Cord blood stem-cell-derived dendritic cells generate potent antigen-specific immune responses and anti-tumour effects

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A B S T R A C T

The aim of the present study was to investigate whether CBSCs [(umbilical) cord blood stem cells] can be a new source of DCs (dendritic cells), which can generate more potent antigen-specific immune responses and anti-tumour effects. CBSCs and PBMCs (peripheral blood mononuclear cells) were collected, cultured and differentiated into DCs. Surface markers, secreting cytokines, antigen-presentation activity, antigen-specific cell-mediated immunity and cytotoxic killing effects induced by these two DC origins were evaluated and compared. CBSCs were expanded ∼17-fold by *ex vivo* culture. The expression of surface markers in CBSC-derived DCs were higher than those in PBMC-derived DCs treated with LPS (lipopolysaccharide). The CBSC-derived DCs mainly secreted IL (interleukin)-6, IL-10 and TNF (tumour necrosis factor)-α, whereas PBMC-derived DCs mainly secreted IL-5 and IFN (interferon)-γ. The CBSC-derived DCs had better antigen-presentation abilities when stimulated with LPS or TNF-α, induced higher numbers of IFN-γ-secreting antigen-specific CD8$^+$ T-cells, as assessed using an ELISpot (enzyme-linked immunosorbent spot) assay, and stimulated more potent antigen-specific CTL (cytotoxic T-cell) activities ($P < 0.01$, one-way ANOVA). CBSC-derived DCs had quicker and greater ERK (extracellular-signal-regulated kinase) and Akt phosphorylation, and weaker p38 phosphorylation, than PBMC-derived DCs when stimulated with LPS. In conclusion, CBSC-derived DCs have the ability to induce stronger antigen-specific immunity and more potent anti-tumour effects and therefore could be a good source of DCs for use in DC-based cancer vaccines and immunotherapy.

INTRODUCTION

DCs (dendritic cells) are the most potent antigen-presenting cells and are required for the initiation of an immune response by stimulating naïve, memory and effector T-cells. They possess a wide spectrum of activities, including the induction of tolerance and prevention of auto-immunity, the induction of an anti-tumour response and protection against infectious agents [1,2]. However, it remains uncertain whether or not these

activities are associated with separate subsets of DCs, their stage of maturation or both [2].

Monocytes are the most commonly used source of DC precursors [3]. When using monocytes as a DC source, medium enriched with GM-CSF (granulocyte/macrophage colony-stimulating factor) and IL (interleukin)-4 are most commonly used [4,5]. There is growing interest in developing methods that allow the generation of large numbers of DCs, as they may be clinically useful in the production of cancer vaccines [1]. HSCs (human stem cells) have been used in order to exploit their potential in both basic developmental biology and clinical applied medicine. HSCs are still of poor therapeutic value for many diseases, mainly because of their scarce availability. Therefore expanding HSCs in *ex vivo* culture would be useful in overcoming the existing limitations and, more importantly, for developing new transplant strategies. In contrast with monocytes, CBSCs [(umbilical) cord blood stem cells] proliferate as another DC source in *in vitro* culture. Balan et al. [6] reported that DCs from fresh UCB (umbilical cord blood) can have an equal efficiency compared with those from frozen UCB. Besides, a CD34$^+$ isolation step is not essential for the generation of mature functional DCs [6].

The development of effective vaccines for cancer treatment represents a major public health issue [7]. Because CTLs (cytotoxic T-cells) are able to recognize and lyse malignant cells, many therapeutic trials have been designed to potentiate CTL responses and, as DCs play a fundamental role in triggering T-cell responses, their use may be promising in new immunotherapeutic strategies [8]. However, the use of autologous DCs for cancer immunotherapy is difficult because of the scarcity of these cells [9] and the possible functional alteration of DCs harvested from tumour-bearing patients. Therefore there is a need for novel immunotherapeutic strategies to overcome the limitations of current protocols.

The aim of the present study was to investigate whether *ex vivo*-expanded of UCB CD34$^+$ cells also have the phenotype and biological activities of DCs originating from PBMCs cultured in DC medium. The results of the present study highlight the differences between CBSC- and PBMC-derived DCs, and the importance of CBSC-derived DCs in the strategy of DC-based cancer immunotherapy.

MATERIALS AND METHODS

Isolation of MNCs (mononuclear cells) from UCB

UCB was obtained from the National Taiwan University Hospital with the mothers' informed consent. The Institutional Review Board approved the study protocol. More than ten UCB samples were collected from respective individuals. MNCs were separated by Ficoll–Paque^{TM} plus with a density of 1.077 g/ml (GE Healthcare) and centrifuged at 1400 *g* for 20 min [8]. The viability of MNCs was checked by Trypan Blue staining, and viable MNCs were cultured and utilized for further experiments.

CD34$^+$ cell preparation from UCB

CD34$^+$ cells from UCB were isolated using the CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec). The purity of the CD34$^+$ cells after isolation was >90% and contamination was <5%.

Isolation of adult PBMCs

Adult peripheral blood was drawn from the peripheral vein after informed consent was received. More than ten adult PBMC samples were tested from respective individuals. The isolation of adult PBMCs was performed as described previously [8]. Briefly, PBMCs were prepared with Ficoll–Paque^{TM} plus solution for separation of cells. After centrifugation, the layer containing the cells was transferred to another centrifuge tube, washed twice in 10 ml of PBS and then centrifuged at 200 *g* for 7 min.

Identification of HLA (human lymphocyte antigen) haplotype

The HLA haplotype was identified using the primary anti-HLA-A2 monoclonal antibody BB7.2 and a FITC-conjugated secondary antibody {Alexa Fluor® 488-conjugated goat anti-[mouse IgG (heavy + light chain)]} [10].

Synthetic peptides

Two HLA-A2-restricted HPV (human papillomavirus) type 16 E7 peptides YMLDLQPETT (amino acids 11–20) and TLGIVCPI (amino acids 86–93) [11] were synthesized by Kelowna. FITC-conjugated short OVA (ovalbumin) peptide [OVA$^{257-264}$ (SIINFEKL)] was purchased from Invitrogen.

Reagents

GM-CSF (granulocyte/macrophage colony-stimulating factor) and IL (interleukin)-4 were purchased from Peprotech, SCF (stem cell factor) was from BioSource, TNF (tumour necrosis factor)-α was from Pepro Tech and LPS (lipopolysaccharide) was from Sigma. The PE (phycoerythrin)-conjugated anti-HLA-DR and anti-CD86 antibodies were from eBioscience Biotechnology, and the FITC-conjugated anti-CD83 and anti-CD1a antibodies were from BD Pharmingen.

Expansion of MNCs from UCB

The MNCs of UCB cells (10^6 cells/ml) were cultured in IMDM (Iscove's modified Dulbecco's medium) (Gibco), 10% FBS (fetal bovine serum) (Gibco), 1% L-glutamate

(Gibco) and 25 mM Hepes (Gibco) with 100 ng/ml SCF. MNCs were incubated at 37°C in humidified 5% CO_2 were seeded into 4 ml of culture medium for 14 days. Fresh medium was replaced every 2 days. The cell number was counted and cells were immunophenotyped by flow cytometric analysis. Cell expansion is expressed as the fold increase (i.e. the absolute number of day 14 cells divided by that of day 0 cells).

Generation of DCs from PBMCs or CBSCs

PBMC-derived DCs were generated as described previously [8]. Briefly, PBMCs were suspended in medium and allowed to adhere to plastic tissue-culture dishes. Following incubation for 2 h at 37°C, the non-adherent cells were removed for further use, and the adherent cells were cultured with GM-CSF (800 units/ml) and IL-4 (500 units/ml) for 6 days in RPMI 1640 (Mediatech). Fresh cytokine-containing medium was replaced every 2 days, and the cells were phenotypically analysed after culture. The cultured cells were collected for further studies and analysis on days 2, 4 and 6. In addition, LPS (100 ng/ml) or TNF-α (50 ng/ml) was added on days 2, 4 or 6, and these LPS- or TNF-α-stimulated cells were collected 24 h later for further studies and analysis.

