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ORIGINAL ARTICLE

Rapid and massive expansion of cord blood-derived cytokine-induced killer cells: an innovative proposal for the treatment of leukemia relapse after cord blood transplantation

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We have used a standardized 21-day expansion protocol to produce cytokine-induced killer (CIK) cells starting from very small amounts of nucleated cells (approximately 15×10^6 cells) isolated from cord blood. Mononuclear cells are stimulated with anti CD3 (OKT3) and IFNγ and then expanded with IL-2. Moreover, we show that washouts of cord blood units bags (at the end of the infusion) may be sufficient to yield almost 500×10^6 CIK by the same expansion protocol. CIK cells show strong cytotoxic activity against a variety of tumor target cell lines including B and T lymphomas and myeloid leukemias. More importantly, expanded cord blood-derived CIK cells are cytotoxic against fresh leukemic blasts and express perforin, granzyme and NKG2D molecule at high levels. The same *in vitro* protocol has already been used to expand CIK cells from peripheral blood of adult donors under GMP conditions and therefore these observations open up the possibility of imagining a future clinical application of leukemia relapse following cord blood transplantation with CIK cells obtained from the same cord blood unit.
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Introduction

Cord blood (CB) transplantation is progressively becoming an extensively used treatment for patients with both malignant and nonmalignant disorders. When compared to unrelated donor bone marrow (BM), CB has the advantages of rapid availability and lower risk of severe acute graft-versus-host disease (GVHD) despite donor recipient human leukocyte antigen (HLA) disparities. Nonetheless, CB transplantation shows still severe limitations when compared to the more traditional BM transplants such as a greater risk of graft failure and delayed neutrophil and platelet recovery, possibly due to an imbalance between the body size of most adult recipients and the number of hematopoietic progenitor cells in each CB unit.[1–3] Furthermore, as recently outlined in two reports of large registry-based studies, the incidence of relapse rate is similar in both CB as well as BM transplants, thus underlying the necessity to ameliorate the graft-versus-leukemia (GVL) effect.[2,4,5] More importantly, in case of leukemia relapse, the CB transplant shows a major limitation in that donor cells are not available to try and plan donor lymphocytes (DLI) or any sort of cellular therapy.

In the last 10 years, a protocol has been firmly established to rapidly and reproducibly expand *in vitro* T cells with NK phenotype characterized by a very high cytolytic potential, starting from human blood from normal donors or from leukemia/lymphoma patients.[6–9] These cells have been called cytokine-induced killer (CIK) cells and can be expanded up to 200- to 1000-fold in 14–21 days of culture after an initial priming with IFNγ and OKT3 followed by repeated stimulation with IL-2. *In vitro* expanded human CIK cells are mostly $CD3^+CD56^+CD8^+$ and show potent cytotoxic activity *in vitro* against a number of tumor cell lines or freshly isolated tumor samples, including AML, CML and B and T lymphoma cells.[7,8,10–13] *In vivo* in animal models, 5×10^6–4×10^7 expanded CIK cells led to significant protection from aggressive syngeneic and allogeneic tumors inoculated at lethal doses.[6,7,9,10,14–16] Furthermore, human CIK cells protected SCID animals from lethal doses of primary human B lymphoma, CML, solid tumors or lymphoma cell lines and proved superior to lymphokine activated killer (LAK) cells.[14–16] CIK cells could also be shown not to interfere with BM engraftment *in vivo* and to have little toxicity at doses of up to 50×10^6 cells. Finally, in several experimental transplant settings, reduced GVHD could be observed *in vivo* possibly reconductable to IFNγ production.[10,14]

Indeed several phase I studies including NHL and Hodgkin and liver cancer patients in the autologous setting have demonstrated that CIK cells could be expanded under

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GMP conditions and that clinical protocols are feasible, showing some clinical activity in the presence of very limited toxicity.[17–19]

Very recently we have explored the use of allogeneic (donor's) CIK cells in a pilot phase I study involving leukemia/lymphoma patients relapsed after allogeneic BM transplant showing no toxicity and limited aGVHD in 3/10 patients.[20]

We asked whether, by using a small fraction of CB or only the washouts of the CB unit bags following infusion, we could obtain functionally active CIK cells in numbers sufficient to plan a clinical trial to try and imagine their possible clinical utilization as a specific cellular therapy for the prevention or treatment of leukemia relapse in patients undergoing CB transplantation.

