time was calculated using the following equation: $T_D = t_p \log 2/(\log N_t - \log N_0)$ where N_0 is the inoculum cell number, N_t is the cell harvest number and t is the time for which the culture was allowed to grow. The doubling time of cells from passage 1 (P1) to P15 was determined.

Cell cycle analysis

UCB-derived adherent cells were permeabilized with 70% alcohol followed by incubation with 10 μg/ml RNase A (Sigma, Shanghai, China). Cells were then incubated with 50 μg/ml propidium iodide (Sigma). DNA content was analysed by FACScan flow cytometry (BD Biosciences, San Jose, CA).

Immunophenotype analysis using fluorescence-activated cell sorting (FACS)

Cell phenotype was analysed by FACScan flow cytometry with CELLQUEST software. At the fourth passage, UCB-derived adherent cells were detached with trypsin/EDTA, washed and resuspended in PBS with 1% FBS. The cells were then stained for 30 min on ice with saturating amounts of anti-human monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC)-conjugated CD31 (FITC-CD31), FITC-CD34, FITC-CD45, FITC-CD154, FITC-MHC II, phycoerythrin-conjugated CD14 (PE-CD14), PE-CD29, PE-CD40, PE-CD73, PE-CD105, PE-CD166, PE-MHC I, phycoerythrin-Cy5-conjugated CD80 (PE-Cy5-CD80) and PE-Cy5-CD86 (eBiosciences, San Diego, CA). Mouse isotypic antibodies served as the control. Cells were all single-labelled and percentage of positive cells was determined.

RNA preparation and reverse transcription–polymerase chain reaction (RT-PCR)

Total RNA was extracted from 1×10^6 cells using trizol reagent according to the manufacturer's instructions. The cDNA was synthesized using a Moloney murine leukaemia virus reverse transcriptase system (Gibco). The PCR amplification was performed following standard procedures and RT-PCR of human β_2-microglobulin was performed as a control for complementary DNA production. The appropriate primers specific for stem cell factor (SCF) and β_2-microglobulin were as follows: SCF (177-base pair PCR product): S: 5′-CTCCTATTTAATCCTC TCGTC-3′; A: 5′-TACTACCATCTCGCTTATCCA-3′. β_2-microglobulin (267-base pair PCR product): S: 5′-TCTGGCCTTGAGGCTATCCAGCGT-3′; A: 5′-GTGGT TCACACGGCAGGCATACTC-3′.

Multilineage differentiation assays

Osteogenic differentiation To promote osteogenic differentiation, cells were plated in six-well plates at a density of 3000 cells/cm^2. When cells reached 70–80% confluency, they were treated with osteogenic medium for 3 weeks with medium changes twice weekly. The osteogenic induction medium consisted of 100 nM dexamethasone, 10 mM glycerophosphate, 0·2 mM ascorbate and 10% fetal calf serum in DMEM. To evaluate mineralized matrix, cells were fixed with 4% formaldehyde and stained with 1% Alizarin red S (Sigma). Osteogenic differentiation was also confirmed by the increase of alkaline phosphatase expression by histochemical staining following the manufacturer's instructions (alkaline phosphatase kit; Sigma).

Chondrogenic differentiation To induce chondrogenic differentiation, a cell pellet consisting of $2{\cdot}5 \times 10^5$ cells was incubated at the bottom of a 15-ml polypropylene tube in 0·5 ml complete chondrogenic differentiation medium for 3 weeks. Medium changes were carried out twice weekly. After the culture period, cryosections were analysed by Safranin O staining and immunocytochemical analysis following the manufacturer's instructions (Cambrex, Walkersville, MD). Chondrogenic medium consists of DMEM, 1% ITS-premix (BD Biosciences), 50 μM ascorbate-2-phosphate, and 40 μM L-proline, 1 mM sodium pyruvate, 0·1 μM dexamethasone and 10 ng/ml transforming growth factor-β1 (Peprotech EC Ltd, London, UK).

Adipogenic differentiation To promote adipogenic differentiation, cells were plated in six-well plates at a density of 3000 cells/cm^2. When cells reached 100% confluency, they were treated with adipogenic medium for 3 weeks. Medium changes were carried out twice weekly and adipogenesis was assessed at weekly intervals. The adipogenic induction medium consisted of DMEM supplemented with 1 μmol/l dexamethasone, 5 μg/ml insulin, 0·5 mmol/l isobutylmethylxanthine, 0·1 mmol/l indomethacin (Sigma) and 10% FBS. Adipogenic differentiation was confirmed by the formation of neutral lipid vacuoles stainable with Oil Red O (Sigma).

Mixed lymphocyte reaction

The BM-MSC served as positive controls and were isolated from BM aspirates taken from the iliac crests of three normal healthy volunteers, aged 19–35 years, after informed consent. The BM-MSC were isolated and culture-expanded according to previously reported methods with slight modification.[26] Briefly, BM-derived MNC were collected by gradient centrifugation and cultured at a density of 1×10^6 cells/cm^2 in culture flasks, and the first change of medium was performed 3 days after initial plating. The medium was changed every 3–4 days thereafter. Two weeks later, on reaching 60–80% confluence, MSC were suspended and replated as described for the UCB-derived adherent cells.

Human PBMC were isolated from healthy donors by Ficoll–Paque (1·077 g/ml, Amersham Biosciences) density gradient centrifugation and resuspended in RPMI-1640 medium supplemented with 10% FBS. The PBMC were depleted of adherent cells overnight and further cultured in PBMC medium. To enhance expression of MHC molecules, UCB-MSC were pretreated with 100 U/ml IFN-γ (Peprotech EC Ltd) for 2 days and then the IFN-γ-treated UCB-MSC (UCB-MSC + IFN-γ) were washed thoroughly (a minimum of five washes) before coculture with PBMC. Expression of MHC II molecules was detected by flow cytometry using monoclonal antibodies specific for MHC II molecules.

To assess the effects of UCB-MSC and UCB-MSC + IFN-γ on mixed lymphocyte culture, 10^5 responder PBMC and 10^5 allogeneic stimulator PBMC were cocultured with different

numbers of the third-party UCB-MSC, BM-MSC or UCB-MSC + IFN-γ (1×10^5, 5×10^4 and 1×10^4/well, respectively). Before coculture, the stimulator PBMC and UCB-MSC, BM-MSC or UCB-MSC + IFN-γ were treated with 25 μg/ml mitomycin C (Sigma) at 37° for 1 hr, followed by five extensive washes with RPMI-1640 medium containing FBS. Cocultures of responder PBMC and allogeneic stimulator PBMC without UCB-MSC, BM-MSC, or UCB-MSC + IFN-γ were used as controls. On day 5, cultures were pulsed with 5 μCi/ml [^{3}H]thymidine ([^{3}H]TdR; Amersham, Buckinghamshire, UK) 18 hr before harvest. Cells were harvested over glass fibre filters and radionuclide uptake was measured by scintillation counting. All experiments were performed in triplicate and were repeated at least twice.

Phytohaemagglutinin activation assay

Responder PBMC were seeded in 96-well flat-bottomed plates at 10^5 cells in a final volume of 100 μl PBMC medium/well. Phytohaemagglutinin (PHA; 2 μg/ml; Sigma) was used instead of stimulator PBMC to induce the proliferation of responder PBMC. The addition of allogeneic UCB-MSC, BM-MSC, or UCB-MSC + IFN-γ and quantification of PBMC proliferation were performed as described for the MLR.

