

## Refreezing of cord blood hematopoietic stem cells for allogenic transplantation: in vitro and in vivo validation of a clinical phase I/II protocol in European and Italian Good Manufacturing Practice conditions

Monica Gunetti<sup>a</sup>, Ivana Ferrero<sup>a</sup>, Deborah Rustichelli<sup>a</sup>, Massimo Berger<sup>a</sup>,  
Loretta Gammaitoni<sup>c</sup>, Fabio Timeus<sup>b</sup>, Wanda Piacibello<sup>c</sup>, Massimo Aglietta<sup>c</sup>, and Franca Fagioli<sup>a</sup>

<sup>a</sup>Stem Cell Transplantation and Cellular Therapy Unit; <sup>b</sup>Pediatric Hematology-Oncology Department, Regina Margherita Children's Hospital, Turin, Italy; <sup>c</sup>Unit of Medical Oncology, Institute for Cancer Research and Treatment-IRCC and Department of Oncological Sciences, University of Turin, Medical School Candiolo-Turin, Turin, Italy

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**Objective.** Several requirements need to be fulfilled for clinical use of expanded hematopoietic stem cells (HSCs). Because most cord blood (CB) samples are frozen in single bags and only an aliquot (~25%) of the blood can be expanded, the thawing and refreezing of samples must be validated in the current European and Italian Good Manufacturing Practice (eIGMP) conditions. Here, we describe in vitro and in vivo validation of the phase I/II protocol for CD34<sup>+</sup> expansion of thawed CB units according to the current Cell Therapy Products (CTPs) Guidelines.

**Materials and Methods.** CB units were thawed and 25% of the total volume was processed for CD34<sup>+</sup> selection by CliniMACS. The 75% of the unit was immediately refrozen. CD34<sup>+</sup> cells were expanded for 3 weeks with stem cell factor, Flt-3/Flk-2 ligand, thrombopoietin, and interleukin-6.

**Results.** In vitro results demonstrated that this culture system induces expansion of thawed CD34<sup>+</sup> (median value = 8.3). In vivo data demonstrated that after culture, the final CTPs maintain their repopulating ability in nonobese diabetic severe combined immunodeficient (SCID) mice. Limiting dilution assays performed by injecting decreasing doses of expanded CD34<sup>+</sup> cells revealed that the frequency of SCID repopulating cells after ex vivo expansion is 1:8,034. Analyses for sterility, viability, cell senescence, and cytogenetic assessment demonstrated that expansion procedures in eIGMP conditions are safe for clinical protocols.

**Conclusions.** This offers promising new options for expansion of allogenic HSCs and also for autologous usage in transplantation and other cell therapy protocols. © 2008 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.

Cord blood (CB) is a useful source of hematopoietic stem cells (HSCs) for transplantation [1]. The first clinical transplantation using CB stem cells was performed in 1988 on a Fanconi anemia patient [2]. Subsequently, clinical applications of CB transplantation on hematological and immunodeficiency diseases were reported [3,4]. CB is richer in severe combined immunodeficient (SCID) repopulating cells (SRCs) and high proliferative potential colony-form-

ing cell (CFC) than bone marrow (BM). The volume of CB collection usually ranges between 100 and 120 mL and every unit consists of 8 to  $12 \times 10^8$  mononuclear cells containing a range of 1 to  $5 \times 10^6$  CD34<sup>+</sup>. This limited number of cells restricts the use of CB units in pediatric transplantation patients. In cord blood transplants in adults, white blood cell (WBC) and mainly platelet engraftment is severely delayed, even in children the graft take is low [5,6]. An increased dose of HSCs can influence transplant outcomes [7]. Different strategies can be applied to overcome cell dose limit. First, some authors showed that the transplantation of two partially human leukocyte antigen-matched CB units is safe, and may overcome the cell-dose barrier that limits use of CB in many adults and

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Offprint requests to: Franca Fagioli, M.D., Department of Pediatrics, University of Turin, P.za Polonia 94, 10126 Turin, Italy; E-mail: franca.fagioli@unito.it

adolescents [8–11]. Secondly, other data demonstrated that the intra-bone marrow transplantation may improve the results of hematopoietic reconstitution [12]. Moreover, ex vivo expansion of CB HSCs would be of considerable clinical utility and preliminary phase I results have been reported [13–15]. In the last 10 years, we have focused on the latter option. We demonstrated that SRCs in CB can be expanded 70-fold after 10 weeks' culture in the presence of stem cell factor, Flt-3/Flk-2 ligand, thrombopoietin, and interleukin 6 (SCF+FL+TPO+IL-6), sustaining the hematopoietic reconstitution over three serial passages in nonobese diabetic (NOD)/SCID mice xenotransplantation models [16–20].

