

Video Article

Expansion, Purification, and Functional Assessment of Human Peripheral Blood NK Cells

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URL: <https://www.jove.com/video/2540>

DOI: [doi:10.3791/2540](https://doi.org/10.3791/2540)

Keywords: Immunology, Issue 48, Natural Killer Cells, Tumor Immunology, Antigen Presenting Cells, Cytotoxicity

Date Published: 2/2/2011

Citation: Somanchi, S.S., Senyukov, V.V., Denman, C.J., Lee, D.A. Expansion, Purification, and Functional Assessment of Human Peripheral Blood NK Cells. *J. Vis. Exp.* (48), e2540, doi:10.3791/2540 (2011).

Abstract

Natural killer (NK) cells play an important role in immune surveillance against a variety of infectious microorganisms and tumors. Limited availability of NK cells and ability to expand *in vitro* has restricted development of NK cell immunotherapy. Here we describe a method to efficiently expand vast quantities of functional NK cells *ex vivo* using K562 cells expressing membrane-bound IL21, as an artificial antigen-presenting cell (aAPC).

NK cell adoptive therapies to date have utilized a cell product obtained by steady-state leukapheresis of the donor followed by depletion of T cells or positive selection of NK cells. The product is usually activated in IL-2 overnight and then administered the following day¹. Because of the low frequency of NK cells in peripheral blood, relatively small numbers of NK cells have been delivered in clinical trials.

The inability to propagate NK cells *in vitro* has been the limiting factor for generating sufficient cell numbers for optimal clinical outcome. Some expansion of NK cells (5-10 fold over 1-2 weeks) has been achieved through high-dose IL-2 alone². Activation of autologous T cells can mediate NK cell expansion, presumably also through release of local cytokine³. Support with mesenchymal stroma or artificial antigen presenting cells (aAPCs) can support the expansion of NK cells from both peripheral blood and cord blood⁴. Combined NKp46 and CD2 activation by antibody-coated beads is currently marketed for NK cell expansion (Miltenyi Biotec, Auburn CA), resulting in approximately 100-fold expansion in 21 days.

Clinical trials using aAPC-expanded or -activated NK cells are underway, one using leukemic cell line CTV-1 to prime and activate NK cells⁵ without significant expansion. A second trial utilizes EBV-LCL for NK cell expansion, achieving a mean 490-fold expansion in 21 days⁶. The third utilizes a K562-based aAPC transduced with 4-1BBL (CD137L) and membrane-bound IL-15 (mIL-15)⁷, which achieved a mean NK expansion 277-fold in 21 days. Although, the NK cells expanded using K562-41BBL-mIL15 aAPC are highly cytotoxic *in vitro* and *in vivo* compared to unexpanded NK cells, and participate in ADCC, their proliferation is limited by senescence attributed to telomere shortening⁸. More recently a 350-fold expansion of NK cells was reported using K562 expressing MICA, 4-1BBL and IL15⁹.

Our method of NK cell expansion described herein produces rapid proliferation of NK cells without senescence achieving a median 21,000-fold expansion in 21 days.

Video Link

The video component of this article can be found at <https://www.jove.com/video/2540/>

Protocol

1. Isolation of PBMCs from Buffy Coat

Peripheral blood mononuclear cells (PBMC) are obtained by buoyant density centrifugation on Ficoll-Paque from healthy donor buffy coat samples derived by leukapheresis.

1. The Ficoll-Paque centrifugation is done as per manufacturer's protocol with minor modifications.
2. Add PBS to a normal-donor blood-bank buffy coat to a final volume of 140 mL (typical buffy coat volume is 40-70 mL).
3. Layer 35 mL of buffy coat sample on 15 mL of Ficoll-Paque (4 tubes).
4. Centrifuge at 400g for 20 minutes without brake.
5. Recover the PBMCs from the Ficoll-Paque: plasma interface, do not discard the RBCs at the bottom of the Ficoll-Paque.
6. Wash PBMCs three times with PBS, centrifuging each time at 400g for 10 minutes.
7. PBMCs can be used directly for NK cell expansion at this stage or NK cells can be isolated by RosetteSep (Section 4)
8. Remaining PBMCs can be frozen in FBS containing 10% DMSO in liquid nitrogen.
9. Aspirate the Ficoll and collect the RBCs from step 5 in to two 50 mL centrifuge tube, wash three times with PBS (added to 50 mL mark), each time aspirate the supernatant skimming the top of the RBC layer to remove granulocytes.

