Chapter 16

Large-Scale Culture and Genetic Modification of Human Natural Killer Cells for Cellular Therapy

Natalia Lapteva, Robin Parihar, Lisa A. Rollins, Adrian P. Gee, and Cliona M. Rooney

Abstract

Recent advances in methods for the ex vivo expansion of human natural killer (NK) cells have facilitated the use of these powerful immune cells in clinical protocols. Further, the ability to genetically modify primary human NK cells following rapid expansion allows targeting and enhancement of their immune function. We have successfully adapted an expansion method for primary NK cells from peripheral blood mononuclear cells or from apheresis products in gas permeable rapid expansion devices (G-Rexes). Here, we describe an optimized protocol for rapid and robust NK cell expansion as well as a method for highly efficient retroviral transduction of these ex vivo expanded cells. These methodologies are good manufacturing practice (GMP) compliant and could be used for clinical-grade product manufacturing.

Key words Natural killer cells, Large-scale expansion, G-Rex, Genetic modification with retroviral vector

1 Introduction

Although natural killer (NK) cell therapies have shown promising results in cancer patients [1–4], they are limited by transient expansion of adoptively transferred NK cells in patients and short persistence after infusion. Therefore, large numbers of cells are required to achieve meaningful clinical results. NK cell doses may range from 1×10^5 cells/kg to 1×10^8 cells/kg and as high as 1×10^{10} NK cells may be necessary for a single infusion of a 100 kg patient [5, 6]. We have adapted a robust method of ex vivo expansion of NK cell using irradiated, HLA-negative K562 feeder cells that are genetically modified with membrane-bound IL-15 and 4-1 BB ligand (K562mbIL15-41BBL) to boost NK cell differentiation, survival, and proliferation [7, 8]. Using this method, we generated large numbers of functional NK cells from unseparated apheresis products or peripheral blood mononuclear cells (PBMCs) after just 10 days of culture in gas-permeable static cell culture flasks (G-Rex).

Srinivas S. Somanchi (ed.), *Natural Killer Cells: Methods and Protocols*, Methods in Molecular Biology, vol. 1441,
DOI 10.1007/978-1-4939-3684-7_16,

Cultures in G-Rexes (optimal 10 mL of medium per cm^2 of surface area of a gas-permeable membrane) typically require no cell manipulation or feeding during the 10 days of culture period. In order to broaden the applications of these activated and expanded NK cells to cancer trials, we also optimized a method for their transduction with retroviral vectors encoding chimeric antigen receptors (CAR), with specificities ranging from CD19 (expressed on many leukemias [9]) to the diasialoganglioside, GD2 (expressed on solid tumors, such as neuroblastoma, sarcomas, and melanoma [10]). These methods for NK cell expansion and retroviral transduction are good manufacture practices (GMP) compliant and are easily utilized in clinical protocols.

2 Materials

1. Cryopreserved or fresh apheresis cells or PBMCs.
2. K562mbIL15-41BBL cell line was kindly provided by Dario Campana (National University of Singapore).
3. Complete-SCGM (C-SCGM): CellGro® Stem Cell Growth Media (CellGenix GmbH) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS, HyClone).
4. Interleukin-2 (IL-2) (Proleukin, Prometheus Laboratories Inc.).
5. Thawing medium: 1× PBS without Ca^{2+} and Mg^{2+} containing 5% HI-FBS.
6. Ficoll-Paque.
7. G-Rex 10 and G-Rex100 culture flask (Wilson Wolf Manufacturing).
8. 1× PBS without calcium chloride and magnesium chloride.
9. 5% human serum albumin (HSA)/Buminate 5% (Baxter).
10. Gamma retroviral vectors encoding a CAR for CD19 or GD2.
11. Non-tissue culture-treated 24-well plates.
12. Retronectin solution (Takara Bio Company).

3 Methods

3.1 Expansion of K562mb15-41BBL Cells in G-Rex Flasks

Expected time to grow $>1.5 \times 10^9$ K562mbIL15-41BBL cells, required for the expansion of NK cells, from one vial with 2×10^7 cryopreserved cells is approximately two weeks. The number of K562mbIL15-41BBL cells required will depend on desired numbers of NK cells (Table 1).