Differentiation and maturation of DC

The PBMC were isolated by Ficoll–Paque density gradient centrifugation and seeded into 75-cm^2 flasks (Costar Corning, Corning, NY) at a density of 1×10^6/cm^2 in RPMI-1640 medium supplemented with 10% FBS. The cells were incubated at 37° in a humidified atmosphere for 2 hr to allow the monocytes to attach. After the adherent cells were collected, monocytes were sorted with magnetic antibody cell sorting (MACS) CD14 microbeads (Miltenyi Biotec, Bergish Gladbach, Germany). The cells were incubated with RPMI-1640 medium supplemented with 10% FBS, 100 ng/ml granulocyte–macrophage colony-stimulating factor (GM-CSF) and 50 ng/ml interleukin-4 (IL-4; Peprotech EC Ltd) for 7 days at 37° to induce dendritic cell (DC) differentiation. To obtain mature DC, the cells were further cultured with 20 ng/ml tumour necrosis factor-α (TNF-α; Peprotech EC Ltd) for another 2 days. Immunophenotyping of immature and mature DC was carried out using the following monoclonal antibodies: FITC-MHC II, PE-CD1a, PE-CD14, PE-CD83 and PE-Cy5-CD86.

Coculture of UCB-MSC with DC

To determine whether UCB-MSC might affect the ability of monocyte-derived DC to stimulate T-lymphocyte proliferation, a T-cell stimulation model was used to evaluate the function of DC. The UCB-MSC and DC were pretreated with mitomycin C as described for the MLR. The DC were cultured in triplicate at 5×10^3, 1×10^4, $1{\cdot}5 \times 10^4$, 2×10^4 or $2{\cdot}5 \times 10^4$ cells per well in flat-bottomed 96-well plates with 1×10^5 allogeneic PBMC. For coculture, UCB-MSC were directly added into the coculture system at a density of 1×10^4 cells/well. Then, 5 μCi/ml [^{3}H]TdR was added to each well 18 hr before the cultures were terminated. The radioactivity was measured with a liquid scintillation counter. To remove UCB-MSC influence on T cells directly, mature DC alone were used in graded doses to stimulate allogeneic responders after they were exposed to UCB-MSC for 2 days. Results are expressed in counts per minute and are shown as means ± SD of triplicate values.

Transwell cultures

Transwell experiments were performed in 24-well transwell plates (0·4 μm pore size; Costar). The UCB-MSC were plated into the lower chamber of 24-well transwell plates at a density of 10^5 cells/well, whereas a total of 5×10^5 responder PBMC were stimulated with 5×10^5 allogeneic stimulator PBMC and were placed either in the same or a separate chamber. After 5 days of culture, cells from the upper chamber were transferred to microtitre wells in triplicate (100 μl/well), pulsed with [^{3}H]TdR, and harvested 18 hr later. T-cell proliferation is expressed as mean counts per minute of three or four wells ± standard deviation.

Statistical analysis

Data were presented as mean ± standard deviation from the experiments. The results were analysed using the statistical package SPSS 13.0 (SPSS Inc., Chicago, IL). The statistical significance was assessed by paired Student's *t*-test and a *P*-value $< 0{\cdot}05$ was considered statistically significant.

Results

Isolation and characterization of UCB-MSC

After UCB-derived MNC were isolated and cultured at a density of 2×10^6/cm^2, attached cells were observed at 5–7 days. Among them, there were two types of adherent cells: multinucleate cells and fibroblastoid cells. The morphology of the multinucleate cells was heterogeneous, either elongated or oval/round shape with smooth borders. Rarely, among the initially adherent cells, a fibroblastic morphology was found, which initiated colony formation within 3 weeks. Single cell-derived clones were culture expanded and gradually reached confluency with a whirlpool-like array (Fig. 1a–c).

Single-cell-derived, clonally expanded fibroblastoid adherent cells derived from UCB showed a high proliferative capacity, which could be readily expanded *in vitro* by serial passage every 3–5 days for over 15 passages, without

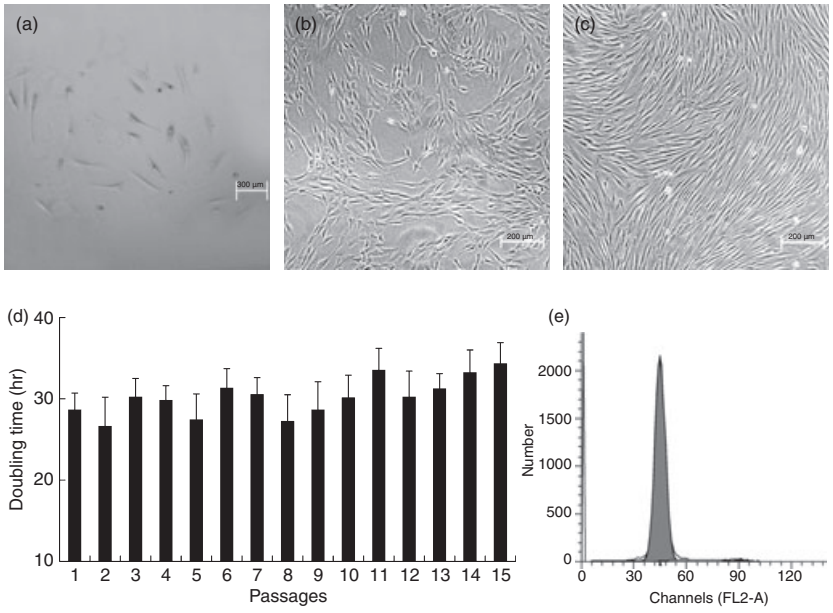


Figure 1. The morphology, population doubling time and cell cycle status of the fibroblastoid cells from umbilical cord blood (UCB). (a) After the initiating plate, the fibroblastoid adherent cells developed the onset of colony formation within 3 weeks (scale bars = 300 μm). (b) Single cell-derived, clonally expanded adherent cells derived from UCB displayed a fibroblastic morphology (scale bars = 200 μm). (c) Single cell-derived clones were culture expanded and gradually reached confluency with a whirlpool-like array (scale bars = 200 μm). (d) Population doubling time of UCB-derived cells (n = 6, P1–P15) was determined. The UCB-derived cells displayed high doubling numbers in all passages analysed and the mean population doubling time remained constant until approximately P15. (e) Representative cell cycle plots from UCB-derived cells. The majority of the UCB-derived cells were retained in the G0–G1 phase, whereas small populations of cells were engaged in the G2–M phase and S phase (Dip G1: 95·15%, Dip G2: 0·62%, Dip S: 4·23%, Apoptosis: 0·04%, Debris: 2·9%, Aggregates: 1·3%).

visible changes in either the growth patterns or morphology. The UCB-derived cells displayed high doubling numbers in all passages analysed and the mean population doubling time (30·2 ± 2·3 hr) remained constant until approximately P15 (Fig. 1d).

Cell cycle analysis demonstrated that the majority of the UCB-derived cells were retained in the G0–G1 phase (median 91·03%; range, 87·6–95·15%), whereas small populations of cells were engaged in proliferation (G2-M phase: median 5·65%, range, 2·94–7·91%; S phase: median 4·78%; range, 4·23–6·75%). Notably, there were no detectable signs of apoptosis (Fig. 1e).

