Bringing natural killer cells to the clinic: ex vivo manipulation

Richard W. Childs¹ and Maria Berg¹

¹Section of Transplantation Immunotherapy, Hematology Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD

Recently, there has been a substantial gain in our understanding of the role that natural killer (NK) cells play in mediating innate host immune responses against viruses and cancer. Although NK cells have long been known to be capable of killing cancer cells independently of antigen recognition, the full therapeutic potential of NK cell–based immunotherapy has yet to be realized. Here we review novel methods to activate and expand human NK cells *ex vivo* for adoptive transfer in humans, focusing on the important phenotypic and functional differences observed among freshly isolated, cytokine activated, and *ex vivo*–expanded NK populations.

Natural killer cell therapy for cancer: a new hope

The ability of natural killer (NK) cells to kill tumor cells without the need to recognize a tumor-specific antigen provides advantages over T cells and makes them appealing for investigation as effectors for immunotherapy.^{1,2} There has recently been a substantial gain in our understanding of the role that NK cells play in mediating innate host immune responses against viruses and cancer. Although NK cells have long been known to be capable of killing cancer cells independently of antigen recognition, the full therapeutic potential of NK cell–based immunotherapy has yet to be realized. Here we review novel methods to activate and expand human NK cells *ex vivo* for adoptive transfer in humans, focusing on the important phenotypic and functional differences observed among freshly isolated, cytokine activated, and *ex vivo*–expanded NK populations.

Ex vivo NK cell activation and expansion

Unlike T cells, NK cells represent only a minor fraction of human lymphocytes. NK cells are defined by their CD3[−]/CD56⁺ phenotype, comprising 5% to 15% of circulating lymphocytes, and are commonly divided into CD56^{dim}CD16⁺ (90%) and CD56^{bright}CD16[−] (10%) subpopulations with distinct effector functions. Recently, investigators have shown that NK cell tumor cytotoxicity can be enhanced by disrupting NK cell signaling through inhibitory receptors such as KIR, PD-1, and NKG2A.^{3,4} Furthermore, exposing tumors to drugs that down-regulate ligands for NK cell inhibitory receptors or up-regulate death receptors for NK cell effector molecules or ligands that bind NK cell–activating receptors can be used as a strategy to sensitize tumors to NK cell–mediated apoptosis. Recently, anti-PD-1 and anti-PD-L1 monoclonal antibodies and the immunomodulatory drug lenalidomide were shown to enhance both NK cell tumor trafficking and NK cell–mediated antibody-dependent cell-mediated cytotoxicity, and cytokine release against tumors while simultaneously suppressing regulatory T cell function.^{5–8} Similarly, anti-CTLA-4 monoclonal antibodies have been shown to augment NK cell antibody-dependent cell-mediated cytotoxicity and TNF α release against melanoma cells by Fc γ RIII (CD16) binding to antibody-bound tumor cells, as well as through regulatory T cell inactivation.^{9–11} These data suggest these agents could be used in conjunction with autologous NK cell infusions as a method to potentiate NK cell–mediated antitumor effects in patients with advanced cancers. Further, recent advances in our ability to expand NK cells *ex vivo* have fueled translational

research investigating a variety of novel methods to bolster immunity against cancer through the use of adoptive NK cell infusions.^{12–16}

Until recently, the therapeutic potential of NK cell–based immunotherapy remained largely unexplored because the small number of NK cells isolated after a typical apheresis procedure and the inability to reliably expand large numbers of NK cells *ex vivo* precluded investigators from pursuing phase 1 trials evaluating for an NK cell dose-response relationship. At present, it is not at all clear what threshold of NK cell numbers is needed to achieve an antitumor effect after adoptive NK cell transfer. Short- and long-term cell cultures containing cytokines without feeder cells, such as IL-15 and IL-2, given alone or in combination with other growth factors, typically result in relatively small *ex vivo* NK cell expansions.^{17–21} Although some investigators have observed differences in their growth rate, in our experience, NK cells obtained from cancer patients proliferate *ex vivo* similarly to those obtained from healthy donors.^{22,23} Because adoptive NK infusions as tumor immunotherapy remain in a proof-of-concept phase, with allogeneic infusions often being given after immunosuppressive chemotherapy or after an HLA-mismatched transplantation, most investigators have pursued methods to expand highly purified NK cells so that both the efficacy and any toxicities of the infused product can be directly attributable to NK cells themselves.^{12,18,24–29} In general, to prevent T-cell and NKT-cell contamination and overgrowth, T cells are usually depleted from PBMCs either before the initiation of NK cell cultures or at the end of the expansion culture.³⁰ Strategies to activate and/or expand NK cells for adoptive infusion in humans with cancer are summarized in Figure 1.

Ex vivo cytokine-activated NK cells

Although culturing NK cells in cytokine-containing medium alone is less effective in expanding NK cells compared with cultures containing feeder cells, such culture conditions are capable of activating NK cells quickly, even after a short overnight incubation, substantially enhancing NK cell cytotoxicity against tumor targets *ex vivo*. Miller et al used a strategy of CD3 depletion of mononuclear cells (using the Miltenyi CliniMACS system) collected by apheresis from haploidentical donors, followed by a brief 8- to 16-hour culture in X-VIVO15 medium containing IL-2 (1000 U/mL), with activated cells being infused into patients after haploidentical stem cell transplantation or after no transplantation³¹ in patients with relapsed hematological malignancies and solid tumors including breast cancer and ovarian cancer.³² Products such

Figure 1. Methods to activate and/or ex vivo expand human NK cells for infusion in patients with cancer.

as these, although devoid of T cells, only contain on average ~ 40% NK cells and may have substantial numbers of contaminating monocytes and B cells. The addition of a CD56⁺ selection after CD3⁺ T-cell depletion typically improves NK cell purity to the 90% range and reduces B-cell contamination to less than 1%.³³ This approach is more effective in avoiding T-cell contamination and is more commonly used in studies exploring adoptive transfer of NK cells after allogeneic haploidentical transplantation, where T-cell contamination could lead to lethal GVHD.^{12,28,34,35} It is important to consider that when CD3 depletion alone is used to enrich for overnight-activated NK cells, B-cell contamination can lead to passenger B-lymphocyte-mediated complications such as EBV-lymphoproliferative disorder and, when products are infused in the context of minor ABO incompatibility, acute hemolytic anemia. To avoid this complication, the University of Minnesota, which has the most experience with the use of IL-2-activated NK cells, now incorporates both CD3⁺ T-cell and CD19⁺ B-cell depletion on overnight IL-2-activated NK cell products before their infusion.³³

Ex vivo NK cell expansion without feeder cells

Some clinical studies of adoptive NK cell transfer have used short-term (12-18 hours) IL-2-activated NK cells.³² IL-2 alone expands small numbers of NK cells, typically 10- to 20-fold after 14 days of cell culture, far less than murine studies predict would be needed to mediate antitumor effects in humans with cancer. Sutlu et al recently developed a unique method to expand moderate numbers of clinical-grade NK cells in an automated bioreactor without the need for feeder cells.³⁶ When this technique was used, human NK cells expanded a median 77-fold after 21 days of cell culture from unsorted PBMCs. These cells were activated ex vivo, being more cytotoxic against K562 target cells than those that were expanded in flasks, and expressed higher levels of the natural cytotoxicity receptor NKp44. Expanded cell cultures contained 10% to 80% (average 38%) CD3⁺/CD56⁺ NK cells with significant numbers of

CD3⁺/CD56⁺ T cells and/or CD3⁺/CD56⁺ NK cell-like T cells, potentially obligating the use of T-cell depletion on these expansion products before they could be used in the allogeneic setting.²⁵ Alici et al similarly showed that highly activated NK cells could be expanded from unseparated PBMCs of myeloma patients using media containing cytokines and the anti-CD3 antibody OKT3 (removed from cultures after day 5).³⁷ Day 20 cultures contained an average of 65% NK cells, which expanded a median 1625-fold (range 502-2658), although T-cell contamination in cultures averaged 22% with this approach.