CBSC-derived DCs were generated as described previously [12] with some modifications. Briefly, CBSCs were cultured with GM-CSF and IL-4 for 6 days as described above. The cultured cells were collected for further studies and analysis on days 2, 4 and 6. In addition, LPS or TNF-α was added on days 2, 4 or 6, and these LPS- or TNF-α-stimulated cells were collected 24 h later for further studies and analyses.

Phenotypic changes of PBMC- and CBSC-derived DCs by flow cytometric analysis

PBMCs and CBSCs were cultured with GM-CSF and IL-4 alone, or stimulated with LPS or TNF-α as described above. These cells were collected, washed and stained with anti-CD1a, -HLA-DR, -CD83 and -CD86 antibodies in FACScan buffer (PBS with 0.2% FBS and 0.5% sodium azide) for 30 min, followed by washing with FACScan buffer. Data acquisition and analysis were performed using a FACScalibur flow cytometer (Becton Dickinson) using CellQuest software.

Detection of cytokines secreted from PBMC- and CBSC-derived DCs by ELISA

Cells (10^6/well) were seeded on to 24-well plates, cultured with GM-CSF and IL-4, and stimulated with or without LPS as described above. The concentrations of IL-5, IL-6, IL-10, IL-12, IFN (interferon)-γ and TNF-α in the cell supernatants were determined by human ELISA kits (BD Biosciences). All measurements were done in triplicate.

Antigen-presentation activity test

To compare the antigen-presentation activities of PBMC- and CBSC-derived DCs stimulated with or without LPS or TNF-α, the cells were seeded on to 24-well plates (10^6/well), cultured with GM-CSF and IL-4, and then co-treated with 1 μg/ml FITC-conjugated OVA[257–264] peptide as described previously [13]. Cells were then washed and assessed by flow cytometry. Unstimulated cells treated with PBS were served as control.

Generation of peptide-specific CTLs

The HLA-A2-positive CBSC- or PBMC-derived DCs were cultured and stimulated as described above. To generate the E7-specific CD8$^+$ T-cells, CBSC- or PBMC-derived mature DCs (2×10^5 cells/well) were cultured and pulsed for 2 h with 10 mM of the particular E7 peptide (amino acids 11–20 or 86–93), washed and incubated with PBMCs (2×10^6 cells/well) from normal volunteers in 24-well plates with human T-cell medium (RMPI 1640, 10% human serum, 5% Hepes, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin) for 6 days. The E7-specific CD8$^+$ T-cells were either used immediately or cryopreserved in liquid nitrogen until testing.

ELISpot (enzyme-linked immunosorbent spot) assays with co-culture of haplotype-compatible peptide-pulsed PBMC- or CBSC-derived DCs and CD8$^+$ T-cells

The ELISpot assay to detect the immune response of antigen-specific CD8$^+$ T-cells was used as described previously [8]. The 96-well filtration plates were coated with an anti-(human IFN-γ) antibody (10 μg/ml; PharMingen) in PBS (50 μl). Following overnight incubation at 4°C, the wells were washed and stored before use. Different numbers of HLA-A2-restricted T-cells (10^6, 3.3×10^5 and 10^5 cells/well) were co-cultured with E7 peptide-loaded (amino acids 11–20 or 86–93) PBMC- or CBSC-derived DCs (T-cell/DC ratio was 3:1) for 48 h. Following culture, the plate was washed and incubated with a biotinylated rat anti-(human IFN-γ) antibody (5 μg/ml; PharMingen) in PBS (50 μl) at 4°C overnight. Following six washes, avidin/alkaline phosphatase (10 μg/ml; Sigma) in PBS (50 μl) was added and the plates were incubated for 2 h at room temperature (25°C). Then the spots were developed by adding BCIP (5-bromo-4-chloroindol-3-yl phosphate/NTB (Nitro Blue Tetrazolium) solution (50 μl; Boehringer Mannheim) and incubated at room temperature for 20 min. The reactions were stopped by discarding the substrate and washing the plates with tap water. The plates were then air-dried and the coloured spots were counted using a dissecting microscope (ImmunoSpot® Analyzers; Cellular Technology).

CTL assays

CTL assays were performed to evaluate whether CBSC-derived DCs generated stronger antigen-specific anti-tumour effects than PBMC-derived DCs. The E7-specific $CD8^+$ T-cells pulsed with peptide-loaded PBMC- or CBSC-derived DCs were used as effector cells to assay for their relative cytotoxic activity. The CaSki tumour cell line was used as a source of target cells, and the HeLa or K562 cell line was used as a negative control. CTL activity was measured using a standard LDH (lactate dehydrogenase) release assay [14,15].

E7-specific $CD8^+$ T-cells stimulated by E7 peptide-pulsed CBSC- or PBMC-derived DCs were mixed together with CaSki, HeLa or K562 cells (10^4 cells/well) at various ratios (5:1, 15:1 and 45:1) in a final volume of 200 μl. Following incubation for 5 h at 37°C, 50 μl of the culture medium was collected to assess the quantity of LDH in the culture medium using a CytoTox assay kit (Promega), according to the manufacturer's protocol. The mean percentage of specific cell lysis of triplicate wells was determined as:

$$\text{Specific release (\%)} = (\text{experimental release} - \text{spontaneous release}/\text{maximum release} - \text{spontaneous release}) \times 100$$

A CTL response was defined as being positive when the degree of cell lysis in a cytotoxicity assay of target cells was at least 15 % higher at the two highest effector cell/target cell ratios than those for the background lysis of target cells.

Western blot analysis

PBMCs and CBSCs were cultured with GM-CSF and IL-4 as described above. Cytokine-cultured PBMC- or CBSC-derived DCs were stimulated further with LPS for 24 h. Western blots were used to detect phospho-ERK (extracellular-signal-regulated kinase), phospho-Akt, and phospho-p38 in PBMC- and CBSC-derived DCs without or with LPS treatment. The cells were lysed in immunoprecipitation assay buffer and were analysed as described previously [16]. Protein extracts were quantified using a BCA Protein Assay Kit (Pierce). Then, 50 μg of each cell lysate was resolved by SDS/PAGE (12 % gel), transferred on to a PVDF/nylon membrane (Millipore), and probed with antibodies specific to ERK, phospho-ERK and Akt (all from Upstate Biotechnology), phospho-Akt (Ser^{473}) (Chemicon International), p38 and phospho-p38 (both from Cell Signaling Technology) or β-actin (Chemicon International). The membrane was then probed with either HRP (horseradish peroxidase)-conjugated goat anti-mouse or goat anti-rabbit antibodies. The specific bands were visualized using an ECL (enhanced chemiluminescence) Western blot system (GE Healthcare) and photographed with Kodak X-Omat Blue autoradiography film (PerkinElmer Life Sciences).

Statistical analysis

All results expressed as means $\pm$ S.E.M. and are representative of at least two different experiments. Results for intracellular cytokine staining with flow cytometric analysis and tumour treatment experiments were evaluated by ANOVA. Comparisons between individual data points were made using a Student's t test. In the tumour protection experiment, the principal outcome of interest was the time to the development of the tumour. $P < 0.05$ was considered statistically significant.

RESULTS

$CD34^+$ cell number in MNCs, other than total MNCs, increase with longer culture times

Before investigating the properties of $CD34^+$ cells in MNCs from UCB, we first evaluated whether these cells could be expanded using the culture protocol. The growth rates of $CD34^+$ cells from UCB in culture medium containing SCF only or a cocktail of growth factors [33] were determined, and the results are shown in Supplementary Table S1 (available at http://www.clinsci.org/cs/123/cs1230347add.htm).

From these preliminary studies, cells from UCB were cultured in SCF alone and were harvested after 14 days of culture.