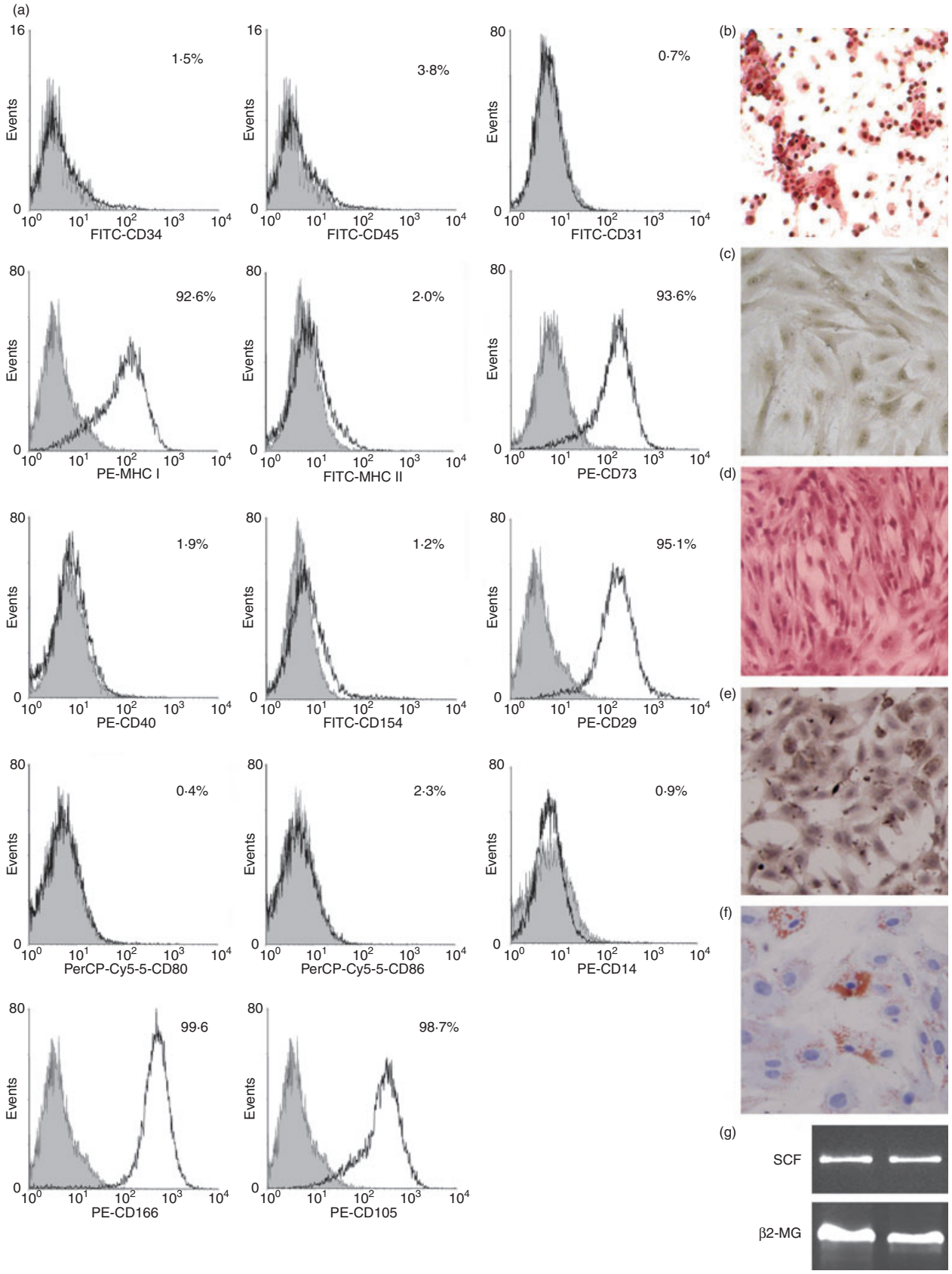
The cell-surface markers of the UCB-derived cells were examined by FACS analysis. As shown in Fig. 2(a), none of the cell populations were found to express CD14, CD34, CD45 (haematopoietic markers) or CD31 (endothelial cell marker), whereas all cell populations expressed CD29, CD105 (SH2), CD73 (SH3) and CD166 (stromal markers).

Cells derived from UCB underwent RT-PCR. Figure 2(g) shows that the fibroblastoid cells from UCB expressed the messenger RNA (mRNA) of SCF.

Tri-lineage differentiation of the UCB-derived cells

To demonstrate the isolation of MSC and to investigate their differentiation potential, the fibroblastoid cells from P3 were cultured under conditions that are favourable for

Figure 2. Phenotype and multilineage differentiation capacity of the fibroblastoid cells from umbilical cord blood (UCB). (a) Cells were cultured for three to five passages, harvested and labelled with antibodies against human antigens CD14, CD29, CD31, CD34, CD40, CD40 ligand, CD45, CD73, CD80, CD86, CD105, CD166, major histocompatibility complex (MHC) I and MHC II, as indicated and analysed by fluorescence-activated cell sorting. Only representative flow cytometric plots are shown. Shaded histogram indicates background signal; open histogram, positive reactivity with the indicated antibody. (b–f) Osteogenic, chondrogenic and adipogenic differentiation. Osteogenic differentiation evidenced by the formation of mineralized matrix shown by Alizarin red staining at original magnification × 40 (b). Osteoblastic differentiation was further confirmed by alkaline phosphatase staining after 21 days of induction (c). Chondrogenic differentiation was shown by Safranin-O staining (d) and by immunohistochemical staining for type II collagen (e). Adipocytic differentiation was detected by the formation of neutral lipid vacuoles stainable with Oil Red O staining (f). Original magnification, × 100 (c–f). (g) Reverse transcription–polymerase chain reaction showed that the fibroblastoid cells from UCB expressed the messenger RNA of stem cell factor.


(a)
1·5%
FITC-CD34
3·8%
FITC-CD45
0·7%
FITC-CD31
92·6%
PE-MHC I
2·0%
FITC-MHC II
93·6%
PE-CD73
1·9%
PE-CD40
1·2%
FITC-CD154
95·1%
PE-CD29
0·4%
PerCP-Cy5-5-CD80
2·3%
PerCP-Cy5-5-CD86
0·9%
PE-CD14
99·6
PE-CD166
98·7%
PE-CD105
Events
(b)
(c)
(d)
(e)
(f)
(g)
SCF
β2-MG

osteogenic, adipogenic and chondrogenic differentiation, respectively.

Under osteogenic conditions, the spindle shape of the fibroblastoid cells flattened and broadened with increasing time of induction and formed mineralized matrix as evidenced by Alizarin red staining. We further subjected the cells to analysis by testing for the expression of alkaline phosphatase, which is an independent indicator of osteogenic differentiation, and the results confirmed by an increased expression of alkaline phosphatase were consistent with those of the Alizarin red staining assay. These results strongly suggest that the fibroblastoid cells can differentiate into osteogenic cells (Fig. 2b,c).

After 3 weeks of differentiation, chondrogenic differentiation was confirmed by the secretion of cartilage-specific proteoglycans stainable with Safranin O. To further confirm chondrogenic differentiation, we evaluated the expression of type II collagen, a gene specifically expressed in chondrogenic cells, using immunohistochemical analysis. We found that most of the cell populations in this assay appeared to differentiate into chondrogenic cells (Fig. 2d,e).

Under adipogenic induction, morphological changes in the fibroblastoid cells as well as the formation of neutral lipid vacuoles were noticeable and visualized by Oil Red O staining at 3 weeks (Fig. 2f).

Immunogenic characterization of UCB-MSC

Immunologically, UCB-MSC have many cell surface markers in common with BM-MSC, including being positive for MHC I but negative for MHC II as well as the costimulatory molecules CD40, CD40 ligand, CD80 and CD86 (Fig. 2a).

To assess whether the lack of MHC II molecule expression was the major factor for the immunological properties of UCB-MSC, UCB-MSC were pretreated with IFN-γ before being used as stimulators. When UCB-MSC were treated with IFN-γ for 48 hr, the expression of MHC II molecules was significantly up-regulated on UCB-MSC from an undetectable range to more than 80% positive expression ($P < 0{\cdot}01$) (Fig. 3a).