Materials and methods

Cells, culture medium and cytokines

Mononuclear (MNC) cells were obtained from umbilical cord blood (UCB) by Ficoll gradient (Lympholyte-H, Cedarlane, Hornby, Ontario, Canada) centrifugation. To generate CIK cells, MNC cells were resuspended in X-Vivo complete medium (Cambrex, Bergamo, Italy) at 5×10^6/ml concentration and rhIFN-γ (Boehringer Ingelheim, Vienna, Austria, 1000 U/ml) were added on day 1. OKT-3 monoclonal antibody (Janssen Cilag, Schaffausen, Swiss, 50 ng/ml) and rhIL-2 (Chiron Corp., Uxbridge, UK, 500 IU/ml) were added on day 2. Fresh complete medium with rhIL-2 were added every 2–3 days. Cells were harvested (solution with 10% DMSO – dimethyl sulphoxide, Sigma-Aldrich Company, Ayrshire, UK) on day 21 and used for phenotyping and cytotoxicity *in vitro* assays.

Alternatively, CB bags after transplant were returned to the laboratory and filled with 20 ml saline (S.A.L.F. S.p.A. Laboratorio Farmacologico, Bergamo, Italy) added with 2% human serum albumin (Kedrion S.p.A., Lucca, Italy). The saline was then removed and the operation repeated two times. Washout solutions were spun at 1500 r.p.m. for 10 min and pellets resuspended in X-Vivo complete medium for the CIK expansion protocol.

This very same protocol is routinely utilized in our GMP facility for the expansion of CIK cells from adults peripheral blood and has been approved by the Italian Istituto Superiore di Sanità in Roma for a phase I study.

Fresh AML blasts were separated from peripheral blood of four different patients in blastic crisis and were ficolled and resuspended in RPMI 1640 complete medium (Cambrex Bio Science, Verviers, Belgium) with 10% heat inactivated FBS (Euroclone, Milano, Italy), L-glutamine (Euroclone, Milano, Italy, 200 mM) and gentamicin sulfate (PHT Pharma, Milano, Italy, 50 μg/ml).

Human cell lines (K562, CEM, JURKAT, BJAB and THP-1) were always kept in RPMI complete medium. For selected experiments, we expanded K562 and CEM lines in RPMI medium in the presence of 10% human AB serum.

Flow cytometry

Cells were stained with mAbs CD3-FITC, CD3-PerCP, CD56-FITC, CD56-PE, CD8-PerCP (Becton Dickinson Biosciences, San Jose, CA, USA) and NKG2D PE (R&D Systems, Minneapolis, MN, USA).

Cells were incubated with abs for 15 min at room temperature. The cells were then washed in phosphate-buffered saline (PBS – Biosera, East Sussex, UK) and then analyzed by flow cytometry using FACScan analyser equipped with an argon-ion laser tuned at 488 nm, and analyzed by Cell Quest program (both from Becton Dickinson).

For intracellular protein Perforin-PE (Becton Dickinson Biosciences-Pharmigen, San Jose, CA, USA), Granzyme B-PE (Sanquin, Amsterdam, Nederland) were used with FIX & PERM cell permeabilization reagents (Caltag Laboratories, Berlingame, CA, USA).

Calcein-AM release cytotoxicity assay

Cytotoxity assays using Calcein-AM have been developed as an alternative to radioactive ^{51}Cr release assays. Calcein-AM is a nonpolar compound that passively crosses the plasma membrane in living cells where it is cleaved by intracellular esterases to reveal a very polar derivative of fluorescein (calcein) that remains trapped in the cytoplasm. Calcein-AM (Fluka, Sigma-Aldrich Company, Ayrshire, UK) was dissolved in DMSO to 1 mM concentration and stored in aliquots at -80°C. Target cells were labeled by incubating 1×10^6 cells in RPMI1640 complete medium in presence of 3.5 μM Calcein-AM for 30 min at 37°C. The labeled cells were then washed with RPMI two times and distributed in TC-Plate 96-well V-shape (Greiner Bio-One, Frickenhausen, Germany) at 5×10^3 cells/well in triplicate. Effector cells were added at the indicated ratio and incubated for 4 h at 37°C. At the completion assay, 100 μl of supernatant was transfered in TC-plate 96-well with flat bottom (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and Calcein-AM release from cells after lysis by effector cells were measured by using microplate reader GENios TECAN; setting of the measurement parameters: exitation wave 485 nm and emission wave 535 nm.

