Published in final edited form as:

Cytotherapy. 2009 ; 11(3): 341–355. doi:10.1080/14653240902807034.

Clinical Grade Ex Vivo-Expanded Human Natural Killer Cells Upregulate Activating Receptors and Death Receptor Ligands and Have Enhanced Cytolytic Activity against Tumor Cells

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Abstract

Background—Cancer immunotherapy involving NK-cell infusions and administration of therapeutic agents modulating the susceptibility of tumors to NK-cell lysis has been recently proposed. Here we provide a method to expand highly cytotoxic clinical grade NK cells *in vitro* for adoptive transfer following bortezomib treatment in patients with advanced malignancies.

Methods—NK cells were expanded with irradiated Epstein-Barr virus-transformed lymphoblastoid cells. Expanded cells were evaluated for their phenotype, cytotoxicity, cytokine secretion, dependence on IL-2 and the ability to retain function after cryopreservation.

Results—A pure population of clinical grade NK cells expanded 490 ± 260 fold over 21 days. Expanded NK cells had increased TRAIL, FasL, and NKG2D expression and significantly higher cytotoxicity against bortezomib-treated tumors compared with resting NK cells. Expanded NK cells, co-cultured with K562 and renal cell carcinoma tumor targets, secreted significantly higher levels of sFasL, IFN γ , GM-CSF, TNF α , MIP-1 α , and MIP-1 β compared with resting NK cells. Secretion of the above cytokines and NK-cell cytolytic function were IL-2 dose dependent. Cryopreservation of expanded NK cells reduced expression of NKG2D and TRAIL and NK-cell cytotoxicity, though this effect could be reversed by exposure of NK cells to IL-2.

Discussion—Here we show a method for the large scale expansion of NK cells with increased expression of activating receptors and death receptor ligands resulting in superior cytotoxicity against tumor cells. This *ex vivo* NK-cell expansion technique is currently being utilized in a clinical trial evaluating the anti-tumor activity of adoptively-infused NK cells in combination with bortezomib.

Keywords

NK cells; expansion; bortezomib; immunotherapy

Introduction

NK cells are innate immune lymphocytes that are identified by the expression of the CD56 surface antigen and the lack of CD3 (1,2). NK cells have the ability to directly kill target cells through the release of granules containing perforin and serine proteases (granzymes) and/or by surface-expressed ligands that engage and activate death receptors expressed on target cells. They can also mediate antibody-dependent cellular cytotoxicity (ADCC) via the membrane receptors FcγRIII (CD16) (3). Unlike T cells, NK cells do not require the presence of a specific tumor antigen to kill cancer cells, rather their recognition of targets is regulated through a balance of activating and inhibitory signals. Even in the presence of an activating ligand, inhibitory ligands can initiate overriding signals that culminate in a net suppression of NK cell function. The inactivation of NK cells by self-HLA molecules is a potential mechanism by which malignant cells evade host NK cell-mediated immunity (4,5).

Recently, we and others observed that the proteasome inhibitor bortezomib (Velcade, PS-341) sensitized malignant cells to tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-dependent NK-cell lysis (6-8). This effect appeared to overcome KIR-mediated suppression of NK cell function, enhancing autologous NK-cell cytotoxicity against patient tumor cells *in vitro*. Based on this finding, we pursued a method for the large scale expansion of clinical grade NK cells to evaluate the anticancer effects of autologous adoptively-infused NK cells following bortezomib treatment in patients with cancer.

Only a few trials investigating adoptive NK-cell infusions in humans with cancer have been conducted to date [Reviewed in (9,10)]. Since NK cells represent only a minor fraction of human lymphocytes, the small number of NK cells isolated following a typical leukapheresis procedure has precluded phase I trials evaluating for NK-cell dose-dependent tumor cytotoxicity in humans with cancer.

Several methods for expansion and activation of NK cells *in vitro* have been investigated, including overnight and long term culture with cytokines (11,12), and the use of PBMC (13), K562 cells (14), and Epstein-Barr virus-transformed lymphoblastoid cell lines (EBV-LCL) as feeder cells (15,16). We previously developed (17) and have now optimized an improved method for the large scale expansion of human NK cells in bags using irradiated EBV-LCL feeder cells and IL-2. EBV-LCL cell line, used in our studies, has been proven previously (18) to be safe for use in clinical trials; cells have met release test criteria for the presence of viral contaminants and infectious EBV. We explored the phenotype, cytotoxic potential against tumor cells and cytokine secretion of these expanded NK cells compared to freshly-isolated cells. We also investigated the effects of IL-2 withdrawal on phenotype and function of expanded cells and, finally, the effects of cryopreservation and thawing.

In the present study we show that NK-cell phenotype and function are modulated following *in vitro* expansion. As a consequence of these changes, NK-cell cytolytic activity against bortezomib-treated tumors was significantly higher with expanded compared to fresh NK cells.