To investigate whether allogeneic UCB-MSC could stimulate the proliferation of PBMC, the PBMC were co-cultured with allogeneic UCB-MSC and pulsed with [^{3}H]TdR at intervals to determine the proliferative response. As shown in Fig. 3(b), although PBMC initiated a very strong proliferative response to allogeneic lymphocytes, allogeneic UCB-MSC failed to elicit any proliferative response, which is consistent with BM-MSC. Moreover, IFN-γ-stimulated allogeneic UCB-MSC did not elicit a stronger proliferative response than untreated UCB-MSC, suggesting that overexpression of MHC II molecules on UCB-MSC did not modify the low immunogenicity of UCB-MSC.

UCB-MSC suppress PBMC proliferation and the function of mature DC

This experiment was designed to investigate whether UCB-MSC could inhibit a MLR that was triggered by allogeneic antigen. The results suggest that proliferation of PBMC induced by allogeneic PBMC was significantly suppressed by UCB-MSC ($P < 0{\cdot}01$). As shown in Fig. 3(c), UCB-MSC suppressed the MLR in a dose-dependent manner, and pretreatment of the UCB-MSC with IFN-γ for 2 days before coculture did not affect this suppressive ability ($P < 0{\cdot}05$). Both types of cells suppressed lymphocyte proliferation to the same extent and the levels were also associated with the cell numbers added.

After confirming that UCB-MSC could inhibit MLR, we then investigated whether UCB-MSC could inhibit PHA-mediated lymphocyte proliferation. The PBMC were stimulated with PHA in the presence or absence of either UCB-MSC or UCB-MSC + IFN-γ. The results demonstrated that UCB-MSC and UCB-MSC + IFN-γ both inhibited the proliferation of PBMC in response to mitogen treatment ($P < 0{\cdot}01$). In addition, the PHA-stimulated proliferation was inhibited by UCB-MSC or UCB-MSC + IFN-γ in a cell number-related manner. Although both cell doses could achieve the inhibition, either UCB-MSC or UCB-MSC + IFN-γ seemed to inhibit the stimulatory effect of PHA more efficiently at a dose of 1×10^5 than 1×10^4 ($P < 0{\cdot}05$).

Highly enriched CD14$^+$ monocytes were incubated for 7 days in medium containing GM-CSF and IL-4. After an additional 2 days of stimulation with TNF-α, mature DC were harvested. As expected, mature DC acquired the size, became non-adherent and clustered, and displayed different protruding veils with abundant cytoplasm, a typical morphology for DC (Fig. 4a). In parallel, DC maturation was accompanied by high expression of the maturation markers MHC II, CD1a, CD83, CD86 and low expression of CD14 (Fig. 4b). To determine whether UCB-MSC might affect the ability of DC to stimulate T-lymphocyte proliferation, we tested the immunosuppressive effects of UCB-MSC on the proliferative response of allogeneic PBMC when stimulated by mature DC, which are considered to be the prototype of professional antigen-presenting cells. Compared with mature DC, UCB-MSC + mature DC have a reduced ability to stimulate the T-lymphocyte proliferation (Fig. 4c). After removing UCB-MSC influence on T cells directly, an inhibition was also shown in the MLR (Fig. 4d). The data suggested that UCB-MSC could suppress the function of mature DC.

UCB-MSC inhibitory effect mediated by cell contact and soluble factors

To investigate the mechanism(s) involved in the suppression of lymphocyte proliferation mediated by UCB-MSC,

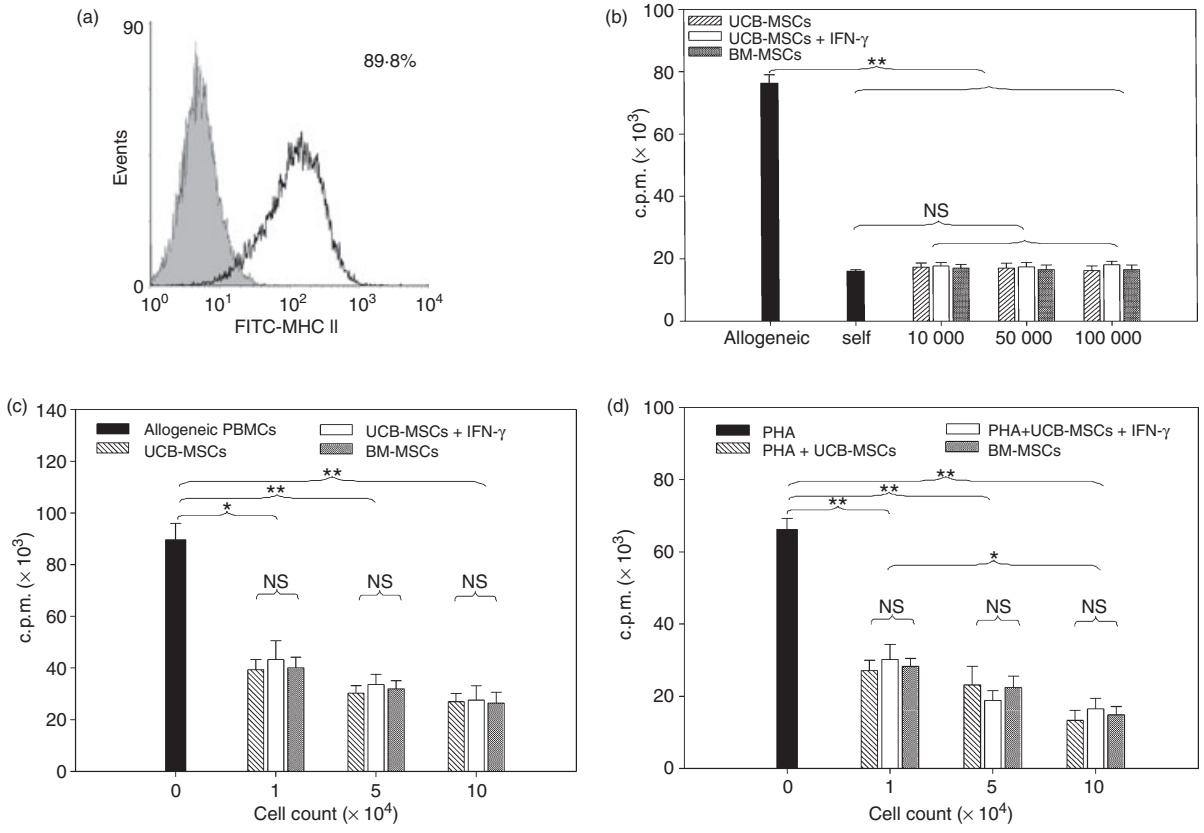


Figure 3. Immunomodulatory properties of umbilical cord blood-derived mesenchymal stem cells (UCB-MSC). (a) After UCB-MSC were treated with interferon-γ (IFN-γ) for 48 hr; the expression of major histocompatibility complex (MHC) II molecules was significantly up-regulated on UCB-MSC from undetectable to more than 80% positive expression. Shaded histogram indicates background signal; open histogram, positive reactivity with the indicated antibody. (b) UCB-MSC failed to stimulate allogeneic peripheral blood mononuclear cells (PBMC). The proliferation of PBMC self-stimulated (self), by allogeneic PBMC (allogeneic), by unstimulated allogeneic bone marrow-derived MSC (BM-MSC; grey bar), by unstimulated allogeneic UCB-MSC (hatched bar), and by stimulated allogeneic UCB-MSC (white bar) was measured using [^{3}H]thymidine incorporation. (c) UCB-MSC and UCB-MSC + IFN-γ inhibited mixed lymphocyte reaction activated peripheral blood mononuclear cells (PBMC) in a cell dose-dependent manner. PBMC were cocultured with equal amounts of allogeneic PBMC and varying amounts of third-party UCB-MSC (hatched bar), BM-MSC (grey bar), and UCB-MSC + IFN-γ (white bar). (d) UCB-MSC and UCB-MSC + IFN-γ inhibit phytohaemagglutinin (PHA)-activated lymphocyte proliferation in a cell dose-dependent manner. The PBMC were cocultured with UCB-MSC (hatched bar), BM-MSC (grey bar) and UCB-MSC + IFN-γ (white bar), which inhibited PHA-activated PBMC proliferation at equal levels. Both UCB-MSC and UCB-MSC + IFN-γ can inhibit the stimulatory effect of PHA more efficiently at the dose of 1×10^5 than the dose of 1×10^4, which is consistent with BM-MSC. The results are representative of three independent experiments. The data are mean ± SD of triplicate cultures. $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$.

