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Srinivas S. Somanchi *Editor*

Natural Killer Cells

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Cover illustration: YTS NK cell activated on glass, fixed, permeabilized and stained for F-actin with phalloidin. The false color image was generated by stimulated emission depletion (STED) super resolution microscopy imaging in 3D then reconstructed and surface rendered to delineate non-contiguous regions of F-actin.
– Emily Mace, Jordon Orange Lab, Center for Human Immunobiology, Baylor College of Medicine and Texas Children's Hospital, Houston, TX, USA

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Preface

Natural killer (NK) cells are the large granular lymphocytes of innate immune system. NK cells play a central role in defense against viral/microbial infections and immune surveillance against tumors. The phenomenon of "natural" or "spontaneous" killing was first observed against tumor cell lines in the early 1970s, and the lymphocytes responsible for this "natural" killing were later characterized in 1975 and dubbed Natural Killer cells. However, little was known about the phenotype of these cells and how they function, and these lymphocytes were often referred to as "null cells."

Our understanding of NK cells has come a long way from their simple portrayal as "null cells"; NK cells are now regarded as a complex subset of lymphocytes that function along the boundaries of innate and adaptive immunity. With the identification of a plethora of activating and inhibitory receptors that are expressed on NK cells, we have gained significant understanding of the orchestration of signaling balance that drives the "natural" killing by NK cells. In addition to direct lysis, NK cells regulate immune responses by other lymphocytes through the secretion of cytokines. The complexity of NK cell behavior is epitomized by the discovery of antigen-specific memory response, a feature normally associated with adaptive immunity. Additionally the diversity and adaptability of NK cells is exemplified by the delineation of (tens of) thousands of phenotypically distinct NK cell subsets in the peripheral blood within an individual that emerge through "adaptable expression" of activating receptors in response to prevailing stimulatory environment. NK cells have therefore emerged as one of the most appealing candidates for adoptive immunotherapy for cancer, and there is a significant momentum to take NK cells from "Bench to Bedside."

Natural Killer Cells: Methods and Protocols provides a collection of research methodologies relevant for both basic and translational research. In this methods book, a compilation of techniques is presented pertaining to new developments in NK cell field such as understanding the influence of NK cell metabolism on its function, identifying complexity of NK cell subsets through mass cytometry, and determining the emergence of memory NK cells in murine model of MCMV infection. This book also includes methods to study - NK cell migration and cytotoxicity through endpoint analysis or live single cell imaging, assessment of calcium mobilization, the role of lipid raft in NK cell signaling and NK cell immunological synapse with target cells or cell-free lipid bilayers using high-resolution microscopy; in order to further our understanding of the intricacies of inhibition and activation, receptor and lytic granule polarization to synapse as well as to study the signaling of individual receptors or class of receptors in isolation. Additionally, detailed protocols are described for genotyping single nucleotide polymorphisms and copy number of Fc receptors expressed on NK cells as well as determining the effect of replication on telomere length. The methods pertaining to translational application of NK cells include ex vivo expansion of NK cells on K562 cell lines genetically modified to express either membrane bound IL-15 or membrane bound IL-21, large-scale NK cell culture, current techniques for engineering NK cells to express chimeric antigen receptors or chemokine receptors

using retroviral vectors, electroporation of mRNA, as well as through the natural phenomenon of trogocytosis. Also included are chapters detailing protocols for siRNA knockdown of gene expression in NK cells and differentiation of gene modified NK cells from hematopoietic stem cells.

Additional chapters include protocols for preclinical evaluation of NK cell adoptive immunotherapies in murine models and methodologies to track adoptively infused NK cells in vivo using fluorescence and MRI imaging. Also included are overview chapters on; bi- and tri-specific killer cell engagers that are emerging as appealing therapeutic molecules for NK cell adoptive immunotherapy, and the regulations and logistics that govern the clinical translation of NK cell adoptive immunotherapies.

Though our understanding of NK cells has dramatically evolved over the past decades, the lytic function against tumors that led to the identification of these lymphocytes remains to be fully exploited for cancer therapy. With the emergence of robust platforms to expand NK cells, and methods to improve tumor recognition and homing, the field is well positioned for propelling our enhanced understanding of these cells towards novel and effective adoptive immunotherapy applications.

I would like to take this opportunity to express my gratitude to all the authors for their time and for sharing their expertise through the contribution of detailed protocols and notes for this book on *Natural Killer Cells: Methods and Protocols* in *Methods in Molecular Biology* series. I hope that the protocols provided here would be valuable resources to researchers not only to understand mechanisms that govern NK cell behavior and diversity but also to systematically evaluate NK cells for adoptive immunotherapy applications.

Houston, TX, USA ***Srinivas S. Somanchi***

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Chapter 1

Tracking Effector and Memory NK Cells During MCMV Infection

Aimee M. Beaulieu and Joseph C. Sun

Abstract

In C57BL/6 mice, NK cells expressing the activating receptor Ly49H proliferate robustly in response to mouse cytomegalovirus (MCMV) infection. The expansion of Ly49H$^+$ NK cells peaks at approximately 1 week post-infection, and is then followed by a distinct contraction phase that ultimately leaves a small but long-lived pool of MCMV-experienced Ly49H$^+$ NK cells that are capable of mediating enhanced memory-like responses during subsequent encounters with MCMV. Here we describe an adoptive transfer model in which the expansion, contraction, and memory cell persistence of transferred Ly49H$^+$ NK cells are tracked in congenic C57BL/6 hosts following MCMV infection.

Key words NK cells, Memory, MCMV, Innate lymphocytes

1 Introduction

Natural killer (NK) cells play a critical role in host immunity to viruses and cancer. Historically NK cells were considered innate immune cells, incapable of immunological memory. However, a number of recent studies have led to a revision of our understanding of NK cell biology and have revealed that NK cells can, under specific circumstances, exhibit features associated with adaptive immunity, including the ability to develop into long-lived "memory" cells with enhanced protective responses to previously encountered viruses and antigens [1–4].

Although adaptive immune responses by NK cells have now been described in both humans and mice, MCMV infection in C57BL/6 mice is arguably the most widely used experimental system to study the development and function of memory NK cells [4]. In C57BL/6 mice, NK cells expressing the activating receptor Ly49H directly recognize and are activated by MCMV-infected cells [5–8]. Recognition is mediated by high-affinity binding between the Ly49H receptor and the MCMV-encoded glycoprotein m157 expressed on the surface of infected cells.

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Receptor-ligand engagement drives a robust proliferative response in the Ly49H$^+$ NK cells [7, 9]. In settings where Ly49H$^+$ NK cells are adoptively transferred into Ly49H-deficient hosts, transferred cells can achieve a 100–1000-fold expansion during the first week after infection [9]. The kinetics of this response closely mirror that of classic CD8$^+$ T cell responses [10], with the expansion of effector Ly49H$^+$ NK cells peaking at around 7–9 days post-infection, followed by a distinct contraction phase that ultimately leaves behind a small, long-lived pool of "MCMV-experienced" NK cells that can exhibit features of immunological memory. In particular, these memory NK cells have been shown to persist for months, if not years, in host animals and, most critically, can mediate more protective immune responses, as compared to naïve NK cells, when re-exposed to MCMV [9, 11]. In this chapter we provide methodology to track the expansion, contraction, and long-term persistence (i.e., the memory phase) of adoptively transferred Ly49H$^+$ NK cells responding to MCMV infection.

2 Materials

2.1 Reagents

Prepare all aqueous solutions using ultrapure water obtained by purifying deionized water using a MilliQ apparatus at a sensitivity of 18 MΩ cm at 25 °C. All solutions and reagents are kept sterile and stored at 4 °C, unless otherwise noted.

1. Complete Dulbecco's Modified Eagle's Medium (cDMEM): 1× DMEM containing 100 U/mL penicillin-streptomycin and 2 mM L-glutamine. Heat-inactivated fetal bovine serum (FBS) can be added to the cDMEM to a final concentration of 3% (cDMEM-3), 5% (cDMEM-5), or 10% (cDMEM-10), as noted in the protocol below.
2. Complete RPMI-1640 medium with 3% FBS (cRPMI-3): RPMI-1640 medium containing 100 U/mL penicillin-streptomycin, 2 mM L-glutamine, and 3% (v/v) heat-inactivated fetal FBS.
3. DMEM/5% milk: Dissolve powdered skim milk in water to a final concentration of 10% (w/v), and then autoclave for 15 min to sterilize. The milk will turn light brown after autoclaving due to caramelization of the milk sugars. Cool the milk solution to room temperature, and then mix in equal volumes with sterile low-glucose 2× DMEM. Prepare fresh 10% milk and DMEM/5% milk immediately prior to use.
4. Trypsin digest medium: Hanks' Balanced Salt solution (HBSS) without calcium and magnesium, containing 0.25% (w/v) trypsin and 0.53 mM EDTA.

5. CMC medium: Make a 1.5% solution (w/v) of carboxymethylcellulose by slowly dissolving 6 g of medium-viscosity carboxymethylcellulose (Sigma, C4888) in hot water while stirring and heating. Autoclave the solution for 20 min to sterilize, and then cool to room temperature. Separately, prepare a sterile solution of low-glucose 2× DME containing 20% heat-inactivated FBS, 200 U/mL penicillin-streptomycin, and 4 mM L-glutamine. The CMC medium is prepared immediately prior to use by mixing equal volumes of the 2× DME solution and the 1.5% carboxymethylcellulose solution.
6. Crystal violet stain: Prepare a 1% stock solution (w/v) of crystal violet in water. Store at room temperature. Immediately prior to staining, make a working stain by mixing 1 mL of the 1% crystal violet stock, 1 mL of 37% (w/v) formaldehyde, and 8 mL of water.
7. Antibodies: Obtain unconjugated purified rat monoclonal antibodies against CD4 (clone GK1.5), CD8 (53.6.72), CD19 (1D3), and Ter119, and fluorophore-conjugated monoclonal antibodies against NK1.1 (PK136), Ly49H (3D10), TCRβ (H57-597), CD3 (145-2C11), and CD69 (H1.2F3). In addition, BioMag Goat Anti-Rat IgG magnetic beads (Qiagen, #310107) and a 15 mL or 50 mL Tube Magnet (Qiagen) are required for enrichment of splenic NK cells by negative selection.
8. ACK lysis buffer: Dissolve 8.29 g of NH_4Cl, 1 g of $KHCO_3$, and 0.0372 g Na_2EDTA in 800 mL of water. Add HCl until pH of 7.2–7.4, and then add water to a final volume of 1000 mL. Filter through 0.2 μM filter to sterilize.
9. Freezing medium: DMEM containing 20% FBS and 10% DMSO.
10. Neat DMEM: Plain Dulbecco's Modified Eagle's Medium without any supplements.
11. Phosphate-buffered saline (PBS).

2.2 Mice

1. Balb/c mice.
2. CD45.1$^+$ B6.SJL mice.
3. Ly49H-deficient mice, generated by breeding BXD-8 mice (which naturally lack a segment of the natural killer gene complex (NKC) locus on chromosome 6 that includes the gene encoding Ly49H) onto a C57BL/6 background, can be obtained from S. Vidal (McGill University, Montreal, Canada) [12].

2.3 Preparation of MCMV Stocks

1. MCMV (Smith strain; ATCC), propagated in vitro in mouse embryonic fibroblasts (MEFs; generation of MEFs is described in Subheading 3.2).

2. Insulin syringes.
3. Dissecting scissors and forceps.
4. Dounce tissue homogenizer with a tight pestle.
5. 15 mL conical tube.
6. 50 mL conical tube.
7. Ice bucket.
8. 1.5 mL microcentrifuge tubes.

2.4 MCMV Plaque Assay

1. MCMV salivary gland stock (described in Subheading 3.1).
2. MEFs (generation of MEFs is described in Subheading 3.2).
3. 15 mL conical tube.
4. 50 mL conical tube.
5. Dissecting scissors and forceps.
6. 10 cm tissue culture dishes.
7. 12-well tissue culture plate.

2.5 Adoptive Transfer

1. Dissecting scissors and forceps.
2. Frosted glass slides.
3. Nylon mesh.
4. 15 mL conical tube.
5. 50 mL conical tube.
6. Ice bucket.
7. Insulin syringes.
8. Multicolor flow cytometer.

3 Methods

3.1 Preparation of Viral Stocks from Balb/c Salivary Glands

Generation of viral stocks is achieved by serial passage of MCMV through BALB/c hosts. Specifically:

1. Infect 4–6-week-old BALB/c mice by injecting approximately 1×10^4 plaque-forming units (PFU) of MCMV (*see* **Note 1**) diluted in 500 μL sterile PBS into the peritoneal cavity of each mouse. Typically five animals are sufficient to generate a stock, but more animals can be used if a larger stock is desired or needed.
2. On day 21, sterilely resect the salivary glands from each infected animal. Pool the salivary glands from three animals and resuspend in 1 mL of ice-cold DMEM/5% milk. Disrupt tissue by "douncing" 20 times using a prechilled Dounce tissue homogenizer. Keep as cold as possible throughout this process.

3. Decant the homogenate into a 15 mL conical tube, and then pellet the tissue debris by centrifuging for 5 min at 805 × *g* at 4 °C.
4. Transfer the supernatant to a prechilled 50 mL conical tube.
5. Pool dounced salivary gland homogenate from up to ten animals into a prechilled Dounce tissue homogenizer. Add 1 mL of ice-cold DMEM/5 % milk to pooled homogenate. Dounce homogenize again 20 times, keeping the homogenate as cold as possible throughout. Decant the homogenate into a 50 mL conical tube, pellet any tissue debris by centrifuging for 5 min at 805 × *g* at 4 °C, and then harvest the supernatant.
6. Combine the supernatants from all animals into a single sample, mix, and aliquot as 35 μL samples into pre-autoclaved 1.5 mL microcentrifuge tubes. Store aliquots at −80 °C.
7. To quantify the stock viral titers, *see* Subheading 2.5 below.
8. The first stock generated by passage of a tissue culture stock through Balb/c hosts is known as "Passage 1." Successive in vivo passages can be generated by repeating **steps 1**–7, except using the salivary gland-derived MCMV stock as the virus source for **step 1** (*see* **Note 2**). Regardless of passage, each stock must always be evaluated as described in **Note 11** in Subheading 3.4, **step 13**, below.

3.2 Generating MEFs for Plaque Assay

1. Set up a breeding pair of C57BL/6 mice and examine the vaginal cavity of the female at least twice per day for the formation of a mucus plug, which indicates successful fertilization and day 0 of the gestation period.
2. On exactly gestational day 17, euthanize the pregnant female by cervical dislocation and open the peritoneal cavity to expose the embryonic sac (*see* **Note 3**).
3. Cut open the embryonic sac and remove each of the intact embryos. Euthanize the embryos by decapitation.
4. Remove and discard the arms, legs, tail, and internal organs of each embryo, and then rinse the remaining body core thoroughly with sterile PBS.
5. Cut the embryonic tissue into small pieces, and place the pooled fragments of all embryos into a single 50 mL conical tube. Add 4 mL of trypsin digest medium per embryo and digest at 4 °C for 16 h.
6. Without disturbing or removing any of the settled tissue fragments or cells, remove and discard all but a small volume (approximately 0.5–2 mL, depending on the number of embryos) of the trypsin digest medium. Incubate the remaining tissue at 37 °C with 5 % CO_2 for 30 min.

7. Add 15 mL of cDMEM-10 and pipet vigorously to break up the tissue. Allow undigested tissue to briefly settle to the bottom (about 2 min), then harvest, and save the supernatant. To the remaining undigested tissue, add another 10 mL of cDMEM-10 and pipet vigorously again. Allow the tissue fragments to briefly settle to the bottom, and then again harvest the supernatant. One last time, add 10 mL of cDMEM-10 to any remaining tissue, pipet vigorously, allow to settle, and then harvest the supernatant.
8. Combine all three supernatant fractions (approximately 35 mL total), which will contain any MEFs liberated during the digest. Allow cells to settle by gravity while sitting on ice (~15 min). Remove all but approximately 2 ml of the medium and resuspend in fresh cDMEM-10 pre-warmed to 37 °C.
9. Seed MEFS into 10 cm tissue culture dishes (three dishes per embryo) in approximately 12 mL of cDMEM-10. Gently pipet up and down in the culture medium to ensure even settling of the MEFs across the dish surface.
10. Incubate the MEFs at 37 °C with 5% CO_2. On the next day, replace the culture medium, with fresh, pre-warmed cDMEM-10 (*see* **Note 4**). Incubate for an additional 3–4 days at 37 °C with 5% CO_2 until the cells are approximately 90% confluent.
11. To harvest the MEFs, remove the culture medium and add approximately 3 mL of the trypsin digest medium. Incubate at 37 °C for 1–2 min, with periodic swirling, until the monolayer begins to detach from the cell culture dishes. Resuspend the cells by gentle pipetting and transfer to a 50 mL conical tube, combining the cells from all dishes. Pellet by centrifugation at $300 \times g$ at 4 °C for 5 min, and then wash two times with ice-cold cDMEM-10. Resuspend in ice-cold cDMEM-10 and use a small aliquot to count the total number of viable cells using a hemacytometer (*see* **Note 5**).
12. The harvested MEFs can be used immediately for the MCMV plaque assay (*see* Subheading 3.3 below), or store aliquots of four to five million cells per mL in freezing medium in liquid nitrogen until further use.

3.3 Plaque Assay to Quantify MCMV Titers

1. Resuspend fresh (or freshly thawed) MEFs in cDMEM-10 at a concentration of 2×10^5 cells per mL, and then add 1 mL of the suspension to each well of a 12-well tissue culture plate. Typically, 12 wells of MEFs are needed to titer a single MCMV sample. Incubate the cells for 16 h at 37 °C with 5% CO_2 until the MEF monolayer is approximately 85% confluent.
2. Thaw an aliquot of the MCMV salivary gland stock (*see* Subheading 3.2, above) and keep on ice. Mix by gently pipet-

ting, and then sterilely add 30 μL of the stock to 270 μL of neat DMEM to create a 10^{-1} dilution of the primary stock. Perform additional tenfold serial dilutions (100 μL plus 900 μL neat DMEM), each in duplicate. It is important to mix each dilution thoroughly and change pipette tips between serial dilutions. Typically, a dilution series ranging from 10^{-2} to 10^{-7} is sufficient.

3. Remove the culture medium from the MEF monolayer and immediately add 250 μL of the MCMV dilution, taking care that the monolayer does not dry out during this process. Each well is inoculated with a single dilution.
4. Incubate for 1.5 h at 37 °C with 5% CO_2, swirling the plate every 15 min to ensure equal distribution of the virus.
5. Remove the virus-containing medium from the MEF monolayer, and then immediately and gently overlay with 2 mL of CMC medium.
6. Incubate for 6–8 days until plaques (*see* **Note 6**) are visible at low magnification under a light microscope.
7. Very carefully remove the CMC medium, being extra cautious not to disturb the MEF monolayer. Rinse each well gently and carefully by slowly adding and removing 2 mL of PBS. Add just enough crystal violet stain to barely cover the monolayer, and then incubate for 2–3 min to stain the monolayer. Use a pipette to carefully remove the stain, and then rinse repeatedly with PBS (again, being extra careful not to disturb the monolayer) until little or no stain comes off during the wash.
8. Count plaques and calculate the MCMV titer. Titer (PFU/mL) = (dilution factor) *multiplied by* (number of plaques per well) *multiplied by* (1/0.25 mL of inoculum per well) (*see* **Note 7**).

3.4 Adoptive Transfer of CD45.1$^+$ Ly49H$^+$ NK Cells and MCMV Infection

Briefly, NK cells, enriched by negative selection from the spleens of CD45.1$^+$ B6.SJL donor animals, are adoptively transferred into Ly49H-deficient recipient animals 1 day prior to infection with MCMV. Specifically:

1. Euthanize 6–8-week-old donor B6.SJL mice (CD45.1$^+$) by CO_2 asphyxiation or cervical dislocation (*see* **Note 8**). Donor and recipient animals should all be sex matched.
2. Resect the spleen from the abdominal cavity and place in ice-cold cRPMI-3. Crush the spleen between frosted glass slides to generate a single-cell suspension (*see* **Note 9**).
3. Transfer to a 50 mL conical tube and pellet the splenocytes by centrifugation at 453 × *g* 4 min at 4 °C. Decant supernatant, wash the pellet with 5 mL of ice-cold cDMEM-3, and then pellet again by centrifugation at 453 × *g* for 3 min at 4 °C.

4. Aspirate off the supernatant and resuspend the cells in ice-cold cRPMI-3 (1 mL per spleen) containing 0.010 mg/mL of αCD4 (clone GK1.5), αCD8 (clone 53.6.72), αCD19 (clone 1D3), and Ter119 purified rat monoclonal antibodies. Incubate the suspension on ice for 20 min.
5. Meanwhile, resuspend the BioMag Goat Anti-Rat IgG magnetic beads by shaking, and transfer the required volume to a conical tube (typically, 2.5 mL of suspended beads for every donor spleen). Place the conical tube containing the beads in a magnet and allow beads to aggregate at the side of the tube (approximately 5 min). Carefully aspirate off the buffer without disturbing the beads. Remove the beads from the magnet, add 10 mL of PBS, and mix by vortexing. Place the tube back in the magnet, allow the beads to aggregate at the side, and then aspirate off the PBS without disturbing the beads. Remove tube from magnet, resuspend the beads in ice-cold cRPMI-3 (0.5 mL per spleen), and place on ice.
6. Pellet the antibody-treated splenocytes from **step 4** by centrifugation at 453×*g* for 3 min at 4 °C, and then wash two times with fresh ice-cold cRPMI-3. After the final wash, resuspend the cells in ice-cold cRPMI-3 (0.5 mL per spleen), and then add the beads from **step 5** above. Mix the beads and cells by vortexing, and then incubate on ice for 20 min. Mix by gentle vortexing periodically throughout the incubation.
7. After 20 min, dilute the suspension in ice-cold cRPMI-3 such that the total volume is approximately 80% of the total tube volume. Place the suspension in the magnet and allow the beads to aggregate at the side of the tube (approximately 5 min). Without disturbing the beads, harvest and transfer the supernatant to a new conical tube. Place the harvested supernatant in the magnet for another 3 min, to allow any remaining beads to aggregate at the side of the tube, and then harvest again without disturbing any beads at the side of the tube. Filter the supernatant through nylon mesh to remove any debris and collect in a new conical tube.
8. Count the total number of viable cells in the supernatant using a hemacytometer.
9. Surface stain a very small aliquot of the cells (~100 μL) with a cocktail containing the following fluorophore-conjugated antibodies: NK1.1 (PK136), Ly49H (3D10), TCRβ (H57-597), and CD3 (145-2C11). Use a flow cytometer to determine the percentage of $TCR\beta^{-}CD3^{-}Ly49H^{+}NK1.1^{+}$ cells (i.e., $Ly49H^{+}$ NK cells) in the sample.
10. Calculate the total number of $Ly49H^{+}$ NK cells in the supernatant by *multiplying* the total number of viable cells determined in **step 8** by the percentage of $Ly49H^{+}$ NK cells determined in **step 9**.

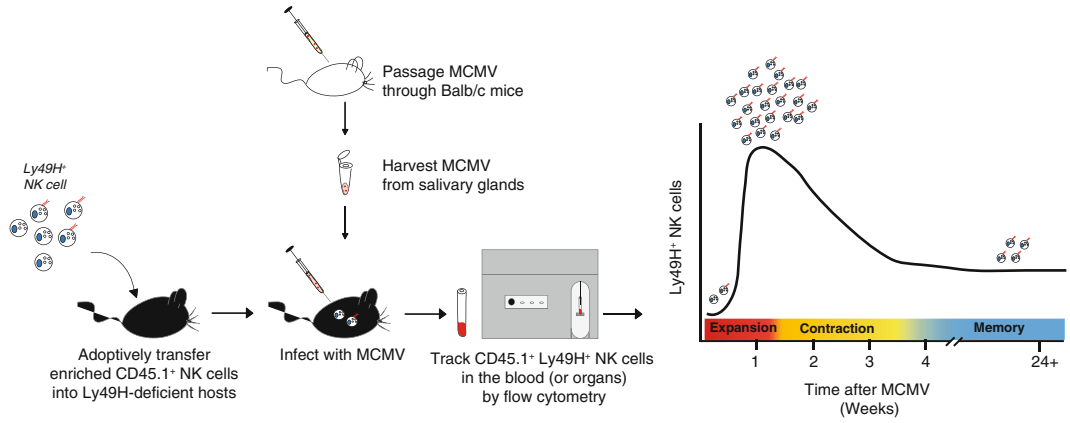

Fig. 1 Summary of experimental methodology. NK cells enriched from the spleens of CD45.1$^+$ mice are adoptively transferred into Ly49H-deficient recipients. Recipients are then infected with MCMV generated by serial passaging through Balb/c mice. The number and percentage of transferred CD45.1$^+$ Ly49H$^+$ NK cells are tracked by flow cytometry throughout the expansion, contraction, and memory phases post-infection

11. Pellet the cells by centrifugation at 453 × *g* for 3 min at 4 °C, and then resuspend in sterile ice-cold PBS at a final concentration of 1×10^6 Ly49H$^+$ NK cells per mL.
12. Using an insulin syringe, intravenously inject 200 μL (containing 2×10^5 CD45.1 Ly49H$^+$ NK cells) into the Ly49H-deficient host animals (*see* **Note 10**).
13. One day after transfer, the host animals are infected by intraperitoneal injection of a predetermined dose of MCMV (*see* **Note 11**) diluted in 500 μL sterile PBS (experimental schema is shown in Fig. 1).

3.5 Tracking the Expansion, Contraction, and Memory Phases of Transferred Ly49H$^+$ NK Cells

The kinetics of the Ly49H$^+$ NK cell response to MCMV is highly reminiscent of a classical CD8$^+$ T cell response, with distinctive effector, contraction, and memory phases. During the first week post-infection, or the effector phase, the Ly49H$^+$ NK cells undergo robust proliferation, with the result that the total numbers can increase 100–1000-fold by days 7–9. Peak expansion then gives way to a distinct "contraction" phase, typically between days 10 and 21 post-infection, during which time most, but not all, of the Ly49H$^+$ NK cells undergo cell death and are cleared from the host. What remains is a small but relatively stable pool of MCMV-experienced Ly49H$^+$ NK cells that can persist in the host for periods of time exceeding 6 months (i.e., the memory phase). The number and percentage of transferred Ly49H$^+$ NK cells during each of these effector, contraction, and memory phases can be experimentally tracked by flow cytometry, as follows.

1. On day 2 post-infection, obtain 50 μL of blood by retro-orbital bleeding. As a control, obtain blood from an uninfected wild-type animal.

2. Transfer the sample(s) to a 96-well plate, and lyse the red blood cells by incubating with 200 μL of ACK lysis buffer for 5 min on ice. Pellet the cells by centrifugation at 805 × *g* for 3 min at 4 °C. Discard the supernatant.
3. Repeat **step 2** at least two more times, until the cell pellet is mostly white in color.
4. Wash cells with 200 μL of RPMI-3. Pellet the cells by centrifugation at 805 × *g* for 3 min at 4 °C. Discard the supernatant. Repeat once.
5. Block Fc receptors by incubating with the 2.4G2 monoclonal antibody in RPMI-3 for 15 min on ice. Pellet the cells by centrifugation at 805 × *g* for 3 min at 4 °C. Wash once with RPMI-3.
6. Surface stain the cells for 20 min on ice with a cocktail containing the following fluorophore-conjugated antibodies in RPMI-3: NK1.1 (PK136), TCRβ (H57-597), CD3 (145-2C11), and CD69 (H1.2F3).
7. Wash two times with ice-cold PBS, resuspend in ice cold RPMI-3 or FACS buffer, and use a flow cytometer to confirm that the $TCR\beta^{-}CD3^{-}NK1.1^{+}$ NK cells have upregulated CD69 (indicating a productive MCMV infection), compared to NK cells from the uninfected control animal.
8. For animals that are productively infected with MCMV, evaluate the numbers and/or percentage of $Ly49H^{+}$ NK cells in the blood or specific organs (e.g., spleen, liver, or lungs) at various time points post-infection. Typically, time points of interest include day 7 (peak of the effector phase), day 14, and day 21 (the contraction phase), and day 28 and day 56 (memory phase) post-infection. Briefly, at each time point, harvest 100–200 μL of blood (or specific organs, if a terminal time point), lyse red blood cells with ACK lysis buffer, and surface stain as described above, using a cocktail containing fluorophore-conjugated antibodies for NK1.1, TCRβ, CD3, Ly49H, and CD45.1. The transferred $Ly49H^{+}$ NK cells ($TCR\beta^{-}CD3^{-}CD45.1^{+}Ly49H^{+}NK1.1^{+}$ cells) can be readily visualized and quantified by flow cytometry.

4 Notes

1. MCMV (Smith strain) can be obtained from the ATCC and maintained by passaging in vitro in MEFs [13].
2. In our experience, "Passage 1" stocks sometimes elicit lackluster $Ly49H^{+}$ NK cell responses in vivo, and "Passage 2" or "Passage 3" stocks are often more useful for the adoptive transfer experiments described below.

3. Use sterile technique and instruments throughout this procedure. Metal instruments, such as scissors and tweezers, should be sterilized by autoclaving and it is recommended that dissections be performed in a biosafety cabinet to maintain sterility.
4. The cultures will typically still contain some tissue debris and non-adherent cells at this stage and changing the medium helps with removal of these debris.
5. Each 10 cm dish will typically yield approximately seven to eight million MEFs.
6. Plaques appear as organized "holes" or lesions, surrounded by a rim of dying cells, in the monolayer and are caused by cell destruction at the focus of infection.
7. As a general rule, plaques are only counted at the dilution(s) that give easily identifiable, well-separated/non-overlapping plaques (ideally 20–50 plaques per well).
8. One donor animal will generally provide sufficient numbers of NK cells for transfer into three to four recipients.
9. Up to ten spleens can be pooled and crushed together if more than one donor animal is used.
10. Adoptive transfer of the NK cells via either tail vein injection or retro-orbital injection yields similar results.
11. It is critical that each MCMV stock is pretested to empirically determine the optimal dose for driving expansion of transferred Ly49H$^+$ NK cells, as follows. First, a pilot group of wild-type C57BL/6 animals are infected by intraperitoneal injection of 0, 5000, 25,000, 50,000, or 100,000 PFU of the MCMV stock. On day 7 post-infection, the number and percentage of splenic Ly49H$^+$ NK cells are determined by flow cytometry (*see* Subheading 3.5, **step 8** above) to identify the dose that drives maximal expansion of Ly49H$^+$ NK cells without causing overt signs of illness or splenic necrosis/atrophy. Next, adoptively transfer 2×10^5 Ly49H$^+$ NK cells into a pilot group of Ly49H-deficient hosts (*see* Subheading 3.4, **steps 1–13**, above) and on the following day infect with a range of MCMV doses, such that the highest dose is the one that was optimal in wild-type mice. In general, a range spanning approximately an order of magnitude is sufficient. For example, if the optimal dose in wild-type mice is 25,000 PFU, the Ly49H-deficient pilot group might be challenged with 25,000, 12,500, 6250, and 3125 PFU. Again, on day 7 post-infection, the number and percentage of transferred Ly49H$^+$ NK cells in the spleen are measured by flow cytometry. The optimal challenge dose for that specific MCMV stock will be the one that drives maximal expansion of the transferred Ly49H$^+$ NK cells without causing overt signs of illness or splenic necrosis or atrophy. In our experience, the optimal dose can vary significantly from

stock to stock (and is particularly dependent on how many times a stock has been passaged through Balb/c hosts), and we have observed optimal dose ranges from 500 to 50,000 PFU depending on the stock used.

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Chapter 2

Application of Mass Cytometry (CyTOF) for Functional and Phenotypic Analysis of Natural Killer Cells

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Abstract

Mass cytometry is a novel platform for high-dimensional phenotypic and functional analysis of single cells. This system uses elemental metal isotopes conjugated to monoclonal antibodies to evaluate up to 42 parameters simultaneously on individual cells with minimal overlap between channels. The platform can be customized for analysis of both phenotypic and functional markers. Here, we will describe methods to stain, collect, and analyze intracellular functional markers and surface phenotypic markers on natural killer cells.

Key words CyTOF, Mass cytometry, Flow cytometry, Natural killer cell, Intracellular cytokine staining

1 Introduction

Mass cytometry is a next-generation flow cytometry platform with several technological advances that offer advantages over fluorescence-based flow cytometry when highly parametric analyses are required. Most notably, mass cytometry does not rely on detection of fluorescence, which requires compensation for spillover into adjacent channels. Instead, through the use of antibodies coupled to metal isotopes, mass cytometry can detect discrete isotope peaks without significant overlap [1, 2], thus ameliorating the need for compensation. In addition, more channels are available. The primary limiting factor is the chemistry required to conjugate the metals to the antibodies with high efficiency. Currently, mass cytometry allows for the detection of 42+ unique parameters rather than the 8–12 parameters that comprise a typical flow cytometry panel.

This increase in total parameters is critical for the study of natural killer (NK) cells, the diverse functional properties of which are influenced by combinatorial expression of multiple phenotypic markers [3, 4]. The functional properties of NK cells can be explored through multiple stimulation conditions including phorbol 12-myristate 13-acetate (PMA) + ionomycin, cytokines

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(such as IL-2, IL-12, IL-15, and/or IL-18), viral stimulation (such as HIV-1-infected or influenza-infected cells), or cell lines deficient in MHC-I expression (such as K562 or 721.221 cells). To analyze intracellular cytokines and chemokines, brefeldin A and monensin are added for the final 4 h of stimulation to maintain the cytokines intracellularly for detection [5]. Because CD107a (a lysosomal protein also known as LAMP-1) is briefly revealed at the cell surface during cytotoxic granule release, anti-CD107a antibodies added during stimulation serve as an indirect measure of cytotoxicity. Most antibodies used in mass cytometry bind directly to the target protein; however, in some cases, two-stage detection is more efficient. For optimal detection of CD107a by mass cytometry, anti-CD107a-APC is added during the stimulation, followed by isotope-conjugated anti-APC antibody as a surface stain.

Antibody–metal isotope pairs are available for purchase from Fluidigm (http://maxpar.fluidigm.com/product-catalog-metal.php). However, optimizing a panel that both explores the desired markers and accounts for isotope spillover and varying degrees of antibody signal intensity often requires a customized panel. Conjugation of antibodies and metal isotopes is an easily performed step that results in increased options for panel design, and has been previously described in detail [6]. Here we describe a method to profile both the phenotypic and functional characteristics of natural killer cells by conjugating antibodies to selected metal isotopes, surface and intracellular labeling of cells and analysis on a mass cytometer. The process of conjugating a custom panel for phenotypic analysis has been described previously; we will mention NK-cell-specific modifications here [3]. This chapter focuses on the addition of conditions for intracellular staining and functional assessment of NK cells, but can also be adapted for other cell types.

2 Materials

All reagents and containers must be free from heavy metal contaminants. No containers exposed to soap that may contain trace metal contaminants should be used. All buffers should be prepared and stored in disposable uncontaminated containers.

2.1 Antibody Conjugation

1. Maxpar® Metal labeling kits (Fluidigm Corporation).
2. Purified monoclonal antibodies (Fig. 1 and Table 1 provide examples), purified IgG or polyclonal. Must not have any carrier protein; otherwise, special order is required. Sodium azide is acceptable.
3. Centrifugal Filter Unit: 50 kDa Amicon Ultra—500 μL V bottom (Millipore) or 30 kDa Amicon Ultra 500 μL V bottom (Millipore).

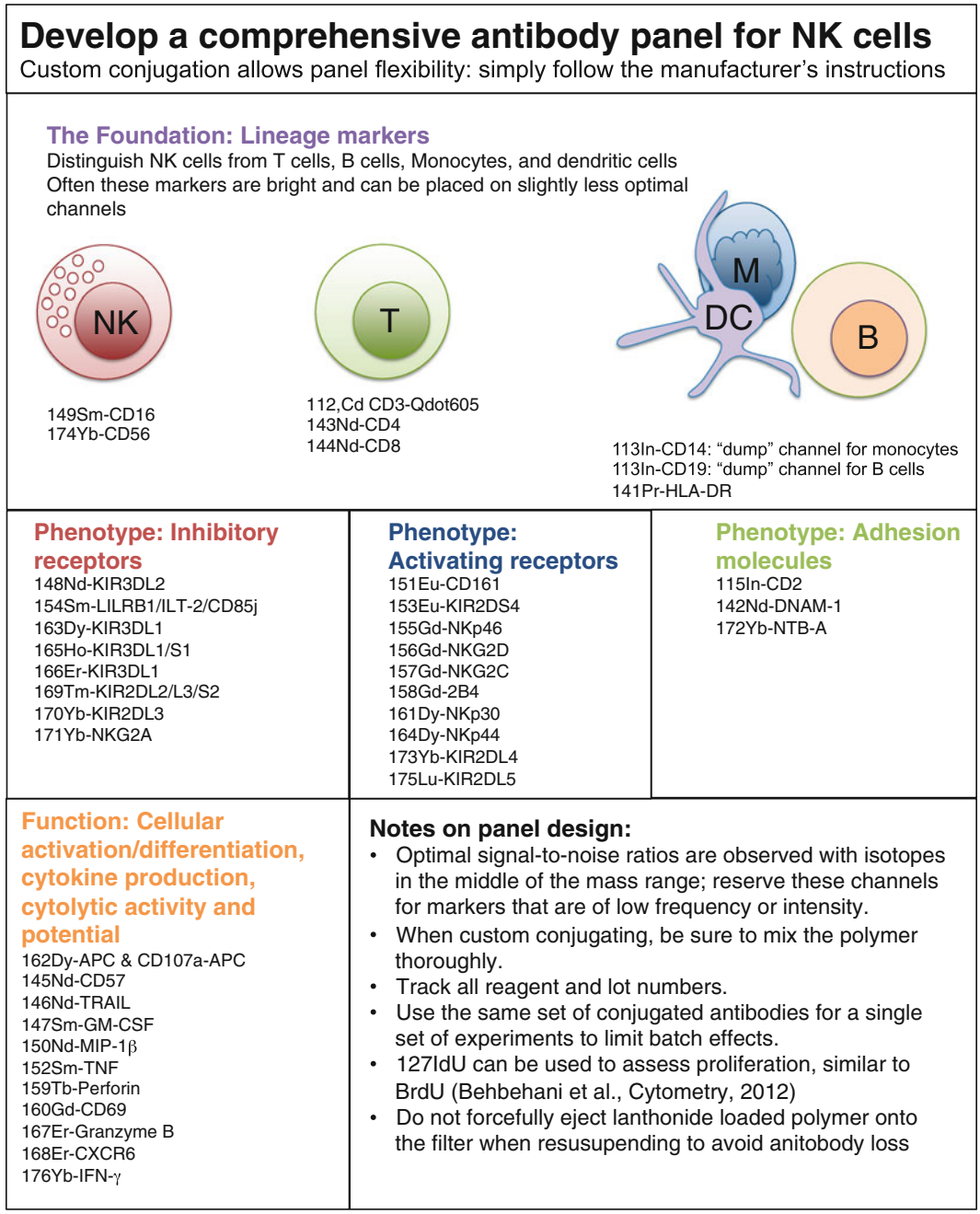

Fig. 1 Schematic display of CyTOF panel development

4. Centrifugal Filter Unit: 3 kDa Amicon Ultra—500 μL V bottom (Millipore).
5. Aerosol Barrier (Filter) Pipette Tips.
6. Bond-Breaker™ TCEP Solution: 0.5 M TCEP (Tris(2-carboxyethyl)phosphine) (Pierce).

Table 1
Example of table to generate antibody surface and intracellular staining cocktails

Isotope	Antibody	Clone	Supplier	Concentration (μg/mL)	Working concentration (μg/mL)	Volume to add[a]
Surface markers						
110,111,112,114Cd	CD3	UCHT1	Life Technologies (Qdot-605)	100 tests/100 μL	0.5 μL/test	10.5
113In	CD14, CD19	M5E2, HIB19	Biolegend	279, 342	2.5, 2.5	9.41, 7.68
115In	CD2	RPA-2.10	BD Biosciences	302	0.625	2.17
141Pr	APC	APC003	Biolegend	252	5.0	20.83
142Nd	DNAM-1	DX11	BD Biosciences	327	5.0	16.06
143Nd	CD4	SK3	Biolegend	171	1.25	7.68
144Nd	CD8	SK1	Biolegend	387	2.5	6.78
145Nd	CD57	HCD57	Biolegend	630	0.625	1.04
146Nd	TRAIL	RIK-2	Biolegend	208	2.5	12.62
148Nd	KIR3DL2		Lanier Lab	266	1.25	4.93
149Sm	CD16	3G8	BD Biosciences	385	1.25	3.41
151Eu	CD161	DX12	BD Biosciences	319	2.5	8.23
153Eu	KIR2DS4	FES172	Beckman Coulter	288	1.25	4.56
154Sm	ILT-2	GHI/75	Biolegend	393	2.5	6.68
155Gd	NKp46	9E2/NKp46	BD Biosciences	385	1.25	3.41
156Gd	NKG2D	1D11	Biolegend	257	5.0	20.43
157Gd	NKG2C	MAB1381	R&D Systems	138	2.5	19.02
158Gd	2B4	2-69	BD Biosciences	324	2.5	8.10
161Dy	NKp30	P30-15	Biolegend	286	2.5	9.18

163Dy	KIR3DL1	DX9	BD Biosciences	415	2.5	6.33
164Dy	NKp44	P44-8	Biolegend	293	1.25	4.48
165Ho	KIR3DL1/S1	Z27.3.7	Beckman Coulter	328	1.25	4.00
166Er	KIR2DL1	143211	R&D Systems	313	1.25	4.19
168Er	CXCR6	56811	R&D Systems	396	5.0	13.26
169Tm	KIR2DL2/L3/S2	GL183	Beckman Coulter	304	1.25	4.32
170Yb	KIR2DL3	180701	R&D Systems	273	2.5	9.62
171Yb	NKG2A	Z199	Beckman Coulter	428	2.5	6.13
172Yb	NTB-A	NT-7	Biolegend	94	1.25	13.96
173Yb	KIR2DL4	181703	R&D Systems	840	1.25	1.56
174Yb	CD56	NCAM16.2	BD Biosciences	436	1.25	3.01
175Lu	KIR2DL5	UP-R1	Biolegend	450	0.625	1.46
					#Vol CyFACS	786.69
Intracellular markers						
147Nd	GM-CSF	BVD2-21C11	Biolegend	119	5.0	44.12
150Nd	MIP-1β	D21-1352	BD Biosciences (Custom)	551	5.0	9.53
152Sm	TNF-α	MAb11	eBioscience	454	5.0	11.56
159 Tb	Perforin	B-D48	Abcam	381	5.0	13.78
160Gd	CD69	FN50	Biolegend	308	2.5	8.52
162Dy	HIVp24	38/5.4A	Abcam	280	10.0	37.50
167Er	Granzyme B	2CF/F5	BD Biosciences	442	5.0	11.88
176Yb	IFN-γ	4S.B4	eBioscience	401	5.0	13.09
					#Vol CyFACS	900.018

[a]Volume to add = (Working Concentration/Concentration) × 50 × Number of Samples (in this case 20) + 1
#Volume CyFACS = (50 × Number of Samples + 1) – Sum Total of the Volume of Antibodies

7. Microcentrifuge, ideally 2 units.
8. Heat block incubator or water bath at 37 °C.
9. PBS-based antibody stabilization solution (Candor Biosciences).
10. Nanodrop for protein quantification.

2.2 Mass Cytometry Labeling

1. Custom-conjugated antibodies or pre-conjugated antibodies purchased from Fluidigm (http://maxpar.fluidigm.com/product-catalog-metal.php).
2. Thermo Scientific™ Nunc™ 96 Deep Well Plates, Polystyrene. 96-well round bottom plates can be used for stimulation and incubation, but cells should be transferred to deep-well plates prior to surface staining.
3. MilliQ dH_2O: No water should have contact with beakers or bottles washed with soap.
4. CyPBS: PBS without heavy metal contaminants, made from 10× PBS using MilliQ purified water, with no contact with glassware washed with soap.
5. CyFACS buffer: 0.1% bovine serum albumin + 2 mM EDTA + 0.1% sodium azide in CyPBS. Filter solution with a 0.2 μM filter.
6. Cisplatin Solution: Prepare 100 mM (Stock solution) in DMSO. Freshly prepare working solution of 10 mM cisplatin in PBS (100 μL cisplatin stock + 900 μL PBS).
7. Lysing Solution: Prepare 1× solution from 10× stock (BD FACS™ Lysing Solution, BD Biosciences) solution using MilliQ deionized water and store in a disposable plastic container.
8. 0.1 μM spin filters (Millipore).
9. Permeabilization buffer: Prepare 1× permeabilization buffer from 10× stock solution (BD FACS™ Permeabilizing Solution 2, BD Biosciences) using MilliQ deionized water and store in a disposable plastic container.
10. Interchelator-PFA solution: Dilute Iradium-Interchelator solution (Fluidigm) 1:10,000 into 2% paraformaldehyde solution. Prepare the 2% Paraformaldehyde solution by diluting 16% Stock Paraformaldehyde (Electron Microscopy Sciences) in CyPBS. Freshly prepare the Interchelator-PFA solution for each use.
11. Complete RPMI medium: RPMI-1640, 10% Fetal Bovine Serum, 1× penicillin-streptomycin and 1× L-glutamine.
12. Refrigerated centrifuge equipped with rotor for spinning 96-well plates.
13. Aspirator with vacuum trap set-up.

2.3 Cell Stimulation

1. Cell Stimulation cocktail contains PMA and ionomycin (500×) (eBioscience): Freshly prepare 1× Working solution in complete RPMI medium (1:500 dilution).
2. CD107a-APC (Biolegend, Clone H4A3).
3. EDTA 0.5 M (Stock): prepare 20 mM working solution.
4. Monensin Solution (1000×) (eBioscience): Freshly prepare 1× Working solution in complete RPMI medium (1:1000 dilution).
5. Brefeldin A Solution (1000×) (eBioscience): Freshly prepare 1× Working solution in complete RPMI medium (1:1000 dilution).

2.4 Running CyTOF Mass Cytometry

1. Ice bucket.
2. Micropipettes.
3. Normalization beads (Fluidigm).
4. Filter-top tubes (BD Biosciences).

3 Methods

All steps may be completed at room temperature (RT) unless otherwise indicated.

3.1 Antibody Conjugation Using MaxPar Metal Labeling Kit

To conjugate antibodies simply follow manufacturer's instructions provided with the MaxPar Metal Labeling Kit using the supplies listed above. An example of a customized panel to profile natural killer cell phenotype and function is shown in Fig. 1. The conjugation protocol has been described in depth previously [2–4]. Custom-conjugating antibodies gives more flexibility in panel design than would be available by purchasing pre-conjugated antibodies. The panel outlined in Fig. 1 contains receptors to identify major cell lineages (such as CD3, CD4, CD8, CD19, CD14, CD56) as well as markers for the major NK cell receptor families including the killer immunoglobulin-like receptors (e.g., KIR2DL1, KIR2DL2/L3/S2), Fc receptors (CD16), natural cytotoxicity receptors (NKp30, NKp44, NKp46), C-type lectins (NKG2A, NKG2C, NKG2D), and markers of maturity and differentiation (e.g. CD57). It also includes NK cell functional cytokines (IFN-γ, MIP-1β, TNF-α) as well as cytotoxicity markers (CD107a, Perforin, Granzyme B). In general, greater signal-to-noise ratios can be expected on isotopes in the middle of the mass range and markers that are of low frequency or low intensity are best reserved for these channels. Please *see* **Notes 1–6** for additional suggestions regarding the conjugation of antibodies.

3.2 Surface and Intracellular Labeling of Cells for Mass Cytometry

1. Isolate human peripheral blood mononuclear cells by Ficoll gradient (*see* **Note 7**).
2. Wash the cells with complete RPMI medium and plate the cells at two million cells in 200 μL in a standard 96-well plate.
3. Add stimulation condition of choice; for example 1× Cell Stimulation cocktail (Subheading 2.3, **item 1**), 1× Brefeldin A and 1× Monensin. To assess degranulation include CD107a-APC at a dilution of 1:25 (25-fold dilution) in this Cell Stimulation cocktail. Incubate the cells at 37 °C for 4 h.
4. During the incubation, prepare the surface and intracellular antibody staining cocktails. This is best performed using a spreadsheet to calculate the quantities based on antibody titrations (Table 1).
5. At the end of the incubation add 20 μL of 20 mM EDTA to each well and mix by pipetting. Incubate for 10 min at room temperature.
6. Spin the plate at 750 × *g* for 3 min, aspirate the supernatant, and add 200 μL CyFACS. Transfer the cells to a deep 96-well plate.
7. Add 300 μL CyFACS to each well and spin the plate at 750 × *g* for 10 min. Aspirate the supernatant.
8. During centrifugation (**step 7**), dilute the 10 mM cisplatin working solution at a ratio of 1:200–1:50 (titration based on prior cisplatin batch optimization) in serum and antibiotic-free RPMI (*see* **Notes 8** and **9**).
9. Resuspend the cells in 400 μL of this diluted cisplatin.
10. Incubate cells for 1 min at RT.
11. Quench (*see* **Note 9**) with 400 μL of serum. Pipette to mix thoroughly.
12. Centrifuge plate at 750 × *g* for 10 min at RT. Aspirate the supernatant.
13. Add 500 μL of CyFACS buffer. Centrifuge plate at 750 × *g* for 10 min at RT. Aspirate the supernatant. After removing plate from the centrifuge, set centrifuge to 4 °C.
14. During centrifugation (**step 13**), centrifuge the surface antibody cocktail in a 0.1 μM Millipore spin filter for 3 min at 10,000 × *g*.
15. Resuspend pelleted cells in 50 μL of the antibody staining cocktail.
16. Incubate for 45 min on ice at 4 °C.
17. Add 500 μL CyFACS buffer to each well. Centrifuge plate at 750 × *g* for 10 min at 4 °C. Aspirate the supernatant.
18. Resuspend each well in 100 μL of 1× Lysing solution (Subheading 2.2, **item 7**) and incubate for 10 min at room temperature (*see* **Note 10**).

