Ex Vivo-expanded Natural Killer Cells Derived From Long-term Cryopreserved Cord Blood are Cytotoxic Against Primary Breast Cancer Cells

Tina Nham, Sophie M. Poznanski,* Isabella Y. Fan,* Fatemeh Vahedi,* Mira M. Shenouda,* Amanda J. Lee,* Marianne V. Chew,* Richard T. Hogg,* Dean A. Lee,† and Ali A. Ashkar**

Summary: With over 600,000 units of umbilical cord blood (CB) stored on a global scale, it is important to elucidate the therapeutic abilities of this cryopreserved reservoir. In the advancing field of natural killer (NK) cell cancer immunotherapy, CB has proven to be a promising and noninvasive source of therapeutic NK cells. Although studies have proven the clinical efficacy of using long-term cryopreserved CB in the context of hematopoietic stem cell transplantations, little is known about its use for the ex vivo expansion of effector immune cells. Therefore, our group sought to derive ex vivo-expanded NK cells from long-term cryopreserved CB, using an artificial antigen presenting cell–mediated expansion technique. We compared the expansion potential and antitumor effector function of CB-derived NK (CB-NK) cells expanded from fresh (n = 4), short-term cryopreserved (< 1-year old, n = 5), and long-term cryopreserved (1–10-year old, n = 5) CB. Here, we demonstrated it is possible to obtain an exponential amount of expanded CB-NK cells from long-term cryopreserved CB. Ex vivo-expanded CB-NK cells had an increased surface expression of activating markers and showed potent antitumor function by producing robust levels of proinflammatory cytokines, interferon-γ, and tumor necrosis factor-α. Moreover, expanded CB-NK cells (n = 3–5) demonstrated cytotoxicity towards primary breast cancer cells (n = 2) derived from a triple-negative breast cancer and an estrogen receptor-positive/progesterone receptor-positive breast cancer patient. Long-term cryopreservation had no effect on the expansion potential or effector function of expanded CB-NK cells. Therefore, we propose that long-term cryopreserved CB remains clinically useful for the ex vivo expansion of therapeutic NK cells.

KeyWords: natural killer cells, ex vivo expansion, long-term cryopreserved, umbilical cord blood, breast cancer immunotherapy

(*J Immunother* 2017;00:000–000)

The first umbilical cord blood (CB) transplantation occurred over 25 years ago and since then, the field of CB banking and therapy has grown tremendously.[1,2] In more recent years, countries like Canada have recognized the need for a national effort in public CB banking.[3] In 2013, The Canadian Blood Services established The National Public Cord Blood Bank, an organization that strives to become a reservoir for a diverse ethnic cross-section of HLA-typed CB units.[4,5] To date, over 600,000 CB units are stored globally, and amidst the rise of CB banking systems, this number is projected to increase.[1] With the recent surge of knowledge in CB cryopreservation, CB cell subpopulations and ex vivo culture technologies, it is important to explore new avenues on how we can utilize cryopreserved CB to its full potential as a therapeutic product.

In the rapidly evolving field of natural killer (NK) cell cancer immunotherapy, CB was discovered to be a promising source for the ex vivo expansion of therapeutic antitumor effector NK cells.[6–12] The ability to obtain vast quantities of lymphocyte effector cells from CB expanded its clinical potential, beyond that of hematopoietic stem cell (HSC) transplantations, for the treatment of hematological diseases. For example, therapeutic, ex vivo-expanded NK cells can be used for adoptive cellular immunotherapy, which can be clinically applied to treat both hematological and solid malignancies.[9] In fact, adoptive cellular immunotherapy is a promising and emerging field that may hold valuable potential for diseases that have poor prognoses, even in light of currently available chemotherapeutic regimens. This includes cancers like triple-negative breast cancer (TNBC), an aggressive type of breast cancer that is estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and human epidermal growth factor receptor 2-negative.[13,14] As TNBCs do not respond to therapies like hormonal-based or trastuzumab-based treatments, which have prolonged disease-free survival for hormone receptor-positive breast cancers, chemotherapy is the treatment of choice. Therefore, it could be important to look at the potential of alternative, immune-based regimens for TNBC, which has few effective treatment options available.[13,14]

Previous studies using alternative expansion protocols have shown limitations to using cryopreserved CB as a source for NK cell expansion, which include low NK cell number and an immature or naïve NK cell phenotype.[7,8,15,16] Recently, however, several types of quick and robust ex vivo culture techniques have been used to overcome these constraints, to generate clinically relevant quantities of mature and activated CB-derived NK (CB-NK) cells in a matter of weeks.[11,12,17] These protocols often involve culturing CB-derived mononuclear cell populations in the presence of IL-2 and may include additional factors, such as IL-15,[18] FLT3,[19] or artificial antigen presenting cells (aAPCS),[12] to further stimulate NK cell growth.[8] After 2–4 weeks, the ex vivo expansion process stimulates growth and activates the cytotoxic profile of NK cells, to yield vast amounts of mature and activated therapeutic effector lymphocytes. NK cell effector function can be further enhanced ex vivo, through stimulation with cytokines such as IL-2, IL-12, IL-15, and IL-18.[9,20–23] The therapeutic cells are

Received for publication July 5, 2017; accepted September 22, 2017.
From the *Department of Pathology and Molecular Medicine, McMaster Immunology Research Centre, McMaster University, Hamilton, ON, Canada; and †Department of Hematology and Oncology, Nationwide Children's Hospital, Columbus, OH.
Reprints: Ali A. Ashkar, McMaster Immunology Research Centre, Rm4015 Michael DeGroote Centre for Learning & Discovery, 1280 Main Street West, Hamilton, ON, Canada L8S 4K1. E-mail: ashkara@mcmaster.ca.

then transferred into autologous or allogeneic cancer patients, where they exert their antitumor functions.[9,24]

In public CB banks, donated CB units are stored indefinitely for possible clinical use.[25] The ability to cryopreserve and store CB for long periods of time creates an appealing therapeutic tool for several reasons. Cryopreserved CB represents a noninvasive, allogeneic, readily available and long-lasting source of valuable hematopoietic cell populations.[2,26–29] For example, hematopoietic progenitor cells were recovered from a 23.5-year old cryopreserved CB units and these units also displayed effective engraftment into immunodeficient mice.[2] It was also demonstrated that hematopoietic progenitor recovery from short-term cryopreserved CB (2–8 wk) was comparable with that of long-term cryopreserved CB (10–15 y).[28,29] Moreover, Mitchell et al[30] findings also showed that a longer period of CB cryopreservation had no effect on clinical outcomes.