we initially evaluated whether UCB-MSC inhibition of T-cell proliferation was dependent on a cell–cell contact mechanism. Experiments were performed in transwell systems in which UCB-MSC (lower compartment) were separated from MLR (upper compartment) physically. It was found that UCB-MSC could exert their suppressive effects on MLR without physical contact, indicating that UCB-MSC were releasing soluble inhibitory factors for T-cell proliferation ($P < 0{\cdot}01$). However, UCB-MSC added directly to the MLR cocultures had stronger inhibitory effects. Moreover, a difference was found between the cell–cell contact and the cell-separate groups ($P < 0{\cdot}05$) (Fig. 5). The data implied that the UCB-MSC inhibitory effect was mediated by both cell contact and soluble factors.

Discussion

Bone marrow-derived MSC have great therapeutic potential for regenerative medicine and cell therapy because of their capacity to self-renew and to undergo multilineage differentiation. They have already been used in various preclinical and clinical studies, including attenuation of graft-versus-host disease, osteogenesis imperfecta, intracoronary transplantation in patients with acute myocardial infarction, and support of haematopoiesis.[27–30] However, aspiration of BM is a highly invasive procedure and the frequency, differentiation potential, and maximal life span of BM-MSC decrease significantly with increasing age.[9–11] Therefore, the search for alternative sources of MSC is of significant importance. It has been reported

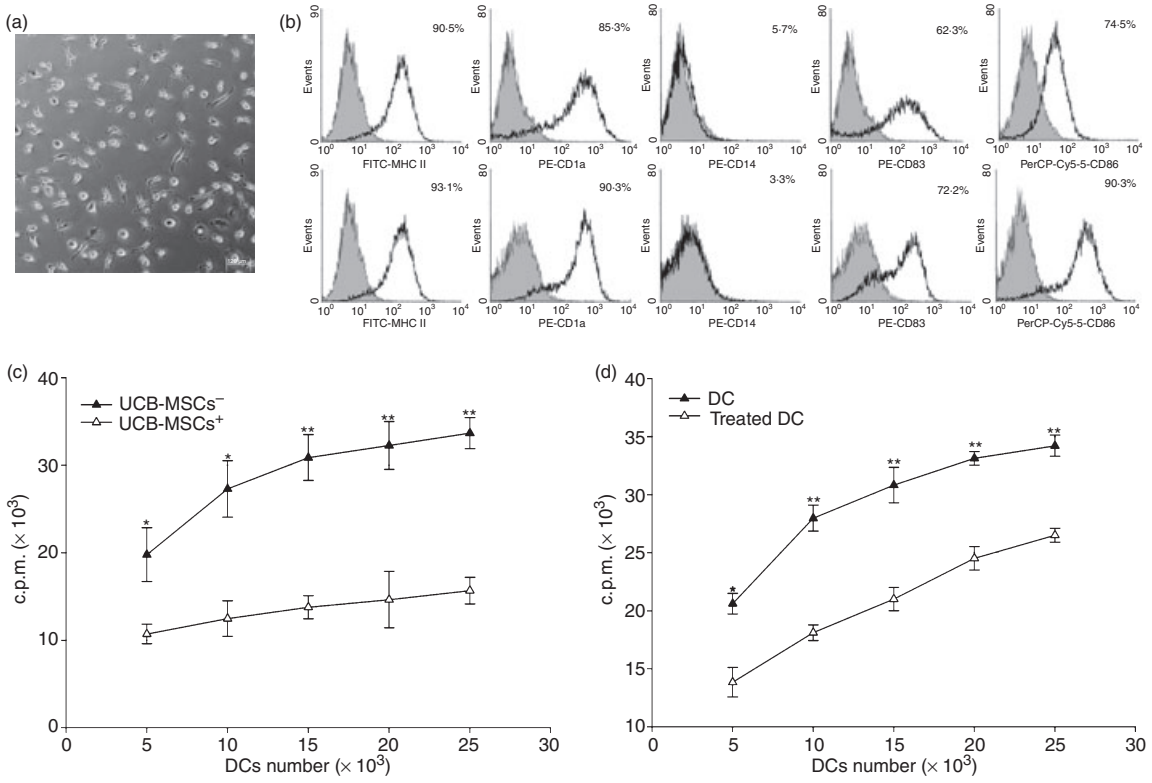


Figure 4. Suppression of umbilical cord blood-derived mesenchymal stem cells (UCB-MSC) on the function of mature dendritic cells (DC). (a) Monocytes cultured in the presence of granulocyte–macrophage colony-stimulating factor and interleukin-4 for 7 days and then tumour necrosis factor-α for another 2 days showed clustered and protruding veils resembling mature DC. Cell morphology was evaluated by phase contrast microscopy (scale bars = 120 μm). (b) Flow cytometry was employed to analyse the surface molecules expressed by immature DC (top row) and mature DC (bottom row). Results shown are representative flow cytometric plots. Shaded histogram indicates background signal; open histogram, positive reactivity with the indicated antibody. (c,d) The UCB-MSC inhibit the ability to stimulate T-lymphocyte proliferation of monocyte-derived DC. Graded doses of mature DC were cocultured with 1×10^5 allogeneic responders in the presence of absence of UCB-MSC for 5 days (c). After UCB-MSC treatment, mature DC alone were used in graded doses to stimulate allogeneic responders (d). The proliferative activity was measured by adding [^{3}H]thymidine to each well 18 hr before the cultures were terminated. The results are representative of three independent experiments. The data are mean ± SD of triplicate cultures. $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$.

that MSC with similar surface expression patterns can be isolated from alternative sources, including trabecular bone, deciduous teeth, fetal pancreas, amniotic fluid, adipose tissue, muscle, skin, placenta, blood, cord blood, synovium, periosteum, and perichondrium.[31–39] Among these, cord blood may be an ideal source because of its accessibility, the lack of risk to the mother or infant and of ethical barriers, and the fact that it is a painless procedure to donors, promising sources for autologous cell therapy and lower risk of viral contamination.[17,40] In particular, we can utilize the established UCB bank to construct a UCB-MSC bank network system for future use as an 'off-the-shelf' therapy.