Representative traces from the flow cytometric analysis of $CD34^+$ cells in MNCs are shown in Figure 1(A). The percentage of $CD34^+$ cells in MNCs increased gradually when the culture interval was extended (Figure 1B). The total number of $CD34^+$ cells in MNCs also increased until culture day 14. However, the total number of $CD34^+$ cells in MNCs reached a plateau after culture for more than 14 days (Figure 1C).

These results indicate that the number of $CD34^+$ cells in MNCs from UCB could be extended in culture medium for at least 14 days.

Differential expression levels of specific surface markers on CBSC-derived DCs compared with those on PBMC-derived DCs cultured with GM-CSF and IL-4

We then evaluated whether cultured $CD34^+$ CBSCs could be differentiated further to DCs, as is the case with adult PBMCs. $CD34^+$ CBSCs and PBMCs were first cultured with GM-CSF and IL-4 and DC surface markers were stained and analysed. Representative traces of the flow cytometric analysis of the surface markers on cultured CBSC- and PBMC-derived DCs at days 0–6 of
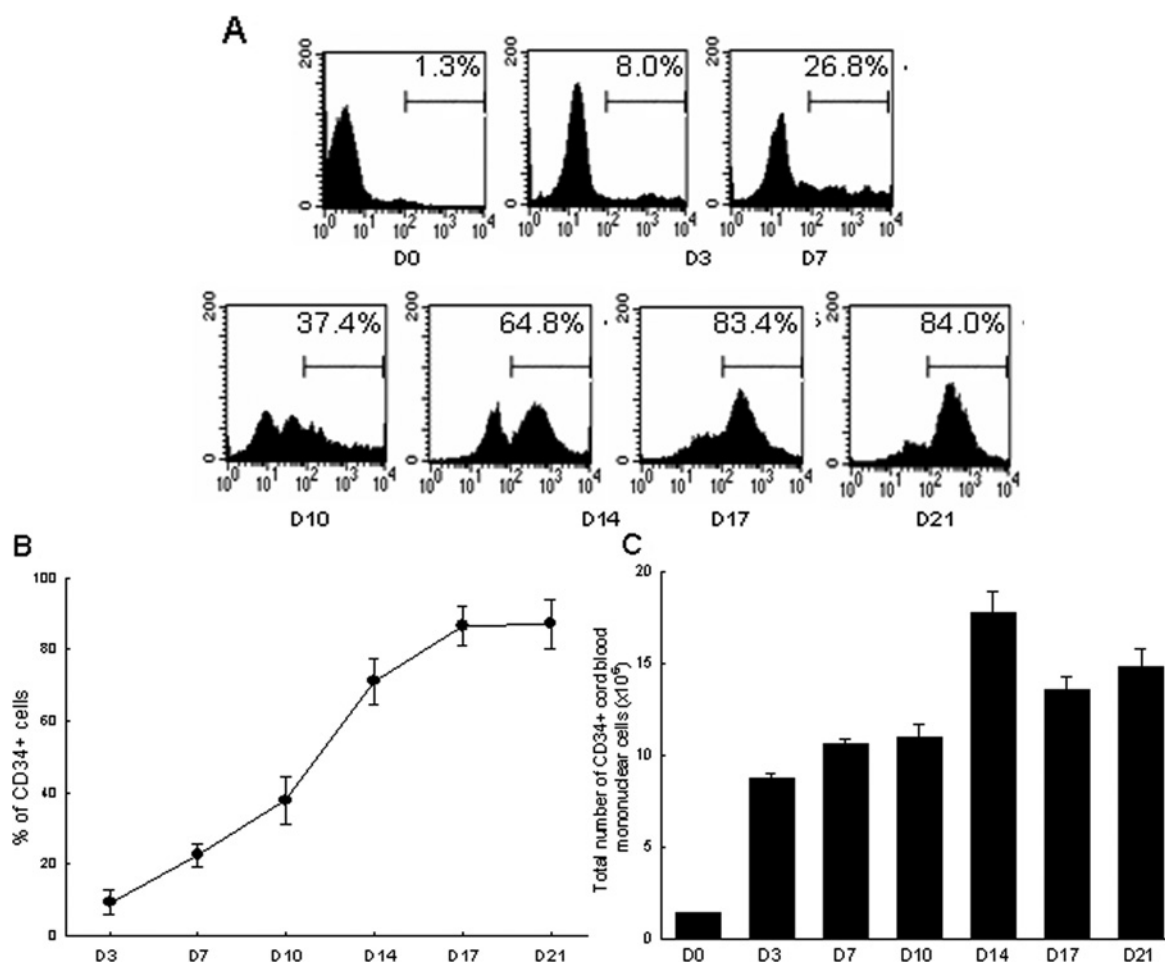
Figure 1   Kinetic changes of cultured cells in UCB

(A) Representative traces from the flow cytometric analysis of CD34$^+$ cells in UCB MNCs at different culture intervals. (B) Quantification of the percentage of CD34$^+$ MNCs in UCB at different culture intervals, showing that these increased with longer culture times. (C) Total number of CD34$^+$ MNCs in UCB at different culture times, showing that the total number of CD34$^+$ MNCs were highest on culture day 14. In (B) and (C), time in days (D) is given on the x-axis.

culture are shown in Figures 2(A) and 2(B) respectively. The expression of surface markers, including CD1a, CD83 and HLA-DR, on cultured CBSC-derived DCs increased significantly after 4 days of culture (Figure 2C), but did not change after this time in the PBMC-derived DCs. Instead, culturing for 6 days was required before the expression of all of the surface markers increased on PBMC-derived DCs (Figure 2D).

These results indicate that, by adding the appropriate cytokines, CBSCs could express DC phenotypes earlier than PBMCs.

CBSC-derived DCs express higher levels of surface markers than PBMC-derived DCs during culture or stimulation with LPS or TNF-α

We then evaluated whether the CBSC- and PBMCs-derived DCs could be matured by LPS or TFN-α.

PBMC-derived DCs at 6 days of culture had enhanced CD1a expression levels when stimulated with LPS or TNF-α (Figure 3A, panel A1), whereas CD1a expression increased significantly on CBSC-derived DCs at 4 days of culture (Figure 3A, panel A2). The expression level of CD83 on PBMC-derived DCs was enhanced significantly by LPS or TNF-α without culture or when cultured for 2, 4 or 6 days (Figure 3B, panel B1). For the CBSC-derived DCs, CD83 expression levels increased after 4 days of culture, regardless of stimulation with PBS, LPS or TNF-α (Figure 3B, panel B2). However, the expression level of CD86 and HLA-DR on CBSC- and PBMC-derived DCs was significantly enhanced by LPS or TNF-α without culture or when cultured for 2, 4 or 6 days (Figures 3C and 3D).