19. Add 500 μL CyFACS buffer to each well. Centrifuge at 750 × *g* for 10 min at 4 °C. Aspirate the supernatant.
20. Add 500 μL CyFACS buffer to each well. Centrifuge at 750 × *g* for 10 min at 4 °C. Aspirate the supernatant.
21. Resuspend cells in 200 μL of Permeabilization solution (Subheading 2.2, **item 10**). Incubate for 10 min at room temperature.
22. Add 500 μL CyFACS buffer to each well. Centrifuge at 750 × *g* for 10 min at 4 °C. Aspirate the supernatant.
23. During centrifugation (**step 22**), centrifuge the intracellular antibody cocktail in a 0.1 μM Millipore spin filter for 3 min at 10,000 × *g*.
24. Resuspend cells in 50 μL of the intracellular antibody staining cocktail.
25. Incubate for 45 min on ice at 4 °C.
26. Add 500 μL CyFACS buffer to each well, centrifuge at 750 × *g* for 10 min at 4 °C and aspirate the supernatant.
27. Repeat **step 26** two additional times.
28. Resuspend cells thoroughly in 100 μL of freshly prepared Interchelator-PFA solution.
29. Incubate overnight at 4 °C.
30. The following day (the same day that the cells will be run on the mass cytometer), add 500 μL of CyPBS buffer to each well. Centrifuge at 1000 × *g* for 10 min at RT. Aspirate the supernatant, leaving 100 μL residual volume in the well.
31. Add 500 μL MilliQ water (metal-free) to each well. Centrifuge at 1000 × *g* for 10 min at RT. Aspirate the supernatant, leaving 100 μL residual volume in each well.
32. Repeat **step 32** twice for a total of three washes in MilliQ water.
33. Resuspend cells in the residual 100 μL MilliQ water after the final wash.
34. Run on mass cytometer after resuspension in approximately 1 mL of MilliQ water with or without normalization beads immediately prior to the run. Pipette cells into a FACS tube through a cell-strainer cap to remove unwanted debris.

3.3 Running Samples on a CyTOF Mass Cytometer

Running samples on a CyTOF mass cytometer is normally performed by Flow Cytometry core services at most Institutions, to procure a detailed step-by-step protocol for operating CyTOF refer to the publication by Leipold M.D. et al. [7]. It is important

to note that when looking for rare cell populations, higher numbers of total cells will need to be run through the mass cytometer. Although the maximum collection rate is estimated at 1000 cells/s, to ensure the avoidance of doublets and nebulizer clots, it is prudent to use low run speeds of 300 cells/s or lower. For assay normalization within and among runs, normalization beads should be used as described in [8]. It is helpful to count the cells in each well in order to appropriately estimate the ideal resuspension volume. Cells should be stored on ice while waiting to run on the mass cytometer.

3.4 Data Analysis

Due to the increase in potential marker combinations allowed for by mass cytometry, manual gating of all possible cellular subsets is not possible. In addition, this approach does not allow for the evaluation of unexpected cellular subsets of interest [2]. However, typical manual gating schema can also be used to major known cell subsets and functions, although there are important differences to consider when analyzing in FlowJo (*see* **Notes 11** and **12** and Figs. 2 and 3). Boolean gating analysis can also be used to identify discreet cellular subsets as described by Horowitz et al. [4]. A number of analysis packages have now been developed in order to analyze CyTOF data in an unbiased manner. Spanning-tree progression analysis of density-normalized events (SPADE) is available through Cytobank and uses density-dependent down sampling, followed by agglomerative clustering, minimum spanning tree construction and upsampling to identify unique cellular subsets from CyTOF [9]. Citrus is an algorithm that identifies clusters of phenotypically similar cells in an unsupervised manner and identifies features (either functional or phenotypic) that are predictive of a selected group of samples with a specific endpoint or treatment relative to a control group [10]. Another tool to visualize these data in two dimensions is viSNE [11]. A complete step-wise protocol for analysis of CyTOF data is beyond the scope of this chapter, and we recommend that the user obtain training from their institution, Cytobank, or Fluidigm in the use of the various algorithms and analysis of these complex data sets and how to apply the programs to their specific research questions.

4 Notes

1. We have had success with IgM conjugations (e.g., CD57) although they are not technically supported by DVS/Fluidigm.
2. Qdot antibodies have a cadmium core that can be detected by mass cytometry on channels Cd111–Cd114. Bright antibodies (e.g. HLA-DR, CD3) are most effectively used on this channel or it can be designated as a dump channel.

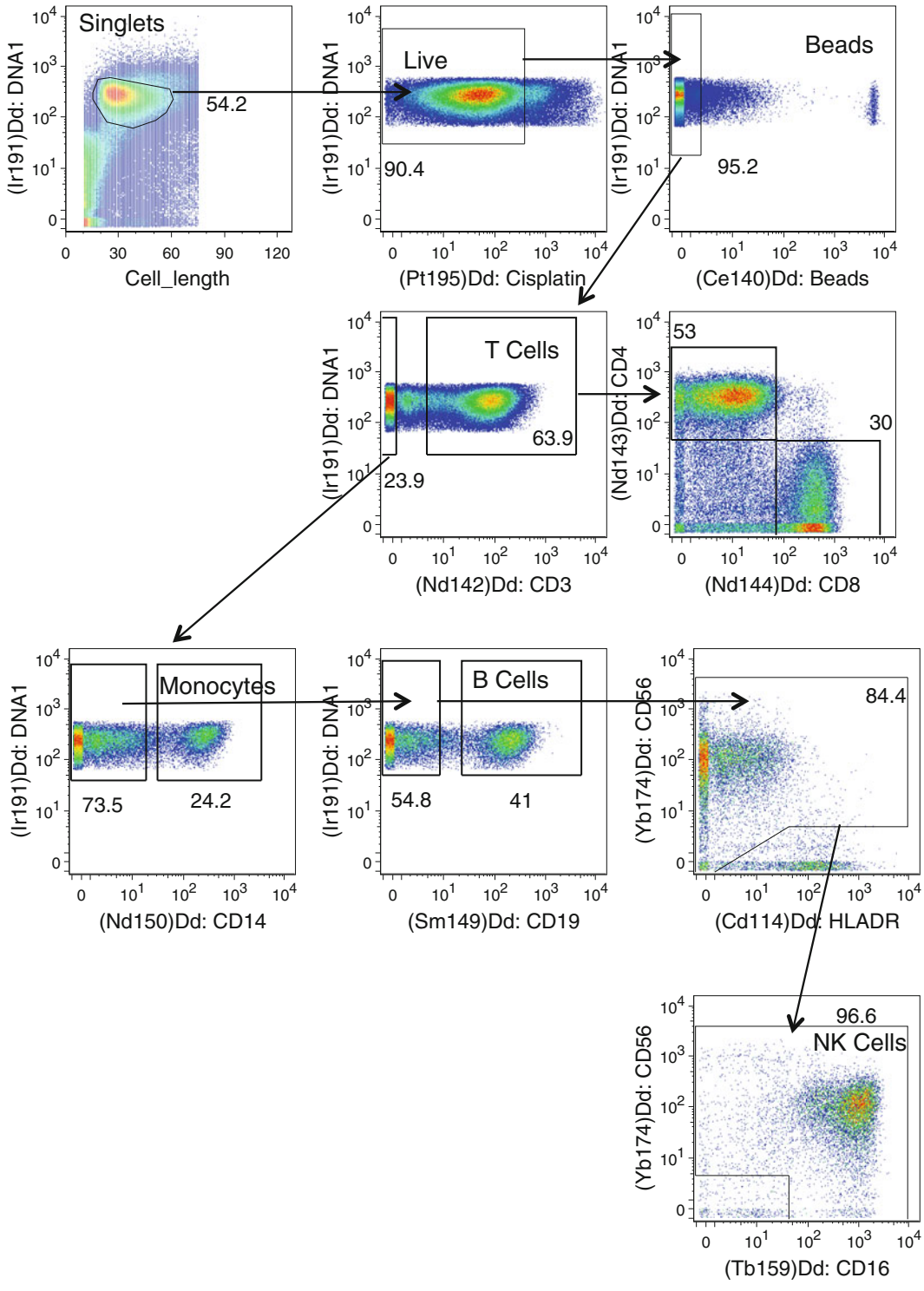

Fig. 2 Gating strategy to identify NK cells and other major cell subsets by mass cytometry. The sequential gates to identify T-cell subsets, monocytes, B cells, and NK cells are shown

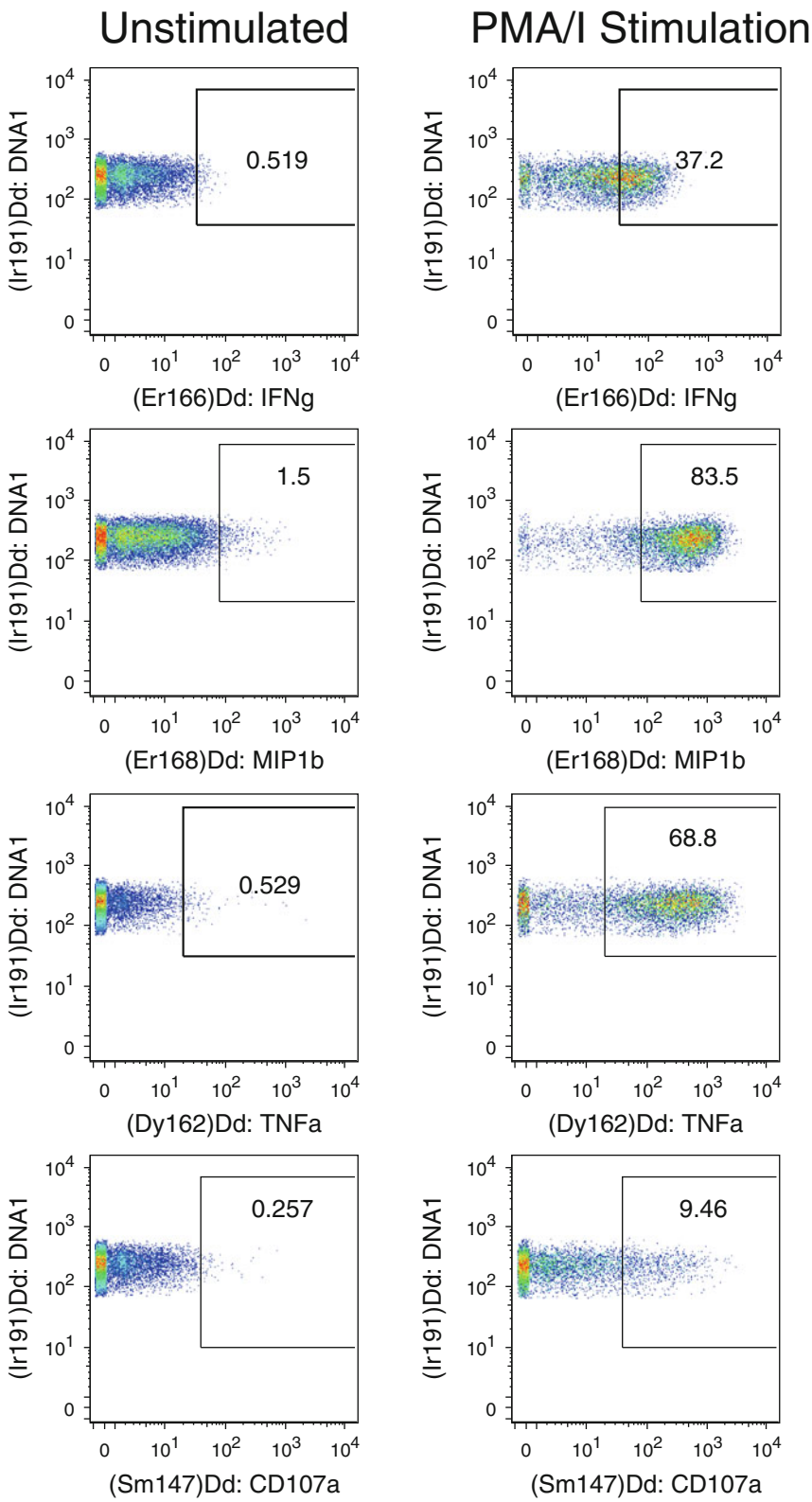

Fig. 3 Representative CyTOF plots of functional markers on stimulated and unstimulated NK cells, gated as in Fig. 2

3. Currently, Fluidigm does not sell kits to conjugate Gd157. Gd157 metals can be procured as 92%+purity from Trace Sciences International, and conjugated with polymer from MaxPar kits.
4. Following conjugation, the total concentration of antibody should be determined by the concentration of A280 protein as measured by Nanodrop. The expected yield is approximately 2/3 of the initial antibody conjugated.
5. Antibodies should be titrated to determine optimal concentration. A typical starting point is 10 μg/mL, with five serial two-fold dilutions to test the range between 0.3125 and 10 μg/mL.
6. Based on the concentration of the antibody following concentration, and the optimal titration as determined empirically, the amount of antibody added per sample can be calculated in order to make the antibody staining cocktail. It is advisable to make extra staining cocktail. Example calculations for 20 samples are shown in Table 1.
7. Peripheral blood mononuclear cells can be used fresh or cryopreserved and thawed prior to use. If the primary focus is NK cell responses, it is not necessary to rest the cells overnight prior to use.
8. Either DOTA-maleimide conjugated to a metal isotope [12] or cisplatin [13] can be used as a live-dead stain. Cisplatin use allows an additional antibody channel because its detection channel cannot be used in conjugations with other antibodies.
9. We have noted some batch-to-batch variability in the concentration of cisplatin required to see a clearly defined dead population. Validation can be performed with samples containing partially heat-killed cells. Cisplatin is a readily available platinum-based chemotherapeutic agent that passively accesses the cell interior of dead cells and rapidly reacts with protein nucleophiles such as R-SH or R-S-CH3 [10]. Cisplatin entry into cells is stopped through the addition of 100% fetal bovine serum that reacts with residual extracellular cisplatin and prevents active transport into live cells.
10. It is acceptable to freeze the plate at −80 °C for up to 1 week after adding FACS Lyse working solution.
11. In order to analyze CyTOF data, settings on FlowJo need to be customized. In order to do so go to the “Preferences” tab, and under workspace select “CyTOF” in the lower right corner and set the value to 20,000. Select the “Define” button under the “Reading Digital Data Files” heading. Select “Side Scatter” and all fluorescence parameters to display with logarithmic staining. Select “Ignore Scaling suggested by the data file”. Add Time and Cell Length as parameters that should

always be linear in the box on the top right. Set the Lowest Standard Log Conversion setting to 1 and the number of decades to display in log-converted data to 6 pulse area parameters and 6 pulse height parameters. Select the Enable Transformation box in addition to the Transform Height Parameters box. Set the number of decades to 5, additional negative display size to 0, and the width basis to −20.

12. Gating strategies to identify cell populations of interest are similar to flow cytometry, but with some important differences given the absence of forward and side scatter (Fig. 2). An example of staining and gating for NK cell functional markers is demonstrated in Fig. 3.

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Chapter 3

Assessment of NK Cell Metabolism

Molly P. Keppel and Megan A. Cooper

Abstract

There has been increasing recognition of the importance of metabolism on immune cell differentiation, homeostasis, and function. Recently, our lab and others have begun to investigate the metabolic requirements for NK cell differentiation and activation. Here, we describe approaches for the in vitro assessment of NK cell metabolism. We present methods for using inhibitors to alter cellular metabolism, measurement of intracellular ATP in NK cells, assessment of real-time glycolysis and oxidative phosphorylation by an extracellular flux assay from Seahorse Biosciences, and some basic protocols for stimulation of NK cells via cytokines and receptors.

Key words Natural killer cells, Metabolism, Interferon-gamma, Glycolysis, Oxidative phosphorylation

1 Introduction

Fundamental cellular processes, including growth and differentiation, are fueled by the generation of intracellular ATP. The two primary, and overlapping, intracellular metabolic pathways that generate intracellular energy/ATP are glycolysis and mitochondrial oxidative phosphorylation (OXPHOS). While the importance of metabolism to sustain basic cellular functions has been well-recognized, work over the past decade has revealed that metabolic pathways can influence immune cell functional responses [1, 2]. For example, differentiation of effector T cells from naïve cells depends on a metabolic switch from oxidative phosphorylation to glycolysis [1]. Further differentiation and persistence of memory T cells depends upon metabolic re-programming to utilize fatty acid oxidation as a mitochondrial fuel [3–5]. Metabolically derived signals are also important for immune cell function, for example, intracellular signaling by mitochondrial-derived reactive oxygen species in antigen-specific T cells [6].

While the role of metabolism has been investigated in several immune cell types, in particular T cells and myeloid cells, similar studies in NK cells have been lacking until recently. There is now

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emerging evidence that NK cell functional responses are also regulated in-part by metabolism. Several recent reports demonstrated the importance of the IL-15-induced mTOR signaling pathway for upregulating glycolytic pathways in activated NK cells [7–9], suggesting that metabolic reprogramming of NK cells may be important during differentiation and activation. Work from our laboratory has found that NK cell production of IFN-γ is highly dependent on glucose-driven OXPHOS when cells are stimulated via ITAM-containing activating receptors, but that stimulation by cytokines, IL-12 + IL-18, is relatively metabolism-independent [10].

NK cells can be activated by two major pathways, recognition of ligands by germline-encoded activating NK receptors, and stimulation through constitutively expressed cytokine receptors. Here, we present basic methods to assess murine NK cell function with metabolic inhibition (*see* **Note 1**). Methods are presented for culturing NK cells with inhibitors of OXPHOS, glycolysis, or fatty acid oxidation. Measurement of NK cell IFN-γ in response to two activating receptors (NK1.1 and Ly49D) and cytokines is provided. We also discuss assays used in our laboratory to measure intracellular NK cell ATP production, which can be used in conjunction with inhibitor assays to investigate the overall metabolic activity of NK cells. Finally, we present methods for the real-time assessment of OXPHOS and glycolysis using an extracellular flux assay from Seahorse Biosciences. The XF analyzers from Seahorse Biosciences measure the real-time oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of cultured cells by frequently sampling small volumes of media from individual wells. OCR is used as a surrogate marker of oxygen consumption, and ECAR is an indirect measurement of anaerobic glycolysis and lactate production. This is a powerful technique that allows for perturbation of the culture system while continuously monitoring NK cell metabolic pathways.

2 Materials

All reagents for use in cell culture should be tissue-culture grade and sterile.

2.1 Magnetic Bead Isolation of NK Cells

1. NK Cell Isolation Kit II, mouse (Miltenyi Biotec, 130-096-892).
2. MACS Buffer: Dulbecco's phosphate buffer solution (PBS) without magnesium and calcium, supplemented with 0.5% BSA or FBS and 2 mM EDTA. Filter sterilize after preparation.
3. LD Columns (Miltenyi Biotec, #130-042-901).
4. Magnet for separation of cells (Miltenyi Biotec, 130-090-976).
5. 70 μm cell strainers.
6. 50 μm cell strainers (Partec, CellTrics®).
7. 1.5, 15, and 50 mL centrifuge tubes.

2.2 Culture of NK Cells with Metabolic Inhibitors (Table 1)

1. Complete R10 Medium: RPMI 1640 (with L-glutamine), supplemented with 10% FBS (heat inactivated), 1× Pen-Strep (100× stock), 1× L-Glutamine (100× stock). Filter sterilize after preparation. Add 2-Mercaptoethanol to media to a final concentration of 50 μM just prior to use.
2. Etomoxir sodium salt hydrate (Sigma E1905): Prepare working stock of 10 mM in PBS. Filter sterilize, and store at −20 °C as aliquots for up to 12 months. Thaw just prior to use. Do not re-freeze.
3. Oligomycin A (Sigma 75351): Prepare working solution of 1 mM in DMSO. Store at −20 °C as aliquots for up to 12 months. Thaw just prior to use. Do not re-freeze.
4. Antimycin A (Sigma A0274): Prepare working solution of 3.75 mM in DMSO. Store at −20 °C as aliquots for up to 12 months. Thaw just prior to use. Do not re-freeze.
5. 2-Deoxy-D-Glucose (2DG, Sigma D8375): Prepare working solution of 500 mM in PBS. Filter sterilize and store at −20 °C as aliquots for up to 12 months. Thaw just prior to use. Do not re-freeze.
6. Spectrophotometer.
7. 24- and 96-well tissue culture-treated plates.
8. 70 μm cell strainers.
9. 50 and 15 mL conical tubes.
10. 0.2 μm filter sterilization units for buffers.

2.3 Assays to Detect Intracellular IFN-γ

1. Dialyzed FBS: Prepare 12 L of PBS with deionized H_2O. Cut approximately 10 in. of 10,000 Molecular Weight Cut-Off Snakeskin dialysis tubing, and secure one end by folding over tubing and attaching clips. Handle tubing with gloves only.

Table 1
Metabolic inhibitors for in vitro use with NK cells

Inhibitor/nutrient	Concentration	Mode of action
Etomoxir	100–300 μM	CPT-1 inhibitor, inhibits fatty acid oxidization
2-Deoxy-D-glucose	1–50 mM	Glucose analog, competitive inhibitor of glucose hexokinase, inhibits glycolysis
Oligomycin	1–100 nM	ATP synthase inhibitor, inhibits OXPHOS
Antimycin	0.1–3.75 μM	Complex III inhibitor, inhibits OXPHOS
Glucose-free media	–	Inhibits glycolysis by limiting glucose availability
Glutamine-free media	–	Inhibits glutaminolysis and subsequent glutamine-fueled OXPHOS by limiting glutamine

The concentrations here are working concentrations that have been optimized for use in vitro use with NK cells

Place 25 mL of FBS through the open end of the tubing. Secure the end by clip, leaving 2–3 in. of empty tubing. Place tubing into 4 L of PBS (in large beaker), add stir bar and place on a magnetic stirrer on low revolution at 4 °C. The tubing should move gently in a circle. Change the PBS two more times over the course of 12–16 h with a minimum of 4 h per buffer change and a total of 12 L of PBS. Then, remove FBS from dialysis tube and filter sterilize. Determine the level of glucose in dialyzed FBS using the Glucose (HK) Assay Kit (Sigma; follow manufacturer's protocol). Glucose level should be below the limits of detection. Store dialyzed FBS in aliquots at −20 °C.

2. Glucose-free R10 (GF media): Glucose-free RPMI 1640 (with L-glutamine) supplemented with 10% dialyzed FBS, 1× Pen-Strep, 1× L-Glutamine, 15% PBS. Filter sterilize after preparation. Add 2-Mercaptoethanol to a final concentration of 50 μM to media just prior to use.
3. Recombinant murine IL-12 (Peprotech #210-12): Prepare 200 ng/mL stock solution in sterile solution of PBS + 0.1% BSA (cell culture grade) or FBS. Aliquot into single-use vials and store at −80 °C (*see* **Note 2**).
4. Recombinant murine IL-15 (Peprotech #210-15): Prepare 2000 ng/mL stock solution in sterile solution of PBS + 0.1% BSA (cell culture grade) or FBS. Aliquot into single-use vials and store at −80 °C (*see* **Note 2**).
5. Recombinant murine IL-18 (MBL #B002-5): Prepare 1000 ng/mL stock solution in sterile solution of PBS + 0.1% BSA (cell culture grade) or FBS. Aliquot into single-use vials and store at −80 °C (*see* **Note 2**).
6. Purified, unconjugated anti-NK1.1 (clone PK136, BioXcell).
7. Purified, unconjugated anti-Ly49D (clone 4E4).
8. Purified, unconjugated isotype control antibody (mouse IgG2A).
9. Anti-CD16 to block FC receptor (2.4G2). We prepare hybridoma supernatant for this application (ATCC® HB-197).
10. Direct flourochrome-conjugated antibodies for flow cytometry: IFN-γ (clone XMG1.2), NK1.1 (clone PK136), CD3 (clone 145-2C11), NKp46 (clone 29A1.4).
11. Brefeldin A, 1000× stock (GolgiPlug™, BD Biosciences).
12. Monensin, 1000× stock (GolgiStop™, BD Biosciences).
13. FACS buffer: PBS supplemented with 2% FBS, 2 mM EDTA. Filter sterilize after preparation.
14. Cytofix/Cytoperm™ solution (BD Biosciences, kits are available with Perm/Wash™ and GolgiPlug™ or GolgiStop™).
15. Perm/Wash Buffer: PBS supplemented with 10 mM HEPES, 0.1% Saponin, 0.1% BSA or FBS (or use BD Perm/Wash™).

16. Viability dye to detect dead or apoptotic cells by flow cytometry. There are a wide variety of choices, some that have been used by our laboratory are Zombie Dye (Biolegend) and Live Dead Fixable Dead Cell Stain (Life Technologies).
17. Paraformaldehyde.
18. Flow cytometer.

2.4 Detection of Intracellular ATP Levels

1. ATPlite Assay (Perkin Elmer #6016941).
2. 96-well, black-walled plates (Falcon).
3. 96-well plate reader capable of detecting luminescence.
4. Trypan blue to count cells.

2.5 Extracellular Flux Assays

This is a specialized assay to be run with an XF-96 Analyzer (Seahorse Biosciences) that might not be available at all research sites.

1. Seahorse NK media: Bicarbonate-free non-buffered RPMI 1640 (Sigma R8755), supplemented with 25 mM glucose, 1 mM sodium pyruvate, and 1% FBS (*see* **Note 3**). Warm the media to 37 °C and adjust the pH to 7.4 with 0.1 N sodium hydroxide. It is important that the media is at pH 7.4 prior to use for most assays.
2. XF96 cell culture microplate (Seahorse Biosciences, #101085-004).
3. XF^{e}96 sensor cartridge (sold in XF^{e}96 FluxPak, Seahorse Biosciences).
4. XF96 Calibrant pH 7.4 (Seahorse Biosciences, #100840-000).
5. Poly-L-lysine (Sigma P4707).
6. XF96 Analyzer, Seahorse Biosciences.

3 Methods

3.1 NK Cell Enrichment

The method below is for the purification of splenic NK cells using a negative selection magnetic bead kit from Miltenyi Biotec. The protocol has been slightly modified based on our laboratory's experience and generally yields approximately $1–2 \times 10^6$ cells/spleen, with a purity of ~80–90% NK1.1$^+$CD3$^-$ NK cells from C57BL/6 mice. It takes 1–2.5 h to enrich, and up to two spleens per column may be enriched.

1. Harvest spleens and isolate splenocytes by pushing them through a 70 μm filter into R10 medium. It works well to place the filter in a 50 mL conical tube and push the spleen through with the plunger from a sterile 3 mL syringe. Centrifuge cells for 5 min at $450 \times g$ at 4 °C.

2. Do not lyse red blood cells.
3. Resuspend splenocytes in 40 μL MACs buffer per 1×10^7 cells (count cells or estimate 100×10^6 cells per wild-type C57BL/6 spleen).
4. From the NK cell isolation kit II, add 2.5 μL of biotin-Ab cocktail per 1×10^7 cells, vortex, and incubate at 4 °C × 5 min (*see* **Note 4**).
5. Add an excess of MACs buffer to wash and centrifuge at $450 \times g$ at 4 °C to pellet cells.
6. Resuspend splenocytes in 80 μL of MACs buffer per 1×10^7 cells.
7. Add 5 μL of anti-biotin beads per 1×10^7 cells, mix by vortex, and incubate at 4 °C × 10 min (*see* **Note 4**).
8. Wash cells with MACs buffer, centrifuge at $450 \times g$ at 4 °C and resuspend in 500 μL of MACs buffer for every $100–200 \times 10^6$ cells.
9. Filter splenocytes through Partecs 50 μM filter into a 1.5 mL Eppendorf tube.
10. Place LD columns in the magnet and clean by adding 2 mL buffer (start washing the columns during anti-biotin bead incubation), discard the eluate (*see* **Note 4**).
11. Place 15 mL collection tubes in ice under the LD columns. Add 500 μL of cells/column, allow cells to completely enter column and then rinse the column three times with 1 mL of MACs buffer, collecting all eluate. Add the next wash when the previous wash has completely gone into the column.
12. Centrifuge eluate at $450 \times g$ for 5 min, which contains the negatively selected NK cells, resuspend NK cells in R10 Medium, and count for use in assays.

3.2 Stimulation of NK Cells

The general stimulation assays below are optimized for stimulating enriched NK cells with cytokines or plate-bound antibody (Table 2). Whole spleen cells can be used, but the plate size and well volumes will need to be increased. Metabolic inhibitors (Table 1) can be added to these assays or other in vitro NK cell stimulation assays to test the effect of altering metabolism on NK cell function. In Fig. 1, we provide an example of our studies demonstrating impaired IFN-γ production with metabolic inhibition when NK cells are stimulated via activating receptors but not cytokines [10].

3.2.1 Antibody Stimulation of NK Cells

Assays here are performed with plate-bound antibody to cross-link the NK cell activating receptors NK1.1 or Ly49D and evaluate NK cell IFN-γ production.

1. In a 24-well plate, add 275 μL/well of antibody diluted in PBS (*see* Table 2 for concentrations) in order to immobilize antibodies on plastic surface for NK cell stimulation.

Table 2
Activation of NK cells with plate-bound antibodies or cytokines and expected percentage of NK cells positive for IFN-γ protein by intracellular flow

Stimulus	Concentration	Expected %IFNγ-positive NK cells
Anti-NK1.1	20 μg/mL	20–40
Anti-Ly49D	40 μg/mL	5–15
IL-12 + IL-15	1–10 ng/mL (IL-12) 10–100 ng/mL (IL-15)	15–40
IL-12 + IL-18	1–10 ng/mL (IL-12) 1–50 ng/mL (IL-18)	40–90

Expected percentage IFN-γ-positive NK cells is based on 6 h antibody assays and 4–6 h cytokine stimulation assays

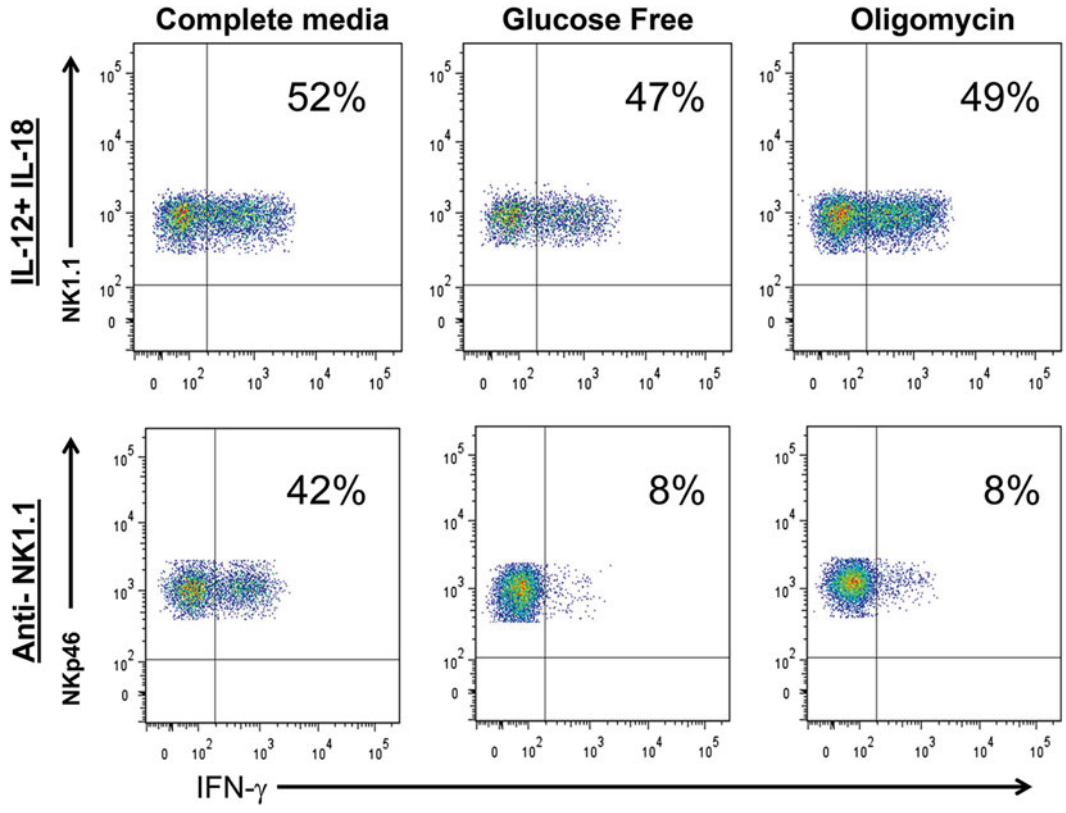

Fig. 1 Impaired NK cell IFN-γ production with metabolic inhibitors. Enriched NK cells were cultured for 6 h with either IL-12 + IL-18 (1 ng/mL each, *top row*) or in plates coated with anti-NK1.1 (*bottom row*) in complete media, glucose-free media, or complete media with oligomycin (100 nM) as indicated. Brefeldin A was added to the cells after 1 h of culture, and at 6 h NK cells were harvested and stained for intracellular IFN-γ. NK cells were stained with a viability dye, and live NK cells were identified as $NK1.1^{+}CD3^{-}$ (for cytokine stimulation) or $NKp46^{+}$ (for anti-NK1.1 stimulation). Cells cultured in glucose-free media or with oligomycin produced significantly less IFN-γ when stimulated by anti-NK1.1, but had no change in IFN-γ when stimulated with IL-12 + IL-18

2. Use similar concentrations of isotype control antibody to coat control wells.
3. Incubate the plate at 37 °C for 90 min.
4. Remove solution from wells with a vacuum pipet, and wash each well twice with 0.5–1 mL of PBS prior to loading cells.
5. Add 1–5 × 10^5 NK cells and inhibitors (*see* Table 2 and **Notes 5** and **6**) to a total volume of 250 μL and place at 37 °C for 1 h.
6. After 1 h of stimulation, add 2.5 μL of a 10× solution of Brefeldin A diluted in media to each well (final concentration of 1×). Mix by swirling plate gently.
7. Culture cells for a total of 6–8 h to evaluate IFN-γ production.
8. Harvest and assay cells for intracellular IFN-γ by flow cytometry.

3.2.2 Cytokine Stimulation of NK Cells

1. In a 96-well round-bottom plate, add 1–5 × 10^5 NK cells and cytokines (*see* Table 2 for concentrations) to a total volume of 250 μL and culture at 37 °C.
2. If intracellular cytokines are being assessed: after 1 h of stimulation, add 2.5 μL of a 10× solution of Brefeldin A diluted in media to each well (*see* **Note 7**). Mix by swirling plate gently.
3. Culture cells for a total of 4–8 h to evaluate IFN-γ production.
4. Harvest and assay cells for intracellular IFN-γ or other readout of interest.

3.2.3 Staining Cells for Intracellular IFN-γ

1. Harvest cells into FACS buffer and place into 5 mL flow tubes. If cells were stimulated in antibody-coated wells, be sure to rinse the wells thoroughly and scrape the wells with a sterile pipette as you harvest to ensure all cells are collected.
2. Centrifuge cells for 5 min at 450 × *g*.
3. Stain cells with a viability dye according to the manufacturer's recommendations, we often use Zombie Dye (Biolegend).
4. Resuspend cells in 50 μL media with anti-CD16 (2.4G2 antibody) and place on ice for 5–10 min to block FC receptor and prevent non-specific staining.
5. Dilute antibodies for surface staining in 2.4G2 hybridoma supernatant for a total volume of ~50 μL antibodies + hybridoma supernatant and add to NK cells (total volume of 100 μL). NK cells can be identified as $NK1.1^+CD3^-$ or $NKp46^+$ (*see* **Note 8**).
6. Stain cells on ice or at 4 °C for 20 min.
7. Add 3 mL of FACS buffer, and centrifuge at 450 × *g* for 5 min.

8. After surface staining, resuspend cells in 200 μL of Cytofix/Cytoperm solution and place at 4 °C for 20 min.
9. Add 3 mL of perm/wash buffer to each tube, and centrifuge at 450 × *g* for 5 min.
10. Resuspend cells in an optimized concentration of anti-IFN-γ antibody diluted in perm/wash buffer for a total volume of 50 μL and stain cells at 4 °C for 20 min.
11. Add 3 mL of perm/wash to each tube, and centrifuge at 450 × *g*.
12. Resuspend in 200 μL of FACS buffer. Cells can be immediately analyzed by flow cytometry or kept in the dark at 4 °C for up to 72 h prior to flow cytometric analysis.

3.3 Measurement of Intracellular ATP in NK Cells

1. Culture NK cells with desired stimulants and inhibitors or use freshly isolated NK cells. Use a minimum of 0.3×10^6 cells/well.
2. Harvest cells from the culture media into a 1.5 mL microcentrifuge tubes and centrifuge at 6000 × *g* for 5 min. Prepare the ATP standard while cells are being pelleted.
3. Dilute the 10 mM ATP stock (provided in ATPlite assay kit) 1:1000 fold (3 μL stock in 3 mL H_2O). Perform serial twofold dilutions in diH_2O to obtain a total of seven standards (including the top standard of 10 μM).
4. Prepare the black-walled flat bottom 96-well plate for cell lysate and standard by add 100 μL of R10 medium to wells that will be used for standards (16 wells).
5. Add 10 μL of each of the seven standards to duplicate wells (containing R10 medium; for a standard curve ranging from 10 to 0.156 μM), adding 10 μL of diH_2O to the last two wells (blank wells).
6. Resuspend cells in 350 μL (~0.1×10^6 cells/100 μL) of room temperature media. If using a larger number of cells, resuspend in a volume that will equal ~1×10^6 cells/mL. If cells are too concentrated, there is a risk of over-shooting the top standard. If cells were cultured in glucose or glutamine-free media, resuspend cells in corresponding media.
7. Add 100 μL of NK cells (~0.1×10^6 cells/well) in triplicates to the 96-well assay plate (black-walled flat bottom).
8. Place remaining cells on ice, and count the cells after the assay is complete. Prepare 1:1 dilution of 25 μL cells + 25 μL trypan blue to count. It is important to have an accurate cell count to normalize data.
9. Add 50 μL cell lysis buffer (provided in the ATPlite Assay kit) to each well, including standard wells, and shake for 5 min on a plate shaker or rotator.

10. Add 50 μL of substrate solution (provided in the ATPlite Assay kit) to each well with cells and shake for 5 min.
11. Cover the plate with foil for 10 min.
12. Read on plate reader for luminescence.
13. Calculate ATP (molarity) for each sample based on the standard curve you generate. Average the three replicates of each sample and divide by the cell count to obtain molarity of ATP per cell.

3.4 Extracellular Flux Assay

There are a number of ways to utilize extracellular flux assays for measurement of cellular metabolism. For example, measurement of baseline OCR or ECAR in resting or stimulated NK cells can tell you whether the cells are primarily using OXPHOS or glycolysis for their metabolism. The instrument can also inject pre-loaded compounds into the wells during the protocol by using up to four different injection ports. Seahorse Biosciences has kits available with compounds and protocols for measuring mitochondrial function (Mito Stress Test Kit), glycolytic capacity (Glycolysis Stress Test Kit), and other metabolic functions. Similar assays can also be easily set up and customized by the experienced user by purchasing the individual compounds for injection (example shown in Fig. 2). In addition to adding compounds such as metabolic inhibitors, it is also possible to customize the assay by adding other compounds such as cytokines or antibodies through the injection ports. Thus, there are many potential applications for these instruments. Our laboratory used extracellular flux assays to analyze the baseline metabolism of freshly isolated NK cells at rest and upon activation [10]. We found that resting naïve NK cells primarily use OXPHOS, but that prolonged high-dose IL-15, which alters the metabolic requirements for NK cell activation, greatly increases overall cellular metabolism and specifically increases anaerobic glycolysis [10].

The protocol here describes our experience with optimizing the adhering NK cells in 96-well plates for use in an XF96 analyzer (Seahorse Biosciences). There are a number of variables to consider when performing these experiments, including: the high numbers of primary NK cells required (5×10^5/well with at least triplicate wells for each condition), adherence of NK cells to the culture well in an even distribution, use of media with minimal buffering capacity (including minimal FBS) since buffers can alter ECAR readings, maintenance of NK cell viability in minimal media during plating and set-up of the assay, and the need for specialized instrumentation. Here, we present a basic protocol for plating NK cells to measure OCR and ECAR under conditions we have optimized to maintain NK cell viability, while minimally perturbing readouts with the use of buffers.

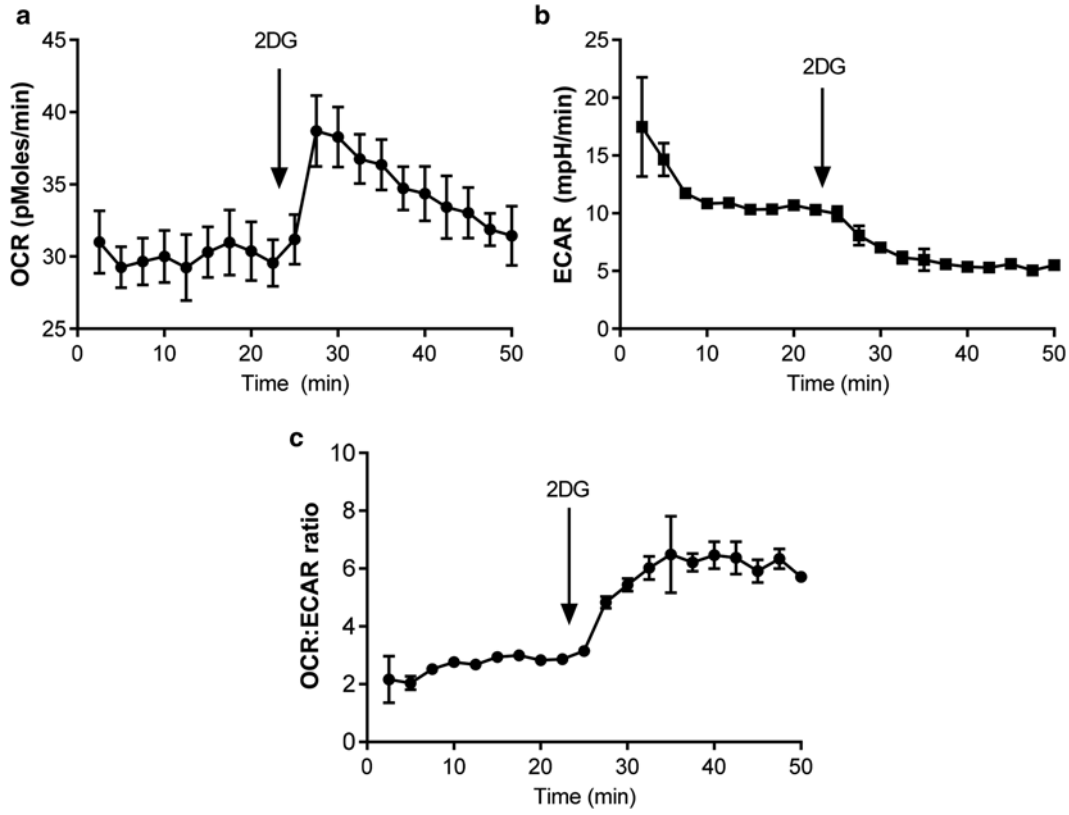

Fig. 2 Measurement of OCR and ECAR in NK cells before and after 2DG administration. Enriched murine NK cells were plated in 96-well plates in Seahorse NK media and OCR and ECAR measured at baseline and after addition of the glycolysis inhibitor 2DG (50 mM). 2DG was pre-loaded into port A of the Seahorse plates and added at the indicated time. With glycolytic inhibition, NK cells increased their OXPHOS (measured by OCR) and decreased glycolysis (measured by ECAR). This is also reflected in the ratio of OCR:ECAR over time. Data represent the mean ±SEM of three replicate wells. Reproduced with permission from *The Journal of Immunology*, "Keppel, M. P., Saucier, N., Mah, A. Y., et al. (2015) Activation-specific metabolic requirements for NK Cell IFN-gamma production. *Journal of immunology* **194**, Feb 15, 2015, 1954–1962", Copyright 2015. The American Association of Immunologists, Inc

3.4.1 Preparation of Instrument and Plate

1. Prepare a template for the XF-96 software for your desired program at least 1 day prior to the experiment.
2. Hydrate the sensor cartridge overnight by adding sterile XF96 Calibrant (as recommended by the manufacturer) and placing in a non-CO_2 incubator at 37 °C.
3. Coat wells of the XF96 cell culture plate with poly-L-lysine by aseptically adding 10 μL of poly-L-lysine to each well.
4. Rock the XF96 cell culture plate gently on a plate rocker for at least 5 min to ensure even coating of the culture surface. Carefully remove the solution by vacuum aspiration.

5. Add 200 μL of sterile tissue culture grade water to each well, aspirate, and repeat to wash the plate.
6. Allow the plate to dry at least 2 h before adding cells. Plates can be coated the night before and kept at 4 °C overnight.

3.4.2 Plating NK Cells and Preparing Sensor Cartridge

1. Purify NK cells by magnetic bead enrichment and culture as desired or use fresh NK cells. Wash cells twice with Seahorse NK media to minimize the effects of residual buffer capacity.
2. Count and resuspend cells at 6.25×10^6 cells/mL.
3. Add 80 μL of cells to each well of the XF96 culture plate (5×10^5 cells/well) in triplicate (*see* **Note 9**).
4. Add 80 μL assay media to wells without cells for negative controls and to have fluid in all wells.
5. Centrifuge the plate with the brake off, this is a two-step process where the orientation of the plate is reversed to optimize even plating of NK cells. Spin up to $40 \times g$, stop the centrifuge as soon as it reaches $40 \times g$ and let the rotor reach a complete stop without braking.
6. Reverse the orientation of the plate and spin again, this time allowing the centrifuge to reach $85 \times g$ before stopping.
7. Carefully dispense 100 μL of Seahorse NK media to each well, trying not to disturb the now-adherent NK cells (*see* **Note 10**).
8. Look at the cells under the microscope to check if they have uniformly adhered, although it may be difficult to visualize a clear image.
9. Place the plate with cells in a non-CO_2 incubator at 37 °C for at least 20 min, while preparing the cartridge and until you are ready to add the plate to the instrument.
10. Remove the hydrated sensor cartridge from the incubator and add your chosen compounds into the appropriate ports. Dilute compounds in pre-warmed Seahorse NK media and add a total volume of 20–25 μL to each port (*see* **Note 11**). There are four ports available for injection of compounds (A–D). For example, adding 2DG (Fig. 2) will inhibit glycolysis and glucose-fueled OXPHOS, and allow you to examine the ability of the cell utilize other fuels for OXPHOS metabolism. In Fig. 2, we plated NK cells in a total volume of 225 μL (80 μL of cells + 145 μL of media). 25 μL of 500 mM 2DG was added to port A, to give a final concentration of 50 mM after injection.
11. Return the cartridge to a non-CO_2 incubator for at least 15 min. After re-warming, place the cartridge in the XF96 instrument to calibrate followed by addition of the culture plate with cells as prompted by the software.

3.5 Data Acquisition and Analysis

1. Assay the cells on the XF96 analyzer with the desired program for your culture system (*see* **Note 12**).
2. Average the OCR and ECAR readings from the triplicate wells at each timepoint. You can graph OCR and/or ECAR over time to visualize changes in OXPHOS and/or anaerobic glycolysis respectively with manipulation of the cells. For example, in Fig. 2 we saw that inhibition of glycolysis with 2DG led to a rapid increase in OCR (OXPHOS) and decrease in ECAR (anaerobic glycolysis), suggesting that NK cells are able to use energy sources other than glucose for OXPHOS, possibly fatty acids or glutamine.

4 Notes

1. *Mouse versus human experiments*: The methods presented here are for the murine system. Similar metabolic assays with human NK cells have not been reported, but the inhibitors and culture methods presented here should translate well to the human system. There are numerous published studies evaluating the activation of human NK cells with cytokines, receptor antibodies, and target cells that could be modified to assess the metabolic requirements of human NK cell activation.
2. *Cytokines*: Aliquot cytokines into small volumes sufficient for experiments and store at −20 °C or −80 °C. After thawing, cytokines should be kept at 4 °C and used within 24 h.
3. *NK Seahorse media*: FBS is not generally recommend by Seahorse Biosciences, but is essential for NK cell viability.
4. *NK cell enrichment*: We prefer using negative rather than positive selection (Miltenyi Biotec) for magnetic bead purification of NK cells, since the NK cells are not "touched" by negative selection. Fluorescence-activated cytometric sorting (FACS) can also be used to purify bulk NK cells, and is useful when a particular NK cell subset is desired. However, in our experience, cell sorting significantly reduces the yield and viability of murine NK cells compared to magnetic bead enrichment and additional starting cells may be required. The values given for biotin-Ab cocktail and anti-biotin beads is less antibody cocktail than recommended in the product insert. These concentrations were optimized in our laboratory to use lower amounts of product. The NK enrichment kit recommends LS columns, but we have found better yield and purity with LD columns.
5. *Glucose-free media*: We have found that NK cells are very sensitive to culture in glucose-free (GF) media and that the addition of PBS to the media enhances NK cell survival during short-term cultures. NK cells are viable in the GF media here for up to 8 h, but longer cultures significantly affect NK cell

viability and most NK cells are dead after 24 h. Prior to culturing NK cells in glucose-free (GF) media, enriched NK cells should be washed in a large volume of PBS (10 mL) prior to culture in GF media to ensure complete removal of glucose contained in R10. NK cell number should be increased to $0.5–1 \times 10^6$ cells per well when using glucose-free media.

6. *Culturing NK cells with metabolic inhibitors*. Working concentrations tested in our laboratory for the inhibitors described here are shown in Table 1. When culturing NK cells with metabolic inhibitors we strongly recommend including a viability dye with your flow cytometry panel as these inhibitors can affect NK cell survival, and it is important to distinguish between live and dead cells (this cannot accurately be done by FSC/SSC gating alone).
7. *Measuring NK cell cytokine production by intracellular flow cytometry*. Brefeldin A is used to inhibit secretion of IFN-γ. Therefore, the readout at the end gives information regarding the IFN-γ production by a single cell over whatever amount of time Brefeldin A was in the culture media. An alternative approach is to look at a "snapshot" of NK cell IFN-γ production by not adding Brefeldin A and assessing cytokine production at a single point in time. We have found that this alternative approach works well for cytokine stimulation, but, in general, the amount of protein stimulated by plate-bound antibodies or target cells is difficult to detect without Brefeldin A. Brefeldin A can be added to the culture for up to 8 h, but becomes toxic to the NK cells after this point.
8. *NK cell markers*: There is no marker that is completely unique to murine NK cells. However, most investigators in the field would consider $NK1.1^+CD3^-$ cells to be bona fide NK cells. The NK1.1 receptor is found in C57BL/6 mice, but many other commonly used mouse strains, including BALB/c, lack this marker. NKp46 is relatively NK-specific and has the advantage of being present on human NK cells and NK cells in nearly all mouse strains [11]. While initially thought to be restricted to NK cells, NKp46 is also found on some innate lymphoid cells (ILCs) [12]; however, these ILC subsets are relatively rare, particularly in the spleen. In strains that lack NK1.1, $DX5^+CD3^-$ is an acceptable alternative to identify NK cells, however NKp46 would be the preferred marker. In summary, NK cells can be identified by flow cytometry as: $NKp46^+$ (all mouse strains), $NK1.1^+CD3^-$ (C57BL/6 strain), or $DX5^+CD3^-$ (strains other than C57BL/6), with DX5 being the least reliable marker. Please note that NK1.1 cannot be used to stain NK cells after activation with plate-bound NK1.1 due to receptor downregulation and/or masking.

