Published in final edited form as:

Cytotherapy. 2015 November ; 17(11): 1582–1593. doi:10.1016/j.jcyt.2015.07.020.

A Novel Method to Expand Large Numbers of CD56⁺Natural Killer Cells from a Minute Fraction of Selectively Accessed Cryopreserved Cord Blood for Immunotherapy Post-transplantation

Sumithira Vasu¹, Maria Berg², Jan Davidson-Moncada³, Xin Tian⁴, Herb Cullis⁵, Richard W. Childs²

¹The Ohio State University, Division of Hematology, Columbus, OH 43210

²Hematology Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland, 20892

³Medical Oncology Branch, National Cancer Institute, Bethesda, MD 20892

⁴Office of Biostatistics Research, National Heart, Lung, and Blood Institute, Bethesda, MD 20892

⁵American Fluoroseal Corporation, Gaithersburg, MD

Abstract

Umbilical cord blood transplantation (UCBT) is increasingly used to treat acute leukemias. UCB-units are thawed and infused in their entirety at transplant, precluding later use as immunotherapy to prevent or treat leukemia relapse. We developed a device which selectively thaws only 1-ml of the UCB-unit leaving the remaining UCB-unit cryopreserved for subsequent transplantation. We also show that large numbers of CD56⁺NK cells can be expanded from these 1-ml fractions of selectively-accessed UCB. Immunomagnetic depletion of CD3⁺ cells of the 1-ml fraction was performed, and the cells were subsequently stimulated with irradiated Epstein-Barr virus-transformed lymphoblastoid cell line (EBV-LCLs) and set to culture in media containing IL-2. When a 1: 20 ratio of TNCs to EBV-LCL feeder cells was used, day 21 and day 35 NK cell cultures initiated from 1 ml of UCB contained a median of 430×10^6 (range: $44\text{--}4321 \times 10^6$) and 6092×10^6 (range: $165\text{--}20947 \times 10^6$) CD3[−]CD56⁺ NK cells. These cells expressed high levels of CD161, LFA-1, CD69, NKG2D, NKp30, NKp44, NKp80 and NKp46. UCB-derived NK cells were highly cytotoxic against K562 leukemia cells, although cytotoxicity was slightly lower than expanded PBMC-derived NK cells. In conclusion, we have developed and optimized a strategy to

Corresponding author: Richard Childs, MD, Clinical Director, National Heart, Lung, and Blood Institute (NHLBI), National Institutes of Health (NIH), Building 10, Room 3E-5330, 10 Center Drive, Bethesda, MD 20892, childsr@nhlbi.nih.gov.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Authorship Contributions

S.V and R.C devised the experiments, wrote the manuscript. S.V., M.B and R.C conducted the experiments. S.V, R.C and H.C developed the SACS device. X.T performed statistical analysis.

Conflict-of-interest disclosure: The authors declare no competing financial interests. A provisional patent application has been filed for the SACS apparatus.

selectively access a small fraction from cryopreserved UCB and show that large numbers of CD56⁺ cells can be expanded from this selectively-accessed fraction. This strategy presents a method to explore whether early adoptive transfer of NK cells expanded from the same UCB-unit used for transplantation can prevent leukemic relapse and decrease graft versus-host disease following UCBT.

Précis

Large numbers of cytotoxic CD56⁺NK cells can be expanded from 1ml of cord blood

Keywords

Natural killer cells; Umbilical Cord Blood; Cryopreserved Cord Blood; Selective Access; Ex-vivo Expansion; Expanded NK cells

Introduction

The number of umbilical cord blood (UCB) transplants being performed annually is increasing, accounting for approximately 11% of all unrelated donor hematopoietic stem cell transplants (HSCT)^{1,2}. The vast majority of these UCB transplants are performed for patients with relapsed leukemias, where transplantation represents the only treatment with curative potential. Unlike unrelated donor peripheral blood stem cell or bone marrow transplants, access to the original UCB donor and/or UCB allograft following transplantation is not feasible, since donors are de-identified and the cryopreserved cord blood unit (CBU) is infused in its entirety following thawing³. This precludes application of T-cell and natural killer-cell (NK) based cellular therapies utilized to prevent and/or treat disease relapse, graft-versus-host disease (GVHD) and infection after transplantation. Importantly, several lines of pre-clinical data suggest adoptive NK-cell infusions must be given early after transplant to simultaneously prevent GVHD and disease relapse by killing host antigen-presenting-cells and leukemic cells. Although a portion of the thawed CBU could be used to expand NK cells for this purpose at the time of transplantation, 3–6 weeks are typically required to expand large numbers of NK cells *in vitro*, which would preclude potential benefits of utilizing NK cells early after transplantation. Although some frozen CBU are partitioned to allow isolation of a fraction of the unit before thawing, these partitioned sections are large, typically comprising 20% of the cord unit which is more than would be necessary to expand large numbers of NK cells *ex vivo* for adoptive infusion. Further, utilizing this portion would reduce the number of cells available for transplantation by 20%, which could be deleterious to the patient, as the outcome of UCB transplantation improves proportionally to the number of total nucleated cells (TNCs) transplanted. In particular, CB transplants containing $< 2.5 \times 10^7$ TNCs/kg are associated with an unacceptably high mortality rate^{4,5}. Therefore, expanding NK cells from the 20% frozen fraction of the unit could only be used as an approach in adult patients who have a relatively large CBU where the remaining number of TNCs would remain above this critical threshold. Currently, no method exists that is capable of selectively accessing and/or partially thawing a small to minute portion of a frozen CBU without compromising the integrity, sterility, and viability of the unthawed portion. Such a methodology could facilitate adoptive cellular immunotherapy using NK cells expanded

from the same CBU used for transplantation aimed at potentiating graft-versus leukemia (GVL) effects and preventing GVHD.

To overcome this obstacle, we developed a device that provided selective access to cryopreserved samples (SACS device) to access a small portion of a cryopreserved CBU to expand NK cells *ex vivo*. Importantly, this device maintains the integrity, sterility and viability of the non-accessed portion of the CBU, which contains a sufficient number of viable TNCs to allow for its subsequent use as the primary graft source. Here we report on an optimized method that uses the SACS device to expand large numbers of highly cytotoxic NK cells *ex vivo* from a 1 ml “SACed” component of a CBU.

Materials and methods

Cord blood units

De-identified frozen 25 ml CBU were obtained from the Cord blood Transplantation registry (COBLT) of the National Heart, Lung and Blood Institute. These frozen units were stored in liquid nitrogen (LN₂) in bags from PALL Corporation which had two compartments: a 20ml compartment with 80% of the unit and a 5ml compartment with 20% of the unit. Both compartments were heat-sealed at the time of collection prior to cryopreservation and contained distinct sectioned portions comprising a single CBU.