These results indicate that CBSC-derived DCs expressed higher levels of surface markers than PBMC-derived DCs when cultured with GM-CSF and IL-4. Thus immature PBMC- and CBSC-derived DC
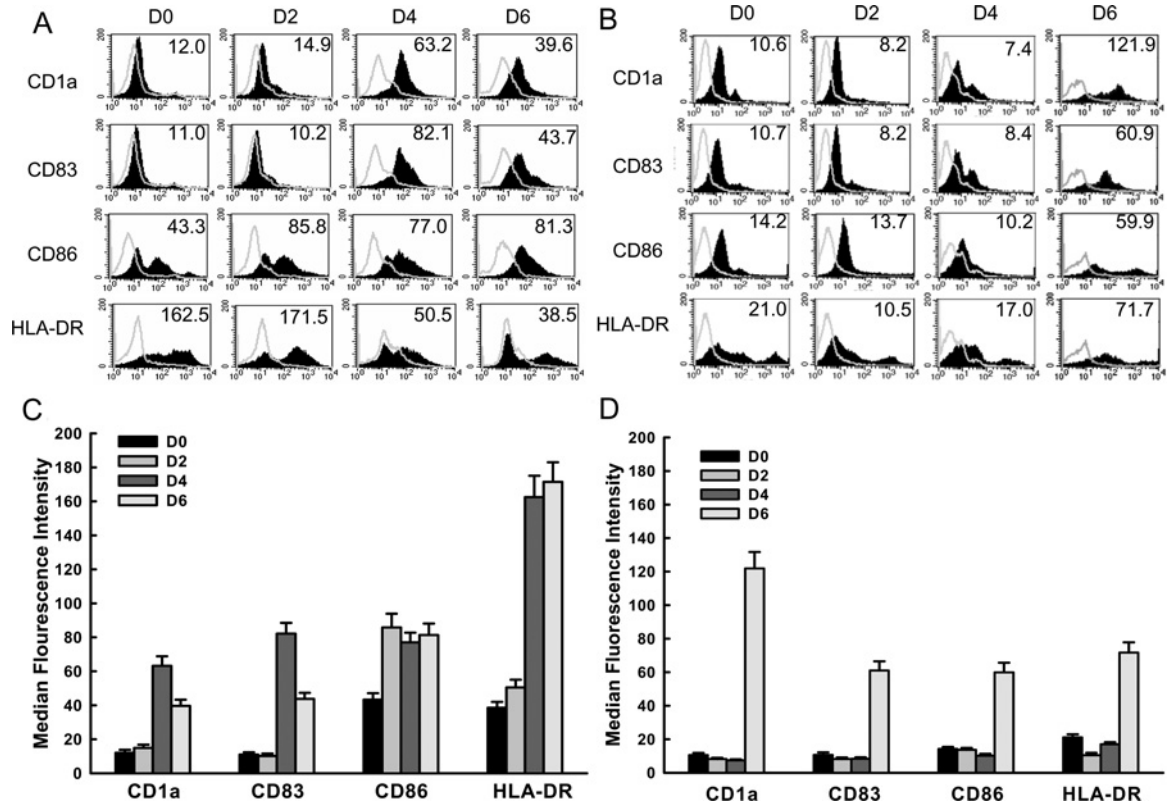
Figure 2   Kinetic changes of various surface markers on cells cultured with GM-CSF and IL-4 at different culture intervals

(A and B) Representative traces from the flow cytometric analysis of the expression levels of the surface markers CD1a, CD83, CD86 and HLA-DR on CBSC-derived DCs (A) and PBMC-derived DCs (B). The values indicate the MFI. (C and D) Kinetic changes in the expression of the surface markers on CBSC-derived DCs (C) and PBMC-derived DCs (D). The expression levels of CD1a, CD83, CD86 and HLA-DR increased with longer culture intervals in CBSC-derived DCs, whereas CD1a, CD83 and CD86 levels of increased in PBMC-derived DCs, but remained lower than those on CBSC-derived DCs. D, day.
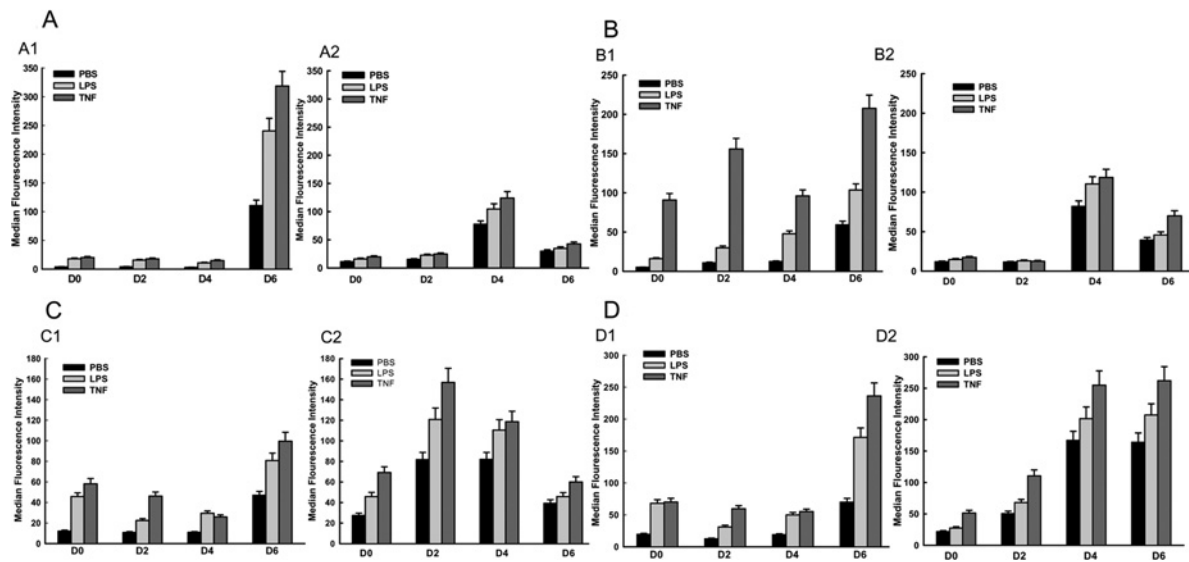
Figure 3   Kinetic changes of various surface markers on GM-CSF- and IL-4-cultured cells at different culture intervals following stimulation with LPS or TNF-α

Kinetic changes of (A) CD1a, (B) CD83, (C) CD86, and (D) HLA-DR surface-marker expression levels on PBMC- (panels A1, B1, C1, D1) and CBSC- (panels A2, B2, C2, D2) derived DCs stimulated with PBS, LPS or TNF-α are shown. TNF-α and LPS enhanced the expression levels of surface markers compared with PBS in both PBMC- or CBSC-derived DCs, but TNF-α enhanced the expression levels more than LPS. Time in days (D) is given on the x-axis.
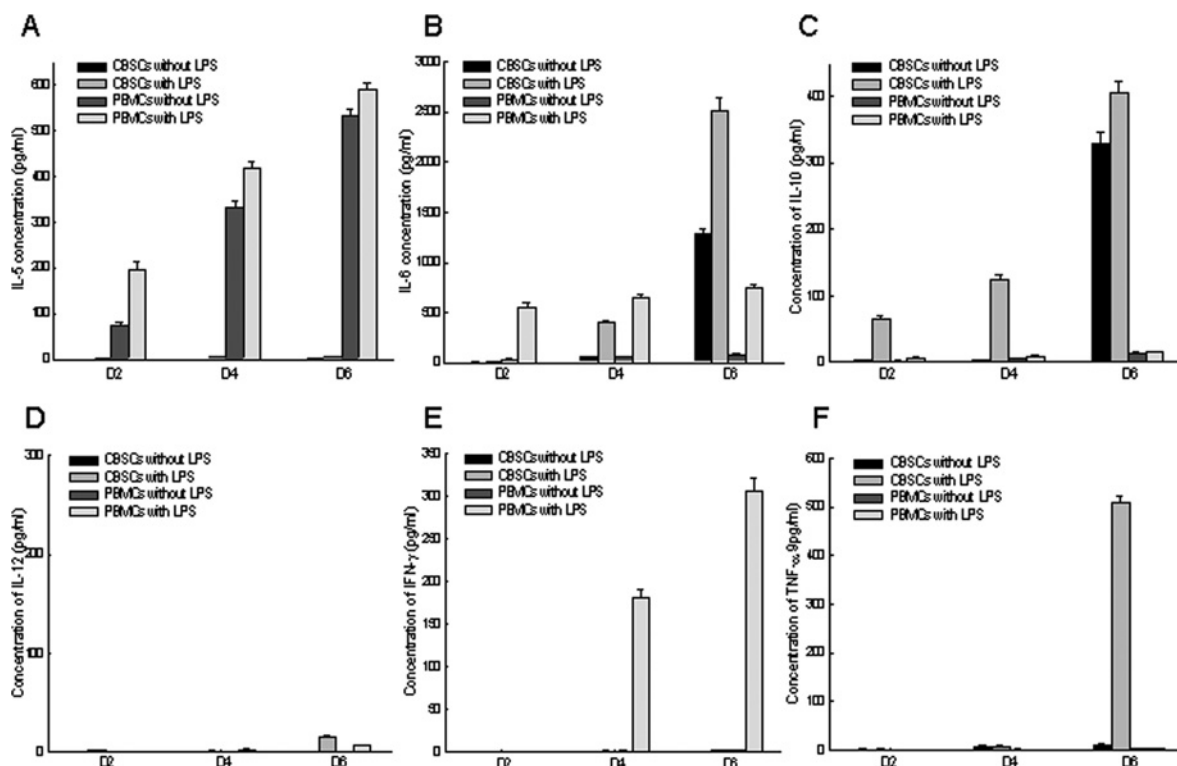
Figure 4   Concentrations of cytokines from CBSC- or PBMC-derived DCs at different culture intervals without or with LPS stimulation