Materials and Methods

Cell isolation, culture, and cryopreservation

Human NK cells were isolated from peripheral blood mononuclear cells (PBMC) obtained from multiple different healthy volunteers and one patient with metastatic sarcoma. Depletion of CD3⁺ T cells and a subsequent positive selection of CD56⁺ cells were performed on a CliniMACS system (Miltenyi Biotec, Inc., Auburn, CA). The cells were analyzed immediately after purification for phenotypic markers and cytotoxicity and were then either expanded or cryopreserved for future analysis. For NK expansions the following parameters were tested:

autologous/allogeneic PBMC vs. EBV-LCL as feeder cells; culture vessels (flasks vs. bags); feeder cell irradiation doses (25, 50 and 75 Gy); feeder-to-NK cell ratios (ratios of 90:1, 50:1, 20:1, 10:1, 5:1, and 1:1 feeder-to-NK cells respectively) and plasma (obtained from NK cell donors or from PBMC donors) vs. serum (2, 5 and 10% of pooled AB plasma, AB serum and 6 different lots of commercial AB serum).

NK cell expansions were performed as follows:

1. Expansions in flasks (small scale expansions): twenty million 100 Gy-irradiated and washed EBV-LCL cells were co-cultured with 10^6 magnetic bead-purified NK cells in upright 75 cm² tissue culture flasks in 15 ml of X-VIVO 20 (Lonza, Walkersville, MD), supplemented with 10% heat inactivated human AB serum (Gemini Bio-Products, West Sacramento, CA), or 10% heat inactivated AB single donor or pooled plasma or serum [obtained from The Department of Transfusion Medicine (DTM) in NIH], 500 IU/mL rhIL-2 (50 ng/mL, Tecin™, Hoffmann-La Roche Inc., Nutley, NJ), and 2 mM GlutaMAX-1 (Invitrogen, Carlsbad, CA) at 37°C and 6.5% CO₂. The effect on NK-cell proliferation of varying the percentage of CO₂ from 5 to 8% was systematically investigated. NK-cell proliferation was greatest at 6.5% CO₂ (data not shown). Therefore, all NK-cell expansions, both small scale and large scale, were performed in incubators using 6.5% CO₂. After 5 days of culture half of the culture medium was replaced. Starting on day 7, NK cells were diluted to 0.6×10^6 cells/mL with growth medium containing IL-2 every 24-72 hours for up to 28 days. In some experiments, following 14 days of culture, 1.0×10^6 expanded NK cells were co-cultured with 20×10^6 of irradiated feeder cells and the culture was expanded for an additional 14 days.
2. Expansions in bags (large scale expansions): in the DTM under good manufacturing practice (GMP) conditions $12\text{-}24 \times 10^6$ magnetic bead-purified NK cells were combined with $120\text{-}240 \times 10^6$ irradiated EBV-TM-LCL cells in 100-140 mL of medium containing rhIL-2 obtained from NIH Pharmacy Development Service (NIH PDS Bethesda, MD) in Baxter 180 cm² 300 mL bags (Fenwal Lifecell, Baxter Healthcare Corporation, Deerfield, IL). Four to 5 days after the initiation of the culture half of the medium was replaced. Two days later, the concentration of NK cells was adjusted to 10^6 cells/mL using growth medium containing IL-2. Expanding cells were counted and diluted every 24-72 hours until day 28.

The GMP-certified human EBV transformed B-cell line EBV-TM-LCL was obtained from Fred Hutchinson Cancer Research Center (FHCRC, Seattle, WA), and had been originally supplied to FHCRC by the Beckman Research Institute of the City of Hope (Duarte, CA), and maintained in our laboratory in RPMI 1640, 10% heat inactivated human AB serum (Gemini Bio-Products), 2 mM GlutaMAX-1, and 15 mM HEPES (Invitrogen). For large scale expansions of NK cells EBV-TM-LCL cells were maintained in DTM in 162 cm² flasks or 300 mL cell culture bags at $0.2\text{-}1.0 \times 10^6$ /mL in the above medium supplemented with 10% heat inactivated human AB type plasma. 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), 10% FBS (Quality Biological, Inc., Gaithersburg, MD), and 2 mM GlutaMAX-1.