9. *Plating NK cells for Seahorse*: We recommend plating NK cells in at least three replicate wells for each condition and averaging data from across the wells during analysis.
10. *Loading Seahorse plate*: The volume of media that you load on the plate prior to running may be adjusted based on the number of compounds you plan to add to the ports, but the total culture volume before injection of compounds should be between 150 and 225 μL.
11. *Adding compounds to the Seahorse plate*: Compounds should be added at a concentration such that they will be at the desired final concentration after injection into the culture well. Uniform loading of the ports is essential for reproducible results, and we recommend using a calibrated multi-channel pipettor to load the compounds.
12. *Seahorse program*: The instrument will record OCR and ECAR readings at pre-programmed time intervals. Programs can be altered with regards to the frequency with which the media is sampling (mix, wait, and measurement times), the number of measurements taken, and the timing of injection of compounds from the pre-loaded ports. For example, in Fig. 2, OCR and ECAR readings were taken with a 30 s mix time and 2 min measure time (no wait time). Ten measurements were taken to establish a baseline followed by injection of port A (2DG) and ten additional measurements.

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Chapter 4

Genotyping Single Nucleotide Polymorphisms and Copy Number Variability of the FCGRs Expressed on NK Cells

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Abstract

Natural killer (NK) cells are one of the main effector immune cells involved in antibody-dependent cell-mediated cytotoxicity (ADCC). Upon recognition of cell-bound IgG antibodies, which occurs through Fc gamma receptors (FCGRs) expressed on the cell surface of NK cells, NK cells become activated and lyse target tumor or infected cells. The FCGRs, FCGR3A and FCGR2C, expressed on the surface of NK cells have single nucleotide polymorphisms (SNPs) that result in differential activity of NK cells. In addition to SNP genetic variation within each of these genes, the FCGRs are subject to copy number variation (CNV), which leads to variable protein expression levels on the cell surface. Studies have found that *FCGR* genotype for *FCGR3A* and *FCGR2C* is associated with variation in the response to immunotherapy.

Due to high sequence homology within *FCGR3* and *FCGR2* families, there are difficulties associated with genotyping these specific receptors related to cross-amplification of non-targeted *FCGRs*. To improve specificity for both *FCGR3A* and *FCGR2C*, Rnase-H (RH) primers were designed to amplify specifically *FCGR3A* (while not co-amplifying *FCGR3B*) and *FCGR2C* (while not co-amplifying *FCGR2B*). In addition, fluorescently labeled locked nucleic acid (LNA) probes provide additional precision for determination of the SNPs within both *FCGR3A* and *FCGR2C*. For CNV determination, separate fluorescently labeled probes for *FCGR3A*, and for *FCGR2C*, can be used with the same RH primers for each gene. These probes can be combined in the same well with control primers/probe for a known diploid gene and used to calculate the copy number of both *FCGR3A* and *FCGR2C*. Here we provide new detailed methodology that allows for the specific amplification of these *FCGRs* in a single PCR reaction, allowing for genotyping of both the SNPs and CNVs using real-time PCR.

Key words *FCGR3A*, *FCGR2C*, NK cells, ADCC, SNP, Immunotherapy, Cancer immunology, CNV

1 Introduction

The innate immune effector cells, natural killer (NK) cells, are major contributors in the fight against certain diseases and infections (viral infections, various forms of cancer, etc.). NK cells can be targeted to "damaged" cells via antibody recognition through Fc gamma receptors [FCGR3A (CD16A) and FCGR2C (CD32C)] expressed on their cell surface, which recognize the Fc portion of

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the IgG antibodies [1]. Engagement of the FCGRs expressed on NK cells activates the NK cell to kill the target cell, a process termed antibody-dependent cell-mediated cytotoxicity (ADCC) [2, 3].

The FCGRs are either activating or inhibitory, and are expressed on a variety of immune cells. They include variants such as FCGR1, FCGR2A, FCGR2B, FCGR2C, FCGR3A, and FCGR3B. Both FCGR2C and FCGR3A are expressed on NK cells, and both have been shown to be subject to genetic variability within the population [4]. Along with some of the other FCGRs, both FCGR2C and FCGR3A have been found to be copy number variable (CNV), and, depending on the individual, can deviate from the "standard" two copies (inheriting one from maternal genome, one from paternal genome), with two copies occurring the most commonly among the population [5–8]. Additionally, *FCGR2C* and *FCGR3A* each have single nucleotide polymorphisms (SNPs); dependent upon the SNP that a person has for each of these genes, the different SNPs create variability in the ability of the FCGRs to interact with the Fc portion of the IgG antibodies.

FCGR3A has a SNP at amino acid position 158 that encodes for either a valine (V) or a phenylalanine (F), resulting in altered affinity of the receptor for IgG (rs396691) [1, 9, 10]. Alternatively, FCGR2C has a SNP in Exon 3 (rs759550223) that results in either expression, or non-expression, of the protein on the cell surface [11, 12]. *FCGR2C* with a "C" at nucleotide position 202 results in an open reading frame (ORF) and expression on the cell surface. In contrast, a "T" at nucleotide position 202 results in a stop codon and the protein is not expressed on the cell surface [11–13]. Determining the SNP variant for each FCGR expressed on NK cells may help to predict the function of that individual's NK cells in ADCC, and thus potentially indicate how a person may respond to ADCC-mediating antibody therapies that aim to target NK cells toward cells targeted for destruction.

For such genotyping, amplifying the area of DNA containing the SNP or CNV region has been difficult as the *FCGR*s have high sequence homology. In particular, *FCGR3A* and *FCGR3B* have very few nucleotide differences in the region that surrounds the amino acids that encode the FCGR3A-158-V/F SNP. In fact, the nucleotides at position *FCGR3A*-559-G/T translate to the amino acid SNP of FCGR3A-158-V/F SNP. Typing for these is complicated by the fact that the gene for *FCGR3B* has a "G" nucleotide at position 559 within its sequence. Since both *FCGR3A* and *FCGR3B* can have a "G" nucleotide at the region of this SNP, if *FCGR3B* is incorrectly amplified it will further confound the accuracy of the SNP determination for *FCGR3A*. Several studies have addressed the difficulties associated with accurately determining the FCGR3A-158-V/F SNP [14–18]. Designing unique methodology to specifically amplify *FCGR3A*, allowing for accurate determination of its SNP and as well as CNV associated with this gene,

is of great importance when trying to associate clinical outcome with the SNP and CNV status for FCGR3A.

We developed Rnase-H (RH) primers to specifically amplify *FCGR3A*, while not co-amplifying *FCGR3B* [19]. We used locked nucleic acid (LNA)-labeled probes to increase both the sensitivity and the specificity of the probe sequences, and also allow for improved precision of SNP determination [20, 21]. The use of Rnase-H-dependent PCR (rhPCR) [integrated DNA technologies (IDTDNA)] allows for improved specificity of the intended PCR product (in this case *FCGR3A*). RH primers include an RNA base that specifically matches a unique nucleotide of *FCGR3A*, but not the sequence in *FCGR3B* (Fig. 1) [19]. By designing the real-time PCR reaction to include, within one of the primers, an RNA base that matches *FCGR3A*, but does not match *FCGR3B*, we were able to specifically amplify *FCGR3A* while not co-amplifying *FCGR3B* as confirmed by real-time PCR in a single PCR reaction (data not shown). Likewise, *FCGR2C* was created from the unequal crossover of *FCGR2A* and *FCGR2B*, and thus shares high sequence homology to each of those *FCGR* variants, with sequence homology surrounding the region of the SNP to *FCGR2B* [11–13]. Thus, in a similar manner to *FCGR3A*, RH primers were designed to specifically amplify *FCGR2C* while not co-amplifying *FCGR2B*, with *FCGR2C* LNA-SNP probes used to determine the genetic variability of *FCGR2C* within individuals. Furthermore, separate CNV probes were created for both *FCGR3A* and *FCGR2C*, to be used in combination with the same RH primers for both *FCGR3A* and *FCGR2C*, respectively. Both the *FCGR3A*

Fig. 1 Sequence similarities between *FCGR3A* and *FCGR3B* can be resolved for specific amplification using an RNA base (*red font*) at the nucleotide position where the sequences differ (*red box*). The RNA base matches the sequence of *FCGR3A*, but does not match the sequence of *FCGR3B*. When Rnase Enzyme is included in the PCR reaction, the mismatch of the RNA base from *FCGR3B* will result in cleavage of the PCR product of *FCGR3B* not allowing it to amplify. Alternatively, as *FCGR3A* matches the RNA base, the cleavage will not occur and PCR product will continue to amplify. The 3′ of the RH primer has a mismatched base to *FCGR3A* followed by a spacer to ensure maximum efficiency of the end block

RH primer/CNV probe and the *FCGR2C* RH primer/CNV probe can also be used in combination with primers/CNV probe of a control gene that is known to be diploid within the human genome (*RNASE P*). By combining the query gene CNV reaction in the same tube as a control gene that is diploid, normalization to the control gene can be done for each sample to accurately analyze the amplification reaction.

By improving the specificity of the PCR reaction for each FCGR expressed on NK cells (FCGR3A and FCGR2C) through the use of RH primers for each gene, greater accuracy should be obtainable for the genotyping results. This improved genotyping accuracy should enable *FCGR* genotyping to be evaluated with respect to a variety of in vivo settings where the function of distinct FCGR SNP variants might influence disease susceptibility, severity, or response to therapy.

2 Materials

All work should be conducted in sterile conditions. If possible, prepare reagents in a PCR hood using nuclease-free materials (filter tips, sterile water, sterile microfuge tubes, sterile strip tubes, and/or real-time PCR plates).

2.1 Premade Reagents

1. Genotyping Master Mix (Applied Biosystems/Life Technologies) (*see* **Note 1**).
2. Express qPCR Master Mix (Invitrogen/Life Technologies) (*see* **Note 1**).
3. Rnase-H Enzyme and Rnase-H Buffer [Integrated DNA Technologies (IDTDNA)]. Dilute enzyme to 25 mU/μL: add 2 mL Rnase H2 Enzyme Dilution Buffer to 50 U of Rnase-H Enzyme. Aliquot into 0.5 mL sterile tubes at 100 μL/tube.

2.2 Instrument Requirements and Software

1. Real-time PCR machine with settings capable of assessing SNP allelic discrimination, such as "Genotyping" analysis found in the StepOnePlus Real Time PCR system (Applied Biosystems/Life Technologies) (*see* **Note 2**).
2. CNV analysis software, such as CopyCaller® Software (Applied Biosystems/Life Technologies).

2.3 Primers and Probes

1. All primers (IDTDNA) used in this protocol are listed in Table 1 (*see* **Note 2**). Resuspend stock primers and probes at 100 μM. Separately, create a working solution of each primer and probe by diluting each primer and probe to 10 μM (90 μL of sterile DNase/Rnase-free water + 10 μL of the 100 μM stock primer or probe). Store all primers and probes at −20 °C.

Table 1
Sequences of primers and probes as ordered through IDTDNA

FCGR3A Rnase-H forward primer	5′-TCCAAAAGCCACACTCAAAGArCAGCGC - C3 spacer-3′
FCGR3A reverse primer	5′ GATGGTGATGTTCACAGTCTCT 3′
FCGR3A-G SNP probe	5′ FAM TCC+CA+A+C+AA+G+CC 3′ Iowa Black FQ Quencher
FCGR3A-T SNP probe	5′ ATTO532N TCC+CA+A+A+AA+GC+CC 3′ Iowa Black FQ Quencher
FCGR3A-CNV probe	5′ FAM AAGCCCCCT/ZEN/GCAGAAGTAGGA 3′ Iowa Black FQ Quencher
FCGR2C forward primer	5′ TATTCCTGGCTCCTGTTGC 3′
FCGR2C Rnase-H reverse primer	5′ TGTCAGAGTCACAGAGTCCTC**rU**TGGAC - C3 spacer-3′
FCGR2C-C SNP probe	5′ ATTO532N CAC+T+G+GGG+CT 3′ Iowa Black FQ Quencher
FCGR2C-T SNP probe	5′ FAM TCCAC+T+A+GGG+CT 3′ Iowa Black FQ Quencher
FCGR2C CNV probe	5′ FAM AGCC+C+C+AGTGG 3' Iowa Black FQ Quencher
RNASE P forward primer	5′ AGATTTGGACCTGCGAGCG 3′
RNASE P reverse primer	5′ GAGCGGCTGTCTCCACAAGT 3′
RNASE P CNV probe	5′ HEX TTCTGACCT/ZEN/GAAGGCTCTGCGCG 3′ Iowa Black FQ Quencher

Unmodified/standard primers were purified by standard desalting, Rnase H primers were purified by HPLC. For Rnase H primers, at least ten base pairs precede the RNA base (bolded). The RNA base is then followed by five more base pairs, which are specific for the intended PCR product (*FCGR3A* or *FCGR2C*), and then a C3 spacer was added. All probes include a fluorescent probe on the 5′ end of the DNA sequence (hexachlorofluorescein (HEX), FAM, or ATTO™532), refer to **Note 2**. LNA-modified bases are indicated by the "+" symbol within the DNA sequence. The ZEN®-modified CNV probes include "/ZEN/" within the DNA sequence

2. All Gene Block (gBlock) control primers (IDTDNA) are listed in Table 2. Resuspend gBlock control sequences and dilute to serve as controls for each SNP. Resuspend control sequences to a concentration of 1 ng/μL with nuclease-free water. Perform serial dilutions (add 1 μL of 1 ng/μL gBlock DNA to 99 μL sterile water, vortex to mix well = 10^{-2} ng/μL; add 1 μL of 10^{-2} ng/μL gBlock DNA to 99 μL sterile water, vortex to mix well = 10^{-4} ng/μL; add 1 μL of 10^{-4} ng/μL gBlock DNA to 99 μL sterile water, vortex to mix well = 10^{-6} ng/μL = 1 fg/μL; add 1 μL of 1 fg/μL gBlock DNA to 99 μL sterile water, vortex to mix well = 10^{-2} fg/μL) in nuclease-free water to a final concentration of 10^{-2} fg/μL (*see* **Note 3**). For "heterozygous" controls, create a mixture of 1:1 *FCGR3A*-F-CNT with *FCGR3A*-V-CNT, and create a mixture of 1:1 *FCGR2C*-C-CNT with *FCGR2C*-T-CNT.

Table 2
Control sequences for *FCGR3A* and *FCGR2C*. SNP assays

Gene block control	Sequence
FCGR3A-V-CNT	5′-GACTTCTACATTCCAAAAGCCACACTCAAAGACAGCGGCTCCTAC TTCTGCAGGGGGCTTGTTGGGAGTAAAAATGTGTCTTCAGAGACT GTGAACATCACCATCACTCAAGGTGAGACATGTGCCACCCT-3′
FCGR3A-F-CNT	5′-GACTTCTACATTCCAAAAGCCACACTCAAAGACAGCGGCTCCTAC TTCTGCAGGGGGCTTTTTGGGAGTAAAAATGTGTCTTCAGAGACT GTGAACATCACCATCACTCAAGGTGAGACATGTGCCACCCT-3′
FCGR2C-C-CNT	5′-CAGCAGCTCCCCCAAAGGCTGTGCTGAAACTCGAGCCCCAGTGG ATCAACGTGCTCCAAGAGGACTCTGTGACTCTGACATGCCGGGGG ACTCACAGCCCTGAGAGCGACTCCATTCCGTGGTTCCACAATGGG AATCTCATTCCCACCCACACGCAGCCCAGCTACAGGTTCAAGGCC AACAACAATGACAGCGGGGAGTACACGTGCCAGACTGGCCAGAC CAGCCTCAGCGACCCTGTGCATCTGACTGTGCTTTCTGGTCAGT GGAGGAAGGCCCCAGGGTGGACCTGGGCCAGGATGGATGAAATC TGCTTTCAGGCAG-3′
FCGR2C-T-CNT	5′-CAGCAGCTCCCCCAAAGGCTGTGCTGAAACTCGAGCCCTAGTGG ATCAACGTGCTCCAAGAGGACTCTGTGACTCTGACATGCCGGGGG ACTCACAGCCCTGAGAGCGACTCCATTCCGTGGTTCCACAATGGG AATCTCATTCCCACCCACACGCAGCCCAGCTACAGGTTCAAGGCC AACAACAATGACAGCGGGGAGTACACGTGCCAGACTGGCCAGACC AGCCTCAGCGACCCTGTGCATCTGACTGTGCTTTCTGGTCAGTGG AGGAAGGCCCCAGGGTGGACCTGGGCCAGGATGGATGAAATCTG CTTTCAGGCAG-3′

The sequences below can be ordered as "Gene Blocks" from IDTDNA to serve as positive controls for SNP assays

3 Methods

All work should be conducted in sterile conditions, taking care to not cross-contaminate samples or reagents. If possible, prepare all reactions in a PCR hood, using sterile, nuclease-free materials filter tips, sterile water, sterile microfuge tubes, sterile strip tubes, and/or real-time PCR plates. Keep all reagents on ice during assay setup procedures.

3.1 SNP Genotyping Methodology: FCGR3A-V158F and FCGR2C*- C/T*

1. Prepare the following reaction master mix as shown in Table 3 (*see* **Note 4**). The final concentration of the primers should be 0.3 μM of each primer and 0.25 μM of each probe included in the master mix of each reaction. Refer to **Note 5** for reaction volumes; refer to **Note 6** for Rnase H Enzyme concentrations; and refer to **Note 7** for master mix tips.
2. Pipette 4 μL of the reaction master mix into each well or tube.
3. Dilute all DNA samples, including the positive controls, to 5 ng/μL. Add 1 μL of 5 ng/μL DNA sample to the appropriate well per the predesigned template (*see* **Note 8**).

Table 3
Master mix for *FCGR3A* SNP and *FCGR2C* SNP

FCGR3A			***FCGR2C***		
	Volume (μL) reaction	**Final concentration**		**Volume (μL) reaction**	**Final concentration**
2× Genotyping master mix (ABI)	2.5	1×	2× Express genotyping master mix (Invitrogen)	2.5	1×
RH *FCGR3A* forward primer (10 μM)	0.15	0.3 μM	*FCGR2C* forward primer (10 μM)	0.15	0.3 μM
FCGR3A reverse primer (10 μM)	0.15	0.3 μM	RH *FCGR2C* reverse primer (10 μM)	0.15	0.3 μM
FCGR3A-G probe (10 μM)	0.125	0.25 μM	*FCGR2C*-C probe (10 μM)	0.125	0.25 μM
FCGR3A-T probe (10 μM)	0.125	0.25 μM	*FCGR2C*-T probe (10 μM)	0.125	0.25 μM
RH enzyme in buffer (25 U/μL)	0.2	1 U/μL	RH enzyme in buffer (25 U/μL)	0.2	1 U/μL
DNA (5 ng/μL)	1	N/A	DNA (5 ng/μL)	1	N/A
Sterile water	0.75	N/A	Sterile water	0.75	N/A

Multiply the number of reactions (# of unknowns = samples × # replicates/sample + # of no template samples + # control samples) × 1.1. Refer to **Notes 7** and **8**.

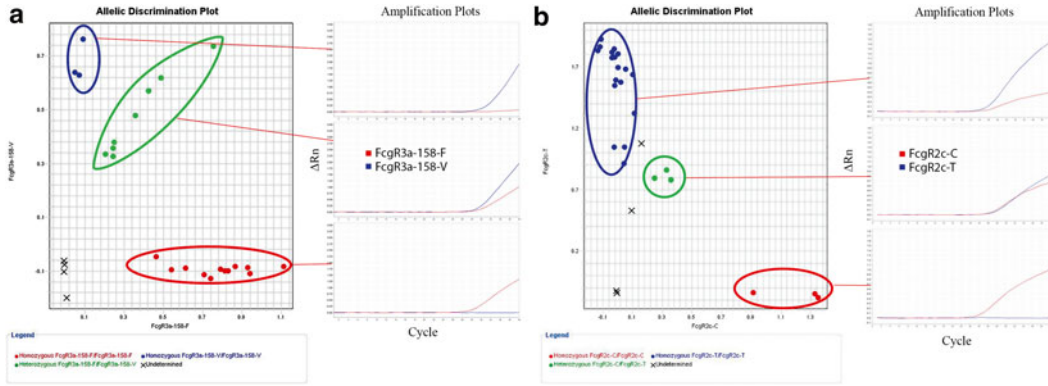

Fig. 2 Example of allelic discrimination plot results for *FCGR3A* and *FCGR2C*. (**a**) Each allelic variant is separated into quadrants of the plot. Amplification of the ATTO532-T probe, but not of the FAM-G probe, indicates a V/V genotype (*blue*). Amplification of the FAM-G probe, but not of the ATTO532-T probe, indicates a F/F genotype (*red*). Amplification of both FAM-G probe and ATTO532-T probe indicates a V/F genotype (*green*). No template controls are noted by an "X". Additionally, samples for which the genotype could not be determined based on the algorithm within the software are also labeled as "X" in the center of the plot. (**b**) Each allelic variant is separated into quadrants of the plot. Amplification of the FAM-T probe, but not of the ATTO532-C probe, indicates a T/T genotype (*blue*). Amplification of the ATTO532-C probe, but not of the FAM-T probe, indicates a C/C genotype (*red*). Amplification of both FAM-T probe and ATTO532-C probe indicates a C/T genotype (*green*)

4. Add 1 μL of sterile water to the no template well. Refer to **Note 9**. If known sample control genotypes are available for each SNP being analyzed, load 1 μL of 5 ng/μL DNA for each known sample control (*see* **Note 10**).
5. Seal/cover the tubes or plate securely.
6. Place the sample in the real-time PCR machine. Follow the real-time PCR machine's manufacturer settings for SNP genotyping settings.
7. Adjust the reaction tube volume to read 5 μL. Set the cycling conditions to those as shown in Table 5 (*see* **Note 11**).
8. If known sample control genotypes are available, indicate which samples are known in the sample setup.
9. After the run is complete, analyze for genotype determinations according to the real-time PCR machine's manufacturer settings, or refer to Fig. 2a (*FCGR3A*) and 2b (*FCGR2C*) for example of expected qPCR results (*see* **Notes 10, 12** and **13**).

3.2 CNV Genotyping Methodology: FCGR3A *and* FCGR2C

1. Prepare the following reaction master mix as shown in Table 4 (*see* **Note 4**). The final concentration of the primers should be 0.3 μM of each primer and 0.25 μM of each probe included in the master mix of each reaction. Refer to **Note 14** for template suggestions; refer to **Note 5** for reaction volumes; refer to **Note 6** for Rnase H enzyme suggestions; and refer to **Note 7** for master mix tips.

Table 4
Master Mix for *FCGR3A* CNV and *FCGR2C* CNV

FCGR3A			***FCGR2C***		
	Volume (μL) reaction	**Final concentration**		**Volume (μL) reaction**	**Final concentration**
2× Genotyping master mix (ABI)	2.5	1×	2× Express genotyping master mix (Invitrogen)	2.5	1×
RH *FCGR3A* forward primer (10 μM)	0.15	0.3 μM	*FCGR2C* forward primer (10 μM)	0.15	0.3 μM
FCGR3A reverse primer (10 μM)	0.15	0.3 μM	RH *FCGR2C* reverse primer (10 μM)	0.15	0.3 μM
*FCGR3A*CNV probe (10 μM)	0.125	0.25 μM	*FCGR2C* CNV probe (10 μM)	0.125	0.25 μM
RNASE P forward primer (10 μM)	0.15	0.3 μM	*RNASE* P forward primer (10 μM)	0.15	0.3 μM
RNASE P reverse primer (10 μM)	0.15	0.3 μM	*RNASE* P reverse primer (10 μM)	0.15	0.3 μM
RNASE P CNV probe (10 μM)	0.125	0.25 μM	*RNASE* P CNV probe (10 μM)	0.125	0.25 μM
RH enzyme in buffer (25 U/μL)	0.2	1 U/μL	RH enzyme in buffer (25 U/μL)	0.2	1 U/μL
DNA (5 ng/μL)	1	N/A	DNA (5 ng/μL)	1	N/A
Sterile water	0.45	N/A	Sterile water	0.45	N/A

Multiply the number of reactions (# of unknowns = samples × # replicates/sample + # of no template samples + # control samples) × 1.1. Refer to **Notes 7**, **14** and **15**.

Table 5
Real-time PCR machine cycling conditions for *FCGR3A* (SNP and CNV) and *FCGR2C* (SNP and CNV) reactions

FCGR3A			*FCGR2C*		
Cycle	**Temperature (°C)**	**Time**	**Cycle**	**Temperature (°C)**	**Time**
1	95	10 min	1	95	3 min
2	95	30 s	2	95	5 s
3	60	1 min	3	63	15 s
Repeat cycles 2–3 × 40–50 more times			Repeat Cycles 2–3 × 40–50 more times		

Refer to **Note 11** for cycle suggestions, and refer to **Note 13** for annealing temperature suggestions

2. Pipette 4 μL of the reaction master mix into each well or tube.
3. Dilute all DNA samples, including the positive controls, to 5 ng/μL. Add 1 μL of 5 ng/μL DNA sample to the appropriate well. Plate samples in triplicate per the predesigned template (*see* **Note 15**).
4. Add 1 μL of sterile water to the no template well (*see* **Note 9**). If known sample control genotypes are available for each CNV being analyzed, load 1 μL of 5 ng/μL DNA for each known sample control (*see* **Note 14**).
5. Seal/cover the tubes or plate securely.
6. Place the sample in the real-time PCR machine. Follow the real-time PCR machine's manufacturer settings using standard cycling conditions settings.
7. Adjust the reaction tube volume to read 5 μL. Set the cycling conditions to those as shown in Table 5. If known sample control genotypes are available, indicate which samples are known in the sample setup.
8. After the run is complete, analyze CN according to the real-time PCR machine's manufacturer settings. For a StepOnePlus, refer to the CopyCaller software manual for analysis. Adjust the threshold for each curve according to the settings as suggested in the software protocol, export results, and analyze in the CNV analysis software.

4 Notes

1. It is important to store reagents properly and avoid multiple freeze–thaw cycles in order to prevent reagent degradation. As reagents become degraded, it is difficult to discriminate between genotype variations (both SNP and CNV).

2. The real-time PCR instrument should be capable of duplex reads of, at the minimum, VIC and fluorescein (FAM). ATTO™532 is an *N*-Hydroxysuccinimide (NHS) Ester that can be substituted for VIC and, depending on the real-time PCR machine, may require calibration. Hexachlorofluorescein (HEX) is another substitute for VIC, and calibration may be necessary depending on the instrument. HEX and ATTO™532 calibration mixes are each available from IDTDNA.
3. It may be necessary to dilute the control gBlock DNA sequences for each gene (*FCGR3A* and *FCGR2C*). The goal is to have the control gBlock sequences amplify around the same cycle as the genomic unknown DNA sequences. After initial dilutions are made, run a small test plate to verify the amplification of the gBlock DNA sequences (Table 2) with a small subset of genomic DNA samples, that are each diluted to 5 ng/μL, to determine if the samples amplify at the same cycle. Optimize the gBlock DNA concentration further if necessary by performing further 1:10 serial dilutions.
4. The Real Time PCR master mix used for the *FCGR3A* RH primer/probe reaction mix worked well with the Genotyping Master Mix from ABI. When using the same *FCGR3A* RH primer/probe reaction with the Express qPCR Master Mix from Invitrogen, the results were more difficult to interpret. Alternatively, the Real Time PCR master mix used for the *FCGR2C* RH primer/probe reaction mix worked well with the Express qPCR Master Mix from Invitrogen, but when using the same *FCGR2C* RH primer/probe reaction with the Genotyping Master Mix from ABI, the amplification of the separate alleles was not distinguishable. Thus, the Real Time PCR master mix used may affect the interpretability of the results.
5. The total reaction volume per tube as prepared in this protocol is 5 μL. This total reaction volume can be increased if desired by adjusting all reaction components accordingly to maintain the correct final concentration of the reaction components. If the reaction volume is increased, the concentration of DNA, however, can remain 5 ng of DNA/reaction.
6. The amount of Rnase H Enzyme and Buffer may vary depending on the real-time PCR master mix used, and may require optimization. The amount of Rnase H Enzyme/Buffer were optimized for the PCR master mix and primer/probe combinations. Depending on the dilution/lot number of the Rnase H Enzyme, optimization may be required.
7. When making the master mix for each reaction, always include additional reaction components to account for pipetting errors. Multiply the total number of samples [including: (unknown samples) × (the number of replicates), the number of no templates,

and the number of positive control samples] by at least 1.1. For example, if analyzing ten samples, prepare master mix for 11; if analyzing 96 samples, prepare master mix for 106 samples.

8. It is highly recommended to predesign a template for the PCR reaction in a template table, such as in Excel. Run samples at least in duplicate if possible. Include at least one no template negative control. Also, if possible include ≥1 known sample controls for each genotype (e.g. DNA from a sample known to encode FCGR3A-158-F/F; DNA from a sample known to encode FCGR3A-158-V/F; and DNA from a sample known to encode FCGR3A-158-V/V).
9. The negative control samples should not show amplification.
10. The known sample controls then can be selected during assay setup allowing for the analysis software to calibrate each reaction read. Also, the user can compare the amplification curves of unknown samples to known controls if the software is unable to differentiate the genotype (Fig. 2a, b). If known sample genotypes are unavailable, refer to **Note 3** and Table 2.
11. If desired for SNP genotyping assays, extend the cycling conditions out to 50 cycles instead of 40 cycles.
12. SNP determinants can be made by referring to the amplification plot of the amplified SNPs without allelic discrimination software. When using Allelic Discrimination Software, select samples that serve as positive controls for each allele that is amplified (each homozygote and heterozygotes). Be sure when selecting the positive control samples that the amplification of each allele is associated with the correct probe (for example, if "Allele 1" is associated with the genotype for the "FAM"-labeled probe, then the positive control "Allele1/Allele 1" should be "FAM"-labeled probe). Also, if the allelic discrimination plot yields genotype results that the software algorithm cannot interpret (Fig. 2a, b "X" labeled "Undetermined"), it may be necessary to refer to the amplification plot to determine the genotype of undeterminable samples. This can be done by referencing the amplification of known genotypes
13. It is crucial to have good positive controls (Table 2). The *FCGR2C*-T probe, although specific for T, does non-specifically amplify *FCGR2C*-C even when there is no *FCGR2C*-C present in the reaction mixture. Due to this, in order to determine the allelic discrimination of unknown samples, it is important to refer to control primer reaction (in particular for *FCGR2C*-T). Depending on the real-time PCR instrument, it may be helpful to optimize the PCR reaction by performing an annealing temperature gradient. It may be necessary to decrease the annealing temperature slightly (to 58 or 59 °C) to improve the specificity of this reaction.

14. Again, it is highly recommended to predesign a template for the PCR reaction assay setup in a template table, refer to **Note 8** above. The confidence in the data will be improved by including more DNA samples. During the analysis of CNV, if a known control for the number of copies of each gene (*FCGR3A* or *FCGR2C*) exists, it should be included in each run for use during copy number analysis. For these analyses, without a proper copy number control, the expected number of copies for both *FCGR3A* and *FCGR2C* can be set as two copies per gene. If possible, using healthy donor samples to standardize this assay for future studies can help to standardize the assay. The number of copies for this analysis can then be based on the expected copy number for such healthy donor samples. As such, it is best to use at least ten different DNA samples for this type of analysis on one plate. By including an adequate number of individual DNA samples, more reliable results can be obtained.
15. The CNV assay is more reliable if each sample is repeated at least twice, on two separate plates. Each time that a sample is tested in a CNV assay, it should be prepared in triplicate sub-replicates on each plate.

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Chapter 5

Measurement of Average Telomere Length in Ex Vivo Expanded Natural Killer Cells by Fluorescence In Situ Hybridization (FISH) and Flow Cytometry

Sourindra N. Maiti

Abstract

Natural killer (NK) cells are a subset of cytotoxic lymphocytes that play a critical role in innate immune surveillance against infections and tumors through cytokine secretion and target cell lysis. NK cells function without any need for prior antigen exposure. Thus, more recently NK cells are considered a promising source of lymphocytes for adoptive tumor therapy. However, because NK cells represent only a small lymphocyte fraction, expand poorly ex vivo, and have limited life spans, clinical scale generation of NK cells for tumor immunotherapy was a challenging issue. To overcome this challenge, numerous expansion platforms have been developed. However, ex vivo expansion of NK cells could lead to proliferation-induced senescence. Telomeres at the end of chromosomes play a crucial role in maintaining the integrity of the chromosome and are lost at each cell division in somatic cells and have emerged as important cellular elements in aging and cancer. Because telomere length is known to decrease in adult human NK cells and is associated with proliferation-induced senescence, it is important to determine the effect of NK cell expansion systems on telomere length. In this chapter, a detailed protocol is provided to analyze the telomere length of expanded NK cells using a commercially available Flow FISH kit.

Key words Telomere length, aAPC, NK cells, Flow cytometry, Flow FISH

1 Introduction

Telomeres at the end of the chromosomes in all vertebrates are composed of a sequence of six nucleotides (TTAGGG) repeated from a few hundred to thousand times [1]. Telomeres protect the chromosomes end and in most somatic cells, telomere repeats are lost after each cell division [1]. Telomerase reverse transcriptase enzyme compensates this loss by adding the hexameric repeats to the chromosome ends [1]. Mutations in the genes involved in telomere maintenance are associated with failure to maintain telomere length [2–4]. Like almost all vertebrate cells in human innate immune Natural Killer (NK) lymphoid cells, telomere shortening and decreased telomerase activity occur with aging [5, 6]. Immature

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$CD56^{bright}CD16^{-}$ NK subset possesses longer telomere compared to mature $CD56^{dim}CD16^{-}$ NK cell subsets [7]. Cytokine IL-2 was shown to increase the telomerase activity in NK cells thereby maintaining the telomere length [8]. Clinical scale generation of ex vivo expanded NK cells from human pluripotent stem cells and or from peripheral blood for adoptive cancer therapy, cultured in the presence of K562 cell line engineered to express 4-1BBL and membrane-bound IL-21, was shown to increase telomerase expression, and therefore telomere length [9–11], compared to K562 cells expressing 4-1BBL and membrane-bound IL-15.

Several methods to measure telomere length are available, each with distinct advantages and disadvantages. While terminal restriction fragment length (TRF) analysis by Southern blot is the gold standard, amount of time and DNA needed for Southern analysis is a limiting factor [12]. In addition, variable distance between terminal restriction sites in the DNA and actual telomere repeats among chromosomes overestimate the average telomere length by several kb [12]. PCR methodologies to measure average and chromosome-specific telomere length have been also described [12]. Telomere length using directly labeled (CCCTAA)3 peptide nucleic acid (PNA) probes in a cell population on individual metaphase chromosomes by flow cytometry is described and widely used [12]. The basic principle of Q-FISH is PNA-labeled probe at low ionic strength that can anneal only to complementary single-stranded DNA sequences and thus PNA-labeled (CCCTAAA)3 probe hybridize to $(TTAGGG)_n$ target sequences and not sub-telomeric sequences that allow an estimation of the telomere length without inclusion of sub-telomeres, a clear advantage to traditional TRF analysis [12]. Further improvements through the inclusion of control cells with known telomere length in every sample tube and automated multicolor Flow FISH have been used to measure the average median telomere length in a very sensitive and fast manner [12].

In this chapter, a simple method is described to assess telomere length by Flow FISH method using a PNA probe-based kit. This is a convenient, fast, and sensitive method for detection of telomeres in hematopoietic cells [13, 14] and is optimal for estimation of telomere length, as the fluorescence intensity of the cells directly correlates to the length of the telomeres [15, 16].

2 Materials

1. PBS.
2. Control cells (1301 cell line from Sigma-Aldrich).
3. Sample cells (primary and expanded/activated NK cells).
4. Burker chamber.

5. Heating block, adjustable to 40 and 82 °C.
6. 1.5 mL microcentrifuge tubes, micropipettes, and tips.
7. Microcentrifuge, adjustable to 500 × *g*.
8. Flow Cytometer (capable of excitation at 488 nm and emission at 530 and 617 nm).
9. Telomere PNA kit/FITC for Flow Cytometry (Dako) (*see* **Note 1**).
10. Working Wash Solution and DNA Staining Solution—Take the vials 3 and 4 from 4 °C (telomere PNA kit) and leave at room temperature (RT) to dissolve any crystals formed. After crystals are dissolved, dilute Wash Solution (vial 3) 1:10 in DNAse/RNAse-free molecular grade water. Store diluted Wash Solution at 4 °C for 6 months. Dilute DNA Staining Solution (vial 4) 1:10 in DNAse/RNAse-free molecular grade. Prepare DNA Staining Solution fresh for each experiment and protect from light.
11. Flow Cytometer.
12. Flow cytometry software such as FlowJo (Tree Star Inc), FCS Express (De Novo Software), and BD FACSDIVA™ (BD Biosciences) to name a few.

3 Methods

3.1 Telomere Length Assay

3.1.1 Day 1

Pre-warm a heating block or water bath to 82 °C. Place 4 from the PNA kit/FITC at RT and leave until day 2 to freshly prepare DNA Staining Solution. On day 1, make sure to have 1–2 × 10^6 extra NK cells (or other test cells) and 1301 cells for DNA ploidy measurement using Propidium Iodide staining. The procedure is described below for performing the assay in duplicates.

1. Wash NK cells and control 1301 cells in PBS.
2. Count cells in Burker chamber (*see* **Note 2**).
3. Mix 2 × 10^6 NK cells and 2 × 10^6 control 1301 cells (*see* **Notes 3** and **4**).
4. Add PBS to a total volume of 6 mL.
5. Mix well and split 1.5 mL of cells into four 1.7 mL microcentrifuge tubes (1 × 10^6 cells/tube), close lid, and label tubes 1, 2, 3, and 4.
6. Centrifuge cells at 500 × *g* for 5 min and aspirate the supernatant.
7. Add 300 μL of hybridization solution contained in vial 1 of the kit, to tubes 1 and 2, and mix immediately by vortexing; these are the control samples (duplicate).

8. Add 300 μL of telomere PNA probe/FITC contained in vial 2 of the kit, to tubes 3 and 4 and mix immediately by vortexing; these are the test samples (duplicate).
9. Incubate the tubes at 82 °C for 10 min (*see* **Note 5**) in a pre-warmed heating block/water bath. Remove the tubes from the heating block/water bath, mix by vortexing, and store overnight at room temperature in the dark.

3.1.2 Day 2

Pre-warm a heating block/water bath to 40 °C. Ensure that the contents of vials 4 stored overnight at room temperature have completely dissolved without any crystals before making DNA Staining Solution (as described in Subheading 2, **item 10**).

1. To the control and test group tubes 1 through 4, add 1 mL of Wash Solution and mix by vortexing.
2. Incubate the tubes at 40 °C for 10 min.
3. Mix by vortexing and centrifuge tubes at 500 × *g* for 5 min and discard the supernatant.
4. Repeat the wash steps (**steps 1** through **3**) one more time.
5. To the washed samples, add 0.5 mL of DNA staining solution.
6. Mix contents by vortexing.
7. Transfer the samples to flow cytometry tubes, label tubes accordingly.
8. Incubate at 4 °C in the dark, for 2–3 h (*see* **Note 6**).
9. Optionally, stain NK cells and 1301 cells with Propidium Iodide, to serve as controls for determining the DNA ploidy as per protocol described before [17]. This step is recommended to ascertain the ploidy of the test cells. However, in this example this step is optional as the ploidy of 1301 cell line is a known Tetraploid cell line and healthy lymphocytes are diploid.

3.2 Analysis and Interpretation of Results

3.2.1 Flow Cytometry

1. Turn on the flow cytometer and allow time for the instrument to warm up.
2. Run the samples and adjust the FSC and SSC voltages to appropriately display sample in the scatter plot, draw gate around live cell population (G_1).
3. Use the test negative control sample (tubes 1, 2) to adjust the PMT voltage for FL1 channel (logarithmic scale, for telomere probe PNA/FITC) and FL3 channel (linear scale, for Propidium Iodide DNA staining) to position the live cells within the scale (*see* **Note 7**).

4. Also, plot the cells from G_1 on FL3-A vs. FL3-W and select the population representing singlets.
5. Acquire and save forward scatter, side scatter, FL1-H vs. FL3-H data.

3.2.2 Data Analysis and Calculation of Relative Telomere Length

1. Open the saved files in any flow cytometry analysis software such as FlowJo, BD FACSDIVA™, FCS express, etc.
2. Generate the FL1 vs. FL3 dot plot.
3. To assess the Relative Telomere Length (RTL), draw a gate around the cell population in the G_0/G_1 phase. The DNA staining solution in the kit identifies G_0/G_1 cells. It is important to correctly gate on the cells in the G_0/G_1 phase of the cell cycle where the cell has one copy of the genome. If cells in S or G_2/M phase are not excluded completely in the gating, the estimated RTL will not be per genome equivalent.
4. Samples hybridized with the telomere PNA probe/FITC (tubes 3 and 4) should exhibit a fluorescence signal in FL1, which is higher than the background/autofluorescence of the same samples hybridized without the probe (tubes 1 and 2) in hybridization solution.
5. Analyze the NK cells and 1301 control cells separately using Dako staining kit to accurately determine the cellular DNA content, to compensate for DNA ploidy, or using propidium iodide staining as described before [17]. The premise of the DNA staining dyes is that they are stoichiometric, i.e., they bind in proportion to the amount of DNA present in the cell. Control 1301 tetraploid cells (should be seen in FL3) and long telomeres (should be seen in FL1). In principle, any other cell types that are easily distinguished from the samples can be used.
6. Calculate the RTL as the ratio between the telomere signal of each sample and the control cells with correction for the DNA index (*see* **Note 8**) of G_0/G_1 cells using the formula;

   RTL = (MFI sample cells with probe − MFI sample cells without probe) × DNA index of control cells × 100/(MFI reference cells with probe − MFI reference cells without probe) × DNA index of sample cells.

   The resultant RTL value indicates the average telomere fluorescence per cell in the sample—NK cells as percentage of the telomere fluorescence per cell in 1301 cell line (control). This data can be used to determine the effect of different culture condition on NK cell telomere length (Fig. 1).

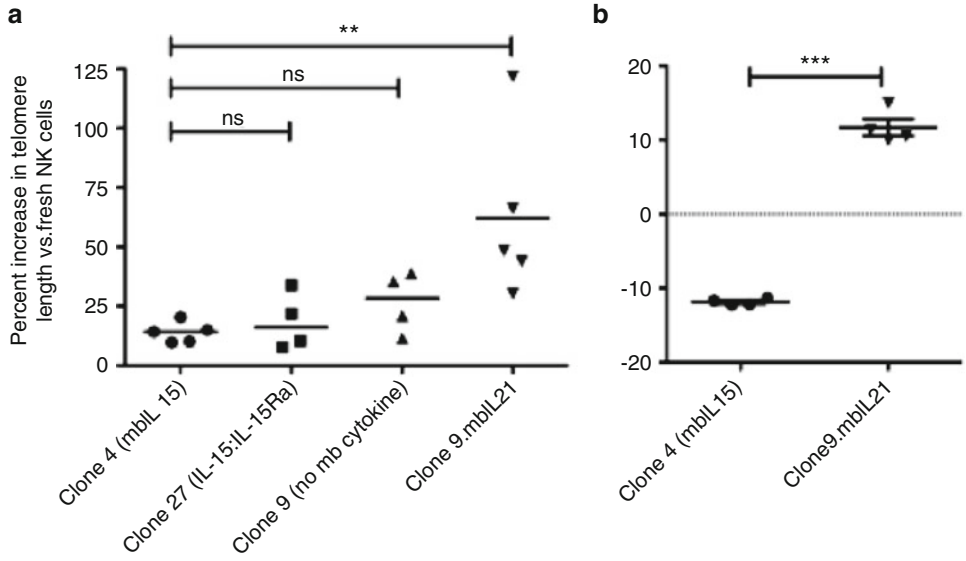

Fig. 1 Analysis of telomere length by Flow FISH. Telomere length of NK cells is analyzed before and after expansion on K562 based feeder cells. The data compares change in telomere length (**a**) after 7 days of culture with irradiated K562 bearing no cytokine (Clone 9), mbIL15 (Clone 4), mbIL21 (Clone 9.mbIL21), or IL-15 fused with a linker to IL15Rα (Clone 27) and statistical analysis was done using One-way ANOVA with Dunnett's multiple comparisons test, and (**b**) after 21 days of culture with irradiated K562 bearing mbIL15 (Clone 4) or mbIL21 (Clone 9.mbIL21) statistics was performed using two-tailed *t*-test the data. The data in both panels was normalized to unexpanded NK cells from each individual NK cell donor. Reproduced from PLOS ONE (2012) "*Denman, C. J., Senyukov, V. V., Somanchi, S. S., et al. (2012) Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells. PloS One* 7, *10.1371/journal.pone.0030264*"

4 Notes

1. The reagents of the kit should be stored at 4 °C. Reagents in vials 1, 2, and 4 are light sensitive and must be protected from light. Vials 1 and 2 contain 70% formamide and are toxic, thus be handled carefully and must be disposed as hazardous waste.
2. To achieve a good reproducibility, it is important that the initial cell count for the control cells is accurate. If the cells are counted in a Burker chamber, at least 64 fields should be counted.
3. The tetraploid 1301 cell line should be preferred as control cells as they have very long telomeres (>30 kb).
4. Cells must not be fixed before use in the assay.
5. The temperature for the denaturation step must be between 80 and 84 °C, temperatures below 75 °C will impair the results.
6. DNA staining should be done for a minimum of 2–3 h. Longer incubation can also be done (up to 24 h) when the samples are incubated in dark at 4 °C.

7. It is mandatory that proper quality control of the flow cytometers is performed since variation between individual flow cytometer is taken into account when results from different cytometers are compared. Make pre-adjustments of the PMTs of the relevant fluorescence detectors to assure that cells hybridized with and without telomere PNA probe/FITC are displayed on scale distinctively. Linearity of the instrument can be checked by using Dako Fluorospheres calibration beads, code K0110. This will give a good estimation of the linearity of the instrument.
8. The DNA index value to be entered in the formula for a normal diploid cell in G_0/G_1 phase is 1; The DNA index for 1301 cell line, a Tetraploid cell line, in G_0/G_1 phase is 2. If the control cell line used for the assay (instead of 1301) is also a diploid cell, then the DNA index correction is not required.

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Chapter 6

In Vitro Assessment of Human Natural Killer Cell Migration and Invasion

Karin Tomin, Ronald H. Goldfarb, and Per Albertsson

Abstract

Cell invasion assays are important to obtain valuable functional data relating to tissue migration and invasion of effector lymphocytes. Boyden chamber invasion assays represent a reductionist system that allows for easy manipulation using various extracellular matrix (ECM) components addressing migratory functions or invasion into/through a three-dimensional matrix where migration and invasion inhibitors as well as stimulators can be added. Presented here is a description using the Transwell® system where invasion and migration can be studied. It constitutes an inner cell culture well with a (PET) polycarbonate filter with 3–8 μm pores that allow for cells to transverse to the bottom chamber where they can be recorded by various methods (Fig. 1). The polycarbonate filters may be coated with ECM proteins for migration and adhesive studies or covered with a thick layer that occludes the pores to address matrix degradation, i.e., *invasion as described in this chapter.*

Key words Invasion assay, Migration, Lymphocytes, Natural killer cells, IL-2 activation, Extracellular matrix, Matrigel

1 Introduction

In order to reach and infiltrate a tumor mass, circulating NK cells need activating signals which enable their slowing down in the circulation, i.e., rolling along the endothelial lining. Followed by the homing process, invasive and migratory NK cells can traverse the microvascular endothelial lining including the sub-endothelial dense basement membrane (BM). Ultimately, migration through the ECM in the sub-endothelial compartment needs to successfully take place to finally make close contact with the malignant cells [1, 2]. A multitude of assays have been described to study lymphocyte migration in vitro in three-dimensional (3D) matrices [3–10].

The migration in 3D matrices is described as amoeboid, i.e., a migration mode characterized by a rounded or ellipsoid cell shape with unstable non-focalized adhesion sites and lack of stress fibers

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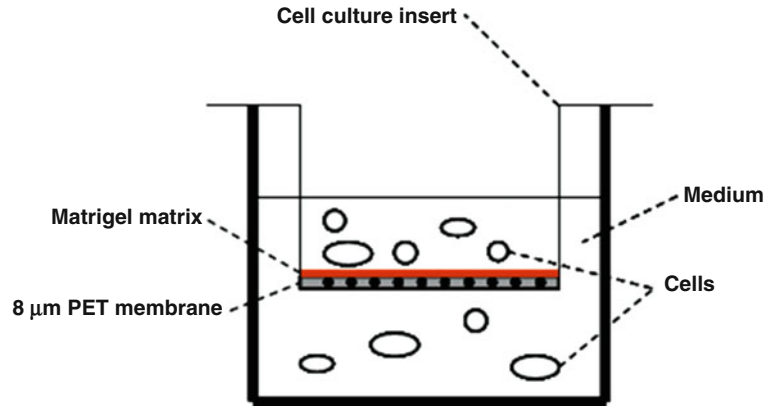

Fig. 1 Schematic drawing of a Matrigel® invasion chamber

that allows the cells to migrate and squeeze through narrow matrix gaps [11]. In rodent experimental tumor models, NK cells have been shown to deform into extremely thin formations during extravasation and intra-tumor migration, ~3 μm, where the nucleus ultimately is size limiting [12, 13]. In the basement membrane, such gaps are not preexisting and therefore the invading cells need to create them themselves, e.g., through the use of matrix-degrading proteolytic enzymes (Fig. 1).

The Boyden invasion assay we here describe uses a reconstituted model basement membrane (Matrigel®) produced by the Engelbreth-Holm-Swarm mouse sarcoma; it has a similar biochemical composition as intact BM, i.e., main structural components are laminin, collagen IV entactin, and heparan sulfate [14]. It is however important to acknowledge the presence of a variety of biological active molecules including growth factors as well as proteases [15]. This must be taken into account when planning experiments and multiple controls are often needed and careful interpretation of the results is warranted. There are structural differences of Matrigel compared to authentic BMs, having a probable lower level of cross-linking but on the other hand it is thicker (Fig. 2a), whereby the relevance of Matrigel as a model of native BM can be questioned.

Nevertheless, the model has merits as it is easy, reproducible, and allows for a fair deal of high throughput and yields quantitative data that can be used to support molecular biology and morphological findings. For example, in vitro studies of freshly isolated NK cells from human, mice, and rats have shown that their migration through Matrigel depends in part on MMPs (Fig. 3) [16–20]. Stimulation by cross-linking of activating receptors has also been reported [21], as well as the importance of cytokines such as IL-2 and IL-18; these invasion data were supported by molecular biology and biochemistry data, e.g., zymography and Western blotting [20, 22].