SACS device

The SACS device consists of 2 thin heating elements that utilize pneumatic compression to rapidly seal off a portion of a frozen CBU (Figure 1). We used CBUs that were stored in two distinct, heat-sealed connected compartments as described above. Part A refers to the 80% portion of the CBU and Part B refers to the 20% portion of the CBU. At the time of retrieval of a cryopreserved CBU from LN₂, part A and part B was rapidly separated using scissors and part A was returned to LN₂ storage. Part B was then positioned in a cooled aluminum block holder molded to the contours of the 5ml component (the block was retrieved immediately prior to use from a LN₂ freezer) and placed between the jaws of the heating elements of the SACS device. Within 1–3 minutes, this computer-controlled device seals off an approximately 1-ml part of the CBU, referred to as part C. Immediately following partitioning of part B from part C, part B was returned in its frozen state to LN₂ storage. Parts B and A were subsequently removed from LN₂ 2 weeks later and were thawed and assessed for viability in comparison to part C. The 1-ml part C was subsequently thawed in a water bath at 37° C using procedures described in COBLT(<https://web.emmes.com/study/cord/sop.htm>)⁶. Part C was accessed in a sterile fashion through a needle attached to a syringe containing thawing media. We used 10 ml of dextran with 5% human serum albumin as thawing media for 1 ml of cells contained in Part C. Cells were gently mixed with the dextran/albumin solution by alternatively injecting and withdrawing thawing media into the syringe over 2–3 minutes. The contents of part C were then transferred into a 50-ml conical tube and were centrifuged at 880g for 20 minutes at 4°C. After removal of supernatant, the cell pellet was re-suspended in 5 ml of dextran/albumin for assessment of viability and cell count.

Processing of Part C

Part C was accessed and processed as described above. Once the cell count was assessed, immunomagnetic depletion of CD3⁺ cells was performed using the CliniMACS system (MiltenyiBiotec, Inc., Auburn, CA). Cells were centrifuged at 300g for 10 minutes. After removal of supernatant, the cell pellet was re-suspended in 80 µl of buffer per 10⁷ cells. 20µl of CD3 microbeads were added per 10⁷ total cells. After a 15-minute incubation at 4–8°C, cells were washed and centrifuged again at 300g for 10 minutes. After the supernatant was removed, the cell suspension was applied onto LS columns. Columns were washed and the total effluent was collected and cell count assessed using trypan blue. After CD3 depletion, some units underwent flow cytometry staining to quantitate CD3 and CD56 expression on cells contingent upon the availability of cells.

Culture of cell lines

The GMP-certified human EBV-transformed B-cell line EBV-LCL was obtained as a gift from the Fred Hutchinson Cancer Research Center (FHCRC, Seattle, WA, USA) and was supplied originally to FHCRC by the Beckman Research Institute of the City of Hope (Duarte, CA, USA) and maintained in the laboratory in RPMI-1640, 10% heat-inactivated human AB serum (Gemini Bio-Products, West Sacramento, CA), 2 mM GlutaMAX-1 and 15 mM HEPES (Invitrogen, Carlsbad, CA).

The human erythroid leukemia cell line K562 (ATCC, Manassas, VA) and human renal cell carcinoma cell lines (RCC) established in our laboratory were cultured in DMEM (Lonza, Walkersville, MD), 10% FBS (Quality Biological, Inc., Gaithersburg, MD), and 2 mM GlutaMAX-1.

Expansion of NK cells from UCB

Ten or twenty million 100Gy-irradiated and washed EBV-LCL feeder cells were co-cultured with 10⁶ CD3-depleted TNCs obtained from part C of the CBU in upright 75cm² tissue culture flasks in 15 ml of X-VIVO 20 (Lonza), supplemented with 10% heat-inactivated human AB serum (Gemini Bioproducts, West Sacramento, CA), 500 IU/ml rhIL-2 (50ng/mL, Tecin™, Hoffman-La Roche Inc., Nutley, NJ), and 2mM GlutaMAX-1 (Invitrogen, Carlsbad, CA) at 37°C and 6.5% CO₂. Half of the medium was replaced on culture days 5 and 8. Starting on day 10, NK cells were diluted to maintain cultures at a concentration of 0.6 × 10⁶ cells/mL, with growth medium containing IL-2 added every 24–72 hours for up to 40 days of cell culture. We also evaluated if the *ex vivo* expansion method utilized in this study could expand NK cells from a more primitive progenitor population contained within UCB that did not express CD16 or CD56. For these experiments, thawed UCB cells were sorted into CD3⁺CD14⁺CD20⁺CD16⁺CD56⁺CD34⁺ and CD3⁺CD14⁺CD20⁺CD16⁺CD56⁺CD34⁺ populations and these cells were set up in expansion cultures with irradiated EBV-LCLs using the same media and culture conditions as described for the CD3-depleted UCB fractions above.

Expansion kinetics assessment

Fold expansion of NK cells was calculated by dividing the number of CD56⁺ cells in cultures on days 14, 21 and 35 respectively by number of CD56⁺ cells placed in culture on

day 0. Cell viability was assessed by trypan blue staining except on days 0 and 14 when flow cytometry staining with 7-AAD was used.

Expansion of NK cells from peripheral blood mononuclear cells (PBMC)

Human NK cells were isolated from PBMC obtained from healthy adult volunteers. Depletion of CD3⁺T cells with subsequent selection of CD56⁺ cells was performed on a CliniMACS system. The CD56⁺ cells were then co-cultured with irradiated EBV-LCLs as described previously for cells obtained from UCB ^{7,8}.