Recently, Frei et al reported on a method to expand clinical-grade NK cells from CD3-depleted apheresis products in feeder cell-free medium containing nicotinamide (NAM), a specific inhibitor of NAD⁺-dependent enzymes.³⁸ In MEM α medium supplemented with IL-2, IL-15, and NAM (at concentrations of 2.5-7.5 mM), NK cells cultured in G-Rex flasks (Wilson Wolf) expanded by 60- to 80-fold in 2 weeks. Remarkably, no medium changes or manipulation of the cell cultures were required during the 2-week expansion process, with expanded cells containing a highly pure population of activated NK cells (> 95% CD3⁺/CD56⁺). NK cells expanded using this approach underwent typical phenotypal and functional changes observed with cytokine-induced NK cell activation, including up-regulation of TRAIL and enhanced cytotoxicity against K562 and other tumor cell lines, compared with fresh NK cells. Remarkably, NK cells expanded with NAM-containing media were found to substantially up-regulate surface expression of the homing receptor CD62L (L-selectin), which typically decreases during ex vivo NK cell activation with cytokine-alone-containing medium. Adoptive infusion of human NK cells expanded using this technique into irradiated NOD/SCID mice had improved homing to the BM compared with NK cells expanded in medium without NAM, perhaps the consequence of CD62L up-regulation.

Ex vivo NK cell expansion using feeder cells

Several different methods (Figure 1) using feeder cells or APCs have been developed recently to expand large numbers of highly activated NK cells *ex vivo*, providing the opportunity to study the full potential of adoptive NK cell immunotherapy in humans. Irradiated PBMCs, Epstein-Barr virus-transformed lymphoblastoid cell lines (EBV-LCL), gene-modified K562 cells expressing NK cell-stimulatory molecules such as 4-1BB ligand and membrane-bound IL-15, and other irradiated tumor cell lines are most commonly used as feeder cells in NK cell expansion cultures (Table 1).^{2,26,39-44} Typically, these cultures are initiated using an enriched NK cell population isolated from apheresis products that have undergone CD3⁺ T-cell depletion with or without CD56⁺ selection to maximize NK cell purity. Utilization of feeder cells in NK cell cultures can dramatically enhance NK cell expansion numbers *ex vivo*, and this technique has been used recently for the large-scale expansion of clinical-grade NK cells.^{27,30,41,45} Substantial phenotypic and functional differences are observed between freshly isolated, IL-2-activated, and *ex vivo*-expanded NK populations, which theoretically could have an impact on their therapeutic efficacy after adoptive infusion.^{30,41,45,46} Criteria for the release of cytokine-activated and *ex vivo*-expanded NK cell products vary among institutions and depend upon the clinical setting in which they are used. For example, protocols using allogeneic NK cell infusions given from HLA-mismatched donors will often restrict contaminating total T cells/kg to $< 1.5 \times 10^5$ to prevent the possibility of severe GVHD,^{28,47} whereas NK cells given in the context of an autologous infusion may be more permissive of T-cell contamination. With expanded NK cell products, many centers will perform sterility cultures 24 hours before and the day of product release, as well as a gram stain, PCR for mycoplasma, testing for endotoxin, and flow cytometry. At the National Heart, Lung, and Blood Institute (NHLBI), NK cells expanded using EBV-LCL are required on the day of release to contain at least 90% NK cells (CD3⁻/CD56⁺), have less than 5% contaminating CD3⁺ T cells and CD19⁺ B cells, and a viability of at least 70% as measured by 7-amino-actinomycin D (7-AAD) staining.⁴⁸

NK cells expanded using irradiated EBV-LCL feeder cells.

EBV-LCL cells have been known for decades to induce NK cell proliferation *in vitro*.^{39,49,50} Preclinical data from our laboratory established that large numbers of highly pure NK cells (99% CD3⁻/CD56⁺) could be expanded from PBMCs enriched for NK cells using Miltenyi immune-magnetic beads in which cells first underwent CD3⁺ T-cell depletion followed by CD56⁺ NK cell selection.⁴¹ Optimal NK cell expansions were obtained by coculturing enriched NK cells with a clinical-grade 100 Gy-irradiated EBV-LCL feeder cell line (TM-LCL 20:1 feeder-to-NK cell ratio) in X-VIVO 20 medium containing 10% human AB serum and 500 IU/mL of IL-2, with NK cells continuing to expand in cultures for up to 28 days. Using this technique, expansions of NK cells in the range of 800- to 1000-fold could be achieved in 2 weeks in a closed system using Baxter PL732 bags. EBV-LCL feeder cell eradication from 2-week cell cultures was confirmed by absence of detectable EBV-encoded early small RNAs. *Ex vivo* expansion led to a significant increase in NK cell surface expression of CD56, TRAIL, FasL, NKG2D, NKp30, NKp44, and NKp46 compared with resting NK cells (Figure 2A). Expression of perforin did not change, although there was a small but consistent increase in the intracellular expression of granzymes A and B and surface expression of LFA-1, NKG2C, CD244, and CD158b. Compared with nonexpanded NK cells, expanded NK cells also secreted (either spontaneously or after coculture with tumor targets K562 and

RCC cells) higher levels of IFN γ , IL-2, FasL, and TRAIL. The net effect of changes in NK cell phenotype and cytokine secretion resulted in expanded NK cells having markedly higher levels of cytotoxicity against K562 and various other tumor cell lines compared with resting or overnight IL-2-activated NK cells (Figure 2B).⁴⁶ Whether enhanced cytotoxicity occurred due to an increase in expression of NK cell activating receptors or was more the consequence of expanded NK cells having increased levels of molecules that induce tumor apoptosis (ie, TRAIL, FasL, granzymes, etc) is unclear.

Based on these preclinical data, we scaled this method up for a clinical trial with an expansion approach that used Good Manufacturing Practice (GMP) conditions and a third-party irradiated allogeneic EBV-LCL feeder line (initially TM-LCL produced by the Beckman Research Institute at City of Hope, and then later SMI-LCL produced by the PACT group for the NHLBI) with IL-2-containing medium to expand large numbers of highly purified, highly activated NK cells from patients with cancer using a "closed bag" culture system. We are currently performing a clinical phase 1 trial investigating the safety and efficacy of infusing large numbers of these *ex vivo*-expanded autologous NK cells after treatment with bortezomib to sensitize tumors to NK cell cytotoxicity.^{14,51,52} In this study, patients undergo a 15 L apheresis to isolate NK cells that were enriched using the Miltenyi CliniMACS to deplete CD3⁺ T cells followed by CD56⁺ selection. These enriched NK cells are then frozen in multiple aliquots and can be used for subsequent thawing and *ex vivo* expansion. First, $5\text{--}12 \times 10^7$ enriched NK cells are placed in culture and are then expanded *ex vivo* in Baxter bags over 14-27 days using the SMI-EBV-LCL feeder cell line as above. After treatment with bortezomib, cohorts 1 through 4 received a single infusion of *ex vivo*-expanded NK cells on day 0 in a dose-escalating fashion (up to a dose of 1×10^8 NK cells/kg). Cohorts 5-7 received 1×10^8 NK cells/kg on day 0 and a second escalating dose of NK cells (from the same NK cell culture) infused on day +5 (up to a dose of 1×10^9 NK cells/kg, respectively). Patients with stable disease or regression were eligible to receive additional cycles of therapy. A total of 78 adoptive NK cell infusions have been given to 26 patients with a variety of different cancers.⁴⁸ Among the 82 NK cell cultures initiated, 78 (95%) expanded successfully to achieve the target NK cell dose, 2 cultures failed to expand sufficiently to meet the targeted NK cell number, and 2 cultures were discarded due to contamination (one each with fungal and bacterial contamination). NK cells on the day of harvest expanded a median 198-fold (range 58-750), 895-fold (range 149-6647), and 3637-fold (range 1298-20 196) on culture days 14-16, 19-22, and 24-27, respectively (Figure 3A). NK cells at harvest contained a median 99.7% (range 92%-100%) CD3⁻/CD56⁺ NK cells and had a median 87% (range 71%-93%) viability assessed by 7-AAD on the day of harvest (Figure 3B). This study has established that large numbers of highly pure clinical-grade NK cells can reproducibly be expanded *ex vivo* using irradiated EBV-LCL feeder cells with NK cells expanding a median 3637-fold after 19-22 days of *ex vivo* culture. With the exception of thyroiditis and one patient who developed transient hypoxia after the infusion of 2.5×10^8 NK cells/kg on day +5, infusions of these *ex vivo*-expanded NK cells have been well tolerated. This study continues to dose escalate, with additional cohorts intended to establish whether expansions up to a dose of 1×10^9 NK cells/kg are technically feasible and can be infused safely into patients. However, because the Baxter PL732 bag is no longer being produced and culture volumes at these higher NK cell-expansion numbers