Most studies that have evaluated the capacities of long-term cryopreserved CB however, have done so in the context of hematopoietic progenitor cell recovery for HSC transplantations.[2,28,29,31] Less is known about the feasibility of using long-term cryopreserved CB for the ex vivo expansion of alternate, valuable cell types, like therapeutic effector CB-NK cells, for adoptive cellular transfer. To garner a better understanding of long-term cryopreserved CB and its potential for ex vivo cell expansion strategies, we compared the expansion potential, phenotype, and cytotoxic effector function of expanded CB-NK cells derived from fresh, short-term cryopreserved (< 1-year old), and long-term cryopreserved (1–10-year old) CB. Moreover, we evaluated CB-NK cell effector function against breast cancer by examining its cytotoxicity against a TNBC cell line, MDA-MB-231, and against primary breast cancer cells derived from TNBC and ER^+/PR^+ breast cancer donors.

MATERIALS AND METHODS

Ethics Statement

Research involving human samples and CB collection was approved by the Hamilton Integrated Research Ethics Board. Breast biopsies and CB units were obtained from donors with written, informed consent. Experiments involving the use of NOD-$Rag1^{-/-}$-$\gamma^{-/-}$ (NRG) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and approved by the McMaster's Animal Research Ethics Board.

Cells and Cell Lines

Eight units of long-term cryopreserved CB-NK cells were used in this study. Five units were used to measure NK cell expansion, phenotype, cytokine production, and cytotoxicity against MDA-MB-231 breast cancer cells. Among these, 3 units were cryopreserved for 10.5 years and 2 were cryopreserved for 2.5 years. An additional 3 units of 1.25-year old long-term cryopreserved CB were used to determine CB-NK cell cytotoxicity against primary breast cancer cells from 2 donors.

K562-based "Clone 9.mIL21" aAPCs were maintained in complete RPMI-1640 medium (Sigma-Aldrich, Oakville, ON, CA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin-streptomycin and 0.01M HEPES (Thermo Fischer Scientific, Burlington, ON).

MDA-MB-231 cells (Cell Biolabs, San Diego, CA) were cultured in complete Dulbecco's modified Eagle medium high glucose medium, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin/streptomycin and 1× MEM nonessential amino acids (Thermo Fischer Scientific).

Isolation and Cryopreservation of CB-derived Mononuclear Cells

Mononuclear cells were collected from fresh CB using Lymphoprep (Stemcell Technologies, Vancouver, BC) as described previously.[32,33] CB-derived mononuclear cells were resuspended in CryoStor CS2 (Stemcell Technologies) for cryopreservation in liquid nitrogen.

Ex Vivo aAPC-based Expansion of CB-NK Cells

CB-derived mononuclear cells were cocultured with twice the amount of irradiated aAPCs and 100 IU/mL of IL-2 (PeproTech, Rocky Hill, NJ) as described previously.[12,32] Complete RPMI-1640 media and IL-2was added every 2–3 days during after hemidepletion. Cocultures were counted, as mentioned previously, and replenished with irradiated aAPCs on a weekly basis.

Antibodies

The following antibodies were used according to the manufacturer's instructions. From BD Biosciences (San Jose, CA): CD3, NKp46, CD69. From BioLegend (San Diego, CA): CD45, CD56, CD16, NKG2D, NKp30, NKp44, CD25. From eBioscience (Santa Clara, CA) Fixable Viability Dye eFluor 780.

Cytokine Stimulation of Expanded CB-NK Cells

After 3 weeks of ex vivo expansion, expanded CB-NK cells were resuspended at 1×10^6 cells/mL in complete RPMI-1640 media and stimulated with a control of IL-2 (100 ng/mL) or a cytokine-activated cocktail of IL-2 (100 ng/mL), IL-12 (10 ng/mL), IL-15 (20 ng/mL) (PeproTech), and IL-18 (100 ng/mL) (Medical & Biological Laboratories, Nagano, Japan) for 24 hours.

Enzyme-linked Immunosorbent Assay (ELISA)/ Cytokine Detection

Supernatants were collected after 24 hours from cytokine-stimulated, expanded CB-NK cells. Interferon (IFN)-γ and tumor necrosis factor (TNF)-α cytokine levels in supernatants were measured using the respective R&D Systems (Minneapolis, MN) human DuoSet ELISA Kits, as per manufacturer's instructions.

Cytotoxicity Assays

CB-NK Cell Cytotoxicity Against MDA-MB-231 Cells

MDA-MB-231 cells were labeled with 5 μM carboxyfluoresceinsuccinimidyl ester (CFSE) (Sigma-Aldrich). CFSE-labeled tumor cells were incubated with expanded only, or expanded and cytokine-activated CB-NK cells at various effector: target (E:T) ratios for 5 hours at 37°C. Cells were stained with Fixable Viability Dye eFluor 780.

CB-NK Cell Cytotoxicity Against Primary Breast Cancer Cells

Tumors from breast biopsies were obtained and processed into 1 mm^2 pieces and subcutaneously injected into NRG mice. Body weight and tumor size were monitored and mice were sacrificed at endpoint. Tumor was resected and processed into 1 mm^2 pieces. 20 mL of 3 mg/mL Collagenase

A (Sigma-Aldrich) was added to tumor pieces and placed in a 37°C incubator shaker for 25 minutes. After a 25 minute incubation period, the cell suspension was filtered using a Falcon 40 μm nylon strainer (Thermo Fisher Scientific). The live cell fraction was selected for using Lymphoprep gradient centrifugation and cells were subsequently resuspended in complete Dulbecco's modified Eagle medium. Expanded CB-NK cells were labeled with 5 μM CFSE (Sigma-Aldrich) and coincubated with the tumor cells for 5 hours at 37°C. Cells were stained with Fixable Viability Dye eFluor 780.

Flow Cytometric Analysis of Cytotoxicity Assays

The percentage of viability dye-positive cells (% experimental lysis) was determined and percent specific lysis was calculated using the following formula: % specific lysis = 100×[(% experimental lysis−% basal lysis)/(100−% basal lysis)].

Flow Cytometry

Cells were stained with conjugated anti-human antibodies as described previously.[32] Samples were run on the BD LSRII flow cytometer and FlowJo software (Tree Star, Ashland, OR) was used for analysis. The $CD3^-$ $CD56^+$ (NK cell) population was analyzed for surface marker expression and fluorescence minus one controls were utilized for all experiments.

Statistical Analyses

Statistical comparisons were performed using GraphPad version 6.0 software. The 2-tailed unpaired Student t-test or ANOVA ($*P<0.05$; $**P<0.01$; $***P<0.001$; $****P<0.0001$) were used where indicated. Error bars represent SEM.