The percentage of specific Calcein-AM release was calculated to the following equation:

$$\%\ \text{Specific Release} = \frac{(\text{Test release}) - (\text{Spontaneous release})}{(\text{Maximal release}) - (\text{Spontaneous release})} \times 100$$

Spontaneous release was obtained by incubating cells in media alone and maximal release was obtained after treatment with 3% TRITON X-100 (Merck, Darmstadt, FR, Germany).

Results

In a first series of experiments, we tried to expand CIK cells from aliquots of freshly collected CB. Aliquots (10–15 ml) were separated over Ficoll gradient and then mononuclear cell recovery was evaluated (data not shown). In order

to compare different experiments, we repeatedly used 15.0×10^6 total MNC and put them in culture standard conditions for CIK expansion for 21 days. The starting populations contained a mean of 7.8×10^6 $CD3^+$ cells and a very small amount of CIK cells (CD3/CD56 double positive) varying from 0.2×10^6 to 0.4×10^6, as shown in Table 1. After 21 days expansion the mean total number of mononucleated cells was approximately 800×10^6 thus representing a mean 53.5-fold expansion (Table 1). $CD3^+$ cells account for more than 97% at the end of the expansion period (Figure 1) and a mean absolute number of 777.3×10^6 $CD3^+$ cells could be obtained, thus reflecting a mean 101-fold expansion with respect to the starting population (Table 1).

The kinetics of expansion in culture of CIK cells are shown in Figure 1a. Absolute numbers of CIKs obtained varied from 264×10^6 to 872×10^6 cells (Table 1), representing a mean 77% of cells with respect to the total expanded population (Figure 1b), indicating a mean 1860-fold expansion (Table 1). The final CIK population is therefore contaminated by a mean 19.5% of $CD3^+/CD56^-$ cells and a mean 3.3% $CD3^-/CD56^+$ cells (Figure 1b), very consistent with what was previously reported during CIK expansion from adult's peripheral blood.

To confirm that the CB-derived CIK cells were comparable with what was previously reported for adult CIK, we analyzed their phenotype and cytotoxic activity. CIK cells are, as previously described for adult derived CIK cultures, mostly $CD8^+$ (more than 87%), with less than 5% $CD4^+$ T cells (data not shown). Moreover, they express the activation marker NKG2D at high levels: indeed in four separate preparations, over 97% CIK cells were positive for NKG2D with a mean 209 MFI (Figure 2a). As expected, the NKG2D 'activation' marker was also detectable at

Table 1 Cell expansion obtained from cord blood samples

Sample #	*Cells utilized at start (day 0)*			*Cells recovered at the end of the expansion protocol (day+21)*		
	A Total MNC $\times 10^6$	*B Total* $CD3^+ \times 10^6$	*C Total* $CD3^+/CD56^+ \times 10^6$	*A′ Total MNC* $\times 10^6$	*B′ Total* $CD3^+ \times 10^6$	*C′ Total* $CD3^+/CD56^+ \times 10^6$
1	15.0	6.0	0.4	990.0	949.0	872.0
2	15.0	9.0	0.3	768.0	744.0	570.0
3	15.0	5.0	0.2	360.0	350.0	264.0
4	15.0	11.0	0.4	1100.0	1066.0	816.0
Mean ± s.d.	15.0	7.8 ± 2.8	0.3 ± 0.1	804.5 ± 326.9	777.3 ± 314.4	630.5 ± 277.3
Fold increase (range)				A′/A 53.5 (24–66)	B′/B 101.5 (70–152)	C′/C 1860 (1320–2180)