Flow cytometry

The phenotype of freshly-isolated or day-14 expanded NK cells was assessed by flow cytometry on a FACSCalibur™ (BD Biosciences, San Jose, CA) with the following antihuman monoclonal antibodies (MAb) purchased from BD Biosciences Pharmingen (San Diego, CA): anti-CD56-allophycocyanin (APC) (clone-B159), anti-CD16-fluorescein isothiocyanate (FITC) (clone-3G8), anti-CD3-phycoerythrin (PE) (clone-UCHT1), anti-CD45-FITC; clone-2D1, anti-CD14-PE; clone-M5E2, anti-CD25-PE; clone-M-A251, anti-NKG2D-APC; clone-1D11, anti-CD11a/LFA-1-PE; clone-G43-25B, anti-FasL-biotin; clone-NOK-1, anti-CD34-FITC; clone-581, anti-CD226-PE (DNAM-1; clone-DX11), anti-CD161-PE; clone-DX12, anti-CD69-PE; clone-FN50, anti-CD336-PE (NKp44; clone-p44-8.1), anti-CD337-PE (NKp30; clone-p30-15), anti-CD335-PE (NKp46; clone-9E2), anti-perforin-FITC; clone-dG9 and anti-CD158b-PE (KIR2DL2/3; clone-CH-L); cell viability was determined by staining with Via-Probe™ (7AAD). Intracellular staining was performed on permeabilized cells fixed using BD Cytotfix/Cytoperm™. Anti-granzyme A-FITC; clone-CB9, anti-granzyme B-PE; clone-GB11, and anti-TRAIL-PE; clone-RIK-2 were purchased from Abcam Inc. (Cambridge, MA). Anti-NKG2A-APC (CD94/CD159a; clone-131411), Anti-NPK80-PE (clone-239127), anti-NKG2C-PE (CD94/CD159c; clone-134591) and anti-KIR2DL1-PE (clone-143211) were purchased from R&D Systems (Minneapolis, MN). Cells were stained with their corresponding isotype-matched control antibodies. CD45 staining excluded nucleated red blood cells (RBCs) and CD14 staining and side scatter were used to identify granulocytes, monocytes and lymphocytes.

Cytotoxicity assays

Standard ⁵¹Cr-release assays were performed as described previously with the following modifications⁷: after a 5-h incubation of NK cells with target-cells at various effector -to-target ratios, 25 µl of culture supernatants were transferred onto Luma plates (Perkin Elmer, Wellesley, MA) and analyzed using a MicroBeta scintillation counter (Perkin Elmer). Target cells were K562 leukemia cells or human renal cell carcinoma (RCC) cells that were either untreated or treated with 10 nM bortezomib for 18 hours prior to cytotoxicity assay to sensitize these tumor cells to TRAIL-mediated killing ⁷. RCC cells were seeded into 10 cm² tissue culture dishes in 12 mL culture medium; 24 hours later 10 nM bortezomib was added. After 16–18 hours, RCC cells were trypsinized, washed in DMEM and used in cytotoxicity assays.

Cytotoxicity assays were performed on all UCB-derived NK cells expanded *ex vivo* for 14 days and were compared to the cytotoxicity of day-14 expanded NK cells derived from adult PBMCs.

Statistical analysis

Descriptive statistics such as median, range, mean and standard error of mean (SEM) were presented. The logarithmic transformation was applied to the skewed data to normalize the distribution. Paired t-tests were used to compare the expansion kinetics of UCB-derived NK cells and to compare the cytotoxicity against different target cells for NK cells derived from UCB or adult PBMC. Welch's t-tests assuming unequal variances were used to compare the cytotoxicity assays of UCB-derived NK cells and NK cells derived from adult PBMCs. Analysis was performed using SAS 9.3 statistical software (SAS Institute Inc, Cary, NC) and a two-sided P-value <0.05 was considered statistically significant.

RESULTS

Isolation of 1 ml of cryopreserved cord blood for expansion of NK cells

The SACS apparatus was first optimized on PALL UCB bags containing frozen media alone. Thereafter, 10 CBU from different donors that had been cryopreserved in 25 ml PALL bags were utilized. The SACS apparatus successfully segmented and isolated a small 1mL fraction (part C) from part B in 10/10 CB units at a median 1 minute and 24 seconds (range 1 minute to 3 minutes and 5 seconds)(Figure 1). Part C was subsequently thawed and processed as described in the methods section. Cells obtained from part C were counted and assessed for viability, using the gating strategy shown in Supplementary Figure 1. The gating strategy shown in Supplementary Figure 1 was used only for freshly thawed cord blood units to account for the large number of nucleated red blood cells. Thawed C components contained a median 48×10^6 viable cells (range 25 to 93×10^6 cells). Flow cytometry performed upon thawing consistently showed 28–35% CD3⁺ cells and 2–10% CD56⁺ cells (data not shown). Following CD3 depletion of part C, cell counts ranged from 0.8×10^6 to 4×10^6 cells (median 1.8×10^6 cells).

Expansion kinetics of UCB-derived NK cells

Conditions to expand NK cells from a small cord blood volume were first optimized using 1 ml of UCB obtained from either the 5ml or 20ml fractions of a thawed CBU. Robust NK cell expansions were achieved with 1ml of UCB using either a 1:10 ratio or a 1:20 ratio of TNCs to feeder cells (Figure 2). Day 12–14 cell cultures derived from part C of the CBU consisted of a relatively pure population of CD3⁺CD56⁺NK cells, with the majority of these NK cells being double positive for CD16 and CD56. Of note, in contrast to NK cells expanded from adult PBMCs that stopped dividing by day 28, the majority of these NK cell cultures continued to expand for up to 40 days before proliferation ceased. When the 1:10 TNC/feeder cell ratio was used with a normalized starting CD3 depleted cell count of 1×10^6 cells, a median 99×10^6 (range: $3\text{--}151 \times 10^6$), 243.5×10^6 (range: $13\text{--}976 \times 10^6$), and 1839×10^6 (range: $110\text{--}44064 \times 10^6$) total CD56⁺ cells were obtained on days 14, 21, and 35 respectively (Table 1). When the EBV-LCL feeder ratio was changed to a TNC/feeder ratio of 1:20 with a similar starting population of 1×10^6 cells, a median 17.4×10^6 (range: 6.1–

135×10^6), 430×10^6 (range: $44\text{--}4321 \times 10^6$) and 6092×10^6 (range: $165\text{--}20947 \times 10^6$) total CD56⁺ cells were obtained on days 14, 21, and 35 respectively (Figure 2). The cells were harvested between days 35–42. Median fold expansion of CD3⁺CD56⁺ cells at harvest using the 1:10 ratio was 1839 (range: 110–44064), while the median fold expansion using the 1:20 ratio was 6092 (range: 165–20947); there were no statistically significant differences in expansion between the two ratios (Figure 2). When data from all 12 expansion cultures were combined (using either a 1:10 or 1:20 TNC/feeder cell culture ratio) and adjusted to a cell dose which would be delivered to a 70 kg recipient, the median number of NK cells obtained on days 14, 21, and 35 were 0.4×10^6 (range: $0.04 \times 10^6\text{--}2.2 \times 10^6$), 4.6×10^6 (range: $0.2 \times 10^6\text{--}61.7 \times 10^6$) and 28.9×10^6 (range: $1.6 \times 10^6\text{--}630 \times 10^6$) NK cells/kg respectively.