1. Pre-warm 9 mL of C-SCGM.
2. Thaw a vial of 2×10^7 K562mbIL15-41BBL master cell bank (MCB) in a 37 °C waterbath.

Table 1
Example of the estimated K562mbIL15-41BBL feeder cells and apheresis cells required for initiation of a large-scale NK cell culture

Number of NK cells required for infusion	Fold of NK expansion	% of $CD56^+CD3^-$ cells in apheresis product	Number of cells to be seeded in one G-Rex100	# of G-Rexes required	Total number of K562mbIL15-41BBL cells required +20%
1×10^{10}	~100	10%	5×10^7 apheresis cells and 5×10^7 of irradiated K562mbIL15-41BBL	20	1.2×10^9

3. Transfer and resuspend thawed cells into 9 mL of warm C-SCGM.
4. Centrifuge cells at $400\times g$ for 5 min, aspirate supernatant.
5. Resuspend the cells in 30 mL of C-SCGM and count viable cells.
6. Transfer thawed K562mbIL15-41BBL cells into one G-Rex10 for 3–4 days in 30 mL of total volume (*see* **Note 1**).
7. On day 3–4 of culture, remove 20 mL of supernatant from G-Rex10 and count the cells.
8. When K562mbIL15-41BBL numbers reach 15×10^6 cells/G-Rex10, transfer all the cells into one G-Rex100. Add C-SCGM medium to 400 mL of total volume per one G-Rex100. If less than 15×10^6 cells were counted, add 20 mL of fresh C-SCGM to the cells in G-Rex10.
9. When growth of K562mbIL15-41BBL cells in a G-Rex100 flask reaches more than 300×10^6, split cells between three G-Rex100 flasks. Transfer between 50×10^6 and 100×10^6 cells per one G-Rex100. Continue to culture and count the cells every 2–3 days (*see* **Note 2**).

3.2 Ficoll Gradient Separation of Cryopreserved Peripheral Blood or Apheresis Cells

1. Warm Thawing medium. The volume of thawing medium should be approximately 10× the volume of the cryopreserved product (*see* **Note 3**).
2. Thaw the apheresis cells in a 37 °C water bath (*see* **Note 4**).
3. Transfer cells from the vial/bag into appropriate size centrifuge tubes containing three volumes of warmed Thawing Medium. Centrifuge at $400\times g$ for 10 min.
4. Aspirate supernatant and resuspend the cells in three volumes of Thawing Medium.
5. Overlay 30–40 mL of cells onto 15 mL lymphocyte separation medium (Ficoll-Paque) and centrifuge for 30 min at $400\times g$ with centrifuge brake set to OFF.

6. Harvest mononuclear cell layer into 2 volumes of Thawing Medium and centrifuge at 450 × *g* for 10 min.
7. Resuspend the apheresis cells in one to four volumes of C-SCGM and centrifuge at 400 × *g* for 5 min.
8. Resuspend the apheresis cells in C-SCGM containing 500 U/mL IL-2 and pool all cells into one sterile container.
9. Analyze an aliquot of cells by flow cytometry to determine the frequency of $CD56^{+}CD3^{-}$ NK cells.
10. Keep apheresis cells in the incubator (37 °C and 5 % CO_2) until the results of flow cytometry are available (*see* **Note 5**).

3.3 Harvest and Irradiate of K562mbIL15-41BBL Feeder Cells

1. Count and calculate the required number of expanded K562mbIL15-41BBL cells. K562mbIL15-41BBL cells are used at a 10:1 ratio of K562 to NK. Use Table 1 as a reference (*see* **Note 6**).
2. Aspirate supernatants, harvest and pool K562mbIL15-41BBL cells. Irradiate K562mbIL15-41BBL cells with 100 Gray using a validated irradiator (*see* **Note 7**).
3. Wash the irradiated K562mbIL15-41BBL cells in C-SCGM (with 500 U/mL IL-2) and re-count the cells (expect 20% loss).