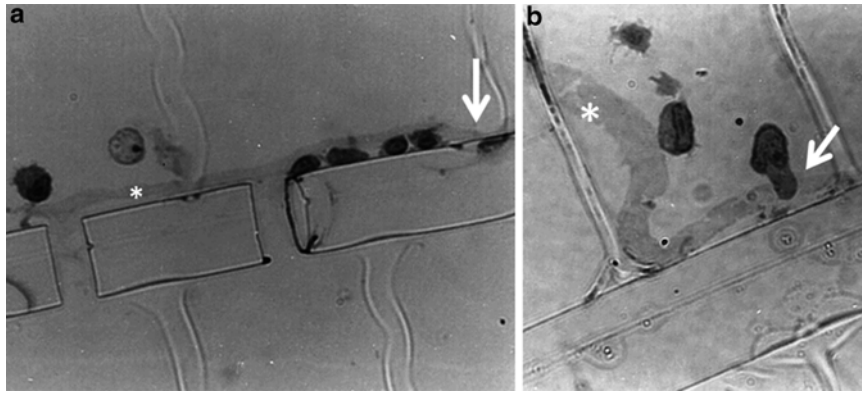

Fig. 2 (**a**) Light microscopy micrograph of freshly isolated human NK cells that have penetrated the Matrigel matrix (visible as a shaded membrane, *asterisks*). The four cells can be seen migrating underneath the Matrigel. A possible point of entrance is marked with *arrow*. (**b**) One possible reason for the sometimes observed large variation in results between identical treated wells is noted: here the intact membrane has detached from the well (*asterisk*) and in this case it could also be a post fixation artifact. One cell is seen on top of the membrane in the process of matrix degradation (*arrow*). Original magnification ×125

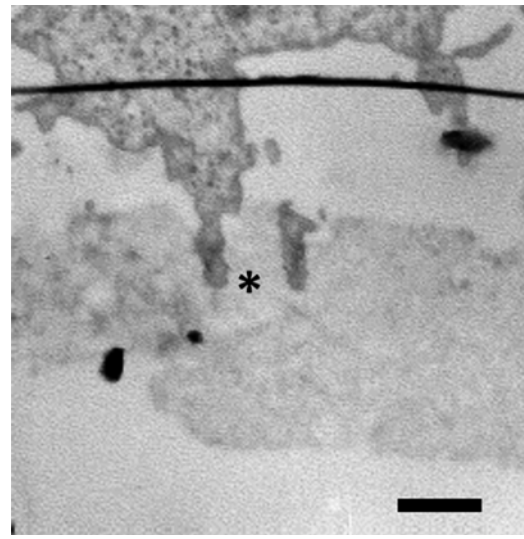

Fig. 3 An electron micrograph depicting a freshly isolated human NK cell in the process of matrix degradation by inserting cell protrusions into the matrix (*asterisk*). We believe that it is likely that matrix-degrading enzymes are used attached or connected to the NK cell surface. Note the approximately 3 μm thick Matrigel® coating. Bar = 1 μm

2 Materials

All cell culture procedures should be performed under sterile conditions and the cells to be propagated at 37 °C with 5 % CO_2 in humidified air. Further, all medium and buffers should be filtered through 0.22 μm pore size membranes before use. It is also critical

to ensure that all cell lines employed are regularly tested for mycoplasma contamination (*see* **Note 1**).

2.1 Cells

1. NK 92 [23].
2. YT [24].
3. Freshly isolated human NK cells (*see* **Note 2**).

2.2 Assay Plates

1. Matrigel Invasion Chambers with 8 μm pore size PET membranes, for 24-well culture plates, from BD Biosciences (*see* **Note 3**).
2. Control invasion chambers with same pore size but without coating.
3. Culture ware for propagating cell line.
4. 96 well plate.

2.3 Reagents

1. Complete Medium (for Freshly isolated Natural killer cells and NK-92 cells): X-Vivo™ supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 5% human fresh frozen AB-plasma (heat inactivated at 60 °C for 45 min), and 100 U/ml IL-2. Media for YT cells: RPMI-1640 supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% Fetal bovine serum (FBS).
2. Recombinant human IL-2 (Proleukin®, Novartis), diluted in RPMI-1640 to obtain a stock solution of 100,000 U/ml.
3. Opti-MEM®: A serum-free medium from GIBCO, with added 2 mM L-glutamine and antibiotics.
4. PBS, Buffer.
5. alamarBlue® (from Serotec) or similar tetrazolium salt (e.g., MTT, XTT) to measure cell number.

2.4 Instruments

1. Forceps, to sterile handle the inserts.
2. Micropipettes.
3. Centrifuge tubes for serial dilution.
4. Cell culture incubator at 37 °C with 5% CO_2.
5. Cell culture laminar flow hood.
6. Fluorescence plate reader or spectrophotometer.

3 Methods

3.1 Matrigel Invasion Assay

1. Culture NK-92 suspension cells in its complete medium and propagate to maximum density of 0.8×10^6/ml with a 1:2 split of saturated culture every 2 days.

2. Culture YT suspension cells in complete medium, propagate ~$0.1–0.2 \times 10^6$/ml with split 1:3–5 every third day. Will grow up to maximum density of approx. $0.6–0.8 \times 10^6$/ml.
3. Primary NK cells should be relaxed after separation in its complete medium including 5 % human fresh frozen AB-plasma for 1–2 h prior to start of assay (*see* **Note 4**).
4. Make sure that enough cells (~10×10^6) are ready for harvesting. Wash cells twice with PBS. Seed a flask or a well of cells that allow harvesting of >0.2×10^5 cells (*see* **Note 5**) to run a standard curve at end of assay 24–48 h later.
5. Hydrate commercial Matrigel-invasion chambers with 0.5 ml of Opti-MEM® for 2 h at 37 °C (*see* **Note 6**).
6. Use at least triplicate well for each stimulation (e.g.,, leukokines like IL-18, IL-2 [20, 22], or cross-linking of activating receptor [21]) or inhibition (e.g., protease inhibitors like the MMP inhibitors: batimastat or GM6001 [17, 18, 20]) concentration studied. Treat vehicle control wells identically (*see* **Note 7**). Also use triplicate control wells with no Matrigel coating for each condition (*see* **Note 8**).
7. Add to top well 500,000 (*see* **Note 9**) NK cells (*see* **Note 10**) in 0.3 ml serum-free Opti-MEM®, 2 mM L-glutamine. Antibiotics can be used in this media.
8. Add 1.5 ml Opti-MEM® medium to the bottom well (*see* **Note 11**). Add the investigated compound, e.g., matrix metallo proteinase inhibitors (10 μM batimastat or GM6001) at the same concentration to both bottom and top wells.
9. If migration of NK cells in response to a stimuli or chemoattractant is the objective for the study, such chemoattractant are sometimes added only in the lower well (*see* **Note 12**).
10. Incubate the plates at 37 °C for 48 h (*see* **Note 13**).
11. At the end of the incubation time, aspirate and discard medium in top wells; remove any remaining cells on the upper surface and in the remaining membrane by carefully cleaning with cotton swabs.
12. Remove any invading cells still attached to the lower side of the membrane by carefully flushing with a pipette using the bottom well medium and return to the bottom well.
13. Label migrating cells present in the bottom chambers using 10 % alamarBlue®.
14. Incubate at 37 °C for 4 h (*see* **Note 14**).

3.2 Detection of Transmigrated Cells

1. Prepare standard curve for determining the number of migrating NK cells. Each cell line used in migration assay with and without any specific stimulation needs its own standard curve (*see* **Note 15**).

2. Harvest cells seeded at start of assay (in Subheading 3.1, **step 4**), wash once, and determine the cell counts.
3. Resuspend NK cells at 1×10^6 cells/ml and perform a twofold serial dilution nine times using Opti-MEM®.
4. Prepare triplicates of 100 µl in a 96 well plate of each cell concentration. This will give you a standard curve ranging from 100,000 cells down to 195 cells. Use pure Opti-MEM® for zero cell control.
5. Add 11 µl of alamarBlue® to each well (to 10% final concentration).
6. Incubate at 37 °C for 4 h (*see* **Note 14**).
7. Read in a microplate fluorescence reader using an excitation wavelength 560 nm and an emission wavelength of 590 nm (*see* **Note 16**).
8. Plot a standard curve using the obtained fluorescence intensities and infer the number of invading NK cells from this standard curve.
9. Express data as number of invaded cells or as percent invasion (*see* **Note 17**).

4 Notes

1. Cell cultures should be screened for mycoplasma contamination preferably using a PCR technique. Intracellular mycoplasma can alter the expression of a large array of genes and thereby bias results. Use only the cell culture that tests negative for mycoplasma.
2. Freshly isolated human NK cells can be separated from venipuncture blood 10–20 ml. However, to get enough cells for multiple testing, one should use buffy coats obtained from a blood bank at a nearby hospital. A Ficoll/Hypaque centrifugation followed by an immunomagnetic purification using the MACS NK cell separation kit from Miltenyi Biotec is a stable separation method. Importantly, a thorough control of purity should be performed and >90% $CD56^+$ and <1% $CD3^+$ cells is a satisfying result.
3. We have used commercial precoated inserts from BD Biosciences. If self-coated plain PET Transwells® are used, one may expect a higher degree of variability between individual wells due to different thickness of coating or incomplete coating that may occur. The integrity of the coating can be checked by measuring an increased electrical resistance across the membrane (using, e.g., Millicell® ERS-2 Voltohmmeter) compared to background resistance measured across uncoated filter.

Self-coating gives the possibility to address different concentrations of the protein membrane and also to use custom-made mixtures of matrix proteins.

4. We have normally done the assay using fresh cells but primary NK cells cultured properly either using cytokine stimulation or on expansion platforms using feeder cells can likely be used in this assay. Freshly isolated NK cells will fail to thrive without cytokine stimulation we have used 10 U/ml of IL-2 for the full duration of the assay to prevent excessive cell loss. This concentration does not activate the cells, i.e., does not turn on interferon-γ production.
5. Each standard curve needs 200,000 cells. Seed approx. 300,000–400,000 cells in 1.5 ml assay-medium including stimulant/inhibitor and keep in assay condition.
6. We regularly use the 24 well format of invasion chambers as a good trade-off, permitting some central microscopy control of the inserted well (cell culture area 0.3 cm^2) and the possibility to run a fair amount of samples. Larger size wells with corresponding inserts (6 or 12 well plates) will permit more detailed microscopy. For instance, one may record for multiple fields of view for blinded morphometric analysis. This will however be less practical if many variations need to be tested. Smaller well size is also available (48 and 96 well plates).
7. Always use vehicle control (having the same concentration and volume). This is especially important when strong solvents, e.g., DMSO, that are needed to keep the compound under investigation in solution. For example, hydroxamate MMP inhibitors may crystallize which can be detected using a light microscopy.
8. It is not unusual to get high standard deviation due to an outlier well (*see* Fig. 2b). We therefore recommend to running triplicate wells, as a good balance to economy and to allow for the ability to handle several different concentrations of the stimuli or inhibitor. It is further important to repeat experiments on three or more different cell batches to make sure that the obtained results are stable/reproducible.
9. The optimal number of cells to add depends on the intrinsic migratory capacity of the used cells. However, one must take into account the sought outcome, i.e., stimulation or inhibition, so that a good resolution can be obtained. We therefore recommend that titration experiments are performed where the amount of added cells are varied. For inhibition studies, a high percentage of invading cells in the control is preferred, at the very least 20–30%. If too low, a small effect of the inhibitor might be obscured due to the often quite high standard deviation obtained (*see* **Notes 3** and **8**). Conversely, for simulation

studies, a too high percentage of invading cells should be avoided and a control invasion above 50% should be avoided. *See* also **Note 13** regarding titration of assay conditions.

10. We have noted a fairly good invasive capacity using freshly isolated human NK cells. This however poses the problem that each time NK cells from a new donor are assessed. We therefore regularly used the human immature NK cell line YT, which is interleukin-2 (IL-2) independent and of very low invasive capacity [24] and the more invasive human immature IL-2-dependent NK-92 cell line [23].
11. One needs to ensure that no air is trapped underneath the insert well, as this will effectively inhibit any invasion. Cautions should be taken to avoid putting down the insert in a prefilled well. The bottom well is best filled when the insert is in place as this will minimize the risk of trapping air.
12. Serum or a specific chemoattractant can be added to the lower well. It is possible to calculate the stability of such a gradient, knowing the volumes of the two compartments and the exchange surface (i.e., the area of all the pores) and the concentration of the compound in the lower well. Rarely, however, this gradient can be expected to be present for at most some 8 h when using pore size 8 μm and 24 well size. Therefore, if chemoattraction is to be studied, one should use dedicated assays for this.
13. Also, the time of the assay has to be titrated to allow for an optimal resolution, depending on the addressed endpoint (stimulation or inhibition) (*see* **Note 9**). It is not recommended that assay is run for more than approximately 48 h as thereafter the nutrients may need to be supplemented. This will dramatically increase well-to-well variability and also proliferation may occur further complicating the interpretation.
14. The time of incubation with alamarBlue® can be varied. It is our experience that increasing time will only increase the background. For human NK cells, we have observed that a period of 4 h appears to be optimal.
15. All stimulants or inhibitors need to be assayed for possible cytotoxic effects, using at the very least Trypan blue dye exclusion. It is nonetheless preferably to also use a metabolic assay, e.g., alamarBlue® as mentioned herein or the MTT/XTT assay or similar tetrazolium salt-based proliferation/viability assay.
16. We use alamarBlue® from Serotec as it can be read using either fluorescence or spectrophotometry (i.e., absorbance). We prefer the fluorescence method as in our hands it gives slightly more stable results and has a better resolution at low cell numbers.

17. The issue of how to report the number of invading cells may be debated. We prefer to present the results as the number of invading cells through Matrigel coated inserts as percentage of the number of migrating cells in simultaneously run uncoated control wells (Fig. 4c). This will then to some extent minimize the possible (albeit likely low) impact of gravitation, and also hint toward a distinction of migratory vs. invasive (matrix degradation) capacity. It is noteworthy that these issues need to be clarified in separate and different experimental conditions, e.g., migratory cell assays [9] using migration under agarose and on matrix compounds in 2D.

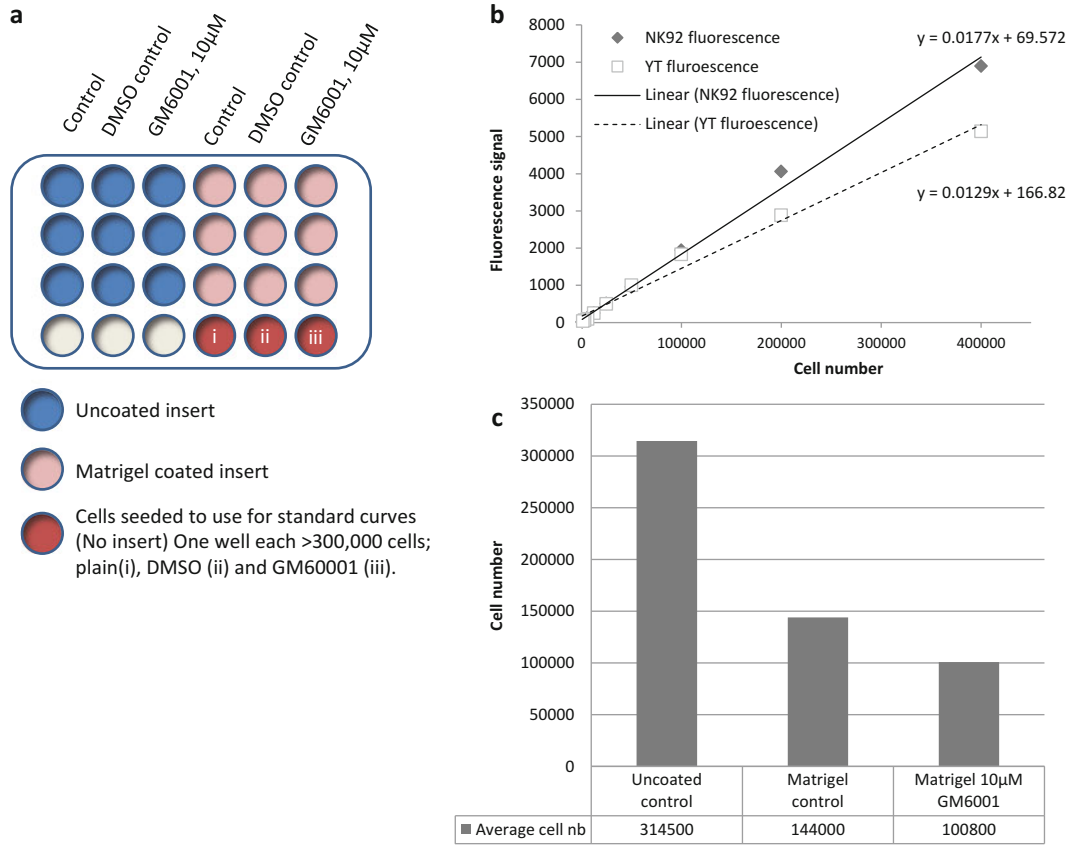

Fig. 4 (**a**) An example of an experimental setup including vehicle control. (**b**) Standard curves for NK-92 and YT cells. (**c**) Hypothetical results from an inhibitory experiment, 500,000 cells added in 48 h assay. In plain inserts, 314,500 cells could be recovered in lower chamber. In Matrigel-covered inserts, 144,000 cells could be recovered. Thus, ~63% migratory cells (314,500/500,000) and the invasion is ~46% (144,000/314,000), i.e., Matrigel transmigration. The MMP inhibition by 10 µM GM6001 is ~30% as 100,800 cells were recovered (1 – (100,800/144,000))

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Chapter 7

Microfluidic-Based Live-Cell Analysis of NK Cell Migration In Vitro

Saravanan Nandagopal, Francis Lin, and Sam K.P. Kung

Abstract

Effector functions and cellular properties of natural killer (NK) cells are regulated by cellular and extracellular factors shaped by the microenvironments. NK cells express specific chemokine and non-chemokine receptors to aid preferential migrations or localizations in tissues. Good understanding of how NK-cell migratory properties are regulated in physiological and pathological microenvironments will provide further insights into the development of NK cell-based therapeutic approaches. In contrast to the commonly used conventional in vitro migration assays such as Trans-well assays that measure movements of a population of the migratory cells, microfluidic-based devices support live-cell imaging of cell migrations under a well-defined chemical gradient(s) at microscale. Subsequent analyses at single-cell level provide quantitative measurements of cell-migration parameters such as speed and Chemotactic Index, and permit distinguishing chemotaxis, chemokinesis, and chemo-repulsion. Our recent work established the use of a Y-shaped microfluidic device to study NK cell migrations in vitro. In this chapter, we described the detailed method of acquiring and analyzing NK cell migration in the microfluidic devices.

Key words Natural killer cell, Bone marrow-derived dendritic cells, Microfluidic device, Chemotaxis, Chemo-repulsion, NK-DC crosstalk

1 Introduction

Cell migration is an important biological function for both unicellular and multicellular organisms [1]. In our immune system, tight regulation of immune cell(s) migration is essential for cell development/differentiation, inflammation, and immunosurveillance [2, 3]. Natural killer (NK) cells are motile bone marrow-derived lymphocytes that are capable of killing "non-self" cells, and/or producing cytokines/chemokines that can profoundly shape the quality and magnitude of adaptive immune responses [4–6]. Regulation of NK cell migration under physiological and pathological microenvironments is complex, and involves expression of specific chemokine and non-chemokine receptors [7–9]. Understanding NK-cell migratory properties in inflamed peripheral

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tissues or tumor sites will provide new insights into the development of NK cell-based therapeutic approaches.

Conventional Trans-well assay has been one of the most commonly used in vitro migration assays in immunology research because of the ease of experimental setup and its relatively high-throughput capacity in evaluating chemoattractant of interest [10]. In contrast, microfluidic devices for cell migration studies support live-cell imaging of cell movement, configurations of stable chemical gradient to mimic complex physiological microenvironment, and quantitative analyses of cell migration at a single-cell level [11–14]. In this chapter, we described the detailed method of acquiring and analyzing NK cell migration in a Y-shaped microfluidic device we have previously established (Fig. 1) [14]. In this system, fluidic channels were coated with fibronectin to facilitate cell adhesion to the device. Chemokines or conditioned medium of interest was infused slowly into the channel through the designated inlets using a syringe pump. Cell migration was recorded by time-lapse microscopy, tracked, and analyzed to extract quantitative cell motility and directional migration parameters (Figs. 1, 2, and 3).

2 Materials and Facilities

2.1 Microfluidic Device Design and Mask Fabrication by Photo-Lithography

1. Specialized software such as Freehand or AutoCAD for device design.
2. Transparency mask with high-resolution printing of the design.
3. Isopropyl Alcohol (IPA).
4. Oxygen plasma machine (Harrick Plasma, model PDC 001).
5. 3″ Silicon wafers (Silicon Inc).
6. Negative photoresist (SU-8 2075) and developer (MicroChem).
7. Spinner and hotplate.
8. Contact mask aligner.
9. Clean room with a lithography area and fume hood.

2.2 Polydimethylsiloxane (PDMS) Device Fabrication by Soft Lithography

1. Polydimethylsiloxane (PDMS) and curing agent (Sylgard 184) (Dow Corning).
2. Digital weighing scale.
3. Tri beaker.
4. Plastic knife and syringes.
5. Desiccator with vacuum setup.
6. Baking oven.
7. Distilled (DI) water.
8. Clean bench.

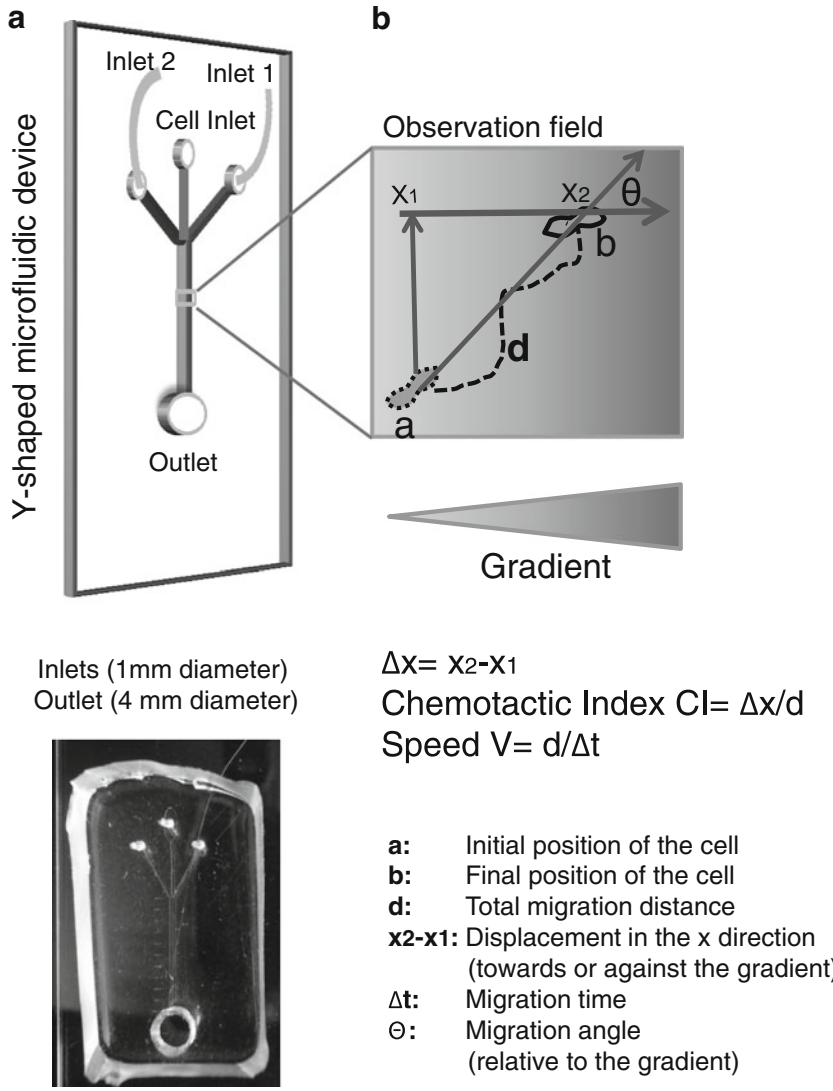

Fig. 1 Schematic illustration of Y-shaped microfluidic platform for cell migration analysis. (**a**) Illustration of Y-shaped microfluidic device. A real picture of the device is shown in the *bottom panel*. (**b**). Illustration of quantitative cell migration data analysis

9. Oxygen plasma machine (Harrick Plasma, model PDC 001).
10. Hole puncher.
11. Glass cover slides (75 mm × 25 mm).
12. Dust-off or Nitrogen gas spray gun.
13. Silanizing agent solution (tridecafluoro-1,1,2,2-tetrahydrooctyl trichlorosilane).

2.3 Fibronectin Coating of the Microfluidic Device

1. Fibronectin (BD Biosciences) is reconstituted in distilled water to a final concentration of 1 mg/mL.
2. Phosphate-buffered saline (PBS), pH 7.4.

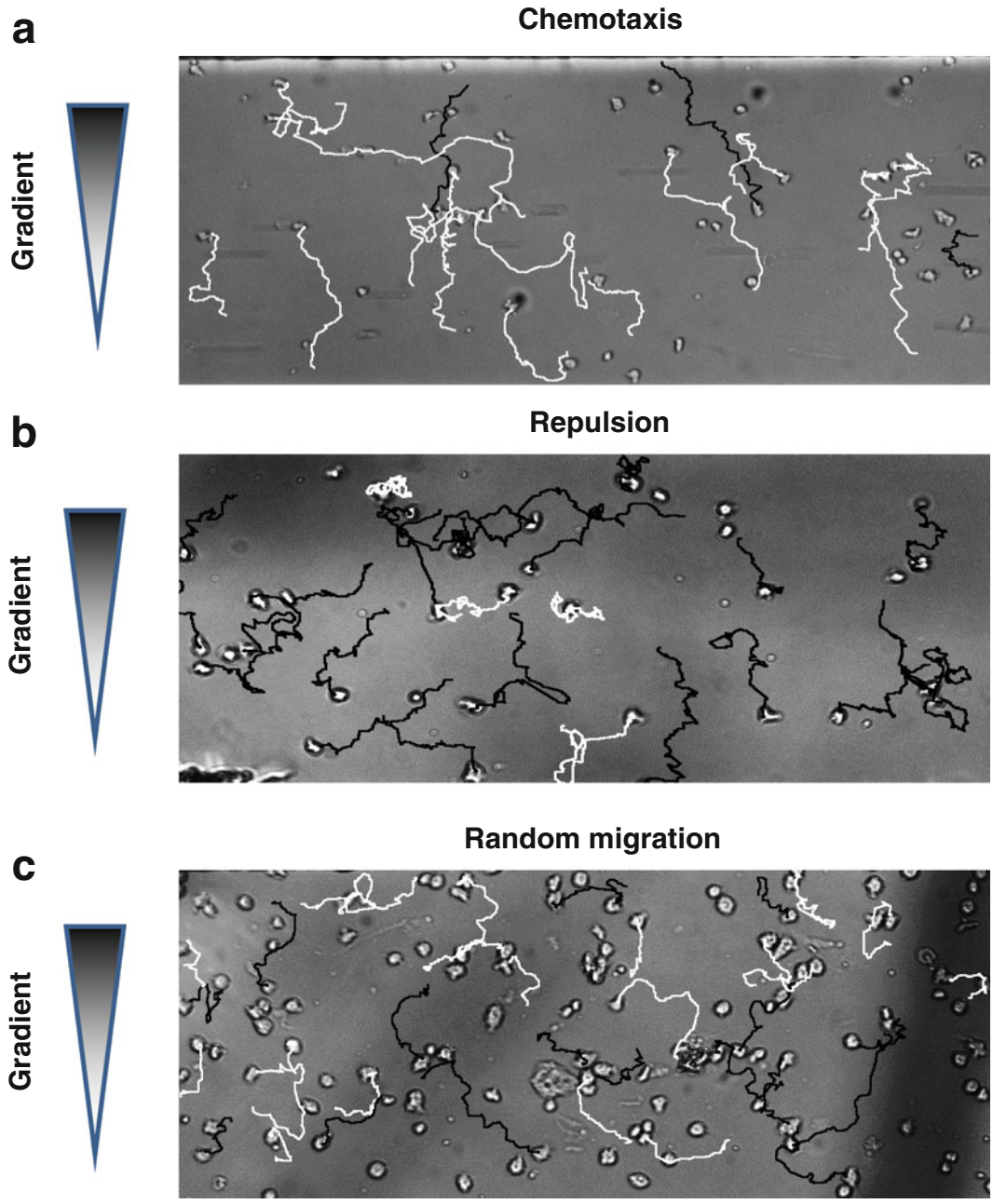

Fig. 2 Examples of NK cell migration images with overlapped cell tracks. (**a**) Toward the gradient. (**b**) Away from the gradient, or (**c**) in a random mode. *White tracks* for cells migrated toward the gradient; *black tracks* are cells migrated away from the gradient

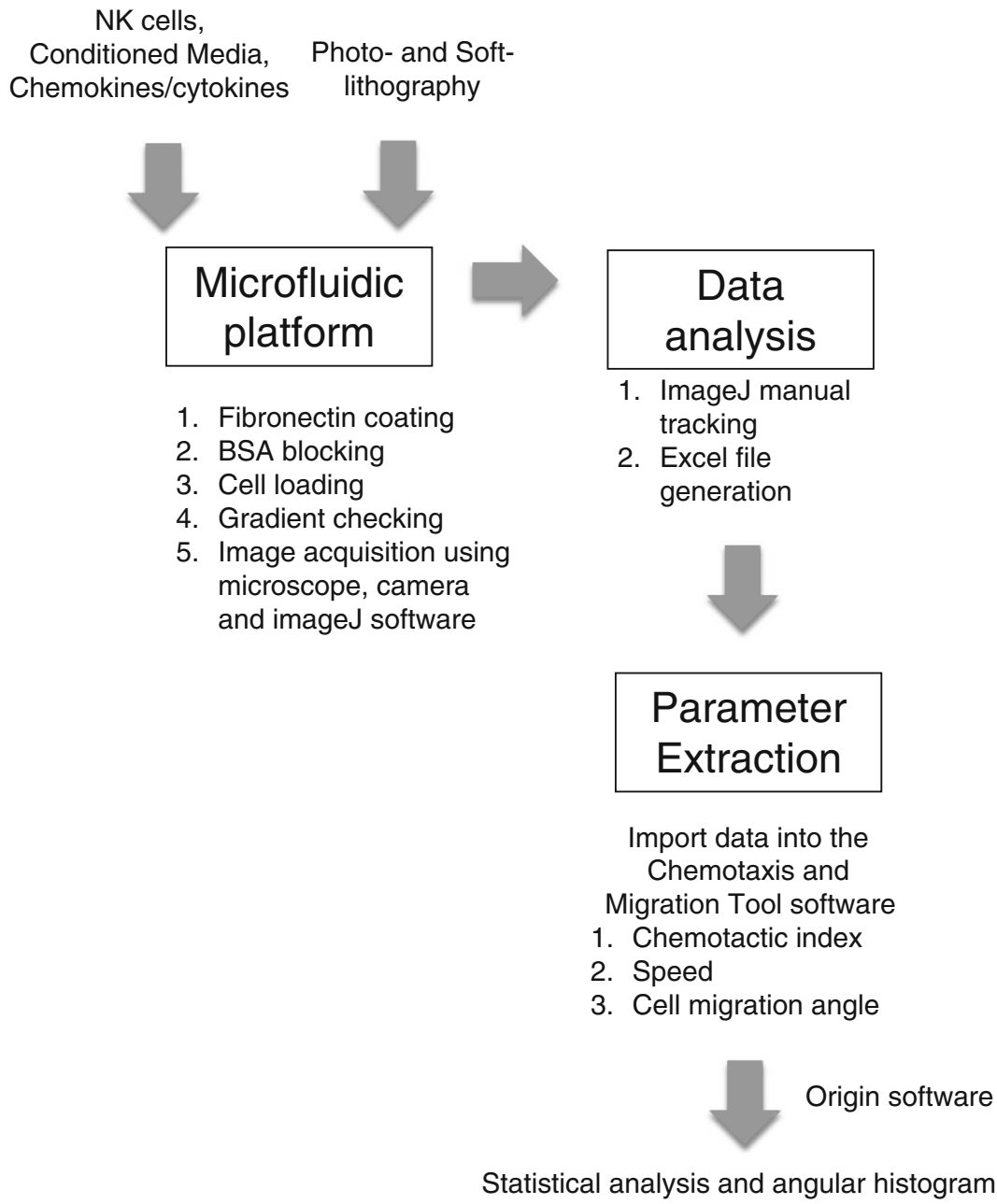

Fig. 3 A flow chart outlining key procedures in microfluidic data acquisition and analyses. In this Y-shaped microfluidic system, fluidic channels were coated with fibronectin to facilitate NK-cell adhesion to the device. Chemokines or conditioned medium of interest was injected slowly into one or both of the channels through the designated inlets using a syringe pump. Cell migration was recorded by time-lapse microscopy, tracked manually in ImageJ software, and further analyzed for quantitative cell migration parameters using the Chemotaxis and Migration Tool software and Origin software

3. Roswell Park Memorial Institute (RPMI) 1640 culture medium (HyClone), supplemented with 0.4% BSA (Sigma).
4. 5-mL syringes.

2.4 Microscopy and Image Acquisitions

1. Thermal-Clear Heater (H15227, Minco).
2. Fluorescence microscope (BX60, Olympus) with a CCD camera (Optikon) for capturing time-lapse images.
3. Power supply.
4. Sensorless temperature controller (CT198, Minco).
5. Digital thermometer.
6. Tri-valve (Ozone lab).
7. Polyethylene tubes (PE-20).
8. Hamilton pump and syringes.
9. 5-mL plastic syringes.
10. 18 gauge and 27 gauge needles.
11. Fluorescein isothiocyanate (FITC)–dextran, with an average molecular weight 10,000 Da (Sigma Aldrich), was reconstituted in RPMI 1640 with 0.4% BSA to a final stock concentration of 20 μg/mL.
12. NIH ImageJ software.

2.5 Data Analyses

1. NIH ImageJ software.
2. Chemotaxis and Migration Tool (ibidi).
3. Origin 8.5 (OriginLab).

2.6 Natural Killer Cells Purification, Activation, and Expansion In Vitro

1. RPMI 1640 culture medium, supplemented with 10% FBS (HyClone), 1% penicillin/streptomycin/glutamate (PSG, Life Technologies), and 1.6 mM 2-Mercaptoethanol (Sigma).
2. EasySep Mouse NK Negative Selection Kit (StemCell Technologies).
3. IL-2 (Peprotech) is dissolved in autoclaved distilled water at 0.1 mg/mL, further diluted with RPMI 1640 culture medium to obtain a stock solution of 100 U/mL. The cytokine is stored in aliquots at −70 °C until uses.
4. NK Cell migration media: Prepare migration media by supplementing 0.4% BSA in RPMI 1640 at 1:9 v/v.

2.7 Culture of Bone Marrow-Derived DC In Vitro

1. RPMI 1640 culture medium, supplemented with 10% FBS, 1% penicillin/streptomycin/L-Glutamine, and 1.6 mM 2-Mercaptoethanol, and 20 ng/mL GM-CSF.
2. Recombinant Granulocyte-monocyte colony stimulatory factor (GM-CSF) (Peprotech) is dissolved in autoclaved distilled water at 0.1 mg/mL, further diluted with RPMI 1640 culture

medium to obtain a stock solution of 1 μg/mL. The cytokine is stored in aliquots at −70 °C until uses.

3. Lipopolysaccharide LPS (Sigma), used in activating immature DC to maturation.

3 Methods

3.1 Microfluidic Device Design

1. Design the Y-shaped microfluidic device as described in detail previously [14] (*see* **Note 1**). The dimensions for the main microfluidic gradient channel are 100 μm × 350 μm × 10 mm (depth, width, and length, respectively).
2. Send out the design to an external service for printing the design onto a transparency film with at least 2500 dpi resolution as the optical mask.
3. Use the printed transparency mask for fabrication of the microfluidics device mold by photolithography (*see* **Note 2**).

3.2 Fabrication: Photolithography and Soft Lithography (See Note 3)

1. Clean silicon wafers by Oxygen plasma for 30 min.
2. Place the cleaned wafer to the spinner. Carefully add about 3 mL of SU-8 2075 at the centre of the wafer. Spin the wafer at 500 rpm for 5 s, followed by a 2000 rpm spinning for another 30 s.
3. Bake the coated wafer using a hotplate at 65 °C for 5 min, followed by 95 °C for 10 min.
4. Expose the wafer to UV light through the transparency mask using the contact mask aligner. The UV exposure time in our process is 20 s.
5. Bake the UV-exposed wafer using a hot-plate at 65 °C for 5 min, followed by 95 °C for 10 min.
6. Develop the wafer by immersing the wafer in the SU-8 developer in a glass pan and gently shake for 10 min.
7. Wash the developed wafer with fresh developer solution followed by a second spray/wash with IPA. Dry the wafer with Nitrogen gas.
8. Place the developed wafer in a petri dish.
9. Silanization: This step is to assist release of cured PDMS from the SU-8 patterns during soft lithography. Take a few microliters of silanizing agent solution in a micropipette tip and place the tip in an empty 15-mL conical tube with loosened cap. Place the conical tube with the SU-8 patterned wafers (the patterns face up) in a desiccator and apply vacuum for 1 h.
10. Mix PDMS base and curing agent at 10:1 ratio (weigh using the digital scale) using a plastic knife in a tri-beaker for ~10 min.

Approximately 20 mL of mixed PDMS is required for each wafer (*see* **Note 4**). Degas for ~15 min using a vacuum desiccators. Use dust off to remove any remaining bubbles.

11. Pour the mixed PDMS to the SU-8 mold in a petri dish (100 mm in diameter; 15 mm in height). Use dust off to remove any remaining bubbles.
12. Cure the PDMS in an oven at 80 °C for 3 h.
13. After baking, remove the petri dish from oven and allow it to cool to room temperature. Cut and peel off the PDMS replica from the SU-8 mold.
14. Punch three inlets (approximately 1 mm in diameter) using a blunt-punching needle and the outlet (4 mm in diameter) using hallow cylindrical puncher.
15. Plasma bond the PDMS replica to a glass cover slide to complete the microfluidic device assembly (Fig. 1) (*see* **Note 5**).
16. Infuse DI water into the microfluidic channels to keep the channels hydrophilic.
17. Store the device in fridge at 4 °C until use.

3.3 Fibronectin Coating and Blocking of Devices

1. Dilute 50 μL of stock fibronectin solution to 1.5 mL of 1× PBS.
2. Use a 5-mL syringe to fill the microfluidic channels with diluted fibronectin through one of the inlets.
3. Leave the device at room temperature for 1 h.
4. After the fibronectin coating, block the channel with the migration medium (0.4% BSA in RPMI) for 1 h at room temperature.
5. The devices are ready for cell migration experiment.

3.4 Culture of Activated NK Cells

1. Isolate primary NK cell preparation that is free of T and NKT cells contamination using splenocytes and mouse NK negative selection kit (StemCell Technologies) (*see* **Note 6**).
2. Culture and activate purified NK cells (LAK cells) in IL-2 (1000 U/mL) for 4 days. Harvest the NK cells on day 4 of migration studies [15].

3.5 Culture of Bone Marrow-Derived DC In Vitro

1. Extract the mouse bone marrow cells from the femur and tibiae.
2. Obtain single-cell suspension. Place the bone marrow cells at 1×10^6 cells/mL in RPMI 1640 tissue medium (0.5 mL of cell suspension per well, using a 24-well plate).
3. Replace the "old" culture medium with 500 μL fresh GM-CSF-containing medium on day 2.
4. Immature DC will be developed in vitro on Day 7. If mature DC is needed, add LPS (at 1 μg/mL final concentration) to

the cells on Day 7. Mature DC will be obtained on Day 8. Collect the immature and mature DC for phenotyping (using CD11c, CD40, CD80, CD86 markers) [16]. Collect and use cell-free conditioned media from these immature and LPS-mature DC cultures in the migration studies [15]. Commercially available chemokines can be used to create single or opposing gradients in place of DC conditioned media depending on research question.

3.6 Migration Studies Using the Microfluidic-Based Setup

1. Add 30 μL of stock FITC-Dextran solution to 2 mL of migration medium in a 15-mL tube. This is used to verify gradient generation in the microfluidic device. Make 2 mL solution of the test samples (such as the conditioned medium from the DC cultures or recombinant chemokine/cytokine) in a separate 15-mL tube (without FITC-Dextran).
2. Load the solutions to a 5-mL syringe and connect the syringe to a three-way valve. A Hamilton syringe and a 27 G needle are fit to the other two ports of the three-way valve. Install the syringe/valve assembly to a syringe pump. Connect one end of the PE-20 tubing to the needle in the syringe/valve assembly (fill the tubing with solutions) and connect the other end of the tubing to the inlets of the microfluidic device for fluid delivery (pump at high flow rate while connecting the tubing to the device) (*see* **Note 7**). Use a new microfluidic device for each experiment.
3. Place the microfluidic device on a Thermal-Clear Heater. Power the heater by a DC power supply and control the temperature using a temperature controller. Calibrate the temperature of the heater to 37 °C using a digital thermometer.
4. Infuse solutions continuously at a low rate (0.2 μL/min). Verify the gradient formation by measuring the fluorescent intensity of FITC-Dextran in the microfluidic channel under the microscope (*see* **Note 8**).
5. Stop the solution infusion during cell loading. Load the NK cells (typically, $0.1–0.4 \times 10^6$ NK cells in 20 μL of migration medium) into the fibronectin-coated microfluidic device from the cell loading inlet. Allow NK cells to settle in the gradient channel for 5 min until no more flowing NK cells are seen in the channel (*see* **Note 9**).
6. Resume the infusion of the migration medium and test sample (cytokine/chemokine or conditioned medium from DC) at a low flow rate (0.2 μL/min) to maintain the gradient. Check again the gradient formation under the microscope.
7. Record time-lapse cell migration images in the observation field, typically ~3 mm downstream of the "Y" junction where the gradient yields a smooth profile (we usually use a 10× objective).

3.7 Images Acquisitions and Data Analyses

1. Capture time-lapse images at 6 frames/min for about 40 min. We controlled the image acquisition using NIH ImageJ (v.1.34s).
2. Analyze NK-cell migratory properties quantitatively using NIH ImageJ software in the following steps. (1) Eliminate background noise using the "despeckle" function in the ImageJ software. (2) Calibrate the distance in the image (in our case, 1 pixel is equal to 0.57 μm) (*see* **Note 10**). Track only those cells that had migrated at least one-cell length (~10 μm) within the microscope image using the Manual tracking function in ImageJ.
3. Export the tracking data in an excel file to the Chemotaxis and Migration tool (ibidi) for further analysis.
4. Calculate Chemotactic Index (C.I.) average speed and migration angle of each cell using the Chemotaxis and Migration Tool (Fig. 1).
   (a) Chemotactic Index (C.I.) is calculated as the ratio of the displacement of a cell toward the gradient (Δx) to the total migration distance (d), using the equation C.I. = $\Delta x/d$, and the results are presented as the average value ± standard error of the mean (s.e.m) of all cells.
   (b) The average speed (V) is calculated as $d/\Delta t$ for each cell and presented as the average value ± s.e.m. of all cells. Δt *is the migration time of tracking.*
   (c) Statistical analysis of cell migration angles is performed using the Chemotaxis and Migration Tool and Origin software to examine the directionality of the cell movement. Specifically, the distribution of migration angles (calculated from x–y coordinates at the beginning and the end of the cell tracks) is summarized in a rose diagram with the angles grouped in defined intervals.
   (d) The percentage of cells that migrated toward the gradient is also calculated (*see* **Note 11**).
5. Compare the parameters between different conditions by the two-sample t test using the Origin software. Data from at least three independent experiments for each condition are used for the comparison.

4 Notes

1. The Y-shaped microfluidic device has two fluidic inlets, one cell inlet and a main gradient channel. A few hundred micrometers are often used for the width of the gradient channel. Please refer to the original paper for details [14].

2. We used the Nano-Systems Fabrication Laboratory (NSFL) at The University of Manitoba for fabricating the SU-8 2075 mold.
3. For details of photolithography and soft lithography, please refer to published work [11, 12, 17].
4. 20 mL is enough for each petri dish. PDMS base and curing agent must be mixed thoroughly.
5. Plasma treatment: Place the PDMS replica (patterns face up) and a glass cover slide in the plasma chamber. Vacuum the chamber and then turn on the plasma for a few minutes' treatment. Adjust the air flow control valve to optimize the plasma intensity inside the chamber. After the treatment, bond the PDMS replica to the cover slide immediately. The plasma treatment promotes permanent bonding between PDMS and glass, and modifies the PDMS surface to hydrophilic.
6. Human NK cells can also be used in the study. We isolated NK cells from PBMC using the human NK isolation kit (StemCell Technologies) and have successfully used the IL-2 activated human NK cells in the migration studies.
7. Air bubble intervention—Inlets are always covered with migration medium to prevent bubbles from entering the channel. Tubing is checked for air bubble before infusing solutions into the microfluidic device.
8. Detailed principle of microfluidic gradient generation has been described previously [11, 13, 14]. The Y-shaped microfluidic device can generate single chemical gradients or overlapping fields of multiple chemicals [18].
9. Cell attachment: Controlling the flow during cell loading is critical for cell attachment. This can be done by adjusting the outlet solution level.
10. Distance calibration of time-lapse images: The pixel value of the time-lapse cell migration images (we used a 10× objective in this study) is calibrated to distance using a calibration slide and the ImageJ software.
11. We typically observe that when more cells migrate toward the gradient (>65%), the average C.I. of all cells is >+0.1; similarly, when more cells (>65%) migrate away from the gradient, the average C.I. of all cells is <−0.1. This helps us distinguish chemotaxis, chemo-repulsion, and random migration.

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Chapter 8

Microwell-Based Live Cell Imaging of NK Cell Dynamics to Assess Heterogeneity in Motility and Cytotoxic Response

Bruno Vanherberghen, Thomas Frisk, Elin Forslund, Per E. Olofsson, Karolin Guldevall, and Björn Önfelt

Abstract

NK cell heterogeneity has primarily been studied either on the population level, measuring average responses, or on the single cell level by flow cytometry, providing static snapshots. These approaches have certain drawbacks, not enabling dynamic observations of single cells over extended periods of time. One of the primary limitations of single cell imaging has been throughput; it has been challenging to collect data for many cells due to their dynamic nature and migrating out of the field of view. Spatially confining cells combined with automated fluorescence microscopy enables the simultaneous monitoring of many NK cells in parallel for extended periods of time (>12 h). Such an approach allows us to dissect how the sum of individual NK cell responses translates to the global average response typically observed.

Key words Natural killer cells, Microchip, Single cell imaging, Fluorescence, Cell migration, Cell motility, Cytotoxicity

1 Introduction

One of the defining features of the immune system is its vast heterogeneity and consequent ability to respond to an almost immeasurable number of threats. Dissecting the mechanisms behind this heterogeneity has, and continues to be, a major focus of immunological research. For immune effector cells, such as T and B cells, it is clear that subpopulations of cells are responsible for immune reactivity. For example, very small numbers of T and B cells, activated by recognition of a foreign peptide presented in the context of MHC, clonally expand for several days to reach a critical mass of highly effective, specialized effector cells. NK cells have also been reported to clonally expand in mice [1] and it is becoming increasingly clear that different subpopulations of NK cells are activated in response to different threats [2, 3].

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Most conventional methods are not suited to measure the efficacy and potency of individual cells, instead measuring the average responses of all cells. One exception is flow cytometry which does permit multiparametric monitoring of single cells. However, flow cytometry is unable to provide dynamic information of single cell responses over time. Microscopy has classically been used to record single cell events but has been hampered by throughput. It has been difficult and time consuming to record sufficient events of highly dynamic, motile cells (i.e., NK cells) in a single experiment to allow an accurate overview of how individual cellular responses compile into the averages traditionally measured. To address this lack in experimental platform, we and others have developed microchip-based approaches permitting spatial confinement of NK and target cells within microwells to enable screening with high-throughput over extended periods of time [4–8]. Here, we describe one of our methods [7, 9, 10] in terms of how to manufacture microchips and a suitable holder. These first steps are not easily performed in a standard biology lab, but microchips and holder could be made to order from companies or received through collaboration. Furthermore, we describe in detail how to load the microchip with fluorescently labeled NK and target cells, setup the microscope, and acquire long-term time-lapse images. Finally, we provide guidelines and pointers into quantification of NK cell responses based on these data.

2 Materials

2.1 Fabrication of Microwell Chips

1. Silicon wafer with low levels of dopant (n- or p-type, resistivity <20 Ω cm), 100 mm diameter, 300 μm thickness, double-sided polished (WRS Materials).
2. Borofloat glass wafers with low alkali content (<10 μg/g Na_2O equivalent), 100 mm diameter, 175 μm thickness, double-sided polished to enable anodic bonding to single crystal silicon wafer (PI-KEM Limited).
3. Properly designed 5-in. photo mask (*see* **Note 1**), chromium on glass.
4. Photo lithography process line including photoresist (e.g., AZ 9260, MicroChemicals GmbH), deposition spinner and mask aligner for UV exposure (e.g., MicroTec MA/BA6, SÜSS MicroTec Lithography GmbH), and developer fluid (e.g., CD26, MicroChemicals GmbH) and developer bath.
5. Silicon dioxide (SiO_2) etcher (e.g., Precision 5000 Mark II, Applied Materials), preferably allowing dry ion plasma etching (compressed CHF_3—15 sccm, compressed CF_4—5 sccm, and compressed Ar—50 sccm, at 350 W and 150 mTorr).

6. Silicon (Si) etcher (e.g., STS ICP, Surface Technology Systems) preferably allowing dry Bosch process of cycling etching and sidewall passivation protection in order to get high aspect ratio wells and walls (SF_6 80 sccm, C_4F_8 70 sccm, O_2 5 sccm at coil power 12 W and platen power 900 W at 90 mTorr).
7. "IMEC" clean: Constituting H_2O:Isopropylalcohol:hydrofluoric acid at a ratio (v/v) of 20:1:1.
8. "7-Up" (aka "Piranha-dip"): 3 H_2SO_4 and 1: H_2O_2 (v/v).
9. Rinsing bath bubbler: deionized water (DI H_2O) with nitrogen (N_2) flow.
10. Oxygen plasma tube (power 1000 W, O_2 flow—100 sccm) for removal of photo resist polymer.
11. Concentrated hydrofluoric acid (HF).
12. Substrate bonder (e.g., SB6, Süss Microtec AG) setup for anodic bonding, enabling substrate heating to 450 °C, voltage of –600 V and current below 50 mA.
13. Substrate dicer (e.g., DFD640, Disco) suitable for silicon-glass dicing, 30 krpm, 0.3 mm/min feed rate, accommodating 110 mm substrate diameter and 500 μm substrate thickness.
14. Rinse and dry substrate machine (e.g., R/D Vertec) with DI H_2O and hot compressed nitrogen (N_2) gas.
15. Dicing film, UV release (e.g., DU-300, Nitto).
16. Dicing blade (e.g., ZH05, Disco).
17. Sonicator (e.g., 2510, Branson).
18. Oxidation furnace equipped with compressed O_2 and H_2 (e.g., Thermco).
19. Mask aligner equipped with UV exposure (e.g., MA6, Karl Suss).

