Published in final edited form as:

Crit Rev Oncog. 2014 ; 19(0): 121–132.

Clinical Grade Purification and Expansion of Natural Killer Cells

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Abstract

Natural killer (NK) cells have been shown to have important functions in anti-tumor responses and therefore have been used as adoptive immunotherapy for cancer. Here, we review the current methods of *ex vivo* activation, enrichment, expansion, and shipment of clinical NK cell products.

Keywords

Natural killer cells; adoptive transfer; manufacture; clinical products

I. INTRODUCTION

In the past three decades, significant efforts have been made by basic and clinical researchers toward elucidating the role of natural killer (NK) cells in tumor immune surveillance and to understand their mechanisms of action.¹ These studies have led to a recent surge in clinical trials of NK cells for the treatment of cancer. The results of these trials have shown that the infusion of NK cells is safe and welltolerated and that infused NK cells can persist at elevated levels in recipients for several months and can induce clinical responses.^{2–4} An ideal protocol for clinical NK manufacturing should produce NK cells with sufficient purity and potency that are available in large numbers and at short notice for multiple potential infusions of the same product. Infused NK cells should be capable of expansion in patients and should exert anti-tumor activity with the potential of complete tumor eradication. A second important attribute of NK cells is their ability to activate adaptive arms of the immune response; therefore, their ability to promote the recruitment, activation and expansion of tumor-specific T cells should also be considered.

II. SOURCES OF NK CELLS AND NK CELL PROTOCOLS

Most cancer clinical trials of NK cells require large numbers of cells for infusion, ranging from 5×10^6 to 5×10^7 CD3[−]CD56⁺ cells per kilogram.^{2,3,5,6} Recently, doses as high as 1×10^8 CD3[−]CD56⁺ NK cells kg^{−1} have been reported.⁷ Because NK cells comprise only a small fraction of lymphocytes (~1–20% compared to ~80% of combined T and B cells), methods

have been developed either to enrich them from large volumes of peripheral blood, such as an apheresis products, or to expand the NK cell population from a smaller number of blood or stem cells (Fig. 1). Approaches that generate NK cells for allogeneic use aim to minimize CD3⁺ T-lymphocyte populations that may cause graft-versus-host disease (GVHD). This often involves depletion of CD3⁺ T cells, which increases the total number of starting cells required, particularly if depletion is performed at the end of the manufacturing procedure.⁸ Most protocols, therefore, use apheresis products ($1 \times 10^9 - 20 \times 10^9$ mononuclear cells) as the starting material; however, expansion from other sources such as buffy coats, cord blood, and embryonic stem cells is also possible.^{9,10} NK cells in peripheral blood and apheresis products can be detected by flow cytometry as CD45⁺CD56⁺CD3⁻ cells (Table 1 and Fig. 2).

In the first “rapid” approach (Table 2), NK cells were enriched from apheresis products by a one or two rounds of depletion of CD3⁺ T cells using CliniMACS’s magnetic beads coated with anti-CD3 antibody (CliniMACS CD3 reagent) with or without overnight activation using IL-2 or IL-15.^{11,12} This method produced up to 2×10^9 NK cells with approximately 20% purity, while contaminating CD19⁺ B cells, and CD14⁺ monocytes comprised greater than 50% of the product. Additional depletion of CD19⁺ B cells with anti-CD19 antibody-coated beads (CliniMACS CD19 reagent) further improved the purity of the NK cells, resulting in an average of 40% CD56⁺CD3⁻ in the final product. An alternative way to enrich for NK cells is to isolate CD56⁺ cells with CliniMACS CD56 reagent (anti-CD56 monoclonal antibody) with or without CD3⁺ T-cell depletion.^{13,14} Without CD3⁺ T-cell depletion, this method yields more than 95% NK cell purity while retaining CD56⁺CD3⁺ natural killer like T (NKT) cells, which also may contribute to anti-tumor immune responses, whereas the inclusion of CD3⁺ T-cell depletion yields up to 99% purity. All of these enrichment procedures lead to loss of the desired NK cells, so the quest for purity should be warranted by improved performance or safety.