Phenotype of NK cells expanded from the 1 ml portion of CBU

Phenotypic data including percentage of CD3⁺, CD16⁺ and CD56⁺ cells on day 0 (immediately following CD3 depletion) and after a 14-day expansion using irradiated EBV-LCL feeder cells are shown in Figure 3A. The low cell numbers on day 0 precluded detailed phenotypic analysis of CD3 and CD56-positive cells; however two units that had a part C with adequate cells for flow cytometry showed CD3 content was 1–10% with CD56 < 1% (Figure 3A). Remarkably, this fraction of CD3 depleted cells which was used to initiate NK cell cultures typically contained very small percentages of CD56⁺ cells (Figure 3A). However, CD56⁺ cells expanded dramatically following co-culture with irradiated EBV-LCL feeder cells. By day 14, expansion cultures contained up to 89% CD56⁺CD3⁺NK cells (median: 84%, range 75–89%) with a smaller population of CD3⁺CD56⁺NK/T-like cells (median: 16%, range 11–25%).

UCB-expanded NK cells had similar expression of CD56, CD16, NKG2D, LFA-1, perforin, and granzymes A and B (Figure 3B) as typically observed with NK cells expanded from adult PBMC (Supplementary Figure 4). The number of CD56⁺ and/or CD16⁺NK cells obtained from thawed UCB post-immunomagnetic CD3 depletion was low, precluding a comparison of the phenotype of expanded vs. non-expanded UCB NK cells. UCB-expanded CD56⁺NK cells expressed high levels of activation markers such as CD161, NKG2D, LFA-1, TRAIL and DNAM-1. These cells also expressed high levels of Fas and CD94 with little to no expression of NKG2C. Further, these cells contained both KIR positive and negative populations indicating the maturation of these cells varied from immature to mature (Figure 3C).

Evaluation of the role of progenitor cells in expansion of NK cells

We observed substantial numbers of NK cells expanded from CD3-depleted UCB that contained only small percentages of either CD56⁺ and/or CD16⁺NK cells. Therefore, we next evaluated if the expansion method utilized here would also expand NK cells from an UCB population that did not express CD16 or CD56. Thawed UCB cells were sorted into CD3⁺CD14⁺CD20⁺CD16⁺CD56⁺CD34⁺ and CD3⁺CD14⁺CD20⁺CD16⁺CD56⁺CD34⁺ populations and set into expansion cultures with irradiated EBV-LCLs using the same media and culture conditions used to expand NK cells from CD3-depleted UCB fractions. Expression of CD16, CD56 and CD34 (Figure 4 A and B) on the flow sorted CD3⁺CD14⁺CD20⁺CD16⁺CD56⁺ populations is shown. Phenotypic analysis of cultures

harvested on day 14 showed a substantial proportion of cells originating from both the CD34⁺ fraction (Figure 4C) and the CD34⁻ fraction (Figure 4D) acquired CD56 expression and remained CD3 negative. By day 28, substantial numbers of CD3⁻/CD56⁺NK cells expanded from the cultured CD34⁺ fraction in contrast to the CD34⁻ fraction where only a small increase in cell numbers from this fraction was observed (Figure 4E).

These data suggest the culture conditions utilized in this study likely resulted in expansion of NK cells not only from a starting population that was CD56 and/or CD16 positive but also from CD34⁺ hematopoietic progenitor cells as well as minor contributions from an immature CD34⁻ progenitor population that had not yet acquired CD56 or CD16.

Cytotoxicity of UCB-derived NK cells in comparison to NK cells expanded from PBMC

NK cell cultures expanded from part C of the CBU had similar high levels of cytotoxicity against K562 leukemia cells as CD56⁺NK cells expanded from adult PBMC at an E: T ratio of 0.5:1 ($P=0.3$) (Figure 5). However at higher E: T ratios, cytotoxicity of PBMC derived NK cells was higher than UCB-derived NK cells ($P=0.01$ for E: T ratio of 1:1 and $P=0.06$ for E: T of 5:1). Cytotoxicity against K562 and RCC cells are shown in Figure 5. Unlike NK cells expanded from PBMC, NK cells expanded from UCB failed to show enhanced cytotoxicity against RCC tumor cells pre-treated with bortezomib compared to untreated RCC cells ($P=0.15$, Figure 5B). However, similar to NK cells expanded from PBMC, surface expression of TRAIL was high on expanded NK cells cultured for 12–14 days (Figure 3B). TRAIL expression gradually declined the longer cultures were maintained, with 92%, 63% and 20% of UCB-derived NK cells expressing TRAIL on days 13, 20, and 34 respectively (Supplementary Figure 2). TRAIL expression also was noted to decline in PBMC derived NK cells when comparing expression on day 14 vs day 22 (Supplementary Figure 3). Because PBMC derived NK cell cultures for clinical use are typically not maintained for more than 3 weeks, we did not evaluate TRAIL expression beyond day 22.

DISCUSSION

The use of UCB as an alternative graft source has increased the availability of potentially curative transplantation procedures for the >50% of patients with relapsed leukemias and other hematological malignancies who do not have an HLA-matched donor^{4,9}. Currently, a global network of UCB banks have an estimated inventory of 600,000 CBU and more than 20,000 units have been released to centers for transplantation of both adults and children with hematologic diseases¹⁰. As a graft source, UCB is unique due to its ease of availability from banked frozen inventories and due to less stringent requirements for HLA matching, as HLA mismatched CBU have a comparable incidence of GVHD as fully HLA matched unrelated donor HSCT^{1,11}. However, CBU contain on average one log fewer CD34⁺ cells compared to BM and PBSC, which results in delayed neutrophil recovery¹¹. Furthermore, once thawed, a CBU is used in its entirety with no opportunity for contacting the donor to donate additional cells such as lymphocytes or hematopoietic progenitor cells which could be used to prevent or treat leukemic relapse or viral infection. One alternative to overcome this limitation has been to utilize either NK cells or T cells from a third CBU for adoptive immunotherapy^{12,13}. However, the use of a 3rd CBU as immunotherapy in this context is

limited by HLA barriers which usually lead to their rejection and by prohibitive expense, given each CBU costs between \$40,000 to \$50,000 U.S. dollars. Here we present a strategy to efficiently expand *in vitro* NK cells from a minute selectively accessed portion of a CBU in advance of thawing the entire unit for transplantation¹³. The device utilized here to access 1ml of UCB to expand NK cells without thawing 96% of the cord unit could also be used to access any desired cellular population for *in vitro* expansion including T cells, antigen presenting cells, and hematopoietic progenitor cells.