In transplant settings, ex vivo manipulation of an aliquot is problematic because CB units have mainly been stored in individual bags. Therefore, refreezing CB progenitors might offer new options. In this context, we demonstrated that successive freeze-thaw procedures do not significantly affect the clonogenic potential and human long-term engraftment in NOD/SCID mice. This suggests the possibility of new approaches in transplants, particularly to optimize the timing of the infusion of expanded and nonexpanded progenitors in CB units cryopreserved in single bags [21,22]. In this article, we describe the in vitro and in vivo validation of the phase I/II protocol for the CD34<sup>+</sup> expansion of thawed cord blood units according to the current European and Italian Guidelines on Cell Therapy Products (CTPs) [23–27]. Our experiments show that thawed and expanded CD34<sup>+</sup> cells maintain their short-term (CFC) and long-term clonogenic (long-term culture-initiating cell [LTC-IC]) activity and are able to engraft in NOD/SCID mice.

Quality control assays performed on final unmanipulated and expanded CTPs demonstrated that thawing a single CB unit, dividing it into two parts, expanding the smaller one, and refreezing the other is safe.

## Materials and methods

### *CB collection and processing*

CB samples were obtained at the end of full-term deliveries after receiving informed consent of healthy donors. Cryopreservation procedures were performed according to an ISO9002 quality system validation program of the Italian Cord Blood Banks Network [28].

Eleven CB units were obtained by umbilical vein catheterization after delivery of full-term newborns. Informed consent was given. CB was collected in PVC MacoPharma (Rho, Milan, Italy) bags containing 21 mL citrate phosphate dextrose. The units were transferred without any prior cell separation into 200 mL Haemofreeze DF700 bags (NPBI, Emmer-Compascuum, The Netherlands) and, after the addition of sterile saline solution plus 5% human albumin, 10% dimethylsulfoxide and 2000 IU heparin to obtain a final volume of 200 mL, were cryopreserved by a standard method (−1°C/min programmed cooling rate, stored in liquid nitrogen). CB units

were thawed at 37°C, gently mixed in melting ice according to the Rubinstein method [29]. 25% of the total volume was drawn through an inserted injection coupler in 150-mL collection bags (Baxter, Deerfield, IL, USA), slowly mixed with the same volume of sterile washing solution (5% Dextran40; Fresenius, Isola della Scala, Verona, Italy, 2.5% human serum albumin; Kedrion, Castelvechio Pascoli, Barga, Lucca, Italy, in 0.9%NaCl) and centrifuged once (400g/10 min). The remaining 75% of the thawed unit was quickly refrozen within 15 minutes, cryopreserving immediately the remaining volume of the thawed CB units, contained in the Haemofreeze DF700 bags, by a standard method (−1°C/min programmed cooling rate, stored in liquid nitrogen).

### *Validation of ex vivo expanded compartment*

*CD34<sup>+</sup> selection.* CB units were thawed and 25% of the total volume (corresponding to 50 mL) was drawn as described here. The supernatant was removed and the sedimented cells were slowly re-suspended in fresh washing solution, kept at room temperature, containing CliniMACS phosphate-buffered saline (PBS)-ethylene diamine tetraacetic acid buffer (Miltenyi Biotec, Bergisch Gladbach, Germany) supplemented with 0.5% human serum albumin, 0.06% anticoagulant citrate dextrose solution (Baxter) to restore the initial volume. This procedure was performed twice. An aliquot of the sample was tested for viability and the initial CD34<sup>+</sup> cells count was performed by flow cytometry. After each washing the supernatant was evaluated for WBC loss. Cells were then labeled with CD34 monoclonal antibody conjugated to superparamagnetic particles CD34 CliniMACS reagent (Miltenyi Biotec) and selected using the CliniMACS system, adjusting the manufacturer's instructions to the low number of nucleated cells and CD34<sup>+</sup> cells content. After selection, CD34<sup>+</sup> purity was evaluated in the positive fraction by the dual platform International Society of Hematotherapy and Graft Engineering protocol [30]. The negative fraction was tested for CD34<sup>+</sup> loss during the procedure. Sterility was tested at the beginning and end of the procedure. Each reagent was checked for the expiration date and, where possible, only reagents employed for in vivo use or European conformity marked and ISO9001 certified were used.

*CD34<sup>+</sup> expansion.* The isolation and expansion protocol of CB stem cells was complied according to the current European and Italian Guidelines on CTPs [23–27]. Expansion procedures were performed in our Stem Cell Transplantation and Cellular Therapy Unit at the Regina Margherita Children's Hospital, ISO9001:2000 certified and the Joint Accreditation Committee of the International Society for Cellular Therapy and European Group for Blood and Marrow Transplantation (JACIE) accredited. After collection, CD34<sup>+</sup> cells were counted and washed to remove the reagents utilized for CliniMACS separation. Cells were expanded in flasks (Corning-Costar, Celbio, Pero, Milano, Italy), in Iscove's modified Dulbecco's medium (Invitrogen, San Giuliano Milanese, Milan, Italy) supplemented with 10% heat-inactivated fetal bovine serum (Defined-Hyclone, Celbio, Pero, Milano, Italy) at the density of  $5 \times 10^4$ /mL in the presence of the following carrier-free growth factors: IL-6 (10 ng/mL; R&D Systems, Abingdon, UK); FL (50 ng/mL; R&D Systems); TPO (10 ng/mL; R&D Systems); SCF (50 ng/mL; R&D Systems). Culture expansion was maintained for 3 weeks. Cells were counted weekly and the culture volume was doubled by adding fresh medium. Cytokines were added every 3/4 days. No antibiotics were added to the cultures. Particular