Because current clinical trials for the treatment of cancer with NK cells demand high NK cell doses and often several infusions, one apheresis product may not contain sufficient numbers of NK cells. Therefore, technically complicated NK cell expansion protocols have been developed. Expansion of NK cells with either IL-2 or IL-15 or both to produce 1,000-fold expansion requires a culture period of up to 12 weeks.¹⁶⁻¹⁸ By contrast, “feeder cell”-based NK expansion approaches are rapid and robust, as large numbers of NK cells become available for infusion within 10–14 days.⁸ “Feeder-cell” methods generally require cytokines as well as irradiated feeder cells, such as EBV-LCLs or genetically modified K562 cells, to produce large numbers of CD3⁻56⁺ NK cells with greater than 70% purity from peripheral blood mononuclear cells (PBMCs).^{8,19,20} For example, Berg et al. developed a method in which CD3-depleted, CD56-enriched PBMCs were cultured in the presence of EBV-LCL feeders and X-VIVO 20 medium supplemented with 10% heat inactivated human AB serum, 500 U mL⁻¹ IL-2 and 2mM Glutamax. NK cells expanded by 490±260-fold over 21 days of culture,¹⁹ with purity of 84.3±7.8% CD56⁺CD16⁺ cells.

Being one of the Centers for Production Assistance for Cellular Therapies (PACT), our group is in charge of the NK-cell manufacturing for treatment of multiple myeloma for investigators at the University of Arkansas for Medical Sciences. We are using a

modification of a rapid NK expansion method described by Dario Campana's group that uses K562 cells genetically modified to express 4-1BBL and membrane-bound IL-15 (K562-41BBL-mbIL-15) as feeders, for the treatment of multiple myeloma.^{20,21} We have optimized this protocol for NK expansion in the G-Rex (Wilson Wolf manufacturing, Minneapolis, MN) and the WAVE bioreactor (GE Healthcare Life Sciences, Piscataway, NJ), producing highly activated and potent NK cells. As starting material we used apheresis products, cryopreserved at $\sim 5 \times 10^7$ total nucleated cells (TNCs) per milliliter in PlasmaLyte (CSS) with 10% dimethyl sulfoxide and 5% human serum albumin in several bags (three to five 70–80-mL aliquots). Cryopreserved bags were shipped from the site of the clinical trial in Little Rock, Arkansas, to our Good Manufacturing Practice (GMP) facility (Baylor College of Medicine, Houston Methodist Hospital and Texas Children's Hospital) in Houston, Texas. On Day 0 of the expansion procedure, we thaw the apheresis cells, wash in PBS supplemented with 5% fetal bovine serum (three times the volume of the frozen aliquot), and use a Ficoll gradient to remove dead cells and debris.²² The NK culture is set up based on the absolute number of $CD56^+CD3^-$ NK cells as assessed by flow cytometry. Each G-Rex100 flask (surface area 100 cm^2 , volume 400 mL) is seeded with a variable number of apheresis cells containing 5×10^6 NK cells supplemented with 5×10^7 irradiated (100 Gy) K562-41BBL-mbIL-15 feeder cells at a 1:10 ratio of NK cells:K562 cells in SCGM medium (CellGenix, Portsmouth, NH) containing 10% FBS. On Day 6 or 7 of culture, NK cells are phenotyped by flow cytometry to confirm the expansion, and the number of NK cells is estimated. Typically, if expanded in G-Rex flasks, we harvested NK cells on days 8 to 10 of culture. Originally, the protocol prescribed a low concentration of 10 U mL^{-1} of IL-2 that produced robust NK cell expansion; however, to determine whether higher doses of IL-2 could increase the yield and cytolytic potency, we tested doses of up to 1000 U mL^{-1} . While high-dose IL-2 did not affect the rate of NK cell expansion or viability, it significantly increased the expression of activation receptors and improved NK cell potency against OPM-2 myeloma cells and K562 cells (Fig. 3, data not shown).