In this study, we revealed that fibroblast-like cells can be isolated from UCB units. The optimal culture conditions for the higher isolating frequency were a storage-process time of < 12 hr, no sign of coagulation or haemolysis, a high inoculum density at 2×10^6 cells/cm^2, and sorting the colonies of fibroblast-like cells with a colony cylinder. The morphology of these cells from UCB resembles that of MSC isolated from BM.[1] Cell cycle analysis demonstrated that the majority of UCB-MSC were retained in the G0–G1 phase, whereas a small population of cells was engaged in proliferation. Our analysis of a variety of cell surface markers on UCB-MSC confirmed the lack of haematopoietic and endothelial markers, such as CD14, CD34, CD45 and CD31, and revealed a similar epitope profile to BM-MSC, such as CD29, CD73, CD105 and CD166. Moreover, the self-renewal capacity of these cells is remarkable, which could be readily expanded *in vitro* by serial passage every 3–5 days for over 15 passages, without visible changes in either growth patterns or morphology. The UCB-MSC displayed high doubling numbers in all passages analysed and the mean population doubling time of UCB-MSC remained approximately constant even after P15. Actually, UCB-MSC did not experience contact-inhibited cell growth and continued to grow by cell multilayering after reaching 100% surface

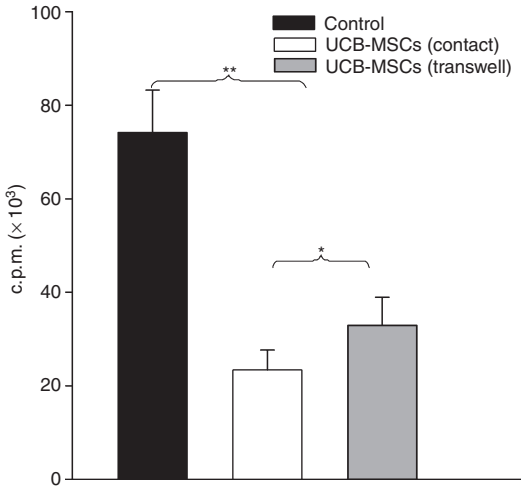


Figure 5. Inhibitory effects of umbilical cord blood-derived mesenchymal stem cells (UCB-MSC) are mediated by cell contact and soluble factors. UCB-MSC were plated into the lower chamber of transwell plates and mixed lymphocyte reactions were set up in the upper chambers. After 5 days of culture, cells from the upper chamber were transferred to microtitre wells in triplicate, pulsed with [^{3}H]thymidine and harvested 18 hr later. The results are representative of three independent experiments. The data are mean ± SD of triplicate cultures. $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$.

confluency. Our results also showed that UCB-MSC have a multilineage differentiation capacity. The capacity of UCB-derived cells to differentiate into osteoblasts that produce mineralized matrices, chondrocytes that produce cartilage-specific proteoglycans, and adipocytes that accumulate lipid vacuoles under *in vitro* conditions is consistent with BM-MSC. In our study, investigations were limited to the mesodermal differentiation capacity. In recent reports, MSC have been shown to differentiate into further mesodermal lineages and endo- and ectodermal lineages as well.[18,41–44] The spectrum of differentiation of UCB-MSC needs to be further investigated to assess their responsiveness toward cardiomyogenic, endothelial, hepatic, neuronal and pancreatic differentiation, which may intensify their use in therapeutic approaches. Altogether, these data illustrate the high similarity between adherent cell populations isolated from BM and UCB, based on the fibroblastic morphology, cell cycle status, great proliferative capacity, differentiation capacity, and the expression of SCF as well as the expression of various phenotypic markers known to be currently tested with MSC.

For a suitable cell source, besides fulfilling the function of the reconstructed tissue, low immunogenicity is advantageous for clinical application. An 'off-the-shelf' product that can be used for any patient as a 'universal' valve substitute would be more appropriate. The limited number of BM-MSC available for autogenous use and the possibility of donor site morbidity, mean that there is a need to identify the use of allogeneic or xenogeneic cells, which may pose the significant problem of anti-graft immune responses.

Although the expansion and differentiation potential of most sources have already been well characterized, immunogenic behaviour has been studied thoroughly only for BM-MSC. In this respect, recent research has shown that BM-MSC are not immunogenic and have immunomodulatory ability. Most reports find that the immunosuppressive properties are broad, effective whether the stimulation is specific or nonspecific, across species, and across different populations of lymphocytes.[21,22,26,45–50] *In vivo*, BM-MSC may improve the outcome of allogeneic transplantation by promoting haematopoietic engraftment and limiting graft-versus-host disease.[24,27,51,52]

As an allogeneic cell source, UCB-MSC may raise the issue of a possible adverse reaction or eventual rejection of the allogeneic MSC by the recipient. An open question is whether UCB-MSC share immune characteristics with BM-MSC. To answer this question, we investigated the immunogenicity and immunomodulatory function of UCB-MSC. First, the expression profiles of MHC class I and II molecules and costimulatory molecules on UCB-MSC were analysed. Similar to BM-MSC,[31,45,53] UCB-MSC express MHC I but do not express MHC II and costimulatory molecules CD80 (B7-1), CD86 (B7-2), CD40, and CD40 ligand, which are mainly expressed on antigen-presenting cells and tissue cells. In addition to the expression of immune molecules, the ability of cells to induce an immune response is also important. Our data demonstrated that UCB-MSC do not induce proliferation of allogeneic PBMC. These results suggest a low immunogenicity of UCB-MSC. The deficiency of complete and functional MHC molecules or costimulatory signalling may be a possible explanation.

To date, it is widely accepted that the identification of foreign cells by the host immune system is generally linked to the recognition of MHC molecules.[54] Although the above data implied that UCB-MSC were non-immunogenic *in vitro*, it is possible that they might be induced to express MHC II *in vivo*, which could in turn stimulate an immune response. To check whether the low immunogenicity was the result of low expression of MHC II, we treated the UCB-MSC with IFN-γ. The results showed that IFN-γ could not restore the low immunogenicity even though the expression of MHC II increased on the UCB-MSC. This demonstrated that pretreatment of UCB-MSC with IFN-γ to up-regulate MHC molecules did not enhance immunogenicity. Although UCB-MSC did not induce lymphocyte proliferation themselves, whether UCB-MSC could inhibit allogeneic PBMC or mitogen-stimulated lymphocyte proliferation needed to be investigated. To test this possibility, MSC were titrated into primary MLR cultures. Data in our experiments show that UCB-MSC have an inhibitory effect on the proliferation of PBMC triggered by allogeneic PBMC or PHA in a

cell-number-dependent manner. Pretreatment of the UCB-MSC with IFN-γ for 2 days before coculture did not affect suppression. These immunosuppressive characteristics are consistent with BM-MSC.[22,24,51,53] Concerning the role of the proinflammatory cytokine IFN-γ in MSC immunomodulation, recent studies have shown that IFN-γ did not ablate MSC inhibition of alloantigen-driven proliferation but up-regulated the immunosuppressive cytokines. Interferon-γ also induced expression of indoleamine 2,3,dioxygenase, which contributes to IFN-γ-induced immunomodulation by MSC.[55,56]

Dendritic cells, the most potent antigen-presenting cells, exist in at least two different stages: immature and mature. Resting or immature DC are poor stimulators of T-cell proliferation and have been shown to induce a state of tolerance both *in vitro* and *in vivo*. However, immature DC switch from an antigen-capturing to an presenting and T-cell-stimulating mode upon exposure to maturation signals. For this reason, mature DC are critical in the initiation of primary immune responses.[57,58] Although UCB-MSC displayed immunosuppressive properties on T-lymphocyte proliferation *in vitro*, immune responses do not rely solely on T cells but also on essential and complicated interactions between antigen-presenting cells and T cells. Therefore, it is highly possible that modulating the antigen-presenting capacity of mature DC by UCB-MSC or the lack of antigen-presenting cells in the proliferative response system is the reason for the immunosuppressive properties. Based on this hypothesis, we tested the immunosuppressive effects of UCB-MSC on the proliferative response of allogeneic PBMC stimulated by mature DC. In this study, we observed that UCB-MSC + mature DC have a significantly reduced ability to stimulate the T-lymphocyte proliferation compared with mature DC alone. After removing the UCB-MSC influence on T cells directly, inhibition was also shown. The data revealed that DC may be one of the targets of MSC displaying immunosuppressive effects. In recent studies, the influence of MSC on DC function has caught the attention of a number of groups. These studies demonstrate that MSC disrupt the three major functions that characterize the transition of DC from immature to mature stages; namely the up-regulation of antigen presentation and costimulatory molecule expression, the ability to present defined antigens, and the capacity to migrate to lymph-node-derived chemokines. Collectively, these data support the hypothesis that MSC profoundly influence host immunity by modulating DC function.[59,60] In the case of UCB-MSC, how they modulate the differentiation and function of DC needs to be further investigated.