2.2 Fabrication of Microwell Chip Holder

1. Aluminum block, sized to accommodate the silicon chip in microscope plate holder.
2. Magnetic disks, 3–5 mm diameter, material stainless steel 400-series.
3. Standard water-resistant two-component epoxy glue.
4. Rod shaped magnets, 3–4 mm diameter, material Nd35.
5. Para-methoxy-*N*-methylamphetamine (PMMA, also known under brand name Plexiglass or acrylic glass) disk, 30 mm diameter, thickness 8 mm.
6. Casting silicon rubber (e.g., polydimethylsiloxane (PDMS), Sylgard 184).
7. Petri dishes 150 mm diameter.
8. Curing oven at 60 °C.

2.3 Setup for Live-Cell Time-Lapse Imaging

1. Inverted microscope for fluorescence and transmitted light detection (*see* **Note 2**) equipped with an incubator for keeping physiological conditions (37 °C, 5% CO_2) and ideally equipped with a motorized stage and suitable software to allow detection at multiple positions in parallel.
2. High-quality air objective allowing detection of the whole well in the field of view (typically 10× or 20× objective).

2.4 Preparing the Microwell Chip for Imaging

1. Microwell chip of desired dimensions (we have used a chip of side-dimensions 22 × 22 mm^2, containing wells of 450 × 450 μm^2) with accompanying holder, PDMS gasket, and magnetic lid.
2. Tweezers.
3. Lid from 33 mm petri dish.
4. 33 mm petri dish with glass bottom (Mattek Corporation).
5. Phosphate-buffered saline (PBS).
6. 25 μg/mL human fibronectin (Sigma Aldrich) solution in PBS.
7. Serum-free RPMI-1640 medium, kept at 37 °C.
8. NK cell fluorescence staining media: Prepare 0.3 μM calcein red-orange in 1 mL of warm (37 °C) serum-free medium.
9. Target cell fluorescence staining media: Prepare 1 μM calcein green AM and 5 μM Far Red DDAO-SE in 1 mL warm (37 °C) serum-free medium.
10. Complete cell culture medium: RPMI 1640 containing 10% fetal bovine serum, 2 mM l-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin (all Sigma Aldrich), kept at 37 °C, 5% CO_2.
11. Complete NK cell culture medium: IMDM containing 10% human AB serum, 2 mM l-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, and 50 μM β-mercaptoethanol (all Sigma Aldrich) warmed to 37 °C, 5% CO_2 prior to starting the experiment.
12. Syringes and 0.2 μm filters.
13. 15 mL conical polypropylene centrifuge tubes.
14. Closed chamber connected to a vacuum pump.

2.5 Cleaning the Microchip for Reuse

1. Microchip of desired dimensions.
2. Tweezers.
3. 60 or 100 mm petri dish.
4. 100 mL glass bottle with cap.
5. Filtered ethanol.
6. MilliQ water.

7. 50 mL falcon tube.
8. Ultrasonic bath sonicator.
9. Closed chamber connected to a vacuum pump.
10. NaOH (5 M).

3 Methods

The following steps (Subheadings 3.1–3.3) that outline the fabrication of the microdevice require specialized equipment and clean room facilities and are, therefore, not possible to perform in an average biology lab. Currently, we are not aware of any supplier that provides a similar device off the shelf but there are several companies that can custom make devices to order. If competence is lacking in-house, it is advisable to get the device fabricated professionally or through academic collaboration.

3.1 Fabrication of Silicon-Glass Microwell Array Chips

1. Clean the silicon wafers in 7-Up at 130 °C for 10 min to remove metallic residues.
2. Rinse the wafers in a rinsing bath bubbler at room temperature for 10 min.
3. Clean the silicon wafers in IMEC clean for 100 s to remove organic residues.
4. Rinse the wafers in rinsing bath bubbler and dry them in R/D machine with DI and hot N_2.
5. Oxidize silicon wafers at 1100 °C in furnace with H_2 and O_2 to 1–2 μm oxide thickness (*see* **Note 3**).
6. Deposit positive photo resist at the center of the wafer and spin at 1500 rpm (equivalent to $125 \times g$ at the periphery of the 100 mm wafer) for 30 s (Fig. 1a). Allow 30 s settling time after deceleration and stop.
7. Soft bake at 90 °C for 10 min in oven.
8. Allow for rehydration for 45 min in ambient atmosphere.
9. Perform masked UV exposure in multiexposure mode, i.e., 4×2.5 s exposures with 15 s delay between each exposure, in a mask aligner (*see* Fig. 1b and **Note 4**).
10. Develop exposed patterns with CD26 developer fluid for 20 s with agitation (Fig. 1c).
11. Perform postexposure baking in a step-wise fashion: ramping from 90 to 120 °C with a duration of 1 h per 10 °C.
12. Etch well pattern through the oxide layer (gas pressure at 15 sccm for CHF_3, 5 sccm for CF_4, 50 sccm for Ar at 350 W) for 7 min in a plasma oxide etcher.

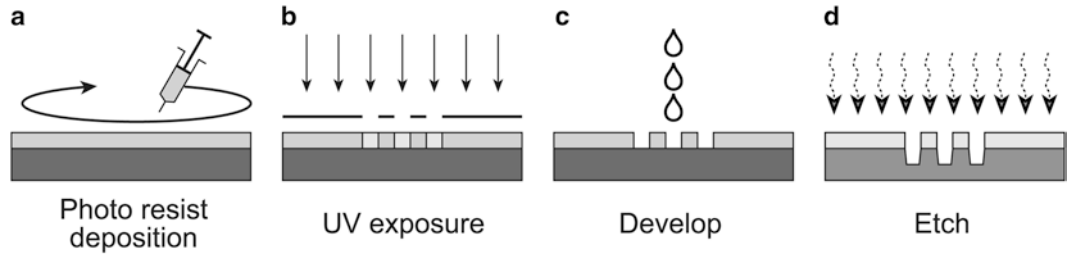


Fig. 1 Schematic of the UV lithography and etching process. (**a**) Photo resist is deposited on wafer rotating at 1500 rpm creating a layer of uniform thickness. (**b**) The wafer with photo resist is exposed to UV light in a mask aligner. (**c**) The photo resist layer is developed through rinsing the wafer with developer liquid under agitation. The photoresist polymerizes and hardens in the unexposed areas, while the photoresist in UV-exposed areas are flushed away. (**d**) The wafer is DRI-etched creating wells where the silicon is exposed

13. Deep etch the silicon wells (DRIE: SF_6 and C_4F_8 alternating Bosch process) during 120 min in a dry ion plasma etcher (*see* Fig. 1d and **Note 5**).
14. Stop etch at bottom oxide layer (*see* **Note 6**).
15. Clean photoresist and etch residuals in oxygen plasma (at 500 sccm O_2, 1000 W) for 15 min.
16. Strip the silicon oxide from the wafers in concentrated hydrofluoric acid (HF) for 3 min followed by rinse and dry.
17. Wet clean wafer during 100 s in freshly mixed 7-UP (upon mixing, the solution reaches approx. 130 °C) followed by rinse in DI H_2O N_2 bubbler followed by rinse and dry in R/D machine.
18. Oxidize wafers again (refer to **step 5**), this time to an approximate surface oxide thickness of 200 nm (*see* **Note 7**).
19. Do the bonding pretreatment through 120 s dipping of the wafers in pure 7-UP, 5 min rinsing in deionized water and 10 min drying in R/D machine.
20. Bond the glass to the silicon wafer with anodic bonding (−600 V at 420 °C for 15 min) (*see* **Note 8**).
21. Attach the glass-silicon stack to UV-release dicing film (60 μm thickness) (*see* **Note 9**).
22. Dice into desired shape with a standard diamond blade (30–100 μm blade thickness) at 30 krpm (0.3–0.5 mm/s feed rate) into chips with your designed footprint (*see* **Note 10**).
23. Release chips from dicing film through UV-light exposure according to manufacturer's recommendation (*see* **Note 11**).
24. Clean the chips in a mixture of ethanol DI H_2O (1:5) with a brief (approximately 30 s) sonication in degassing mode followed by rinsing in a large volume of DI H_2O for >45 min (*see* **Note 12**).

3.2 Fabrication of Microwell Chip Holder and Gasket

1. Mill a cavity the size of the chip in an aluminum block suited for microscope substrate holder, leaving a thin flange at bottom for chip to sit on (*see* **Note 13**).
2. Drill four holes a few millimeters from the chip cavity for magnetic disks.
3. Anodize aluminum to 20 nm thicknesses (*see* **Note 14**).
4. Glue the magnetic disks in the holes of the holder with standard water-resistant two-component epoxy glue.
5. Mill a cavity in the size of the maximum array outline in the PMMA disk.
6. Drill four holes for the magnet rods aligned to the positions of the four holes in the aluminum block.
7. Glue the magnetic rods level to the bottom of the PMMA disk with epoxy glue.
8. For the gasket, mix PDMS (base:curing agent in 10:1 weight ratio) and pour to proper thickness in petri dish (*see* **Note 15**).
9. Degas the liquid PDMS in a vacuum desiccator for 10–20 min to remove bubbles.
10. Cure the PDMS overnight in 60 °C.
11. Cut the PDMS into a fitting gasket with sharp scalpel (*see* **Note 16**).

3.3 Preparing the Microwell Chip for Imaging

1. Filter the fibronectin solution, PBS, serum-free RPMI-1640 medium and complete cell culture medium, using 0.2 μm filter (*see* **Note 17**).
2. Mount the chip into the holder prepared in Subheading 3.2, place the gasket on the chip so that it outlines the edges and gently press it down with a pair of tweezers. Secure the magnetic lid on top, holding the gasket firmly to the chip. Finally, place a lid of a 33 mm petri dish on top of the magnetic lid (*see* Fig. 2 and **Note 18**).
3. Add 1 mL of fibronectin solution to the chip using a 1 mL pipette (*see* **Note 19**).
4. To remove air that is trapped in the microwells below the liquid, gently degas the chip for 10 min in a closed chamber connected to a vacuum pump (*see* **Note 20**).
5. Examine the chip in an inverted microscope to confirm the absence of air bubbles. Any remaining small bubbles can be removed from the microwells through gentle mixing with a pipette.
6. Incubate the chip at room temperature for 50 min.
7. Aspirate 0.5 mL of the fibronectin solution and replace with filtered PBS. This washing step is repeated seven times.

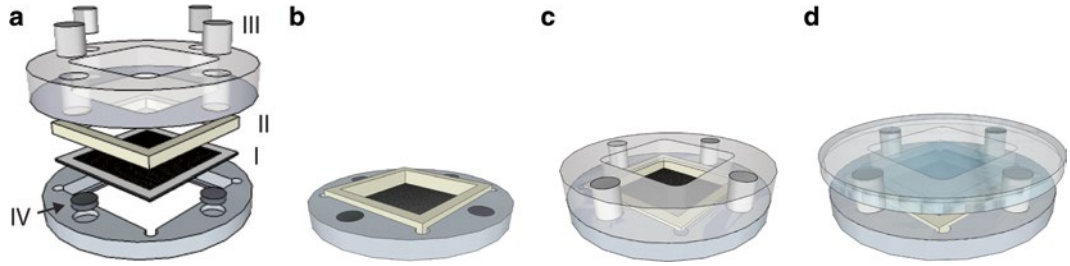


Fig. 2 Schematic of the microchip and holder. (**a**) Exploded view showing the microchip (*I*), PDMS gasket (*II*), magnets (*III*), magnetic disks (*IV*), and holder. (**b**) The microchip and PDMS gasket are placed in the bottom part of the holder. The gasket should be thick enough to stick out 0.5–1 mm above the holder to avoid leakage when the device is assembled. (**c**) Assembled device with the top and bottom of the holder aligned to allow contact between the magnets and disk, clamping the upper and lower parts of the holder together. A basin for cell medium is created above the microwell chip. (**d**) Assembled and loaded device with a plastic lid placed on top to avoid contamination

8. Aspirate 0.5 mL of PBS and replace with complete cell culture medium. This washing step is repeated seven times.
9. Incubate the chip at 37 °C, 5% CO_2.

3.4 Fluorescence Labeling of NK and Target Cells

1. Collect one to two million NK cells and one to three million target cells in two separate 15 mL falcon tubes and wash with warm (37 °C), serum-free RPMI-1640 media. Pellet cells at 300 × *g* for 10 min. Aspirate the supernatant and repeat the washing step two times (*see* **Note 21**).
2. After the last wash, remove the supernatant, resuspend the NK, and target cell pellets in 1 mL of respective fluorescence staining media (Subheading 2.4, **items 8** and **9**) (*see* **Note 22**) and incubate for 10 min at 37 °C, 5% CO_2 (*see* **Note 23**).
3. After staining, add 10 mL of warm (37 °C), filtered, complete cell culture medium to the NK and target cells (*see* **Note 24**).
4. Centrifuge NK and target cells in their separate tubes at 300 × *g* for 10 min, remove the supernatants and resuspend the cell pellets in filtered, warm complete cell culture medium. Repeat this washing step two times.
5. Next, resuspend NK and target cells in their separate tubes in 0.5 mL filtered, warm (37 °C) complete cell culture medium and maintain at 37 °C, 5% CO_2 (*see* **Note 25**).

3.5 Loading of NK and Target Cells on the Microchip

1. Count the target cells and dilute to a density of ~200,000 cells/mL.
2. Aspirate sufficient medium from the chip so that the liquid surface is level with the top of the gasket and then remove an additional 100 µL (*see* **Note 26**).

3. Seed 20,000 fluorescently labeled target cells in 100 μL medium onto the chip followed by gentle mixing. Incubate the chip for 15 min at 37 °C, 5 % CO_2 (*see* **Note 27**).
4. Count NK cells and dilute to a density of 80,000 cells/mL (*see* **Note 27**).
5. Aspirate 100 μL of liquid from the chip and seed 8000 NK cells in 100 μL medium into the chip, followed by careful mixing to avoid detaching target cells that have adhered to the bottom of the wells. Incubate the chip for 5 min at 37 °C, 5 % CO_2.
6. Carefully remove 200 μL of liquid from the chip and replace with 200 μL of complete NK cell culture medium. Repeat this step five times (*see* **Note 28**).
7. Place the petri dish lid on top of the holder containing the seeded NK and target cells and mount into the climate chamber on an inverted microscope stage. Proceed to imaging as fast as possible (*see* **Note 29**).

3.6 Setup for Time-Lapse Imaging

1. Setup and start the heating and incubation system on the microscope in good time prior to mounting the sample and starting the experiment (*see* **Note 30**).
2. Choose an appropriate objective. A 10× or 20× air objective is suitable for imaging microwells of side-dimensions 450 × 450 μm^2 (*see* **Note 31**).
3. Upon mounting the chip holder, make it as flat as possible by gently pressing down all sides simultaneously. Use the eye-piece to roughly set the focus then start the scanning. Use the transmission channel to align the chip in the field of view before mounting the top of the incubation chamber (*see* **Note 32**).
4. Next, adjust the laser and acquisition settings for the different fluorescence channels and transmission image. To this end, mix a small volume of the fluorescently labeled NK and target cell suspensions in a petri dish/chip with a glass bottom, and place on the microscope. Adjust the fluorescence settings of the microscope to optimally detect the different cell tracer dyes (*see* **Note 33**).
5. Remove the sample used to adjust the laser and acquisition settings.
6. Once the microchip has been seeded with cells of interest, mount the chip holder on the microscope as described in **step 3**.
7. Find suitable imaging locations where the cellular distribution is as desired and save the coordinates for these positions (*see* **Note 34**).
8. Set the time interval between image acquisition as well as the total length of time. We usually image every 2 min for over 12 h (*see* **Note 35**).

3.7 Cleaning the Microchip for Reuse

1. Fill an appropriate sized petri dish to approximately half with MilliQ water (*see* **Note 36**).
2. Preferably, do not let the chip dry out after a finished experiment. Hence, work fast to remove the reservoir media, disassemble the holder, and place the used chip in the petri dish face up using the tweezers (*see* **Note 37**).
3. Examine the chip in an inverted microscope to confirm the absence of air bubbles.
4. If necessary, remove air bubbles by gently degassing the chip for 10 min in a closed chamber connected to a vacuum pump. Any remaining small bubbles can be removed from the microwells through gentle mixing with a pipette (or placing the petri dish in a 37 °C incubator for 10–15 min).
5. Prepare a 100 mL glass bottle for each chip by filling it about halfway with MilliQ water.
6. Place the chip in the bottle facing downwards allowing cell debris to fall under the effect of gravity.
7. Sonicate the chip for 1 h depending on how dirty the microchip is (*see* **Note 38**).
8. Remove the chip from the glass bottle and place face up in a petri dish containing fresh MilliQ water. Degas if necessary (the sonication process can cause bubble formation).
9. Move to a laminar flow hood, from now on work under sterile conditions.
10. Prepare a new petri dish and fill it about halfway with filtered EtOH (*see* **Note 39**). Place the chip in the EtOH and sterilize the chip for 30 min.
11. Wash the chip in MilliQ.
12. Store the clean, disinfected chip in a MilliQ-filled 50 mL Falcon tube, or store it dry in a sealed environment.

3.8 Analysis and Quantification of NK Cell Data

3.8.1 Cell Tracking and Quantification

1. To ensure high fidelity, NK cell migration is tracked manually using appropriate software, e.g., Volocity (Perkin Elmer) (*see* **Note 40**). The target cells can also be tracked in a similar way if desired (*see* **Note 41**).
2. Define parameters and characteristics to quantify for the NK cell trajectories (*see* **Note 42**). For example, each NK cell trajectory can be divided into migration and interactions with other cells. Cellular interactions can be subdivided into conjugation and attachment periods. Conjugation is characterized by local flattening at the membrane interface. The postconjugation, attachment phase starts when the NK cell has ended its commitment to the target cell and attempts/starts to migrate away. The following parameters could be scored for each target cell an NK-target cell interaction.

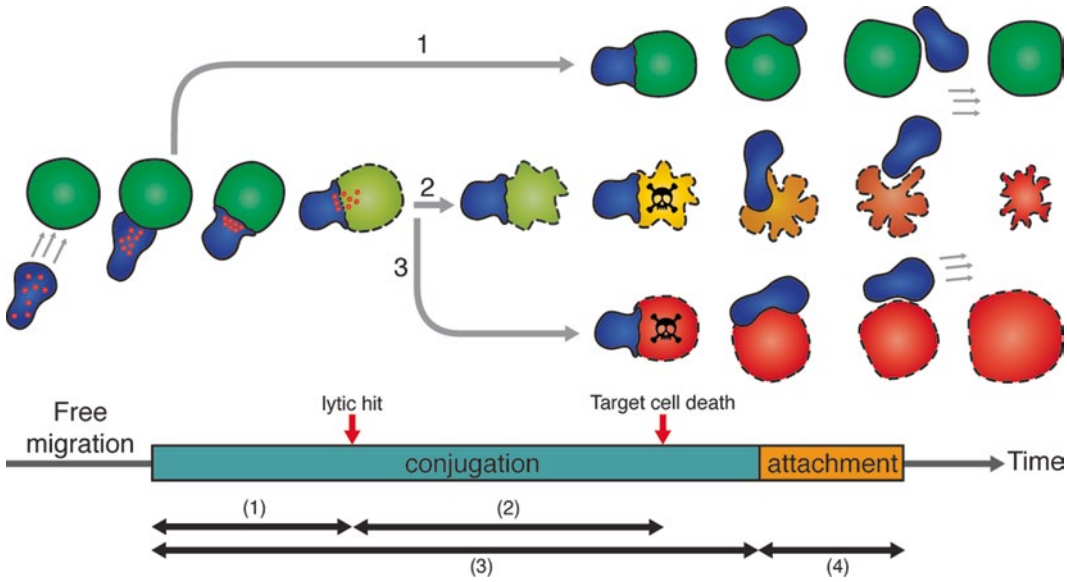


Fig. 3 Schematic of different phases of the interaction between NK cell and target cell. The interactions between NK and target cells can be either nonlytic (following *path 1*) or lytic (*paths 2* or *3*). NK cells can induce either slow (*path 2*) or fast (*path 3*) target cell death. The interaction between NK and target can be divided into phases of free migration, conjugation, and attachment. During the conjugation phase, the NK cell delivers a lytic hit leading to target cell death. Based on this scheme four different times can be defined: (*1*) Time to lytic hit, (*2*) time to target cell death, (*3*) conjugation time, and (*4*) attachment time

(a) Time point when the NK cell contacts the target cell initiating conjugation (Fig. 3).

(b) Time point when conjugation ends and attachment begins (Fig. 3).

(c) Time point when the NK cell is fully separated from the target (end of attachment phase).

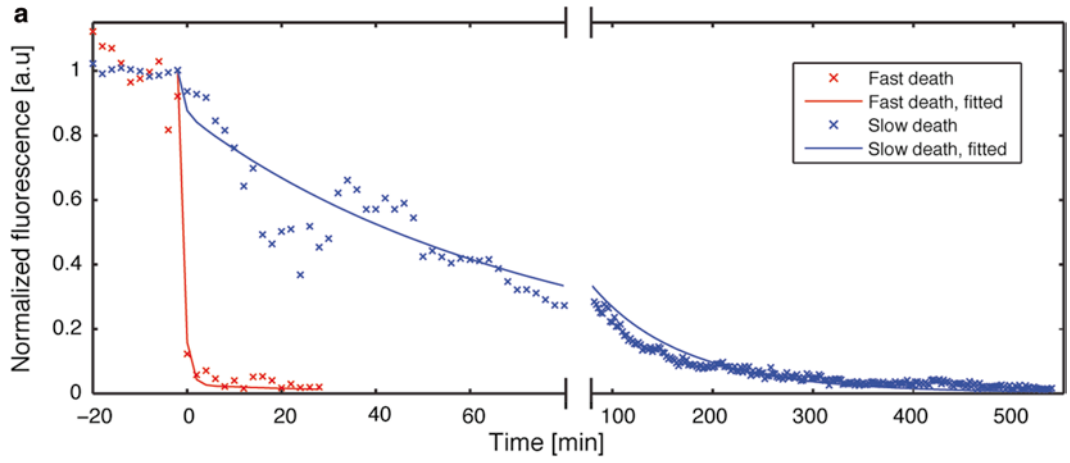
(d) Determine outcome of NK-target cell interaction (killing/nonkilling) and time point at which target cell death occurs.

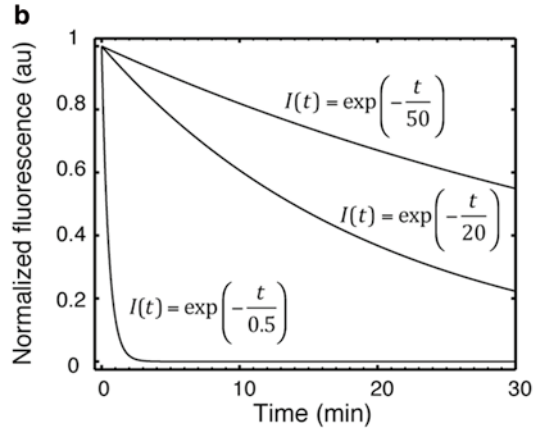
3. Score each individual NK cell track for the defined parameters. If the NK cell engages with another target, restart this process. Continue until all NK cells have been tracked and scored.

3.8.2 Analysis of Target Cell Death by Calcein Fluorescence Decay

1. Quantify the calcein fluorescence intensity decay of individual target cells as a function of time using an appropriate software (*see* **Notes 43** and **44**).
2. The total fluorescence intensity of the target cell during the interaction, $I(t)$, is fitted to the sum of three exponentials:

$$I(t) = a_1 \exp\left(-\frac{t}{\tau_1}\right) + a_2 \exp\left(-\frac{t}{\tau_2}\right) + a_3 \exp\left(-\frac{t}{\tau_3}\right) + C,$$





c

$$I(t)=\alpha_1\exp\left(-\frac{t}{\tau_1}\right)+\alpha_2\exp\left(-\frac{t}{\tau_2}\right)+\alpha_3\exp\left(-\frac{t}{\tau_3}\right)+C$$

$$\tau_{average}=0.5\alpha_1+20\alpha_2+50\alpha_3$$

	α_1	α_2	α_3	$\tau_{average}$ (min)
Fast death	0.97	0.03	0.00	1
Slow death	0.12	0.18	0.70	39

Fig. 4 Analysis of calcein decay profiles. (**a**) Examples of fluorescence decay profiles from a target cell undergoing fast (*red trace*) or slow (*blue trace*) deaths after being exposed to NK cell lytic hits. The time of the lytic hit is defined as the time of the initial drop in the fluorescence curve (here set to time = 0 min). Both curves were fit with a sum of three exponentials (*solid lines*) used to define average lifetimes of target cell death. For display purposes, the intensity profiles have been normalized by the mean fluorescence intensity registered before the lytic hit ($t < 0$). The selected curves had no residual fluorescence after the calcein decay ($C = 0$). Note the broken time axis. (**b**) Graph showing single-exponential decays corresponding to the three lifetimes 0.5, 20, and 50 min. (**c**) Equations for fitting the decay profiles (*top*) and calculating the average lifetime (*middle*). The table (*bottom*) shows the preexponential factors (α_1–α_3) and calculated average lifetimes for the decay curves in (**a**)

where t is the time, a_1–a_3 are preexponential factors, τ_1–τ_3 are lifetimes corresponding to 0.5, 20, and 50 min, and C is a constant corresponding to any background fluorescence remaining after the calcein decay (*see* **Note 45**). Before fitting, the time vector has to be adjusted to start at zero where an initial drop corresponding to the lytic hit is observed (Fig. 4). Curve fitting can be performed in the software of choice.

3. Calculate the average lifetime to compare individual fluorescence decays with each other:

$$\tau_{\text{average}} = \alpha_1\tau_1 + \alpha_2\tau_2 + \alpha_3\tau_3.$$

where α corresponds to relative contributions of the decay from the particular lifetime so that $\alpha_1 + \alpha_2 + \alpha_3 = 1$.

4. Fast decays were defined as $\tau_{\text{average}} \leq 10\,\text{min}$ (*see* **Note 46**).

4 Notes

1. The microwells should be designed with a corner radius in the 5–10 μm range. Wall thickness should be in 30–100 μm range to avoid reentrant etching-caused holes between wells. The mask should carry alignment marks for future additional layers in fabrication. It should also contain dice lines, enabling precise alignment during wafer dicing. Symmetry in *y*-axis is preferred as this minimizes the risk of errors with respect to up-side-down issues. Keep a 10 mm margin around the perimeter of the wafer to avoid etching of wafer edge. Microchips containing wells of varying sizes can be manufactured to suit the experimental purpose. For example, larger wells (i.e., arrays of 450×450 μm^2 wells custom made to fit with a 10× and 20× objective) can be used to image migration properties of cells while smaller wells (i.e., 50×50 μm^2 and 30×30 μm^2) can be used to spatially confine NK and target cells in close proximity to test, e.g., cytotoxicity. For migration studies, it is practical to use adherent target cells while for the cytotoxicity screens, both adherent and nonadherent cells can be used.
2. Both a conventional fluorescence microscope based on camera detection or a laser scanning confocal microscope (LSCM) can be used. If using an LSCM microscope, it can be helpful to open the pinhole to maximize the amount of fluorescence light reaching the detector. This will allow a lower laser intensity to be used, decreasing the risk of cellular phototoxicity.
3. The oxide layer covering the front and backside of wafer has three main purposes: forming a highly etch-resistive masking material for the deep silicon etch, wafer backside etch stop at the end of the deep etch (thus minimizing etch gases escaping through etched wells and attacking wafer holding chuck), and, finally, protecting the bond surface from scratches and contaminants. Layer thickness measurement tools such as laser elipsometers and stylus profilometers can be used to ensure that proper layer thicknesses have been acquired. A versatile inspection tool is a stereomicroscope, which enables rapid optical inspection of chip geometries and surface qualities.

Another effective way to verify that consecutive process steps yield intended results can be done with SEM imaging. Validation of layer thicknesses, well shapes, etching aspect ratio, and surface flatness can easily be carried out through looking at the chips at various angles.

4. The masked UV exposure of the photoresist covered silicon wafer can preferably be done in multiexposure mode, i.e., divide the total exposure time into four or five parts by inserting 15–30 s delays between the exposures. Upon prolonged exposure the thick photoresist will heat up and form bubbles, distorting the pattern integrity and lower the fabrication quality. The delay between exposures will allow the photoresist to cool reducing the risk of heat-induced damage.
5. Etching of silicon with the Bosch process should be carefully tried out and evaluated. By shortening the intervals between alternating etching and sidewall protection (a.k.a. passivation) steps, the risk of "ripples" caused by the etching process is reduced. The aspect ratio (i.e., how "vertical" the walls are) can also be tuned during processing by varying the etching/passivation times and by adjusting the chamber temperature. Adjusting other process parameters like gas flows and chamber vacuum will also have an impact on etching quality. Recipes are usually available from the machine manufacturers.
6. The oxide is transparent so once the etch has reached the oxide the microwell structure becomes visible. When all wells are transparent the etching is finished.
7. Perform a second wet oxidation to form a silicon dioxide layer across the surface of the wafer. This increasing the chip biocompatibility and allows cleaning and reusage of the chips. Oxidation should be done to approximately 50 nm.
8. The bonding quality can be monitored and documented by recording the voltage and especially the current flow through the wafer-glass sandwich. When the bonding current is less than 0.5 mA the bonding process can normally be terminated.
9. Avoid bubbles in the film and make sure the film is firmly attached to the wafer.
10. Two notes on dicing: (1) place dicing film over the wells sealing them and dice with the glass side up so that the wells are not filled with dicing debris during cutting; (2) add dicing lines (5 μm thickness) during the design phase to enable rapid positioning of dicing blade and aligning of dicing street.
11. Peeling off the dicing film from the microwell array chip is preferentially done in a diagonal fashion, with a low angle of incidence, in a rolling manner to avoid breakage of the walls between the wells.

12. This wash is done to remove residuals of the dicing film glue as well as residuals from the acidic bond pretreatment. High-power sonication can damage the silicon, so ensure short time or low power (<250 W) of the sonicator.
13. A flat seat for the chip minimizes problems with chip wobbling that can lead to leakage or focus shifts during imaging. Running the milling tool several times over the same area will ensure that the flange is level.
14. Anodizing the holder block minimizes reflections from stray light during imaging. The aluminum oxide will also protect the bulk material from oxidizing during ion-rich wet experiments.
15. The gasket performs dual functions. Firstly, it adds height to the liquid reservoir above the microwell chip, decreasing risk of dehydration as well as buildup of ion and salt concentrations. Secondly, the stickiness of the silicon rubber will secure the chip to the holder. Ideally, the gasket sticks out about 0.5–1 mm above the side of the holder when the chip is sealed in. If the gasket is too thick, the magnetic attraction might be too low to securely seal the holder.
16. It is easier to first cut the cavity in the middle and then to cut the gasket outer edges than vice versa as the silicone rubber is prone to crack on mechanical stress from sharp objects such as scalpels or razor blades.
17. These solutions are filtered to remove all particles that may be present and can interfere with imaging. Keep all solutions sterile and work in laminar flow hood when handling liquids and cells to prevent bacterial/yeast contamination.
18. The PDMS gasket should stick to the chip to prevent leakage of fluids; this can be seen as a darkening of the PDMS gasket-chip interface when pressed onto the chip. If the gasket is not sufficiently fresh, it will not stick as well to the chip.
19. Other extracellular matrix proteins can be used to coat the microchip.
20. During degassing, "boiling" of the small fluid volume in the chip can cause it to leak out of the chip. For effective, but gentle degassing, when excessive amounts of bubbles appear at the liquid surface, slowly open the air inlet to adjust the pressure inside the chamber and then close it again. This will allow the bubbles to evaporate.
21. *Effector cells*: The effector cells used for the experimental setup will vary for each application. We often use freshly isolated or IL-2 stimulated NK cells obtained using the negative selection kit from Miltenyi Biotech. However, there is no limit on which cell type to use, whether NK, B or T cells that are freshly isolated, cytokine stimulated or treated in some other form. When

using fresh cells, it is preferential to use a kit that negatively selects for the cells of interest. Kits based on positive selection can affect cells by, for example, cross-linking receptors used for isolation or blocking important receptors. *Target cells*: For migration experiments, use adherent target cells. We have commonly used HEK293T cells due to their sensitivity to NK cell mediated lysis. For assays in microchips with smaller well size (i.e., to monitor NK cell cytotoxic function) both adherent and nonadherent cells can be used. During the labeling step, cells are washed with serum-free media as labeling with fluorescent dyes can be serum-sensitive, decreasing staining efficiency.

22. Target cells are labeled with the cytoplasmic viability dye calcein green AM, and the fluorescent dye FarRed DDAO-SE, which forms covalent bonds with cellular structures. In this fashion, target cell death can be monitored by retention of FarRed DDAO-SE, and leakage of calcein green AM. Such a labeling strategy also facilitates automated image analysis. It is important to note that the spectral characteristics (i.e., emission and excitation wavelengths) of fluorophores used for dual labeling should be distant enough to minimize/eliminate fluorescence bleed through.
23. The incubation time for staining will depend on the individual cell type, concentration, and incubation temperature. It should be optimized for each cell type and ideally uses the least amount of dye possible while allowing reliable tracing for the entire length of the experiment. Additionally, there are many different fluorescent dye choices available for staining cells. In our experience, some dyes fluoresce strongly but affect other processes, such as cellular motility. We have found the cell tracker series of dyes, which are free flowing within the cytoplasm once absorbed, provide both long-term fluorescence and serve as a viability marker while not affecting NK cell functionality (such as cellular cytotoxicity and cytokine production).
24. Serum-containing medium is used to stop the staining reaction.
25. Before seeding NK cells into the chip, make sure that the microscope is setup and ready for time-lapse imaging. See the "time-lapse imaging" sect. 3.6 for more detail.
26. If the liquid surface is level with the top of the gasket, it is generally more flat resulting in a more even distribution of cells in the wells. A convex surface results in denser seeding toward the middle of the chip while a concave surface gives a higher cell density at the edges. The easiest way to acquire a flat surface is to level it with the top of the gasket.
27. The specified number of seeded target and NK cells are merely suggestions and should be modified according to the particular

experiment and microchips used. Use warm media to dilute the cells to maintain cells at physiological temperature.

28. The purpose of the washing step is to remove all cells that have not settled into the well, preventing them sedimenting during imaging. Additionally, the media used should contain the necessary nutrients required for the cells. We sometimes use cytokine-activated NK cells; hence, include cytokine (e.g., IL-2) in the cell culture medium.
29. It is important to start the imaging as soon as possible after the chip has been prepared to get as close as possible to time 0. This is important to monitor the entire contact history of the NK cells. The microscope should be setup and ready for time-lapse imaging.
30. It is important to setup the imaging system together with incubation chamber, humidifier, and other apparatus at least 1 h prior to the start of the experiment to ensure that the environment is already at physiological temperature. Additionally, changes in temperature during imaging may cause focus-drift and may affect functionality.
31. One of the reasons to isolate cells within microwells is to spatially confine them, facilitating imaging of motile cells, for example. Hence, the choice of objective is linked to the ability to image the whole microwell, while still able to see the sides of the well.
32. It is important that the chip is in as perfect focus as possible since minor *z*-changes will affect the fluorescence intensity. A good microscope stage which has been zeroed in all directions helps to prevent drift.
33. For long-term live-cell imaging, laser intensity should be kept to a minimum to reduce effects of phototoxicity. However, keep in mind that bleaching and dye leakage can pose problems over long experiments so it might be worthwhile to slightly overexpose cells to compensate for this. Ideally, the setup allows for a full range of fluorescence intensities with a minimal amount of saturated pixels. If using multiple dyes, it is important to check that fluorescence bleed through is minimal between the different laser/dye/filter combinations. Unstained cells should give minimum signal in the fluorescence channels. Also, we have noticed that target cells that were dead when seeded were many times brighter than living cells for certain dyes. Those cells had to be overexposed to not lose information from the cells of interest. Settings will be different depending on your system, we have summarized our laser and filter setup in Table 1.
34. The number of available/possible imaging positions will vary depending on the imaging system used. It is limited by the

Table 1
Fluorescent dyes and LSCM settings

	Fluorescent dye		
	Calcein green AM	**Far red DDAO-SE**	**Calcein red-orange AM**
Acquisition track	1	1	2
Excitation laser (nm)	488	633	543[a]
Emission filter	BP 505–530 nm	LP 650 nm	LP 560 nm[b]

Example of suitable lasers and filters used to detect all three fluorescent dyes when using a conventional LSCM (e.g., Zeiss 510 Meta). To minimize detection of fluorescence bleed through from a different channel, the signal from calcein red-orange is collected individually in a separate track. Calcein green AM and DDAO signals are collected simultaneously with two separate detectors

[a]Also 561 nm can be used if this laser line is available

[b]Also a BP filter can be used to avoid any risk of detecting fluorescence from DDAO

time and optical resolution wanted, the scanning speed of the microscope, the speed of the moving stage, and the desired amount of data. We typically acquire data from eight different positions every 2 min for 12 h. Of note, unless the chip is perfectly flat in the holder, adjust the focus for each position and save it.

35. The time interval and length will depend entirely on the application and experiment. For high-speed high-resolution imaging, image acquisition can be much faster, including z-stacks. For slower processes, the interval can be much longer. However, there is always a trade-off between the number of positions and the resolution.
36. When cleaning more than one chip at a time, use a 100 mm petri dish which holds up to four chips for subsequent degassing. To clean a single chip, use a 60-mm dish. Although the chip fits inside a 33 mm dish it is difficult to remove after cleaning.
37. Do not use ethanol before sterilizing the microchip (**step 9**) as it will fix debris slightly to the glass substrate in the chip making it more difficult to clean.
38. Cell debris from adherent cells might be more difficult to remove and sonication may have to be repeated. However, high-power sonication can damage the silicon, so ensure short time or low power (<250 W) of the sonicator. If the chip is still not sufficiently clean, an extensive cleaning protocol can be applied by immersing the chip in a 5 M NaOH solution for 5–10 min. However, the chip should not be exposed to NaOH for too long, as this will etch the silicon.

39. In our experience, premixed 70 % EtOH used for general disinfection in the cell laboratory contains many small particles which will sediment into the wells decreasing image quality. If a chemical grade EtOH is used, the filtering process may not be necessary.
40. Track each NK cell from the time point it appears (most cells will be present at t_1) until the end of the assay, the cell dies, the cell divides, or disappear out of focus and cannot be detected by either transmission or fluorescence.
41. The described assay is most suitable for adherent target cells. If using nonadherent target cells, e.g., the common NK-target cell line K562, we suggest using smaller wells as described in refs [7, 8].
42. Parameters to score will depend upon the experimental setup and question of interest. It is important to clearly define each parameter prior to scoring. For example, we often define an NK-target cell conjugate as a period of cell–cell contact between an NK and a target cell that is at least 4 min (i.e., spans three time points with a time step of 2 min). Clear definitions from the start will prevent having to rescore interactions. A single NK cell can be conjugated to several targets simultaneously and, thus, several interactions may be scored in parallel. Some NK-target cell interactions will not have a pronounced attachment phase. In this case, $t_{\text{conjugation,end}} = t_{\text{attachment,end}}$.
43. The rate of target cell death is believed to be related to the strength of the lytic hit delivered by the NK cell [7]. Generally, fast death is accompanied by target cell swelling and bursting while slower death is coupled to apoptotic blebbing. The swelling and bursting can be induced by high levels of perforin alone while the blebbing is associated with sublethal levels of perforin in combination with granzymes.
44. If a target cell is clearly separated from other target cells (i.e., no fluorescence overlap) then the fluorescence can be quantified automatically using software such as ImageJ or Volocity. If there is fluorescence overlap, analyze the target cell fluorescence manually. It is important to also analyze target cells that are not contacted by NK cells throughout the experiment to determine the rate of spontaneous calcein loss and bleaching.
45. These three lifetimes were chosen as they gave satisfactory fits to most fluorescence intensity decays and can serve as guidelines. They may need to be adjusted with other data sets.
46. We observed that during NK-target cell conjugate there was a decrease in target cell calcein fluorescence followed either directly, or shortly afterwards, by visual signs of cellular death. This corresponded to the NK cell delivery of lytic granules to the target cell. Target cell death could be split into a fast and a

slow calcein decay based on the value of $\tau_{average}$. It was possible to further subdivide the slow calcein decays manually into separate groups. The method described here allows an overview of the target cell fluorescence profiles of NK cell-induced target cell death. In some target cells, more than one calcein drop could be observed, corresponding to more than one single lytic hit. These target cells can be analyzed manually.

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Chapter 9

Assessment of Natural Killer Cell Cytotoxicity Using Image Cytometry Method

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Abstract

Although natural killer (NK) cells produce various cytokines that regulate other lymphocytes of the immune system, the primary effector function of NK cells is the direct cytolysis of their targets. Hence analyzing the cytotoxic potential of these lymphocytes is fundamental to understanding their biology and their clinical impact. We have previously shown that release-based cytotoxicity assays, such as calcein release assay, could potentially underestimate percent specific lysis if the entrapped reporter is not completely released and demonstrated that an Image cytometry method can overcome this caveat. In this chapter, we describe a detailed methodology to quantitate NK cell cytotoxicity using the Cellometer Vision Image Cytometry system.

Key words NK cells, Cytotoxicity, Image cytometry, Calcein release assay, Cellometer Vision

1 Introduction

As part of the innate immune system, natural killer (NK) cells play a critical role in defense against pathogens and participate in immune surveillance against cancer [1]. While a subset of NK cells ($CD56^{bright}CD16^{-}$) secrete cytokines such as interferon-γ and TNF-α that activate immune response, the majority of NK cells directly lyse their targets [2]. Therefore assessing the cytolysis of target cells is important for studying the biology of NK cells and evaluating their therapeutic efficacy.

Various methods have been developed over the years to facilitate assessment of percent specific lysis by cytolytic lymphocytes. The chromium release assay (CRA) is considered the "gold standard" for assessing cytotoxic potential of NK cells and cytolytic T cells [3–7]. In this assay, target cells are loaded with chromium-51 (^{51}Cr) and mixed with the effector cells. The entrapped ^{51}Cr is released when the target cell membrane is disrupted during cytolysis and the amount of released chromium is quantified at the end of the assay. Toxicity, access, and disposal concerns related to

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working with radioactive material have led to the development of alternative methods to assess cellular cytotoxicity, refer to review by Zaritskaya et al. [8]. One of the appealing alternatives uses the nontoxic fluorescent dye Calcein AM [9]. This assay parallels CRA in that the target cells are loaded with the dye, which is released when the target cell membrane is disrupted. This assay has shown similar percent specific lysis results to that of CRA [10]. Cytotoxicity assay based on calcein release has been previously reported for NK cells [11, 12].

The calcein release assay like CRA is an indirect method as the percent lysis is determined through the quantitation of released reporter. Therefore it is conceivable that any retention of the reporter in the dead cell will negatively impact the accuracy of the percent specific lysis reported by the assay. To this end we have previously shown that the sensitivity of detecting percent specific lysis by calcein release assay could be affected by potential incomplete release of calcein into the supernatant when the target cells undergo apoptotic death [13]. Apoptotic bodies appear as cellular "blebs" that can retain cellular contents including calcein. Therefore we developed an image cytometry-based method using Cellometer Vision image cytometer, a direct method to evaluate cellular cytotoxicity, to overcome the intrinsic limitations of the release-based assays [13]. The Cellometer Vision image cytometer has been previously used to perform rapid and accurate cell counting, morphology analysis, and cell-based assays [14, 15].

In this chapter, we are providing a protocol that uses Cellometer Vision image cytometry to directly assess NK cell cytotoxicity. Since the image cytometry is a direct method, the percent specific lysis is determined by counting the live target cells that remain at the end of the assay. This method also improves the accuracy of the cytotoxicity assay through its ability to exclude the apoptotic bodies from live cell counts based on the size as well as relative fluorescence intensity. The method described here is simple and sensitive for assessment of cellular cytotoxicity.

2 Materials

1. K562 cells (or target cell of choice, *see* **Note 1**).
2. NK cells (primary or activated/expanded).
3. RPMI complete medium: RPMI 1640 medium supplemented with 10 % FBS, 1× Pen-Strep and 1× GLutaMAX.
4. NK cell medium: RPMI 1640, 10 % FBS, 1× Pen/Strep, 1× Glutamax, freshly supplemented with 50IU/ml of IL2 (Proleukin, Novartis Vaccines and Diagnostics Inc) each time just prior to use.

5. Cellometer Vision Image Cytometer (Nexcelom Bioscience).
6. Cellometer Counting Chambers (Nexcelom Bioscience).
7. Acridine Orange and Propidium Iodide.
8. Calcein AM (1mg/ml).
9. FCS Express (DeNovo or other software of choice to analyze .csv files).

3 Methods

3.1 Cytotoxicity Assay Setup

The setup of cytotoxicity assay is described here for K562 cell line, this methodology was tested with other suspension cell lines (721.221, Jurkat and Molm-13). We have not used adherent cell lines in our studies, however the method can be applied to adherent cell lines following appropriate cell dissociation steps using either nonenzymatic cell dissociation buffer or trypsin, as long as the control group (Spontaneous control—group without NK cells) is treated similarly. The source of NK cells for the assay will depend on the research question. The assay is compatible with primary as well as activated and expanded NK cells. Here, we are providing protocol for activated and expanded NK cells, with notes on assay optimization to use primary NK cells as effectors.

3.1.1 Preparing NK Cells

1. If the NK cells used in the study are freshly isolated primary NK cells, rest them overnight in NK cell medium before cytotoxicity assay, or if the NK cells (activated/expanded) are in active culture directly proceed with the assay.
2. If the NK cells are stored frozen, thaw the cells and culture in NK cell medium for 1–2 days prior to the assay for full recovery from thaw.
3. Count the NK cells using trypan blue exclusion method or by Image cytometry using live/dead staining with Acridine Orange and Propidium Iodide (*see* **Note 2**).
4. Recover desired number of NK cells (1×10^6 expanded NK cells per condition per tumor target) (*see* **Note 3**) and spin at $400 \times g$ for 5 min.
5. Resuspend the NK cell pellet in RPMI complete medium at 2×10^6 cells per ml (*see* **Note 4**).
6. Seed 100 μl of NK cells per well in triplicates in a "U" bottom 96 well plate (e.g., A1;B1;C1).
7. Perform 3–5 twofold serial dilutions (*see* **Note 5**), by transferring 50 μl of NK cells to adjacent wells containing 50 μl of RPMI complete medium.
8. Place the plate in CO_2 incubator at 37 °C while the target cells are stained with Calcein AM.

3.1.2 Calcein AM Staining of Target Cells and Assay Setup

1. Maintain K562 cell line in complete RPMI culture medium.
2. To stain K562 with Calcein AM—count and transfer desired number of K562 cells (*see* **Note 6**) into a 15 ml conical tube.
3. Spin cells at 400 × *g* for 5 min.
4. Resuspend K562 cells at a cell density of 1×10^6/ml in RPMI complete medium.
5. Add stock Calcein AM (1 mg/ml) to the cells at 1:500 fold dilution (2 μl/ml of cells).
6. Allow cells to incubate with Calcein AM at 37 °C in a CO_2 incubator for 30 min.
7. Vortex cells every 5 min to promote uniform labeling.
8. Wash the cells to remove excess Calcein AM, using 10 ml of RPMI medium.
9. Spin cells at 400 × *g* for 5 min.
10. Repeat wash step two more times (*see* **Note 7**).
11. Resuspend K562 cell pellet (will appear green), initially based on the prestain cell number (from **step 5**), in RPMI complete medium at a cell density of ~2×10^6 cells/ml.
12. Perform a cell count of the calcein stained K562 cell resuspension and add more medium to adjust the final cell density to 1×10^6 cells/ml (*see* **Note 8**).
13. Remove the 96 well plate from the incubator and add 50 μl of K562 to the wells containing NK cells (50,000 cells/well).
14. Also add 50 μl of calcein stained K562 to six replicate wells (containing 50 μl RPMI medium) designated as Spontaneous control (No-NK cell control).
15. Spin the plate at 100 × *g* for 1 min to initiate cell contact and incubate the plate at 37 °C in a CO_2 incubator for 4 h.
16. After 4 h, gently yet thoroughly mix the contents of each well to uniformly suspend the cells (*see* **Note 9**).
17. Recover 20 μl of cells from each well and load in to Cellometer counting chamber. Take care to avoid bubbles.
18. Image the counting chamber using Cellometer Vision Image cytometer.