Growing NK cells in the G-Rex flasks has many advantages over conventional bags and flasks.²³ These include high cell yield due to superior gas exchange and reduced nutrient deprivation, low harvest volume due to the possibility of aspirating up to 80% of the supernatant without disturbing the cells on the growth surface, and little or no manipulation because, with our seeding density of $1.25 \times 10^4 \text{ NK cells mL}^{-1}$, no medium exchange is required during the entire culture. Typically, one G-Rex100 flask yields up to 5×10^8 NK cells. To achieve much higher cell numbers requires multiple G-Rex flasks, which can be cumbersome and costly. For this reason, we also optimized NK cell expansion in the WAVE bioreactor when higher numbers or NK cell enrichment was required. Initially, a static culture of NK cells is set up in an appropriately sized bag to achieve $3\text{--}5 \times 10^4 \text{ NK cell mL}^{-1}$ together with 100-Gy-irradiated K562-41BBL-mbIL-15 cells (at a 1:10 NK:K562 ratio) in SCGM medium supplemented with 10% FBS and 500 U mL^{-1} IL-2. When total cell concentration reaches $8\text{--}10 \times 10^5 \text{ cell/mL}$, cells are moved into an appropriately sized WAVE bag and diluted to $2 \times 10^5 \text{ cells/mL}$ in fresh culture medium. The culture is rocked at a 6° angle at 6 tilts per minute for an additional 4–5 days and then harvested using the CellSaver5+ instrument (Haemonetics Corporation, Braintree, MA). Side-by-side expansion of NK cells in G-Rex flasks and the WAVE bioreactor yielded products that were similar in

numbers, quality, and potency (as measured by phenotype and in cytotoxicity assays). Interestingly, the WAVE-generated products contain fewer CD3⁺ T cells and a higher frequency of CD56⁺CD3⁻ NK cells, perhaps because T cells prefer static culture (Fig. 4). The clinical trial in multiple myeloma demands both autologous and allogeneic NK cells. In the case of allogeneic products, CD3⁺ T cells are efficiently removed (as well as CD3⁺56⁺ cells) by depletion with the CliniMACS CD3 reagent, reducing the CD3⁺ cell frequency from 19±9% to 0.4±0.5% (N=5). This depletion allows less than 5×10⁵ CD3⁺ CD56⁻ T cells kg⁻¹ for infusion, as mandated by the clinical protocol.

Despite significant progress in the development of expansion and enrichment of NK cells, practical considerations such as logistics, complex multiple component protocols, and significant manufacturing costs drive the interest in alternative sources of NK cells, such as NK cell lines. The human NK-92 cell line was originally purified by Klingemann et al from a patient with NK cell lymphoma.²⁴ NK-92 has broad anti-tumor cytotoxicity and can be expanded *in vitro* with IL-2 for infusion.^{24,25} Because it is an allogeneic cell line with infinite division potential, these cells must be irradiated before infusion for safety. We have also adapted NK-92 culture to the G-Rex and can achieve up to 200-fold expansion of the cells with minimum manipulation in less than 4 weeks of culture.

III. SHIPPING NK CELL PRODUCTS

Remote manufacturing of cell therapy products in centralized facilities is likely to be “the norm” in the future. As a PACT manufacturing facility, we have shipped NK cell products to the clinical site located in another state, and we have had to optimize conditions for overnight cell shipment. We first explored the possibility of shipping expanded NK cells cryopreserved in 10% DMSO, 40% HBSS and 40% human serum albumin solution (containing 25% HSA). Notably, greater than 70% of the cells were viable within several hours of thaw, they were not cytotoxic. While their potency could be regained after overnight culture in IL-2, their viability dropped significantly (Fig. 5). Further, thawed NK cells did not expand in patients.⁵ Therefore, we explored the possibility of shipping NK cells formulated for infusion at 1×10⁷ cells mL⁻¹ in infusion buffer Buminat 5% (Baxter, Deerfield, IL) at room temperature and at 4–15°C (on –20°C frozen insulated ice packs). Shipping formulated cells allows infusion upon arrival at the clinical site. In our trial, the clinical site counts the cells, removes cells in excess of the infusion dose, and performs additional sterility testing. We found that expanded NK cells shipped both at RT and on ice in Fenwal transfer packs retained greater than 90% viability and potency within 48 hours after formulation without cell loss (Fig. 5 and Table 3). We finally chose to ship the cells on ice packs because the temperature was more stable (temperature fluctuated from 8 to 11°C) during shipping on ice packs compared to room temperature conditions, which fluctuated from 5 to 21°C.