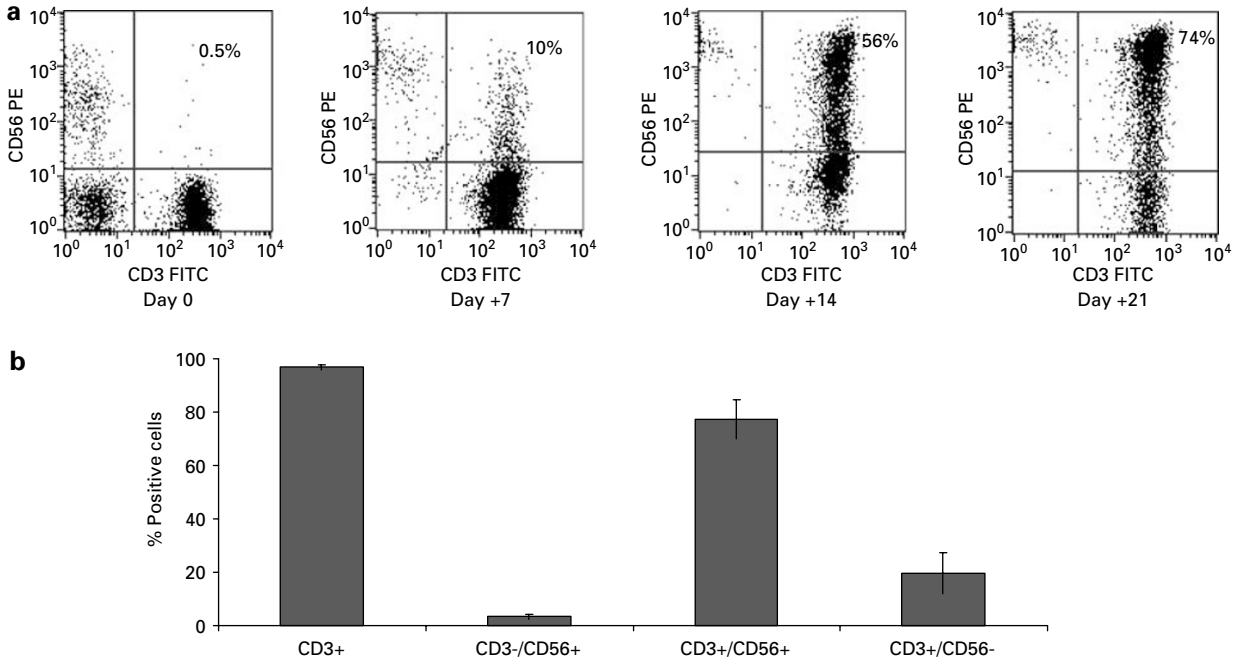

Figure 1 Kinetics of expansion and distribution of phenotypes by CB-derived CIK cultures. (**a**) Phenotypic analysis at days 0, +7, +14 and +21 by double CD3/CD56 staining. The percentages of double positive cells are indicated. (**b**) Distribution of different cell subsets at the end of the culture: results refer to total CD3+, true NK (CD3– CD56+), CIK (CD3+CD56+) and true T (CD3+CD56−).

a

NKG2D expression levels				
Population studied	At day 0		At day +21	
	% Positive cells	MFI	% Positive cells	MFI
CD3+/CD56-	25.5±3.53	49.3±12.42	67.5±10.5	168.2±19.8
CD3-/CD56+	99.5±0.7	51±6.71	99±0.8	174.7±10.6
CD3+/CD56+	ne	ne	97.5±0.6	209.9±12.3

