ELSEVIER

Experimental Hematology

Experimental Hematology 36 (2008) 61–68

Generation of functional natural killer and dendritic cells in a human stromal-based serum-free culture system designed for cord blood expansion

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(Received 29 May 2007; revised 30 May 2007; accepted 7 August 2007)

Objective. **We have previously reported on the ability of a mesenchymal stem cell–based serum-free culture system to expand human cord blood (CB) hematopoietic stem cells along the myeloid pathway and simultaneously generate a $CD7^+CD34^-$ population. In this study, we investigated the ability of the $CD7^+CD34^-$ population to differentiate into natural killer and dendritic cells (DCs).**

Materials and Methods. **CB $CD34^+$ cells were expanded over a mesenchymal stem cell layer in serum-free medium supplemented with stem cell factor, basic fibroblast growth factor, leukemia inhibitor factor, and Flt-3 ligand for 2 weeks. Cultured cells were harvested and $CD7^+CD34^-Lin^-$ cells sorted and plated for 2 additional weeks in either natural killer– or DC-inductive medium.**

Results. **Culture of $CD34^+$ cells for the first 2 weeks in this system resulted in expansion of the stem cell pool and the myeloid component of the graft, and also produced a 58-fold increase in the $CD7^+CD34^-$ cell population. When sorted $CD7^+CD34^-Lin^-$ cells were induced toward a natural killer cell phenotype, further expansion was observed during this time in culture, and differentiation was confirmed by cytotoxic activity and by flow cytometry, with cells displaying CD16 and CD56 in the absence of CD3. Generation of DC cells in culture was also verified by observing both the characteristic dendritic morphology and the dendritic phenotypes $HLA\text{-}DR^{bright}CD123^{bright}CD11c^-$ and $HLA\text{-}DR^{bright}CD11c^+$.**

Conclusion. **These results demonstrate the ability of an ex vivo culture system to drive expansion of human CB hematopoietic stem cells, while promoting the immune maturation of the graft and generation of DC and natural killer cells that could then be utilized for adoptive cancer cellular immunotherapy.** © **2008 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Use of umbilical cord blood (UCB) as a source of hematopoietic stem cells (HSC) for allogeneic stem cell transplantation has increased over the last 18 years to become a standard and safe alternative to bone marrow transplantation [1–3]. This procedure has extended the boundaries of HSC transplantation to patients who otherwise would not be suitable for this therapy [4–6] and offers considerable rational and clinical advantages over other allogeneic hematopoietic stem cell sources [2,3,7]. Some of the advantages of using CB as a source of HSC are due to the prompt availability of frozen UCB units, the decreased risk of transmission of viruses, and the lower incidence and severity of acute graft-vs-host disease. In addition, the relative absence of mature immune effector cells in CB permits use of grafts with one to two human leukocyte antigen (HLA) mismatches, increasing the probability of finding a compatible donor, especially among underrepresented minorities [8]. Nevertheless, UCB transplantation has also been associated with some inherent limitations, such as a greater risk of graft failure due to the inadequate number of stem/progenitor cells available per unit, as well as delayed immune and hematologic reconstitution, because

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0301-472X/08 $–see front matter.
doi: 10.1016/j.exphem.2007.08.031

of the higher prevalence of developmentally immature cells [9–14]. Furthermore, in the case of cancer relapse, CB transplantation shows a major disadvantage because donor lymphocytes and/or dendritic cells (DCs) are not available for subsequent immunotherapy. Thus, the development of methods to overcome these limitations would greatly improve the utility of CB as an alternative source of HSC for transplantation and would allow its widespread use in the clinic. We have previously reported that human mesenchymal stem cells (MSCs) are able to effectively support the ex vivo expansion/maintenance of human UCB HSCs using a serum-free culture system [15]. Some of the major advantages of this culture system include use of preestablished human allogeneic stromal layers, thus overcoming the limitations of using xenogeneic and/or transformed and immortalized human stromal cell lines, this culture system's serum-free conditions, and its ability to expand CB cells along the myeloid pathway, while simultaneously generating a population expressing a marker of early lymphopoiesis, $CD7^+$.

Because several different investigators had reported the ability to generate natural killer (NK), B, dendritic, and T cells [10,16–22] from a $CD7^+CD34^+$ cell population from fresh CB and bone marrow, in the present studies, we examined whether the $CD7^+CD34^-$ population obtained after CB expansion over MSC layers, in serum-free medium, also had lymphocytic differentiative potential. Specifically, we investigated the ability of the $CD7^+$ population generated in our culture system to give rise to two of the major constituents of the immune system, mature NK cells, and DCs that could ultimately be used in cellular immunotherapy.