NK cells comprise approximately 15% of lymphocytes and do not require prior antigen exposure to mediate cytotoxicity^{14,15}. Human NK cells are characteristically CD3⁻ and CD56⁺ and/or CD16⁺. NK cells also express inhibitory receptors such as NKG2A, LIRs, and KIR and exert cytotoxicity based on recognition of “lack-of-self”. Recent data indicate that acute myelogenous leukemia (AML) cells are exquisitely sensitive to killing by allo-reactive NK cells; in the context of haplo-identical transplantation, KIR incompatible NK cells have been shown to mediate potent anti-tumor effects against AML which prevents leukemic relapse while simultaneously reducing the incidence of GVHD^{16–18}. A number of studies are ongoing to determine the best strategy to harness NK-mediated GVL effects against AML ([Clinicaltrials.gov](https://clinicaltrials.gov) NCT01823198, NCT01593670, and NCT02316964)^{16,19–23}. One option being explored is the expansion and infusion of NK cells post-transplant from the same donor used to donate hematopoietic stem cells to bolster antitumor effects against AML and prevent GVHD^{16, 19–23}²⁴. Preclinical studies suggest these NK-cell infusions must be given early after transplant to optimize their ability to prevent GVHD by killing host APC and/or donor alloreactive T cells and to mediate anti-leukemic effects^{25,26}. In this report, we demonstrate a method to expand large numbers of CD3⁻ CD56⁺ NK cells from a minute quantity of UCB using the SACS apparatus and EBV-LCL feeder cells. The approach outlined here provides a strategy that would make these NK cells readily available for infusion at the time the cord unit was thawed for transplantation. Expanded UCB-derived NK cells had high surface expression of both NKG2D and TRAIL and were highly cytotoxic against K562 leukemia cells, with levels comparable to those of NK cells expanded from adult PBMC²⁷.

The isolation and subsequent expansion of desired cellular populations from a small fraction of cryopreserved cell source poses special challenges. Given the small size and limited cellular content of CB units, we sought to develop a highly efficient method to expand CD56⁺ cell populations from a very small quantity of UCB. The SACS device successfully isolated a small portion from a CBU without compromising the sterility or integrity of the remaining non-accessed portion which was maintained in its frozen state. The isolated fraction of UCB, despite its small volume of only 1 ml, had sufficient viability and contained an adequate number of cells to expand up to 28.9×10^6 NK cells/kg (assuming a recipient weight of 70kg). The methodology presented here offers the first viable strategy to expand large numbers of clinical grade NK cells *ex vivo* for adoptive immunotherapy from a minute quantity of UCB in advance of thawing the CBU that will ultimately be used for transplantation.

We noted a significant loss of TNC when we CD3 depleted the selectively accessed cord fraction. Since UCB units typically have about 30% of T cells, the decline in TNC from $48 \times$

10^6 to 1.8×10^6 post CD3 depletion cannot be explained by T-cell depletion alone. We attributed the loss of TNC to predominant loss of neutrophils and nucleated red blood cells during the process of immunomagnetic separation.

Besides NK cells, we have also used the SACS device to isolate small amounts of cord blood to expand ex vivo CD34⁺ progenitor cells and CD3⁺ T cells (unpublished data)²⁸. Hematopoietic progenitors, expanded from these SAC'd portions of cord blood could be used to expedite neutrophil recovery and prevent graft rejection while expanded T cells and NK cells could be used to prevent or treat infection or leukemia relapse.

Over the past five years, several groups have shown the feasibility of expanding NK cells *ex vivo* from thawed CBU^{29–33}. However, approaches used by most investigators have required the entire CBU be used to expand sufficient NK cell numbers. Spanholtz et al used a cytokine based culture system with SCF, IL-15, IL-2, and IL-7 starting with CD34⁺ cells selected from a thawed CBU. They observed more than a 3-log NK-cell expansion from frozen CD34⁺ UCB cells and could reproducibly generate 1.5×10^9 NK cells from the entire CBU after a period of 5 weeks in culture³⁴. Of note, following optimization of our expansion method where a 1:20 ratio of TNCs: EBV-LCL feeders was utilized, we achieved similar NK-cell numbers, with a median 3.8×10^9 NK cells being expanded by day 35. However, in contrast to Spanholtz et al, these NK cell numbers were achieved by expanding from only 1ml (approximately 4%) of the cord unit. Although our technique reliably expanded large numbers of NK cells, we did note significant variability in the final NK cell numbers that could be expanded between units, with some units having exceptionally robust expansions as high as 44,064 fold. Whether variability in HLA/KIR matching vs mismatching between the donor NK cell source and the EBV-LCL line used to expand NK cells may have had impacted the number of NK cells that could be expanded ex vivo is unknown as we did not perform HLA and KIR typing on the cord units used in these experiments. Further, baseline numbers of CD56⁺ and/or CD34⁺ cells in expansion cultures, which were not accessed in our experiments, could also have impacted NK cell expansion kinetics between cord units.

Ayello et al expanded NK cells from an entire CBU where thawed cells were incubated overnight to deplete adherent monocytes. The non-adherent cells were cultured with an antibody/cytokine combination resulting in a four-fold increase in CD3⁺CD16⁺CD56⁺ cells with a median 68×10^6 cells being available at harvest^{35,36}, although these cells had substantial phenotypic and functional heterogeneity³⁷. Culture of resting CD56⁺CD16⁺ cells with IL-2 or IL-15 has been shown to result in CD56 acquisition, suggesting these starting populations represent NK-cell progenitors³⁸. These observations, in the context of our study showing large numbers of CD56⁺CD3⁺NK cells could be expanded from mostly CD56 negative/CD16 negative cells, led us to hypothesize that the culture conditions utilized in this study likely drove CD34⁺ progenitor cells to differentiate and expand into CD56⁺NK cells. Subsequently, we conducted further experiments that showed large numbers of CD3⁺/CD56⁺NK cells could be expanded from CD3⁺CD14⁺CD20⁺CD16⁺CD56⁺CD34⁺ cells, and to a lesser degree CD3⁺CD14⁺CD20⁺CD16⁺CD56⁺CD34⁺ cells, confirming this hypothesis.

Luevano showed that higher NK cell numbers can be derived using frozen UCB CD34⁺ cells³². These expanded NK cells did not express KIR and only contained about 21% CD16⁺ and CD56⁺ cells, suggesting incomplete maturation and hence had not acquired KIR receptors^{39,40}. In contrast to Luevano, we expanded NK cells from a CD3 negative starting population of UCB with expanded NK cells containing more than 75% CD16⁺/CD56⁺ cells and also expressing KIR receptors. Differences in the starting population and in culture methods used to expand cells likely account for why more phenotypically mature NK cells were expanded using our system.