3.2 Acquisition and Analysis of Data by Image Cytometer

3.2.1 Imaging/ Acquiring Data

1. Switch on the Cellometer Vision Image Cytometer and open the Cellometer software.
2. Under Options → Save Options, ensure your software is setup to auto save the data.txt file and auto save your raw sample images. These raw sample images can always be reanalyzed under different assay conditions in the future if needed.
3. Select the appropriate assay from the drop down menu. There are two options for assay type: (1) Brightfield-based

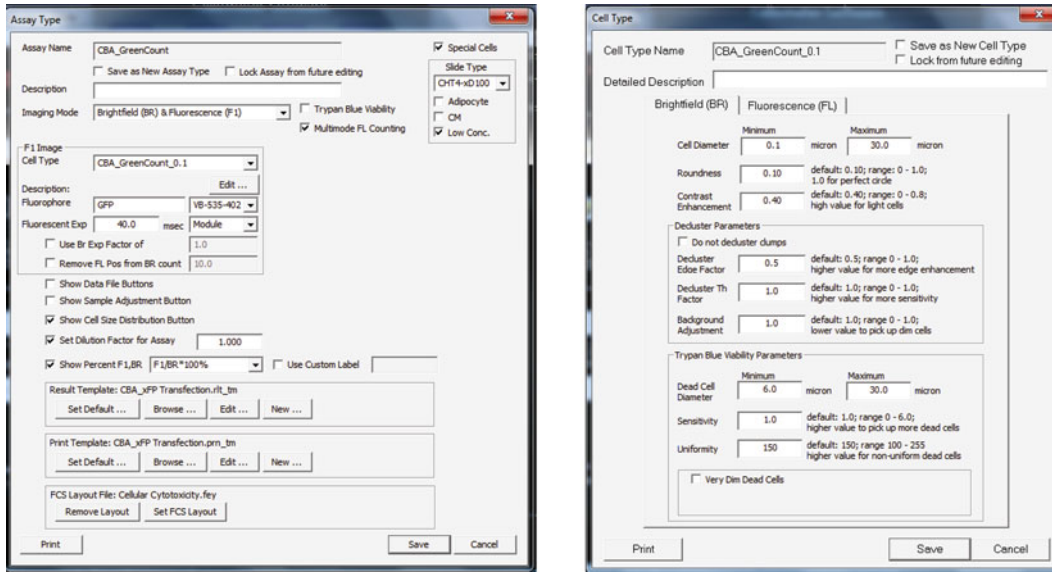

Fig. 1 The parameters for the "CBA_GreenCount" Assay and "CBA_GreenCount_0.1" Cell Type shown here can be used to create an assay based on Brightfield cell recognition with fluorescence quantification and analysis in FCS Express

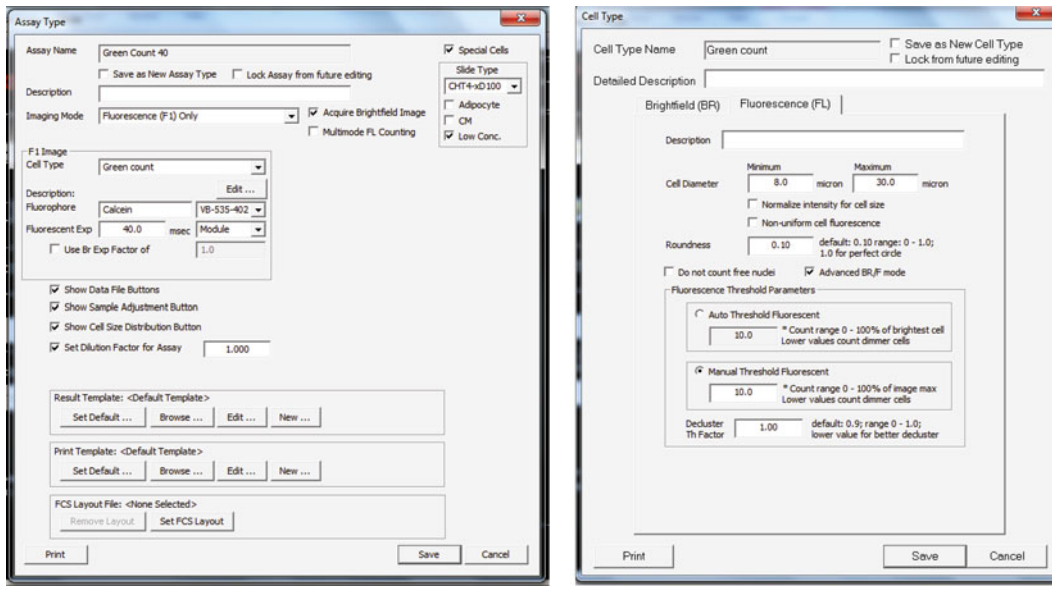

Fig. 2 The parameters for the "Green Count 40" Assay and "Green Count" Cell Type shown here can be used to create an assay for Fluorescence-based cell counting and direct reporting in Cellometer software

cell recognition and fluorescence quantification analysis using FCS Express (Fig. 1) (*see* **Note 10**), or (2) fluorescence-based cell counting leading to cell counts reported directly by Cellometer software (*see* **Notes 11** and **12**) (Fig. 2). If analysis will be done in FCS Express, ensure your software is

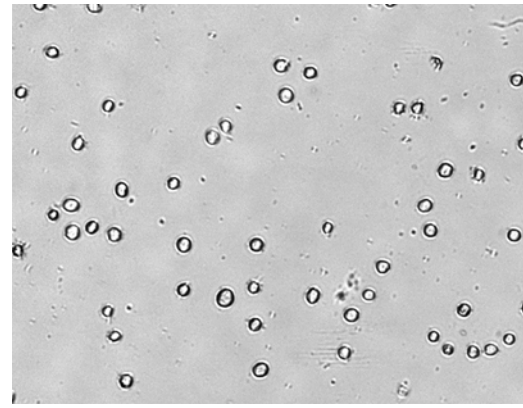

Fig. 3 Example Cellometer Vision image of K562 cells in focus

set to auto export to Nexcelom Data Package. If using a different software package to generate the histograms, you will have the option to export your results to .csv file once the report pops up—do this for each sample.

4. Insert the Cellometer counting chamber, sample side first. Start with one of your Spontaneous Control samples to ensure proper optimization of focus and exposure time.
5. Click "Set Sample ID" and type in a name for your sample.
6. Click "Preview Brightfield Image" and adjust the focus using the focus knob on the Cellometer. Ideal focus is a dark, crisp outline and a white center to your cell (Fig. 3). Once you have a good focus, click "Stop Preview" (*see* **Note 13**).
7. Click "Preview F1 Image" and ensure that the green fluorescence of the Calcein AM is clearly visible without much background (*see* **Note 14**).
8. Once the focus and exposure time are set, click "Count." The Cellometer will capture the images and analyze them to report your results.
9. Close the report and repeat **steps 4–8** until your cell samples from every well have been imaged (*see* **Note 15**).

3.2.2 Data Analysis and Percent Specific Lysis

1. If fluorescence-based cell counting is performed, open the data.txt file with automatically saved cell counts. Open the file in Microsoft Excel or transfer the data to a software package of your choice (skip to **step 3**).
2. If brightfield-based cell counting is performed, the fluorescence quantification can be done in FCS Express. Open FCS Express and load all of the .nxdat Nexcelom Data Files into the data list. Plot the Spontaneous Control samples first and set the gate for the histogram to include the peak of bright, intact K562 cells, and exclude any of the dimmer objects that

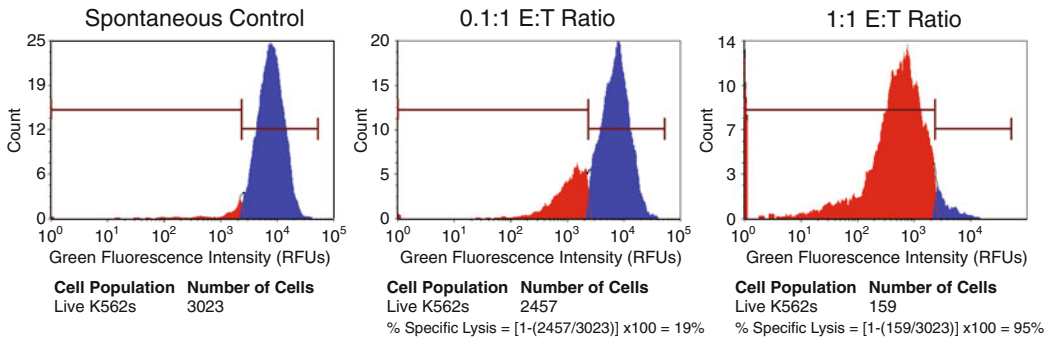

Fig. 4 Example histograms of fluorescence intensity showing correct gating based on the Spontaneous Control. The same gate that was set for the Spontaneous Control is applied to the samples from 1:1 *E*:*T* ratio and 0.1:1 *E*:*T* ratio. Reported cell numbers are used to calculate percent specific lysis. Adapted from PloSOne "Somanchi, S. S., McCulley, K. J., Somanchi, A., et al. (2015) A Novel Method for Assessment of Natural Killer Cell Cytotoxicity Using Image Cytometry. *PLoS ONE* 10, *e0141074*"

may be debris. Apply this same gate to all of your experimental samples and record the reported cell number (*see* **Note 16**).

3. Calculate the average cell count from Spontaneous Control sample replicates.
4. Calculate the percent specific lysis from each of your experimental samples by using the formula $[1-(\text{Count}^{\text{experimental}}/\text{Count}^{\text{avg spontaneous}})\times 100]$. You can then calculate standard deviations based on the replicates (Fig. 4).

4 Notes

1. Currently, we have only tested suspension cell lines. Adherent cell lines can be used for the assay with the inclusion of a trypsinization step (or nonenzymatic dissociation) to ensure complete recovery of target cells (not described here).
2. NK cell viability of >85 % is desired for running a reliable assay, if the viability is lower, perform a Ficoll-Paque centrifugation as per manufacturer's recommendation.
3. For primary NK cells it may be desirable to use higher *E*:*T* ratios starting from 10:1, in this case seed target cells at a density of 25,000 tumor target cells per well, to prevent overcrowding. Therefore, 2×10^6 primary NK cells (including extra for error) will be required per assay condition per tumor target.
4. If primary NK cells are used for the assay, resuspend NK cells at density of 5×10^6 cells/ml, in order to accommodate the 10:1 *E*:*T* ratio when using a target cells at 25,000 cells per well.

5. Perform at least three dilutions for expanded NK cells (starting at *E*:*T* ratio of 2:1), to generate *E*:*T* ratios of 2:1, 1:1, and 0.5:1 ratios (*E*:*T* ratios as low as 0.1:1 can be tested). For primary NK cells (starting at *E*:*T* ratio of 10:1) perform five serial dilutions to generate 10:1, 5:1, 2.5:1, 1.25:1, and 0.6:1 *E*:*T* ratios.
6. Calculate the number target cells required for the assay by multiplying *50,000 cells per well* with *number of replicates* × *number of E:T ratios being tested* × *number of conditions being tested* + 20% error (to accommodate cell loss during wash steps). If primary NK cells are used in the assay, substitute the 50,000 target cells per well with 25,000 cells per well.
7. It is important to completely remove excess calcein from the target cells, to avoid staining NK cells with residual calcein during cytotoxicity assay coculture.
8. When using primary NK cells the targets cells are resuspended at a final cell density of 0.5×10^6 cells/ml (25,000 cells/well).
9. Although it is important to mix thoroughly to suspend the cells completely and uniformly, vigorous pipetting should be avoided to prevent cell death due to pipetting.
10. The brightfield-based cell recognition and fluorescence quantification means that the Cellometer software will identify every object with a dark outline in the captured brightfield images, which will include K562s, NK cells, and also smaller debris. For every identified object, the fluorescence intensity of each pixel in the object is summed up and these values per object are exported into FCS Express and placed into a histogram of fluorescence intensity. Fluorescence-based cell counting will capture brightfield images as a reference, but will only analyze the fluorescence images to identify fluorescent objects that meet specific size and intensity criteria as set in the Cellometer cell type associated with the assay. Brightfield-based counting allows more detailed gating to be performed within the FCS Express software, though Fluorescence-based counting methods should yield comparable results and can be performed on a wider range of Cellometer instruments without the need to use the additional software package.
11. The check mark next to "Low Conc" in the assay type tells the software to capture images from eight fields of view. The cell type setting of cell diameter from 0.1 to 30 μm under the "Brightfield (BR)" tab ensures all objects that appear in the brightfield images are identified by the Cellometer software. Ensure that the "Manual Threshold Fluorescent" value under the "Fluorescence (FL)" tab is set to 0, which allows fluorescence intensities from all objects to be included and exported into the histogram of fluorescence intensity.

12. The cell type setting of cell diameter from 8 to 30 μm under the "Fluorescence (FL)" tab ensures that only intact K562 cells will be counted and smaller fluorescent objects (debris or apoptotic bodies) will be ignored. The cell size should be set based on your Spontaneous Control and may change by target cell line. The other parameter to adjust is the "Manual Threshold Fluorescent" which is set to 10 in this example. Higher thresholds will exclude dimmer objects. This threshold should also be set based on your Spontaneous Control according to Calcein AM staining intensity.
13. Focus on the K562 target cells (which are larger) instead of the NK cells. The target cells are the most important for analysis and hence should determine the focal position.
14. We found that an exposure time of 40 ms was ideal for K562 cells and the described staining procedure. You can adjust the exposure time so that the displayed "Percent of Range" is in the 90 s (100% would indicate saturation). The exposure time should be set based on the brightness of the Spontaneous Control and should not be adjusted for other samples. At higher *E*:*T* ratios, you may not see much fluorescence signal because of the high cytotoxicity; this is normal.
15. We have found the most efficient way to quickly analyze every sample is to have one person mixing the samples from the wells and pipetting onto the Cellometer counting chamber, and one person performing the image capture and analysis.
16. Gating can be set so as only to include high fluorescence intensity objects which will be the intact, live K562 cells. K562s that have interacted with NK cells will have much lower overall fluorescence intensity since the majority of the Calcein AM will have leached out. Apoptotic bodies will be much smaller than a live K562 cell and so their summed up fluorescence intensity will fall below the gating threshold for a much larger, intact, live K562 cell. NK cells will also fall below the gating threshold because they were not stained with Calcein AM, regardless of cell size.

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Chapter 10

Analysis of Intracellular Ca^{2+} Mobilization in Human NK Cell Subsets by Flow Cytometry

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Abstract

In signaling cascades downstream of NK cell activating receptor engagement, Ca^{2+} ions are pivotal second messengers for NK cell cytotoxicity as well as cytokine production. Upon cellular activation, intracellular mobilization of Ca^{2+} ions initially involves depletion of endoplasmic reticulum stores, leading to subsequent Ca^{2+} influx through specific plasma membrane Ca^{2+} release activated Ca^{2+} channels. Multiple probes and assays for detecting intracellular Ca^{2+} concentrations have been developed. With the advance of multiparameter flow cytometry instrumentation, a thorough analysis of signaling in specific NK cell subsets is possible. Here, a flow cytometric method for dynamic measurements of intracellular Ca^{2+} concentrations in human NK cells subsets is detailed and discussed. This assay can be further adapted for specific scientific and diagnostic questions, with implications for various immunopathological conditions.

Key words Human, Natural killer cells, Activation, Ca^{2+} flux, Polychromatic flow cytometry

1 Introduction

Natural killer (NK) cells are lymphocytes that can recognize and eradicate infected and malignant cells [1]. Target cell killing is mediated via directed exocytosis of granules containing cytotoxic proteins. Activation of NK cells typically also involves release of cytokines that promote host resistance to infection. Besides such activities that promote immune responses, NK cells can control excessive immune activation through killing of activated immune cells.

NK cell activity is regulated by a multiplicity of germline-encoded activating and inhibitory receptors [2]. NK cell effector functions impinge on mobilization of intracellular Ca^{2+} ions. Activating NK cell receptors recruit and activate phospholipase C (PLC)-γ at the cell membrane for sustained Ca^{2+} signaling, a prerequisite for exocytosis of cytotoxic granules as well as transcription of cytokine-encoding genes. Of note, engagement of chemokine receptors on NK cells can also induce transient

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intracellular Ca^{2+} mobilization [3], without necessarily leading to cytotoxic granule exocytosis or cytokine production.

Phospholipase C-γ (PLC-γ) can hydrolyze phosphatidylinositol 4,5-bisphosphate to produce the second messengers diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). Whereas DAG remains bound to the membrane, IP_3 can diffuse through the cytosol to bind calcium channels in the smooth endoplasmic reticulum (ER). This causes increases in the cytosolic concentration of Ca^{2+} ions within seconds of activating receptor engagement. Both PLC-γ1 and PLC-γ2 have been implicated in human NK cell signaling [4], with pharmacological inhibition of PLC-γ abrogating signaling downstream of multiple activating receptors [5]. By comparison, PLC-γ2 appears to be the main isoform in mouse NK cells, with PLC-γ2 deficiency leading to dysfunctional responses to malignant and infected cells as well as deficiencies in terminal differentiation and maturation, as shown by a marked general decrease of Ly49 expression [6–8]. Importantly, depletion of Ca^{2+} ions from the ER can in turn activate Ca^{2+} release activated Ca^{2+} (CRAC) channels on the plasma membrane, leading to subsequent influx of Ca^{2+} from the extracellular space that augment intracellular Ca^{2+} concentrations [9]. Molecularly, STIM-1 is a sensor of ER Ca^{2+} concentrations. Upon Ca^{2+} depletion of the ER, STIM-1 oligomerizes, binds to and opens ORAI-1, a CRAC channel. STIM-1 or ORAI-1 deficiency is associated with a combined immunodeficiency syndrome [10, 11]. Upon encounter with susceptible target cells, NK cell cytotoxic granule exocytosis and cytokine production are severely comprised in patients with biallelic *STIM1* or *ORAI1* mutations [12].

Cell permeable Ca^{2+} concentration-sensitive compounds readily enable flow cytometric real-time assessment of Ca^{2+} mobilization. Thus, Ca^{2+} measurements can provide insights to early phases of lymphocyte activation. Dynamic measurements of Ca^{2+} flux in single cells in general [13], and NK cells in particular, have been performed for 30 years [14]. Flow cytometric single-cell measurements of intracellular Ca^{2+} levels provide a snapshot of a cell's Ca^{2+} levels. In each individual cell, Ca^{2+} levels may rapidly oscillate [15]. Flow cytometric measurements of intracellular Ca^{2+} levels in a number of cells per time unit thus reflect average Ca^{2+} levels, with an increasing fraction of cells expected to display high Ca^{2+} levels upon stimulation.

As more complex flow cytometers have been developed, increasing numbers of cell populations can be assessed simultaneously [16]. In this chapter, we detail a method for dynamic, ratiometric detection of Ca^{2+} flux in defined human NK cell subsets, exemplified as $CD56^{bright}$ and $CD56^{dim}$ NK cells, among peripheral blood mononuclear cells (PBMC). Recently, MacFarlane and colleagues have described a similar method for detection of Ca^{2+} flux

in mouse NK cell subsets [17]. Our protocol entails cross-linking biotin-conjugated agonistic mAbs to activating NK cell receptors after staining of PBMC with markers for positive and negative NK cell selection. The extracellular staining panel provides a general guideline, allowing for addition of other markers of interest for further dissection of Ca^{2+} signaling in specific NK cell subsets. As patients with defects in Ca^{2+} flux signaling are associated with severe immunodeficiency syndromes [12], measurement of Ca^{2+} flux in NK cells may not only provide insights into the underpinnings of functional differences in NK cell subsets, but also enable the identification of novel immunodeficiency syndromes affecting NK cell functionality.

2 Materials

2.1 Cells, Media, and Solutions

1. Whole blood collected in sodium heparin vials (10 ml should provide sufficient cell numbers for the described assay) (*see* **Notes 1** and **2**).
2. Lymphoprep (Axis-Shield) stored at room temperature and protected from light.
3. Optional: NK cell negative isolation kit (Miltenyi) (*see* **Note 2**).
4. Complete culture medium: RPMI-1640, supplemented with 10% heat-inactivated FBS and 1 mM l-glutamine.
5. Assay buffer: Hanks buffered salt solution (HBSS, containing 1.26 mM Ca^{2+}) supplemented with 1% heat-inactivated FBS.
6. 100 mM Probenecid (Sigma-Aldrich, St Louis, MO) solution prepared by resuspending Probenecid in 0.1 M NaOH and adjusting to pH 7.4 (*see* **Note 3**).
7. Phosphate buffered saline solution (PBS).

2.2 Antibodies and Fluorescent Reagents for Ca^{2+} Flux Detection

2.2.1 Stimulating Mouse IgG_1 Isotype mAbs (See Note 4)

1. Biotin-conjugated anti-CD16 mAb (clone 3G8, BD Biosciences).
2. Biotin-conjugated anti-CD226 mAb (clone DX11, BD Biosciences).
3. Biotin-conjugated anti-CD244 mAb (clone C1.7, eBioscience).
4. Biotin-conjugated isotype control mAb (clone MOPC-21, BD Biosciences).

2.2.2 Fluorochrome-Conjugated mAbs

1. BrV785-conjugated anti-CD3 mAb (clone OKT3, BioLegend).
2. Qdot605-conjugated anti-CD4 mAb (clone S3.5, Invitrogen).
3. APC-Cy7-conjugated anti-CD14 mAb (clone MφP9, BD Biosciences).
4. APC-Cy7-conjugated anti-CD19 mAb (clone SJ25C1 BD Biosciences).

5. PE-Cy7-conjugated anti-CD56 mAb (clone NCAM 16.2, BD Biosciences) (*see* **Note 5** *for a discussion on additional antibodies*).

2.2.3 Fluorescent Reagents

1. Pacific blue-conjugated streptavidin (Invitrogen).
2. LIVE/DEAD Fixable Near-IR dead Cell Stain (Invitrogen) (*see* **Note 6**).
3. Fluo 4 (Invitrogen) resuspended to 2 μg/ml in DMSO.
4. Fura Red (Invitrogen) resuspended to 2 μg/ml in DMSO.

2.3 Flow Cytometry Hardware and Software

1. The analysis of Ca^{2+} flux in NK cell subsets described here is optimized for a LSR Fortessa (BD Biosciences) equipped with a 405 nm, a 488 nm, a 561 nm, and a 635 nm laser. Table 1 provides a detailed description of the instrument laser and filter configuration for the included fluorochromes.
2. FlowJo software (version 9.9) can be used for analysis of the acquired raw data.

2.4 Other Material

1. Anti-mouse Ig κ compensation beads (BD Biosciences).
2. 280–300 μl 96-well polystyrene conical bottom plate.
3. 50 ml polypropylene falcon tubes for PBMC isolation.
4. 5 ml polystyrene falcon tubes for Ca^{2+} flux samples.
5. 1.2 ml polypropylene FACS tubes for compensation beads.

Table 1
Laser and filter setup, LSR Fortessa

Laser (nm)	Filter	Flourochrome	Target
488	530/30 nm	Fluo 4	Ca^{2+}
488	670/30 nm	Fura Red	Ca^{2+}
650	780/60 nm	APC-Cy7	CD14 + CD19
650	780/60 nm	Near-IR	Dead cell marker
405	450/50 nm	PacB	Biotin
405	610/20 nm	Qdot605	CD4
405	780/60 nm	BrV785	CD3
561	586/15 nm	PE	NKG2C
561	620/14 nm	PE-CD594	CD57
561	710/50 nm	PE-Cy5.5	CD56
561	780/60 nm	PE-Cy7	NKG2A

3 Methods

In this example, intracellular Ca^{2+} concentrations are measured in PBMC. In total, seven fluorochrome channels are used, two for dynamic assessment of Ca^{2+} fluxes (Fluo-4 and Fura Red), one for elimination of dead cells as well as CD14 and CD19 expressing cells (combined dump channel), two additional channels for exclusion of cells expressing CD3 and CD4 (if desired, facilitating analysis of T cell subsets), one for identification and discrimination of NK cell subsets on the basis of CD56 expression, and a final channel for detection of fluorochrome-conjugated streptavidin binding and cross-linking of cells. A number of useable channels have been left open, which can facilitate users' adaption of the method for further analysis of specific subsets. For a 4-laser flow cytometer it is however not recommended to extend beyond 10–12 open channels (*see* **Note 5**). In this assay, NK cells are stimulated streptavidin-mediated cross-linking of biotinylated antibodies prebound to the cell surface.

3.1 Preparation of Peripheral Blood Mononuclear Cells

1. Isolate PBMCs from heparinized whole blood samples by density gradient centrifugation with Lymphoprep according to the manufacturer's instructions (*see* **Note 7**).
2. After centrifugation, harvest the PBMC in a 50 ml Falcon tube and wash with PBS.
3. Centrifuge the cells at $450 \times g$ for 10 min.
4. Discard the supernatant and wash the cell pellet twice in PBS.
5. Optional: Purify NK cells by negative selection using an NK cell isolation kit according to the manufacturer's instructions (*see* **Note 2**).
6. Resuspend cells in complete culture medium at a concentration of $2–5 \times 10^6$ cells/ml.
7. After isolation, maintain effector cells overnight in complete culture medium in an incubator set at 37 °C and 5% CO_2 (*see* **Note 8**).

3.2 Staining, Stimulation, and Acquisition

3.2.1 Ca^{2+} Flux Detection

1. Prepare surface antibody staining solution master mix containing anti-CD3, anti-CD4, anti-CD14, anti-CD19, anti-CD56, and a dead cell marker. Table 2 provides a summary of the suggested antibody panel including recommended dilutions of the fluorochrome-conjugated mAbs (*see* **Notes 9** and **10**).
2. Prepare solutions of the biotinylated antibodies:
   (a) Isotype control mAb.
   (b) Anti-CD16 mAb.
   (c) Anti-2B4 plus anti-DNAM-1 mAbs.

Table 2
Recommended antibody dilutions for staining solution

Laser (nm)	Filter	Flourochrome	Target	*n* of µl/50 µl solution
635	780/60 nm	APC-Cy7	CD14	1
635	780/60 nm	APC-Cy7	CD19	1
635	780/60 nm	Near-IR	Dead cell marker	0.1
405	610/20 nm	Qdot605	CD4	0.2
405	780/60 nm	BrV785	CD3	1
561	710/50 nm	PE-Cy5.5	CD56	0.3
		Assay buffer		43.3

All antibodies should have the final concentration 1 µg/ml (*see* **Note 11**).

3. Spin down the PBMC at 300 × *g* for 4 min and resuspend in ice-cold assay buffer at 1×10^7 cells/ml (*see* **Note 12**).
4. Transfer 200 µl to each used well in a precooled 96-well conical bottom plate, remember to add three samples for negative and single-stained compensation controls for the Fluo-4 and Fura Red.
5. Spin down the plate at 300 × *g* for 4 min in a centrifuge cooled to 4 °C. Remove the supernatants gently with pipettes.
6. Add 50 µl of surface antibody staining solution (**step 1**).
7. Add 50 µl of solutions containing biotinylated antibodies for stimulation (**step 2**) to separate wells as indicated (Fig. 1).
8. Add 100 µl of assay buffer to the compensation controls (Fig. 1).
9. Incubate the cells on ice for 30 min.
10. During the incubation, prepare the dye loading solution and single-stained Ca^{2+} dye controls. (*See* Table 3 for calculations.)
11. To prepare dye loading solution, first aliquot sufficient assay buffer (92 µl/sample) based on number of samples to be tested + five (equating to four controls and an additional sample to ensure sufficient pipetting volume), also include additional volume to accommodate for losses during the procedure (at least 10%).
12. Then add probenecid to the assay buffer (8 µl/92 µl of assay buffer).
13. Aliquot 200 µl to a separate tube (negative control).
14. Aliquot 200 µl to a separate tube and add 1 µl Fura Red (Fura Red control).

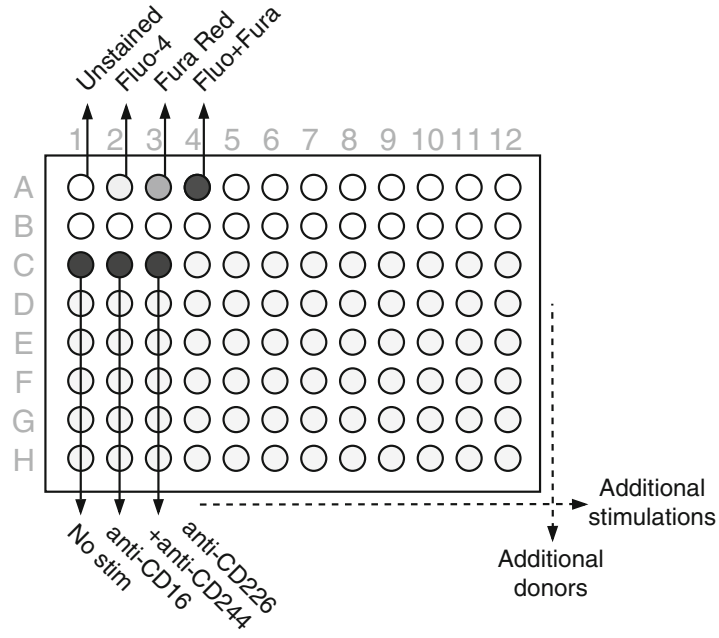

Fig. 1 Overview of suggested plate layout of samples. The *upper left corner*, wells *A1–4*, contain the cells control stained for Fluo-4, Fura Red, or the combination of these. Wells *C1–3* contain Fluo-4 and Fura Red-stained cells precoated with biotinylated antibodies to surface receptors, as indicated, for subsequent streptavidin-mediated cross-linking. The assay can be further expanded. For example, in wells *C4–12*, there is space for additional stimulations whereas *rows D–H* could be loaded with cells from additional donors

Table 3
Reagent calculator for dye loading buffer

Reagent	Amount/sample (μl)	Total amount (μl)
Probenecid	8	$8\times(n+5)$
Assay buffer	92	$92\times(n+5)$
Fluo 4	0.2	$0.2\times(n+1.5)$
Fura Red	0.5	$0.5\times n$(+1 separately for control)

n = number of samples (staining controls not included)
NB! It is advised to include a 10% margin for all buffers. This is not included in the calculations above

15. Aliquot 200 μl to a separate tube and add 0.4 μl Fluo 4 (Fluo 4 control).
16. To the remaining buffer add Fluo 4 (at 0.2 μl/100 μl) and Fura Red (at 0.5 μl/100 μl)—label as dye loading solution.
17. Add 100 μl dye loading solution (**step 16**) to each well containing cells.

18. Add 100 μl of the compensation control solutions (from **steps 13–15**) to the compensation samples.
19. Keep cells on ice for another 30 min.
20. During these 30 min, warm up a water bath to 37 °C. It is recommended to have the water bath within an arm-length distance of the flow cytometer.
21. Spin down the plate at 300 × *g* for 4 min in a centrifuge cooled to 4 °C. Remove supernatants gently by pipetting.
22. Add 250 μl ice-cold assay buffer.
23. Spin down the plate at 300 × *g* for 4 min in a centrifuge cooled to 4 °C. Remove the supernatants gently by pipetting.
24. Keep the cells on ice.
25. Prepare the cross-linking solution by diluting streptavidin-Pacific blue in assay buffer to a concentration of 0.1 μg/ml (*see* **Note 11**).
26. Individually, for every sample immediately prior to flow cytometric analysis (*see* **Note 13**), add 130 μl assay buffer prewarmed to 37 °C to a 5 ml FACS tube and resuspend the cells in another 130 μl prewarmed assay buffer and transfer to the FACS tube, rendering a total sample volume of 260 μl.
27. Preincubate sample for 3 min at 37 °C in the water bath.
28. Set appropriate flow speed (*see* **Note 14**).
29. Run sample for 30 s on flow cytometer to establish a base line level of fluorescence for the Ca^{2+}-sensitive dyes.
30. Take the sample out while the flow cytometer continues to run. Add 10 μl of cross-linking solution, vortex for 2 s, and immediately place the sample back on the flow cytometer and continue acquiring for a total of 4–5 min.

3.2.2 Control Samples for Compensation of Spectral Overlap

1. For compensation of Fluo 4 and Fura Red, use the cell samples unstained or stained with each of the Ca^{2+}-sensitive dyes. *See* Subheading 3.2.1 for details on the staining procedure.
2. For the remaining fluorophores, use single stained or unstained anti-mouse Ig κ compensation beads. Perform the staining procedure in 1.2 ml FACS tubes.
3. Add one drop of anti-mouse Ig κ compensation beads and 200 μl of assay buffer to each tube.
4. Add 1 μl of APC tandem conjugates or 0.3 μl of all other fluorochromes to each tube, with only one fluorochrome-conjugated antibody per tube (*see* **Note 15**).
5. Perform staining for 10 min at room temperature in the dark.
6. Acquire data for each sample on the flow cytometer (*see* **Note 16**). Prior washing of the beads is unnecessary.

3.3 Flow Cytometry Compensation and Analysis

1. For generation of a compensation matrix, use the FlowJo compensation wizard or similar, which accepts multiple negative controls. For Fluo 4 and Fura Red, use unstained and single-stained PBMC as negative and positive controls, respectively. For the remaining fluorochromes, use unstained beads and single-stained beads as negative and positive controls, respectively. After the creation of the compensation matrix, perform a custom biexponential transformation for all channels. Thereafter, visually inspect all fluorochrome combinations, in order to identify and correct possible over- and undercompensation created by the automatic FlowJo compensation algorithm.
2. Following compensation of all samples, identify the NK cell populations of interest by manual gating.
3. Identify lymphocytes by FSC/SSC characteristics.
4. Exclude cell conjugates on FSC-H/FSC-A characteristics.
5. Exclude CD3, CD4, CD14 and/or CD19 positive events.
6. Define NK cells as CD56 positive cells.
7. Divide NK cells based on expression levels of CD56. *See* Fig. 2a for a suggested gating strategy.
8. Derive a ratio parameter by dividing the compensated Fluo 4 by the compensated Fura Red in FlowJo or similar software (*see* **Notes 17** and **18**).
9. Using a kinetics platform in FlowJo or similar software, overlay and compare different responses (*see* **Notes 19** and **20**, Fig. 2b, c).

4 Notes

1. Instead of PBMC isolated from fresh whole blood, PBMC isolated from buffy coats or PBMC thawed from frozen aliquots of cells that were frozen down immediately after isolation can be analyzed with the described assay. In either case, it is recommended to rest the cells overnight as described under Subheading 3.1. This step allows equilibration of intercellular Ca^{2+} concentrations prior to analysis.
2. Robust Ca^{2+} flux measurements require a minimum number of cells of interest per second upon acquisition on the flow cytometer. Low frequencies of NK cells in general or of subsets of interest in particular can hamper analysis. Therefore, prescreening for individuals with high percentages of NK cells in PBMC might facilitate analyses. Prescreening can however also introduce a selection bias. Alternatively, NK cells can be isolated for more focused analysis, facilitating a higher acquisition rate of NK cells in the analysis. In addition, excluding the possibility

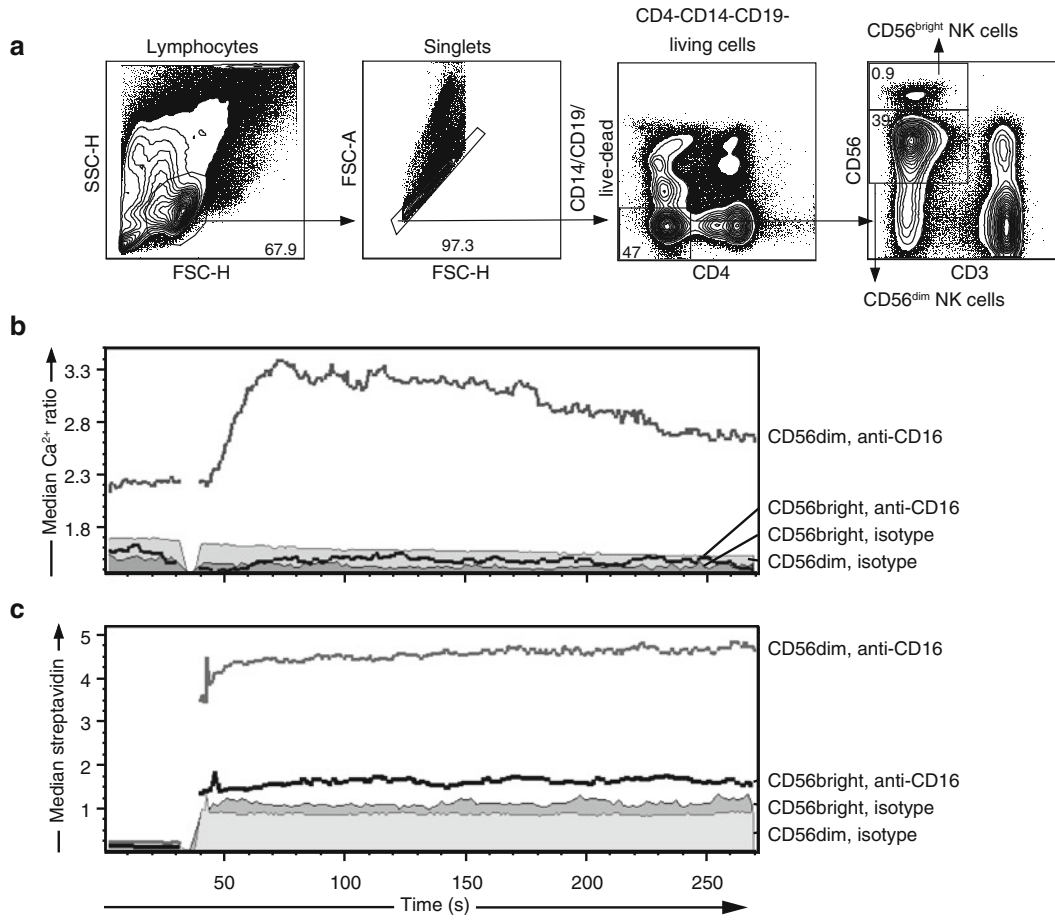

Fig. 2 (**a**) Gating strategy for selection of NK cells. Lymphocytes were selected on forward scatter/side scatter characteristics, doublets were excluded using the relationship between area and peak measurements for forward scatter, Dead, and/or CD4, CD14, or CD19 positive cells were excluded, and NK cells were identified as either $CD3^{-}CD56^{dim}$ or $CD3^{-}CD56^{bright}$. (**b**) Median Fluo-4/Fura Red curves after cross-linking of anti-CD16 and isotype control antibodies are shown for $CD56^{dim}$ and $CD56^{bright}$ NK cells. Gaussian smoothing is applied. (**c**) Median streptavidin-Pacific blue curves after cross-linking of anti-CD16 and isotype control antibodies are shown for $CD56^{dim}$ and $CD56^{bright}$ NK cells. Gaussian smoothing is applied

of NK cell cross talk with other cells that might confound interpretation of data on the responsiveness of NK cells might under some circumstances be necessary. If small NK cell subsets are to be investigated or if cell type cross talk should be avoided, inclusion of the optional NK cell isolation step might be necessary.

3. The Fluo-4 and Fura Red dyes in this assay are acetoxymetyl esters, and are thus uncharged and cell permeable. When entering a cell, esterases cleave off the ester side-chain, leaving a charged fluorescent molecule that no longer can diffuse over

the cell membrane. To prevent organic anion transporters from actively pumping out the dyes, reducing the dye concentrations, probenecid is added. Probenecid acts as a broad inhibitor of organic anion transporters [18].

4. Ideally, we recommend that stimulating antibodies should be of the IgG_1 subclass if antibodies raised in mice are used. The Fc portion of mouse monoclonal antibodies of the IgG_1 subclass do not bind to the human Fc receptor CD16 and do thus not by themselves induce Ca^{2+} flux. This is different for mouse IgG_{2a}, IgG_{2b}, and IgG_3 antibodies that all to different extents can bind and engage human CD16 [19].
5. In our example assay, seven fluorochrome channels are included, which are considered the base line markers needed for NK cell identification in PBMC. A number of useable channels remain unused. These can readily be adopted to facilitate users' specific purposes. For a 4-laser flow cytometer however, it is not recommended to extend over 10–12 open channels. Including more than 10–12 fluorochromes may give rise to complex compensation artifacts. Importantly, all fluorochromes with emission in the 650 nm range that are excited by either violet or blue lasers (and thus interfering with the Fura Red Ca^{2+}-free and Ca^{2+}-bound emission), such as PE-Cy5, APC, Qdot655, BV650 etc., should be avoided.
6. Especially when using previously cryopreserved cells, it is useful to include a marker for dead cell exclusion in the dump gate. If markers that have a spectral overlap with Fura Red, such as 7AAD are used, it is important to confirm that no cells are excluded due to leakage into the dead cell channel.
7. Vials for the collection of blood must contain an anticoagulant. Vials containing sodium heparin are recommended for the collection of blood samples to be used for the analysis of cellular function. Other anticoagulants such as EDTA and citrate deplete intracellular Ca^{2+} stores. Therefore, cells collected in sample vials with such anticoagulants may display functional hyporesponsiveness, unless the cells are sufficiently rested in culture medium to replenish intracellular Ca^{2+} stores. If the samples require transport to a laboratory for diagnostic evaluation, we recommend shipping them at room temperature. Furthermore, for obtaining comparable results of cellular responses from shipped blood relative to results of cells isolated directly from fresh blood, the duration from sample acquisition to isolation of PBMC should not exceed 24 h.
8. For maintenance of the cells, no cytokine stimulation is necessary.
9. Investigators are advised to titrate each fluorochrome-conjugated mAb for optimal staining. The most desirable mAb

concentration is the one that provides the brightest signal of a positive subset together with the dimmest background signal of the negative subset. The titration procedure is to be repeated for each new batch of fluorochrome-conjugated mAb.

10. Caution should be taken as to testing tandem-conjugated antibodies of interest together with probenecid, Fluo-4 and Fura Red, as in our experience quenching of especially the Cy7 portion of PE-Cy7 may occur.
11. To ensure optimal signal-to-noise ratio, it is advised to titrate both the activating antibodies and the streptavidin concentration.
12. As compared to other measurable NK cell functional responses, such as cytokine production or exocytosis, Ca^{2+} flux can be more easily triggered, and a lower threshold for activation is seen [15]. It is thus important to keep all solutions and conditions cold throughout the assay to prevent spontaneous Ca^{2+} flux occurring without the need for cross-linking. This may be especially important when stimulating via the Fc receptor CD16, for which mAb binding without secondary cross-linking is sufficient to instigate signals, increasing intracellular Ca^{2+} concentrations.
13. The total number of samples that can be acquired in one experimental round is limited by the fact that dye is pumped out of the cells over time, resulting in decreased Ca^{2+} dye signals. Moreover, if cell pellets are left for a long time on ice, they may dry out.
14. For robust Ca^{2+} flux measurements, a certain number of cell events need to be acquired per time unit. This can be attained through adjustments of cell concentrations or acquisition speed. Generally, a high cell density and low acquisition speed is beneficial as this reduces turbulence in the flow cell. At the same time, cell densities cannot exceed the capacity of the instrument. Practically, we recommend acquiring at least 500 cells of interest per second (i.e., the smallest subset of interest). On BD Fortessa instruments, a flow speed of approximately 35 μl/min is appropriate.
15. As different batches even from the same conjugate, clone, and company can differ in spectral characteristics, it is advisable to use the same antibodies for the compensation controls as for the antibody panel.
16. Acquisition of compensation control samples should be performed in the same session as the Ca^{2+} flux assay, to ensure that instrument setup, such as PMT and laser voltages, is identical.
17. Fluo-4 is a relatively low-affinity (K_d 335 nM) single-wavelength Ca^{2+} indicator that increases in green fluorescence

upon binding to Ca^{2+}. In contrast, Fura Red is a higher affinity (K_d 140 nM) dual excitation wavelength Ca^{2+} indicator whose blue laser excited red spectrum fluorescence diminishes upon binding to Ca^{2+}. This combination thus enables a ratiometric Ca^{2+} flux measurement with higher sensitivity and stability than with either dye alone [20]. Multiple other Ca^{2+} indicators, such as the dual emission UV-excited indo-1 and fura-2, are commercially available. The strong signal from Fluo-4 and the great stokes-shift of Fura Red in combination with their different Ca^{2+} affinities, their slow photo-bleaching decay, and the wide availability of blue lasers has however rendered this combination the standard for flow cytometric Ca^{2+} flux analyses. It can be further noted that with the use of a 405 laser, the signal for the Ca^{2+}-bound Fura Red can be detected in the spectrum around 630 nm. Inclusion of this signal in a multiparameter Ca^{2+} concentration detection formula could prove to even further increase the sensitivity and stability of the Ca^{2+} measurement.

18. Absolute cellular Ca^{2+} concentrations can be determined as previously described [21].
19. Considerable donor variation in NK cell Ca^{2+} flux magnitude is to be expected. Ideally, an increase of 100–300% from base line levels should be obtained with anti-CD16 mAb stimulation (that in general also has an increased base line compared to isotype controls). A lower increase is expected with most other activating receptor stimuli.
20. In the kinetics platform in FlowJo, a number of alternative visualizations and smoothing options are available. The reader is encouraged to use these in accordance with the scientific question.

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Chapter 11

Using NK Cell Lipid Raft Fractionation to Understand the Role of Lipid Rafts in NK Cell Receptor Signaling

Esther Serrano-Pertierra and Carlos López-Larrea

Abstract

Lipid rafts were first defined as detergent-resistant membranes (DRMs) due to their relative insolubility in non-ionic detergents. Although they should not be confused with lipid rafts, DRMs are a valuable starting point for the study of these membrane domains and the interactions of proteins with rafts.

Here we describe the isolation of DRMs by ultracentrifugation on a sucrose gradient, a method we have used to study the role of lipid rafts in NKG2D-mediated signaling. We also describe raft fractionation of NK cells involving the selective solubility of β-octylglucoside (β-OG). OG is a non-ionic detergent that efficiently dissolves DRMs but does not disrupt protein associations with the cytoskeleton. Using these two techniques may yield useful information about the proteins involved in receptor recruitment into lipid rafts and the interactions of the actin cytoskeleton with lipid rafts.

Key words Lipid raft, Membrane raft, DRM, NK cell, Ultracentrifugation, Sucrose gradient, β-octylglucoside

1 Introduction

Membrane rafts (also called "lipid rafts") are special compartments within the plasma membrane that act as signaling platforms. Due to their specific lipid composition, membrane rafts are relatively resistant to certain detergents (Triton X-100, octylglucoside, NP-40, some of the Brij series), and upon solubilization produce detergent-resistant membrane (DRM) complexes [1] that are believed to correspond almost exactly to the raft microdomains of intact membranes. For this reason, DRMs are widely used as an approximate biochemical equivalent of membrane rafts.

In response to cellular stimuli, lipid rafts alter their composition, including or excluding proteins involved in the activation of various signaling pathways [2]. Membrane rafts are involved in receptor signaling, intracellular transport and protein sorting, and the interaction of cells with pathogens. A functional role of

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membrane rafts has been identified from studies in hematopoietic cells. Indeed, the importance of these structures has been demonstrated in a number of studies in T lymphocytes [3]. Most research has focused on elucidating the roles of membrane rafts in T cell activation, so less is known about these compartments in NK cells. Our current knowledge indicates that NK cell receptors have a different distribution. Activating receptors must be recruited into membrane rafts to ensure their correct organization at the site of target cell contact, and thereby to guarantee their NK cell effector functions. Prevention of this recruitment by inhibitory receptors is known to impair NK cell activation [4–9].

Subsequent studies addressing the structure of membrane rafts have demonstrated the involvement of raft-associated proteins in the formation of raft domains. Interactions of these proteins with the actin cytoskeleton are able to control the membrane raft dynamics that form and maintain these domains [10, 11]. Interactions between FcεRI (the high-affinity receptor for IgE) and lipid rafts that depend on the actin cytoskeleton have been described [12, 13]. Furthermore, Chichili and Rodgers [14] used fluorescence resonance energy transfer (FRET) to demonstrate that the actin cytoskeleton promotes clustering of raft-associated proteins in Jurkat T cells, which, in turn, regulate the signaling of these proteins.

We have investigated the possible role of membrane rafts in NKG2D-mediated signaling. Analysis of DRMs isolated by sucrose gradient ultracentrifugation showed us the recruitment of the receptor to lipid rafts as well as other proteins involved in the signaling of this receptor. Subsequent proteomic analysis revealed other proteins related to the actin cytoskeleton that are actively recruited into these compartments. We then studied raft fractions solubilized with β-OG by western blot and proteomic analyses. These raft fractionation methods have contributed to our understanding of the roles of an actin bundling protein (L-plastin) in NKG2D recruitment into lipid rafts and in the regulation of physiological events such as NK cell migration [15].

2 Materials

Prepare all solutions using ultrapure water and analytical-grade reagents.

2.1 Density Gradient Ultracentrifugation

1. Lysis buffer: 150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 1% detergent (e.g., Brij-98, NP-40, Triton X-100). Store at 4 °C (*see* **Note 1**).
2. Sucrose: Prepare 35% (w/v) sucrose by adding 3.5 g sucrose to 10 ml of lysis buffer. Prepare 80% (w/v) sucrose by adding 1.6 g to 2 ml of lysis buffer (*see* **Note 2**).

3. Protease and phosphatase inhibitors: 10 mM sodium fluoride (NaF) (Sigma), 2 mM EDTA, pH 8.0, 1 mM Pefabloc (Sigma), 1 mM sodium orthovanadate (Na_3VO_4) (Sigma), 5 mM iodoacetamide (Sigma) (*see* **Note 3**).
4. SDS-PAGE sample buffer: 2× concentrated non-reduced sample buffer with 4× concentrated SDS (2N4×SDS). 128 mM Tris–HCl, pH 6.8, 10% glycerol, 4% SDS. Aliquot and store at –20 °C.
5. 40% DTT (dithiothreitol): prepare 1 ml 40% DTT and store at –20 °C.
6. Polyallomer centrifuge tubes (5 ml), (Beckman Coulter; 13×51 mm).

2.2 Raft Fractionation by β-Octylglucoside Selective Solubility

1. TST buffer: 150 mM NaCl, 0.2% Triton X-100, 25 mM Tris–HCl, pH 7.4.
2. β-octylglucoside (β-OG): Prepare a 0.6-M stock for use at a final concentration of 60 mM.
3. Protease and phosphatase inhibitors: 1 mM NaF, 0.2 mM Na_3VO_4, 1 mM Pefabloc (Sigma), 1 mM phenylmethanesulfonyl fluoride (PMSF) (Sigma), 1 μg/ml aprotinin (Sigma).
4. SDS-PAGE sample buffer: (5× concentrated Laemmli buffer) 60 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 5% beta-mercaptoethanol, 0.01% bromophenol blue.

2.3 Protein Purification from DRM Fractions

1. Chloroform (Merck Millipore).
2. Methanol (Merck Millipore).
3. Tris–NaCl buffer: 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% SDS.

3 Methods

The methods for isolating rafts described here have been optimized for the NKL cell line and for human primary NK cells in raft fractionation by β-OG selective solubility. It should also be useful for other NK-like cell lines with some small modifications depending on the proteins of interest.

3.1 Density Gradient Ultracentrifugation

This technique requires a large number of cells. Initially, 5×10^7 cells are recommended, the number subsequently being scaled up if necessary. Harvest the cells during their exponential growth phase and perform the required treatment/stimulation (e.g., activation of the membrane receptor of interest) before lysis.

1. Centrifuge cells at 400×*g* for 5 min at 2 °C.
2. Remove supernatant thoroughly. Place pellet on ice (*see* **Note 4**).

3. Resuspend the pellet in ice-cold lysis buffer (0.5 ml/5×10^7 cells) and transfer the lysate to a precooled Eppendorf tube (*see* **Note 5**).
4. Incubate for 30 min on ice (*see* **Note 6**).
5. Add equal volume of ice-cold 80% sucrose to the cell lysate (to adjust the cell lysate to 40% sucrose).
6. Place the lysate/40% sucrose (1 ml) at the bottom of a 5 ml polyallomer centrifuge tube.
7. Carefully overlay with 3.5 ml ice-cold 35% sucrose.
8. Overlay with 0.5 ml lysis buffer without any sucrose (0% sucrose layer).
9. Calibrate the tubes and place them in the ultracentrifuge (*see* **Note 7**).
10. Centrifuge at 250,000 × *g* for 18–20 h (overnight) at 2 °C (*see* **Note 8**), ending with a slow deceleration profile.
11. Remove the tubes from the rotor carefully, without disturbing the layers of sucrose. A band above the 0–35% sucrose interface, which corresponds to the raft fraction, should be visible.
12. Collect 8 fractions (600 μl each) from the top of the gradient (Fig. 1), keeping the tube steady and upright.
13. Take a volume of each fraction (e.g., 50 μl) and dilute it 1:1 in 2N4×SDS sample buffer. Store the rest at −20 °C or at −80 °C over the long term (*see* **Note 9**).
14. Reduce the samples by adding 1% DTT and boiling for 1 min at 99 °C, if required.
15. Prepare the samples for SDS-PAGE and immunoblotting or store the samples at −20 °C until use (*see* **Note 10**).