Materials and methods

CB cell isolation

CB samples were obtained from the Pediatric Stem Cell Transplant Program at Duke University Medical Center (Durham, NC, USA) after maternal donor consent. Samples were collected into sterile bags containing citrate-phosphate dextrose anticoagulant and diluted 1:3 in Iscove's modified Dulbecco's medium (GIBCO Laboratories, Grand Island, NY, USA) before separation of mononuclear cells (MNC). Low-density MNC were separated on a Ficoll-Histopaque density-gradient centrifugation (1.077 g/mL; Sigma, St. Louis, MO, USA) and washed twice in Iscove's modified Dulbecco's medium. CB MNC from each donor were enriched for $CD34^+$cells using the Direct CD34 Progenitor Isolation Kit (Miltenyi Biotec Inc., Auburn, CA, USA).

Human bone marrow Stro-1$^+$ stroma layer cell cultures

Heparinized human bone marrow was obtained from healthy donors after informed consent. Low-density bone marrow MNC were separated on a Ficoll-Histopaque density-gradient centrifugation (1.077 g/mL; Sigma). For each donor, Stro-1^+ cells were isolated magnetically. Briefly, bone marrow MNC were incubated for 30 minutes at 4°C with Stro-1 antibody (R&D Systems, Minneapolis, MN, USA), washed, incubated with rat anti-mouse IgM beads for 15 minutes, and separated using a MiniMacs column (Miltenyi Biotec, Inc.). Cells were cultured in gelatin-coated T25 flasks with mesenchymal stem cell basal medium (Poietics; Cambrex Bioscience, Baltimore, MD, USA) supplemented with MSCGM SingleQuot Kit. Stroma layers were cultured to confluence and then γ-irradiated with a ^{137}Cs source as described previously [15,23]

Ex vivo expansion of CD34$^+$

$CD34^+$cells were cultured in QBSF-60 serum-free medium with L-glutamine (Quality Biological, Inc, Gaithersburg, MD, USA) supplemented with 100 ng/mL stem cell factor (SCF), 10 ng/mL leukemia inhibitor factor, 5 ng/mL basic fibroblast growth factor, and 100 ng/mL Flt-3 ligand (FL) (all cytokines from PeproTech Inc., Rocky Hill, NJ, USA), on irradiated Stro-1^+ stromal layers, at 37°C in a 5% (v/v) CO_2 incubator. Every 3 days, half of the medium was replaced with fresh medium and half of the cultures were harvested for the following analyses: cell count, viability using trypan blue stain 0.4% solution (GIBCO Laboratories), and phenotype by flow cytometry.

Differentiation assays

NK cells. Ex vivo expanded $CD34^+$ cells (as described here previously) were harvested at day 12 of culture and $CD7^+CD2^-CD3^-CD5^-CD16^-CD56^-CD34^-$ cells were sorted on a FACSVantage (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA) and cultured in HAM'S F12 medium (GIBCO Laboratories) supplemented with 5 μM β-mercaptoethanol (Sigma), insulin, transferrin, and selenium (Sigma), 10 ng/mL SCF, interleukin (IL)-7, IL-15, FL, and 1000 U/mL IL-2 (all cytokines from PeproTech Inc.), seeded on irradiated Stro-1^+ stromal layers. Every 3 days half of the medium was changed and the cells counted and analyzed by flow cytometry (for presence of CD3, CD16, and CD56).

After 2 weeks in culture, cytotoxic assays (CytoTox96 Non Radioactive Cytototoxic Assay; Promega, Madison, WI, USA) were performed to demonstrate the cytolytic activity of this cell population.

DCs. $CD34^+$ cells ex vivo expanded for 12 days were harvested and sorted for $CD7^+CD2^-CD3^-CD5^-CD14^-CD16^-CD56^-CD34^-$ cells, on a FACSVantage. Sorted cells were then cultured in the absence of stroma in RPMI-1640 (GIBCO Laboratories) supplemented with 5 μM β-mercaptoethanol (Sigma), 200 U/mL IL-4, 0.2 U/mL granulocyte/macrophage colony-stimulating factor, and 10 ng/mL FL (all cytokines from PeproTech Inc.). Every 3 days, cells were harvested and analyzed for proliferation, morphology, and phenotype (presence of CD1a, CD11c, CD83, and CD123); half of the medium was replaced with fresh supplemented medium. Morphological analysis was conducted after cytocentrifugation onto slides and staining with Wright Giemsa.