Several groups have investigated the functional properties of expanded UCB NK cell populations^{41,42}. Prior studies have shown that UCB derived CD56⁺ cells have low cytotoxic activity compared to their peripheral blood counterparts⁴³, however their function can be augmented by IL-2 or IL-15. Xing et al showed that culturing UCB cells in IL-2 could reverse their impaired lytic immunological synapse formation⁴⁴. The ability of cytokines to modify low baseline cytotoxic function of both UCB NK cells and T cells has also been demonstrated by others^{45–47}. Consistent with this observation, we observed that NK cells expanded from UCB using EBV-LCL feeder cells and IL-2 containing media were highly cytotoxic against K562 leukemia targets, comparable to that observed with NK cells expanded from adult PBMC⁷.

Previously, we reported that irradiated EBV-LCL cells and IL-2 containing media could be used under GMP conditions to expand NK cells from PBMC for adoptive infusion in humans with cancer⁸. To avoid T-cell contamination, we have utilized a starting population of PBMC that are CD3 depleted and CD56 selected. Data from the current study have established that it would be impractical to attempt to expand NK cells from a similar population of UCB since only a minority of CB TNCs are CD56⁺^{7,8}. Further, CD56 selection would remove progenitor cell populations such as CD34⁺ cells, which we have shown in this study contribute to the overall number of CD56⁺NK cells expanded from CD3 depleted CB utilizing the current expansion culture conditions. Unlike other methods which utilized the entire cord blood unit for NK expansion, we were not able to do detailed phenotypic analysis on all one ml fractions prior to culture. The CD56 subset appeared to be less than 1% at the start of the culture, and we did see robust expansion of a pure population of NK cells after day 14. Other groups have reported on expansion of NK cells. Shah et al reported 1848-fold expansion from fresh and 2389-fold expansion from cryopreserved CB with >95% purity for NK cells (CD56⁺/CD3⁻ and less than 1% CD3⁺ cells using an artificial antigen presenting cell based culture system after 14 days⁴⁸. Satwani et al showed the effects of short-term culture with anti-CD3 + IL-2 + FLT-3 + IL-15 on cord blood (CB) NK cell and NK-cell subset expansion and function. Along with significant increase in NK cells, they also demonstrated up to 46 fold expansion of NK cells bearing KIR receptors. They also noted a significant increase in both NK and lymphokine-activated killer (LAK) cytotoxicity with IL-2 + anti-CD3 + FLT-3 + IL-15 (100ng/mL)⁴⁹.

NK cells expanded using the current strategy contained a relatively pure population of NK cells that had minimal T-cell contamination. Deriving a population of CD56⁺ cells which are devoid of CD3 cells is important in the context of using these cells in a clinical transplant trial, as contaminating T cells could potentially induce GVHD.

Potential limitations of the study include the variability in expansion kinetics observed between CBUs and an observed decline in NK-cell TRAIL expression which occurred with prolonged duration in culture. Also, significant heterogeneity exists in methods to freeze UCB, which could significantly impact post-thaw viability. The SACS device was used only on units that had a 20ml fraction sealed with a 5 ml fraction (commonly called 80-20 units); there are some units that were cryopreserved earlier in the course of cord blood banking which do not have a separation into 20 ml and 5 ml fractions. While the SACS device has the capability to selectively thaw units without a physical separation, formal testing of the device on these types of units needs to be conducted to ensure a more global reproducibility of the application of the SACS device on the majority of cord units housed in public banks.

In summary, we show that by selectively accessing a small 1ml portion of CB, we could expand large numbers of highly cytotoxic CD3⁻CD56⁺NK cells using EBV-LCL feeder cells. The findings presented in this study describe a novel method for large-scale expansions of GMP-grade NK cells from a minute fraction of UCB. This methodology potentially could be used to facilitate an investigational transplant approach in which a single CBU is transplanted in conjunction with an infusion of large numbers of expanded CD56⁺NK cells from the same CBU to prevent GVHD and leukemia relapse.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Division of Intramural Research (DIR) of the National Heart, Lung, and Blood Institute (NHLBI), National Institutes of health (NIH).

References

1. Barker JN, et al. Searching for unrelated donor hematopoietic stem cells: availability and speed of umbilical cord blood versus bone marrow. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation*. 2002; 8:257–260. [PubMed: 12064362]
2. Barker JN, et al. Transplantation of 2 partially HLA-matched umbilical cord blood units to enhance engraftment in adults with hematologic malignancy. *Blood*. 2005; 105:1343–1347. [PubMed: 15466923]
3. Wagner JE, et al. Transplantation of unrelated donor umbilical cord blood in 102 patients with malignant and nonmalignant diseases: influence of CD34 cell dose and HLA disparity on treatment-related mortality and survival. *Blood*. 2002; 100:1611–1618. [PubMed: 12176879]
4. Barker JN, Byam C, Scaradavou A. How I treat: the selection and acquisition of unrelated cord blood grafts. *Blood*. 2011; 117:2332–2339. [PubMed: 21149636]
5. Barker JN, Scaradavou A, Stevens CE. Combined effect of total nucleated cell dose and HLA match on transplantation outcome in 1061 cord blood recipients with hematologic malignancies. *Blood*. 2010; 115:1843–1849. [PubMed: 20029048]
6. Kurtzberg J, et al. Results of the cord blood transplantation (COBLT) study unrelated donor banking program. *Transfusion*. 2005; 45:842–855. [PubMed: 15934981]
7. Berg M, et al. Clinical-grade ex vivo-expanded human natural killer cells up-regulate activating receptors and death receptor ligands and have enhanced cytolytic activity against tumor cells. *Cytotherapy*. 2009; 11:341–355. [PubMed: 19308771]