Freshly-isolated or expanded NK cells were cryopreserved in PlasmaLyte A medium (Baxter) supplemented with 4% human serum albumin (HSA, Talecris Biotherapeutics, Inc., Research Triangle Park, NC), 6% pentastarch (Hypoxyethylstarch, NIH PDS), 10 µg/mL DNase I (Pulmozyme, Genentech, Inc., South San Francisco, CA), 15 U/mL heparin (Abraxis Pharmaceutical Products, IL), and 5% DMSO at $20\text{-}50 \times 10^6$ cells/mL per vial. Thawing medium contained X-VIVO 20, 10% human AB serum or plasma, 4% HSA, and 10 U/mL

heparin. Cells were thawed at 37°C, slowly diluted with 10 mL of thawing medium, and left at room temperature for 1-2 hours before being centrifuged to avoid cell breakage. Thawed cells were used for expansion experiments, in cytotoxicity assays and for flow cytometry 1.5-2 hours following thawing.

Flow cytometry analysis of resting and expanded NK cells

The phenotype of freshly-isolated or expanded NK cells was assessed by flow cytometry on a FACSCalibur™ (BD Biosciences, San Jose, CA) with the following anti-human monoclonal antibodies: anti-CD56-APC (clone B159), anti-CD16-FITC (clone 3G8), anti-CD3-PE (clone UCHT1), anti-CD25-PE (clone M-A251), anti-NKG2D-APC (clone 1D11), anti-CD244-PE (2B4, clone 2 69), anti-CD48-FITC (clone TŪ145), anti-CD11a/LFA-1-PE (clone G43-25B), anti-FasL-biotin (clone NOK-1), anti-perforin-FITC (clone δG9), and-CD158b-PE (KIR2DL2/3, clone CH-L); cell viability was determined by staining with Via-Probe™ (7AAD). Intracellular staining was performed on cells that were permeabilized and fixed using BD Cytotfix/Cytoperm™. Above antibodies and reagents were purchased from BD Biosciences Pharmingen (San Diego, CA) and were used according to manufacturer's specifications. 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) and anti-NKG2C-PE (CD94/CD159c, clone 134591) were purchased from R&D Systems (Minneapolis, MN). Anti-KIR3DL1-PE (clone DX9) was obtained from BioLegend Inc. (San Diego, CA). Cells were also stained with their corresponding isotype-matched control monoclonal antibodies.

Cytotoxicity assays

Standard ⁵¹Cr-release assays were performed as previously described (17) with the following modifications: after 5-hour 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).

Assay for cytokine production by NK cells co-cultured with K562 and RCC target cells

One hundred thousand NK cells expanded for 14 days in Baxter bags under GMP conditions or NK cells that were expanded in tissue culture flasks were washed twice in X-VIVO 20 medium and plated into triplicate wells in 96-well tissue culture plates with 10⁴ K562 or RCC target cells in 200 µL of X-VIVO 20 containing 10% human AB serum and 2 mM GlutaMAX-1. RCC cells were left untreated or were treated with 10 nM bortezomib (Millennium Pharmaceuticals, Cambridge, MA) for 16 hours prior to co-culture with expanded NK cells. After 5 hours of incubation at 37°C supernatants were collected, centrifuged, and cleared supernatants were stored at -20°C. Beadlyte® Human Multi-Cytokine Beadmaster™ Kit and Beadmates™ were obtained from Millipore Corporation (Billerica, MA) and used according to manufacturer's specifications. Data was acquired on a Luminex IS100 (Luminex Corp, Austin, TX) and analyzed using MasterPlex QT 3.0 (MiraiBio Group, Hitachi Software Engineering America, South San Francisco, CA). The same culture supernatants were also analyzed by Quantikine® ELISA (R&D Systems, Minneapolis, MN) according to manufacturer's instructions.

IL-2 withdrawal from expanded NK cells

NK cells which were expanded for 13 and 19 days with EBV-LCL feeder cells were washed twice in X-VIVO 20 and cultured at 10⁶ cells/mL in medium without IL-2 or in media containing 5, 50, or 500 IU/mL of IL-2 for 24 hours. Cells were assessed for viability with 7AAD and CD56, CD16, TRAIL and NKG2D expression by flow cytometry. Lytic capability of NK cells incubated with 5, 50, or 500 IU/mL of IL-2 or without IL-2 against K562 and RCC

target cells was determined by ^{51}Cr -release assays. Cytokine secretion was measured in culture supernatants with a Millipore Kit or Quantikine® ELISA as above.

Treatment of tumor cells with bortezomib

RCC cells were seeded into 10 cm² tissue culture dishes in 12 mL of culture medium; 24 hours later 10 nM of bortezomib was added. After 16-18 hours, RCC cells were trypsinized, washed in DMEM and used in cytotoxicity assays.

Results

Expansion kinetics of NK cells

Previously, small scale laboratory-based experiments showed that NK cell lines could be expanded *in vitro* using a variety of different methods (16,17). We sought to optimize conditions for large scale NK-cell expansions using GMP conditions for NK-cell-based clinical trials in humans with cancer.