Flow cytometric analysis. The phenotype of fresh and cultured cells was assessed by flow cytometry. Briefly, harvested cells were incubated with fluorescent monoclonal antibodies against CD1a, CD3, CD7, CD11c, CD16, CD34, CD56, CD83, CD123, and HLA-DR (Becton-Dickinson Immunocytometry Systems) for 15 minutes at room temperature. Cells were then washed in phosphate-buffered saline 1% sodium azide (Sigma) and fixed with 4% paraformaldehyde. Isotype controls (Simultest control γ1/γ 1 and Simultest control γ1/γ 2a) were included in

every experiment to evaluate the unspecific binding. Samples were analyzed using a FACScan (Becton-Dickinson Immunocytometry Systems) with CellQuest analysis software (Becton Dickinson).

Cytotoxic assays

Cytototoxic activity of cultured cells was determined with a colorimetric assay that measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. Conversion of a tetrazolium salt into a red formazan product by the released enzyme is then measured in a coupled enzymatic assay, in a plate reader at visible wavelength. In brief, target cells (T) (NK-sensitive cell line K562) were incubated with effector (E) cells, at different E:T ratios (10:1, 5:1, 2.5:1, 1.25:1) for 4 hours; after incubation, supernatants were collected and incubated for 30 minutes with the substrate mix, the reaction was then stopped by adding 1 M acetic acid (Stop solution). Triplicates of each E:T ratio were performed.

Spontaneous LDH release was measured by incubating the target cells in the absence of effector cells. Maximum LDH release was determined by adding lysis solution (0.9 % (v/v) Triton X-100). The amount of LDH released was measured in a plate reader (Bio-Rad Model 3550-UV) and the percent cytotoxicity was calculated as follows, for each E:T ratio:

$$\%Cytotoxicity = \frac{Experimental - Effector\ Spontaneous - Target\ Spontaneous}{Target\ Maximum - Target\ Spontaneous} \times 100$$

Results

$CD7^+$ cells obtained at day 9 in culture are derived from expansion and differentiation of a more primitive $CD34^+CD7^+$ cell population and not only the result of expansion of the preexisting $CD34^-CD7^+$ population at day 0

We reported previously [15,23] that CB-derived $CD34^+$ cells could be expanded ex vivo in an MSC-based serum-free culture system containing SCF, FL, leukemia inhibitor factor, and basic fibroblast growth factor, differentiating primarily towards a myeloid phenotype, while maintaining a population of cells that expressed CD7, a marker of early lymphopoiesis. In this culture system, total CB $CD34^+$–enriched cells expanded 124- to 358-fold, $CD34^+$ cells increased by 35-fold, and $CD34^+CD38^-$ cells by 48-fold by the end of culture. The total fold increase in clonogenic potential was 137.46 ± 2.2 times that of the initial culture [15].

Although it was clear from these studies that a population of cells positive for CD7 was maintained in this culture system, we were unable to determine with certainty whether the population of $CD7^+$ cells obtained after HSC expansion reflected the ability of our culture system to support expansion and differentiation of the more primitive $CD34^+CD7^+$ cell pool, or whether these $CD7^+$ cells were derived from expansion of the small number of preexisting $CD7^+CD34^-$ cells present at day 0.

In order to address this question , we started by expanding CB $CD34^+$ cells under culture conditions identical to those described previously [15,23], and analyzed the kinetics of expansion and differentiation of the $CD7^+CD34^+$ and $CD7^+CD34^-$ cell populations every 3 days. As can be seen in Table 1, during the first 3 days of culture, we first observed a significant increase in the $CD7^+CD34^+$ population from 4.10% ± 0.95% to 24.1% ± 5.12% ($p < 0.001$), while no significant variation in the numbers of $CD7^+CD34^-$ cells (12.3% ± 4.51% to 16.4% ± 2.60%) was observed. From day 3 to day 9, a decrease in CD34 expression was seen within the $CD7^+CD34^+$ cells. This population decreased from 24.1% ± 5.12% to 9.92% ± 1.70%, to give rise to a population of cells possessing a $CD7^+CD34^-$ phenotype. In fact, at day 9, $CD7^+CD34^-$ cells constituted 62.3% ± 6.79% ($5.76 \times 10^6 \pm 0.65 \times 10^6$) of the total cells present in culture (Table 1), corresponding to a 58-fold increase in the $CD7^+34^-$ population.