8. Childs RW, Berg M. Bringing natural killer cells to the clinic: ex vivo manipulation. *Hematology / the Education Program of the American Society of Hematology. American Society of Hematology. Education Program.* 2013; 2013:234–246.
9. Barker JN, et al. Availability of cord blood extends allogeneic hematopoietic stem cell transplant access to racial and ethnic minorities. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation.* 2010; 16:1541–1548. [PubMed: 20800103]
10. Gluckman E, et al. Milestones in umbilical cord blood transplantation. *British journal of haematology.* 2011; 154:441–447. [PubMed: 21726206]
11. Laughlin MJ, et al. Outcomes after transplantation of cord blood or bone marrow from unrelated donors in adults with leukemia. *The New England journal of medicine.* 2004; 351:2265–2275. [PubMed: 15564543]
12. Brunstein CG, et al. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood.* 2011; 117:1061–1070. [PubMed: 20952687]
13. Hanley PJ, et al. Functionally active virus-specific T cells that target CMV, adenovirus, and EBV can be expanded from naive T-cell populations in cord blood and will target a range of viral epitopes. *Blood.* 2009; 114:1958–1967. [PubMed: 19443656]
14. Farag SS, Fehniger T, Ruggeri L, Velardi A, Caligiuri MA. Natural killer cells: biology and application in stem-cell transplantation. *Cytotherapy.* 2002; 4:445–446. [PubMed: 12473220]
15. Farag SS, Fehniger TA, Ruggeri L, Velardi A, Caligiuri MA. Natural killer cell receptors: new biology and insights into the graft-versus-leukemia effect. *Blood.* 2002; 100:1935–1947. [PubMed: 12200350]
16. Ruggeri L, et al. Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. *Blood.* 1999; 94:333–339. [PubMed: 10381530]
17. Velardi A, Ruggeri L, Alessandro Moretta, Moretta L. NK cells: a lesson from mismatched hematopoietic transplantation. *Trends in immunology.* 2002; 23:438–444. [PubMed: 12200065]
18. Venstrom JM, et al. HLA-C-dependent prevention of leukemia relapse by donor activating KIR2DS1. *The New England journal of medicine.* 2012; 367:805–816. [PubMed: 22931314]
19. De Angelis C, et al. Expansion of CD56-negative, CD16-positive, KIR-expressing natural killer cells after T cell-depleted haploidentical hematopoietic stem cell transplantation. *Acta haematologica.* 2011; 126:13–20. [PubMed: 21411985]
20. Ruggeri L, Capanni M, Mancusi A, Martelli MF, Velardi A. The impact of donor natural killer cell alloreactivity on allogeneic hematopoietic transplantation. *Transplant immunology.* 2005; 14:203–206. [PubMed: 15982564]
21. Ruggeri L, et al. Natural killer cell alloreactivity in haploidentical hematopoietic stem cell transplantation. *International journal of hematology.* 2005; 81:13–17. [PubMed: 15717682]
22. Ruggeri L, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science.* 2002; 295:2097–2100. [PubMed: 11896281]
23. Ruggeri L, et al. Natural killer cell recognition of missing self and haploidentical hematopoietic transplantation. *Seminars in cancer biology.* 2006; 16:404–411. [PubMed: 16916611]
24. Curti A, et al. Successful transfer of alloreactive haploidentical KIR ligand-mismatched natural killer cells after infusion in elderly high-risk acute myeloid leukemia patients. *Blood.* 2011
25. Olson JA, et al. NK cells mediate reduction of GVHD by inhibiting activated, alloreactive T cells while retaining GVT effects. *Blood.* 2010; 115:4293–4301. [PubMed: 20233969]
26. Farag SS, et al. High-dose busulfan, cyclophosphamide, and etoposide does not improve outcome of allogeneic stem cell transplantation compared to BuCy2 in patients with acute myeloid leukemia. *Bone marrow transplantation.* 2005; 35:653–661. [PubMed: 15711571]
27. Lundqvist A, et al. Bortezomib and depsipeptide sensitize tumors to tumor necrosis factor-related apoptosis-inducing ligand: a novel method to potentiate natural killer cell tumor cytotoxicity. *Cancer research.* 2006; 66:7317–7325. [PubMed: 16849582]
28. Rebecca Lopez CZ, Sumithira Vasu, Herb Cullis, Richard Childs. A Novel Method to Expand Viral Reactive CTL From a Selectively Accessed Portion of a Cryopreserved Cord Unit to Prevent Viral

- Infections After Transplantation. American Society of Hematology Annual Meeting, Vol. Blood. 2010; 116:958a.
29. Lehmann D, et al. Ex vivo generated natural killer cells acquire typical natural killer receptors and display a cytotoxic gene expression profile similar to peripheral blood natural killer cells. *Stem cells and development*. 2012; 21:2926–2938. [PubMed: 22571679]
 30. Spanholtz J, et al. Clinical-grade generation of active NK cells from cord blood hematopoietic progenitor cells for immunotherapy using a closed-system culture process. *PloS one*. 2011; 6:e20740. [PubMed: 21698239]
 31. Luevano M, et al. The unique profile of cord blood natural killer cells balances incomplete maturation and effective killing function upon activation. *Human immunology*. 2011
 32. Luevano M, et al. Frozen cord blood hematopoietic stem cells differentiate into higher numbers of functional natural killer cells in vitro than mobilized hematopoietic stem cells or freshly isolated cord blood hematopoietic stem cells. *PloS one*. 2014; 9:e87086. [PubMed: 24489840]
 33. Luevano M, Madrigal A, Saudemont A. Generation of natural killer cells from hematopoietic stem cells in vitro for immunotherapy. *Cellular & molecular immunology*. 2012; 9:310–320. [PubMed: 22705914]
 34. Spanholtz J, et al. High log-scale expansion of functional human natural killer cells from umbilical cord blood CD34-positive cells for adoptive cancer immunotherapy. *PloS one*. 2010; 5:e9221. [PubMed: 20169160]
 35. Ayello J, et al. Characterization of natural killer and natural killer-like T cells derived from ex vivo expanded and activated cord blood mononuclear cells: implications for adoptive cellular immunotherapy. *Experimental hematology*. 2009; 37:1216–1229. [PubMed: 19638292]
 36. Ayello J, et al. Characterization of cord blood natural killer and lymphokine activated killer lymphocytes following ex vivo cellular engineering. *Biology of blood and marrow transplantation: journal of the American Society for Blood and Marrow Transplantation*. 2006; 12:608–622. [PubMed: 16737934]
 37. Verneris MR, Miller JS. The phenotypic and functional characteristics of umbilical cord blood and peripheral blood natural killer cells. *British journal of haematology*. 2009; 147:185–191. [PubMed: 19796267]
 38. Gaddy J, Broxmeyer HE. Cord blood CD16+56– cells with low lytic activity are possible precursors of mature natural killer cells. *Cellular immunology*. 1997; 180:132–142. [PubMed: 9341743]
 39. Grzywacz B, Kataria N, Blazar BR, Miller JS, Verneris MR. Natural killer-cell differentiation by myeloid progenitors. *Blood*. 2011; 117:3548–3558. [PubMed: 21173117]
 40. Grzywacz B, et al. Coordinated acquisition of inhibitory and activating receptors and functional properties by developing human natural killer cells. *Blood*. 2006; 108:3824–3833. [PubMed: 16902150]
 41. Eissens DN, et al. Selective expansion of human natural killer cells leads to enhanced alloreactivity. *Cellular & molecular immunology*. 2014; 11:160–168. [PubMed: 24240123]
 42. Lin SJ, Yang MH, Chao HC, Kuo ML, Huang JL. Effect of interleukin-15 and Flt3-ligand on natural killer cell expansion and activation: umbilical cord vs. adult peripheral blood mononuclear cells. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology*. 2000; 11:168–174. [PubMed: 10981526]
 43. Dalle JH, et al. Characterization of cord blood natural killer cells: implications for transplantation and neonatal infections. *Pediatric research*. 2005; 57:649–655. [PubMed: 15718362]
 44. Xing D, et al. Cord blood natural killer cells exhibit impaired lytic immunological synapse formation that is reversed with IL-2 ex vivo expansion. *J Immunother*. 2010; 33:684–696. [PubMed: 20664358]
 45. Lanier LL. Natural killer cell receptor signaling. *Current opinion in immunology*. 2003; 15:308–314. [PubMed: 12787756]
 46. Lin SJ, Chao HC, Kuo ML. The effect of interleukin-12 and interleukin-15 on CD69 expression of T-lymphocytes and natural killer cells from umbilical cord blood. *Biology of the neonate*. 2000; 78:181–185. [PubMed: 11044766]