When allogeneic PBMC were used as feeder cells, NK cells were most efficiently expanded by 25 Gy-irradiated feeder cells added to cultures at a 20:1 ratio in culture medium containing 500 IU/mL IL-2 and 10% single donor or pooled plasma in upright culture flasks or Baxter bags at a starting density of 1.0×10^6 NK cells/mL in 6.5% CO₂. Under these conditions, up to 100 fold increase in cell number was achieved in 15 days, and after a second round of expansion for an additional 14 days, increases of up to 200 to 400 fold could be achieved, although results varied depending on the NK cell donor (Fig. 1A). Cryopreservation and subsequent thawing of purified NK cells before the start of expansion did not affect the expansion kinetics of NK cells compared to NK cells that were isolated and expanded fresh from the blood.

We next evaluated if EBV-LCL (EBV-TM-LCL) that had been previously manufactured under GMP conditions would achieve more efficient and consistent NK cell yields. Freshly-isolated or cryopreserved and thawed non-expanded NK cells were cultured in upright 75 cm² flasks in the presence of irradiated EBV-TM-LCL cells at a 20:1 feeder-to-NK cell ratio. NK cells from 5 normal donors cultured for 16 days expanded 815 to 3,267 fold (Fig. 1B).

To facilitate conditions for expanding NK cells on a larger scale under GMP, we next optimized NK cell expansions in bags rather than flasks. NK cells isolated from 4 normal donors and a sarcoma patient who had previously undergone an autologous transplant were co-cultured with EBV-TM-LCL feeder cells. The total yield of NK cells in bags was comparable to yields obtained when NK cells were grown in flasks (Fig. 1C).

Phenotype of resting vs. expanded NK cells

We next evaluated for phenotypic changes associated with expanding NK cells *in vitro*. Resting and expanded NK cells were analyzed by flow cytometry at baseline and ≥ 10 days following *in vitro* expansion.

NK cells enriched from PBMCs by immunomagnetic bead selection contained 1-30% monocytes, <1% CD3⁺ T cells, no CD56⁺/CD3⁺ cells, no CD19⁺ B cells and 70-92% CD56⁺/CD3⁻ NK cells. Resting CD56⁺ NK cells did not express TRAIL, FasL or NKG2C, while NKG2D, LFA-1, CD244, CD48, perforin and granzymes A and B were constitutively expressed. CD25 expression varied amongst donors but was typically low or absent on resting NK cells.

NK cells obtained from 9 different donors and expanded over 10-22 days had a mean expression of CD56⁺/CD16⁺ and CD56⁺/CD16⁻ of 84.3±7.8% (range of 66.5-97.5%) and 14.7±7.7% (range of 2.1-31.9%) respectively and did not contain CD56⁻/CD16⁺ populations. After expansion, there was a substantial increase in NK-cell surface expression of CD56, TRAIL, NKG2D, CD48, and CD25; on expanded vs. resting NK cells from 3 different donors, CD56 expression increased from a median 85.3±3.4% to 99.3±0.3% [mean CD56 fluorescence intensity (MFI) increased from 70.4±39.9 to 470.6±66.6], TRAIL expression increased from a median 0.6±0.4% to 80.8±15.4% (mean TRAIL MFI increased from 6.0±5.1 to 37.9±3.2), NKG2D surface expression increased from a median MFI of 48.3±16.3 to 432.0±70.9, CD48 surface expression increased from a median MFI of 36.9±9.1 to 121.0±38.8, and CD25 expression increased from a median 2.3±1.6% to 48.6±19.7% (mean CD25 MFI increased from 4.8±1.8 to 20.7±6.5). Expression of perforin did not change, although there was a small but consistent increase in surface expression of LFA-1, FasL, NKG2C, CD244 and intracellular expression of granzymes A and B respectively (Fig. 2A). Surface expression of the NK-cell inhibitory receptor CD158b increased in expanded NK cells; compared to fresh NK cells, the MFI of CD158b increased 1.7±0.4 and 3.7±0.0 fold in NK cells expanded for 10 and 22 days respectively. The MFI of NKG2A and KIR3DL1 remained unchanged, although the percent of expanded NK cells expressing NKG2A increased 3.7±1.8 fold (Fig. 2B).

Cytotoxic function of expanded NK cells

We next evaluated the lytic effects of expanded vs. resting non-expanded NK cells against K562 and RCC cell lines.

NK cells expanded in culture from 10 to 21 days consistently demonstrated increased cytotoxicity against K562 and RCC cells compared to resting NK cells (Fig. 3A). At a 1:1 effector-to-target ratio, lysis of RCC cells was significantly higher with expanded NK cells (27.6±9.3%) compared to resting NK cells (3.4±2.1%) (p=0.005).