attention was paid to reagent selection. When possible the reagents and the materials utilized were suitable for clinical use, or CE marked and ISO9001 certified. The Corning Costar flasks are tissue culture–tested, endotoxin-free, and apyrogen. The Invitrogen Iscove's modified Dulbecco's medium is CE marked. The Defined Hyclone serum derived from a pooled bovine serum came from United States Department of Agriculture and was produced under current European and Italian Good Manufacturing Practice (eIGMP) conditions and is European Medicines Agency-conforming. The serum was collected under sterile conditions and filtered three times through 0.4- $\mu$ m pores during processing and was Pharmacopeia-tested for endotoxin level, sterility (bacteria and fungi), mycoplasma, and viral genome (10 tests). The ex vivo cytokines from R&D System were carrier-free and produced in conditions analogous to eIGMP. These growth factors were also tested for endotoxin level and sterility, and were filtered on 0.2- $\mu$ m pores. The PBS 1 $\times$  used to resuspend the cytokines, and to perform cell washing after ex vivo expansion was from Euroclone and was cell culture–tested (sterility, apyrogenicity, endotoxin level). Our ISO9001:2000 and JACIE quality system checked the certification of the material and guaranteed the traceability of every batch of material and the correct supply of the reagents utilized for the procedures. Furthermore, our quality system guaranteed the correct utilization and cleaning of instruments and locations necessary for stem cell manipulation. All the operators are properly trained for manipulation in eIGMP conditions, and are routinely updated. All the instruments and the location were routinely cleaned. As requested, before and after every new procedure we performed an extraordinary cleaning as reported in our operative instruction manual. Each laboratory procedure was evaluated by numeric indicators that allow the whole quality system to be checked, as requested by ISO9001:2000 and JACIE standards.

#### *Quality control assays for the extensive ex vivo manipulated CD34<sup>+</sup> CB cells*

At every medium addition during the expansion period, the culture was subjected to the following.

**Sterility evaluation.** Sterility was evaluated at every manipulation stage using the standard colorimetric system VITAL 200 (Biomérieux, Rome, Italy) on an aliquot of supernatant medium. Mycoplasma contamination analysis was carried out by conventional polymerase chain reaction (PCR) using mycoplasma-specific primers that target the 16S rDNA gene in all mycoplasma species (Reagent Set Mycoplasma, Euroclone; Celbio, Pero, Milano, Italy) on 100  $\mu$ L supernatant medium. To detect the Gram-negative bacterial endotoxin, the Limulus Amebocyte Lysate Kinetic-K-QLC kit (Cambrex, Verviers, Belgium) was used. The assay, which has a sensitivity ranging from 50 endotoxin units (EU)/mL to 0.005 EU/mL, was assessed on 100  $\mu$ L supernatant medium by incubating the samples and the calibrators at 37°C in the presence of the limulus amebocyte lysate for 1 hour and 40 minutes and reading the plates at 405 nm.

**Viability evaluation.** Viability was evaluated by optic microscopy, after trypan-blue staining. Propidium iodide (PI) exclusion was also evaluated on 50,000 cells incubated for 10 minutes with 200  $\mu$ L solution containing 0.5  $\mu$ g/mL PI in PBS 1 $\times$ . The cyto-

fluorimetric analysis (Beckman Coulter Epics XL, Milan, Italy) of PI-negative cells was performed acquiring 20,000 events.

**Flow cytometry.** CD34<sup>+</sup> cells were evaluated using the standard dual-platform International Society of Hematology and Graft Engineering protocol. Briefly, 50,000 cells were stained for 20 minutes with anti CD45 fluorescein isothiocyanate (FITC) and CD34 phycoerythrin (PE; Beckman Coulter, CA, USA). Labeled cells were thoroughly washed with PBS 1 $\times$  and analyzed on an Epics XL cytometer with the XL2 software program. At least 20,000 events were acquired.

**CFC and LTC-Icassays.** Basal thawed isolated CD34<sup>+</sup> cells (1.5  $\times$  10<sup>3</sup> cells) or an aliquot of thawed expanded cells were plated in triplicate in methylcellulose medium containing SCF + GM-CSF + G-CSF + IL-3 + IL-6 + erythropoietin (Miltenyi). After 14 days' incubation at 37°C in 5% CO<sub>2</sub>, colony-forming unit granulocyte-macrophage, burst-forming unit- erythroid, and multilineage (colony-forming unit granulocyte-erythrocyte-monocyte-megakaryocyte) progenitors were scored.

The LTC-IC content of the initial and expanded CD34 population was determined by assaying for secondary CFCs in methylcellulose culture after 5 weeks' stromal (irradiated SL/SL and M210-B4 cell lines) coculture.