RESULTS

A Similar Log-scale Expansion of Ex Vivo-expanded CB-NK Cells Can be Generated From Fresh, Short-term Cryopreserved, and Long-term Cryopreserved CB

Little is known about the ability to generate therapeutic effector CB-NK cells from long-term cryopreserved CB. Therefore, our group sought to use an aAPC-based protocol for the ex vivo expansion of CB-NK cells from long-term cryopreserved CB.[12,32] Our results demonstrate that a log-scale expansion of CB-NK cells can be generated from long-term cryopreserved CB after 28 days (Fig. 1). Moreover, there were no significant differences in the total fold expansion between expanded CB-NK cells derived from fresh, short-term cryopreserved, or long-term cryopreserved CB (Fig. 1).

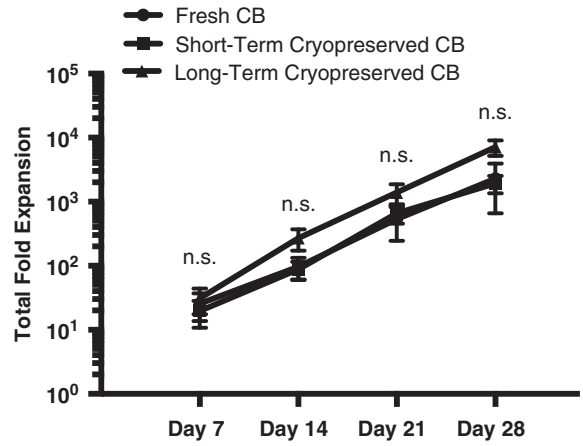

FIGURE 1. Ex vivo expansion generates a similar, robust log-scale expansion of CB-NK cells derived from fresh, short-term cryopreserved, or long-term cryopreserved CB after 28 days. Total fold expansion of NK cells from fresh (n = 4), short-term cryopreserved (n = 5), or long-term cryopreserved (n = 3) CB was measured after 28 days. CB indicates cord blood; NK, natural killer; n.s., not significant.

The Ex Vivo Expansion Process Significantly Upregulates the Surface Expression of Activating Markers on Expanded CB-NK Cells, Compared With Resting CB-NK Cells

In addition to inducing massive CB-NK cell proliferation and expansion, the aAPC-based CB-NK cell expansion protocol has also been shown to stimulate NK cell activation by generating a cytotoxic phenotype and function.[12,32] Therefore, we assessed NK cell phenotype by evaluating the surface expression of various activating and maturity markers, which are involved in mediating the balance of NK cell activation and inhibitory signals on CB-NK cells before (resting CB-NK cells) and after ex vivo expansion (expanded CB-NK cells) (Fig. 2). Although CB-NK cells were derived from fresh, short-term cryopreserved, or long-term cryopreserved CB, there were no significant differences in the surface marker expression across these groups (Fig. 3). Expanded CB-NK cells significantly upregulated the surface expression of activating receptors including CD69, and CD25 and the natural cytotoxicity receptors NKp30 and NKp44. In addition, there was a trend towards an increasing number of expanded CB-NK cells that expressed the activating marker NKG2D.

Ex Vivo-expanded CB-NK Cells Derived From Fresh, Short-term Cryopreserved, and Long-term Cryopreserved CB Produce Similar and Robust Levels of IFN-γ and TNF-α Upon Combined Stimulation With IL-12/IL-15/IL-18

In response to activation with dendritic cell-derived or macrophage-derived factors like IL-12, IL-15, and IL-18, NK cells are potent producers of the antitumor cytokines IFN-γ and TNF-α.[9,24] In order to assess the effector function of expanded CB-NK cells, we measured their ability to produce proinflammatory cytokines upon cytokine stimulation. Our results show that expanded CB-NK cells derived from fresh, short-term cryopreserved, and long-term cryopreserved CB potently produce similar levels of IFN-γ and TNF-α upon stimulation with a combination of IL-12, IL-15, and IL-18 (Fig. 4). There were no significant differences in IFN-γ (Fig. 4A) and TNF-α (Fig. 4B) production from expanded CB-NK cells derived from fresh, short-term cryopreserved or long-term cryopreserved CB upon cytokine stimulation.

Expanded CB-NK Cells Derived From Fresh, Short-term Cryopreserved, and Long-term Cryopreserved CB Mediate Cytotoxicity Against a TNBC Cell Line, MDA-MB-231, and have Increased Cytotoxicity Upon Cytokine Activation With IL-12/IL-15/IL-18

In addition to being potent producers of proinflammatory and antitumor cytokines, we also sought to assess the functionality of expanded CB-NK cells through their ability to mediate direct cytotoxicity against a TNBC cell line,