Treatment of RCC cells with proteasome inhibitor bortezomib has previously been shown to upregulate surface expression of the TRAIL death receptor DR5 (TRAIL-R2), which sensitizes tumors to NK-cell cytotoxicity (6-8). Therefore, we compared lysis by resting vs. expanded NK cells against bortezomib-treated vs. untreated RCC cells. Lysis of RCC cells by both resting and expanded NK cells was augmented by pre-treating tumor cells with bortezomib for 16 hours. However, in contrast to resting NK cells, there was a dramatic increase in bortezomib-treated tumor killing by expanded NK cells; at a 1:1 effector-to-target ratio, resting NK cells lysed 3.4±2.1% and 5.0±2.7% (p=0.44, unpaired t-test) of untreated and bortezomib-treated RCC tumor cells respectively compared to NK cells expanded for 12-18 days which killed 27.6±9.3% and 55.8±8.3% (p=0.001, unpaired t-test) of untreated vs. bortezomib-treated RCC tumor cells respectively (Fig. 3B).

Cytokine secretion profiles of NK cells

We next compared cytokine secretion profiles of freshly-isolated vs. expanded NK cells. Resting cells spontaneously produced very low levels of TRAIL, MIP-1α, MIP-1β, and high amounts of IL-1ra. Co-culture with K562 target cells for 5 hours in the absence of IL-2 induced NK cell secretion of TNFα, IFNγ, GM-CSF, FasL, MIP-1α, MIP-1β, and IL-1ra but not TRAIL. NK cells expanded for 14 days spontaneously secreted IL-2, IFNγ, GM-CSF, FasL, TRAIL, MIP-1α, MIP-1β but not IL-1ra (the only cytokine secreted by resting but not expanded NK cells). With the exception of IL-2 and TRAIL, the secretion of the above cytokines was augmented by co-culturing expanded NK cells with K562 and RCC target cells (Fig. 4A and B). RCC cells pretreated with bortezomib stimulated NK cells to produce higher levels of TNFα, whereas the secretion of other cytokines remained unchanged (Fig. 4B). There was no

spontaneous TNF α release from K562 and RCC cells. In these experimental conditions, neither resting nor expanded NK cells produced IL-1 α , IL-1 β , TNF β , IL-10, G-CSF, and IL-13.

The effect of IL-2 deprivation on expanded NK cells

Whether exogenous IL-2 would be required to support NK-cell cytotoxicity and proliferation following adoptive NK-cell infusions in humans is unclear. Thus, we evaluated the effects of IL-2 withdrawal and add-back on the phenotype and function of expanded NK cells. TRAIL expression on expanded NK cells declined rapidly in association with IL-2 deprivation; there was a decline in both the percentage of NK cells expressing TRAIL ($68.3 \pm 23.5\%$ to $26.3 \pm 13.1\%$) and in TRAIL MFI (52.2 ± 24.0 to 18.4 ± 2.9) within 16-24 hours of IL-2 removal from the medium. TRAIL expression was restored by the subsequent addition of IL-2 back into the medium, and was IL-2 dose dependent (data not shown). Similar to TRAIL, the MFI of NKG2D expression in expanded NK cells declined significantly (2.1 ± 0.2 fold) 24 hours following IL-2 removal from the medium. After the addition of IL-2, NKG2D expression was restored in a dose-dependent manner.

Reductions and subsequent increases in TRAIL and NKG2D surface expression that occurred with the removal and addition of IL-2 directly correlated with NK-cell cytotoxicity against K562 and RCC target cells (Fig. 5A). Culturing previously expanded NK cells in media containing no or low doses of IL-2 (0-5 IU/mL IL-2) for 24 hours resulted in a substantial decline in NK-cell cytotoxicity against K562 and RCC target cells compared to cultures containing 50-500 IU/mL of IL-2 where cytotoxicity was maintained. Likewise, spontaneous secretion of FasL and TRAIL and multiple cytokines, including GM-CSF, TNF α and IFN γ was also IL-2 dose dependent, declining rapidly in cultures in which the concentration of IL-2 was decreased or where IL-2 was removed. (Fig. 5B). Expanded NK cells did not secrete IL-1 α , IL-1 β , IL-10, G-CSF and TNF β regardless of IL-2 content in culture medium. In one of 4 donors, IL-13 was detected (260-280 pg/mL) in cultures of expanded NK cells when 50 and 500 U/mL of IL-2 were added for 24 hours (data not shown).