**Additional quality control at the end of the ex vivo expansion procedures.** Sterility, clonogenic potential and CD34<sup>+</sup> count were evaluated as described above. We also analyzed the presence of the more primitive stem cell subpopulation CD34<sup>+</sup>CD38<sup>-</sup>CD33<sup>-</sup> after 3 weeks' culture. For this analysis we used the following monoclonal antibody CD33FITC, CD38PE (Becton-Dickinson, San Jose, CA, USA) and CD34PC5 (Beckman Coulter, Fullerton, CA, USA) in association with PI 0.5  $\mu$ g/mL to exclude the dead cells (PI<sup>+</sup>) from the analysis.

**Animals.** NOD/SCID mice were supplied by Jackson Laboratories (Bar Harbor, ME, USA), maintained at the animal facilities of Immunogenetic and Experimental Oncology Center, Torino and handled according to institutional regulations, under sterile conditions in microisolator cages. Mice were transplanted 24 hours after total body irradiation with 350 cGy, through a single intravenous injection of CB CD34<sup>+</sup> cells obtained at the initiation of culture or harvested from the expansion cultures as described later. For limiting dilution assay purposes, mice were transplanted with decreasing numbers of expanded CD34<sup>+</sup> cells progeny. Mice were sacrificed at 2 weeks (short-term engraftment) and at 6 to 8 weeks (long-term engraftment) from transplantation in order to assess the number and types of human cells detectable in murine BM.

**Analysis of human cell engraftment in murine BM.** BM cells were flushed from the femurs and tibias of each mouse and flow cytometric analysis performed using a Beckman Coulter Epics XL cytometer after staining the cells with human-specific monoclonal antibodies, such as CD45FITC (Beckman Coulter), CD71 FITC (Beckman Coulter), and glycophorin A-PE (Chemicon, Hampshire, UK). The presence of  $\geq$ 0.1% of human CD45<sup>+</sup>, CD71<sup>+</sup> and glycophorin A<sup>+</sup> cells in the BM of NOD/SCID mice defined a positive engraftment. We confirmed the results by PCR for the  $\alpha$ -satellite of human chromosome 17. A dose of 0.5  $\times$  10<sup>6</sup> of total

BM collected from sacrificed mice was seeded in methylcellulose medium containing specific human cytokines SCF + GM-CSF + G-CSF + IL-3 + IL-6 + erythropoietin (Miltenyi). After 14 days' incubation at 37°C in 5% CO<sub>2</sub>, human CFC progenitors were scored.

**Evaluation of CD34<sup>+</sup> telomere length.** In order to determine cell senescence during culture, telomere length was analyzed at each passage by flow fluorescence in situ hybridization [31]. Two-hundred thousand basal and expanded nucleated CB cells were labeled with a telomere-specific conjugated (C<sub>3</sub>TA<sub>2</sub>)<sub>3</sub> peptide nucleotide acid probe (Applied Biosystems, Foster City, CA, USA). As a control, the same number of cells was analyzed without a probe. The same numbers of nucleated cells from a pool of CB was used as examples of long telomeres. Stained cells were analyzed on a FACSCalibur cytometer (Beckon Dickinson). At the beginning of each experiment, fluorescence signals from four different populations of FITC-labeled microbeads (Quantum 24 FITC Premix Low Level; Flow Cytometry Corporation, Fishers, IN, USA) were acquired. The voltage and amplification of the FL1 parameter were set in such a way that blank; 6,288; 12,825; and 26,904 molecular equivalents of soluble fluorochrome units per bead corresponded to channel numbers ranging from 10 to 15, 150 to 160, 450 to 460 and 800 to 820, respectively in the FL1 channel on a linear scale. The resulting calibration curve ( $y=0.029x$ ) was used to convert telomere fluorescence into molecular equivalents of soluble fluorochrome units in order to compare the experimental results.

**Karyotype analysis.** CD34<sup>+</sup> cells were grown for 24 hours in Iscove's modified Dulbecco's medium supplemented with 10 ng/mL IL-3 and 50 ng/mL GM-CSF to allow cells to proliferate. Then, 100 ng/mL Colcemid (Invitrogen Corporation, Carlsbad, CA, USA) was added to block the cells during metaphase. The cells were washed, lysed with hypotonic 0.075 M KCl and fixed in a solution of methanol/acetic acid (3:1). Chromosome preparations were made by the air-drying method and the analysis was performed on 10 Giemsa-stained metaphases using MackType software (Nikon Corporation, Tokyo, Japan).

**Bovine proteins limitation.** In order to reduce bovine protein content, at the end of the culture in fetal bovine serum, we performed three extensive washings of the cells in PBS 1× (Euroclone), and then we resuspended the expanded cells in an adequate volume of

PBS + 2% human serum albumin. We evaluated the quantity of bovine serum albumin (BSA) by enzyme-linked immunosorbent assay before and after the extensive washings. As reported, we also evaluated sterility before and after the washing procedures.

**Transport simulation.** We validated the delivery of the final CTPs from our stem cell unit to clinical units taking part in the protocol, by simulating transport of 1 hour at room temperature. As reported in our internal standard operating procedures (SOP) concerning tagging, we closed and tagged the collection bags, suitable for human cells, containing the expanded cells, in a secondary diathermic bag with a sterile compartment (Kimberly Clark, Roswell, GA, USA), which was then put in an appropriately tagged rigid plastic biobox. We checked temperature before, during, and after the simulated transport. We also evaluated the sterility and viability before and after the procedures.