10. The RBCs can be used immediately for RosetteSep purification of NK cells (refer to **Section 4**) or stored in equal volume of Alsever's solution at 4°C for later use (The RBCs can be store for a maximum of 4 weeks).

2. NK Cell Expansion

The NK cell expansion can be initiated using PBMCs or purified NK cells. The amount of PBMCs used for expansion can be varied based on the amount of NK cells desired at the end of a three week expansion, refer to representative results section for details. (See Note 1)

STIMULATION 1

Day 0

1. For each 5×10^6 PBMCs to be expanded, count and irradiate 10×10^6 K562 Cl9 mL21 using a gamma irradiator at 100 Gy.
2. Post irradiation, wash the cells with PBS and resuspend in NK cell expansion media (NKEM).
3. Seed 5×10^6 PBMCs with 10×10^6 irradiated K562 Cl9 mL21 (1:2 ratio) in 40 mL of NKEM in a T75 flask and place it upright in an incubator at 37°C and 5% CO₂.

Days 3 and 5

4. Recover cells by centrifugation at 400g for 5 min and replace half of the media with fresh NKEM (adding fresh IL2 for the entire media volume) and continue culture.

STIMULATION 2

Day 7

1. Count the number of cells in culture at the end of one week.
2. Set aside 5×10^5 cells for phenotyping by flow cytometry (See Note 2)
3. For each 5×10^6 cells to be restimulated, count and irradiate 5×10^6 K562 Cl9 mL21 using a gamma irradiator at 100 Gy.
4. Add an equal number of irradiated K562 Cl9 mL21 (1:1 ratio) and resuspend in NKEM at 2.5×10^5 total cells/mL (See Note 3).
5. Seed cells in T75 flasks (maximum 50 mL per flask).

Days 10 and 12

6. Count the number of cells.
7. Change entire media with fresh NKEM based on the cell numbers (See Note 3).

Day 14

8. At the end of two weeks of expansion count the number of cells in culture.
9. Set aside 5×10^5 cells for phenotyping by flow cytometry (See Note 2)
10. If expansion was started from PBMCs the NK cells can be purified at this stage of expansion using the RosetteSep purification protocol (refer to Section IV). If expansion was started from purified NK cells proceed to stimulation 3.
11. After purification set aside 5×10^5 cells for phenotyping by flow cytometry to verify purity of the NK cells (as in Step 8).
12. Proceed with Stimulation 3 using all of the purified NK cells (See Note 4).

STIMULATION 3

1. Resuspend NK cells with irradiated K562 Cl9 mL21 (1:1 ratio) in NKEM based on cell numbers (See Note 3).

Days 17 and 19

2. Count the number of cells.
3. Change media with fresh NKEM based on the cell numbers (See Note 3).

Day 21

4. At the end of three weeks of expansion count the number of cells in culture.
5. Recover 1×10^6 cells for flow cytometry analysis for full NK cell phenotyping antibody panel (See Table 1).
6. Freeze cells in FBS containing 10% DMSO at a maximum density of 5×10^7 cells per vial for future use.

3. NK Cell Cytotoxicity Assay

1. Thaw a vial of NK cells and seed in NKEM, one day prior to performing cytotoxicity assay to allow recovery.
2. For each NK cell cytotoxicity assay using a single target cell line, 6×10^5 NK cells and 3×10^5 target cells are required (See Note 5).
3. Prepare CAM-Media by diluting Calcein-AM (stock 1 mg/mL in DMSO) in NKEM (See Note 6).
4. Resuspend 10^6 target cells in 1 mL of CAM-media (See Note 7).
5. Incubate for 1 h at 37 °C, with occasional shaking.
6. Resuspend NK cells at 1×10^5 cells/ mL and add 200uL of NK cell suspension to each of the 3 wells of a U-bottom 96-well plate corresponding to 10:1 E:T ratio shown in Figure 1. (See Note 8)
7. Add 100uL of complete media to all remaining wells except for "Maximum".