The effect of cryopreservation on phenotype and function of expanded NK cells

In order to assess the impact of cryopreservation, the phenotype and cytolytic function against K562 and RCC cells of expanded vs. cryopreserved NK cells was compared. Lysis of untreated and bortezomib-treated RCC cells by expanded thawed NK cells was significantly lower compared to lysis by non-frozen expanded NK cells (Figure 6A). Lysis of K562 cells by thawed NK cells was also diminished, although this effect was only evident at 1:1 and 0.5:1 effector-to-target ratios (data not shown). Decreased cytotoxicity of thawed NK cells against tumor targets correlated with their reduced surface expression of both TRAIL and NKG2D, together with an increase in the percentage of cells that were either negative or had dim expression of CD16 (Fig. 6B). Expanded NK cells maintained in culture contained $89.2 \pm 2.7\%$ CD56 $^{+}$ /CD16 $^{+}$ ($88.0 \pm 9.6\%$ co-expressed TRAIL) and $7.4 \pm 0.3\%$ CD56 $^{+}$ /CD16 $^{-}$ ($62.4 \pm 10.9\%$ co-expressed TRAIL) while thawed cells were $57.9 \pm 24.6\%$ CD56 $^{+}$ /CD16 $^{+}$ double positive and $35.4 \pm 20.6\%$ CD56 $^{+}$ /CD16 $^{-}$ with only $27.7 \pm 4.9\%$ of double positive cells co-expressing TRAIL. Incubation of thawed cells in medium containing 500 IU/ml of IL-2 for 6 hours increased NK cytolytic function and surface expression of NKG2D and TRAIL to about 50% of baseline (data not shown) while 16 hour treatment with IL-2 restored NKG2D and TRAIL and cytotoxicity to levels seen with non-frozen cells (Fig. 6A and B). Although the addition of IL-2 to medium was able to restore NK-cell cytotoxicity, the viability of thawed NK cells (assessed by 7AAD staining) declined from 93-97% immediately after thawing to 38-50% at 16 hours. This decline in thawed NK-cell viability did not correlate with the time NK cells were maintained in culture prior to cryopreservation. These results suggest that expanded NK cells that have been cryopreserved may require culturing in IL-2-containing medium following thawing to restore function prior to infusion in patients. However, the substantial decline in

viability of thawed NK cells rescued with IL-2-containing medium highlights the limitation of this approach.

Discussion

Although there has been increased interest in exploring the anti-tumor effects of adoptively-infused NK cells in cancer patients, the small number of cells isolated following a typical apheresis procedure has precluded trials assessing for a relationship between NK cell dose and tumor response. Here we present a functionally closed *in vitro* system using irradiated EBV-LCL feeder cells resulting in the large scale expansion of highly cytotoxic clinical grade NK cells.

In contrast to NK cell expansion protocols that require culturing in plastic flasks and multiple rounds of stimulation with feeder cells, the expansion technique presented here utilized sterile bags, required only a single round of stimulation with irradiated EBV-LCL feeder cells, and achieved substantial NK expansions in the range of 250-850 fold over a 2-3 week interval. With a starting population of 200 million immunomagnetic bead-purified CD3⁺/CD56⁺ NK cells isolated after a typical 15 liter apheresis, this expansion technique would achieve a final NK-cell product in the range of 3×10^{10} cells, a number that would seem sufficient for phase I studies. To address the safety of using EBV-LCL cells for NK-cell expansion, 3 expanded NK-cell products were tested by *in situ* hybridization for EBV-encoded early small RNAs (EBER) and were all found to be negative. The TM-LCL feeder cell line used here to expand NK cells was previously used by others to expand T-cell lines *in vitro* utilizing GMP-compliant components (18,19). To avoid expanding T cells which proliferate rapidly under these culture conditions (data not shown), a 2 step CD3⁺ T cell depletion followed by a CD56⁺ selection was used to enrich for an NK- cell population that typically had <0.5% T-cell contamination.

The most efficient large scale NK-cell expansions were achieved when cells were cultured in Baxter "Lifecell" bags. In contrast, cultures generated in Teflon-coated bags resulted in relatively limited NK-cell expansions (data not shown). The viability and expansion rates of NK cells were at their greatest 9 to 15 days following the initiation of cell cultures and declined after 21 days. Several attempts to re-expand NK cells with EBV-LCL feeder cells after cells had been cultured for ≥ 14 days were mostly unsuccessful. Regardless of culture vessels used for expansions of NK cells, the phenotype and lytic activity of the expanded cells were similar.

Although NK cells can be activated by IL-2, IL-2 alone fails to expand NK cells *in vitro*. In contrast, NK cells stimulated with EBV-LCL feeders expanded dramatically, had an activated phenotype, and as a consequence of upregulated expression of NKG2C, NKG2D, FasL, TRAIL and granzymes A and B, were significantly more cytotoxic against tumor cells compared to fresh NK cells. We also observed that expression of CD244 (2B4) and CD48 was augmented in expanded compared to resting NK cells. The function of CD48 and CD244 on expanded human NK cells is not entirely understood. Although one study reported increased expression of CD244 could have an inhibitory effect on the function of NK cells (20), murine data have shown that homotypic interactions between these molecules prevent fratricide and enhance NK cell expansion and cytolytic activity (21).