**Analyses performed on twice thawed unmanipulated CB units.** After the second thawing, the 75% unmanipulated unit was validated for sterility, viability, flow cytometry analyses, and clonogenic activity as previously reported.

#### Statistical analysis

The SRC frequency in a population of cells was determined by injecting cohorts of mice with several dilutions of cells. The SRC frequency was calculated from the proportions of negative mice in each cohort, using L-calc<sup>T</sup> software program (Stem Cell Technologies, Vancouver, BC, Canada), which uses Poisson statistics and the method of maximum likelihood.

Bovine protein limitation data and WBC data in the mice BM after sacrifice were analyzed for differences with Student's *t*-test, with the levels of statistical significance set at  $p < 0.005$ .

## Results

### Validation of the expanded units

**In vitro data.** Our data on cell proliferation, MNC, CD34<sup>+</sup>, CFC, and LTC-IC fold-increase demonstrated that our culture system induces expansion of thawed CD34<sup>+</sup> cells (median value:  $8.3 \times 10^6$  after 3 weeks of expansion (range,  $5 \times 10^6$ – $22.8 \times 10^6$ ), whereas after thawing the median value is  $0.9 \times 10^6$  (range,  $0.3 \times 10^6$ – $1.5 \times 10^6$ )] (Tables 1 and 2).

**Table 1.** Summary of CD34<sup>+</sup> evaluation during ex vivo expansion of 11 thawed cord blood units

Cord blood no.	25% of thawed unit (mL)	CD34 <sup>+</sup> number after thawing ( $\times 10^6$ )	CD34 <sup>+</sup> number after 3 weeks of expansion ( $\times 10^6$ )	CD34 <sup>+</sup> fold increase after expansion	Total no. of CD34 <sup>+</sup> to infuse ( $\times 10^6$ )
1	50	1.24	7.7	6.2	11.4
2	50	1.1	10.7	9.7	14
3	50	0.9	7.1	7.9	9.8
4	50	1.5	11.2	7.5	15.7
5	50	1.1	25	22.8	28.4
6	50	0.43	4.8	11.1	6.8
7	50	1	8.3	8.3	13.6
8	50	0.3	3	8.9	4.3
9	50	0.4	3.6	8.1	5.5
10	50	0.3	1.5	5	2.7
11	50	0.8	7.5	9.1	10.7

**Table 2.** Summary of CD34<sup>+</sup> subpopulation, MNC, CFC, and LTC-IC evaluation during ex vivo expansion of 11 thawed cord blood units

Cord blood no.	CD34 <sup>+</sup> CD38 <sup>-</sup> CD33 <sup>-</sup> subpopulation after thawing ( $\times 10^6$ )	CD34 <sup>+</sup> CD38 <sup>-</sup> CD33 <sup>-</sup> subpopulation after 3 weeks of expansion ( $\times 10^6$ )	Absolute no. of MNC after 3 weeks of expansion ( $\times 10^6$ )	CFC fold increase after 3 weeks of expansion	LTC-IC fold increase after 3 weeks of expansion
1	0.4	2.3	77	1.8	1081
2	0.3	2.5	80	13	812.6
3	0.1	1.6	40	5.9	320.5
4	0.3	1.8	86	3.1	507.3
5	0.4	7.5	165	507.3	1373.6
6	0.1	1.2	45	157.2	125.5
7	0.4	2.3	45	95.7	76.4
8	0.06	0.5	11	18.9	78
9	0.1	0.8	12	41.9	40
10	0.07	0.4	9	28.2	45.7
11	0.3	2.2	59	18.2	97

CFC = colony-forming cell; LTC-IC = long-term culture-initiating cells; MNC = mononuclear cells.

CD34<sup>+</sup>CD38<sup>-</sup>CD33<sup>-</sup> content after 3 weeks of expansion ranged from  $0.4 \times 10^6$  to  $7.5 \times 10^6$  (median value:  $1.8 \times 10^6$ ), whereas after thawing, it ranged from  $0.06 \times 10^6$  to  $0.4 \times 10^6$  (median value:  $0.3 \times 10^6$ ) (Table 2).

*In vivo data.* LDA assays performed on basal cryopreserved CD34<sup>+</sup> CB cells demonstrated that a dose of 10,000 of these cells failed to engraft in NOD/SCID mice. Frequency of SRCs in basal cryopreserved CD34<sup>+</sup> is 1:34,861 (confidence interval, 1:74,847–1:16,237). In vivo experiments performed on thawed and expanded CD34<sup>+</sup> cells demonstrated that after the culture procedure, the final CTP maintain the short-term and long-term in vivo repopulating ability in NOD/SCID mice. LDA assays performed by injecting decreasing doses of expanded CD34<sup>+</sup> cells revealed that the progeny of 10,000 cultured cells engrafts in the BM NOD/SCID mice. The frequency of SRCs after ex vivo expansion is 1:8,034 (confidence interval, 1:20,587–1:3,137) (Table 3). Human engraftment was confirmed by PCR for  $\alpha$ -satellite of human chromosome 17 and WBC was higher in the BM of mice injected with low doses of cells ( $p = 0.008$ ) (data not shown).