Expanded $CD7^+CD34^-$ obtained in culture can be further differentiated into cell types that can be used for cellular immunotherapy

In order to investigate whether the $CD7^+34^-$ population obtained after expansion in culture could be further differentiated into functionally mature NK cells and DCs that could ultimately be used in cellular immunotherapy, we sorted, respectively, $CD7^+$ CD2, CD3, CD5, CD16, CD34, CD56-negative cells or $CD7^+CD2^-CD3^-CD5^-CD14^-CD16^-CD56^-CD34^-$ cells, at day 12 of culture and replated these cells in specific media inductive of NK or DC differentiation.

Table 1. Flow cytometric analysis of cord blood $CD34^+$–enriched cells

Day	$CD34^+$ 7^- (%)	$CD34^+7^+$ (%)	$CD7^+$ 34^- (%)
0	79.4 ± 6.81	4.10 ± 0.95	12.3 ± 4.51
3	71.9 ± 7.60	24.1 ± 5.12	16.4 ± 2.60
6	51.7 ± 4.48	19.5 ± 4.56	47.7 ± 3.71
9	30.1 ± 1.27	9.92 ± 1.79	62.3 ± 6.79

Relative percentage of CD34 and CD7 cells with time in culture. Data are presented as mean percentage ± SEM (n = 5).

$CD7^+CD34^-$ obtained in culture are able to differentiate into NK cells

Cells were sorted from the initial culture system at day 12 based on CD7 positivity and CD2, CD3, CD5, CD16, CD34, CD56 negativity, and plated over new stromal layers and cultured for 12 additional days, in the presence of media inductive of NK cell differentiation, as described in the Materials and Methods section. Every 3 days, cultures were evaluated for cell expansion, and phenotypic analysis; a cytotoxic assay was performed on the last day of culture. Between day 0 and day 3 of culture, a twofold increase in total cell numbers was observed, increasing from $1.28 \times 10^6 \pm 0.45 \times 10^6$ cells to $2.84 \times 10^6 \pm 1.42 \times 10^6$ cells ($n = 5$); no further increase was found between day 3 and day 9, with the cell count reaching a plateau at approximately $1.10 \times 10^6 \pm 0.23 \times 10^6$ (Fig. 1).

Immunophenotypic characterization showed that the cells acquired a phenotype consistent with that of NK cells as early as day 3 of culture, becoming $CD16^+CD3^-$ and $CD56^+CD3^-$, as shown in Figure 2. On day 3, the percentages of $CD16^+CD3^-$ cells and $CD56^+CD3^-$ cells were 19.6% ± 9.31% and 11.9% ± 4.78%, respectively. At day 6, the percentage of cells exhibiting a phenotype of $CD16^+CD3^-$ was 21.1% ± 9.77%, and at day 12, it was 24.8% ± 12.0%, while the percentages of $CD56^+CD3^-$ cells were 24.5% ± 10.8% on day 6 and 25.3% ± 4.18% on day 12 (Table 2). Of note is that, although the culture conditions we employed were not ideal for T-cell differentiation, a small $CD3^+$ population was obtained between day 3 and day 12 in culture, 1.89% ± 0.25% and 8.75% ± 0.48%, respectively (Table 2).

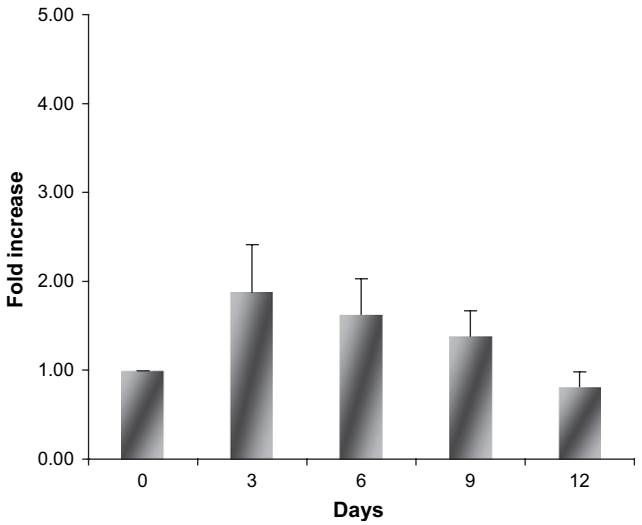

Figure 1. Fold increase of total cell number of viable sorted $CD7^+CD34^-Lin^-$-cells under natural killer cell–inductive conditions (data are expressed as mean fold expansion ± SEM; n = 5).