The concentrations of cytokines in cell supernatants of the cultured CBSC- and PBMC-derived DCs stimulated without or with LPS were detected by ELISA. (A) IL-5, (B) IL-6, (C) IL-10, (D) IL-12, (E) IFN-γ and (F) TNF-α. PBMC-derived DCs secreted higher concentrations of IL-5 and IFN-γ compared with CBSC-derived DCs, whereas CBSC-derived DCs secreted higher concentrations of IL-6, IL-10 and TNF-α than PBMC-derived DCs. Time in days (D) is given on the x-axis.

phenotypes could be shifted to a more mature phenotype by stimulation with LPS or TNF-α.

## CBSC-derived DCs secrete immune-related cytokines

To evaluate whether the cytokines secreted by CBSC- and PBMC-derived DCs were different in the absence or presence of LPS stimulation, the concentrations of various cytokines in the cell culture supernatants were determined by ELISA. PBMC-derived DCs secreted higher concentrations of IL-5 (Figure 4A) and IFN-γ (Figure 4E) than CBSC-derived DCs. In contrast, CBSC-derived DCs secreted higher concentrations of IL-6 (Figure 4B), IL-10 (Figure 4C) and TNF-α (Figure 4F) compared with PBMC-derived DCs. However, neither CBSC- nor PBMC-derived DCs secreted high amounts of IL-12, even when stimulated with LPS (Figure 4D).

These results indicate that the cytokines secreted by CBSC- and PBMC-derived DCs differ during the culture period.

CBSC-derived DCs have better antigen-presentation abilities than PBMC-derived DCs

To evaluate whether the PBMC- and CBSC-derived DCs had the ability to present antigens, DCs were collected after different culture intervals, pulsed with FITC-conjugated HLA-A2-compatible OVA[257–264] peptide and analysed by flow cytometry. Representative traces from the flow cytometric analysis are shown in Figure 5(A). The MFIs (median fluorescence intensities) of CBSC-derived DCs pulsed with FITC-conjugated HLA-A2-compatible OVA[257–264] peptide were significantly higher ($P < 0.05$, Mann–Whitney U test) than those of PBMC-derived DCs or the control group from 2 days of culture with GM-CSF and IL-4 (Figure 5B).

When pulsed with FITC-conjugated HLA-A2-compatible OVA[257–264] peptide, the MFIs of LPS-treated CBSC-derived DCs were significantly higher ($P < 0.05$, Mann–Whitney U test) than those of PBMC-derived DCs or the control group (Figure 5C). In addition, when pulsed with FITC-conjugated HLA-A2-compatible OVA[257–264] peptide, the MFIs of
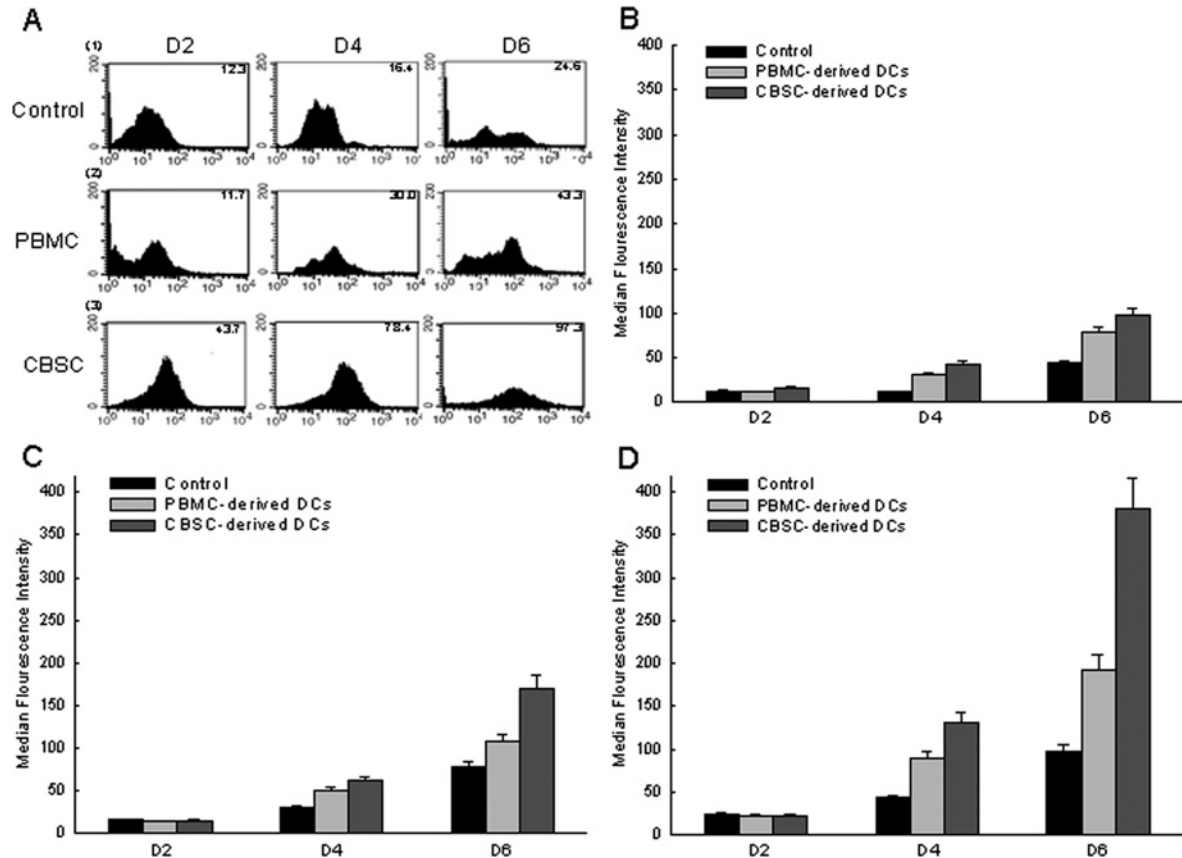
Figure 5   Antigen-presentation activities of CBSC-derived DCs and PBMC-derived DCs pulsed with HLA-compatible OVA peptide

(A) Representative flow cytometric traces for the antigen-presentation activity of PBMC- and CBSC-derived DCs pulsed with FITC-conjugated OVA[257–264]. (B–D) Quantification of the antigen-presentation activity of PBMC- and CBSC-derived DCs at various intervals treated with (B) PBS, (C) LPS and (D) TNF-α. The MFI values increased when the number of days in culture increased. The MFI values of CBSC-derived DCs were higher than those of PBMC-derived DCs, regardless of stimulation with PBS, LPS or TNF-α. When stimulated with TNF-α or LPS, both CBSC- and PBMC-derived DCs produced significantly higher MFIs than those stimulated with PBS. In (B–D), time in days (D) is given on the x-axis.

TNF-α-treated CBSC-derived DCs were also significantly higher ($P < 0.05$, Mann–Whitney U test) than those of PBMC-derived DCs or the control group (Figure 5D).