Table 1. Methods to isolate, activate, and expand NK cells for clinical applications

Study	Starting population	Feeder cells*	Culture conditions	Fold NK expansion	Purity	Used clinically
Escudier et al ⁴⁰	Kidney cancer patients: PBMCs, CD3-depleted × 2, monocyte free	LCL cell line (LAZ388)	Multicell plates, then transferred into bags	43 ± 26 in 13-21 d	90%	Yes
Carlens et al ¹⁷	Healthy donors: unseparated PBMCs	None (cytokines alone)	6-well plates	193 (21-277) in 21 d	55% (7-92)	No
Luhm et al ²⁶	Allogeneic NK cells (negative selection beads)	Allogeneic PBMCs	Vuelife (Teflon) bags	80-100 in 14-15 d	75%	Yes
Passweg et al ¹²	Allogeneic HSCT donors: CD3-/CD56+ PBMCs	N/A	Not expanded and not activated	N/A	Unavailable	Yes
Koehl et al ¹⁸	Allogeneic HSCT donors: CD3-/CD56+ PBMCs	None (cytokines alone)	Unavailable	5 in 12 d	95%	Yes
Klingemann et al ¹⁹	Healthy donors: CD56--selected PBMCs	None	Unavailable	5-40 in 14 d	Unavailable	No
Harada et al ⁴²	Patients with malignant glioma: unseparated PBMCs	Wilms tumor cell line (HFWT)	24-well plates, transferred to 75 cm ² flasks	113 in 14 d	96%	Yes
Alici et al ³⁷	Patients with multiple myeloma: unseparated PBMCs	None	Flasks	1625 (502-2658) in 20 d	65%	No
Berg et al ⁴¹	Healthy donors: CD3-/CD56+ PBMCs	Allogeneic PBMCs	Flasks, Baxter bags	100 in 15 d	98%	No
Berg et al ⁴¹	Healthy donors: CD3-/CD56+ PBMCs	EBV-LCL (TM-LCL)	Baxter bags	300-930 in 15 d	98%	Yes
Fujisaki et al ⁶³	Healthy donors: unseparated PBMCs	K562-mb 15-41BBL	Teflon bags	90.5 (33-141) in 7 d	83.1% (72.9%-85.9%)	No
Rubnitz et al ³⁵	Allogeneic donors: CD3-/CD56+ PBMCs	N/A	Not expanded and not activated	N/A	Unavailable	Yes
Sutlu et al ³⁶	Healthy donors: unseparated PBMCs	None	Flasks, bags, bioreactor	1100 flasks, 530 bags, 77 bioreactor (in 20 d)	44% flasks, 31% bags, 38% bioreactor	No
Siegler et al ²⁴	Healthy donors: CD3-/CD56+ PBMCs	Autologous NK-depleted PBMCs fraction	Baxter bags	117 ± 20 in 19 d	99%	No
Bachanova et al ³²	Haploidentical donors: CD3-depleted PBMCs	None (incubated 8-16 h in IL-2)	Unavailable	N/A	43 ± 11%	Yes
Dezell et al ⁶²	CD34-selected cells from UCB	Stromal cells	Multicell plates	64-fold greater than heparin based	67 ± 20% stage IV NKs	No
Dezell et al ⁶²	CD34-selected cells from UCB	None (heparin based)	Multicell plates	N/A	62 ± 20% stage IV NKs	No
Parkhurst et al ⁶⁵	Autologous cancer patients: CD3-depleted PBMCs	Autologous PBMCs	175 cm ² flasks, then transferred into bags	278-1097 in 21-26 d	91%-97%	Yes
Curti et al ²⁸	Haploidentical donors: CD56+-selected PBMCs	N/A	Not expanded and not activated	N/A	Unavailable	Yes
Zhang et al ²⁹	Healthy donors: negatively selected NK cells from PBMCs	K562-41BBL-expressing, restimulated weekly	Unavailable	1000 in 21 d	Unavailable	No
Spanholtz et al ⁶¹	CD34 selected cells from UCB	None	Bags, transferred to a Bioreactor on day 14	2000 in 14 d	90%	No
Frei et al ³⁸	Healthy donors: CD3-depleted PBMCs	None (NAM and cytokines in media)	G-Rex flasks	60-80 in 14 d	>95%	No
Imai et al ⁴⁴	Healthy donors: unseparated PBMCs	K562-mb 15-41BBL	Teflon bags	309-12 409 in 21 d	76%-98%	No
Garg et al ²²	Healthy donors and myeloma patients: unseparated PBMCs	K562-mb 15-41BBL, restimulated on day 7	Unavailable	804 (HD), 351 (MM), 20-10 430 in 14 d	88.3%	No
Lapreva et al ³⁰	Healthy donors: unseparated PBMCs	K562-mb 15-41BBL	G-Rex flasks	175 (39-255) in 8 d	70 ± 11%	No
Denman et al ⁵⁶	Healthy donors: unseparated PBMCs	K562-mb 15-41BBL, restimulated weekly × 3†	75 cm ² flasks	47 967 ± 42 230 in 21 d	Unavailable; T-cells depleted on d 14	No
Liu et al ⁵⁷	Neuroblastoma patients: unseparated PBMCs	K562-mb 15-41BBL, restimulated weekly†	6-well plates, then transferred into 25- and 75-cm ² flasks	2363 ± 443 in 14 d	83% ± 3%	No
Kim et al ²⁷	Cancer patients: CD3-/CD56--selected PBMCs	Autologous NK-depleted PBMC fraction	24-well plates	300 using healthy donor feeders and 169 using patient feeders	98%	No

HSCT indicates hematopoietic stem cell transplantation; UCB, umbilical cord blood; N/A, not applicable; HD, Hodgkin disease; and MM, multiple myeloma.

*All feeder cells were gamma irradiated.

†K562-CD64-CD84-41BBL-truncated CD19-mbL21.

CD3-/CD56+ indicates a 2-step purification of NK cells; CD3 depletion and a subsequent CD56 positive selection.

Figure 2. Phenotype and function of freshly-isolated, IL-2-activated and expanded NK cells. (A) Phenotype of freshly-isolated versus expanded NK cells. (B) Cytotoxicity of NK cells against tumor cells. Used with permission from Berg et al.⁴¹

require large volumes of medium (> 20 L), the ability of G-Rex100 containers to support these expansions at higher NK cell concentrations is currently being evaluated.

NK cells expanded using irradiated PBMCs. Irradiated allogeneic PBMCs have been used for years to expand T cells for adoptive infusion in humans, including tumor-infiltrating lymphocytes and

Figure 3. Fold expansion of ex vivo-expanded clinical grade NK cells and their characteristics on day of infusion. (A) NK cells were adoptively infused into cancer patients on the day of harvest. A total of 78 NK cell cultures expanded using irradiated EBV-LCL feeder cells were infused 14-27 days after culture initiation. (B) Phenotype/purity and viability of 78 clinical-grade NK cell products expanded over 14-27 days from cancer patients using EBV-LCL feeders. Used with permission from Reger et al.⁴⁸

antigen-specific T-cell lines and clones. Irradiated allogeneic PBMCs can likewise be used as feeder cells to expand NK cells ex vivo, although expansion numbers achieved with PBMCs are lower than those achieved using EBV-LCL or genetically modified K562 cells.^{26,41} We found that when allogeneic PBMCs were used as feeder cells, NK cells isolated from PBMCs using CD3⁺ depletion followed by CD56⁺ selection were most efficiently expanded when 25-Gy-irradiated feeder cells were added to cultures at a 20:1 ratio in culture medium containing 500 IU/mL IL-2 and 10% single donor or pooled AB plasma in upright culture flasks or Baxter bags at a starting density of 1×10^6 NK cells/mL.⁴¹ Under these conditions, up to a 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. Autologous irradiated PBMCs can likewise be used as feeder cells to stimulate ex vivo NK cell expansion.^{24,27,53} Kim et al used an approach in which NK cells were isolated using the Miltenyi 2-step CD3⁺ depletion followed by CD56⁺ selection, with the non-NK cell fraction of cells being irradiated with 25 Gy and then used as feeder cells in NK cell cultures.²⁷ Using this approach, a highly purified population of NK cells could be expanded in AIM-V medium in 14 days, with NK cells expanding 300- and 169-fold when feeder

PBMCs were obtained from healthy donors and cancer patients, respectively.