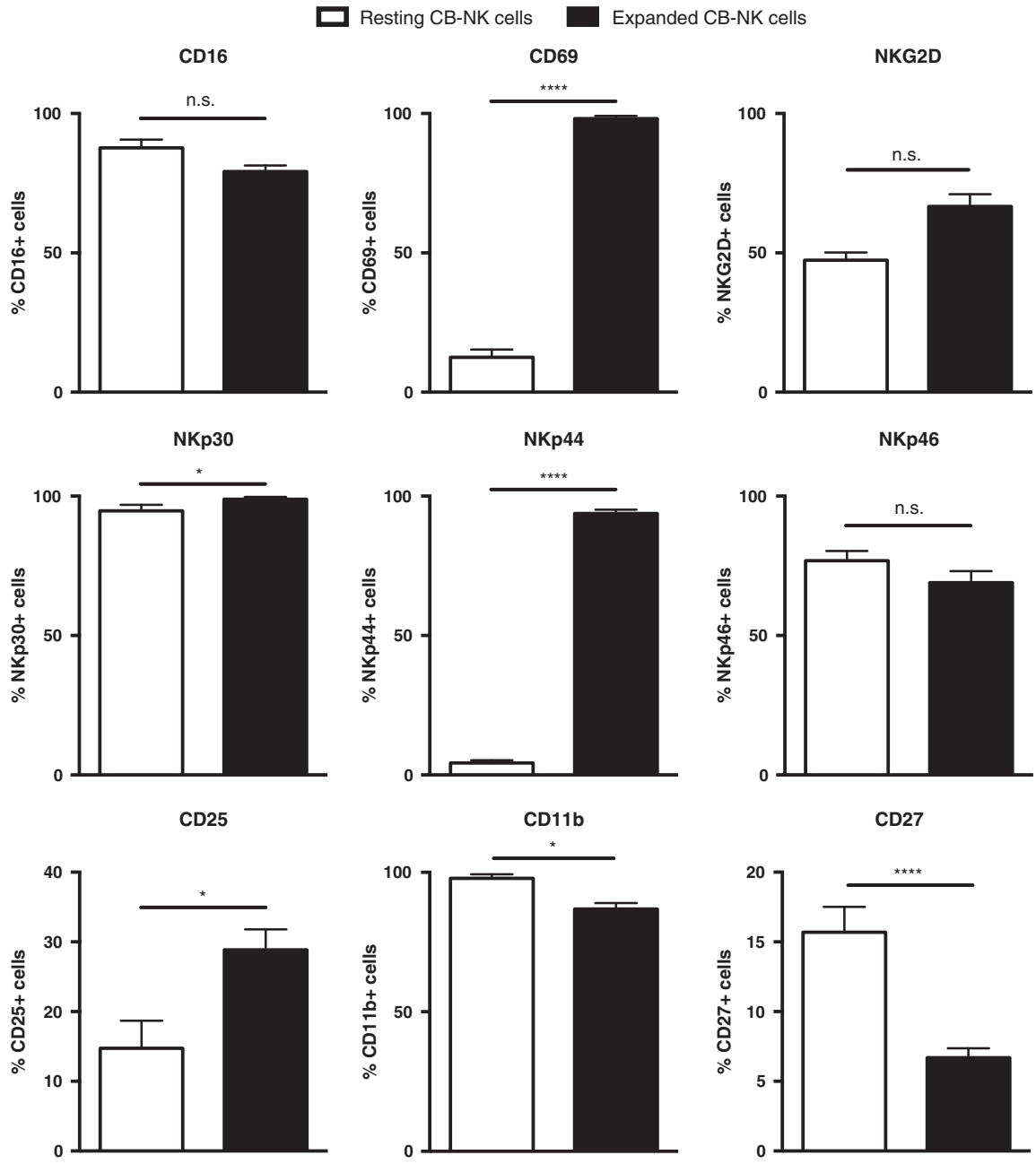

FIGURE 2. Ex vivo expansion upregulates the surface expression of activating markers on expanded CB-NK cells. Resting CB-NK cells (n = 3) and expanded CB-NK cells were stained with conjugated anti-human antibodies for 30 minutes. Flow cytometry was used to assess the surface expression of activating and maturity markers on the $CD56^{+}CD3^{-}$(NK cell) population. Statistical analyses were performed using a 2-tailed unpaired Student t-test (*$P < 0.05$;***$P < 0.001$, ****$P < 0.0001$). CB indicates cord blood; NK, natural killer; n.s., not significant.

MDA-MB-231 (Fig. 5).[34] Expanded CB-NK cells derived from fresh, short-term cryopreserved and long-term cryopreserved CB demonstrated similar levels of cytotoxicity against MDA-MB-231 breast cancer cells and lysed tumor cells in a concentration-dependent manner (Fig. 5A). Upon combined stimulation with IL-12/IL-15/IL-18 for 24 hours, expanded CB-NK cells demonstrated significantly increased percent specific lysis when incubated with MDA-MB-231 breast cancer cells (Fig. 5B). At an E:T ratio of 10:1, the mean percent specific lysis of expanded, cytokine-activated CB-NK cells derived from fresh, short-term cryopreserved, and long-term cryopreserved CB was 77%, 75%, and 78%, respectively. Overall, the length of CB cryopreservation did not affect the cytotoxicity of expanded CB-NK cells against the TNBC cell line.

Expanded CB-NK Cells Derived From Long-term Cryopreserved CB are Cytotoxic Towards TNBC and ER^{+}/PR^{+} Primary Breast Cancer Cells

After testing the efficacy of expanded CB-NK cells against TNBC MDA-MB-231 cells, we also sought to