Cytotoxic activity of $CD7^+CD34^-$-derived NK cells

In order to investigate whether $CD7^+CD34^-$-derived NK cells were functional, we harvested these cells after 12 days of culture and tested them as effector cells against NK-sensitive targets (K562) as described in the Material and Methods section. $CD7^+CD34^-$–derived NK cells were confirmed to be functional as shown by the cytotoxic activity that increased in an E:T ratio-dependent fashion. As shown in Figure 3 at an E:T ratio of 1.25:1, the cytotoxicity was 2.70 ± 0.47 and increased to 19.7 ± 0.47 at an E:T ratio of 10:1, showing that $CD7^+CD34^-$-derived NK cells effectively killed K562 targets.

Expansion and characterization of $CD7^+CD34^-$-derived DCs

In order to investigate whether $CD7^+CD2^-CD3^-CD5^-CD14^-CD16^-CD56^-CD34^-$ cells also had the potential to differentiate into DCs, we cultured these cells in the presence of IL-4, granulocyte/macrophage colony-stimulating factor, and FL and found that a 1.8-fold increase in total cell numbers occurred during the 12 days in culture. These cultures were started with $0.93 \times 10^6 \pm 0.09 \times 10^6$ cells; at day 3, the total number of cells in culture increased to $1.72 \times 10^6 \pm 0.18 \times 10^6$, and at day 9, $1.15 \times 10^6 \pm 0.07 \times 10^6$ cells were still present (Fig. 4).

Differentiation toward a dendritic phenotype was assessed by performing flow cytometry on cultured cells by gating for lineage negativity ($CD3^-$, $CD14^-$, $CD16^-$, $CD19^-$, $CD20^-$, and $CD56^-$), and high expression of HLA-DR. This double-gated population was then evaluated for expression of CD11c and CD123. Using this three-color assay, we were able to separate two populations: HLA-$DR^{bright}CD123^{bright}CD11c^-$, indicative of a plasmacytoid dendritic subset and a HLA-$DR^{bright}CD11c^+$, a myeloid dendritic subset. Differentiation toward a plasmacytoid phenotype was initially seen at day 3 with 4.22% ± 0.94% of the cells displaying a $CD123^+$HLA-DR^+ phenotype. This number increased to 18.6% ± 0.75% by day 6 of culture, and by day 9, 38.5 % ± 4.22% of cells had differentiated into $CD123^+$HLA-DR^+ cells. Differentiation toward a myeloid dendritic subset was achieved more rapidly, with 8.08% ± 0.34% of the cells possessing a $CD11c^+$HLA-DR^+ phenotype at day 3. This population increased to 14.3% ± 1.03% at day 6 of culture, and by day 9, 20.6% ± 1.63% of the cells were $CD11c^+$HLA-DR^+ (Table 3). Furthermore, these cells exhibited a phenotype associated with maturation of DCs, since CD1a- and CD83-positive cells increased progressively during the time in culture. By day 9, 10% of the total cells were CD1a- and CD83-positive (Table 3).

Despite the typical DC phenotype determined by flow cytometry, we wished to further confirm the generation of these cells by performing cytospins for evaluation of cell morphology. As can be seen in Figure 5, these cells displayed the typical DC morphology, characterized by an