These results indicate that CBSC-derived DCs have a better antigen-presentation ability than PBMC-derived DCs, which increases further when the cells were treated with LPS or TNF-α.

CBCS-derived DCs induce higher antigen-specific cell-mediated immunity

To evaluate further whether CBSC-derived DCs also induced a strong antigen-specific immune response in addition to a better antigen-presentation ability, DCs were collected, pulsed with various HLA-A2-compatible HPV type 16 E7 epitopes (amino acids 11–20 or 86–93) and then co-cultured with CD8^{+} T-cells of an HLA-A2 haplotype using ELISpot assays. Representative results

from the ELISpot assays of cells pulsed with amino acids 11–20 and 86–93 of the E7 peptide between CBSC- and PBMC-derived DCs are shown in Figures 6(A) and 6(B) respectively. The number of IFN-γ-secreting CD8^{+} T-cells in the various individuals providing the CBSC-derived DCs, when pulsed with amino acids 11–20 of the E7 peptide, were significantly higher than those in the individuals providing the PBMC-DCs (Figure 6C, panels C1 and C2 respectively; $P < 0.001$, one-way ANOVA). Moreover, the number of IFN-γ-secreting CD8^{+} T-cells in the individuals providing the CBSC-DCs, when pulsed with amino acids 86–93 of the E7 peptide, was also significantly higher than those in the individuals providing the PBMC-DCs (Figure 6D, panels D1 and D2; $P < 0.001$, one-way ANOVA).

These results indicate that CBSC-derived DCs could induce stronger antigen-specific immunity compared with PBMC-derived DCs when pulsed with HLA-A2-compatible epitopes.
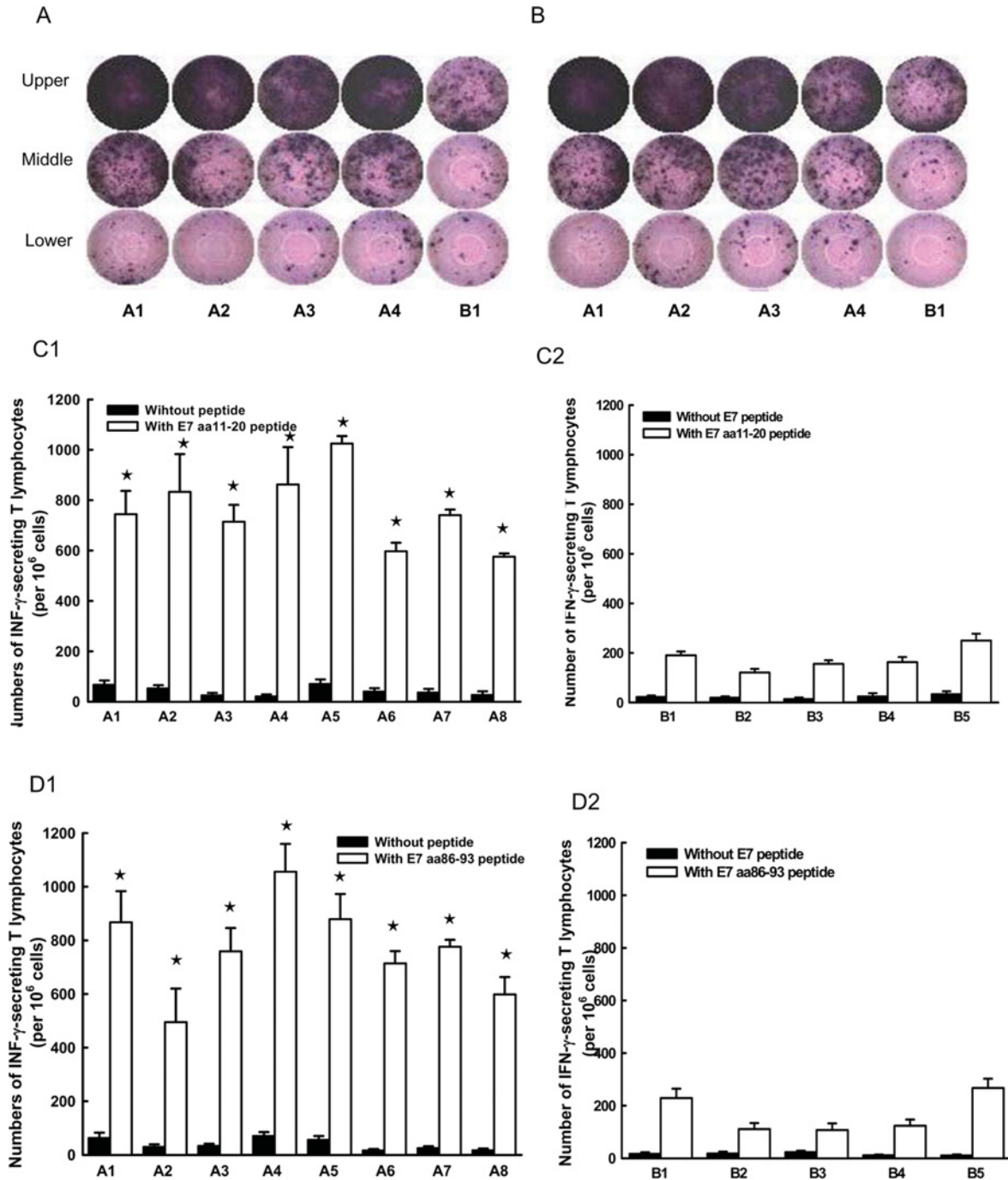
Figure 6   ELISpot assays for CBSC- and PBMC-derived DCs

Representative results from the ELISpot assay of CBSC- and PBMC-derived DCs pulsed with amino acids 11–20 (A) and 86–93 (B) of the E7 peptide. The T-cell/DC ratio was 3:1 in each well. Upper row, 10^6 T-cells/well; middle row, 3.3×10^5 T-cells/well; lower row, 10^5 T-cells/well. (A1–A4) T-cells co-cultured with peptide-loaded CBSC-derived DCs. (B1) T-cells co-cultured with peptide-loaded PBMC-DCs. (C and D) Quantification of the results from the ELISpot assay of CBSC- and PBMC-derived DCs pulsed with amino acids 11–20 (A) and 86–93 (B) of the E7 peptide. (C, panel C1, and D, panel D1), CBSC-derived DCs; (C, panel C2, and D, panel D2), PBMC-derived DCs. The number of IFN-γ-secreting CD8$^+$ T-cells in the individuals (A1–A8) supplying the CBSC-derived DCs, regardless of pulse with amino acids 11–20 and 86–93 (B) of the E7 peptide, were significantly higher than those of the individuals (B1–B5) supplying the PBMC-derived DCs. *$P < 0.01$ compared with without peptide, by one-way ANOVA.
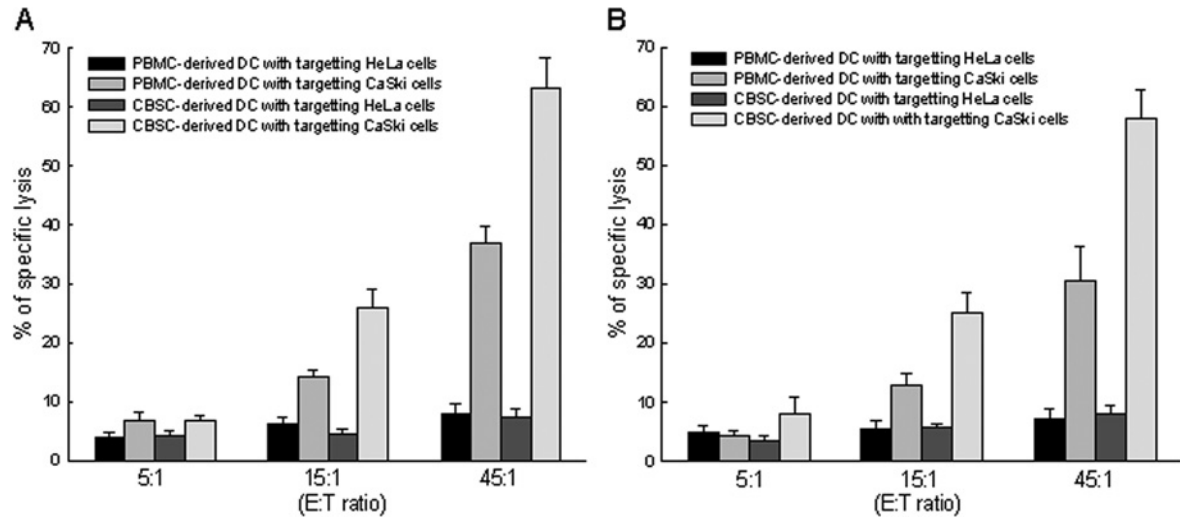
Figure 7   Comparison of the CTL activities stimulated by CBSC- and PBMC-derived DCs