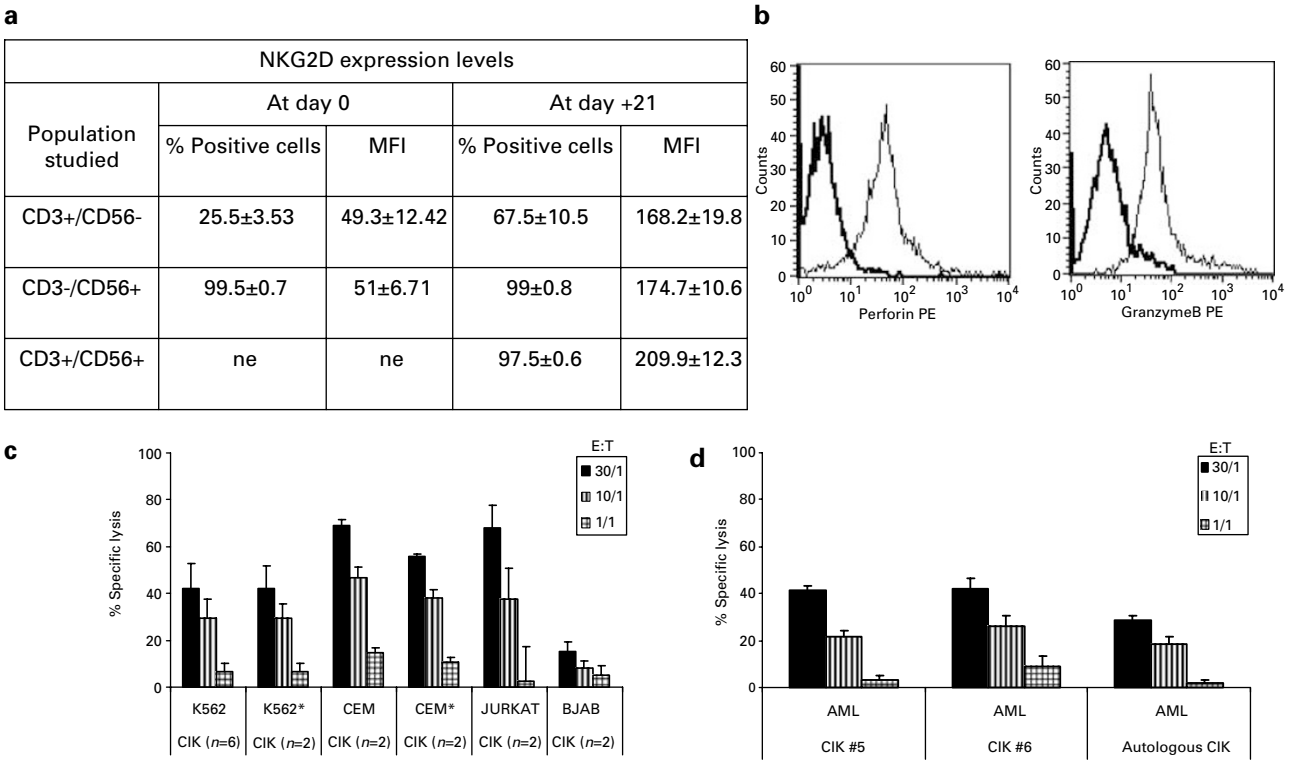

Figure 2 Phenotypic characteristics of CB-derived CIK cells. (**a**) NKG2D expression of different cell populations at the beginning and at the end of *in vitro* expansion. Data refer to the percentages of NKG2D positive cells and relative MFI as detected on CD3+/CD56−, CD3−/CD56+ and CD3+/CD56+ populations. ne = not evaluable. Differences between MFI values at day 0 and day +21 by Student's *T*-test showed statistically significant; $P<0.0001$. (**b**) CD3+CD56+ CIK cells are analyzed for perforin and granzyme expression. One representative case is shown in the figure. (**c**) CB-derived CIK cultures are analyzed for cytotoxicity against K562 (six separate preparations) CEM, JURKAT and BJAB (two separate preparations) at several E:T ratios. CEM* and K562* refer to cell lines expanded in RPMI in the presence of 10% human AB serum. (**d**) Final CB-derived CIK cultures (samples from two separate preparations) are analyzed for cytotoxicity against fresh leukemic blasts from an AML patient at different E:T ratios. For this patient also cytotoxicity from autologous peripheral CIK cells is shown.

comparable levels in more than 67% T and 99% NK cell present in the expanded population at the end of the culture period (Figure 2a). Therefore, the NKG2D marker is significantly upregulated during the culture period in all the analyzed populations, compared to the starting conditions (25% T cells, MFI = 49; and 99% NK cells, MFI = 51). Finally, in all cases CIK cells were perforin and granzyme B positive (a representative case is shown in Figure 2b).