NK cells expanded using irradiated gene-modified K562 cells. The MHC class I deficient chronic myelogenous leukemia cell line K562 can also be used as a feeder cell to induce NK cell proliferation ex vivo.^{29,30,44,45,54} Imai et al retrovirally transduced this line with a construct containing 2 NK cell-stimulatory molecules, the ligand for the costimulatory surface molecule 4-1BB (CD137), and the human IL-15 gene fused to GFP and a gene encoding the CD8 α transmembrane domain, leading to surface-bound IL-15 expression. These genetically modified K562 cells coexpress surface-bound IL-15 and 4-1BB ligand and are highly efficient at inducing NK cell proliferation in vivo. A 7-day coculture of purified CD3-depleted and CD56-enriched NK cells with 100-Gy-irradiated K562-mb15-41BBL in RPMI 1640 or SCGM medium induced a median 21.6-fold expansion of CD3⁺/CD56⁺ NK cells from peripheral blood (range 5.1- to 86.6-fold) without inducing T-cell proliferation, with NK cells expanding more than 277-fold after a 21-day cell culture.⁴⁵ Complete elimination of leukemic feeder cells was confirmed by a Click-iT EdU Alexa Fluor 647 flow cytometry assay and staining of the final product for GFP expression.³⁰ When unfractionated PBMCs (in contrast to purified NK

cells) were stimulated with irradiated K562-mb15-41BBL feeder cells using GMP conditions, NK cells expanded a median 90.5-fold (range 33-141) in 7 days and contained a median 83.1% (range 72.9%-85.9%) CD3⁺/CD56⁺ NK cells. Similar to EBV-LCL-expanded NK cells, K562-mb15-41BBL expanded NK cells with up-regulated surface expression of the natural cytotoxicity receptors NKp30, NKp44, and NKp46, and activating receptors such as 2B4, DNAM-1, and NKG2D were significantly more cytotoxic to K562 targets than freshly stimulated or IL-2-activated NK cells.

Lapteva et al developed a large-scale system to expand clinical-grade NK cells using irradiated K562-mb15-41BBL feeder cells in gas-permeable static cell culture flasks (G-Rex).³⁰ Using unfractionated PBMCs, NK cells expanded in large-scale experiments by up to 128-fold (range 70-128) after an 8-day culture in SCGM medium and contained a median 77% (range 52%-88%) pure CD3⁺/CD56⁺ NK cells. This method did result in moderate T-cell contamination, with NK cell cultures containing up to 34% (range 4%-34%) CD3⁺ T cells. These investigators showed that supernatants taken from NK cell expansion cultures induced substantial MHC class I up-regulation on K562-mb15-41BBL cells, which stimulated CD8⁺ T cells *ex vivo*, including T cells that were found to have alloreactivity. However, contaminating T cells could be completely removed from NK cell cultures using CliniMACS CD3⁺ depletion, and this step would be necessary if this method were used to expand allogeneic donor NK cell products. This system required no manipulation after setup of the initial cell culture and achieved higher fold increases in NK cell numbers in G-Rex flasks than was observed with the use of gas-permeable bags.

Several investigators have shown that K562-mb15-41BBL feeder cells are capable of expanding NK cells isolated from allogeneic donors and patients who have received heavy prior treatment with chemotherapy for cancers such as acute myelogenous leukemia, gastric cancer, and multiple myeloma.^{22,37,45,55} Adoptive transfer of NK cells expanded from myeloma patients using K562-mb15-41BBL feeder cells into myeloma-bearing immune-deficient mice has been shown to result in NK cell *in vivo* proliferation for up to a month and inhibition of myeloma tumor growth, suggesting a potential therapeutic application for these cells in humans with myeloma.

Investigators have recently reported that additional genetic modification to K562 cells can be used to further enhance their potential to expand NK cells. Denman and Lee developed a K562 cell line that was genetically modified to express CD64 (FcγRI), CD84 (B7-2), CD137L (4-1BBL), truncated CD19, and membrane-bound IL-21 (mbIL-21) rather than mbIL-15.^{56,57} Although NK cells stimulated with K562 cells expressing either mbIL-15 or mbIL-21 had similar phenotypes, mRNA expression profiles, and tumor cytotoxicity profiles, mbIL-21-expanded NK cells had longer telomeres, increased expression of CD160, superior cytokine secretion of IFN-γ and TNF-α, and better *ex vivo* expansion. By day 21, NK cells expanded from unseparated PBMCs increased a median 31 747-fold using mbIL-21 K562 cells compared with only a 325-fold expansion using mbIL-15 K562 feeders. Remarkably, NK cell cultures stimulated weekly × 3 using mbIL-21-expressing K562 cells in IL-2-containing medium continued to expand for up to 6 weeks in culture, whereas NK cells expanded using mbIL-15 K562 typically became senescent at 4 weeks.

NK cells can also be expanded *ex vivo* from primitive stem cell populations including hematopoietic progenitor cells^{58,59} and human

embryonic stem cells.⁶⁰ Spanholtz et al recently developed a fully closed large-scale system to expand clinical-grade NK cells from CD34⁺ cells enriched from umbilical cord blood⁶¹ using a stromal-cell-free method. NK cells expanded from CD34⁺ cells using this technique were >90% pure (CD3⁺/CD56⁺) and were highly activated, displaying high expression of activating receptors and high degrees of cytotoxicity against K562 cells. Despite these findings, the use of stromal cells in expansion cultures may offer advantages to stromal-free conditions. Dezell et al have shown that NK cell expansion from hematopoietic progenitors using stromal cells appears to increase the yield of NK cells compared with stromal-free conditions, perhaps by differentiating less committed progenitors into the NK cell lineage.⁶² Despite this potential advantage, stromal-based expansions are logistically more challenging to use under GMP conditions, potentially limiting their application in the clinical setting.