3.4 Expansion of NK Cells in G-Rex 100s

1. Prepare C-SCGM containing 500 U/mL IL-2. Four hundred mL of medium is required per one G-Rex100.
2. Aliquot apheresis cells or PBMCs containing 5×10^6 NK cells per each G-Rex100.
3. Aliquot 5×10^7 K562mbIL15-41BBL (100-gray irradiated, washed and recounted from Subheading 3.3) into each G-Rex100.
4. Add C-SCGM medium to 400 mL per G-Rex100 and 500 U/mL IL-2 (200,000 units total).
5. On day 6 of culture, test glucose in one representative G-Rex100. Take 10–50 μL of culture supernatant and place it on a testing strip of a validated glucose self-monitoring device, designed for use by diabetics (e.g., Accu-Chek Aviva, Roche). If glucose is below 70 mg/dL, aspirate ~300 mL of the medium from the representative G-Rex100, count the cells and estimate the total number of cells in all G-Rexes seeded. Harvest the cells when the required total number of NK cells is achieved. If cell numbers are not sufficient, replace 300 mL of supernatant from each G-Rex with fresh C-SCGM supplemented with IL-2 (final concentration 500 U/mL). If glucose is above 70 mg/dL, return all G-Rexes to the incubator (*see* **Note 8**).
6. Harvest NK cells by aspirating 300 mL of supernatant from each G-Rex. Resuspend the sheet of cells in each G-Rex and pool in centrifuge tubes. If cells are to be used clinically, the cells should be pooled in a suitably sized container before trans-

fer to centrifuge tubes. Aliquots of pooled cells and pooled cell culture medium should be saved for quality control testing.

7. Wash harvested NK cells three times with 300–400 mL of PBS supplemented with 2.5 % HSA. Concentrate the cells in 5 % HSA (5 % Buminate) at 1×10^7 cells/mL and transfer the cells into transfer packs for infusion. Cells are stable in this condition for up to 48 h if refrigerated at 4 °C or shipped on –20 °C frozen ice packs, wrapped in plastic bags and absorbent materials to prevent possible leakage and/or direct contact with ice packs.
8. Allogeneic clinical NK cell products should undergo depletion of $CD3^+$ T cells prior formulation. Deplete $CD3^+$ T cells using CD3 CliniMACS reagent with a CliniMACS system (Miltenyi Biotec) according to the manufacturer's instructions.

3.5 Transduction of Human NK Cells with Retroviral Vectors

1. Perform expansion of NK cells as described above in Subheadings 3.1–3.4. This "primary expansion" ensures the proper activation and proliferation of NK cells that allows for optimal retroviral transduction efficiency by day 3 to 4 of expansion.
2. Coat wells of a 24-well *non-tissue culture*-treated plate with 0.5 mL/well of a retronectin solution (7 μg/mL in 1× PBS): incubate plate overnight at 4 °C for binding of the retronectin molecule to the plate.
3. Next day, aspirate retronectin-containing PBS from plate(s), wash ×1 with cold C-SCGM, then add viral supernatant (1 mL/well) to the retronectin-coated plate(s) (*see* **Note 9**).
4. Spin in ultracentrifuge at $2000 \times g$ for 1 h. After spin, aspirate all of the viral supernatant completely from each well.
5. Harvest NK cells from G-Rex flasks as above in Subheading 3.4, count NK cells, and bring them to a 2.5×10^5 cells/mL concentration in fresh C-SCGM containing 500 U/mL IL-2.
6. Plate 2 mL/well of NK cell solution into each well of the retronectin/retrovirus-coated plate, giving a final concentration of 5×10^5 NK cells/well.
7. Spin at $1000 \times g$ for 5 min.
8. Culture plates for 48–72 h (known as "transduction culture").
9. Harvest NK cells from transduction culture at 48–72 h by repeat pipetting. Phenotype and assess transduction efficiency by flow staining of cells with anti-CD56 and CD3 mAbs and construct selection marker of choice. *See* Fig. 1 for a representative flow plot showing transduction efficiency of several CAR constructs.
10. If further expansion of the genetically modified NK cells is required (i.e., "secondary expansion"), re-seed NK cells in G-Rex flasks with feeder cells as per Subheading 3.4. Figure 2