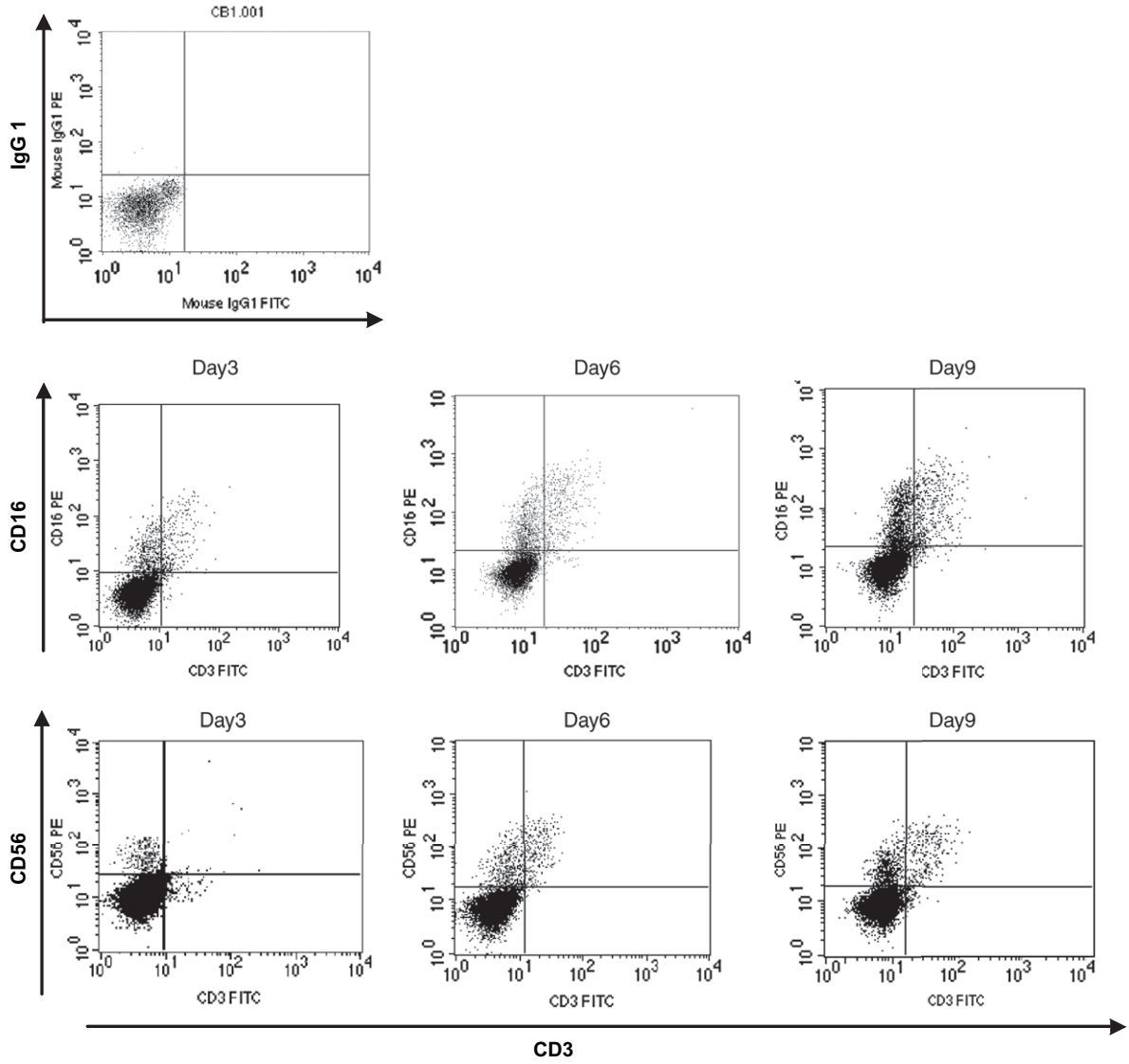

Figure 2. Flow cytometric plots of cultured cells in natural killer cell–inductive medium with time in culture. The dot plot on the top left represents the isotype control; the top three plots show expression of CD16 and CD3; the bottom three plots display the expression of CD56 and CD3. These plots show day 3, 6, and 9 in culture.

irregular form, extending long and thin processes in many directions from the cell body [24].

Discussion

Use of CB as a viable alternative to marrow and peripheral blood transplantation, in adults who lack a matched related or unrelated donor, is limited by the very intrinsic biologic nature of CB. The inadequate number of primitive HSC and the predominance of immature immune cells lead to delayed engraftment and abnormal immune reconstitution, potentially resulting in higher mortality posttransplantation than with HSC from adult sources.

Thus, the main focus for CB transplantation continues to be the pediatric patient, because a very high percentage of adults referred for UCB transplantation are ineligible for the procedure, based on the recommended cell dose for their given body weight [20].

Table 2. Flow cytometric analysis of cells cultured under natural killer cell–inductive conditions in the presence of stroma and stem cell factor, Ftl-3 ligand, interleukin-2, -7, and 15

Day	$CD16^+CD3^-$ (%)	$CD56^+CD3^-$ (%)	$CD3^+$ (%)
0	0.00	0.00	0.00
3	19.6 ± 9.31	11.9 ± 4.78	1.89 ± 0.25
6	21.1 ± 9.77	24.5 ± 10.8	3.44 ± 0.23
9	27.0 ± 12.1	28.5 ± 6.86	7.50 ± 0.73
12	24.8 ± 12.0	25.3 ± 4.18	8.75 ± 0.48

Each time point represents the mean percentage ± SEM (n = 5).

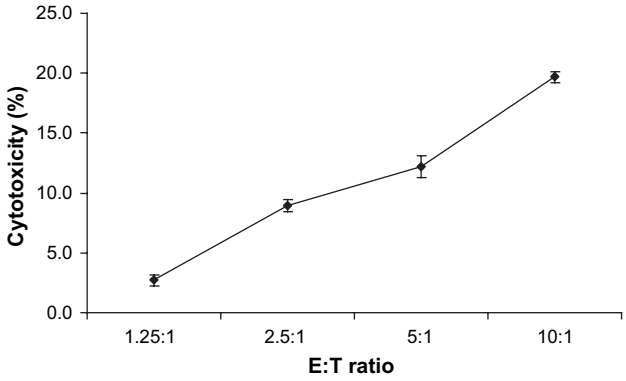

Figure 3. Cytolytic activity of $CD7^+CD34^-$-derived natural killer cells. Cytotoxic activity was analyzed by colorimetric assay. Cultured cells were incubated with K562 cells for 4 hours at 37°C, the supernatants were collected and absorbance was read at 490 nm (data are presented as mean percentage of triplicates ± SEM; n = 3).