In this report, the mechanisms underlying these effects are largely unknown but are likely to be mediated by direct cell-to-cell interactions and soluble factors.[22,47,48,51,61] By comparative analysis of the typical coculture and transwell system, the relative contribution of intercellular contact or soluble factors could be estimated. In transwell systems, cocultures of UCB-MSC with PBMC separated by a membrane also exert an inhibitory effect, indicating that various soluble factors may account for UCB-MSC-mediated inhibition. On the other hand, UCB-MSC added directly to the MLR cocultures had stronger inhibitory effects compared with the inhibitory effects of separate cell groups. It was found that there were differences between the cell–cell contact and the separate cell groups. The data implied that the UCB-MSC inhibitory effect was mediated by both cell contact and soluble factors. It will be of further interest to expand such studies to identify the soluble factors or the molecules expressed on the cell surface that play a role in immune regulation.

In summary, the present study describes the isolation of MSC from human UCB and demonstrates that UCB-MSC share most of the characteristics with BM-MSC, including morphological phenotypes, the expression of various phenotypic markers, multilineage differentiation capacity, immunogenicity and immunoregulatory potency, which were not changed by IFN-γ treatment. The UCB-MSC are unable to stimulate the proliferation of allogeneic PBMC, which implied that UCB-MSC are not completely immunogenic. In addition, cells can modulate the immune system, not only acting directly on T cells but also at the very first step of the immune response through the inhibition of DC function *in vitro*. All the evidence implied the retention of the immunoprivileged status of UCB-MSC, which seems to be mediated by both cell contact and soluble factors. Together with the distinct advantages of UCB, such as accessibility, painless procedures for donors, no ethical controversy, higher proliferation capacity and lower risk of transmitting infections, this immunoprovilege may render UCB-MSC a suitable alternative to BM-MSC for allogeneic transplantation in cell-based therapeutic strategies. Furthermore, UCB banking for the transplantation of haematopoietic stem cells has emerged, which considering the relatively low success rate of isolating MSC from UCB, provides available and suitable donors to establish a UCB-MSC bank network system for patients requiring allogeneic transplantation. Preliminary observations suggest that UCB from unrelated donors is a feasible alternative source of stem cells for transplantation. Consequently, further studies are required to fully evaluate the immunogenic property and immunomodulatory function of UCB-MSC *in vivo*.

Acknowledgements

This study was financially supported by the National '863' (2006AA02A122) Project Foundation.

References

1 Pittenger MF, Mackay AM, Beck SC *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* 1999; **284**:143–7.

2 Pittenger MF, Martin BJ. Mesenchymal stem cells and their potential as cardiac therapeutics. *Circ Res* 2004; **95**:9–20.

3 Satake K, Lou J, Lenke LG. Migration of mesenchymal stem cells through cerebrospinal fluid into injured spinal cord tissue. *Spine* 2004; **29**:1971–9.

4 Shang Q, Wang Z, Liu W, Shi Y, Cui L, Cao Y. Tissue-engineered bone repair of sheep cranial defects with autologous bone marrow stromal cells. *J Craniofac Surg* 2001; **12**:586–93.

5 Ferrari G, Cusella-De Angelis G, Coletta M, Paolucci E, Stornaiuolo A, Cossu G, Mavilio F. Muscle regeneration by bone marrow-derived myogenic progenitors. *Science* 1998; **279**:1528–30.

6 Shintani S, Murohara T, Ikeda H *et al.* Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. *Circulation* 2001; **103**:2776–9.

7 Noort WA, Kruisselbrink AB, In't Anker PS *et al.* Mesenchymal stem cells promote engraftment of human umbilical cord blood-derived CD34(+) cells in NOD/SCID mice. *Exp Hematol* 2002; **30**:870–8.

8 Angelopoulou M, Novelli E, Grove JE, Rinder HM, Civin C, Cheng L, Krause DS. Cotransplantation of human mesenchymal stem cells enhances human myelopoiesis and megakaryocytopoiesis in NOD/SCID mice. *Exp Hematol* 2003; **31**:413–20.

9 Mueller SM, Glowacki J. Age-related decline in the osteogenic potential of human bone marrow cells cultured in three-dimensional collagen sponges. *J Cell Biochem* 2001; **82**:583–90.

10 Stenderup K, Justesen J, Clausen C, Kassem M. Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. *Bone* 2003; **33**:919–26.

11 Rao MS, Mattson MP. Stem cells and aging: expanding the possibilities. *Mech Ageing Dev* 2001; **122**:713–34.

12 Gluckman E, Rocha V, Boyer-Chammard A *et al.* Outcome of cord-blood transplantation from related and unrelated donors. Eurocord Transplant Group and the European Blood and Marrow Transplantation Group. *N Engl J Med* 1997; **337**:373–81.

13 Grewal SS, Barker JN, Davies SM, Wagner JE. Unrelated donor hematopoietic cell transplantation: marrow or umbilical cord blood? *Blood* 2003; **101**:4233–44.

14 Warkentin PI; Foundation for the Accreditation of Cellular Therapy. Voluntary accreditation of cellular therapies: Foundation for the Accreditation of Cellular Therapy (FACT). *Cytotherapy* 2003; **5**:299–305.

15 Mareschi K, Biasin E, Piacibello W, Aglietta M, Madon E, Fagioli F. Isolation of human mesenchymal stem cells: bone marrow versus umbilical cord blood. *Haematologica* 2001; **86**:1099–100.

16 Wexler SA, Donaldson C, Denning-Kendall P, Rice C, Bradley B, Hows JM. Adult bone marrow is a rich source of human mesenchymal 'stem' cells but umbilical cord and mobilized adult blood are not. *Br J Haematol* 2003; **121**:368–74.

17 Erices A, Conget P, Minguell JJ. Mesenchymal progenitor cells in human umbilical cord blood. *Br J Hematol* 2000; **109**:235–42.

18 Goodwin HS, Bicknese AR, Chien SN, Bogucki BD, Quinn CO, Wall DA. Multilineage differentiation activity by cells isolated from umbilical cord blood: expression of bone, fat, and neural markers. *Biol Blood Marrow Transplant* 2001; **7**:581–8.

19 Lee OK, Kuo TK, Chen WM, Lee KD, Hsieh SL, Chen TH. Isolation of multipotent mesenchymal stem cells from umbilical cord blood. *Blood* 2004; **103**:1669–75.

20 Kakinuma S, Tanaka Y, Chinzei R *et al.* Human umbilical cord blood as a source of transplantable hepatic progenitor cells. *Stem Cells* 2003; **21**:217–27.

21 Augello A, Tasso R, Negrini SM, Amateis A, Indiveri F, Cancedda R, Pennesi G. Bone marrow mesenchymal progenitor cells inhibit lymphocyte proliferation by activation of the programmed death 1 pathway. *Eur J Immunol* 2005; **35**:1482–90.

22 Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2005; **105**:1815–22.

23 Deng W, Han Q, Liao L *et al.* Allogeneic bone marrow-derived flk-1+ Sca-1– mesenchymal stem cells leads to stable mixed chimerism and donor-specific tolerance. *Exp Hematol* 2004; **32**:861–7.