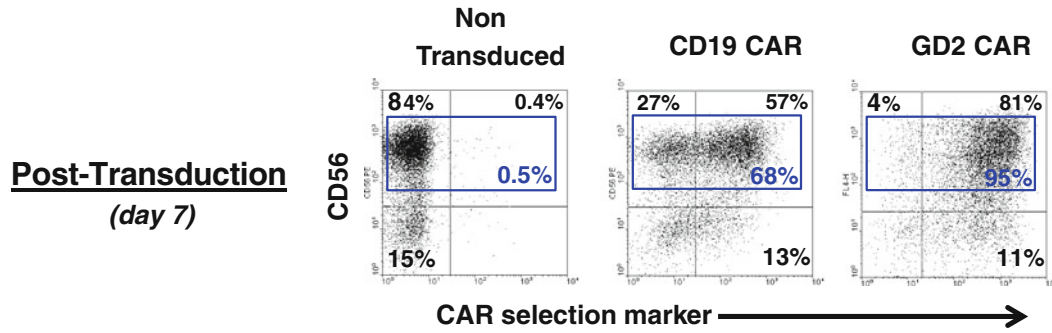

Fig. 1 Generation of expanded and activated NK cells transduced with CAR molecules. NK cells were transduced with retroviral vectors expressing CAR molecules against CD19 and GD2, respectively, with truncated surface molecules as selection markers. After a 3-day transduction as outlined in Subheading 3.5, approximately 65–95 % transduction efficiency was obtained for the various CAR constructs on the NK cell surface. Mock transduction with an empty retroviral vector was used as a "non-transduced" control

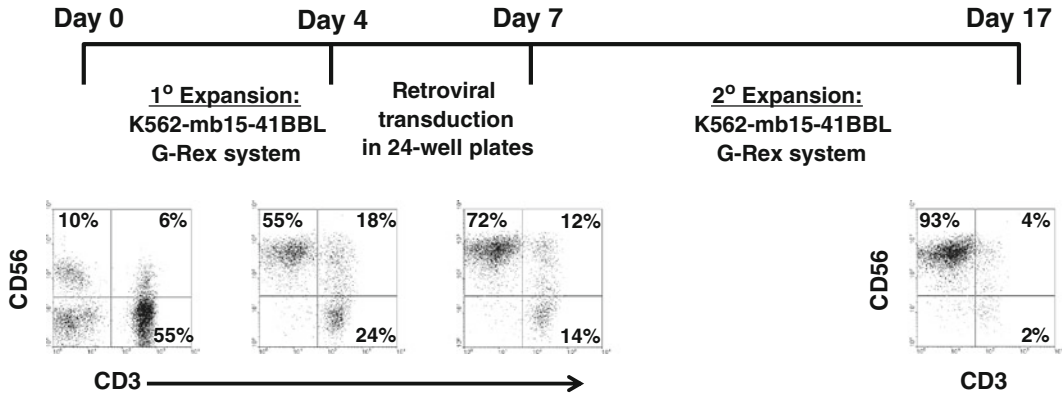

Fig. 2 NK cell expansion/transduction schema and phenotype analysis. PBMC obtained from normal donors were co-cultured in the presence of sub-lethally irradiated K562 mb15-41BBL and IL-2 in G-Rex flasks for 4 days and transduced with retroviral constructs expressing CARs for 3 additional days. Transduced NK cells were then secondarily expanded for an additional 10 days. Cells were harvested at indicated time-points and analyzed for phenotype by flow cytometry. NK cells are characterized by a $CD56^{+}CD3^{-}$ phenotype. Shown is a representative donor of more than 20 different normal donors examined

depicts the overall expansion/transduction schema employed herein and shows phenotypic analysis of NK cell expansion over time.

4 Notes

1. Seed one G-Rex10 with at least 5×10^5 and not more than 1×10^7 of K562mbIL15-41BBL cells. Total culture volume in one G-Rex10 is 30 mL.