While this strategy is feasible from a manufacturing point of view, it requires tight coordination with the patient and the clinical site. There is a concern that unforeseen events could render a patient ineligible at the last minute. We therefore also tested whether we could ship expanded NK cells in G-Rex flasks in their culture medium. In this case, cells could be kept in the incubator for a day or two while waiting for the infusion, allowing more

flexibility at the clinical site. This approach proved very successful, but when shipped in culture medium, cells needed to be kept on gel packs pre-heated to 37°C to prevent loss of viability and clumping, which occurred on ice packs. The major caveat with this strategy is that the clinical site must have cell-processing labs capable of washing and formulating the final product for infusion.

IV. RELEASE CRITERIA FOR CLINICAL PRODUCTS FOR EXPANDED NK CELLS

For any cell therapy product, NK cells must comply with basic standard release criteria as well as product-specific release criteria, which have not yet been standardized. The release criteria for our expanded NK cells are shown in Table 4 and include requirements that products for autologous use should contain at least 50% CD56⁺CD3⁻ cells, whereas products for allogeneic use should contain greater than 70% of CD56⁺CD3⁻ cells and no more than 5×10^5 CD3⁺CD56⁻ T cells per kilogram of patient weight. There should be less than 0.1% of K562- 41BBL-mbIL-15 feeder cells in the final product as measured by flow cytometry, and feeder cell inability to proliferate is confirmed using a modified “Click-iT assay” (Life Technologies, Grand Island, NY). Identity to the apheresis donor is confirmed by HLA-A and -B locus typing. Notably, we found that autologous products required DNase (Bensonaze Nuclease ultrapure, Sigma-Aldrich, St. Louis, MO) treatment prior to DNA extraction to eliminate the DNA released by killed K562 cells. Allogeneic products did not require DNase treatment because they undergo CliniMACS depletion of CD3⁺ T cells, a process that effectively removes K562 cell debris, including DNA.

We also measure the potency of each NK cell product using K562 cells as a surrogate targets in standard chromium⁵¹ release cytotoxicity assays. Because K562 cells are robustly killed even by unstimulated NK cells, it may not be an informative potency assay, and it would be even more valuable to measure potency with patient-derived myeloma cells. However, the primary tumor cells are difficult to obtain and culture. Therefore, this test would be difficult to implement as a standard release criterion.

In conclusion, in the last three decades significant progress has been made in the production of sufficient clinical grade NK cells for therapeutic applications. Additionally, pre-clinical and clinical studies are required to characterize the biodistribution, persistence, expansion and activity of these cells in mouse models and patients in order to select the right product for the specific indication. The mechanisms underlying the fate of NK cells after infusion are still not well understood, and more effort must be dedicated to identify the products that have significant expansion potential and that persist in patients. Reproducible *in vivo* expansion would allow lower infusion cell doses, and the choice of *in vivo* supplementary cytokines requires more explanation. Studies are necessary to identify the best way to cryopreserve the NK cells so that sufficient cells can be grown at one time to allow multiple infusions. This would also facilitate third-party applications. Future clinical studies with NK cells will likely include genetic modifications to enhance and expand specificity and function, while combinatorial treatments with T cells and small molecules should potentiate their function and take advantage of their ability to recruit and activate the adaptive immune response. All studies should include detailed patient immune monitoring post infusion to

measure expansion, cytokine secretion and to identify epitope-spreading that could be a marker of activation of adaptive responses.