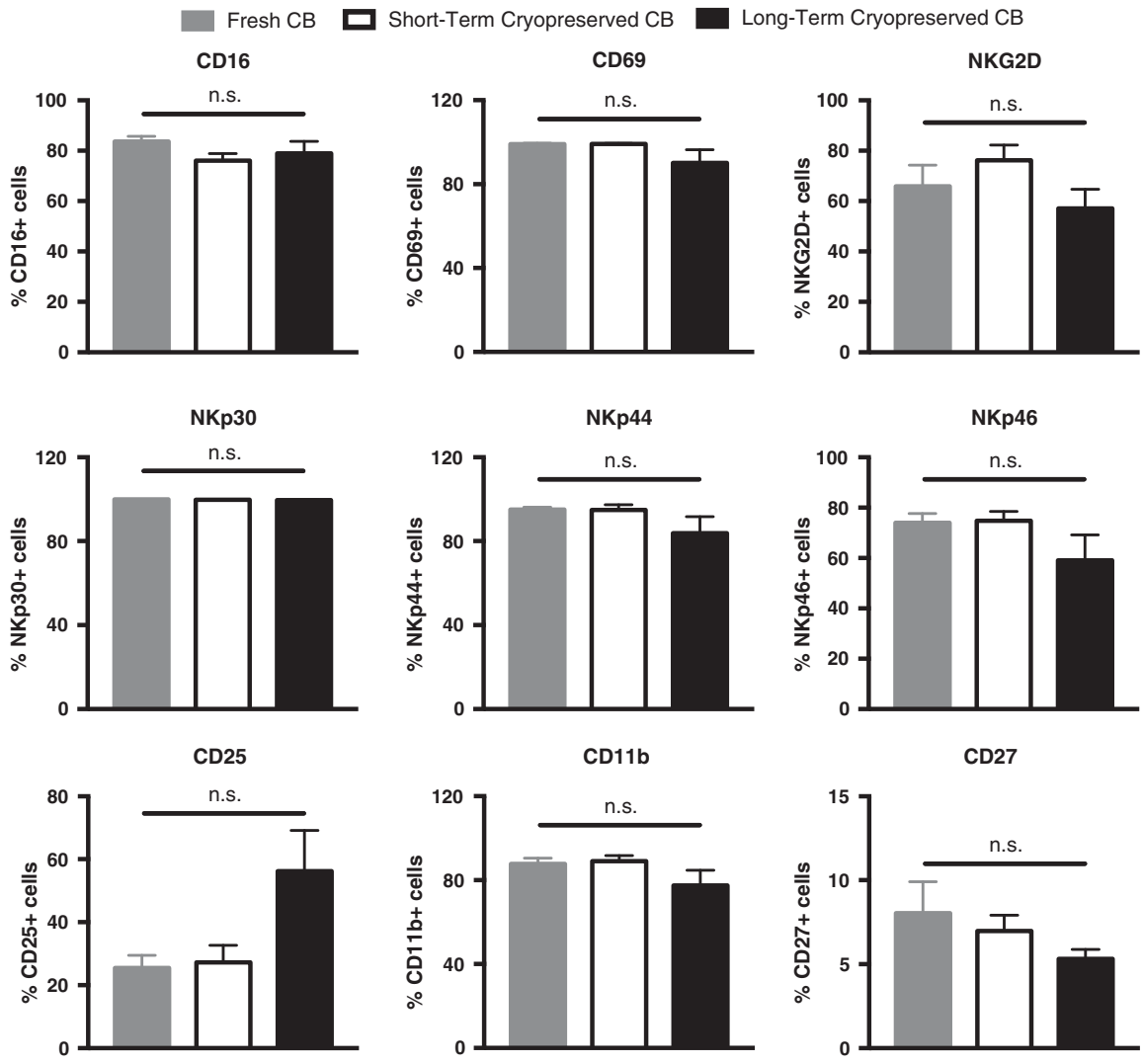

FIGURE 3. Ex vivo-expanded CB-NK cells derived from fresh, short-term cryopreserved, and long-term cryopreserved CB units share a similar expression of activating and maturity markers. Expanded resting (n = 4), short-term cryopreserved (n = 5), and long-term cryopreserved CB-NK cells (n = 5) were stained with conjugated anti-human antibodies for 30 minutes. Flow cytometry was used to assess the surface expression of activating and maturity markers on the $CD56^{+}CD3^{-}$(NK cell) population. Statistical analyses were performed using a 1-way analysis of variance. CB indicates cord blood; NK, natural killer; n.s., not significant.

determine their effector function against primary breast cancer cells, which are more representative of clinical-grade breast cancers. Therefore, we obtained single-cell suspensions of primary breast cancer cells from breast biopsies of 2 patients, breast cancer patient A and B, diagnosed with TNBC and ER^{+}/PR^{+} breast cancer, respectively. Our results indicate expanded CB-NK cells were able to lyse primary breast cancer cells in a concentration-dependent manner (Fig. 6). At the 10:1 E:T ratio, expanded CB-NK cells demonstrated variable cytotoxicity towards TNBC (Fig. 6A) and ER^{+}/PR^{+} (Fig. 6B) breast cancer cells. The mean percent specific lysis of the expanded CB-NK cells at a 10:1 ratio ranged from 19% to 27% against TNBC, compared with 74% to 92% against ER^{+} and PR^{+} breast cancer. Overall, our results indicate long-term cryopreserved CB yields therapeutic expanded NK cells that are cytotoxic against primary breast cancer cells, in vitro.

DISCUSSION

In this current study, we sought to elucidate the potential of using long-term cryopreserved CB as a source of therapeutic NK cells for adoptive NK cell immunotherapy. As such, we used an aAPC-based protocol for the ex vivo expansion of CB-NK cells. We assessed whether the long-term cryopreservation of CB affected the expansion potential and cytotoxic effector function of expanded CB-NK cells. Our findings show that a log-scale expansion of therapeutic effector NK cells can be obtained from long-term cryopreserved CB using an aAPC-based ex vivo expansion technique (Fig. 1). Expanded CB-NK cells produced robust levels of antitumor cytokines, IFN-γ, and TNF-α, and demonstrated cytotoxic function towards the TNBC cell line MDA-MB-231 (Figs. 4, 5). Moreover, expanded CB-NK cells derived from long-term cryopreserved CB demonstrated cytotoxicity against both TNBC and ER^{+}/PR^{+}

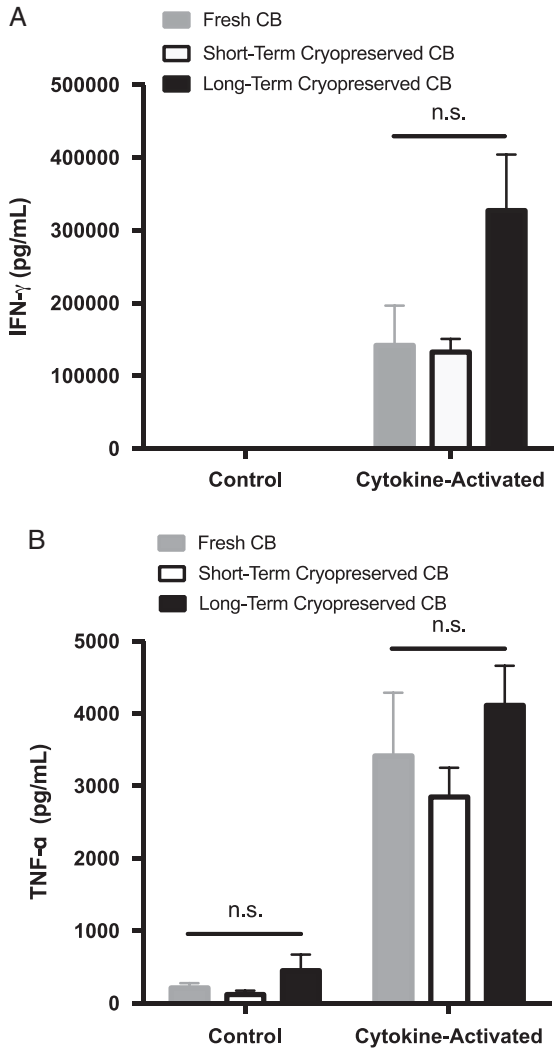

FIGURE 4. Expanded CB-NK cells derived from fresh, short-term cryopreserved, and long-term cryopreserved CB potently produce anti-tumor cytokines, IFN-γ and TNF-α, upon cytokine activation. Expanded CB-NK cells were stimulated with IL-2 (100 ng/mL) (control) or a combined cocktail of IL-2 (100 ng/mL), IL-12 (10 ng/mL), IL-15 (20 ng/mL), and IL-18 (100 ng/mL) (cytokine activated) for 24 hours. Supernatants were collected from expanded CB-NK cells derived from fresh (n = 4), short-term cryopreserved (n = 5), and long-term cryopreserved (n = 3) CB and assayed for IFN-γ (A) and TNF-α (B) cytokine levels using the appropriate enzyme-linked immunosorbent assay kits. CB indicates cord blood; IFN, interferon; NK, natural killer; n.s., not significant; TNF, tumor necrosis factor.