Differences among fresh, IL-2-activated, and *ex vivo*-expanded NK cells

Although cultures using irradiated EBV-LCL or genetically modified K562 feeder cells substantially increase the number of NK cells that can be expanded compared with cytokine alone containing cultures, concerns exist that extensive *ex vivo* expansion might significantly reduce the *in vivo* proliferative potential and long-term viability of these populations after adoptive transfer in humans. We investigated for differences in phenotype, tumor cytotoxicity and *in vivo* persistence between short-term IL-2-activated and long-term-expanded NK cells.^{41,46} CD3⁺/CD56⁺ NK cells that were isolated from donors by immunomagnetic bead selection were either activated with IL-2 (500 U/mL) for 12-16 hours or were expanded *ex vivo* over 14 days using irradiated EBV-LCL feeder cells in IL-2-containing medium (500 U/mL). Short-term IL-2-activated NK cells did not expand in number, in contrast to EBV-LCL-stimulated NK cells, which expanded up to 1000-fold by culture day 14. FACS analysis revealed that expanded NK cells had significantly higher expression of TRAIL, NKG2D, and the natural cytotoxicity receptors NKp30, NKp44, and NKp46 compared with freshly isolated and IL-2-activated NK cells, with no differences in telomere lengths between the 3 populations as assessed by Flow-FISH. Expanded NK cells were significantly more cytotoxic against K562 cells compared with overnight IL-2-activated NK cells (Figure 2B) and had increased cytotoxicity against a TRAIL-sensitized kidney cancer tumor cell line (Figure 4). Using bioluminescent imaging, the 5-day *in vivo* longevity was found to be similar among freshly isolated, IL-2-activated, and expanded NK cells labeled with DiR and then infused into immunodeficient CB.17 SCID-beige mice receiving twice daily IL-2 (100 000 units ip).⁴⁶ Although NK cells expanded for 14 days using EBV-LCL maintained similar telomere lengths and longevity *in vivo* as nonexpanded short-term IL-2-activated NK cells, these data should be interpreted with caution because results may vary depending on the methods used to expand NK cells and the culture duration. Extensive culturing of NK cells beyond 4 weeks typically leads to their senescence when using either EBV-LCL feeders or K562-mb15-41BBL feeder cells. Although transduction of the hTERT gene into expanded NK cells can overcome this limitation,⁶³ its application in the clinical setting is impractical. Remarkably, Denman et al found NK cells expanded with mbIL-21-expressing K562 cells for 21 days had longer mean telomere lengths than NK cells expanded with mbIL-15 K562 cells, even though the mbIL-21-stimulated NK cells had the greatest amount of *ex vivo* proliferation.⁵⁶ Therefore, altering methods used to expand NK cells could potentially

Figure 4. Cytotoxicity of human NK cells against tumor cells and persistence in vivo following adoptive transfer in immunodeficient mice. Left: NK cytotoxicity versus TRAIL-sensitized kidney cancer cells. Right: NK cell persistence in vivo. NK cells were labeled with DiR near infrared dye and imaged every 6-12 hours after intraperitoneal NK cell infusion into CB.17 SCID-beige mice. IL-2 (100 000 U) was administered intraperitoneally every 12 hours. Used with permission from Berg et al.⁴⁶

overcome problems related to NK cell senescence associated with long-term culturing.

Pitfalls of NK cell expansion

Up-regulation of FAS

Cellular activation increases cell susceptibility to apoptosis and activation-induced cell death. The effects of ex vivo expansion of NK cells in terms of their susceptibility to programmed cell death mediated through Fas and other cellular death receptors has been poorly characterized. Fas expression appears to be similar on overnight IL-2-activated NK cells compared with fresh NK cells. In contrast, Fas expression increases when NK cells are maintained in IL-2 for more than 2 days or when NK cells are expanded with either EBV-LCL or K562 cells, increasing their susceptibility to rhFasL-mediated apoptosis.⁶⁴ Culturing with rhFasL increased NK cell apoptosis a median 5.6-fold ($P = .0001$) with 10- to 14-day-expanded NK cells compared with a 1.2-fold increase ($P = .32$) with fresh NK cells and no increase with overnight IL-2-activated NK cells. In contrast to Fas, surface expression of the TRAIL death receptors DR4 and DR5 do not appear to be affected by NK cell activation or in vitro expansion. These data suggest that expanded NK cells are more susceptible to Fas-mediated apoptosis compared with fresh and overnight IL-2-activated NK cells, potentially enhancing their susceptibility in vivo to apoptosis. Methods to expand activated NK cells while avoiding up-regulation in Fas surface expression remain an active area of investigation.

Homing

Whether adoptively infused NK cells are able to mediate clinically meaningful antitumor effects will ultimately be determined by their ability to home and traffic to the microenvironment where the tumor resides. Therefore, maintaining and/or enforcing the expression of homing receptors in expanded NK cell populations is required to achieve cell delivery via the vasculature to any target tissue. To move from the bloodstream into inflamed tissue sites, leukocytes must attach to the vascular endothelium, migrate between adjacent endothelial cells, and penetrate the basement membrane.⁶⁵ The molecular mechanism underlying these events involves a series of sequential adhesive interactions between activated leukocytes that

are chemoattracted to endothelial cells that are activated by inflammatory mediators such as IL-1 and TNF- α . The initial step in emigration from postcapillary venules is a low-affinity interaction between leukocyte ligands with selectins expressed on endothelium cells referred to as “rolling” or “tethering.” Furthermore, the interaction of chemokine receptors expressed on lymphocytes with chemokines secreted by the tumor or infected cells play a critical role in their ability to home to their target cells.⁶⁶

At present, little is known regarding the ability of ex vivo-expanded NK cells to migrate to lymph nodes, the BM, and other environments where tumors reside. The expression of chemokine receptors on NK cells may be critical to this process. NK cells expanded with genetically modified K562 cells contain predominantly CD56⁺/16⁺ bright NK cell populations, which do not express CCR7, a chemokine receptor that is known to facilitate NK cell homing to lymph nodes.⁶⁷ Although IL-18 can up-regulate CCR7, it does so in only a minority of NK cells.⁶⁸ Somanchi et al recently demonstrated that mbIL-21-expressing K562 feeder cells can be further genetically modified to express other transgenes, the products of which can be rapidly and transiently expressed in NK cells via trogocytosis by coculturing with expanded NK cells. K562 cells expressing mbIL-21 and CCR7 (clone9.CCR7) rapidly transferred CCR7 to expanded NK after a brief 1-hour culture, with up to 80% of NK cells acquiring CCR7 surface expression (Figure 5).⁶⁹ Although surface expression was transient, declining to baseline by 72 hours, NK cells that became positive for CCR7 had improved NK cell migration toward the CCR7 ligands CCL19 and CCL21 in transwell experiments and had increased homing into the lymph nodes of athymic mice. The relative ease of this approach to modify NK cells potentially makes it a viable strategy for scale-up under GMP conditions to explore its ability to improve ex vivo-expanded NK cell homing in humans.

Although NK cells cultured with irradiated EBV-LCL can be expanded by more than 1000-fold and are more cytotoxic to tumor cells compared with resting or IL-2-activated NK cells, we have observed these expanded cells undergo a substantial reduction in surface expression of CD62L, which could hinder their ability to be

Figure 5. NK cell trogocytosis as a method to increase NK cell surface expression of CCR7. Used with permission from Somanchi et al.⁶⁹

recruited from the circulation into the BM and secondary lymphoid tissues, where hematological malignancies reside. As discussed previously, investigators have reported an NK cell expansion technique that uses NAM in the medium, which appears to substantially increase CD62L expression on NK cells, leading to their improved homing into the spleens and BM of immune-deficient mice.³⁸ Furthermore, work conducted in our laboratory has shown that treatment of NK cell cultures using EBV-LCL feeders with NAM added to culture medium on day +7 resulted in the expansion of NK cells that contained substantially higher CD62L surface expression compared with cultures without NAM.⁷⁰ These NAM-cultured NK cells showed improved homing to mouse BM 24 hours after infusion into immune-deficient NSG mice compared with mice receiving expanded NK cells that were not cultured in NAM. These data suggest that NK cell expansion techniques that incorporate NAM-containing media could be used as a method to improve NK cell trafficking to the BM, potentially enhancing the antitumor effects of adoptively transferred NK cells against a variety of hematological malignancies.

Dependence on cytokines

Methods used to activate and/or expand NK cells ex vivo may affect the need for exogenous cytokine administration with IL-2 or IL-15 to support in vivo NK cell proliferation and cytotoxicity. Ex vivo-expanded NK cells have enhanced cytokine secretion profiles and are significantly more cytotoxic to tumor cells, although maintenance of this activated state is often dependent on the persistence of IL-15 or IL-2. We found that day 14 EBV-LCL-expanded NK cells had a rapid decline in both the percentage of NK cells expressing TRAIL and NKG2D within 16-24 hours of IL-2 removal from the medium. TRAIL and NKG2D expression was restored by the subsequent addition of IL-2 back into the medium and was IL-2 dose dependent.⁴¹ Reductions and subsequent increases in TRAIL and NKG2D surface expression that occurred with the removal and addition of IL-2 were directly correlated with

NK cell cytotoxicity against tumor cells. Culturing previously expanded NK cells in medium containing no or low doses of IL-2 (0-5 IU/mL) for 24 hours resulted in a substantial decline in NK cell cytotoxicity against K562 and other tumor target cells compared with 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. These data suggest that cytokine dependence may occur with extensive ex vivo NK cell expansion, although these effects too may be dependent on the method used to expand NK cells and the degree of ex vivo-induced NK cell proliferation.