Acknowledgments

We are thankful to all members of the cell processing and vector production facilities and the flow cytometry laboratory of Center for Cell and Gene Therapy for their help with this work. This work was supported in part by NIH-NHLBI- N01 HB37163, NHLBI-1U54 HL081007 and NIH-NCI PO1 CA94234, NIH-NCI PO1 CA55819, and NIHNCI RO1 CA134522.

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ABBREVIATIONS

AML	acute myeloid leukemia
B-ALL	acute B-cell lymphoblastic leukemia
C-ALL	common acute lymphoblastoid leukemia
H-SCT	hematopoietic stem cell transplant
IL	interleukin
N/A	not applicable

NK cell	natural killer
PBMC	peripheral blood mononuclear cells
SCGM	stem-cell growth medium

FIG. 1.

Clinical-grade NK cells are usually derived from fresh or frozen apheresis products. (A) A CliniMACS device can enrich products for CD56⁺ cells via depletion of non-NK-cell subsets, positive selection using anti-CD56 beads or both strategies may be used on the same product for higher NK cell purity. Following CliniMACS enrichment NK cells are infused directly or after overnight activation with IL-2. Alternatively, NK cells may be expanded from a portion of the apheresis product *in vitro* using solely (B) a cytokine cocktail or (C) adding in feeder cells for more rapid expansion to allow the product to be available for therapy sooner.

FIG. 2.

Identification of NK cells in an apheresis product by flow cytometry. The lymphocyte/monocyte populations are gated on (A) FSC and SSC dot plots and then (B) sub-gated as CD45⁺ cells. (C) NK cells are identified as CD56⁺CD3⁻ cells in gated populations.

FIG. 3.
Enhanced killing of OPM-2 myeloma cells by NK cells expanded with K562-41BBL-mbIL-15 cells with high-dose (500 and 1000 U mL⁻¹) IL-2

FIG. 4.

Comparison of NK cell expansion in G-Rex flasks and WAVE bioreactor. PBMCs from healthy donor containing 5×10^6 NK cells were co-cultured with K562-41BBL-mbIL-15 cells for 9 days. Cells were moved to WAVE on day 4 of culture. (A, B, C) Similar numbers and fold of NK expansion were observed in G-Rex and WAVE cultures. (D) Potency (killing K562 cells) of both products was also comparable. Similar expansion was obtained for three donors and the representative data for one donor is shown.

FIG. 5.

Characterization of cryo-preserved and fresh expanded NK cell products. (A, B) Expanded and cryopreserved NK cells stay viable within hours post thaw; however, killing K562 cells do not. Cryopreserved cells regain their potency after overnight incubation, however, lose their viability. Freshly formulated NK cells (N=3) remain potent after shipment at ambient temperatures or on frozen ice packs (C).

TABLE 1

NK cell content in apheresis products

Apheresis product source	TNC ($\times 10^6$)	CD45+CD56+CD3- NKs (%)	Absolute number of NKs ($\times 10^6$)
Healthy donor	6222	4	249
Healthy donor	21600	4	864
Healthy donor	16328	7	1143
Healthy donor	21200	10	2120
Myeloma patient	7181	17	1221
Myeloma patient	11368	13	1478
Myeloma patient	10560	16	1690
Myeloma patient	35088	10	3509
Myeloma patient	14190	36	5108
Average $\pm$ StDev	15971 $\pm$ 9018	13 $\pm$ 10	1931 $\pm$ 1499