8. Add 100 μ L of 2% Triton X-100 to "Maximum".
9. Perform serial dilutions of the NK cells for the 5 subsequent E:T ratios by transferring 100 μ L of cells each time, mix well. Discard 100 μ L from the last wells (E:T ratio of 0.3125:1).
10. After 1 hour of calcein loading, wash target cells in NKEM twice, centrifuging for 5 min at 1200 rpm. (See Note 9)
11. Re-count the target cells and resuspend at 1×10^5 cells/ mL.
12. Add 100 μ L of target cells to each well (1×10^4 /well). Centrifuge for 1 min at 100g to initiate cell contact.
13. Incubate at 37 °C and 5% CO₂ for 4 hours.
14. Mix the culture gently by pipetting with a 100 μ L pipetter in order to uniformly suspend the released calcein, spin down plate at 100g for 5 minutes to pellet the cells and transfer 100 μ L of the supernatant to a new plate taking care to avoid bubbles. Pop any bubbles that may form using fine needle.
15. Read the plate using a fluorescent plate reader (excitation filter 485 nm, emission filter 530 nm). Bottom read is recommended.
16. Calculate Percent Specific Lysis according to the formula $[(\text{test release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})] \times 100$.

4. NK Cell Purification by RosetteSep

1. Take 100-fold excess of RBCs to that of PBMCs or expanded cells, into a 50 mL tube (100:1 RBC:PBMC).
2. If using fresh RBCs proceed directly to next step or if the RBCs were stored in Alsever's solution, count the number of RBCs and wash appropriate (100 fold excess) amount of RBCs with PBS supplemented with 2% FBS three times, centrifuging at 1200 rpm for 10 minutes each time.
3. Combine RBCs with PBMCs from step 1.7 or expanded cells from step 2.10 in PBS + 2% FBS to a final volume of 1 mL per 5×10^7 of PBMCs or expanded cells.
4. Add 1 μ L of RosetteSep Human NK Cell Enrichment Cocktail per 1×10^6 of PBMCs or expanded cells.
5. Mix well and incubate at room temperature for 20 minutes with gentle mixing every 5 minutes.
6. Add equal volume of PBS + 2% FBS mix gently and layer on top of Ficoll-Paque.
7. Repeat the steps of Ficoll-Paque centrifugation described for PBMC isolation (Section I).
8. Count the NK cells recovered after purification and set aside 5×10^5 cells for phenotyping by flow cytometry for NK cell purity (Step 8).

5. Notes

NOTE 1. NK cells can be expanded directly from PBMCs, or from RosetteSep purified NK cells. We have noted similar expansion efficiency, but some donors may have very low NK cell numbers resulting in difficulty purifying by RosetteSep prior to expansion.

NOTE 2. We routinely use CD56-FITC, CD16-PE, and CD3-PE-Cy5 for phenotyping during the expansion, enumerating NK cells as those which are CD3-negative and CD16- or CD56-positive.

NOTE 3. At each media change or stimulation, resuspend cells at 2.5×10^5 /mL to keep PBMC/NK cell numbers at or under 2 million per mL at peak stages of expansion. This will prevent depletion of nutrients and help achieve maximal expansion and survival.

NOTE 4. The NK expansion rate is donor dependent and at the end of stimulations 1 or 2 part of the cells can be frozen and part expanded further depending on the experimental need. We have had good success in using the frozen cells for expansions at a later time.