Cryopreservation/thawing

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. NK cells isolated from fresh PBMCs can be cryopreserved, have viability in the range of 80% to 90% upon thawing, can be activated with overnight culture in IL-2, and expand well ex vivo with the use of various feeder-cell-based expansion methods. Lapteva et al found that NK cells expanded with IL-15- and 4-1BBL-expressing K562 feeder cells maintained excellent stability after cryopreservation, with an average 91% viability (range 85%-94%) upon thawing; stability data were confirmed for up to 12 months on expanded NK cells maintained in liquid nitrogen.³⁰ However, subsequent studies by the same group have shown low recovery (~30% in some cases) of cryopreserved cells after thawing and overnight culture and no in vivo expansion after infusion into patients.^{23,47} Furthermore, despite their excellent post-thaw viability, potency assays showed that freshly thawed NK cells failed to lyse K562 cells, with overnight culturing in IL-2-containing media (10 μ g/mL) being required to rescue NK cell cytotoxic function. Similarly, we found that NK cells expanded

Figure 6. Effects of freeze/thawing on the cytotoxicity and phenotype of expanded NK cells. Used with permission from Berg et al.⁴¹

using EBV-LCL feeders that were frozen and then thawed maintained excellent viability (>90%),⁴¹ although the cytolytic capacity of these cells to kill K562 and renal cell carcinoma cells was substantially lower than that of expanded NK cells that had never undergone cryopreservation. Thawed NK cells were found to have lower surface expression of TRAIL and NKG2D and were more likely to contain populations that were dim or negative for CD16. The cytotoxicity of expanded NK cells that were frozen and then thawed could be rescued by culturing in IL-2-containing medium, although (Figure 6) the viability of thawed NK cells (assessed by 7-AAD staining) declined from 93% to 97% immediately after thawing to 38% to 50% at 16 hours. These results suggest that expanded NK cells that have been cryopreserved may require reculturing in IL-2-containing medium after thawing to restore function before infusion in patients.

Future directions: engineering a better NK cell

The development of an efficient method that results in stable genetic modifications to NK cells could be used to characterize NK cell differentiation, acquisition of self-tolerance, and tumor trafficking in vivo, as well as to manipulate NK cells to improve their homing and enhance their cytotoxicity against infectious diseases and tumors. For example, retroviral transduction of primary NK cells to express endogenous IL-15 has been proposed as a method to avoid the need for exogenous cytokine administration after the adoptive infusion of expanded NK cells.⁷¹ The genetic disruption of expres-

sion of inhibitory receptors on NK cells such as KIR or NKG2A could potentially be used as a method to overcome the tumor evasion that occurs as a consequence of MHC class I expression. Lentiviral vectors (LVs) encoding shRNA targeting various regions of the NKG2A transcript have been used to silence NKG2A expression in the NK cell line NKL.^{4,72} NKG2A-silenced NK cells had enhanced killing of HLA-E-expressing tumors ex vivo and in vivo after their infusion into tumor-bearing mice. Although LVs have been used to efficiently transfer genes into human T cells and the NK cell lines such as NK92 and NKL, LV transduction of fresh and ex vivo-expanded human NK cells has been more challenging.⁷³ We used an LV-expressing enhanced green fluorescence protein driven by a murine stem cell virus long terminal repeat promoter to transduce CD3⁻ and CD56⁺ and/or CD16⁺ human NK cells that were either resting, IL-2 activated, or expanded ex vivo using an irradiated EBV-LCL feeder cells.⁴ Resting NK cells were difficult to transduce with LVs, even at high multiplicities of infection, with transduction efficiencies in the range of only 3% to 14%. The efficiency of LV transduction was improved when the NK cells were prestimulated ex vivo with IL-2, IL-15, or IL-21. Transduction efficiencies improved to 21% ± 0.2% in NK cells cultured for 24 hours in medium containing IL-2 (200 U/mL) and to 28.7% ± 12.9% when NK cells underwent ex vivo expansion over 10 to 14 days using irradiated EBV-LCL feeder cells and medium containing IL-2 (500 U/mL). Transduced NK cells maintained stable enhanced green fluorescent protein transgene expression ex

vivo, which peaked 5 days after LV transduction and remained stable for an additional 9 days. The phenotype, cytokine production, and cytotoxicity of NK cells compared with tumor targets were not altered by LV transduction.⁴ Sutlu et al have shown that the efficiency of lentiviral transduction of NK cells can be improved using both IL-2 and IL-21 in the culture medium. Furthermore, these investigators found that the transduction efficiency of freshly isolated NK cells cultured in IL-2- or IL-21-containing medium could be increased to the 50% range by inhibiting NK cell innate immune receptor signaling by adding BX795 to the medium, an inhibitor of TBK1/IKKε that acts as a common mediator in the signaling pathways of the receptors RIG-I, MDA-5, and TLR3.⁷⁴

Similar to T cells, transduction of chimeric antigen receptors (CARs) into NK cells has been explored recently as a method to induce tumor-specific NK cell killing. Shimasaki et al used a novel method of electroporation to introduce anti-CD19-4-1BBL-CD3ζ mRNA into nonexpanded and expanded NK cells, obtaining efficiencies of 40.3% and 61.3%, respectively, and have optimized this method for future clinical application.⁷⁵ CARs specific for antigens expressed on B-cell malignancies such as CD19 and CD20, HER2/ErbB2GD2 on breast tumors, and GD2 on neuroblastoma tumors, when transduced into primary or expanded NK cells, have been shown to overcome tumor resistance to killing by autologous NK cells. These data, as well as recent data showing the efficacy of T-cell-based CAR therapy targeting CD19 in B-cell malignancies, suggest that NK cells modified to express CARs after mRNA or viral transduction represent a therapeutic tool worthy of exploration in the clinical setting.^{43,44,57,73,76-79} The ability of CAR-expressing NK cells to home to the tumor may be a critical determinant of their efficacy in humans. As discussed previously, expanded NK cells may lack or down-regulate molecules that are critical for NK cell homing from the circulation.⁷⁰ Therefore, the use of expansion techniques that modify the phenotype of NK cells to improve their ability to home to the BM and lymph nodes may be necessary to optimize the therapeutic potential of CAR-expressing NK cells.^{38,67} The genetic manipulation of NK cells is technically challenging, expensive, and remains relatively inefficient despite the above described advances. Expansion techniques that engineer NK cells to express desired surface molecules via the trogocytosis method pioneered by Somanchi et al might offer a more efficient, safer, and more practical approach than genetic modification of NK cells via viral transduction.

Disclosures

Conflict-of-interest disclosure: The authors declare no competing financial interests. Off-label drug use: None disclosed.

Correspondence

Richard W. Childs, MD, Section of Transplantation Immunotherapy, National Institutes of Health, Bldg 10-CRC, Rm 3-5330, 10 Center Dr, Bethesda, MD 20892; Phone: 301-451-7128; Fax: 301-480-2664; e-mail: childsr@nhlbi.nih.gov.