In order to overcome this shortcoming, several different approaches have been put forward to increase the number of total mononuclear cells available for transplantation into patients [11]. Use of multiple cords has been quite successful, but it remains uncertain whether this approach will provide the patient with a normal and prompt immune reconstitution, or if it will be able to minimize the relapse of the primary disease. Another successful approach relies on ex vivo expansion of whole CB unit prior to transplantation or the combination of a partially expanded unit with the unmanipulated fraction. However, it seems that most of the available ex vivo expansion methods have failed to improve engraftment due to expansion of the more mature cells instead of the primitive HSC, alteration in stem cell homing, cell cycling, and even induction of apoptosis [20]. Also, it has been reported that although ex vivo expansion of CB does not seem to impair T-cell development, it does appear to decrease DC differentiation, contributing to altered immune function posttransplantation.

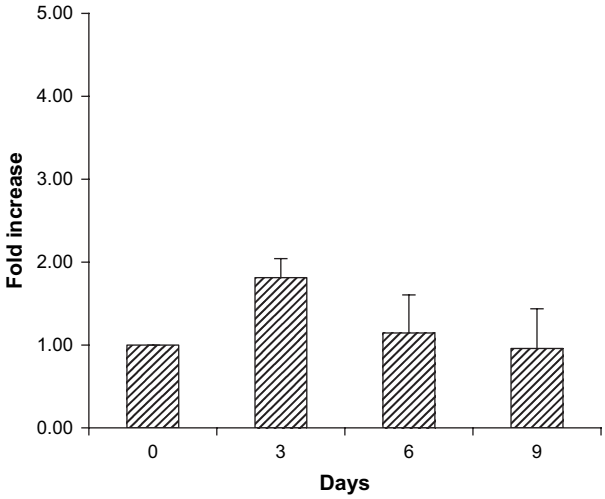

Figure 4. Fold increase of viable cells under dendritic cell–inductive conditions after cell sorting (data are presented as mean fold increase ± SEM; n = 3).

Table 3. Expression of dendritic cell markers CD1a, CD83, CD11c, CD123, and HLA-DR in $CD7^+CD34^-$-derived dendritic cells during culture

Day	$CD123^+$ $HLA\text{-}DR^+$ (%)	$CD11c^+$ $HLA\text{-}DR^+$ (%)	CD1a (%)	CD83 (%)
0	0.00	0.00	0.00	0.00
3	4.22 ± 0.94	8.08 ± 0.34	2.90 ± 0.35	0.57 ± 0.35
6	18.6 ± 0.74	14.3 ± 1.03	7.55 ± 2.51	7.53 ± 0.63
9	38.5 ± 4.22	20.6 ± 1.63	9.26 ± 2.98	10.7 ± 0.85

Data are expressed as mean percentage ± SEM (n = 3).

Thus, development of efficient methods that could allow CB manipulation ex vivo in a way that promoted expansion of primitive HSC without losing grafting ability and simultaneously allowed maturation of the immune cellular component of the graft would make this already valuable source of HSC available to a wider range of transplant applications. Use of all-trans retinoic acid, epigenic modification, and copper chelation [25–28] are all novel methods that may prove useful to allow expansion of the primitive pool of HSC in CB without inducing engraftment defects.

We have previously reported development of a stroma-based serum-free culture system [15,23] in which cocultivation of CB $CD34^+$–enriched cells with stroma in a cytokine cocktail (SCF, FL, basic fibroblast growth factor, and LIF) resulted in a 48-fold increase of the $CD34^+CD38^-$ cell population with a total fold increase in clonogenic potential of 137.46 ± 2.2 at day 24. Kinetic analysis of this culture system showed that the presence of stroma was the primary contributing factor for the observed decrease of cell death [29]. Although the overall differentiative potential of the cells in culture was biased toward the myeloid lineage,