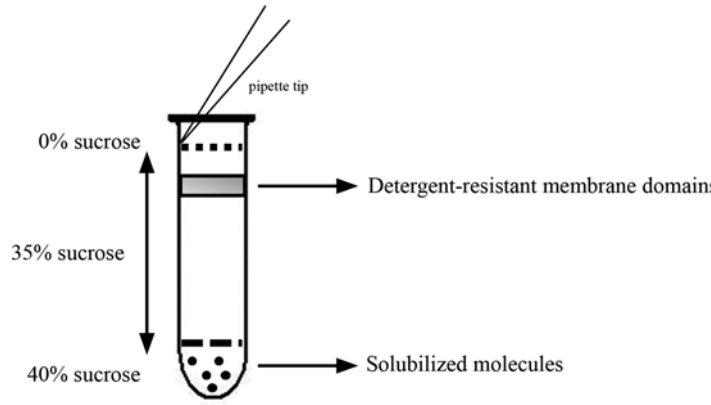

Fig. 1 Schematic representation of the distribution of DRMs after ultracentrifugation on a sucrose gradient. Collecting the fractions is a critical step and should be done carefully, placing the tip of the pipette as shown and aspirating gently in order not to mix the fractions

3.2 Raft Fractionation by β-Octylglucoside Selective Solubility

This protocol is optimized for NKL and human primary NK cells, using up to 7×10^6 cells per condition. After cell lysis, it is important to perform all steps on ice or at 4 °C. This means using a refrigerated centrifuge and refrigerated pipette tips.

1. Centrifuge cells at $400 \times g$ for 5 min.
2. Wash once with PBS. Centrifuge cells at $400 \times g$ for 5 min. Remove supernatant.
3. Add 100 µl of TST buffer with inhibitors (1 mM NaF, 0.2 mM Na_3VO_4, 1 mM Pefabloc, 1 mM PMSF, 1 µg/ml aprotinin) and transfer the lysate to a pre-cooled Eppendorf tube.
4. Incubate for 20 min on ice with occasional agitation.
5. Centrifuge the lysate at $15{,}500 \times g$ for 3 min at 4 °C (*see* **Note 11**).
6. Separate supernatant and keep on ice. This is the soluble fraction (fraction 1).
7. Add 100 µl of TST buffer with 60 mM β-OG to each pellet.
8. Carefully pipette up and down until the pellet is thoroughly disaggregated.
9. Incubate for 15 min at 4 °C.
10. Incubate for 10 min at 37 °C.
11. Centrifuge at $15{,}500 \times g$ for 3 min at 4 °C.
12. Separate the supernatant and keep on ice. This is the raft fraction (fraction 2).
13. Add 100 µl TST to the final pellet (fraction 3).
14. Add 5× Laemmli sample buffer to the collected fractions 1, 2 and 3, to a final concentration of 1× (*see* **Note 12**).
15. Sonicate the fraction 3 before boiling (*see* **Note 13**).
16. Boil the samples for 5 min at 95 °C.
17. Prepare the samples for SDS-PAGE and western blot or store the samples at −20 °C until use (*see* **Note 14**).

3.3 Protein Purification from DRM/Rafts

If purification of protein is desired from the raft fractions for example for subsequent proteomic analysis, follow chloroform–methanol protein precipitation method as detailed below. This method, based on that described by Wessel and Flügge [16] is recommended for removal of salt, detergents, and the sucrose that is contained in DRM fractions.

1. Transfer the required volume of sample to a new tube. The following steps indicate the quantities for a starting volume of 100 µl.
2. Add 400 µl of methanol and vortex very well for at least 1 min.
3. Add 100 µl of chloroform and vortex for at least 1 min.

4. Add 300 μl of distilled water (dH_2O) and vortex for at least 1 min.
5. Centrifuge at 14,000 × *g* for 1 min.
6. Remove the top aqueous layer; the protein is the interface (*see* **Note 15**).
7. Add 400 μl of methanol and vortex for at least 1 min.
8. Centrifuge at 14,000 × *g* for 2 min.
9. Remove as much methanol as possible without disturbing the pellet.
10. Dry the samples in a rotational vacuum concentrator at room temperature (*see* **Note 16**).
11. Resuspend the proteins in the most appropriate buffer according to desired subsequent analysis (*see* **Note 17**).

4 Notes

1. Prepare and store the lysis buffer with Tris–HCl and NaCl. Add the 1% detergent to the required amount of lysis buffer just before use.
2. 80% sucrose is very viscous. Place it in a 37 °C bath until it has completely dissolved. To pipette the 80% sucrose, cut the end of a tip and resuspend several times. Prepare fresh sucrose. Add inhibitors to sucrose solutions before use.
3. Add the protease and phosphatase inhibitors before use.
4. Optionally, cells may be washed with ice-cold PBS before cell lysis.
5. Cells are usually lysed in ice-cold lysis buffer, and the lysate then mixed with 80% sucrose (1:1). Nevertheless, the cell pellet may be lysed directly in lysis buffer containing 40% sucrose (to avoid generating a lysate that is too viscous to pipette).
6. During the incubation, prepare the 80% and 35% sucrose solutions.
7. This step is critical when using an ultracentrifuge. Tubes must contain precisely the same mass. If only one sample is being produced, another gradient must nevertheless be prepared even though it does not contain an experimental sample. Handle and load the tubes with great care.
8. These parameters depend on the ultracentrifuge model and the rotor that will be used. A speed of approximately 40,000–50,000 rpm is required.
9. This is a suggestion, but depends on what subsequent analysis of the fractions is desired. Nevertheless, it is advisable to check

the distribution of the raft markers such as Annexin2, Lyn, or Flotillin by western blot. Thawing–freezing cycles should be avoided as far as possible. The proteins in the samples may also be precipitated for concentration and sucrose removal. The chloroform–methanol protein precipitation method is recommended [16].

10. Run all the collected samples and check the presence/absence of the proteins of interest. Low-density DRM fractions (i.e., fractions 2–3) correspond to raft fractions (Fig. 2a). It is also important to use an antibody as a control for the membrane fraction (e.g., anti-Lyn or anti-Flotillin-1).
11. During this centrifugation, prepare the required amount of TST buffer with 60 mM β-OG.
12. It is recommended to store the soluble and the insoluble fractions (fractions 1 and 3, respectively) and use controls for each fraction (e.g., anti-Transferrin receptor for the soluble fraction, and anti-β-actin for the insoluble fraction) in western blot analyses.
13. Use a sonicator for 3 × 5 s, within a 10-s intermission between each burst. Sonication produces localized heat in the solution; keep the tube on ice at these intervals. Make sure that the pellet is well submerged in TST buffer, otherwise it may produce a foam that reduces the efficiency of sonication.

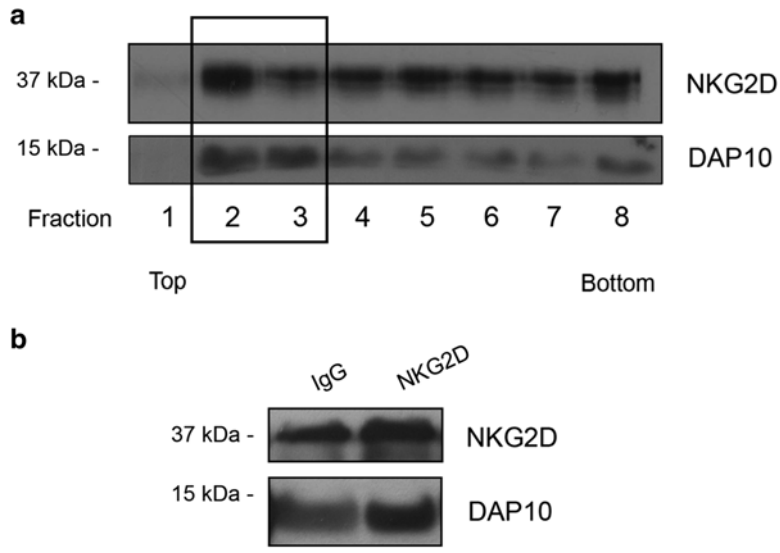

Fig. 2 (**a**) Isolation of DRMs by sucrose gradient ultracentrifugation. NKL cells (7.5×10^7 cells/condition) were solubilized upon stimulation in lysis buffer containing 1 % Brij-98. The fractions were analyzed by western blot to determine the distribution, in this case, of NKG2D and DAP10. The presence of the proteins in the low-density fractions (i.e., 2 and 3) indicates their recruitment into DRMs. (**b**) Raft fractionation by β-octylglucoside selective solubility. Western blot analysis of the distribution of NKG2D and DAP10 in DRM fractions under control conditions (IgG) and upon stimulation of NKG2D

14. Run the raft fractions to check the presence/absence of the proteins of interest. Western blot analysis enables the relative protein levels in raft fractions to be quantified and the results under different experimental conditions compared (Fig. 2b). Use an antibody as a control for the membrane fraction (e.g., anti-annexin2).
15. In case of disturbing the interface containing the proteins, mix well again by vortexing for at least 1 min and centrifuge.
16. This step usually takes no more than half an hour, check every 10 min the samples. If a rotational vacuum concentrator is not available in the laboratory, air dry the pellet overnight (16–18 h) at room temperature.
17. For in-gel digestion for mass spectrometry (MS) analysis, resuspend the protein in Tris–NaCl buffer (e.g., in 20 μl) and store at −20 °C until use. An aliquot should be stored separately and used for bicinchoninic (BCA) protein content analysis (Pierce BCA protein assay kit, as per manufacturer's protocol). Run the desired amount of protein in a SDS-PAGE gels by adding 5× Laemmli sample buffer to a final concentration of 1× and boiling the samples for 5 min at 95 °C.

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Chapter 12

High- and Super-Resolution Microscopy Imaging of the NK Cell Immunological Synapse

Emily M. Mace and Jordan S. Orange

Abstract

Recent advances in imaging technology have enabled significant advances in the study of NK cell cytotoxic effector function through quantitative analysis of the NK cell immunological synapse. This can include the use of high- and super-resolution microscopy to quantify dynamics of cytoskeletal elements and the role they play in the regulation and execution of NK cell directed secretion. Here we describe a protocol for the recapitulation of the NK cell lytic synapse on glass, the acquisition of microscopy images, and suggested approaches for the processing and analysis of microscopy data.

Key words Natural killer cell, Immunological synapse, Super-resolution microscopy, Confocal microscopy, Image analysis

1 Introduction

Natural killer (NK) cells provide critical human host defense against malignancy and viral infections, particularly through the directed secretion of specialized secretory lysosomes termed lytic granules. This targeted lysis is a tightly regulated process as NK cells largely identify susceptible cells through germline encoded receptors. Therefore, their innate ability is harnessed and only released following passage through multiple regulatory checkpoints [1]. Key control of the cytotoxic process is contributed largely by cytoskeletal components, namely microtubules and F-actin. At each of the main stages of function, recognition, effector and termination, the cytoskeleton modulates NK cell responsiveness through the immunological synapse formed between it and the target cell.

The study of these processes, their regulation, and their contribution to disease has been driven largely by cell biological techniques. In particular, the use of high- and super-resolution microcopy to dissect the molecular contribution of cytoskeletal elements has been very productive. While detailed description of these techniques and their application is beyond the scope of this

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introductory targeted primer, perspectives on the use of microscopy to study the immunological synapse have been well reviewed elsewhere [2, 3]. This protocol focuses specifically on the application of confocal microscopy or stimulated emission depletion (STED) microscopy to the study of an NK cell activated on glass coated with antibody to activating receptors.

The use of glass coated with antibody (or recombinant protein) has been shown to recapitulate the target cell interface while still allowing for high-resolution examination of the synapse. In addition, it allows for the selective ligation of receptors of interest. Under activating conditions, specifically the engagement of an adhesion receptor and an activating receptor, the NK cell will undergo degranulation at the plane of the glass, accompanied by the molecular events associated with exocytosis, including the expansion of the actin meshwork to enable or facilitate lytic granule passage, the exposure of CD107a (LAMP1) to the outer membrane and the release of lysosomal contents, namely perforin, granzymes and esterases [4, 5]. The choice of which receptor(s) to engage is dependent upon the cell origin (primary or cell line) and, if a cell line, the expression of receptors on the cell surface. The primary adhesion molecule on all NK cells is LFA-1, the αLβ2 integrin (CD11a/CD18). Ligation of LFA-1 can occur through the use of a non-blocking anti-CD18 antibody, or through the use of recombinant ICAM-1, LFA-1's most common physiological ligand. We use anti-CD18 (clone 1B4), which is commercially available in purified form from several suppliers. For activation of primary NK cells, we most commonly use anti-NKp30 in combination with anti-CD18, which is sufficient for NK cell activation and degranulation. Alternatively, anti-NKG2D may be used [4]. For cell lines, the choice is more dependent upon the line. For example, the NK-92 cell line [6] expresses the natural cytotoxicity receptor NKp30, and engagement of NKp30 in combination with LFA-1 results in robust activation and degranulation. Engagement of CD28 on YTS or YT cells has a similar effect, whereas for the NKL cell line NKG2D or CD16 engagement is commonly used [4].

Other methods to recapitulate the synapse include the use of supported lipid bilayers, which allows for imaging at the plane of the glass, yet is restricted to live cell imaging. The use of target cells has also been described, although it can be difficult to keep these oriented for imaging in the XY dimension at the synapse and the added depth of the target precludes imaging at the plane of the glass, a requirement for such techniques as total internal reflection microscopy (TIRF). While all systems have their advantages and drawbacks, the recent report of similar behavior of T cells on lipid bilayer and glass lends confidence to the consistency of each system to study synaptic events [7].

Close examination of the NK cell lytic synapse has revealed some unexpected findings and underscored the importance of

rigorous biophysical analysis. These findings include the interaction between activating and inhibitory receptors in microclusters, which shows that microclusters of inhibitory receptors at the NK cell synapse undergo reorganization following activating receptor ligation [8]. The movement of lytic granules prior to degranulation at the synapse has been described by TIRF yet is incompletely understood [9]. Finally, our group and others recently described the presence of an actin mesh throughout the lytic synapse [4, 5]. This F-actin network creates granule-permissive clearances in response to activating signal, and its opening specifically requires the depolymerization of F-actin [10]. These findings were possible only because of the parallel use of multiple high- and super-resolution techniques and, again, underscore the importance for such type of analysis.

In this protocol we will describe the activation of NK cells on glass and the subsequent steps to the acquisition and preliminary processing of data obtained using confocal or STED microscopy (*see* example in Fig. 1). In this case, the technique described utilizes a commercially available Leica SP8 laser scanning confocal microscope with STED capabilities. STED exploits the use of a high energy, toroidal shaped depletion beam that selectively darkens the photons produced around a single fluorophore and thus allows for sub-diffraction limited imaging [11]. In the case of the Leica system, this beam is positioned at 592 nm and/or 660 nm, potentially enabling depletion in the 488–630 nm range and allowing use of commercially available fluorophores. A further advantage of this system is the tunable white light laser and tunable PMTs or detectors. This allows for manipulation of both excitation and detection of emission. While this protocol describes the use of this system, the techniques described will be germane to the application of other confocal microscopes.

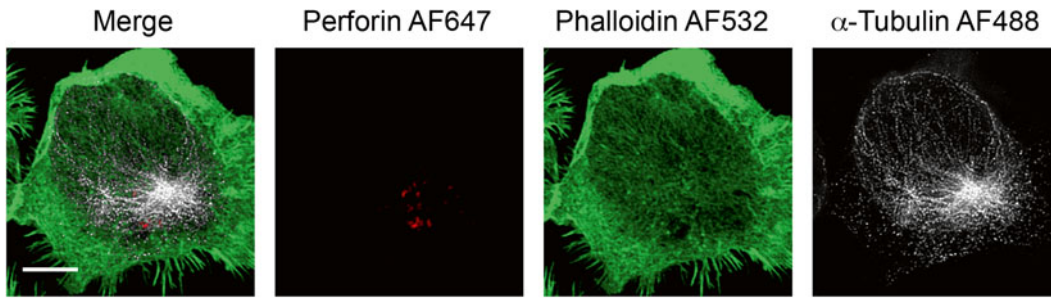

Fig. 1 Representative image of the cytoskeleton of an NK cell activated on glass. NK92 cells were activated on glass then fixed, permeabilized and stained for F-actin, a-tubulin and perforin as described in the protocol. Images were acquired on a Leica SP8 using both confocal (perforin) and STED (α-tubulin, F-actin) modules. Images were deconvolved with Huygens (SVI) software. Scale bar = 5 μm

2 Materials

1. ProLong anti-fade reagent (Life Technologies).
2. Antibodies for coating the coverslips for activation: purified anti-NKp30, anti-CD18, anti-NKG2D, anti-CD16, etc. (as required by experimental setup).
3. Antibodies for staining sample: anti-perforin, phalloidin, anti-α-tubulin, etc. (as required by experimental setup).
4. Bovine serum albumin (BSA) (Sigma-Aldrich).
5. Phosphate buffered saline (PBS).
6. Cotton tipped applicator.
7. Super PAP pen (hydrophobic barrier pen) (Life Technologies).
8. BD Cytofix/Cytoperm (BD Biosciences).
9. Triton X-100 (Electron Microscopy Sciences).
10. Laboratory tissue wipers.
11. Thermo Scientific™ Colorfrost™ Plus Slides (Fisher Scientific).
12. #1.5 coverslips.
13. NK-92 cell line (ATCC).
14. Ficoll-Paque (for isolation of primary NK cells).
15. RosetteSep (STEMCELL Technologies) for enrichment of primary NK cells.
16. NK92 medium: Myelocult (STEMCELL Technologies) supplemented with 100 U/ml IL-2 and penicillin–streptomycin.
17. NK cell medium: RPMI 1640, 10% FBS, penicillin–streptomycin, HEPES, nonessential amino acids, sodium pyruvate, L-glutamine.
18. Staining buffer: 1% BSA PBS with 0.1% Saponin.
19. Cytofix/Cytoperm buffer: BD Cytofix/Cytoperm with 0.1% Triton X-100.
20. Leica SP8 laser scanning confocal microscope with STED module.
21. Volocity software (PerkinElmer).
22. Huygens software (Scientific Volume Imaging).
23. 50 ml sterile conical tubes.
24. Saponin (Sigma-Aldrich)

3 Methods

3.1 NK Cell Culture

1. NK-92 cell line should be maintained in Myelocult media (STEMCELL Technologies) with 100 U/ml IL-2.
2. Cells should be passaged 1:10 every 2–3 days and maintained at a density of $1–3 \times 10^5$ cells/ml.

3.2 Primary NK Cells Isolation and Purification

1. Enrich and isolate peripheral blood mononuclear cells (PBMCs) from peripheral blood by Ficoll-Paque method.
2. Incubate 15 ml fresh peripheral blood with 750 μl RosetteSep (STEMCELL Technologies) for 20 min.
3. Dilute with 15 ml of PBS and carefully layer on 15 ml of Ficoll-Paque in a 50 ml tube.
4. Spin the cells at $400 \times g$ for 20 min with no brake.
5. Carefully recover the PBMCs from the Ficoll-Paque interface.
6. Wash NK cells once with complete NK cell medium and resuspend at a concentration of 1×10^6. In our experience it is recommended to use cells immediately after isolation (*see* **Note 1**).

3.3 Coating Slides with Activating Antibody

1. Use a Super PAP pen to mark a circle or square (approximately 1.5 cm diameter) on one 22 mm × 22 mm #1.5 coverslip per condition to be tested (including single stained controls), in order to create a shallow hydrophobic barrier, to contain cells and staining buffer.
2. Resuspend primary antibody against receptors of interest (i.e., CD18, NKp30, NKG2D) (*see* **Note 2**) at 5 μg/ml in phosphate buffered saline (PBS). Vortex briefly to mix. Prepare approximately 200 μl per condition.
3. Gently pipette 100–200 μl of coating antibody on to coverslip inside the hydrophobic barrier (may be done under non-sterile conditions) and incubate at 37 °C 5% CO_2 for 30 min.
4. Just prior to seeding NK cells, gently dip the coverslips in a 50 ml conical tube of PBS to wash off unbound activating antibodies. Coverslips should be used immediately following coating (*see* **Note 3**).

3.4 Preparing NK Cells and Activation on Coverslips

1. Harvest 2×10^5 NK cells (primary or cell lines) from culture per condition (per each coverslips prepared in Subheading 3.3).
2. Wash NK cells once with prewarmed NK cell medium, centrifuge at $400 \times g$, remove the supernatant, and resuspend in NK cell medium at a density of 1×10^6/ml.
3. Gently pipette 100–200 μl cells onto coverslips over the immobilized antibodies and incubate at 37 °C 5% CO_2 for desired time (*see* **Note 4**).
4. Gently dip coverslips in a 50 ml conical tube containing PBS to remove unbound cells.
5. Gently pipetting 200 μl of prewarmed Cytofix/Cytoperm buffer, to fix and permeabilize the cells on coverslips.
6. Incubate for 10–30 min in humidified chamber (e.g., slide box with moist paper towel) in the dark at room temperature.

3.5 Antibody and Phalloidin Staining

1. Following fixation and permeabilization, wash cells gently in staining buffer (by gently dipping coverslips in 50 ml conical tube).
2. Prepare antibodies for staining in staining buffer. Include single stained controls (*see* **Note 5**).
3. Stain first with primary antibody to structure of interest (i.e., microtubules or perforin) for 30–60 min.
4. Rinse gently in staining buffer and dab edges of hydrophobic region with cotton swab.
5. Stain with secondary antibody for 30–60 min and wash as in **step 1** in Subheading 3.5 (*see* **Note 6**).
6. Repeat for subsequent antibody stains.
7. Stain for phalloidin to detect F-actin last (if phalloidin staining is desired).
8. Maintain humidity during staining by performing all stains in humidified chamber.

3.6 Mounting Coverslips on Slides

1. Use mounting media that is compatible with the imaging technique (*see* **Note 7**).
2. Gently pipette approximately 10–20 μl of mounting media on each glass slide, avoiding formation of bubbles. Should bubbles form, gently pipette these off.
3. Gently lay the coverslip down on the slide, so that the surface on which the cells are fixed is in contact with the mounting media.
4. Allow coverslips to set for 24–48 h and then seal edges of coverslips with nail polish.

3.7 Image Acquisition

1. Prior to acquiring images turn on the Leica SP8 confocal microscope. Initialize LASAF software and allow lasers to warm up for 30–60 min.
2. Place slide on microscope and adjust focus with eyepieces.
3. Acquire first channel and set laser power for optimal signal without pixel saturation. Adjust gain and, if necessary, exposure time (*see* **Note 8**).
4. Repeat setup for each channel to be imaged. Acquire single stained controls using each setting and adjust as necessary to reduce spectral overlap into other channels.
5. Acquire images. For quantitative imaging it is recommended to acquire at least 20 cells per condition, although this can be best dictated by the experiment in question and using traditional appropriately powered statistical sample size calculations. To preserve fluorescence during sequential scan it is best

to acquire images with longer excitation wavelengths first. For best resolution of those components at the synapse, focus finely to ensure imaging at the plane of the glass.

6. Should the application of STED be desired, align the depletion laser prior to beginning STED imaging using automatic STED alignment in LASAF software.
7. Apply STED laser by activating laser to 50% to begin with and determine conditions that give best resolution. Variables to be adjusted at this stage may include laser power, gain, STED laser power and time gating (where applicable). A visual stepwise demonstration of this process has been published and common pitfalls are shown in Fig. 2 [12].
8. Save and export images as .LIF files.

3.8 Processing and Analyzing Images (See Note 9)

1. Open images in Volocity (direct import) or FIJI (using Bioformats plug-in) for image analysis and processing.
2. Determine threshold based on background fluorescence (*see* **Note 10**). In general, it is best to analyze data in its most unmanipulated form possible.
3. Following selection of objects based on threshold, the mean fluorescence intensity and area of these objects can be calculated using the Measurement feature in both Volocity and FIJI software.
4. Choose representative images for display and export as .TIF files at 300 dpi (*see* **Note 11**).

4 Notes

1. Isolate primary NK cells from peripheral blood prior to the experiment. For imaging experiments it is best to use a negative selection kit such as RosetteSep (STEMCELL Technologies). Primary NK cells should not be maintained or expanded in cytokines if the cytoskeleton is to be studied (unless this is the purpose of the experiment) as these have been shown to influence signaling pathways following activation [13].
2. Ensure that if primary-secondary antibody combinations are used, they were not raised in the same species as the antibody used to coat the coverslip.
3. Coverslips can be coated overnight at 4 °C but should be prewarmed at 37 °C prior to use. It is preferable to coat immediately prior to the experiment but the user may prefer to save time by precoating.
4. The duration of activation should be chosen based on the function of interest. For example, F-actin accumulation will

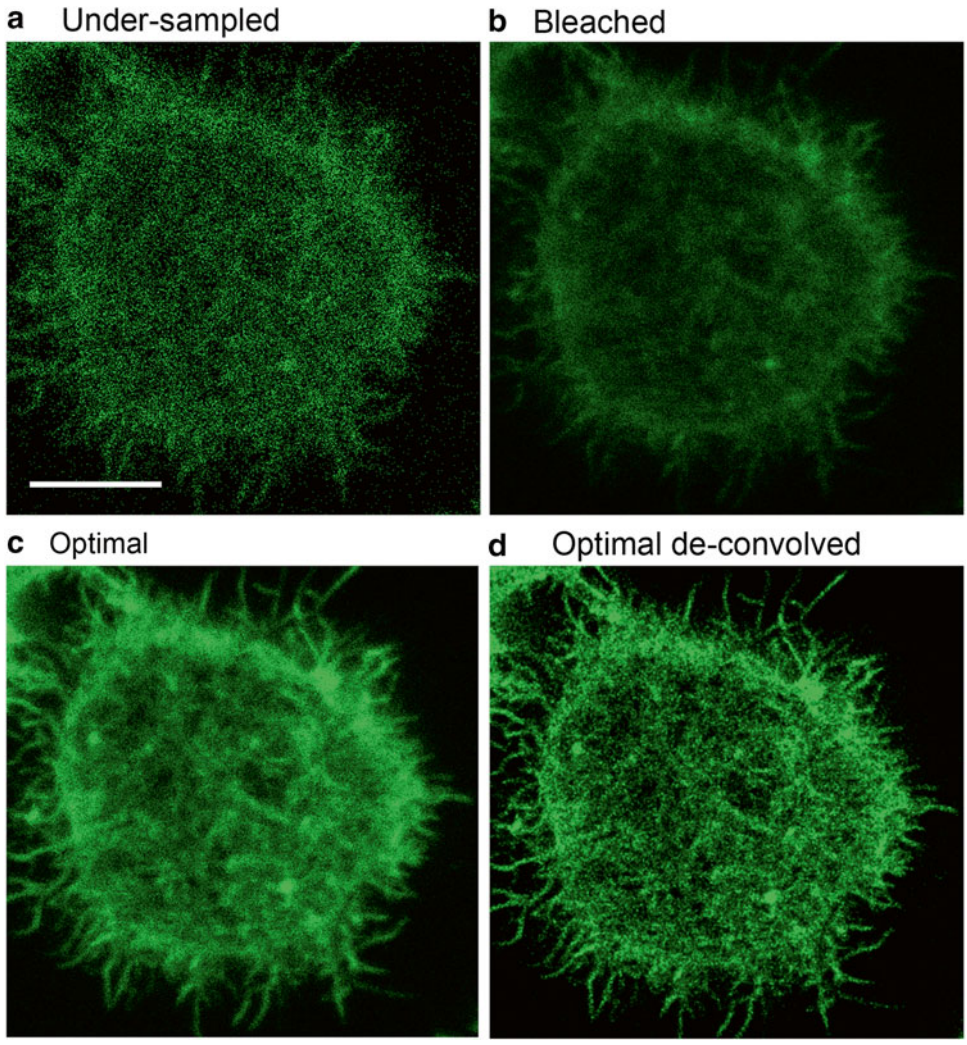

Fig. 2 Acquisition by STED imaging and common pitfalls. NK92 cells were activated on anti-CD18 and -NKp30 coated glass for 20 min then fixed, permeabilized and stained for F-actin with Phalloidin Alexa Fluor 488. (**a**) An example of loss of image information due to under-sampling. (**b**) An example of loss of resolution due to bleaching/over-sampling (**c**) conditions optimized (**d**) optimized conditions lead to greater improvement in resolution with deconvolution. Scale bar = 5 μm. Image reproduced with permission: "Mace, E. M., Orange, J. S. Visualization of the Immunological Synapse by Dual Color Time-gated Stimulated Emission Depletion (STED) Nanoscopy. *J. Vis. Exp.* (85), e51100, doi:10.3791/51100 (2014)"

occur relatively quickly (5–10 min), where as complete polarization of lytic granules may take 25–30 min.

5. We recommend staining each antibody sequentially, although phalloidin may be combined with the final secondary antibody. Single stained controls should be prepared in order to ensure lack of spillover of signal into other channels of interest. Antibody concentration will require titration depending on

the sample and the antibody. For many antibodies dilutions of 1:100 or 1:200 are suitable but titration of primary antibodies in particular is highly recommended. Generally, 30 min of staining time per antibody is sufficient. In the case of low avidity antibodies staining time may be increased to 1 h.

6. Recommended secondary antibodies for STED include Alexa Fluor 488, tetramethylrhodamine, Alexa Fluor 532, and Horizon V500. For STED, avoid streptavidin–biotin combinations.
7. The use of hard-set medias is preferred, namely ProLong, ProLong Gold or ProLong Diamond. Vectashield is not compatible with STED. Mowiol is acceptable. 2, 2 thiodioethanol cannot be used in combination with phalloidin.
8. Pixel size is a function of several variables on a laser scanning confocal microscope. These include the image format (number of pixels contained in the image), the amount of zoom used and the objective. Data collected as part of a single experiment should be obtained with the same pixel size and acquisition settings throughout. For STED, a pixel size of less than 40 nm is highly recommended.
9. The analysis performed will be dependent upon the experiment. However, common measurements may include mean fluorescent intensity and area as these can reflect the relative density of protein of interest. In general, image processing for visualization should be limited to the application of a threshold to remove background signal. All forms of nonlinear thresholding (gamma) should be avoided. The threshold should be chosen based on fluorescent intensity of the background and should generally be fixed at this intensity for all images and analyses.
10. For STED images, deconvolution can dramatically improve image quality and enable more accurate analysis. Deconvolution recovers information lost through imaging and can be performed mathematically through software. Huygens (SVI) is provided with Leica LASAF software and uses the theoretical point spread function estimated by the parameters used in your experiment to deconvolve both confocal and STED data. We generally use default parameters for deconvolution although optimizing the signal to noise ratio may be necessary.
11. Images portrayed should reflect the mean of values obtained and be processed in the same way as during analysis. If possible, each channel should be shown separately and together as a merge. Scale bars should be shown. It is recommended that in an aggregate data summary the representative image be specifically denoted as to where it resides within the aggregate.

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Chapter 13

The Planar Lipid Bilayer System Serves as a Reductionist Approach for Studying NK Cell Immunological Synapses and Their Functions

Grant Bertolet and Dongfang Liu

Abstract

The immunological synapse (IS) is the junction between an immune cell (e.g., a T or NK cell) and another cell (e.g., an antigen-presenting cell (APC), or a tumor cell). The formation of the IS is crucial for cell-mediated immunity, and as such, an understanding of both the composition of the IS and the factors that drive its formation are essential for understanding how and when NK cells eliminate susceptible target cells. The supported lipid bilayer (SLB) system is a highly effective tool for directly studying the IS. SLBs confer three main advantages: (1) they allow for synapse formation on a level horizontal surface, allowing for direct visualization of the IS under high resolution imaging systems, (2) they mimic the surface of a target cell by providing a fluid mosaic into which surface proteins can be embedded while permitting free motion in two dimensions, which is important for studying the dynamics of synapse formation, and (3) they allow investigators to determine the exact composition of the bilayer, thus in turn allowing them to answer very specific questions about the IS. It is our hope that this chapter will furnish readers with an awareness of the applications of the SLB system for studying the IS in NK cells, and also of a basic knowledge of how to use this system for themselves.

Key words Natural killer, NK, Immune synapse, Immunological synapse, Immunosynapse, IS, Supported lipid bilayer, SLB, Confocal microscopy

1 Introduction

NK cells act as important conductors of cell-mediated immunity. Their ability to kill diseased or malignant cells without prior sensitization makes them a vital part of the immune response. However, similar to cytotoxic T lymphocytes (CTLs), NK cells must form stable contacts with their targets and in order to "read" them and decide whether to kill or to spare. The contact zone between an effector cell and its target is a transient structure composed of different clusters of surface receptors or proteins, which, altogether, is called the immunological synapse (IS). The IS was first imaged directly by confocal microscopy in combination with glass-supported

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DOI 10.1007/978-1-4939-3684-7_13,

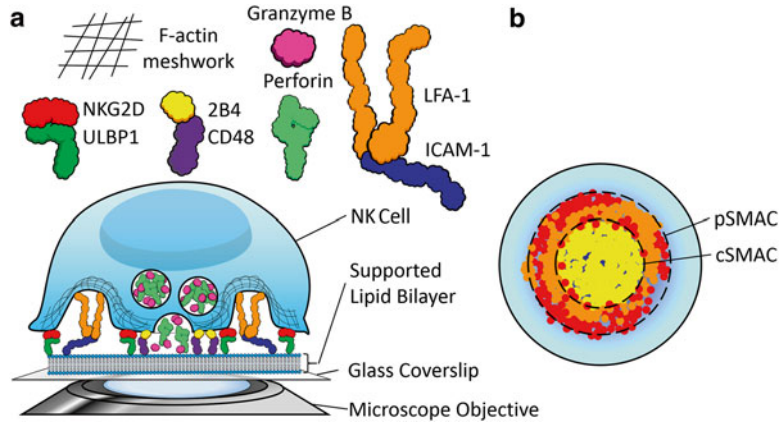


Fig. 1 Cartoon illustrating a model of the NK cell synapse formed on the supported lipid bilayer (SLB). Ligands for various NK cell surface proteins are anchored in the SLB, which itself rests on a flat rigid glass surface. NK cells are added atop this bilayer, which they recognize much as they would the surface of a target cell, and an immunological synapses (IS) forms between the surface proteins on the NK cell and the ligands embedded in the SLB. The planar orientation of the bilayer allows for easy and direct imaging of the resulting IS. (**a**) The particular synapse shown here is a model interpreted from the findings of Liu et al. [3]. On a lipid bilayer containing (1) ULBP1 (*green*), (2) CD48 (*green*), and (3) ICAM-1 (*blue*), these proteins will be bound by (1) NKG2D (*red*), (2) 2B4 (*yellow*), and (3) LFA-1 (*orange*), respectively, causing a reorganization of the F-actin cytoskeleton and polarization of perforin-positive lytic granules to the synapse. These lytic granules will then be extruded from the cell through regions of F-actin hypodensities, like salt granules through the pores in a shaker. (**b**) The "head-on" view of the IS provided by the SLB system allows for the visualization of the IS' characteristic "bull's-eye" structure, which in this case consists of centralized aggregated 2B4 surrounded by an outer ring of LFA-1 and NKG2D. These regions are known as the cSMAC and pSMAC, respectively

planar lipid bilayers (SLBs) [1]. SLBs are phospholipid bilayers supported on flat transparent surfaces (typically a thin glass coverslip), and into which can be embedded any desired proteins, including those necessary for synapse formation. The result is a flat surface that mimics the surface of a target cell, and which thus allows direct visualization of the IS formed on the SLB by immune cells [2] (Fig. 1).

The use of SLBs has proved highly valuable in the study of NK cell synapses. In 2009, Liu et al. used the SLB system to study synapse structure as well as the trafficking of cytolytic granules within the intercellular space formed by the IS [3]. The authors observed both well-organized natural cytotoxicity synapses mediated by NKG2D, 2B4, and LFA-1 and disorganized antibody-dependent cellular cytotoxicity (ADCC) synapses mediated by CD16 and LFA-1. These findings were, within the same study, validated by a cell–cell conjugation system in vitro, illustrating their translatability from the SLB to actual intercellular events. Additionally, Liu et al. discovered a hallmark for the cytotoxic IS, bidirectional vesicular traffic at center of NK synapse [3]. Thus, SLBs have been successfully used to study NK activation.

SLBs can be used in studying NK cell inhibition as well as activation. In 2012, Liu et al. used the same SLB system containing

human leukocyte antigen (HLA)-E, a ligand for the inhibitory receptor CD94/NKG2A, to discover a novel role of the adaptor protein Crk in controlling NK cell inhibition [4]. Abeyweera et al. also used this system to visualize NK cell inhibitory synapses and the roles played by immunotyrosine inhibition motif (ITIM) bearing receptors in their formation [5, 6].

Recent advances in fluorescent microscopy (such as the advent of commercially available super-resolution optical microscopes) have further increased the utility of the SLB system. Using stimulated emission depletion (STED) microscopy in combination with the SLB system, Zheng et al. demonstrated that perforin-containing granules accumulate at regions of low-density F-actin at the IS when CD16 activating receptors are ligated by bilayer-embedded antibodies against CD16 [7], a finding that corroborates earlier observations made by Rak et al. and Brown et al. using antibodies fixed on a glass slide [8, 9].

The aim of this chapter is: (1) to familiarize readers to SLB technology and (2) to provide a detailed instructional protocol on how to carry out this technique. The use of SLBs provides an unparalleled look into the IS, and the fact that investigators can alter the composition of the SLB at will allows for a straightforward reductionist approach towards testing the effect of individual receptors on synapse formation. It is therefore our hope that this chapter will broaden both awareness of and expertise in this technology, which will in turn lead to further advancements in our understanding of the NK cell immunological synapse.

2 Materials

2.1 Lipid Preparation

1. 18:1 (Δ9-Cis) PC (DOPC): 1,2-dioleoyl-sn-glycero-3-phosphocholine (10 mg/mL in chloroform) (Avanti).
2. 18:1 Biotinyl Cap PE: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl) (sodium salt) (10 mg/mL in chloroform) (Avanti).
3. Compressed argon gas.
4. A lyophilizer.
5. Lyophilizer tubes (Labconco).
6. Chromatography tubes (Santa Cruz).
7. Octyl-β-D-glucopyranoside (Sigma-Aldrich).
8. Sonicator (Avanti).
9. Cleaning solution: 1 L of 95% ethanol, 120 mL pure H_2O, and 60 g potassium hydroxide, KOH.
10. Dialysis tubing 6.4 mm diameter with a molecular weight cut-off of 12–14 kDa. (Spectrum® Labs).

11. Dialysis tube closures.
12. Dilution buffer: Prepare 25 mL solution of 25 mM Tris–HCl, pH 8.0 (from 1 M stock solution) and 150 mM NaCl (from 5 M stock solution), deoxygenate the solution with argon gas and then add 2% (by weight) n-octyl-β-D-glucopyranoside (OG) detergent. After preparation, filter the solution with a 0.2 μm cellulose acetate membrane and store it at 4 °C (*see* **Note 1**).
13. Dialysis buffer: Same as **item 12** in Subheading 2.1, but without OG detergent. 25 mM Tris, pH 8.0, 150 mM NaCl (8 L, aliquoted into 8 1 L screw cap bottles). Deoxygenate the buffer with argon gas and store at 4 °C (*see* **Note 1**).
14. Compressed argon gas.
15. Screw cap microcentrifuge tubes (water tight).

2.2 Determination of Protein Seeding Density on the Lipid Bilayer

1. Fluorescently labeled biotinylated protein.
2. 96-well V-bottom polystyrene plate (untreated).
3. Non-functionalized silica beads (Bangs Laboratories).
4. FACS tubes.
5. Quantum™ MESF beads (Bangs Laboratories).
6. 1.5 mL microcentrifuge tubes.
7. DOPC (400 μM diluted in dilution buffer; *see* Subheading 3.1).
8. Diluted Biotin-PE lipid (80 μM diluted in 4 mM DOPC and dilution buffer; *see* Subheading 3.1).
9. Vortex.
10. Blocking buffer: 5% casein in PBS, pH 7.3. Dissolve 18 g of casein 250 mL of ultrapure water in a beaker while stirring at RT. While stirring, add 1.26 mL 10 N NaOH in 100 μL aliquots. Allow to stir for 2–3 h at RT and then place at 4 °C overnight at a slow stir.
11. Wash buffer: HEPES buffered saline (HBS), 1% human serum albumin (HSA).
12. Streptavidin: 333 ng/mL in wash buffer.

2.3 Assembling the Supported Lipid Bilayer

1. Piranha solution: 3 parts sulfuric acid (H_2SO_4), 1 part of 30% hydrogen peroxide (H_2O_2).
2. 25 × 75 mm glass coverslip.
3. DOPC (400 μM solution in dilution buffer; *see* Subheading 3.1).
4. Diluted Biotin-PE: (80 μM diluted in 4 mM DOPC and dilution buffer; *see* Subheading 3.1).
5. Hemostat.
6. 6 lane chamber slide: ibidi Sticky-Slide VI 0.4 (ibidi).
7. Blocking buffer (*see* Subheading 2.3, **item 10**).

8. Wash buffer: HEPES buffered saline (HBS), 1 % human serum albumin (HSA).
9. Streptavidin.
10. D-biotin (25 mM) (Add company name/no need for catalog number or vendor information).
11. Type F immersion oil.
12. A Leica TCS STED microscope.

3 Methods

There are three major processes that go into working with SLBs. The first involves the preparation of the raw lipids, and this can be further subdivided into the initial prep stage and the subsequent dialysis of the dried lipids. The next phase focuses on determining how densely the bilayers are "seeded" with protein of a given molarity. The final phase deals with the actual assembly of the SLB within the imaging chamber and subsequent addition of the surface proteins and the cells to it. This methods section will detail how our lab in particular works with the SLB system, but the entire process is very amenable to customization. An effort has been made to indicate in the notes some of the places in our protocol where significant flexibility in materials or design is possible.

3.1 Lipid Preparation

1. Prior to beginning the lipid preparation, submerge glass chromatography tubes to be used in the following steps, into cleaning solution for 20 min. Then rinse the tubes three times with distilled water.
2. Beginning with 10 mg/mL initial stock concentrations of 1,2-di oleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phospho-ethanolamine-N-cap biotinyl (Biotin-PE), aliquot 629 μL of DOPC and 88 μL Biotin-PE into separate clean glass chromatography tubes (*see* **Note 2**).
3. Evaporate the chloroform with a stream of argon in a chemical fume hood and seal each chromatography tube with paraffin film (*see* **Note 3**).
4. Lyophilize the liposomes overnight to ensure complete removal of any remaining chloroform (*see* **Note 4**).
5. After lyophilization, dissolve the dried DOPC and Biotin-PE lipids in 2 mL and 200 μL of dilution buffer (*see* Subheading 2.1, **item 10**), respectively, to make a 4 mM solution of each.
6. Mix the Biotin-PE lipids with the DOPC lipids (*see* **Note 5**). Add 0.2 mL of 4 mM Biotin-PE and 1 mL of 4 mM DOPC to 8.8 mL of the dilution buffer to make a final concentration of 80 μM Biotin-PE.

7. Next, dilute 1 mL of remaining 4 mM DOPC to 400 μM by adding 9 mL dilution buffer.
8. Ensure homogeneity of the lipid solutions by sonicating until the solution clears, which typically takes about 10 min.
9. Finally, displace any oxygen in the top of the tube with argon and seal with paraffin film.
10. Now, dialyze the phospholipids to remove the OG detergent. To begin, clean and sterilize the tubing by soaking the newly cut sections in 200 mL of distilled water for 2 min at RT, followed by 5 min at a boil.
11. Knot one end of each tube and rinse out the inside with a small volume of dilution buffer. Then squeeze out as much of the wash buffer as possible.
12. Inside a laminar flow hood, add the diluted phospholipids prepared in Subheading 3.1 into each tube—pure DOPC in one and the DOPC/Biotin-PE mixture in the other—and clamp the open ends with a small dialysis tube closure so that we exclude all air (*see* **Note 6**).
13. Submerge the tubes inside the 1 L bottles containing dialysis buffer (Subheading 2, **item 11**) and add a magnetic stirring bar into each.
14. Deoxygenate each bottle with argon, re-cap tightly, and place each of them on a magnetic stir plate set to spin at medium speed overnight at 4 °C.
15. Replace the old buffer from each bottle with new buffer every 12 h, 3 times for each bottle.
16. An hour before the dialysis is scheduled to finish, label and deoxygenate 20 small screw-cap, water-tight microcentrifuge tubes to aliquot the dialyzed lipids.
17. Finally, after 36 h of continuous dialysis, take both bottles back into a laminar flow hood and remove the dialysis tubes (*see* **Note 7**).
18. Cut the dialysis tubing below the clip, and then carefully divide the dialyzed lipid solution into 1 mL aliquots using the preprepared tubes, which should be kept on ice during this process.
19. When at last all the aqueous lipid solution has been aliquoted, deoxygenate each tube with argon, seal with paraffin film, and store them at 4 °C.

3.2 Determination of Protein Seeding Density on the Lipid Bilayer

Prior to preparing the lipid bilayer for any experiment, one will need to establish how densely protein of a given molarity will seed the bilayer. This is an important consideration, as differing balances of surface proteins on the bilayer can lead to significantly different experimental outcomes [10], and experimental questions that seek

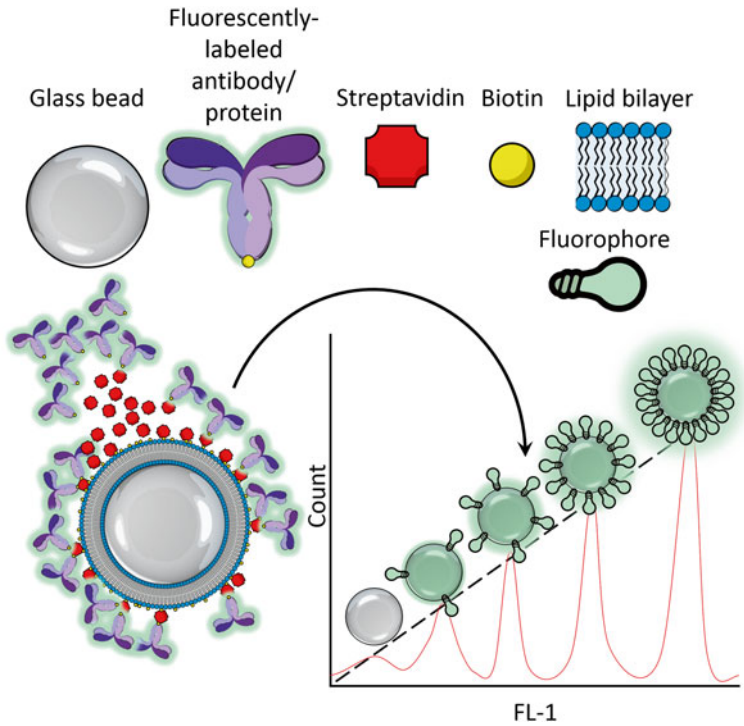
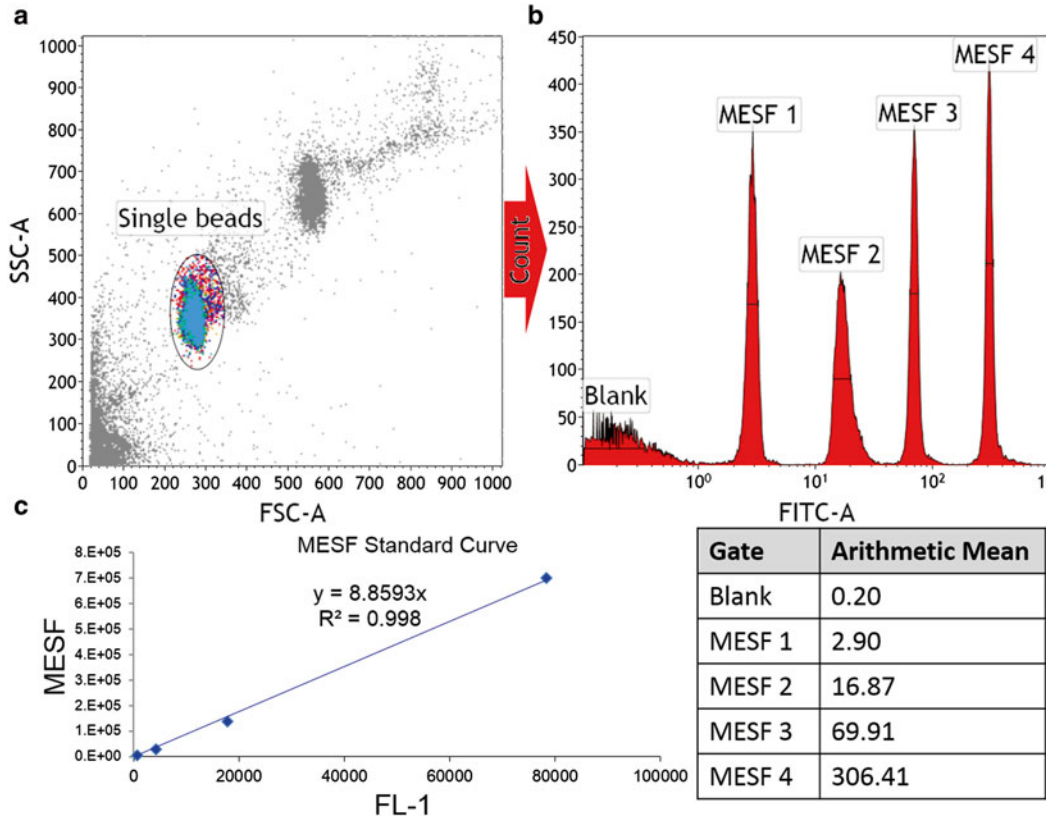


Fig. 2 Overview of the principle behind determining the seeding density of a given protein on the SLB. Silica beads of known diameter are coated with the biotinylated phospholipids, followed by streptavidin, and then with biotinylated, fluorescently labeled protein of various concentrations. These coated beads are then assayed by flow cytometry, and their fluorescent values are plotted against a standard curve established from a series of beads labeled with known numbers of the same fluorophore. From this, the number of molecules of equivalent soluble fluorochromes (MESF) can be correlated with the protein concentration, and dividing by the number of fluorophores per unit protein, the average density of protein coating each bead can be derived. From there, one simply needs to compute the surface area of the bead from its diameter to determine the seeding density of protein per square micrometer of the bilayer as a function of concentration

to gauge the effect of one protein on another must take this into account. The basic principle involves coating silica beads with lipids, which are then coated with fluorescently labeled protein. The labeled beads are then read on a flow cytometer and compared to a standard series of beads prelabeled with the same fluorophore at known MESF (Molecules of Equivalent Fluorochrome—the standard unit of fluorescent intensity) values (*see* Fig. 2).