References

1. Ljunggren HG, Malmberg KJ. Prospects for the use of NK cells in immunotherapy of human cancer. *Nat Rev Immunol*. 2007; 7(5):329-339.
2. Srivastava S, Lundqvist A, Childs RW. Natural killer cell immunotherapy for cancer: a new hope. *Cytotherapy*. 2008; 10(8):775-783.
3. Benson DM Jr, Bakan CE, Zhang S, et al. IPH2101, a novel anti-inhibitory KIR antibody, and lenalidomide combine to enhance the natural killer cell versus multiple myeloma effect. *Blood*. 2011;118(24):6387-6391.
4. Furutani E, Su S, Smith A, Berg M, Childs R. siRNA inactivation of the inhibitory receptor NKG2A augments the anti-tumor effects of adoptively transferred NK cells in tumor-bearing hosts [abstract]. *Blood (ASH Annual Meeting Abstracts)*. 2010;116(21):1015.
5. Zhu D, Corral LG, Fleming YW, Stein B. Immunomodulatory drugs Revlimid (lenalidomide) and CC-4047 induce apoptosis of both hematological and solid tumor cells through NK cell activation. *Cancer Immunol Immunother*. 2008;57(12):1849-1859.
6. Hayashi T, Hideshima T, Akiyama M, et al. Molecular mechanisms whereby immunomodulatory drugs activate natural killer cells: clinical application. *Br J Haematol*. 2005;128(2):192-203.
7. Benson DM Jr, Bakan CE, Mishra A, et al. The PD-1/PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: a therapeutic target for CT-011, a novel monoclonal anti-PD-1 antibody. *Blood*. 2010;116(13):2286-2294.
8. Davies FE, Raje N, Hideshima T, et al. Thalidomide and immunomodulatory derivatives augment natural killer cell cytotoxicity in multiple myeloma. *Blood*. 2001;98(1):210-216.
9. Callahan MK, Postow MA, Wolchok JD. Immunomodulatory therapy for melanoma: ipilimumab and beyond. *Clin Dermatol*. 2013;31(2):191-199.
10. Laurent S, Queirolo P, Boero S, et al. The engagement of CTLA-4 on primary melanoma cell lines induces antibody-dependent cellular cytotoxicity and TNF-α production. *J Transl Med*. 2013;11:108.
11. Intlekofer AM, Thompson CB. At the bench: preclinical rationale for CTLA-4 and PD-1 blockade as cancer immunotherapy. *J Leukoc Biol*. 2013;94(1):25-39.
12. Passweg JR, Tichelli A, Meyer-Monard S, et al. Purified donor NK-lymphocyte infusion to consolidate engraftment after haploidentical stem cell transplantation. *Leukemia*. 2004;18(11):1835-1838.
13. Wennerberg E, Sarhan D, Carlsten M, et al. Doxorubicin sensitizes human tumor cells to NK cell- and T-cell-mediated killing by augmented TRAIL receptor signaling. *Int J Cancer*. 2013;133(7):1643-1652.
14. Lundqvist A, Yokoyama H, Smith A, Berg M, Childs R. Bortezomib treatment and regulatory T-cell depletion enhance the antitumor effects of adoptively infused NK cells. *Blood*. 2009;113(24):6120-6127.
15. Velardi A. Natural killer cell alloreactivity 10 years later. *Curr Opin Hematol*. 2012;19(6):421-426.
16. Murphy WJ, Parham P, Miller JS. NK cells—from bench to clinic. *Biol Blood Marrow Transplant*. 2012;18(1 Suppl):S2-7.
17. Carlens S, Gilljam M, Chambers BJ, et al. A new method for in vitro expansion of cytotoxic human CD3-CD56+ natural killer cells. *Hum Immunol*. 2001;62(10):1092-1098.
18. Koehl U, Sörensen J, Esser R, et al. IL-2 activated NK cell immunotherapy of three children after haploidentical stem cell transplantation. *Blood Cells Mol Dis*. 2004;33(3):261-266.
19. Klingemann HG, Martinson J. Ex vivo expansion of natural killer cells for clinical applications. *Cytotherapy*. 2004;6(1):15-22.
20. McKenna DH Jr, Sumstad D, Bostrom N, et al. Good manufacturing practices production of natural killer cells for immunotherapy: a six-year single-institution experience. *Transfusion*. 2007;47(3):520-528.

21. McKenna DH, Kadidlo DM, Cooley S, Miller JS. Clinical production and therapeutic applications of alloreactive natural killer cells. *Methods Mol Biol.* 2012;882:491-507.
22. Garg TK, Szmania SM, Khan JA, et al. Highly activated and expanded natural killer cells for multiple myeloma immunotherapy. *Haematologica.* 2012;97(9):1348-1356.
23. Szmania S, Lapteva N, Garg TK, et al. Expanded natural killer (NK) cells for immunotherapy: fresh and made to order [abstract]. *Blood (ASH Annual Meeting Abstracts).* 2012; 120(21):1912.
24. Siegler U, Meyer-Monard S, Jäger S, et al. Good manufacturing practice-compliant cell sorting and large-scale expansion of single KIR-positive alloreactive human natural killer cells for multiple infusions to leukemia patients. *Cytotherapy.* 2010;12(6): 750-763.
25. Berg M, Childs R. Ex-vivo expansion of NK cells: what is the priority—high yield or high purity? *Cytotherapy.* 2010;12(8):969-970.
26. Luhm J, Brand JM, Koritke P, Hoppner M, Kirchner H, Frohn C. Large-scale generation of natural killer lymphocytes for clinical application. *J Hematother Stem Cell Res.* 2002;11(4): 651-657.
27. Kim EK, Ahn YO, Kim S, Kim TM, Keam B, Heo DS. Ex vivo activation and expansion of natural killer cells from patients with advanced cancer with feeder cells from healthy volunteers. *Cytotherapy.* 2013;15(2):231-241 e231.
28. 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(12):3273-3279.
29. Zhang H, Cui Y, Voong N, et al. Activating signals dominate inhibitory signals in CD137L/IL-15 activated natural killer cells. *J Immunother.* 2011;34(2):187-195.
30. Lapteva N, Durett AG, Sun J, et al. Large-scale ex vivo expansion and characterization of natural killer cells for clinical applications. *Cytotherapy.* 2012;14(9):1131-1143.
31. 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(8):3051-3057.
32. Bachanova V, Burns LJ, McKenna DH, et al. Allogeneic natural killer cells for refractory lymphoma. *Cancer Immunol Immunother.* 2010;59(11):1739-1744.
33. Skeate R, Singh C, Cooley S, et al. Hemolytic anemia due to passenger lymphocyte syndrome in solid malignancy patients treated with allogeneic natural killer cell products. *Transfusion.* 2013;53(2):419-423.
34. Stern M, Passweg JR, Meyer-Monard S, et al. Pre-emptive immunotherapy with purified natural killer cells after haploidentical SCT: a prospective phase II study in two centers. *Bone Marrow Transplant.* 2013;48(3):433-438.
35. 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(6):955-959.
36. Sutlu T, Stellan B, Gilljam M, et al. Clinical-grade, large-scale, feeder-free expansion of highly active human natural killer cells for adoptive immunotherapy using an automated bioreactor. *Cytotherapy.* 2010;12(8):1044-1055.
37. Alici E, Sutlu T, Björkstrand B, et al. Autologous antitumor activity by NK cells expanded from myeloma patients using GMP-compliant components. *Blood.* 2008;111(6):3155-3162.
38. Frei GM, Persi N, Lador C, et al. Nicotinamide, a form of vitamin B3, promotes expansion of natural killer cells that display increased in vivo survival and cytotoxic activity [abstract]. *Blood (ASH Annual Meeting Abstracts).* 2011; 118(21):4035.
39. Hercend T, Meuer S, Reinherz EL, Schlossman SF, Ritz J. Generation of a cloned NK cell line derived from the "null cell" fraction of human peripheral blood. *J Immunol.* 1982; 129(3):1299-1305.
40. Escudier B, Farace F, Angevin E, et al. Immunotherapy with interleukin-2 (IL2) and lymphokine-activated natural killer cells: improvement of clinical responses in metastatic renal cell carcinoma patients previously treated with IL2. *Eur J Cancer.* 1994;30A(8):1078-1083.
41. Berg M, Lundqvist A, McCoy P Jr, 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(3):341-355.
42. Harada H, Watanabe S, Saijo K, Ishiwata I, Ohno T. A Wilms tumor cell line, HFWT, can greatly stimulate proliferation of CD56+ human natural killer cells and their novel precursors in blood mononuclear cells. *Exp Hematol.* 2004;32(7):614-621.
43. Shook DR, Campana D. Natural killer cell engineering for cellular therapy of cancer. *Tissue Antigens.* 2011;78(6):409-415.
44. Imai C, Iwamoto S, Campana D. Genetic modification of primary natural killer cells overcomes inhibitory signals and induces specific killing of leukemic cells. *Blood.* 2005;106(1): 376-383.
45. Fujisaki H, Kakuda H, Shimasaki N, et al. Expansion of highly cytotoxic human natural killer cells for cancer cell therapy. *Cancer Res.* 2009;69(9):4010-4017.
46. Berg M, Lundqvist A, Betters D, Childs RW. In vitro expanded NK cells have increased natural cytotoxicity receptors, TRAIL and NKG2D expression, and superior tumor cytotoxicity compared to short-term IL-2-activated NK cells [abstract]. *Blood (ASH Annual Meeting Abstracts).* 2009;114(22):463.
47. Szmania S, Garg TK, Lapteva N, et al. Fresh ex vivo expanded natural killer cells demonstrate robust proliferation in vivo in high-risk relapsed multiple myeloma (MM) patients [abstract]. *Blood (ASH Annual Meeting Abstracts).* 2012;120(21):579.
48. Reger RN, Berg M, Lundqvist A, et al. A phase I trial of adoptively transferred ex-vivo expanded autologous natural killer (NK) cells following treatment with bortezomib to sensitize tumors to NK cell cytotoxicity [abstract]. *Blood (ASH Annual Meeting Abstracts).* 2011;118(21):1001.
49. Perussia B, Ramoni C, Anegón I, Cuturi MC, Faust J, Trinchieri G. Preferential proliferation of natural killer cells among peripheral blood mononuclear cells cocultured with B lymphoblastoid cell lines. *Nat Immun Cell Growth Regul.* 1987;6(4): 171-188.
50. Rabinowich H, Sedlmayr P, Herberman RB, Whiteside TL. Increased proliferation, lytic activity, and purity of human natural killer cells cocultured with mitogen-activated feeder cells. *Cell Immunol.* 1991;135(2):454-470.
51. Lundqvist A, Abrams SI, Schrupp DS, et al. Bortezomib and decapeptide sensitize tumors to tumor necrosis factor-related apoptosis-inducing ligand: a novel method to potentiate natural killer cell tumor cytotoxicity. *Cancer Res.* 2006;66(14):7317-7325.
52. Lundqvist A, Su S, Rao S, Childs R. Cutting edge: bortezomib-treated tumors sensitized to NK cell apoptosis paradoxically