TABLE 2

Summary of NK clinical protocols using CliniMACS-based procedures

Indication	CliniMACS procedure	Culture conditions	Cytokines used for NK stimulation	Outcome (at the time of the report)	Reference
Multiple myeloma (N=10)	CD3 ⁺ T cell removal with CD3 reagent	AIM-V with 2% HSA	300 U mL ⁻¹ IL-2 overnight	Transient CR in ~50% patients	(12)
Refractory or progressive leukemia post H-SCT (N=3 AML and ALL)	Two rounds of CD3 ⁺ T cell depletion with CD3 reagent followed by CD56 ⁺ NK enrichment with CD56 reagent	X-VIVO 10 medium with 5% heat-inactivated human fresh frozen plasma	1000 U mL ⁻¹ IL-2 for 10-14 days	patient 1 died of infection +152 days complete remission; patient 2 relapsed and died+80 days; patient 3 TTP, died +45 days	(14)
Hematologic malignancies (after autologous SCT N=13)	Depletion of CD3 ⁺ T cells with CD3 reagent	X-VIVO 15 with 10% heat inactivated human AB serum	1000 U mL ⁻¹ IL-2	7 patients in remission and 6 patients relapsed	(15)
Acute myeloid leukemia (N=19)	Depletion of CD3 ⁺ T cells	X-VIVO 15 with 10% heat inactivated human AB serum	1000 U mL ⁻¹ IL-2	5 patients in remission	(2)
High risk hematological malignancies (N=5)	CD3 ⁺ T cell removal with CD3 reagent followed by CD56 ⁺ NK cell enrichment with CD56 reagent	N/A; cells were infused immediately	None	4 patients in remission	(13)
Relapsed AML, T-ALL, cALL, B-ALL (N=8)	Depletion of CD3 ⁺ T and CD19 ⁺ B cells with CD3 and CD19 reagents	RPMI with 10% human AB serum overnight	10 ng mL ⁻¹ IL-15	All patients died from relapse/ progression	(11)

Abbreviations: AML, acute myeloid leukemia; B-ALL acute B-cell lymphoblastic leukemia; C-ALL, common acute lymphoblastoid leukemia, IL, interleukin; N/A, not applicable; H-SCT, hematopoietic stem cell transplant; TTP, thrombotic-thrombocytopenic purpura.

TABLE 3

Viability of NK cells (N=3) 48 hours post-formulation at 1×10^7 cell mL^{-1} in 5% human serum albumin (HSA)

	Fresh	Ambient	Frozen ice packs
Recovery (%)	N/A	91.3 $\pm$ 2	91.3 $\pm$ 10
Viability (%)	91.9 $\pm$ 2.6	83.6 $\pm$ 2.8	84.9 $\pm$ 1.2
CD56 ⁺ CD3 ⁻ NKs (%)	64.1 $\pm$ 16.1	65.3 $\pm$ 14.2	67.8 $\pm$ 14.2

N/A: not applicable.

TABLE 4

Release criteria for freshly formulated expanded NK cell products

Test	Specimen	Specification	Availability at the time of infusion
Viability by 7-AAD	Cell product	>70% viable	Yes
Endotoxin (LAL assay by EndoSafe)	Cell product	<5.0 EU mL ⁻¹	Yes
Gram stain	Cell product	No organisms seen	Yes
Mycoplasma (MycAlert-Lonza)	Cells on day 3 of culture	Negative	Yes
Feeder cells (flow for GFP ⁺ K562-41BBL-mbIL-15 cells)	End of production cells	<0.1% GFP ⁺ cells	Yes
Phenotyping	End of production cells	>50% CD56 ⁺ CD3 ⁻ for auto products >70% CD56 ⁺ CD3 ⁻ for allo products	Yes
Phenotyping (allogeneic products only)	End of production cells	<5×10 ⁵ CD3 ⁺ CD56 ⁻ cells per kg	Yes
Potency (Chromium release assay with K562 targets)	Cells on Day 6-12 of culture	>20% lysis at 20:1 of effector to target ratio	Yes
HLA typing* Low resolution for HLA-A and B loci	Final product	Identical HLA to donor	Yes
Sterility Bactec Aerobic, Anaerobic and Fungal	Final product	Negative	No
21 CFR 610.12 Bacterial sterility	Final product	Negative	No

* To shorten the timeline between cell formulation and infusion we have recently removed HLA typing of the product from the release criteria.