*Viability assay.* During the 3 weeks of culture, the viability evaluated with trypan blue and PI was >98% (Tables 4 and 5).

*Cell senescence and cytogenetic evaluation.* Flow fluorescence in situ hybridization analyses performed on basal and expanded CD34<sup>+</sup> cells revealed that ex vivo expansion did not induce telomere shortening lower than 3 kb (Tables 4 and 5). Karyotype analyses performed at the beginning and the end of cultures demonstrated that our culture system did not modify the chromosome pattern (Tables 4 and 5).

*Sterility.* Microbiological tests for anaerobic and aerobic bacteria and fungi were negative from the beginning until the end of culture. Limulus amoebocyte lysate test performed

during the expansion were complied (<0.5 EU/mL). PCR analyses for identification of mycoplasma contamination were negative during the 3 weeks of culture (Tables 4 and 5).

*Bovine proteins limitation.* In order to reduce the bovine protein content in the final CTPs, we performed a series of extensive washings with PBS 1 $\times$ . In six samples, we analyzed the BSA content before and after these procedures and verified that the cell suspension contained <2 ng/mL bovine proteins ( $p = 0.001$ ) after three washings (Fig. 1).

Collectively, all these data represent the reference values that have to be reached in order to consider the validity of the final cell-therapy product.

**Table 3.** Summary of in vivo engraftment of thawed and expanded CD34<sup>+</sup> cells compared to unmanipulated CD34<sup>+</sup> cells

CD34 <sup>+</sup>	No. of positive mice/injected mice	SRC frequency
No. of basal		
$1 \times 10^4$	0/5	1:34,861
$2 \times 10^4$	3/7	(confidence interval, 1:74,847–1:16,237)
$3.5 \times 10^4$	4/6	
$5 \times 10^4$	4/5	
No. of expanded		
Short-term engraftment		
$5 \times 10^4$	6/6	Unevaluable
$10 \times 10^4$	2/2	
Long-term engraftment		
$1 \times 10^4$	4/4	1:8,034
$2 \times 10^4$	2/3	(confidence interval, 1:20,587–1:3,137)
$5 \times 10^4$	2/2	

The presence of 0.1% of human CD45<sup>+</sup>, CD71<sup>+</sup>, and GpA<sup>+</sup> cells in the bone marrow (BM) of nonobese diabetic severe combined immunodeficient (SCID) mice was defined as positive engraftment. Human engraftment was confirmed by polymerase chain reaction for  $\alpha$ -satellite of human chromosome 17. Human colonies were revealed seeding  $0.5 \times 10^6$  and  $1 \times 10^6$  of whole murine BM in metilcellulose specific medium (data not shown). SRC = SCID repopulating cells.

**Table 4.** Summary of results obtained during the validation process of the expansion of CD34<sup>+</sup> cells isolated by CliniMacs from 25% of 11 thawed cord blood units

Cord blood no.		Sterility	Mycoplasma	Endotoxin level (EU/mL)	Viability (%)	Karyotype	Telomere length (kb)
1	Basal	NC	NC	ND	59	Normal	9.3
	+1 Week	NC	NC	<0.4	100	ND	ND
	+2 Week	NC	NC	<0.4	100	ND	ND
	+3 Week	NC	NC	<0.4	100	Normal	7.7
2	Basal	NC	NC	ND	86	Normal	7.2
	+1 Week	NC	NC	<0.4	100	ND	ND
	+2 Week	NC	NC	<0.4	100	ND	ND
	+3 Week	NC	NC	<0.4	100	Normal	7.8
3	Basal	NC	NC	ND	56	Normal	9.1
	+1 Week	NC	NC	<0.4	100	ND	ND
	+2 Week	NC	NC	<0.4	100	ND	ND
	+3 Week	NC	NC	<0.4	100	Normal	8.6
4	Basal	NC	NC	ND	90	Normal	8.7
	+1 Week	NC	NC	<0.4	100	ND	ND
	+2 Week	NC	NC	<0.4	100	ND	ND
	+3 Week	NC	NC	<0.4	100	Normal	8.5
5	Basal	NC	NC	ND	77	Normal	7.9
	+1 Week	NC	NC	<0.4	100	ND	ND
	+2 Week	NC	NC	<0.4	100	ND	ND
	+3 Week	NC	NC	<0.4	100	Normal	7.9

EU = endotoxin units; NC = no contamination; ND = not determined.