NOTE 5. In order to allow room for error we recommend using a minimum of 7×10^5 NK cells resuspended in 700 μ L of NKEM and 4×10^5 Calcein-AM stained target cells resuspended in 4 mL of NKEM for setting up the cytotoxicity assay. If using multichannel pipette for seeding target cells higher volumes of cells (up to 6×10^5 in 6 mL) may be required based on the size of media basin being used. Also the recommended NK cell numbers are specifically for the E:T ratios shown in the protocol, for using higher E:T ratios increase the NK cell numbers per mL accordingly (eg. For a 40:1 E:T ratio use 4×10^6 cells/ mL)

NOTE 6. We recommend performing a preliminary Calcein-AM loading titration for the target cell line of choice, using the following dilutions of 1:500, 1:400, 1:300, 1:200 and 1:100 to achieve optimal difference between maximum and spontaneous release.

NOTE 7. When using an adherent cell line as target, first prepare single cell suspension using non-enzymatic cell dissociation buffer. If performing ADCC, prepare a duplicate tube of target cells in CAM-media.

NOTE 8. If performing ADCC, add same NK cells to 3 wells corresponding to 10:1 E:T for ADCC. Repeat for additional donors. Repeat for additional target cells.

NOTE 9. If performing an ADCC experiment, after 45 minutes of calcein loading, add 10 μ g of antibody specific for inducing ADCC against the target cells. After 15 more minutes, wash target cells in complete medium twice, centrifuging for 5 min at 1200 rpm. Resuspend cells at 1×10^5 cells/ mL and proceed with the next step in the protocol.

6. REPRESENTATIVE RESULTS

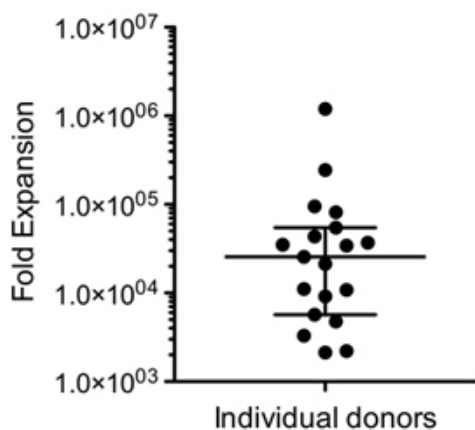


Figure 2. When the expansion is performed as per the scheme described above, using 5×10^6 PBMCs as starting material, typical NK cell yields range from 1×10^9 to 10^{10} cells (donor dependent variability). The figure shows NK cell fold expansion ($n=19$) compared to NK cells present in the original product (median \pm quartile).

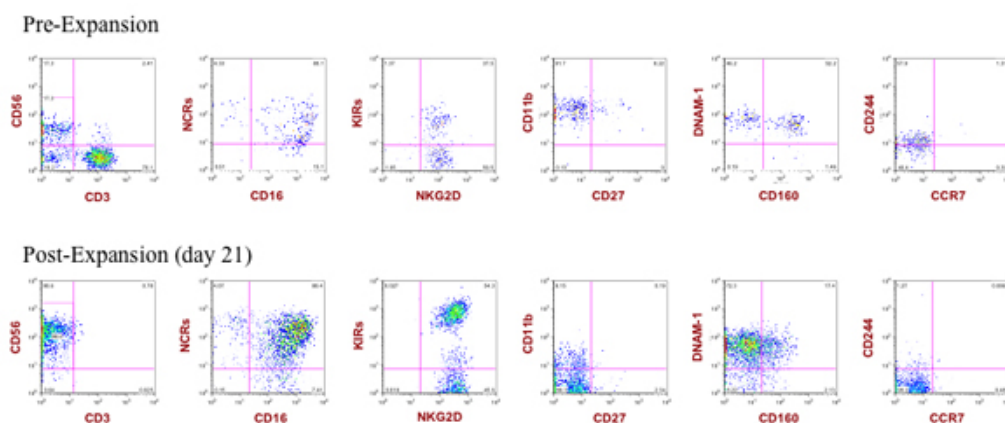


Figure 3. The expanded NK cells express various NK cell receptors that are comparable to the unexpanded primary NK cells with a few exceptions (CD11b, CD160 and CD244).