(A) Specific lysis of cytotoxic T-cells cultured with amino acids 11–20 of the E7 peptide-pulsed PBMC- or CBSC-derived DCs targeting CaSki or HeLa cells. The percentage specific lysis was significantly higher ($P < 0.01$, one-way ANOVA) in CBSC-derived DCs with targeting CaSki cells than those in the PBMC-derived DCs at an effector/target ratio (E:T ratio) of 45:1. However, the percentage specific lysis was not different between CBSC-derived DCs and PBMC-derived DCs with targeting HeLa cells when pulsed with amino acids 11–20 of the E7 peptide. (B) Specific lysis of cytotoxic T-cells cultured with amino acids 86–93 of the E7 peptide-pulsed PBMC- or CBSC-derived DCs targeting CaSki or HeLa cells. When pulsed with amino acids 86–93 of the E7 peptide, the percentage specific lysis was also significantly higher ($P < 0.01$, one-way ANOVA) in CBSC-derived DCs compared with PBMC-derived DCs at an effector/target ratio (E:T ratio) of 45:1. However, the percentage-specific lysis was not different CBSC-derived DCs and PBMC-derived DCs with targeting HeLa cells when pulsed with amino acids 86–93 of the E7 peptide.

CBSC-derived DCs have more potent CTL activities than PBMC-derived DCs

To determine whether CBSC-derived DCs have more potent antigen-specific CTL activities than PBMC-derived DCs, CD8$^+$ T-cells pulsed with HLA-A2-compatible E7 peptide (amino acids 11–20 or 86–93)-loaded CBSC- or PBMC-derived DCs were used as effector cells and CaSki tumour cells were used as target cells in CTL assays. The percentage of specific lysis was significantly higher ($P < 0.01$ one-way ANOVA) in CBSC-derived DCs with targeting CaSki cells than that for PBMC-derived DCs when pulsed with amino acids 11–20 of the E7 peptide (at an effector/target cell ratio of 45:1; Figure 7A). Findings for PBMC-derived DCs were similar to that reported previously by Cheng et al. [8]. There was no difference between the CBSC-derived DCs or PBMC-derived DCs targeting HeLa cells (effector/target cell ratio of 45:1; Figure 7A).

When pulsed with amino acids 86–93 of the E7 peptide, the percentage of specific lysis was also significantly higher ($P < 0.01$ one-way ANOVA) in CBSC-derived DCs compared with PBMC-derived DCs (effector/target cell ratio of 45:1; Figure 7B). There was no difference between the CBSC-derived DCs or PBMC-derived DCs targeting HeLa cells (effector/target cell ratio of 45:1; Figure 7B). The CTL activities to CaSki cells were significantly higher ($P < 0.01$ one-way ANOVA) than those to HeLa cells in both CBSC-derived DCs and PBMC-derived DCs when pulsed with either amino acids 11–20 or 86–93 of the E7 peptide (Figure 7).

These results indicate that CBSC-derived DCs have more potent tumour killing effects than PBMC-derived DCs.

CBSC- and PBMC-derived DCs have different effects on signal transduction pathways

To identify whether there is any difference in the activation of signalling pathways in the differentiation and maturation processes of CBSC- and PBMC-derived DCs, the phosphorylation of ERK, Akt and p38 were examined by Western blot analysis. As shown in Figures 8(A) (CBSC-derived DCs) and 8(B) (PBMC-derived DCs), the phosphorylation of ERK remained unchanged in both CBSC- and PBMC-derived DCs from day 0 to day 6, whereas the phosphorylation of Akt increased in both cell types over this time period, particularly in CBSC-derived DCs. The phosphorylation of p38 increased in PBMC-derived DCs (Figure 8B), but decreased gradually in the CBSC-derived DCs over the same period (Figure 8A).

The activation of signalling pathways in DCs was evaluated further following stimulation with LPS. LPS increased the phosphorylation of ERK in CBSC- and PBMC-derived DCs (Figures 8A and 8B respectively),
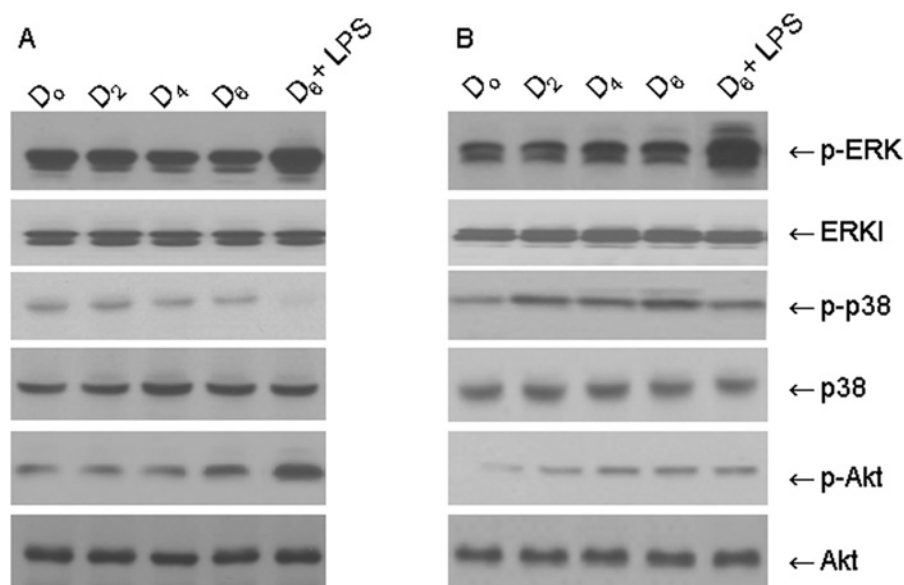
Figure 8   Phosphorylation of ERK, p38 and Akt in CBSC-derived DCs (A) and PBMC-derived DCs (B)

Cells were cultured for up to 6 days and on day 6 (D6) of culture the cells were then stimulated further with LPS for 24 h. The cells were lysed and the lysates were assayed by Western blotting with the phospho-specific antibodies. In CBSC-derived DCs, the phosphorylation of Akt increased from day 0 to day 6, but the phosphorylation of p38 was decreased when stimulated by LPS on day 6 of culture. In PBMC-derived DCs, the phosphorylation of Akt and p38 increased from day 0 to day 6; however, the phosphorylation of the three proteins did not change when stimulated with LPS at day 6 of culture.

with phospho-ERK being more prominent in PBMC-derived DCs. However, neither phospho-p38 nor phospho-Akt levels changed after stimulation with LPS in PBMC-derived DCs (Figure 8B). In CBSC-derived DCs, the level of phospho-p38 decreased with LPS stimulation, whereas phospho-Akt increased (Figure 8A).

These results indicate that differential activation of signalling pathways occurred during the differentiation and maturation processes of CBSC- and PBMC-derived DCs.