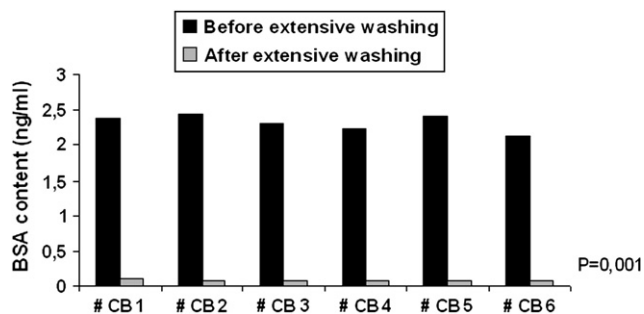
*Transport validation.* We simulated transport at room temperature as described in Materials and Methods. We checked temperature in the biobox containing the final CTPs, before, during and after transport. We also evaluated

the sterility and viability before and after this procedure. In every case, sterility assays for aerobic, anaerobic bacteria, and fungi were negative after the transport simulation procedure. During the simulation temperature ranged between

**Table 5.** Summary of results obtained during the validation process of the expansion of CD34<sup>+</sup> cells isolated by CliniMacs from the 25% of 11 thawed cord blood units

Cord blood no.		Sterility	Mycoplasma	Endotoxin level (EU/mL)	Viability	Karyotype	Telomere length (kb)
6	Basal	NC	NC	ND	66.2	Normal	12.3
	+1 Week	NC	NC	<0.4	100	ND	ND
	+2 Week	NC	NC	<0.4	100	ND	ND
	+3 Week	NC	NC	<0.4	100	Normal	11.1
7	Basal	NC	NC	ND	61	Normal	10.8
	+1 Week	NC	NC	<0.4	100	ND	ND
	+2 Week	NC	NC	<0.4	100	ND	ND
	+3 Week	NC	NC	<0.4	100	Normal	10.25
8	Basal	NC	NC	ND	77	Normal	10.2
	+1 Week	NC	NC	<0.4	100	ND	ND
	+2 Week	NC	NC	<0.4	100	ND	ND
	+3 Week	NC	NC	<0.4	100	Normal	9.1
9	Basal	NC	NC	ND	71	Normal	10.8
	+1 Week	NC	NC	<0.4	100	ND	ND
	+2 Week	NC	NC	<0.4	100	ND	ND
	+3 Week	NC	NC	<0.4	100	Normal	10.9
10	Basal	NC	NC	ND	75	Normal	9.9
	+1 Week	NC	NC	<0.4	100	ND	ND
	+2 Week	NC	NC	<0.4	100	ND	ND
	+3 Week	NC	NC	<0.4	100	Normal	9.6
11	Basal	NC	NC	ND	76	Normal	10.3
	+1 Week	NC	NC	<0.4	100	ND	ND
	+2 Week	NC	NC	<0.4	100	ND	ND
	+3 Week	NC	NC	<0.4	100	Normal	10.1

EU = Endotoxin units; NC = no contamination; ND = not determined.



**Figure 1.** Bovine proteins limitation analyses. The quantity of bovine serum albumin (BSA) by enzyme-linked immunosorbent assay (ELISA) before and after three extensive washing of final cell therapy products (CTPs) in phosphate-buffered saline was performed by ELISA assay. In six samples, CTPs contained <2 ng/mL bovine proteins ( $p = 0.001$ ).

23° and 25°C and the viability was conserved (data not shown).

**Validation of unmanipulated CB units.** After thawing CB units, 25% of the unit was expanded and the remaining unmanipulated 75% of the collection was immediately refrozen. In order to confirm that this procedure is safe, we evaluated CD34<sup>+</sup>, CD34<sup>+</sup>CD38<sup>-</sup>CD33<sup>-</sup> content, clonogenic activity, sterility, and viability after the second thawing procedure of six refrozen unmanipulated units. All data about WBC, CD34<sup>+</sup>, CD34<sup>+</sup>CD38<sup>-</sup>CD33<sup>-</sup>, CFC, and LTC-IC referred to viable cell counts after washing (Table 6). Our results demonstrated that twice thawed unmanipulated CB stem cells maintain their clonogenic potential. Sterility tests performed after the second thawing were negative.

## Discussion

In this article, we described the *in vitro* and *in vivo* validation of phase I/II protocol for the CD34<sup>+</sup> expansion of thawed CB units according to the current European and Italian Guidelines on CTPs [23–27]. This might offer new options for transplants in various oncohematological diseases affecting patients with no BM related donor. Between the various strategies to overcome cell dose limit in adult

patients, in the last years double-unit CB transplantation has provided a successful option and has shown a high short-term engraftment rate, even if with a wide range of neutrophil recovery [8]. The mechanisms underlying the engraftment with this approach are partially unknown, but the results are, in the meantime, good, with relatively short follow-up periods [32]. A theoretic concern about the use of two CB units is the doubling of the biologic risk, even if extremely low, related for example to the presence of pre-leukemic clones (detected in >1% of a large unselected series of CB samples) [33] or to the possibility of donor late-onset inherited metabolic disorders, undiagnosed during standard CB bank follow-up.

Ex vivo expansion of CB HSCs is another approach to overcome the cell dose limit in the case of high-weight recipients. It represents a flexible technique able to manipulate not only the hematopoietic, but also the immune component of the graft [34,35], and it could be particularly useful in autologous or sibling CB transplants.

It is also possible to associate double-UCB transplantation and expansion. Shpall et al. [36] are currently performing a randomized controlled trial in which patients receive either two unmanipulated cords or one unmanipulated and one ex vivo expanded [36].