47. Lin SJ, Kuo ML. Cytotoxic function of umbilical cord blood natural killer cells: relevance to adoptive immunotherapy. *Pediatric hematology and oncology*. 2011; 28:640–646. [PubMed: 21970456]
48. Shah N, et al. Antigen presenting cell-mediated expansion of human umbilical cord blood yields log-scale expansion of natural killer cells with anti-myeloma activity. *PloS one*. 2013; 8:e76781. [PubMed: 24204673]
49. Satwani P, et al. Interleukin (IL)-15 in combination with IL-2, fms-like tyrosine kinase-3 ligand and anti-CD3 significantly enhances umbilical cord blood natural killer (NK) cell and NK-cell subset expansion and NK function. *Cytotherapy*. 2011; 13:730–738. [PubMed: 21413839]

Figure 1.
Manipulation of an umbilical cord blood unit.

Figure 2.

Fold expansion of cord blood-derived CD3⁻/CD56⁺ cells relative to the starting number of CD3⁻/CD56⁺ NK cells placed in culture. NK cells were expanded using irradiated EBV-LCL feeder cells at TNC: LCL ratios of 1:10 and 1:20. Data for 6 units are displayed with box-and whisker plots. The shaded box was drawn from the lower quartile to the upper quartile, with the median and mean indicated by a middle bar and a dot, respectively. The whiskers extended out from the box to the smallest and the largest data points.

A

Phenotype of cord blood on day 0 after CD3⁺ depletion Phenotype of day-14 expanded CD56⁺ cells

B.

Figure 3.

Phenotype of NK cells expanded from a 1-ml fraction of CD3-depleted UCB. (A). Phenotype of cord blood cells on day 0 (top panel) and 14 days (bottom panel) after in vitro NK-cell expansion using irradiated EBV-LCL feeder cells. (B). Phenotype of CD3⁻/CD56⁺ day-12 expanded NK cells. Shaded histograms represent isotype controls; open histograms represent expression of proteins in viable cells. (C). KIR receptor profile of CD3⁻/CD56⁺ day-12 expanded NK cells.

Figure 4.

Phenotype and expansion rate of UCB cells sub-populations. UCB cells were thawed, subjected to a density gradient centrifugation, and flow-sorted into CD3⁻CD14⁻CD20⁻CD16⁻CD56⁻CD34⁺ or CD3⁻CD14⁻CD20⁻CD16⁻CD56⁻CD34⁻ populations. Expression of CD16 and CD56 (A) and CD34 (B) on CD3⁻CD14⁻CD20⁻CD16⁻CD56⁻CD34⁻ populations is shown. Following flow-sorting, CD34⁺-positive and CD34⁻-negative cells were cultured with irradiated EBV-LCL and IL-2 containing media. Phenotypic analysis of cultures originating from the CD34⁺ fraction (C) and from the CD34⁻ fraction (D) harvested on day 14. (E). Fold expansion of NK cells originating from CD34⁻-negative and CD34⁺-positive fractions.

Figure 5.

Cytotoxicity of day-14 expanded NK cells derived from normal adult PBMC (A) compared to the cytotoxicity of day-14 expanded cord blood NK cells (B). Target cells were K562 leukemia cells or human renal cell carcinoma cells that were untreated or treated with 10 nM bortezomib for 18 hours prior to the cytotoxicity assay. Three Effector: target (E: T) ratios of 0.5:1, 1:1 and 5:1 were used. (C) Cytotoxicity of NK cells derived from normal adult PBMC

or cord blood against K562 target cells. The mean $\pm$ SEM based on cells from 3 adult PMBC donors and from 4 different cord blood units are shown in (A)–(C).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1
Expansion of cord blood-derived CD56⁺ cells from Part C of the cord blood unit

Expansion of cord blood-derived NK cells from Part C of the cord blood unit. NK-cell purity represents the percent of NK cells that were CD3 negative and CD56 and/or CD16 positive.

Unit Identifier	Ratio of TNC:LCL	TNCs - Day 0 ($\times 10^6$)	CD3 ⁺ /CD56 ⁺ cells at day 14 ($\times 10^6$)	CD3 ⁺ /CD56 ⁺ cells at day 21 ($\times 10^6$)	CD3 ⁺ /CD56 ⁺ cells at harvest Day 35-42 ($\times 10^6$)	NK cell purity
W159300034350	1:10	1	150.6	975.6	44064	94%
	1:20	1	16.9	295	998	98%
W159300020009	1:10	1	8.8	39.2	265	90%
	1:20	1	34.6	564.6	20947	92%
W159300072020	1:10	1	3	13	2801	88%
	1:20	1	11	44	1239	84%
W159300060006	1:10	1	123	146	110	79%
	1:20	1	18	212	165	82%
W159300041989	1:10	1	121	709.4	14214	92%
	1:20	1	6	710.6	18829	90%
W15930012711	1:10	1	77	341	877	96%
	1:20	1	135	4321	10946	97%
Median	1:10 (n=6)	1	99.1	244	1839	91%
	1:20 (n=6)	1	17.4	430	6092	91%
P value to compare paired data between the two ratios						P=0.62
			P=0.45	P=0.19	P=0.68	