DISCUSSION

The results of the present study show that CD34+ monocytes from UCB can be expanded by approximately 17-fold using an *ex vivo* method. DCs from UCB express DC-specific markers, such as CD1a, CD83, CD86 and HLA-DR, earlier than those from peripheral blood. Moreover, CBSC-derived DCs secreted different cytokines compared with PBMC-derived DCs. CBSC-derived DCs had better antigen-presentation abilities, induced stronger antigen-specific immunities and had more potent tumour killing effects than PBMC-derived DCs. CBSC-derived DCs also had increased Akt phosphorylation and reduced p38 phosphorylation, characteristics in the differentiation and maturation processes of DCs, compared with the PBMC-derived DCs.

DCs can be utilized as active specific cell-based cancer immunotherapeutic agents. They are very potent antigen-presenting cells that play a key role in the initiation of the immune response [17] and are considered as promising tools for cancer immunotherapy [18]. Active adaptive immunotherapy through DCs induces antigen-specific T-cell responses against tumour antigens. DCs are immature cells in almost every organ and tissue at the interface of potential pathogen entry sites. They can be triggered by a danger signal, such as a pathogen, tissue or signs of inflammation, and then they start to mature and up-regulate chemokine receptors [18].

Mature DCs can prime CD8+ cells (CTLs) for MHC class I and CD4+ (helper cells) for MHC class II, which circulate and infiltrate tumour tissues to kill metastatic or residual tumour cells [19]. The generation of immune responses against tumour antigens following DC immunization has been demonstrated, with favourable clinical responses reported [20]. Many studies have shown that tumour-associated antigens loaded with DCs are able to induce anti-tumour responses in animal models [21,22]. Clinical trials, including in melanoma and prostate cancer, have reported both immunological and clinical responses [1,23,24].

The major limitation of DC-derived immunotherapy is in terms of acquiring sufficient numbers of DCs. The differentiation of DCs from adult peripheral blood monocytes is the most widely used model for studying DC biology [3]. The major drawback for this

DC-derived method is the limitation of acquiring DCs, as monocytes can only differentiate, but not proliferate, into DCs following the incubation with GM-CSF and IL-4. Strategies to expand the amount of DCs has become an important issue to overcome this limitation for basic DC research and the clinical application of DCs.

UCB HPCs (haemopoietic progenitor cells) have been demonstrated to possess significant advantages over their peripheral blood counterparts in terms of proliferative capacity and immunological reactivity. UCB is a useful source of allogenic haemopoietic stem cells for bone marrow reconstitution with a low risk of severe graft-versus-host disease, owing to the naïve nature of circulating neonatal T-cells and/or the presence of peripheral tolerance-inducing T-cells or DCs [25–27]. The stem cell compartment of UCB, in contrast with adult PBMCs, is less mature, and therefore has higher proliferative potential *in vitro* and *in vivo*, and is associated with an expanded lifespan with longer telomeres [25–27].

A major limitation in using UCB cells is the limited number of HPCs and haemopoietic stem cells. The low number of HPCs in UCB needs to be overcome by *ex vivo* expansion using growth factors acting on early HPCs [28,29]. Much interest is currently focused on improving *in vitro* methods to expand HPCs without compromising their original development and engrafting potential. In the present study we first tried to expand HPCs using an *in vitro* culture protocol that supports the self-renewal of HPCs (Figure 1). After confirming that almost no differentiated lymphoid cells or DCs were identified among the cells harvested after 14 days of expansion (Figure 2), the subsequent culture of such expanded CD34$^+$ cells under DC differentiation conditions led to the production of DCs (Figure 2).

The ability of DCs to prime naïve T-cells, leading to both effector T-cell differentiation and memory T-cell expansion, makes DCs promising tools for cancer immunotherapy [30]. The clinical use of DCs requires a repeated vaccination to induce relatively high frequencies of tumour-antigen-specific CTLs and a complete response. Repeated vaccination in turn requires large numbers of DCs and hence the *ex vivo* generation of large numbers of DCs is essential [31]. Autologous DC cultures have limitations because the yield is low and the ability of DC differentiation is dependent on a patient's individual status. The *ex vivo* DC generation from CD34$^+$ cells derived from UCB provides an alternative source. This is an attractive model because of the high proliferation potential of CD34$^+$ cells. Jiang et al. [32] reported *in vitro* culture systems to generate DCs from CD34$^+$ HPCs. Because of the CD34$^+$ cell amplification before differentiation into DCs, an overall production of approximately 15-fold more DCs can now be finally

obtained even without amplification. Arrighi et al. [33] used another CD34$^+$ cell expansion system with SCF, FLT3 ligand and thrombopoietin, and subsequent culture with GM-CSF and TNF-α, to induce DC differentiation [33]. They found that there was no further cell growth during DC culture after expansion.

The results of the present study confirm that UCB can be a better source than adult peripheral blood for DCs as a vaccine for cancer immunotherapy. An important feature of DCs is their capacity to stimulate the proliferation of T-cells and induce specific immunity. We have found that CBSC-derived DCs stimulate stronger antigen-specific immunity than PBMC-derived DCs (Figure 6). Furthermore, CBSC-derived DCs secrete more TNF-α, have better antigen-presentation ability and have more potent CTL activities (Figure 4, 5 and 7). The explanation is that CBSC-derived DCs are more potent antigen-presenting cells than PBMC-derived DCs. The markers of mature DCs, such as CD1a, CD80, CD86 and HLA-DR, are expressed earlier in CBSC-derived DCs than those in PBMC-derived DCs when stimulated with LPS or TNF-α (Figure 3), indicating that CBSC-derived DCs are more mature DCs after stimulation with LPS or TNF-α. These results are consistent with other findings showing higher expressions of co-stimulatory molecules and HLA-DR [34–36]. Since UCB-derived DCs offer multiple advantages over adult peripheral cells, UCB may provide a future source of DCs for cancer immunotherapy.

In conclusion, we report in the present study the development an *in vitro* culture system to expand UCB CD34$^+$ cells and then to differentiate them into DCs. These CBSC-derived DCs have higher levels of co-stimulatory molecules, secrete more amounts of immuno-modulating cytokines, have better antigen-presentation abilities, induce stronger antigen-specific immunity and stimulate more potent CTL activities than PBMC-derived DCs. As such, CBSCs have the potential of improving the strategy of DC vaccines for cancer immunotherapy.

AUTHOR CONTRIBUTION

Chen participated in the interpretation of the results and discussion. Wen-Fang Cheng designed the experiments and wrote the paper with the input of all of the other authors.

FUNDING

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Cord blood stem-cell-derived dendritic cells generate potent antigen-specific immune responses and anti-tumour effects

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Table S1   Comparison of the growth rates of CD34$^+$ UCB cells under culture conditions using SCF alone or a cocktail of growth factors

The percentage of CD34$^+$ UCB cells and the total number of CD34$^+$ UCB cells ($\times 10^6$ CD34$^+$ cells from 10^8 original MNCs from UCB) are shown. *$P < 0.05$ and **$P < 0.01$, as determined by one-way ANOVA.

Days in culture	Medium addition . . .	Percentage of CD34$^+$ UCB cells		Total number of CD34$^+$ UCB cells	
		SCF	Growth factor cocktail	SCF	Growth factor cocktail
0		1.4 ± 0.2	1.4 ± 0.2	1.0	1.0
3		9.4 ± 3.3	10.5 ± 3.0	8.75 ± 0.29	9.57 ± 0.30
7		22.6 ± 3.1	28.5 ± 2.8	10.58 ± 0.33	11.86 ± 0.24*
10		37.9 ± 6.6	55.7 ± 5.6	10.92 ± 0.72	14.90 ± 0.60*
14		71.2 ± 6.4	85.2 ± 4.6*	17.73 ± 1.13	21.37 ± 1.38*
17		86.6 ± 5.7	90.5 ± 4.8	13.51 ± 0.77	25.31 ± 1.87**
21		87.2 ± 6.7	92.6 ± 4.9	14.82 ± 0.99	31.24 ± 2.04**

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