The present study was designed to infuse at time 0 expanded and not expanded cells. In the two previous phase I UCB ex vivo expansion studies [13,14], the expanded fraction was infused, respectively, 10 and 12 days after the unmanipulated one. Both studies failed to show shorter recovery time in comparison with historical controls. We assume that the simultaneous infusion of expanded and unmanipulated material could allow better recovery.

As most CB samples are frozen in single bags, and only an aliquot (~25%) of the blood can be expanded, several requirements need to be met before clinical use of CB HSCs. We have previously shown that successive freezing and thawing procedures do not affect the *in vitro* or *in vivo* activity [21,22]. In this work, we set up standard values for validation of the final CTPs in eIGMP conditions. Use of bovine serum as a reagent for the expansion protocol is justified, as our previous data demonstrated, as it improves stem cell expansion rather than serum-free

**Table 6.** Summary of the validation analyses performed on six twice thawed 75% of the unmanipulated cord blood units

Cord blood no.	Twice thawed 75% unmanipulated CB unit (mL)		Viability after thawing and washing (%)	Total WBC ( $\times 10^6$ )	Total CD34 <sup>+</sup> ( $\times 10^6$ )	Total CD34 <sup>+</sup> CD38 <sup>-</sup> CD33 <sup>-</sup> ( $\times 10^6$ )	Total CFC ( $\times 10^6$ )	Total LTC-IC ( $\times 10^6$ )
	Sterility							
1	150	NC	87	890	2.4	1.6	1.2	0.2
2	150	NC	70	770	3.3	2	0.7	0.1
3	150	NC	93	480	0.95	0.8	0.7	0.1
4	150	NC	80	1110	4	2.1	0.7	0.1
5	150	NC	76	780	1.2	0.8	0.5	0.1
6	150	NC	84	710	1.7	0.6	0.6	0.1

CB = cord blood; CFC = colony-forming cells; LTC-IC = long-term culture-initiating cell; NC = no contamination; WBC = white blood cells.

media or human serum [20]. We selected an extremely controlled serum produced in eGMP conditions. To avoid anaphylactic shock, no antibiotics were added to the cultures. Our results demonstrated that the frequency of SRC after *ex vivo* expansion is 1:8,034 (confidence interval, 1:20,587–1:3,137). As we have previously reported [18] the frequency of basal SRCs in cryopreserved CD34<sup>+</sup> is 1:34,861 (confidence interval, 1:74,847–1:16,237), therefore, this protocol allows the expansion of thawed SRCs. Furthermore, quality-control assays on final unmanipulated and expanded CTPs have shown that thawing a single CB unit, dividing it into two parts, expanding the smaller one, and refreezing the other is safe and could be exploited to devise new options in expansion procedures and quality controls so that both unmanipulated and expanded CB units could be infused at the same time.

To date, published data of expanded CB clinical trials use different culture systems. However, compared to our protocol, none of the other studies reported the contemporaneous infusion of CB units [13–15]. Although these authors demonstrated the feasibility and safety of expansion procedures, but the positive effect on a faster engraftment remained unclear. The authors utilized only some of the cytokines that, as we reported [16–20], are able to promote SRCs expansion. In one study, the SCF and an analogue of TPO, but not the FL, were used. The two other works utilized FL, but avoided TPO and SCF. Moreover, all of the authors avoided the presence of IL-6 and, by contrast, they used G-CSF, GM-CSF, and IL-3, which, as reported [16–20,37,38], can trigger proliferation and maturation followed by a detrimental effect on stem cells' self-renewing ability and thus on the maintenance of SRCs. A further difference between our study and these three phase I/II protocols is that they infused the expanded CB units on days +10 to 12 after transplanting unmanipulated units. This delay might not allow a faster granulocyte and platelet engraftment, due to the expanded compartment.

Finally, Peled et al. [39] reported the feasibility of a pre-clinical study, currently in a phase I clinical trial. They expanded CB stem cells for 3 weeks but, unlike our protocol, they used CB CD133<sup>+</sup> cells cultured in the presence of SCF + FL + IL-6 in association with the copper chelator tetraethylenepentamine [39].

In conclusion, our data demonstrate that *ex vivo* manipulation, performed in eGMP conditions according to the current European and Italian Guide Lines related to CTPs, is safe and useful for clinical applications involving frozen HSCs. This could open new options for expansion of allogenic and autologous HSCs in order to enhance the possibility of transplanting a larger number of patients with hematologic malignancies and also to implement other cell-therapy protocols using expanded HSCs. In this context, an important issue is that the *ex vivo* expansion procedures, contrary to the unmanipulated cells, could condition cultured stem cells through particular lineages. In the hema-

tological transplantation field, our culture system is able to induce the expansion of myeloid cells rather than that of lymphoid lineage. During culture, in addition to expansion of CD34<sup>+</sup> cells, also primitive hematopoietic stem cells, such as CD34<sup>+</sup>CD38<sup>-</sup>CD33<sup>-</sup>, and SRC, are expanded. A further step could be to verify the possibility, using this expansion protocol, to induce specific differentiation of hematopoietic stem cells into nonhematopoietic cells of various tissue lineages that could be utilized to treat patients affected by degenerative diseases.

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