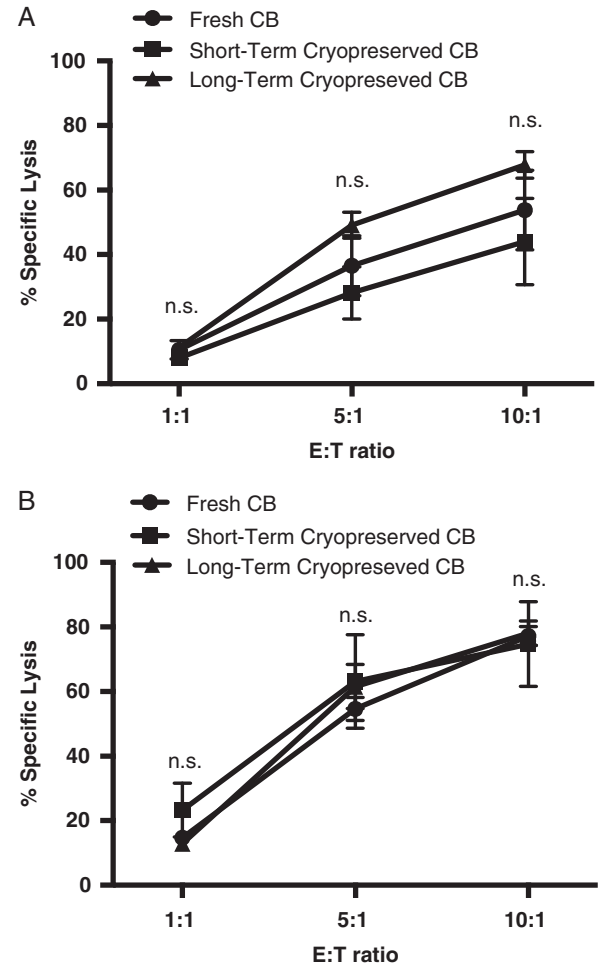

FIGURE 5. Expanded CB-NK cells derived from fresh, short-term cryopreserved, and long-term cryopreserved CB exhibit similar cytotoxicity towards MDA-MB-231 breast cancer cells and increased cytotoxicity upon cytokine activation. A, Expanded CB-NK cells derived from fresh (n = 4), short-term cryopreserved (n = 5), and long-term cryopreserved (n = 3) CB and (B) expanded CB-NK cells derived from fresh (n = 4), short-term cryopreserved (n = 4), and long-term cryopreserved (n = 3) were stimulated with a combined cocktail of IL-2 (100 ng/mL), IL-12 (10 ng/mL), IL-15 (20 ng/mL), and IL-18 (100 ng/mL) for 24 hours. Expanded CB-NK cells were coincubated with carboxyfluoresceinsuccinimidyl ester-labeled MDA-MB-231 breast cancer cells for 5 hours. Statistical analysis was performed using a 1-way analysis of variance. CB indicates cord blood; E:T ratio, effector: target ratio; NK, natural killer; n.s., not significant.

primary breast cancer cells (Fig. 6). Overall, our results show that the length of CB cryopreservation does not affect CB-NK cell expansion or effector function, as there were no significant differences in the expansion, phenotype, and antitumor abilities of CB-NK cells expanded from fresh, short-term cryopreserved, and long-term cryopreserved CB.

Similar to previous studies that have utilized the K562-based "Clone 9.mIL21" aAPCs for ex vivo NK cell expansion from peripheral blood and CB,[12,32] our results show it is possible to obtain a log-scale expansion of CB-NK cells derived from fresh, short-term cryopreserved, and long-term cryopreserved CB (Fig. 1). As there were no significant differences in the mean total fold expansion of CB-NK cells from the 3 groups of CB, this suggests that the cryopreservation and thawing process of CB does not affect the expansion potential of CB-NK cells.

Resting, unmanipulated CB-NK cells have previously been identified as phenotypically immature and poorly cytotoxic, the latter which can be due to an impaired ability to form immunologic synapses with their respective target cells among other factors.[7,15,16,35] Previously, it was found that ex vivo NK cell expansion with IL-2 could reverse the poor cytolytic ability of CB-NK cells by creating a stronger synapse between NK cells and their targets through the recruitment of adhesion molecules and perforin.[35] Similarly,

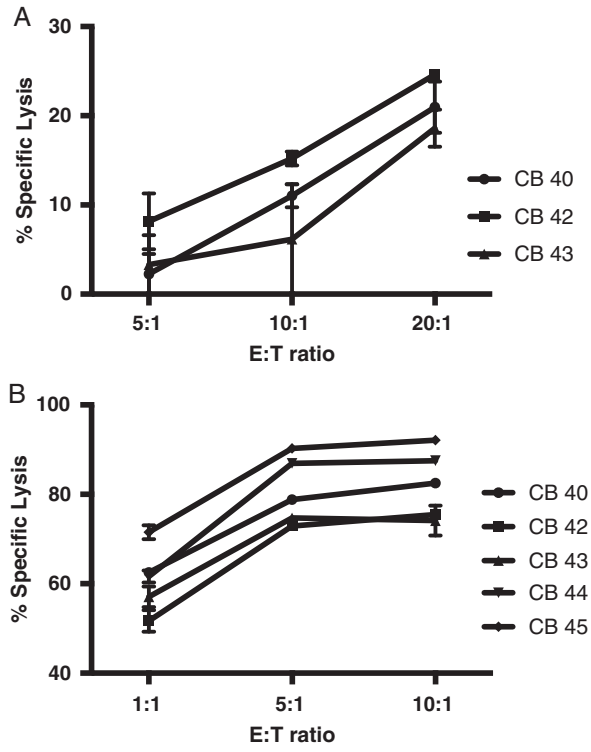

FIGURE 6. Expanded CB-NK cells derived from long-term cryopreserved CB are cytotoxic towards triple-negative breast cancer and estrogen receptor-positive/progesterone receptor-positive primary breast cancer cells, derived from BCP A and B, respectively. Primary breast tumors were obtained from (A) BCP A and (B) BCP B through breast biopsies and processed into single-cell suspensions. Primary breast cancer cells were immediately coincubated with carboxyfluoresceinsuccinimidyl ester-labeled expanded CB-NK cells (from 3 CB donors: 40, 42, 43) at various E:T ratios for 5 hours. BCP indicates breast cancer patient; CB, cord blood; E:T ratio, effector: target ratio; NK, natural killer; n.s., not significant.

our results indicate that the aAPC-based expansion used in this study yields CB-NK cells that exhibit effective cytotoxicity against MDA-MB-231 breast cancer cells (Fig. 5A). This enhanced cytotoxicity could also be attributed to the increased surface expression of multiple NK cell activating markers on expanded CB-NK cells including CD69, NKp30 and NKp44, and CD25 (Fig. 2).

In addition, different combinations of IL-2, IL-12, IL-15, and IL-18 have been shown to induce higher proliferative responses, cytokine secretion, and cytotoxic effects in both CB-derived and peripheral blood-derived NK (PB-NK) cells.[9,24,36] In agreement with these data, our results indicate that aAPC-expanded CB-NK cells preactivated with IL-12/IL-15/IL-18 demonstrate increased IFN-γ and TNF-α secretion and cytotoxicity towards MDA-MB-231 breast cancer cells (Figs. 4, 5B). Recently, it has been reported that the adoptive transfer of IL-12/IL-15/IL-18-stimulated PB-NK cells induced a clinical response in patients with acute myeloid leukemia.[37] Our results indicate that expanded CB-NK cells derived from cryopreserved CB respond robustly to cytokine stimulation and thus may be a feasible source of NK cells for such therapies.