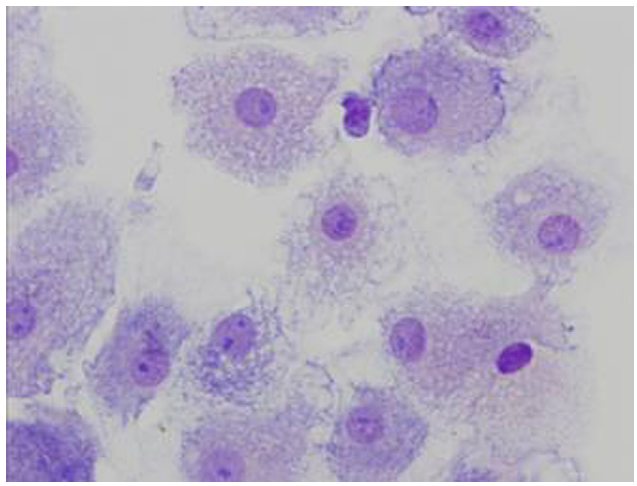

Figure 5. Cytospin preparation from cell cultures in dendritic-inductive media, at day 12 showing large cells with a typical dendritic morphology.

we also observed an increase in the percentage of $CD7^+$ cells [15]. Because the $CD7^+$ subpopulation of $CD34^+CD38^-$ CB cells has been reported as a clonogenic, primitive, and progenitor population with NK, B, and DC potential [17,30,31], we hypothesized that the stroma-based serum-free culture system reported by da Silva et al. [15] would be able to expand primitive HSC while simultaneously leading the expanded cells into the immunologic maturity needed for successful transplantation. Furthermore, because there is no mechanism at this time to use CB for adoptive cancer cellular immunotherapy after CB transplantation, in the current work, we investigated the potential of the $CD7^+CD34^-$ cells obtained during HSC expansion to give rise to NK and DC populations. We focused our attention on the $CD7^+CD34^-$ population, because concomitant with the HSC expansion phase, a 58-fold increase was seen in the $CD7^+CD34^-$ cells, corresponding to 62.3%± 6.79% of the total cells within 12 days of culture. In order to evaluate the differentiative potential of the $CD7^+CD34^-$ cells that were not yet committed toward NK or DC lineage, $CD7^+CD34^-$ cells obtained in culture were depleted of mature cells by cell sorting with lineages markers, before culturing under conditions inductive of NK or DC differentiation. At day 9 of culture in the presence of stroma and a cocktail of cytokines known to induce NK differentiation (SCF, IL-2, IL-7, IL-15, and FL), we obtained a population, 28.5 ± 6.86 which were $CD56^+CD3^-$ cells, a phenotype consistent with that reported for NK cells [32]. In order to demonstrate the functionality of the differentiated cells, the cytolytic activity of this cell population was also evaluated, and proved to be similar to freshly isolated NK cells from cord blood and bone marrow [33], as well as NK cells derived from a $CD34^+CD7^+$ population [30]. Although the culture conditions were not ideal to generate T cells, by day 12, 8.75% ± 0.48% of the cells in culture expressed CD3, a marker of T cells.

The DC population has been the target of several studies because of their relevance in the immune system as potent initiators of adaptive immune response by presenting antigens to T cells and regulating the production of cytokines [16,34]. In the present study, we were also able to differentiate lineage-depleted $CD7^+CD34^-$ into DCs. After 9 days of culture, we were able to observe two distinct dendritic populations: a plasmacytoid-like ($CD123^+HLA\text{-}DR^+$; 38.5% ± 4.22%) and a myeloid-like ($CD11c^+HLA\text{-}DR^+$; 20.6% ± 1.63%) population. Because DCs can also be evaluated for their maturation/activation based on surface markers such as CD83 [35], we also examined expression of this marker in our cultures. After 9 days in culture, 10.7% ± 0.85% of cells expressed a phenotype of mature/activated DC ($CD83^+$). Furthermore, morphologic analysis of these cells showed an irregular form with long and thin processes characteristic of DCs. In conclusion, we have developed an in vitro culture system that fulfills the essential criteria for paving the way to the more widespread usage of CB for HSC transplantation. This culture system reproducibly drives ex vivo expansion of human CB HSCs, while simultaneously promoting maturation of the immune cellular component of the graft by generating, at earlier time points, a known lymphoprogenitor population $CD34^+$ $CD7^+$, and at later time points, considerable numbers of $CD34^-CD7^+$ cells that are able to differentiate into NK cells and DC and could thus be used for adoptive cancer cellular immunotherapy after CB transplantation.

Acknowledgments

This work was supported by grants HL70566, HL73737, NAG9-1340 from National Institutes of Health and NASA. Ana M. Frias is a recipient of a scholarship SFRH/BD/8970/2002 awarded from Fundação para a Ciência e a Tecnologia, Portugal.

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