- acquire resistance to antigen-specific T cells. *J Immunol.* 2010;184(3):1139-1142.
53. Parkhurst MR, Riley JP, Dudley ME, Rosenberg SA. Adoptive transfer of autologous natural killer cells leads to high levels of circulating natural killer cells but does not mediate tumor regression. *Clin Cancer Res.* 2011;17(19):6287-6297.
54. Baird K, Fry T, Merchant M, et al. Early clinical experience of aAPC Activated NK-DLI following allogeneic PBSCT in pediatric solid tumors patients [abstract]. *Blood (ASH Annual Meeting Abstracts).* 2012;120(21):3013.
55. Voskens C, Watanabe R, et al. Ex-vivo expanded human NK cells express activating receptors that mediate cytotoxicity of allogeneic and autologous cancer cell lines by direct recognition and antibody directed cellular cytotoxicity. *J Exp Clin Cancer Res.* 2010;29:134.
56. 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(1):e30264.
57. Liu Y, Wu HW, Sheard MA, et al. Growth and activation of natural killer cells ex vivo from children with neuroblastoma for adoptive cell therapy. *Clin Cancer Res.* 2013;19(8):2132-2143.
58. Miller JS, Alley KA, McGlave P. Differentiation of natural killer (NK) cells from human primitive marrow progenitors in a stroma-based long-term culture system: identification of a CD34+7+ NK progenitor. *Blood.* 1994;83(9):2594-2601.
59. Luevano M, Madrigal A, Saudemont A. Generation of natural killer cells from hematopoietic stem cells in vitro for immunotherapy. *Cell Mol Immunol.* 2012;9(4):310-320.
60. Woll PS, Grzywacz B, Tian X, et al. Human embryonic stem cells differentiate into a homogeneous population of natural killer cells with potent in vivo antitumor activity. *Blood.* 2009;113(24):6094-6101.
61. 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(6):e20740.
62. Dezell SA, Ahn YO, Spanholtz J, et al. Natural killer cell differentiation from hematopoietic stem cells: a comparative analysis of heparin- and stromal cell-supported methods. *Biol Blood Marrow Transplant.* 2012;18(4):536-545.
63. Fujisaki H, Kakuda H, Imai C, Mullighan CG, Campana D. Replicative potential of human natural killer cells. *Br J Haematol.* 2009;145(5):606-613.
64. Betters DM, Smith AL, Berg M, Lundqvist A, Childs RW. In vitro expanded natural killer (NK) cells are more susceptible to Fas-mediated apoptosis compared to fresh and overnight IL-2 activated NK cells. *Cancer Res.* 2011;70(8 Suppl):1271.
65. Sackstein R. The lymphocyte homing receptors: gatekeepers of the multistep paradigm. *Curr Opin Hematol.* 2005;12(6):444-450.
66. Sackstein R, Merzaban JS, Cain DW, et al. Ex vivo glycan engineering of CD44 programs human multipotent mesenchymal stromal cell trafficking to bone. *Nat Med.* 2008;14(2):181-187.
67. Somanchi SS, Senyukov VV, Denman CJ, Lee DA. Expansion, purification, and functional assessment of human peripheral blood NK cells. *J Vis Exp.* 2011;(48). pii: 2540.
68. Mailliard RB, Alber SM, Shen H, et al. IL-18-induced CD83+CCR7+ NK helper cells. *J Exp Med.* 2005;202(7):941-953.
69. Somanchi SS, Somanchi A, Cooper LJ, Lee DA. Engineering lymph node homing of ex vivo-expanded human natural killer cells via trogocytosis of the chemokine receptor CCR7. *Blood.* 2012;119(22):5164-5172.
70. Mamcarz E, Berg M, Peled T, Frei G, Reger R, Childs R. Nicotinamide (NAM) increases surface expression of CD62L on in vitro expanded NK cells [abstract]. *Pediatr Blood Cancer.* 2013;60(S2):Abstract 513.
71. Alici E, Sutlu T, Sirac Dilber M. Retroviral gene transfer into primary human natural killer cells. *Methods Mol Biol.* 2009;506:127-137.
72. Figueiredo C, Seltsam A, Blasczyk R. Permanent silencing of NKG2A expression for cell-based therapeutics. *J Mol Med (Berlin).* 2009;87(2):199-210.
73. Boissel L, Betancur M, Lu W, et al. Comparison of mRNA and lentiviral based transfection of natural killer cells with chimeric antigen receptors recognizing lymphoid antigens. *Leuk Lymphoma.* 2012;53(5):958-965.
74. Sutlu T, Nystrom S, Gilljam M, Stellan B, Applequist SE, Alici E. Inhibition of intracellular antiviral defense mechanisms augments lentiviral transduction of human natural killer cells: implications for gene therapy. *Hum Gene Ther.* 2012;23(10):1090-1100.
75. Shimasaki N, Fujisaki H, Cho D, et al. A clinically adaptable method to enhance the cytotoxicity of natural killer cells against B-cell malignancies. *Cytotherapy.* 2012;14(7):830-840.
76. Altvater B, Landmeier S, Pscherer S, et al. 2B4 (CD244) signaling by recombinant antigen-specific chimeric receptors costimulates natural killer cell activation to leukemia and neuroblastoma cells. *Clin Cancer Res.* 2009;15(15):4857-4866.
77. Muller T, Uherek C, Maki G, et al. Expression of a CD20-specific chimeric antigen receptor enhances cytotoxic activity of NK cells and overcomes NK-resistance of lymphoma and leukemia cells. *Cancer Immunol Immunother.* 2008;57(3):411-423.
78. Pegram HJ, Kershaw MH, Darcy PK. Genetic modification of natural killer cells for adoptive cellular immunotherapy. *Immunotherapy.* 2009;1(4):623-630.
79. Kruschinski A, Moosmann A, Poschke I, et al. Engineering antigen-specific primary human NK cells against HER-2 positive carcinomas. *Proc Natl Acad Sci U S A.* 2008;105(45):17481-17486.