2. Because genetically modified K562 cells may down-regulate expression of 4-1 BBL and membrane bound IL-15 after prolonged culture, plan the day of NK initiation based on the doubling time of K562 cells. Our master cell bank's doubling time is about 48 h. Do not allow K562mbIL15-41BBL cells to become static, i.e., 8×10^8 per G-Rex100s.
3. Frozen or fresh peripheral blood mononuclear cells (PBMCs) can be used as a starting cell population for NK cell expansion. Expect the same expansion rates for PBMCs and apheresis cells.
4. Work rapidly to reduce the toxicity of DMSO.
5. Because the percentage of the $CD56^+CD3^-$ NK cells is donor-specific, the total nucleated cell number (containing 5×10^6 NK cells) to be seeded in each G-Rex100 will vary between different products.
6. The expected NK cell expansion is about 100-fold by days 8 or 10 of culture. Therefore, to obtain 1×10^{10} NK cells, we typically seed 20 G-Rex100s with 5×10^6 NKs per G-Rex100. One G-Rex flask should be seeded with 5×10^6 NKs and 5×10^7 K562mbIL15-41BBL cells. To estimate the number of apheresis cells required to produce a certain number of NK cells, a preliminary small-scale culture can be performed in a G-Rex10 with the cells from a reference vial containing an aliquot of the cryopreserved apheresis product. Seed each G-Rex10 with apheresis cells or PBMCs containing 5×10^5 $CD56^+CD3^-$ cells and 5×10^6 100-gray irradiated K562mbIL15-41BBL cells in 40 mL of C-SCGM supplemented with 500 U/mL IL-2. The fold of expansion will be the same in small (G-Rex10)- and large (G-Rex100)-scale cultures.
7. Confirmation of efficacy for feeder cell irradiation is required for clinical NK cell products. To this end, we have adapted flow cytometry-based Click-iT cell proliferation assay [11].
8. Typically fresh C-SCGM contains ~400–450 mg/dL glucose. NK cells are ready for harvest when glucose drops below 100 mg/dL. The cells can be harvested earlier if the required cell number and purity has been achieved. Most of our clinical products were harvested on day 8 of culture. If seeded at the densities specified by this protocol, we recommend harvesting between days 8 and 10 of culture. Cultures harvested earlier than day 7 may contain K562mbIL15-41BBL feeder cells detectable by flow cytometry, particularly if the rate of NK cell expansion is low. After day 10 of culture the NK cells start to lose viability and activation markers expression and a second re-stimulation with K562mbIL15-41BBL is required to boost them.
9. Viral supernatants were generated as follows: 293T cells were transfected in the presence of GeneJuice with vector plasmid containing gene of interest + RDF, an expression plasmid

supplying the RD114 envelope + PeqPam-env, a gagpol expression plasmid, for 48 h at 37 °C. Culture supernatant (i.e., viral supernatant) was aspirated, sterile filtered, and snap frozen for further use in transduction cultures, as above in Subheading 3.5, **Step 3**. For clinical use however, such transient supernatants would not be suitable and a clinical grade virus producer cell line would be required.

References

1. Miller JS, Soignier Y, Panoskaltsis-Mortari A et al (2005) Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. Blood 105:3051–3057
2. Curti A, Ruggeri L, D'Addio A et al (2011) Successful transfer of alloreactive haploidentical KIR ligand-mismatched natural killer cells after infusion in elderly high risk acute myeloid leukemia patients. Blood 118:3273–3279
3. Rubnitz JE, Inaba H, Kang G et al (2015) Natural killer cell therapy in children with relapsed leukemia. Pediatr Blood Cancer 62: 1468–1472
4. Bachanova V, Cooley S, Defor TE et al (2014) Clearance of acute myeloid leukemia by haploidentical natural killer cells is improved using IL-2 diphtheria toxin fusion protein. Blood 123:3855–3863
5. Shah NN, Baird K, Delbrook CP et al (2015) Acute GVHD in patients receiving IL-15/4-1BBL activated NK cells following T-cell-depleted stem cell transplantation. Blood 125:784–792
6. Szmania S, Garg TK, Lapteva N et al (2012) Fresh ex vivo expanded natural killer cells demonstrate robust proliferation in vivo in high-risk relapsed multiple myeloma (MM) patients. Blood (ASH Annual Meeting Abstract) 120
7. Imai C, Iwamoto S, Campana D (2005) Genetic modification of primary natural killer cells overcomes inhibitory signals and induces specific killing of leukemic cells. Blood 106:376–383
8. Fujisaki H, Kakuda H, Shimasaki N et al (2009) Expansion of highly cytotoxic human natural killer cells for cancer cell therapy. Cancer Res 69:4010–4017
9. Weiland J, Elder A, Forster V et al (2015) CD19: a multifunctional immunological target molecule and its implications for Blineage acute lymphoblastic leukemia. Pediatr Blood Cancer 62:1144–1148
10. Suzuki M, Cheung NK (2015) Disialoganglioside GD2 as a therapeutic target for human diseases. Expert Opin Ther Targets 19:349–362
11. Lapteva N, Durett AG, Sun J et al (2012) Large-scale ex vivo expansion and characterization of natural killer cells for clinical applications. Cytotherapy 14:1131–1143