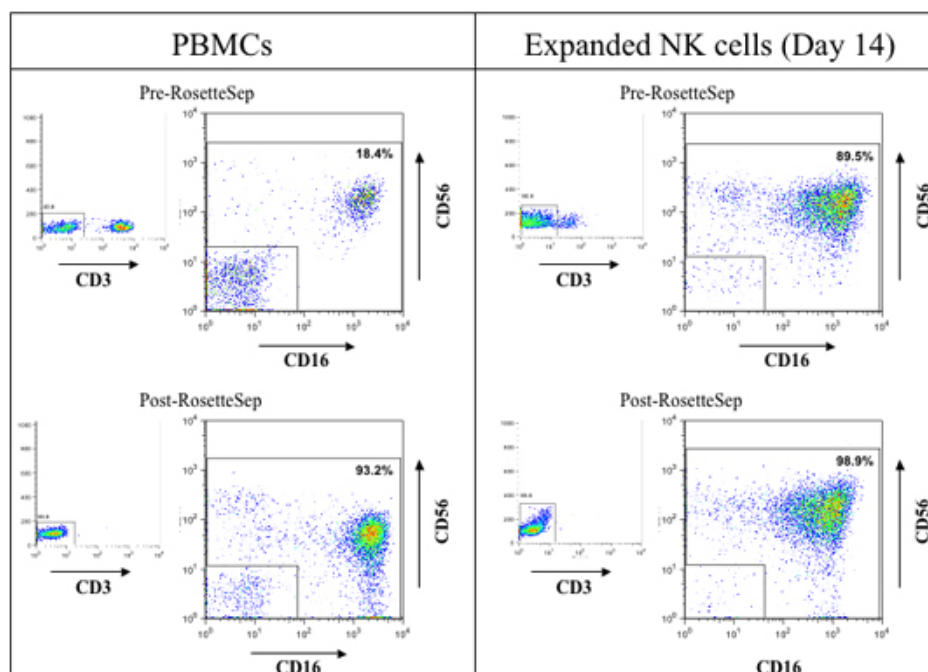


Figure 4. PBMCs recovery from Buffy coat is donor dependent and can range from 300×10^6 to 800×10^6 . NK cells may comprise 2% - 18% of the PBMCs. For RosetteSep purification of expanded cells, recovery of pure NK cells on day 14 ranges from 40-70%. By following the recommended protocol of expansion and purification, NK cell purity of 99% can be expected.

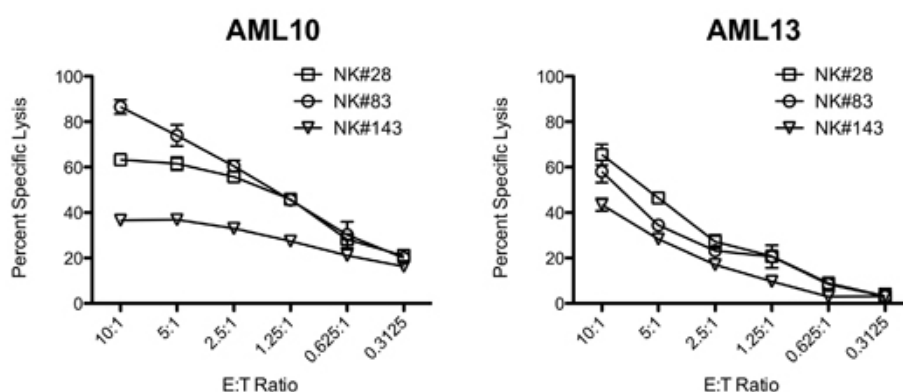


Figure 5. Expanded NK cells have demonstrated cytotoxicity against a range of tumor cell lines including neuroblastoma, AML, osteosarcoma and melanoma (representative AML killing shown as percent specific lysis).

Disclosures

No conflicts of interest declared.

Acknowledgements

The authors would like to thank Laurence Cooper, Harjeet Singh, and Lenka Hurton for their work in creating the initial K562 aAPC and mL21 fusion vectors.

Funding for this work was provided by the UT MD Anderson Physician Scientist Program, the St. Baldrick's Foundation, and the Legends of Friendswood.

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