Lastly, our results show that expanded CB-NK cells can mediate cytotoxicity towards primary breast cancer cells, which better represent true forms of clinical-grade breast cancer, compared with a breast cancer cell line. It is interesting to note that, although expanded CB-NK cells were derived from the same cryopreserved CB units, expanded CB-NK cells had variable cytotoxicity between TNBC and ER^+/PR^+ breast cancer (Fig. 6B). Expanded CB-NK cells exhibited increased cytotoxicity against the hormone-positive breast cancer in comparison with the TNBC breast cancer. The observed heterogeneity in cytotoxicity may be attributed to the varying levels of resistance against NK cell immunity by different breast cancers. The breast cancer microenvironment has been shown to evade NK cell immunity by dampening NK cell cytotoxicity in numerous ways including the expression of nonclassic HLA-I antigen, HLA-G[38,39]; the production of immunosuppressive cytokines like TGF-β; the downregulation of stress ligands expressed on tumor cells; and, the downregulation of NK cell activating receptors like NKG2D.[40,41] Although there was variability in the NK cell-mediated cytotoxicity of the 2 primary breast cancer types, the expanded CB-NK cells were still shown to lyse breast cancer cells in a concentration-dependent manner in an in vitro setting.

In conclusion, our findings show an alternative use for long-term cryopreserved CB in its potential to generate clinically relevant quantities of therapeutic effector NK cells against breast cancer, which contributes to the recent advances and successes in the field of NK cell immunotherapy for other types of cancers. Previous clinical trials have reported the success of adoptive NK cell immunotherapy for hematological malignancies like acute myeloid leukemia, which demonstrated complete remissions in elderly patients or patients with poor prognoses and event-free survival for 18 months in pediatric patients.[42–44] Moreover, others have successfully directed NK cell functions against neuroblastoma and glioblastoma.[45–47] To date, breast cancer clinical trials that have used ex vivo IL-2- or IL-15-activated NK cells have shown improved patient outcomes, albeit with limited clinical efficacy.[48–51] Therefore, it is also of interest to investigate the clinical efficacy of a APC-expanded NK cells in breast cancer patients.

It is also important to explore other ways we can utilize the vast quantities of long-term cryopreserved CB our systems can currently store. With over 600,000 CB units stored globally, approximately only 30,000 CB transplantations have been performed; it is unclear what happens to publicly or privately banked CB samples that are stored for extensive periods of time.[1] As our data provides novel evidence that activated therapeutic effector CB-NK cells can be expanded, ex vivo, from long-term cryopreserved CB and demonstrate cytotoxicity against primary breast cancer cells, we propose that long-term cryopreserved CB is clinically useful for NK cell immunotherapy, in addition to HSC transplantations. The ability to store CB samples that maintain functionality after decades of cryopreservation raises the possibility of housing vast, diverse, and long-lasting donor libraries, where readily available sources of therapeutic NK cells can be harvested.

CONFLICTS OF INTEREST/ FINANCIAL DISCLOSURES

None reported. All authors have declared there are no financial conflicts of interest with regard to this work.

REFERENCES

1. Ballen KK, Gluckman E, Broxmeyer HE. Umbilical cord blood transplantation: the first 25 years and beyond. *Blood.* 2013;122:491–498.