Six different CB-derived CIK populations tested were found to be very cytotoxic *in vitro* against myeloid and T lymphoid targets (K562, CEM, JURKAT), as shown in Figure 2c. More importantly, two different populations (CIK cultures #5 and #6) were also tested against one freshly isolated sample of AML blasts and found to be cytotoxic (Figure 2d). Interestingly, it was possible to compare the activity of two CB-derived CIK cells with the cytotoxic activity of the autologous adult's CIK cells derived from the peripheral blood of this AML patient. As indicated in Figure 2d, the CB-derived CIK cells were at least as active as the autologous CIK cultures. To exclude that the observed cytotoxicity could be ascribed to bovine xenoantigens possibly present in the medium utilized to grow the cell lines, we have also expanded K562 and CEM lines in RPMI medium in the presence of 10% human AB serum and show that they could be lysed at comparable efficiency by CIK cells (Figure 2c). Finally, CIK cells are poorly lytic against the NK resistant BJAB cell line (Figure 2c).

In a second set of experiments, we tested whether the frozen CB units utilized for the transplant could indeed be utilized for *in vitro* CIK expansion. At the end of the infusion, the empty bags were returned to the laboratory and here filled several times with sterile saline, and the washouts were removed, collected and pelleted. In the three cases analyzed (Wo #1, 2 and 3) 12×10^6, 24×10^6 and 30×10^6 total nucleated cells were recovered, respectively (Table 2) showing that the number of cells obtained was comparable to that utilized in the first set of experiments. Considering the overall cellularity of the three CB units utilized, we have recovered 0.7, 6.4 and 2.4%, respectively, of the initial nucleated cells (Table 2 and data not shown).

The mean total number of $CD3^+$ cells was 3.5×10^6 and a small amount of CIK cells were also present varying from 0.2×10^6 to 0.6×10^6 (Table 2). After 21 days of culture, we recovered a mean number of 618×10^6 CD3 and 473×10^6 CIK cells with an overall fold increase of 182 times for the T population and of 1485-fold for the CIK cells, thus showing very comparable results with previously studied

Table 2 Cell expansion obtained from washouts of cord blood unit bags

Sample	*Total cells present in the cord blood unit bags*		*Total cells recovered after washout (day 0)*			*Total cells recovered at the end of the expansion protocol (day +21)*		
	NC $\times 10^6$	*CD3*$^+$ $\times 10^6$	*A NC* $\times 10^6$	*B CD3*$^+$ $\times 10^6$	*CD3*$^+$/*CD56*$^+$ $\times 10^6$	*A′ NC* $\times 10^6$	*B′ CD3*$^+$ $\times 10^6$	*C′ CD3*$^+$/*CD56*$^+$ $\times 10^6$
Wo #1	1700	790	12.0	3.7	0.4	474.0	444.0	378.0
Wo #2	468	89	30.0	2.8	0.6	695.0	690.0	507.0
Wo #3	1000	67	24.0	4.0	0.2	750.0	720.0	533.0
Mean $\pm$ s.d.	1056 $\pm$ 618	315.3 $\pm$ 411	22 $\pm$ 9	3.5 $\pm$ 0.6	0.4 $\pm$ 0.2	640 $\pm$ 146	618 $\pm$ 152	473 $\pm$ 83
Fold increase (range)						A′/A 31.3 (23–39)	B′/B 182 (120–246)	C′/C 1485 (845–2665)