Compared to non-expanded NK cells, expanded NK cells secreted either spontaneously or following co-culture with tumor targets (K562 and RCC cells) higher levels of IFN γ , IL-2, FasL and TRAIL. In contrast, non-expanded NK cells secreted higher levels of IL-1 α which was not produced by expanded cells. An unexpected and previously unobserved finding was that TNF α secretion increased when NK cells were co-cultured with bortezomib-treated RCC cells. The biologic significance of this finding is unknown, although TNF α can be directly cytotoxic to tumor cells and can have a positive immunoregulatory function, inducing dendritic

cell maturation, activation, and Ag cross-presentation resulting in augmented T-cell cytokine secretion (22,23). Contrary to previous reports, only very low levels of IL-10 were detected in expanded NK cells cultured in IL-2. In contrast to when IL-12 is combined with IL-2, IL-2 alone is a weak stimulator of IL-10 secretion. IL-10 has been shown to have anti-inflammatory effects, inhibiting macrophage and DC activation and maturation and secretion of multiple pro-inflammatory cytokines (24,25). Therefore, lack of IL-10 secretion would seem desirable when expanded NK cells are used in the context of tumor immunotherapy.

The net effect of changes in NK cell phenotype and cytokine secretion resulted in expanded NK cells having markedly higher levels of cytotoxicity against tumor cells compared to non-expanded cells. Although, it is likely that both an increase in expression of activating receptors and molecules that induce tumor apoptosis (i.e. TRAIL, FasL, granzyme B, etc.) in expanded NK cells contributed to their enhanced cytotoxicity, blocking experiments to define the exact contribution of individual pathways to augmented NK-cell cytolytic function were not performed in this analysis.

Previously, we and others have shown that the proteasome inhibitor bortezomib enhances TRAIL-mediated cytotoxicity against tumor cells *in vitro* (26,27) and *in vivo* (6-8). In experiments conducted in this study, we observed that lysis of bortezomib-treated RCC tumors was dramatically higher with expanded compared to resting NK cells, providing strong evidence that increased surface expression of TRAIL on expanded NK cells substantially augmented their tumor lysis at least in part via TRAIL apoptotic pathways. In contrast, it is unlikely that changes in NK cell inhibitory receptors played any role in augmenting NK-cell cytotoxicity, as CD158b/KIR2DL2/3, KIR3DL1 and NKG2A expression remained unchanged or increased slightly following NK cell expansion.

The changes in phenotype and maintenance of cytotoxicity against tumor cells by expanded NK cells were dependent on IL-2. Withdrawal of IL-2 from expanded NK cell populations rapidly resulted in substantial reductions in NK-cell killing of tumor cells. Whether the exogenous administration of IL-2 would be required to maintain high levels of NKG2D, TRAIL, and tumor cytotoxicity *in vivo* of adoptively-infused expanded NK cells is currently being investigated in an animal model.

The ability to cryopreserve and subsequently thaw NK cells while maintaining their cytolytic activity could logistically facilitate clinical trials evaluating multiple rounds of adoptive NK cell infusions. Although expanded NK cells that were frozen then subsequently thawed maintained high viability, their cytolytic capacity was substantially lower than that of expanded NK cells that had never undergone cryopreservation. Thawed NK cells had lower surface expression of TRAIL and NKG2D and were more likely to contain populations that were dim or negative for CD16. These findings suggest thawed adoptively-infused NK cells might have reduced cytotoxic potential compared to expanded NK cells that are maintained fresh in culture. Importantly, the cytotoxicity of expanded NK cells that were frozen then thawed could be rescued by culturing in IL-2-containing medium for 16 hours, although the overall viability of these populations was lower than that of non-thawed cells.

In conclusion, we show a method for the large scale production of *in vitro*-expanded NK cells using irradiated EBV-LCL feeder cells and a functionally closed “bag-based” culture system. *In vitro*-expanded NK cells had altered cytokine secretion profiles, were phenotypically distinct from non-expanded NK cells and were significantly more cytotoxic to tumor cells.

Expanded cells had increased surface expression of the NKG2D and TRAIL and greatly enhanced TRAIL-mediated cytotoxicity against bortezomib-treated tumors compared to non-expanded NK cells. Based on these findings, a phase I trial has recently been initiated in patients with advanced metastatic tumors and hematological malignancies to investigate the safety and

anti-tumor effects of escalating doses of adoptively-infused *ex vivo*-expanded autologous NK cells. NK-cell doses in this trial will range from 5×10^6 to 10^8 NK cells/kg and will be given every 3 weeks following treatment with bortezomib and concomitant with IL-2 administration.