24 Bartholomew A, Sturgeon C, Siatskas M *et al.* Mesenchymal stem cells suppress lymphocyte proliferation *in vitro* and prolong skin graft survival *in vivo*. *Exp Hematol* 2002; **30**:42–48.

25 Maitra B, Szekely E, Gjini K, Laughlin MJ, Dennis J, Haynesworth SE, Koc ON. Human mesenchymal stem cells support unrelated donor hematopoietic stem cells and suppress T-cell activation. *Bone Marrow Transplant* 2004; **33**:597–604.

26 Le Blanc K, Tammik L, Sundberg B, Haynesworth SE, Ringden O. Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. *Scand J Immunol* 2003; **57**:11–20.

27 Ringden O, Uzunel M, Rasmusson I *et al.* Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease. *Transplantation* 2006; **81**:1390–7.

28 Horwitz EM, Prockop DJ, Fitzpatrick LA *et al.* Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. *Nat Med* 1999; **5**:309–13.

29 Chen SL, Fang WW, Qian J *et al.* Improvement of cardiac function after transplantation of autologous bone marrow mesenchymal stem cells in patients with acute myocardial infarction. *Chin Med J (Engl)* 2004; **117**:1443–8.

30 Lazarus HM, Koc ON, Devine SM *et al.* Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. *Biol Blood Marrow Transplant* 2005; **11**:389–98.

31 Lee RH, Kim B, Choi I, Kim H, Choi HS, Suh K, Bae YC, Jung JS. Characterization and expression analysis of mesenchymal stem cells from human bone marrow and adipose tissue. *Cell Physiol Biochem* 2004; **14**:311–24.

32 Shih DT, Lee DC, Chen SC, Tsai RY, Huang CT, Tsai CC, Shen EY, Chiu WT. Isolation and characterization of neurogenic mesenchymal stem cells in human scalp tissue. *Stem Cells* 2005; **23**:1012–20.

33 Trubiani O, Di Primio R, Traini T, Pizzicannella J, Scarano A, Piattelli A, Caputi S. Morphological and cytofluorimetric analysis of adult mesenchymal stem cells expanded *ex vivo* from periodontal ligament. *Int J Immunopathol Pharmacol* 2005; **18**:213–21.

34 In't Anker PS, Scherjon SA, Kleijburg-van der Keur C, de Groot-Swings GM, Claas FH, Fibbe WE, Kanhai HH. Isolation of

mesenchymal stem cells of fetal or maternal origin from human placenta. *Stem Cells* 2004; **22**:1338–45.

35 In't Anker PS, Noort WA, Scherjon SA *et al.* Mesenchymal stem cells in human second-trimester bone marrow, liver, lung, and spleen exhibit a similar immunophenotype but a heterogeneous multilineage differentiation potential. *Haematologica* 2003; **88**:845–52.

36 De Bari C, Dell'Accio F, Tylzanowski P, Luyten FP. Multipotent mesenchymal stem cells from adult human synovial membrane. *Arthritis Rheum* 2001; **44**:1928–42.

37 Young HE, Steele TA, Bray RA *et al.* Human reserve pluripotent mesenchymal stem cells are present in the connective tissues of skeletal muscle and dermis derived from fetal, adult, and geriatric donors. *Anat Rec* 2001; **264**:51–62.

38 Zvaifler NJ, Marinova-Mutafchieva L, Adams G, Edwards CJ, Moss J, Burger JA, Maini RN. Mesenchymal precursor cells in the blood of normal individuals. *Arthritis Res* 2000; **2**:477–88.

39 Arai F, Ohneda O, Miyamoto T, Zhang XQ, Suda T. Mesenchymal stem cells in perichondrium express activated leukocyte cell adhesion molecule and participate in bone marrow formation. *J Exp Med* 2002; **195**:1549–63.

40 Rubinstein P, Rosenfield RE, Adamson JW, Stevens CE. Stored placental blood for unrelated bone marrow reconstitution. *Blood* 1993; **81**:1679–90.

41 Kogler G, Sensken S, Airey JA *et al.* A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. *J Exp Med* 2004; **200**:123–35.

42 Silva GV, Litovsky S, Assad JA *et al.* Mesenchymal stem cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a canine chronic ischemia model. *Circulation* 2005; **111**:150–6.

43 Lee KD, Kuo TK, Whang-Peng J *et al. In vitro* hepatic differentiation of human mesenchymal stem cells. *Hepatology* 2004; **40**:1275–84.

44 Moriscot C, de Fraipont F, Richard MJ, Marchand M, Savatier P, Bosco D, Favrot M, Benhamou PY. Human bone marrow mesenchymal stem cells can express insulin and key transcription factors of the endocrine pancreas developmental pathway upon genetic and/or microenvironmental manipulation *in vitro*. *Stem Cells* 2005; **23**:594–603.

45 Le Blanc K, Tammik C, Rosendahl K, Zetterberg E, Ringden O. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. *Exp Hematol* 2003; **31**:890–6.

46 Rasmusson I, Ringden O, Sundberg B, Le Blanc K. Mesenchymal stem cells inhibit lymphocyte proliferation by mitogens and alloantigens by different mechanisms. *Exp Cell Res* 2005; **305**:33–41.

47 Beyth S, Borovsky Z, Mevorach D, Liebergall M, Gazit Z, Aslan H, Galun E, Rachmilewitz J. Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. *Blood* 2005; **105**:2214–9.

48 Krampera M, Glennie S, Dyson J, Scott D, Laylor R, Simpson E, Dazzi F. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. *Blood* 2003; **101**:3722–9.

49 Klyushnenkova E, Mosca JD, Zernetkina V, Majumdar MK, Beggs KJ, Simonetti DW, Deans RJ, McIntosh KR. T cell responses to allogeneic human mesenchymal stem cells: immunogenicity, tolerance, and suppression. *J Biomed Sci* 2005; **12**:47–57.

50 Glennie S, Soeiro I, Dyson PJ, Lam EW, Dazzi F. Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells. *Blood* 2005; **105**:2821–7.

51 Tse WT, Pendleton JD, Beyer WM, Egalka MC, Guinan EC. Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. *Transplantation* 2003; **75**:389–97.

52 Le Blanc K, Ringden O. Immunobiology of human mesenchymal stem cells and future use in hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* 2005; **11**:321–34.

53 Majumdar MK, Keane-Moore M, Buyaner D, Hardy WB, Moorman MA, McIntosh KR, Mosca JD. Characterization and functionality of cell surface molecules on human mesenchymal stem cells. *J Biomed Sci* 2003; **10**:228–41.

54 Modo M, Mellodew K, Rezaie P. *In vitro* expression of major histocompatibility class I and class II antigens by conditionally immortalized murine neural stem cells. *Neurosci Lett* 2003; **337**:85–88.

55 Ryan JM, Barry F, Murphy JM, Mahon BP. Interferon-gamma does not break, but promotes the immunosuppressive capacity of adult human mesenchymal stem cells. *Clin Exp Immunol* 2007; **149**:353–63.

56 English K, Barry FP, Field-Corbett CP, Mahon BP. IFN-gamma and TNF-alpha differentially regulate immunomodulation by murine mesenchymal stem cells. *Immunol Lett* 2007; **110**:91–100.

57 Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998; **392**:245–52.

58 Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K. Immunobiology of dendritic cells. *Annu Rev Immunol* 2000; **18**:767–811.

59 English K, Barry FP, Mahon BP. Murine mesenchymal stem cells suppress dendritic cell migration, maturation and antigen presentation. *Immunol Lett* 2008; **115**:50–58.

60 Jiang XX, Zhang Y, Liu B, Zhang SX, Wu Y, Yu XD, Mao N. Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. *Blood* 2005; **105**:4120–6.

61 Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, Grisanti S, Gianni AM. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 2002; **99**:3838–43.