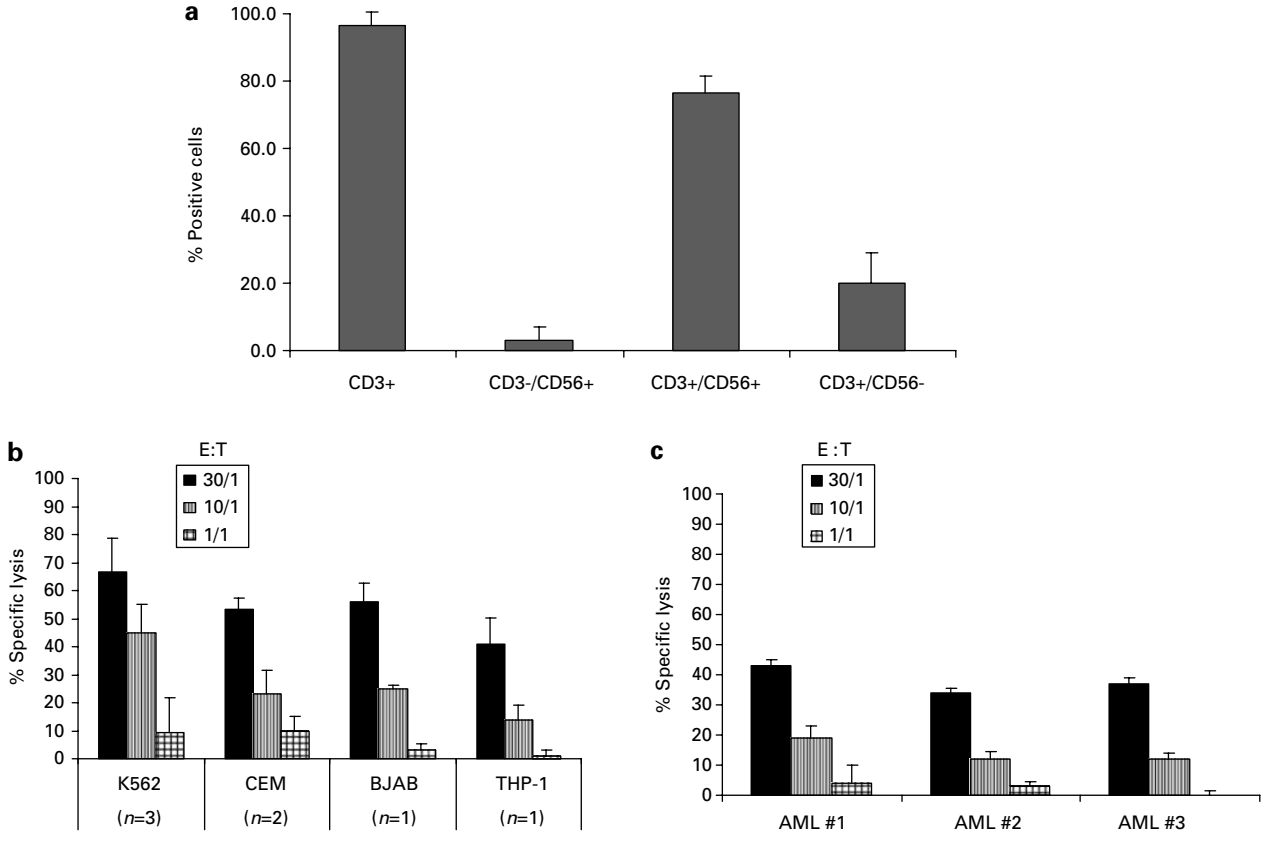

Figure 3 Phenotypic characteristics of CIK cells obtained by CB bags washouts. (**a**) Distribution of different cell subsets at the end of the culture: results refer to total CD3+, true NK (CD3− CD56+), CIK (CD3+CD56+) and true T (CD3+CD56−). (**b**) CIK cultures are analyzed for cytotoxicity against K562 , CEM, BJAB and THP-1 cell lines at different E:T ratios (*n* refers to the number of the different preparations analyzed). (**c**) One CIK culture is analyzed for cytotoxicity against three separate freshly isolated AML blasts at different E:T ratios.

mononuclear cells (Tables 1 and 2). The distribution of the main cellular subsets obtained at the end of the culture was also absolutely comparable with the one observed on the CB-derived CIK cells (Figure 3a, CIK cells were present for more than 75%) and they showed strong cytotoxic activity *in vitro* against K562, CEM, BJAB and THP-1 cell lines at the different E:T ratios tested (Figure 3b). Finally, and more importantly, all the expanded populations showed measurable cytotoxicity against freshly isolated AML blasts: one typical example is shown in Figure 3c indicating the cytotoxic activity of the sample Wo #3 against three separate target cells.

Discussion

CB transplantation is gaining increasing attention for the several advantages that it can offer with respect to the traditional adult BM transplant. In particular, as recently reviewed, CB is an abundant source, can fully represent the genetic repertoirs of minorities, is rapidly available and easily shipped.[1,2] Nonetheless one major disadvantage lies in the unavailability of the donor for patients experiencing graft failures nor of DLI for recipients who suffer leukemia relapse.