Acknowledgments

This research was supported by the intramural research program of NIH, National Heart, Lung, and Blood Institute, Hematology Branch. We wish to acknowledge ACKC (Action to Cure Kidney Cancer) and The Dean R. O'Neill Memorial Fellowship for generous contributions supporting this research. The authors would also like to thank Dr. EJ Read, Dr. David Stroncek, Dr. Hanh Khuu, Vicki Fellows and Virginia David-Ocampo from the Department of Transfusion Medicine in NIH for their valuable contribution to the development of clinical grade NK-cell expansion protocols, Dr. Stefania Pittaluga (NIH/NCI) for performing EBER testing of NK cells, and Drs. Shelly Heimfeld, Brenda Sandmaier and Kimberly Boyt from Fred Hutchinson Cancer Research Center. The authors have no conflicting financial interests.

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Figure 1. Expansion kinetics of NK cells grown *ex vivo* under various conditions. NK cells were isolated from PBMC by immunomagnetic bead selection of CD56⁺/CD3⁺ cells. Irradiated PBMC were used as feeder cells for expansion of NK cells from 4 healthy donors in flasks (NK1772 and NK1257) and in Baxter bags (NK0772 and NK0155) [A]. NK cells from 5 healthy donors were co-cultured with irradiated EBV-TM-LCL cells in flasks at 20:1 feeder cells to NK cells ratio (B). NK cells grown in Baxter bags in the presence of EBV-TM-LCL feeder cells at 10:1 feeder cells to NK cells ratio in the Department of Transfusion Medicine under GMP conditions (C).

Figure 2. Flow cytometry analysis of freshly-isolated resting and expanded NK cells

NK cells were stained with indicated monoclonal antibodies immediately after isolation and after 12 days of expansion. Cell surface expression on viable cells is shown. Data from a representative experiment in 1 out of 3 donors are shown. The shaded areas represent negative isotype controls.

Figure 3. Specific lysis of K562 and RCC cell lines by resting non-expanded vs. expanded NK cells and the effect of bortezomib on NK-cell cytolytic function

^{51}Cr -release assays were performed using freshly-isolated, cryopreserved and thawed NK cells in parallel with 12 day-expanded cells from the same donor at the indicated effector-to-target ratios (E:T). Experimental results for 2 out of 3 donors are shown as mean $\pm$ SD (A). RCC tumor cells were treated with 10 nM bortezomib for 16 hours or left untreated. Percent specific lysis of tumor cells by freshly-isolated or expanded NK cells for 1:1 NK-to-target cell ratio from 3 donors was determined in a 5-hour ^{51}Cr -release assay (B).

Figure 4. Cytokine secretion profile of NK cells after co-culture with K562 and RCC target cells Resting NK cells or NK cells from 2 normal donors, expanded for 14 days, were cultured for 5 hours in 96-well plates in 200 μ L of NK cell growth medium (no IL-2) in triplicate either without target cells or with 10^4 K562 at 10:1 NK-to-target cell ratio (A). Expanded NK cells were cultured as above with K562 or RCC cells, which were untreated or treated with 10 nM bortezomib for 16 hours. Representative data for one out of 3 donors are shown (B). Cytokine content in cell-free supernatants was measured both by the Beadlyte[®] Human Multi-Cytokine Beadmaster[™] Kit and R&D Quantikine ELISA. ELISA data are shown for FasL, MIP-1 α and MIP-1 β , Luminex data are shown for other cytokines.

Figure 5. Cytotoxicity of expanded NK cells and their cytokine production after IL-2 deprivation
 IL-2 dose-dependent lysis of K562 and RCC cells with and without bortezomib treatment. The percent specific lysis of target cells was determined in a 5-hour ^{51}Cr -release assay. Data for NK cells from 2 normal donors are presented as mean $\pm$ SD (A). Cytokine secretion and TRAIL and sFasL release by expanded NK cells from one of 3 donors after culture in medium without IL-2 or with varying doses of IL-2. NK cells were expanded for 12 days, washed twice in X-VIVO 20 medium, and incubated at 10^6 cells/mL for 24 hours. Cell-free culture supernatants were harvested and assayed for cytokine secretion as described in the figure 4 legend (B).

Figure 6. Cytolytic function of expanded NK cells after cryopreservation and thawing; correlation with CD56/CD16, TRAIL and NKG2D expression

Expanded NK cells were cryopreserved, then subsequently thawed and analyzed in parallel with cells maintained in culture or thawed cells incubated for 16 hours in medium with 500 U/mL of IL-2. Chromium release assay data for 1:1 ratio of NK cells from 2 normal donors to K562 cells and untreated or bortezomib-treated RCC target cells are presented as mean $\pm$ SD (A). Flow cytometry analysis of expanded NK cells. Cell viability was assessed by Trypan Blue stain exclusion and 7AAD staining. Representative flow cytometry data for NK cells from one of the above donors are shown. Dot plots show the percent of viable cells and the gates, and numbers in histograms represent the mean fluorescence intensity (B).