2. Broxmeyer HE, Lee MR, Hangoc G, et al. Hematopoietic stem/progenitor cells, generation of induced pluripotent stem cells, and isolation of endothelial progenitors from 21- to 23.5-year cryopreserved cord blood. *Blood*. 2011;117: 4773–4777.
3. Gassas A. Cord stem-cell transplantation in Ontario: do we need a public bank? *Curr Oncol*. 2011;18:e121–e125.
4. Armson BA, Allan DS, Casper RF. Umbilical cord blood: counselling, collection, and banking. *J Obstet Gynaecol Can*. 2015;37:832–844.
5. Allan D, Petraszko T, Elmoazzen H, et al. A review of factors influencing the banking of collected umbilical cord blood units. *Stem Cells Int*. 2013;2013:463031.
6. Kotylo PK, Baenzinger JC, Yoder MC, et al. Rapid analysis of lymphocyte subsets in cord blood. *Am J Clin Pathol*. 1990;93: 263–266.
7. Luevano M, Daryouzeh M, Alnabhan R, et al. The unique profile of cord blood natural killer cells balances incomplete maturation and effective killing function upon activation. *Hum Immunol*. 2012;73:248–257.
8. Cany J, Dolstra H, Shah N. Umbilical cord blood-derived cellular products for cancer immunotherapy. *Cytotherapy*. 2015; 17:739–748.
9. Cheng M, Chen Y, Xiao W, et al. NK cell-based immunotherapy for malignant diseases. *Cell Mol Immunol*. 2013;10: 230–252.
10. Spanholtz J, Preijers F, Tordoir M, 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.
11. Luevano M, Domogala A, Blundell 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.
12. Shah N, Martin-Antonio B, Yang H, 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.
13. Garnock-Jones KP, Keating GM, Scott LJ. Trastuzumab: a review of its use as adjuvant treatment in human epidermal growth factor receptor 2 (HER2)-positive early breast cancer. *Drugs*. 2010;70:215–239.
14. Yao H, He G, Yan S, et al. Triple-negative breast cancer: is there a treatment on the horizon? *Oncotarget*. 2017;8:1913–1924.
15. Wang Y, Xu H, Zheng X, et al. High expression of NKG2A/CD94 and low expression of granzyme B are associated with reduced cord blood NK cell activity. *Cell Mol Immunol*. 2007;4: 377–382.
16. Dalle JH, Menezes J, Wagner E, et al. Characterization of cord blood natural killer cells: implications for transplantation and neonatal infections. *Pediatr Res*. 2005;57 (pt 1):649–655.
17. Boissel L, Tuncer HH, Betancur M, et al. Umbilical cord mesenchymal stem cells increase expansion of cord blood natural killer cells. *Biol Blood Marrow Transplant*. 2008;14: 1031–1038.
18. Tomchuck SL, Leung WH, Dallas MH. Enhanced cytotoxic function of natural killer and CD3+ CD56+ cells in cord blood after culture. *Biol Blood Marrow Transplant*. 2015;21:39–49.
19. Li Y, Schmidt-Wolf IG, Wu YF, et al. Optimized protocols for generation of cord blood-derived cytokine-induced killer/natural killer cells. *Anticancer Res*. 2010;30:3493–3499.
20. Ni J, Miller M, Stojanovic A, et al. Sustained effector function of IL-12/15/18-preactivated NK cells against established tumors. *J Exp Med*. 2012;209:2351–2365.
21. Lauwerys BR, Renauld JC, Houssiau FA. Synergistic proliferation and activation of natural killer cells by interleukin 12 and interleukin 18. *Cytokine*. 1999;11:822–830.
22. Romee R, Leong JW, Fehniger TA. Utilizing cytokines to function-enable human NK cells for the immunotherapy of cancer. *Scientifica (Cairo)*. 2014;2014:205796.
23. Ferlazzo G, Pack M, Thomas D, et al. Distinct roles of IL-12 and IL-15 in human natural killer cell activation by dendritic cells from secondary lymphoid organs. *Proc Natl Acad Sci USA*. 2004;101:16606–16611.
24. Vivier E, Ugolini S, Blaise D, et al. Targeting natural killer cells and natural killer T cells in cancer. *Nat Rev Immunol*. 2012;12: 239–252.
25. Armitage S. Cord blood banking standards: autologous versus altruistic. *Front Med (Lausanne)*. 2015;2:94. doi: 10.3389/fmed.2015.00094.
26. Brunstein CG, Setubal DC, Wagner JE. Expanding the role of umbilical cord blood transplantation. *Br J Haematol*. 2007;137: 20–35.
27. Mugishima H, Harada K, Chin M, et al. Effects of long-term cryopreservation on hematopoietic progenitor cells in umbilical cord blood. *Bone Marrow Transplant*. 1999;23:395–396.
28. Kobylka P, Ivanyi P, Breur-Vriesendorp BS. Preservation of immunological and colony-forming capacities of long-term (15 years) cryopreserved cord blood cells. *Transplantation*. 1998;65:1275–1278.
29. Yamamoto S, Ikeda H, Toyama D, et al. Quality of long-term cryopreserved umbilical cord blood units for hematopoietic cell transplantation. *Int J Hematol*. 2011;93:99–105.
30. Mitchell R, Wagner JE, Brunstein CG, et al. Impact of long-term cryopreservation on single umbilical cord blood transplantation outcomes. *Biol Blood Marrow Transplant*. 2015;21: 50–54.
31. Parmar S, de Lima M, Worth L, et al. Is there an expiration date for a cord blood unit in storage? *Bone Marrow Transplant*. 2014;49:1109–1112.
32. Denman CJ, Senyukov VV, Somanchi SS, et al. Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells. *PLoS One*. 2012;7:e30264.
33. Somanchi SS, Senyukov VV, Denman CJ, et al. Expansion, purification, and functional assessment of human peripheral blood NK cells. *J Vis Exp*. 2011:48. pii:2540.
34. Chavez KJ, Garimella SV, Lipkowitz S. Triple negative breast cancer cell lines: one tool in the search for better treatment of triple negative breast cancer. *Breast Dis*. 2010;32:35–48.
35. Xing D, Ramsay AG, Gribben JG, 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.
36. Alnabhan R, Madrigal A, Saudemont A. Differential activation of cord blood and peripheral blood natural killer cells by cytokines. *Cytotherapy*. 2015;17:73–85.
37. Romee R, Rosario M, Berrien-Elliott MM, et al. Cytokine-induced memory-like natural killer cells exhibit enhanced responses against myeloid leukemia. *Sci Transl Med*. 2016;8: 357ra123.
38. Campoli M, Ferrone S. Tumor escape mechanisms: potential role of soluble HLA antigens and NK cells activating ligands. *Tissue Antigens*. 2008;72:321–334.
39. Konig L, Kasimir-Bauer S, Hoffmann O, et al. The prognostic impact of soluble and vesicular HLA-G and its relationship to circulating tumor cells in neoadjuvant treated breast cancer patients. *Hum Immunol*. 2016;77:791–799.
40. Coudert JD, Zimmer J, Tomasello E, et al. Altered NKG2D function in NK cells induced by chronic exposure to NKG2D ligand-expressing tumor cells. *Blood*. 2005;106:1711–1717.
41. Lee JC, Lee KM, Kim DW, et al. Elevated TGF-beta1 secretion and down-modulation of NKG2D underlies impaired NK cytotoxicity in cancer patients. *J Immunol*. 2004;172: 7335–7340.
42. Miller JS, Soignier Y, Panoskaltsis-Mortari A, et al. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. *Blood*. 2005;105:3051–3057.
43. Curti A, Ruggeri L, D'Addio 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;118:3273–3279.

44. Rubnitz JE, Inaba H, Ribeiro RC, et al. NKAML: a pilot study to determine the safety and feasibility of haploidentical natural killer cell transplantation in childhood acute myeloid leukemia. *J Clin Oncol*. 2010;28:955–959.
45. Yang RK, Kalogriopoulos NA, Rakhmilevich AL, et al. Intratumoral treatment of smaller mouse neuroblastoma tumors with a recombinant protein consisting of IL-2 linked to the hu14.18 antibody increases intratumoral CD8+ T and NK cells and improves survival. *Cancer Immunol Immunother*. 2013;62:1303–1313.
46. Han J, Chu J, Keung Chan W, et al. CAR-engineered NK cells targeting wild-type EGFR and EGFRvIII enhance killing of glioblastoma and patient-derived glioblastoma stem cells. *Sci Rep*. 2015;5:11483.
47. Delgado DC, Hank JA, Kolesar J, et al. Genotypes of NK cell KIR receptors, their ligands, and Fcgamma receptors in the response of neuroblastoma patients to Hu14.18-IL2 immunotherapy. *Cancer Res*. 2010;70:9554–9561.
48. deMagalhaes-Silverman M, Donnenberg A, Lembersky B, et al. Posttransplant adoptive immunotherapy with activated natural killer cells in patients with metastatic breast cancer. *J Immunother*. 2000;23:154–160.
49. Geller MA, Cooley S, Judson PL, et al. A phase II study of allogeneic natural killer cell therapy to treat patients with recurrent ovarian and breast cancer. *Cytotherapy*. 2011;13:98–107.
50. Burns LJ, Weisdorf DJ, DeFor TE, et al. IL-2-based immunotherapy after autologous transplantation for lymphoma and breast cancer induces immune activation and cytokine release: a phase I/II trial. *Bone Marrow Transplant*. 2003;32:177–186.
51. Roberti MP, Rocca YS, Amat M, et al. IL-2- or IL-15-activated NK cells enhance cetuximab-mediated activity against triple-negative breast cancer in xenografts and in breast cancer patients. *Breast Cancer Res Treat*. 2012;136:659–671.