CIK cells are naturally occurring cytotoxic cells active against a variety of leukemia and lymphoma targets and

with low or absent activity against normal BM stem cells and tissues.[6–9] Their *in vitro* expansion is easily obtained applying standardized protocol which can be utilized under strict adherence in GMP conditions, as already established for the autologous setting.[17–19] This paper presents for the first time the idea that a salvage CIK infusion may be potentially planned for patients experiencing leukemia relapse after CB transplantation. Indeed we demonstrate that simply washing the CB unit bag at the end of the infusion is sufficient to recover a number of functionally active CIK cells, which could be infused.

With a comparable perspective, others have previously reported that *in vitro* expansion of CB lymphocytes in IL-2 can be obtained with increasing cytotoxicity[20] and retaining the naive repertoire,[21] but in both cases the entire CB unit was utilized for experimental procedure. Indeed, in a single case the CB cell residues have been used to collect $CD4^+$ lymphocytes, which were subsequently expanded in IL-2 and used to treat the mixed chimerism in a newborn with Omenn syndrome.[22]

In this experience, we have shown that using a very small percentage of total nucleated cells present in a CB unit (from 0.7 to 6.4%) in 21 days a mean number of 473×10^6 CIK cells can be obtained, indicating that this procedure could give reasonable number of cells without hampering the CB transplant.

The CIK cells obtained from CB have an overlapping phenotype with the traditional CIK cells described so far obtained from adult peripheral blood. More importantly, they express NKG2D and perforin, the two proposed molecules which have been demonstrated to play a major role in CIK-mediated cytotoxicity so far.[23] Moreover, the CB-derived CIK cells are shown here to be able to kill both myeloid and T lymphoid leukemic targets and, more importantly, fresh AML blasts.

So far, CIK cells have been used in a phase 1 study at 10×10^6/kg or above in the autologous setting.[17–19] Owing to the allogeneic condition of the CB transplant, a lower dosage of CB-derived CIK cells may suffice to show an antileukemic effect with limited toxicity. Indeed, in a very recent pilot phase I study with allogeneic (donor's) CIK cells to treat leukemia lymphoma patients relapsed after allogeneic BM transplantation, we have observed a very reduced aGVHD toxicity (grade I/II in 3/10 patients)[20] confirming the preclinical observations on a very limited GVHD activity of human CIK cells in animal models.[10,14] In our phase I study, we have used exactly the same CIK expansion protocol here utilized for CB lymphocytes, showing its compliance with full GMP criteria.[20]

In consideration of the time constraints of the microbiological detection required for the full application of the European GMP rules, a total time frame of 5 weeks has to be planned between the availability of the CB bag to the laboratory and the planned infusion, which is probably compatible with a timely use of CB-derived CIK cells for the treatment or prevention of leukemia relapse.

Moreover, an early infusion of these *in vitro* expanded $CD3^+$ cells may also contribute to speed the hematopoietic engraftment which still represents a major problem in CB transplantation.[3]

A variable proportion of common T cells contaminate our preparation of CIK cells at the end of the 21 days expansion. These T cells always contaminate the CIK preparations both from mouse and men and did not seem to influence the cytotoxic activity of CIK cells when purified nor play an active role in exacerbating graft-versus-host reactions in animal models and humans.[10,13,17] However, the naive origin of such cells, if derived from the CB, could potentially have a different immune reactivity *in vivo*, even if the CB has always been described as less alloreactive, contributing to the less severe GVHD observed.[1–3] Furthermore, in a recent experience with Il-2 expanded CB lymphocytes, there has been shown the maintenance of the T-cell receptor repertoire and the naive phenotype.[21] On the other hand, the *in vitro* amplification step may also contribute nonspecifically to a reduction in the alloreactivity as previously repeatedly reported [24–27] and recently confirmed.[28]

Overall, we propose the expansion of CIK cells under GMP conditions starting from the washouts of the CB unit bags used for the transplant as a possible cell therapy approach for the relapse of a CB transplanted leukemia patient.

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