1. Start by creating a graded series of protein dilutions. A typical range begins with 100 μM and is serially diluted tenfold 5 times, with a total volume of 50 μL for each dilution. The protein(s) must be both fluorescently labeled and possess an anchoring mechanism, such as biotinylation (*see* **Note 2**).

2. In a 96-well, V-bottom plate, add 1 μL of silica beads at a concentration of $0.5–1.0 \times 10^6$ beads/μL (diluted in PBS) into 6 wells, one for each protein dilutions, plus one for blank (negative control).
3. Next mix together pure DOPC lipids (400 μM) with diluted Biotin-PE lipids (80 μM), at a 1:1 ratio, for a total volume of $2 \times n + 2$, where n is our number of wells in a 1.5 mL microcentrifuge tube.
4. Add 2 μL of the lipid mixture into the wells containing the beads and the blank well.
5. Then, gently pulse the plate on a vortex in order to mix and promote interaction of the lipids with the silica beads (*see* **Note 8**).
6. Add 150 μL of blocking buffer (*see* **Note 9**). Block for 10 min, then wash.
7. To wash, fill each well to capacity (250 μL) with wash buffer, centrifuge at $1000 \times g$ for 2 min, carefully remove the top 200 μL, and repeat twice more for a total of three times.
8. Add 50 μL of streptavidin at a concentration of 333 ng/mL. Gently pulse the plate on a vortex and then let it sit on a rotary shaker at RT for 15 min in order to let the streptavidin interact with biotin. Wash off the excess, using the same procedure as in **step 6**.
9. At this point, add protein (*see* **Note 10**) and allow it to incubate with the streptavidin on the rotary shaker for 20–30 min at RT in the dark. Then wash off the excess, unbound protein with wash buffer as in **step 6**.
10. After the final wash, add 50 μL of wash buffer (for a total volume of 100 μL) and transfer the contents from each well to a separate FACS tube. Bring the volume up to 500 μL with wash buffer.
11. Next add one drop of beads from each bottle of an MESF series into a single tube and bring the volume up to 300 μL with wash buffer.
12. Run the MESF tube on the cytometer and adjust the voltage of excitation laser so that each peak in the series is visible in a histogram measuring the intensity of each fluorophore. There should be as many distinct peaks as there were bottles in the series.
13. Then draw a linear gate spanning the width of each peak at its half-maximal intensity, and assign the mean fluorescent intensity (MFI) for each of these gates to that series of beads (*see* Fig. 3).
14. Record the data from the MESF series, then read each tube of the protein dilution series. Once this is done, establish an intensity-to-molecule ratio for the fluorophore from the MESF series, and then intrapolate the measured intensity of the protein-labeled beads into the number of fluorophores (*see* Table 1).



Gate	Arithmetic Mean
Blank	0.20
MESF 1	2.90
MESF 2	16.87
MESF 3	69.91
MESF 4	306.41

Fig. 3 How to calculate the MESF standard curve. (**a**) Run a tube containing equal parts of each tube in the standard MESF series on a flow cytometer, then gate on the single beads in an SSC vs FSC dot plot. (**b**) Plot the singlet population on a unidimensional histogram with the fluorescent channel on the *X*-axis, and draw a gate spanning the width of each peak at its half-maximal intensity. Record the arithmetic mean for each gate. (**c**) Subtract the arithmetic mean for each peak from the blank value, then plot these against the MESF values (which can be found from the manufacturer). From this, a linear relationship between MESF and fluorescence can be obtained, which can then be used to calculate the MESF of your protein-labeled lipid-coated beads

15. Since this gives only the number of fluorophores, to determine the number of protein monomers, divide the values obtained from **step 14** by the average number of fluorophores on each monomer (which can be determined using a spectrophotometer and the instructions are provided by the fluorophore's manufacturer). This will tell what concentration of protein to add to achieve a given density of protein on the lipid bilayer (*see* **Note 11**).

3.3 Assembling the Supported Lipid Bilayer

This section is similar to Subheading 3.3. The primary difference is that this section describes assembling the lipid bilayer on a flat glass surface and incorporates an extra step to eliminate any potential nonspecific binding before adding cells. A graphical overview of the entire process can be seen in Fig. 4.

1. Clean a standard 25 × 75 mm glass coverslip by submerging into freshly prepared Piranha solution (*see* **Note 12**) for 20 min (*see* **Note 13**).

Table 1
Example worksheet for computing the seeding density of a given fluorescently labeled protein (adapted from the one provided by Bangs Laboratories with the purchase of any of their MESF kits)

Quantum MESF Standard Beads					
Fluorophore:	AF488	Tube Number	FL-1	FL-1 minus blank	MESF value
Lot:		B	48.6	0	0
[redacted]		1	741	692.4	3843
		2	4320	4271.4	27657
		3	17937	17888.4	136386
MESF:FL-1 ratio:	8.86	4	78422	78373.4	699951

Fluorescently-Labeled Protein-Conjugated Lipid-Coated Beads						
Protein:	OKT3	Concentration (nM)	FL-1	FL-1 minus blank	Calculated MESF	Seeding Density (units/μm²)
Labeling Efficiency:	0.5	0	8.34	0	0	0
Mean bead diameter:	5.06	20	40.4	32.06	568	7.06
Bead surface area:	80.44	100	54.5	46.16	818	10.17
		200	275	266.66	4725	58.74
		1000	578	569.66	10094	125.49

A linear relationship between fluorescent intensity (FL-1) and number of fluorophores is established from the standard MESF series as described in Fig. 3. This yields a standard linear equation of the form y = m * x, where y is the number of fluorophores and x is the measured FL-1. By inputting the measured FL-1 values of the protein-labeled lipid-coated beads into the equation, one can determine the MESF values. These values can then be divided by the labeling efficiency of the protein (moles of fluorophore per moles of protein) to obtain the number of fluorescently labeled protein monomers per bead. Dividing this number by the surface area of the bead, which can be calculated from its diameter, yields the density of the labeled protein per unit area as a function of its initial molar concentration

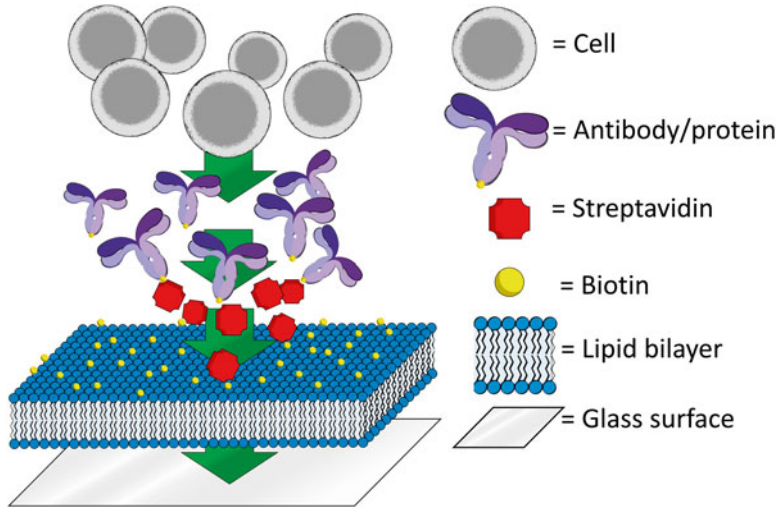


Fig. 4 Overview of the process of constructing the SLB. The first step of the process is to add biotinylated lipids to a pretreated glass surface. Then, streptavidin is added to provide a substrate for biotinylated proteins (here generically represented by an antibody). These proteins are strongly anchored onto the bilayer by the biotin–streptavidin interaction, but remain laterally mobile. Finally, cells are added to the bilayer, where they form protein-dependent contacts and, subsequently, immune synapses

2. While the coverslip is being cleaned, mix the DOPC stock with the diluted Biotin-PE at a 1:1 ratio as in the Subheading 3.3. The volume needed varies depending on how many experimental conditions are to be tested. Use 3 μL per condition (*see* **Note 14**).
3. After the coverslip is cleaned, use a plastic hemostat to remove the coverslip from the solution and wash off the Piranha solution with copious distilled water. Then stand the coverslip—still clamped by the hemostat—on one end and allow it to air-dry (*see* **Note 15**, also Fig. 5).
4. Using a 6-lane flow chamber slide, turn the slide bottom-up and place two 1.5 μL drops of mixed lipids within each lane for each experimental variable to be tested (*see* **Note 16**).
5. Quickly place the coverslip smoothly and firmly over the bottom of chamber (*see* **Note 17**, also Fig. 6). Follow up by pressing down around and in between each lane using a hemostat (or some other tool with a fine blunt tip) in order ensure a firm seal between the adhesive and the coverslip.
6. Then, inject 100 μL of 5 % casein into each lane that will be used.
7. Follow with 100 μL of streptavidin, and allow to incubate at RT for 10 min.
8. Wash out the streptavidin by flowing 1 mL of wash buffer through the chamber 3 times (*see* **Note 18**, also Fig. 7).
9. Then add protein, suspended in 100 μL of wash buffer, and allow to incubate between 20–30 min at RT in the dark. The exact concentration of the protein will depend on the final desired density of the protein on the bilayer, as well as if any other proteins are to be added simultaneously.
10. Wash as in **step 8**.

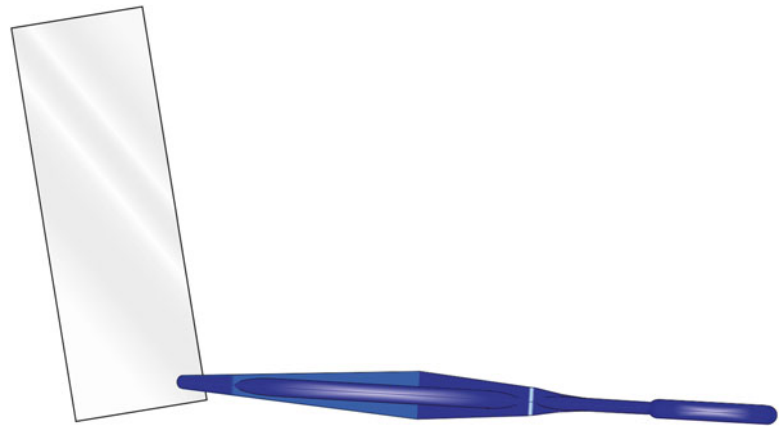


Fig. 5 Illustration of how to position a coverslip vertically in order to air-dry, following cleaning with Piranha solution and subsequent rinsing with distilled water

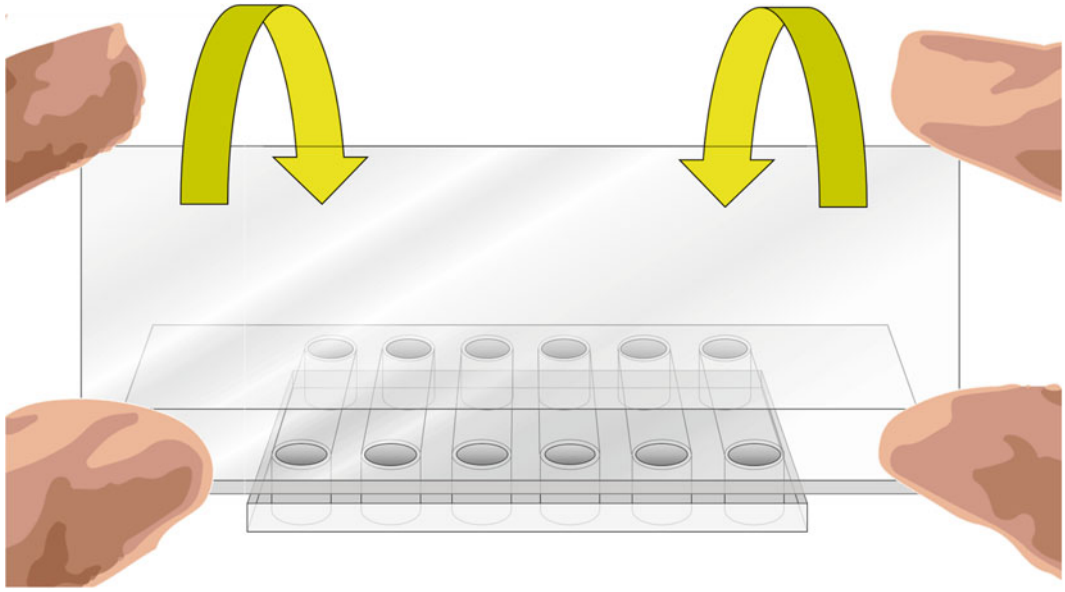


Fig. 6 Illustration of how to place the coverslip over the bottom of the flow chamber. With ungloved hands, grasp the edges of the coverslip between your thumb and index finger as pictured. Line up the near edge of the coverslip with the near edge of the chamber, them gently and evenly lay down the coverslip away from yourself until it lies flat against the chamber

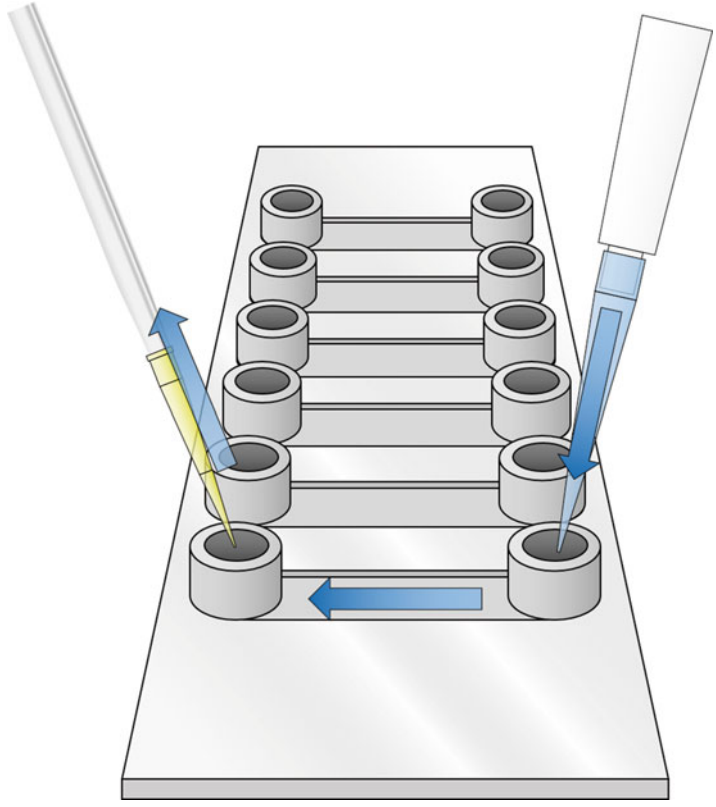


Fig. 7 Illustration of how to wash one chamber of the flow cell. A 1 mL pipette is placed at one end, and wash buffer is slowly passed through as suction is applied to the other end

11. At this point, block any unfilled streptavidin binding sites by adding 100 μL of D-biotin suspended in wash buffer at a 25 nM concentration to each lane. We let this sit for 10 min at RT in the dark, and finish by washing as in **step 8**.

At this point, the chambers are ready for the cells to be added. Depending on the experiment, this may or may not include an incubation period at 37 °C. The initial steps of synapse formation happen very rapidly (within 10 min). However, synapse formation can and will continue to develop over a period of hours. How long the cells are allowed to incubate on the bilayer before imaging them will depend on what stage of synapse formation is desired to be studied.

The chamber slide can be imaged just like a normal microscope slide using confocal or TIRF (total internal reflection fluorescence) microscopy.

4 Notes

1. To save time, the dilution and dialysis buffers can be prepared and stored in advance while the lipids lyophilize.
2. SLBs can be made in a variety of compositions, and can use a variety of different mechanisms in order to anchor surface proteins. These include glycophosphatidylinositol (GPI) anchors, polyhistidine tags, and biotin–streptavidin conjugation. Our lab currently employs the biotin–streptavidin system for its versatility and for the strength of the biotin–streptavidin interactions.
3. During this step, and all subsequent steps of the procedure in which one must expose the lipids to the air, the samples should be handled, deoxygenated, and resealed quickly to minimize oxygenation of the lipids, which will degrade them.
4. If speed is desired, 60–90 min lyophilization should suffice.
5. We recommend diluting the Biotin-PE lipids with the DOPC because the biotinylated phosphate head groups of the Biotin-PE lipids have more steric hindrance than the normal DOPC lipids. Thus, proteins embedded in a bilayer composed of pure Biotin-PE lipids may display impaired lateral mobility.
6. Complete air exclusion requires the sacrifice of a small volume of the sample by clamping just below the "waterline."
7. When removing the dialysis tubes from the bottle, it is important to do this in a sterile environment such as a biosafety cabinet. As the tubes will be dripping wet, though, you may want to put down some paper towels or a bench diaper beforehand.
8. We strongly emphasize "gently" when vortexing the plate. Too strong a vortex will send the beads violently flying out of the wells, requiring you to start over.

9. We block with casein in order to saturate any nonspecific binding sites (such as those created by static charge or hydrophobic/hydrophilic forces) on the coated beads with an inert, invisible protein, so that when we add our labeled, biotinylated protein, only the specific streptavidin sites will be available.
10. While there is no universally typically used proteins in these studies, our lab commonly works with anti-CD16 (clone 3G8), anti-PD-1 (clone EH12), and ICAM-1. These proteins provide positive, negative, and adhesive signaling, respectively. The proteins are stored frozen in PBS, and diluted in wash buffer, typically at final dilutions of 100 ng/mL, 80–100 ng/mL (depending on assay), and 100–500 ng/mL, respectively.
11. This process will need to be repeated for each fluorophore one intends to use, as the intensity of one fluorophore is not equivalent to another.
12. Sulfuric acid and Piranha solution are both extremely corrosive, and should always be handled with proper PPE in the confines of a chemical fume hood. Always add the hydrogen peroxide to the sulfuric acid slowly—never vice versa.
13. Treating glass coverslips with Piranha solution has two purposes: the first is to thoroughly clean the coverslip of any organic residues; the second is to hydroxylate the surface of the glass, making it highly hydrophilic (and thus an attractive substrate for the hydrophilic phosphate head groups of our lipids).
14. Be sure to deoxygenate your stock tubes with argon after opening them. Also, keep them on ice when transporting to and from the refrigerator.
15. This process can be expedited by using a compressed air canister to "blow-dry" the coverslip.
16. The drops need not be this exact volume or number. Each drop forms a lipid "island" within the lane. We have found that this volume is good for forming a single drop of sufficient size to capture a large number of cells, but small enough that it does not merge with either side of the lane, which will disrupt the bilayer. The two drops are meant to further increase our "capture" area, but in practice, we often observe that 90% of the cells that do form synapses do so on the first "island" anyway.
17. A trick we often use in this step is to remove our gloves, which can otherwise get caught between the coverslip and the chamber, and place the coverslip down with our bare fingers. Be sure when doing this, however, that you only grasp the edge of the coverslip. We find it also helps to line up the near edge first and gently lay down the coverslip away from yourself.
18. In order to flow a large volume of fluid through the chamber, grab a pipette and inject the wash buffer in one end while hold-

ing a vacuum line tipped with a narrow pipette tip at the other, as illustrated. Hold the vacuum tip right at the surface of the exit port, and allow the fresh intake of buffer from the other side to raise the volume until it comes in reach of your vacuum.

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Chapter 14

Expansion of NK Cells Using Genetically Engineered K562 Feeder Cells

Minh-Trang Thi Phan, Seung-Hwan Lee, Sang-Ki Kim, and Duck Cho

Abstract

Natural killer (NK) cells can be expanded upon activation by proliferative cytokines (such as IL-2 and IL-15). The NK cell expansion can be greatly enhanced by proteins from feeder cells such as tumor cell lines or PBMCs. Therefore, coculture systems of irradiated feeder cells and NK cells in media containing IL-2 and IL-15 have been developed to generate large numbers of NK cells, although NK cell expansion protocol using anti-CD3 antibody (OKT-3) without feeder cells has also been developed. Commonly used feeder cell lines are RPMI8866, Epstein-Barr lymphoblastoid cell line (EBV-LCL), and K562. Stimulation with NK-sensitive K562 cells is known to augment NK cell proliferation to IL-2, IL-15, and IL-21 in combination.

Recently, remarkable NK cell-expansion rates are achieved when genetically engineered (GE) feeder cells are used. Dr. Dario Campana's group found that membrane-bound IL-15 and 4-1BBL, coexpressed by K562 cells, acted synergistically to augment K562-specific NK stimulatory capacity, resulting in vigorous expansion of peripheral blood $CD56^+$ $CD3^-$ NK cells without concomitant growth of T lymphocytes. Here, we describe an in vitro expansion method of human NK cells among PBMCs by coculturing with GE_K562 cells.

Key words Natural killer cell, Expansion, K562, Feeder cells

1 Introduction

NK cells are defined phenotypically as $CD56^+$ $CD3^-$ lymphocytes, comprising 5–20% of peripheral blood mononuclear cells (PBMCs) in humans. Based on their phenotype and function, two NK cell subsets have been characterized: $CD56^{dim}$ $CD16^+$ NK cells which represent approximately 90% of circulating NK cells and have high cytotoxic activity, and $CD56^{bright}$ $CD16^{neg/dim}$ NK cells which constitute approximately 10% and have the capacity to produce abundant cytokines and chemokines [1, 2].

NK cells can directly kill target cells via the perforin–granzyme pathway, antibody-dependent cellular cytotoxicity (ADCC), and death receptor-ligand induced apoptosis. The killing of target tumors and virus infected cells by NK cells is regulated by the

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balance between activating and inhibitory receptors, including killer cell Ig-like receptors (KIRs), NKG2A, NKG2D; and natural cytotoxic receptors NKp30, NKp44, NKp46 [3, 4]. In addition to direct cytotoxicity, NK cells produce an array of cytokines and chemokines in response to target cells to modulate immune responses. On the basis of their antitumor killing activity and cytokine production, NK cells are one of the attractive tools for targeting cancer in immunotherapy [3, 5]. However, the selective expansion of NK cells to yield relevant amounts of these lymphocytes has been a major hurdle in the development of methods for clinical therapeutic use. Recently expansion and activation of NK cells has been achieved using cytokines (IL-2, IL-15) and feeder cells (tumor cell lines or PBMCs) that selectively activate NK cells in the cell-cell contact dependent manner. Among widely used feeder cells, GE_K562 (i.e., K562-mb15-41BBL) cells are shown to be the best candidate for remarkable NK cell expansion [6–8]. The NK cell expansion protocol using the GE_K562 cells was originally developed by Dr. Dario Campana group and then modified by others [6, 8–11]. Here, we describe an in vitro expansion method of human NK cells among PBMCs by coculturing with irradiated GE_K562 cells. Notably, this method with slight modification can also be used for the expansion of canine NK cells [12].

2 Materials

1. Vacutainer blood collection tubes with sodium heparin (BD)
2. Whole blood remaining in the leukoreduction system (LRS) chambers of Trima Accel (Gambro BCT) after platelet pheresis from healthy donors.
3. 1× phosphate buffered saline (PBS).
4. Fetal bovine serum (FBS, Gibco), with or without heat inactivation (30 min, 56 °C).
5. Lymphoprep 1.077 g/ml (Axis-Shield PoC AS, Oslo, Norway).
6. NK cell media: RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated FBS, 4 mM L-glutamine (Gibco), and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin, Lonza).
7. Complete RPMI 1640 media: RPMI 1640 medium supplemented with 10% heat-inactivated FBS, and 1% antibiotics.
8. 0.2 μm disposable filter systems (Corning).
9. Interleukin-2 (Peprotech Inc.).
10. Interleukin-15 (Peprotech Inc.).
11. 24-well tissue culture plate.
12. T75 tissue culture flask.

13. Sterile autoclaved micropipette tips (10, 100, and 1000 μL).
14. 15 and 50 ml sterile centrifuge tubes.
15. Sterile microcentrifuge tubes, 1.5 ml.
16. Cesium-137 Gammacell-3000 Elan irradiator (Best Theratronics).
17. GE_K562 (K562-mb15-41BBL cells, a gift of Dr. D. Campana, National University of Singapore).
18. FACS buffer: 1% FBS in 1× PBS.
19. Fixation buffer: 1% formaldehyde in 1× PBS.
20. Mouse anti-human CD3 conjugated with FITC (BD Pharmingen).
21. Mouse anti-human CD56 conjugated with PE-Cy5 (BD Pharmingen).
22. Mouse anti-human CD16 conjugated with PE (BD Pharmingen).
23. Mouse anti-human CD69 conjugated with PE (BD Pharmingen).
24. Mouse anti-human NKG2D conjugated with PE (BD Pharmingen).
25. Mouse anti-human NKp30 conjugated with PE (BD Pharmingen).
26. Mouse anti-human NKp44 conjugated with PE (BD Pharmingen).
27. Mouse anti-human NKp46 conjugated with PE (BD Pharmingen).
28. Mouse anti-human CD158b conjugated with PE (BD Pharmingen).
29. FACS Calibur cytometer and BD CellQuest software (BD Immunocytometry System).
30. Freezing solution: 10% dimethyl sulfoxide (DMSO) and 90% heat-inactivated FBS.

3 Methods

3.1 Preparation of Genetically Engineered K562 Feeder Cells

1. GE_K562 (i.e., K562-mb15-41BBL, a gift of Dr. D. Campana, National University of Singapore) cells are cultured in T-75 flasks containing 25 ml of complete RPMI 1640 media at 37 °C in a humidified incubator containing 5% CO_2.
2. Harvest feeder cells, centrifuge at 400 × *g* for 3 min. Resuspend the cell pellet in 5 ml complete RPMI 1640 media.
3. Irradiate feeder cells at 100 Gy with a Gammacell 3000 Elan irradiator (*see* **Notes 1** and **2**).

3.2 Isolation of Peripheral Blood Mononuclear Cells

1. Whole blood from healthy donors are diluted with PBS at 1:2 ratio (10 ml blood: 20 ml PBS), overlay onto 15 ml Lymphoprep and centrifuge at 1200 × *g* for 25 min at room temperature with no brake (acceleration 1; deceleration 0).
2. Harvest cells from the interface (buffy coat layer) and wash 3 times with PBS at 400 × *g* for 7 min.
3. Resuspend the cell pellet by tapping and then add NK culture media, count the cells with trypan blue exclusion method.

3.3 Culture of NK Cells from Isolated PBMCs

1. *On Day 0*: Seed 3×10^6 freshly prepared PBMCs and 0.5×10^6 100 Gy-irradiated GE_K562 in 1 ml NK cell media into 24 well plate (*see* **Note 3**) (An overview is presented in Fig. 1).
2. Add 1 ml NK cell media with 20 U/ml IL-2 to the well. Total media volume is 2 ml/well, final concentration of IL-2 is 10 U/ml. Mix well by gently pipetting.
3. Incubate at 37 °C, 5% CO_2 incubator.
4. *On Days 3 and 5*: change media; remove half of the media and add 1 ml fresh media containing sufficient fresh IL-2 to make 10 U/ml for 2 ml final volume. (*See* **Note 4**)
5. *On Day 7*: Count the number of cells in culture at the end of 1 week. If the cell number is more than 4×10^6 cells/ml, split the cell from one to four wells. If not, split the cell from one to two wells.
6. The concentration of IL-2 is increased to 100 U/ml and an additional 5 ng/ml of IL-15 is added to the media. In this method, NK cells are stimulated with GE_K562 cells only once at Day 0 during the entire 21 day period (*see* **Notes 5** and **6**)
7. Assess the purity of NK cells using a fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD3 and a PE-Cy5-conjugated mouse anti-human CD56 monoclonal antibodies for every week.
8. *Day 10 and 12*: change media; remove half of the media and add 1 ml fresh media containing 200 U/ml IL-2 and 10 ng/ml IL-15.
9. *Day 14*: Count the number of cells in culture at the end of 2 weeks. If the cell number is more than 4×10^6 cells/ml, split the cell from one to four wells. If not, split the cell from one to two wells. Check the purity of expanded NK cells after 2 weeks (as in **step 7**).
10. *Day 17 and 19*: change media; remove half of the media and add 1 ml fresh media containing 200 U/ml IL-2 and 10 ng/ml IL-15.
11. *Day 21*: Count the number of NK cells by trypan blue exclusion method, and check the purity of expanded NK cells using a FITC-anti-human CD3 and a PE-Cy5 anti-human CD56 monoclonal antibodies at the end of 3 weeks. Overall fold

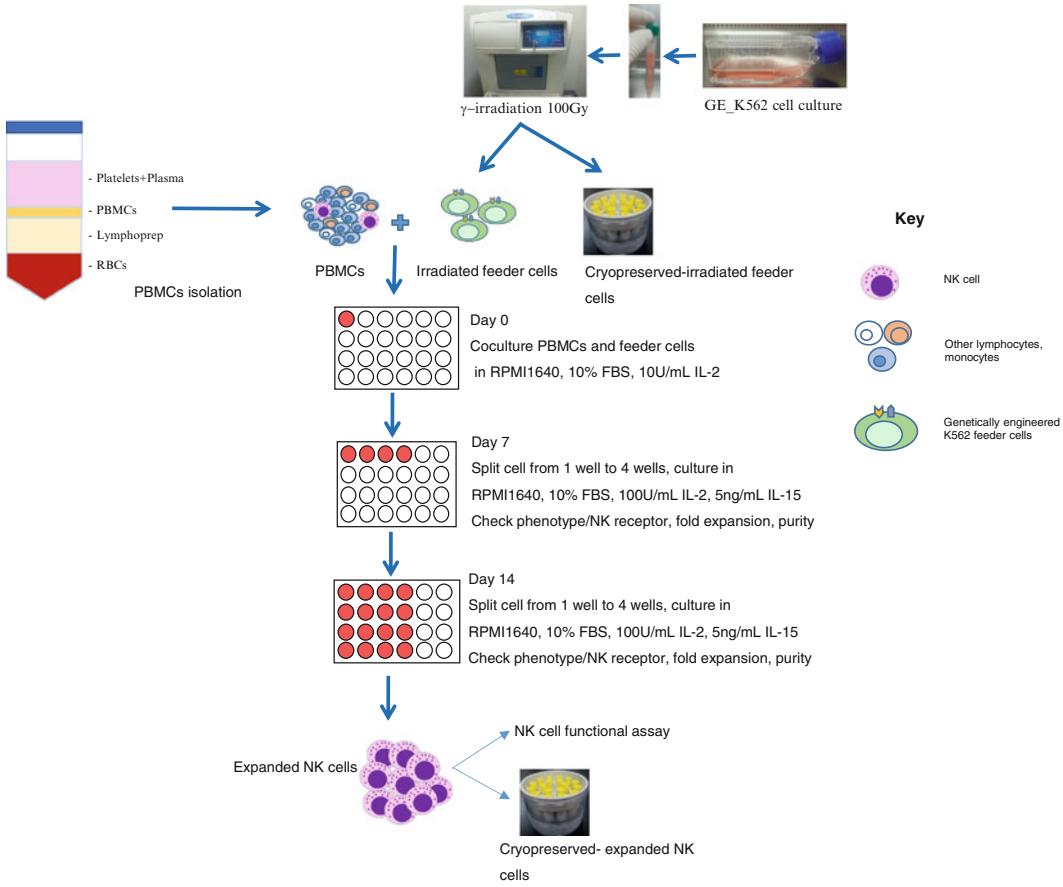


Fig. 1 Isolated PBMCs from healthy donors were cocultured in 24-well plate with cryopreserved-thawed irradiated GE_K562 feeder cells at a ratio of 6:1 (PBMCs:GE_K562) in NK cell media at 3×10^6 PBMCs/2 ml. NK phenotype, purity and fold expansion are checked every week. After a period of 2 or 3 weeks, sufficient numbers of expanded NK cells with high purity (>90 %) were obtained

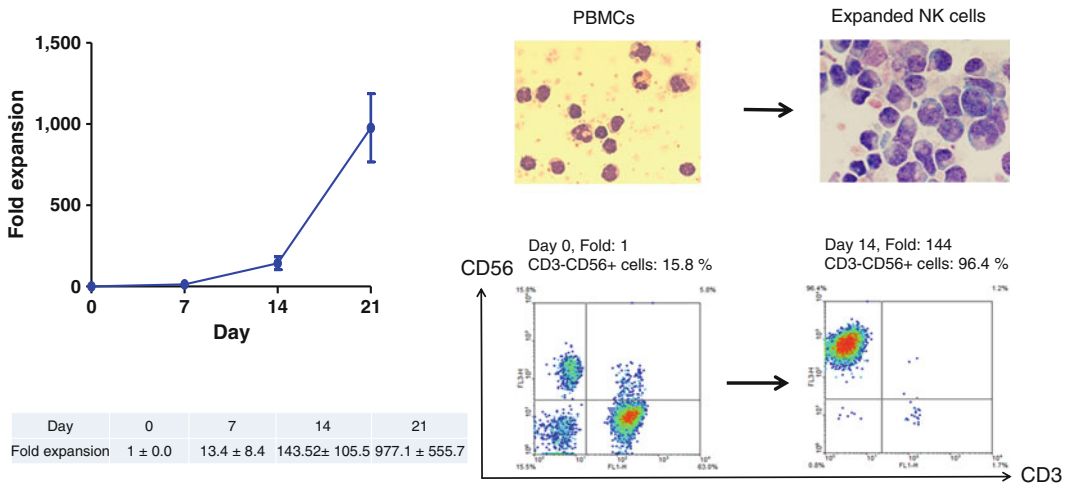
expansion and purity of NK cells that can be achieved by this protocol is shown in Fig. 2.

12. Harvest all expanded NK cells, which may be used directly or cryopreserved for later use.

3.4 Receptor Expression on Cultured NK Cells

Surface expression of NK cell activating and inhibitory receptors on cultured NK cells are checked on days 0, 7, 14, and 21 by flow cytometry.

1. Recover 2×10^5 cells per staining condition, and spin at $400 \times g$ for 5 min.
2. Remove the supernatant and resuspend cells in 100 μL FACS buffer per 2×10^5 cells.
3. Transfer 100 μL FACS buffer containing cells to FACS tubes.



Day	0	7	14	21
Fold expansion	1 ± 0.0	13.4 ± 8.4	143.52± 105.5	977.1 ± 555.7

Fig. 2 The morphological and flow cytometric analysis of NK cells before (day 0) and after (day 14) expansion was performed by May-Grünwald-Giemsa staining and flow cytometry. PBMC (day 0) and expanded NK cells (day 14) were stained with CD3, CD56 monoclonal antibody and analyzed by FACS. These photomicrographs were prepared with May-Grünwald-Giemsa-stained cytocentrifuged slides. The large granular lymphocytes indicate the expanded NK cells

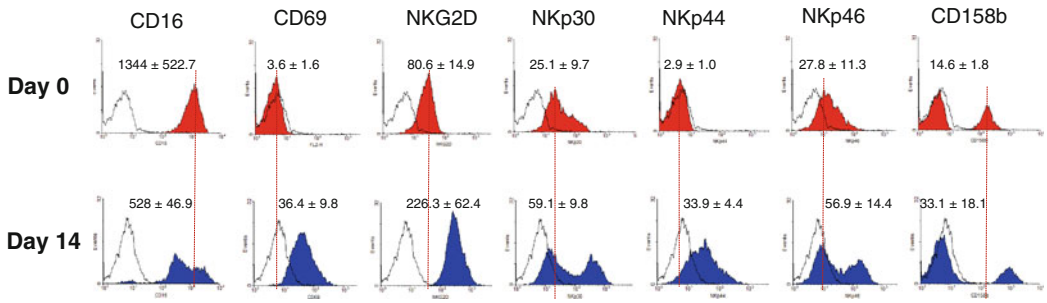


Fig. 3 Changes of expression of surface receptors on natural killer (NK) cells expanded using GE_K562 feeder cells. Phycoerythrin (PE)-conjugated antibody to human CD16, CD69, natural killer group-2, member D (NKG2D), NKp30, NKp44, NKp46, and CD158b (G) antibodies were used to analyze the NK cell receptors on day 0 (*upper*) and day 14 (*lower*). The peak at the extreme left of each figure shows the isotope control

4. Add 5 μL of fluorescence-conjugated target Abs or isotype control Ab, incubate on ice for 15 min. (Standard surface expressions of receptors assessed on expanded NK are CD16, CD69, NKG2D, NKp30, NKp44, NKp46, and CD158b as well as appropriate isotype controls).
5. Wash the cells after staining with 1 ml FACS buffer and spin at 400 × *g* for 5 min.
6. Repeat the washing step two more times.
7. Remove supernatant and resuspend stained cells in fixation buffer and analyze them by flow cytometry (Fig. 3).

4 Notes

1. In order to prevent cancer feeder cell overgrowth, several methods such as fixing with 10% formalin, treatment of methanol–acetic acid (3:1) mixture, heating, freezing–thawing, and irradiation have been used. Among them, irradiation (at dose from 30 Gy to 100 Gy) is a safe and effective method to prevent overgrowth in the coculture with NK cells [13, 14]. Our group uses 100 Gy for irradiation.
2. Both cryopreserved and freshly prepared irradiated feed cells can be used for the NK cell expansion. We usually have good success in NK cell expansion by using cryopreserved irradiated GE_ K562 cells (K562-mb15-41BBL cells). Irradiated feeder cells can be cryopreserved with freezing medium for later use. The NK cell expansion rate, NK cytotoxicity, NK cell receptors, and level of interferon-γ secretion are similar in NK cell expanded by using freshly irradiated feeder cells and cryopreserved irradiated feeder cells [10].
3. Direct cell contact between feeder cells and NK cells might be a substantial factor for NK cell expansion. In a 24-well plate, we start with 3×10^6 fresh PBMCs and 0.5×10^6 irradiated GE_ K562 for optimal proliferation of NK cells. The optimal cell density of PBMCs: GE_K562 ratio is critical factor for successful NK cell expansion.
4. To diminish effect of spontaneous evaporation during culture, we remove 950 μL and add 1000 μL of fresh media containing cytokines in 24-well plates when changing media half.
5. The NK cell expansion protocol using GE_K562 (K562-mb15-41BBL cells) requires a low concentration of IL-2 (i.e., 10 U/ml of IL-2) during the first week, and 100 U/ml of IL-2 after the first week.
6. It was reported that more than 95% of GE_K562 (K562-mb15-41BBL) cells cannot survive at day 3 of NK cell expansion [10]. Therefore, the feeder cells might lose the function of genetically engineered molecules (i.e., membrane bound form of IL-15). However, in our protocol, we do not add GE_ K562 cells every week, in contrast with other groups [7, 8]. Instead of adding GE_K562 cells every week, we add 5 ng/ml of IL-15 to the media after 7 days of culture and IL-15 every 2 days through the culture period. In the presence of 5 ng/ml of IL-15 as well as 100 U/ml of IL-2, NK cells vigorously proliferated over a period of 3 weeks [9, 10].
7. This protocol is a slight modification of the original one described in [6, 7].

Acknowledgments

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Chapter 15

Ex Vivo Expansion of Human NK Cells Using K562 Engineered to Express Membrane Bound IL21

Srinivas S. Somanchi and Dean A. Lee

Abstract

Natural killer (NK) cells have gained significant attention for adoptive immunotherapy of cancer due to their well-documented antitumor function. In order to evaluate the therapeutic efficacy of NK cell adoptive immunotherapy in preclinical models with a potential for clinical translation, there is a need for a reliable platform for ex vivo expansion of NK cells. Numerous methods are reported in literature using cytokines and feeder cells to activate and expand human NK cells, and many of these methods are limited by low-fold expansion, cytokine dependency of expanded NK cells or expansion-related senescence. In this chapter, a robust NK cell expansion protocol is described using K562 cell line gene modified to express membrane bound IL21 (K562 mb.IL21). We had previously demonstrated that this platform enables the highest fold expansion of NK cells reported in the literature to date (>47,000-folds in 21 days), and produces highly activated and pure NK cells without signs of senescence, as determined by telomere shortening.

Key words NK cells, mbIL21, Expansion, Adoptive immunotherapy

1 Introduction

Over the last decade, there has been tremendous interest in applying natural killer (NK) cell adoptive immunotherapies for various cancers [1–7]. The natural ability of these cells to lyse tumor cells without prior sensitization [8], their pivotal role in antitumor responses and immunosurveillance against cancer [9, 10], along with the findings that NK cells do not cause graft-vs-host disease (GVHD), and that in fact prevent T cell induced GVHD through lysis of host APCs [11] while maintaining graft vs tumor effect [12] have all been central to the overall appeal of NK cells for adoptive immunotherapy for cancer.

However, the progress of NK cell immunotherapy for cancer has been slow, mainly due to the limitation in obtaining sufficient number of NK cells for robust preclinical and clinical evaluations. In normal human peripheral blood, the NK cells comprise 1–32.6% (with a median of 7.6%) of peripheral blood lymphocytes [13] and

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until recently, there were no efficient methods to expand NK cells ex vivo. Although leukapheresis had been used to obtain NK cells (following T cell depletion and IL-2 activation), sufficient for a single infusion of $<2\times10^7$ cells/kg [14], with good antitumor effect [3, 5, 15], the technical difficulties in reliably obtaining large NK cell numbers while maintaining high NK cell purity remained an important hurdle of this technique.

Therefore, there has been significant interest in developing methods for ex vivo expansion of NK cells. Several methods have been developed using single or multiple cytokines to support NK cell expansion in culture [16–22] or with the support of feeder cells [23–25], including genetically modified K562 [26–28]. These methods enabled expansion of NK cells ex vivo from ~35 folds in 12 days [23] to 1625 folds in 20 days [2], with one exception, where >15,000 fold expansion was achieved in 35 days [21] from umbilical cord blood hematopoietic stem and progenitor cells.

Previously, we developed a robust NK cell expansion platform using K562 cell line gene modified to express membrane bound IL21 (K562 mbIL21) as feeder cells [29], based on the reported role of IL-21 in NK cell expansion and regulation [30]. In order to minimize the potential for cytokine dependency we supplemented the NK cell culture with a low dose of soluble IL-2 (50 IU/mL). This platform supported the highest fold expansion of NK cells reported in the literature—with a mean NK cell expansion of 47,967-folds in 21 days [31]. This methodology has since been widely used, to study NK cell biology in disease [32, 33], to expand NK cells from human umbilical cord blood [34], to expand NK cells for in vitro and preclinical evaluation in neuroblastoma disease model [35, 36], to expand NK cells differentiated from inducible pluripotent stem cells and human embryonic stem cells [37, 38] as well as to expand NK cells from rhesus macaques [39]. In this chapter we have provided a detailed protocol for ex vivo expansion of human NK cells using K562 mb.IL21 cell line as feeder cells, recommendations for phenotypic analysis and protocol for calcein release assay to assess cytolytic function of the expanded NK cells.

2 Materials

2.1 Isolation of PBMCs

1. Buffy coat sample from healthy donor (Regional Blood Bank).
2. 250 mL sterile storage bottles or T75 flasks.
3. Ficoll-Paque Plus (GE Healthcare Life Sciences).
4. 50 mL conical tubes.
5. Wash buffer: 2% fetal bovine serum (FBS) in PBS (store at 4 °C).
6. RPMI complete medium: Prepare RPMI 1640 medium supplemented with 10% FBS, 1× Pen-Strep, and 1× GlutaMAX, filter-sterilize through a 0.2 μ media filter and store at 4 °C.

2.2 RosetteSep Purification of NK Cells

1. Wash buffer: 2% fetal bovine serum (FBS) in PBS (store at 4 °C).
2. RosetteSep™ Human NK cells enrichment Kit (Stemcell Technologies).
3. Alsever's solution (Sigma).
4. Ficoll-Paque Plus (GE Healthcare Life Sciences).
5. 50 mL conical tubes.
6. NK cell medium: RPMI 1640 medium, 10% FBS, 1× Pen-Strep, 1× GlutaMAX, filter-sterilize through a 0.2 μ media filter and store at 4 °C, freshly supplement with 50 IU/mL of IL2 (200 IU/μL stock) (Proleukin, Novartis Vaccines and Diagnostics Inc.).
7. Freezing medium: 90% FBS and 10% DMSO.

2.3 CD3 Depletion of PBMCs

1. Human CD3 MicroBeads (Miltenyi Biotec).
2. LD columns (Miltenyi Biotec).
3. MidiMACS Separator (Miltenyi Biotec).
4. MACS buffer: Prepare MACS buffer by supplementing PBS (Ca^{2+} and Mg^{2+} free) with 0.5% BSA and 2 mM EDTA (pH 7.2), store at 4 °C.
5. 5 mL FACS tubes.
6. 15 and 50 mL conical tubes.
7. NK cell medium: RPMI 1640 medium, 10% FBS, 1× Pen-Strep, 1× GlutaMAX, freshly supplement with 50 IU/mL of IL2 (200 IU/μL stock) (Proleukin, Novartis Vaccines and Diagnostics Inc.).
8. Freezing medium: 90% FBS and 10% DMSO.

2.4 NK Cell Expansion and Phenotype

1. K562 mb.IL21 (Dr. Dean A. Lee's Laboratory, MD Anderson Cancer Center).
2. GammaCell 1000 Irradiator.
3. RPMI complete medium: Prepare RPMI 1640 medium supplemented with 10% FBS, 1× Pen-Strep, and 1× GlutaMAX, filter-sterilize through a 0.2 μ media filter and store at 4 °C.
4. NK cell medium: RPMI 1640 medium, 10% FBS, 1× Pen-Strep, 1× GlutaMAX, filter-sterilize through a 0.2 μ media filter and store at 4 °C, freshly supplement with 50 IU/mL of IL2 (200 IU/μL stock) (Proleukin, Novartis Vaccines and Diagnostics Inc.).
5. 50 and 250 mL conical tubes.
6. T-75 and T-150 flasks.
7. Freezing medium: 90% FBS and 10% DMSO.
8. Antibody Cocktails: Prepare antibody cocktails as per Table 1. Prepare bulk cocktails sufficient for 25–50 samples depending on usage, and store in the dark at 4 °C.

Table 1
List of antibodies and recommendation for staining combination for phenotypic analysis of expanded NK cells using four-color flow cytometer

	Antibody cocktail	Volume/ sample (μL)[a]		Antibody cocktail	Volume/ sample (μL)[a]
Tube 1	Isotype FITC[b]	5	*Tube 7*	KIR2DL1-FITC	5
	Isotype PE[b]	5		KIR2DL2/3-PE	5
	Isotype Pe-Cy7[b]	5		NKp46-PE-Cy7	5
	Isotype APC[b]	5		KIR3DL1-APC	5
Tube 2	CD56-FITC	5	*Tube 8*	CD56-FITC	5
	CD16-PE	5		CD11b-PE	5
	CD3-PE-Cy7	5		CD3-PE-Cy7	5
	NKp46-APC	5		CD27-APC	5
Tube 3	CD56-FITC	5	*Tube 9*	CD56-FITC	5
	NKp44-PE	5		CD11a-PE	5
	CD3-PE-Cy7	5		CD3-PE-Cy7	5
	NKp30 Alexa Fluor 647	5		TRAIL-APC	5
Tube 4	CD56-FITC	5	*Other surface and intracellular markers to consider*:		
	CD244-PE	5	CD2		
	CD3-PE-Cy7	5	CD28		
	NKG2D-APC	5	CD57		
Tube 5	CD56-FITC	5	CD62L		
	CD226-PE	5	CD69		
	CD3-PE-Cy7	5	CD25		
	CD160 Alexa Fluor 647	5	Chemokine receptors		
Tube 6	CD56-FITC	5	Chemokines (CCL3, CCL4, CCL5)		
	KIR2DL1/DS1-PE	5	Granzyme B		
	KIR2DL2/3-PE	5	Granzyme K		
	KIR3DL1-PE	5	Perforin		
	CD3-PE-Cy7	5	IFN-γ		
	NKG2A-APC	5	TNF-α		

[a]The antibody volumes recommended are for a "100 test size," use 2 μL of antibody for a "25 test size" antibody vials
[b]More than one set of isotype controls may be necessary depending on the specific isotype of the antibodies used in each antibody cocktail

2.5 Assessment of NK Cell Cytotoxicity

1. K562 and/or other tumor cell lines.
2. Calcein AM (1 mg/mL, Life Technologies).
3. RPMI complete medium: RPMI 1640, 10% FBS, 1× Pen/Strep, 1× GlutaMAX.
4. Triton X-100.
5. "U" and flat clear bottom 96-well plates.
6. Fluorescence spectrophotometer.

3 Methods

3.1 Isolation and Purification of NK Cells

NK cell expansion on K562 mb.IL21 activating and propagating cells (AaPC) can be performed either directly from PBMCs derived from peripheral blood, buffy coat or cord blood sources. However, to avoid T cell contamination in the expanded NK cell product, the NK cells can be expanded either from purified NK cells or from T cell (CD3) depleted PBMCs. Currently, CD3 depleted PBMC product is used for expansion of NK cells on K562 mbIL21 for clinical application. For laboratory use and preclinical animal models of adoptive immunotherapy, NK cells are expanded from purified NK cells. This protocol describes PBMC isolation by Ficoll-Paque density centrifugation, NK cell purification by RosetteSep™ method and CD3 depletion by CD3 MicroBead method, which are routinely used in our laboratory. However, it is important to note that there are other commercial reagents available for NK cell purification and CD3 depletion.

3.1.1 Isolation of PBMCs

This protocol describes isolation of PBMCs using Ficoll-Paque density centrifugation as per manufacturer's protocol, with minor modifications. Use sterile reagents and plasticware for PBMC isolation and perform the protocol in tissue culture hood.

1. Transfer the blood sample (peripheral blood, buffy coat or cord blood sample) into a T75 cell culture flask (kept upright) or 250 mL sterile bottle and dilute the blood sample with equal volume of PBS (*see* **Note 1**).
2. Add 15 mL of Ficoll-Paque to 50 mL conical tube. Use as many 50 mL tubes as needed to accommodate the blood sample.
3. Slowly layer up to a maximum of 35 mL of diluted blood sample on to Ficoll-Paque in each 50 mL tube (*see* **Note 2**).
4. Centrifuge the sample at 400*g* for 20 min at room temperature, without brakes.
5. Carefully transfer the tubes to biosafety cabinet without disturbing the layer of PBMCs at the interface.
6. Aspirate and discard the top 25 mL (*see* **Note 3**).
7. Carefully recover the PBMCs from the Ficoll-Paque interface using a P-1000 pipette and combine into a single fresh 50 mL conical tube (*see* **Note 4**). Save the RBCs for RosetteSep™ purification of NK cells (described in Subheading 3.1.2).
8. To wash PBMCs, add wash buffer to 50 mL mark and spin the tube at 400*g* for 5 min (*see* **Note 5**), aspirate the supernatant.
9. Repeat the wash step two more times.

10. Resuspend the PBMCs in 30 mL of complete medium, recover a 10 μL aliquot into a 0.5 mL microcentrifuge tube, dilute ~20 folds with PBS, and perform cell count and viability using trypan blue exclusion method.
11. Set aside about 3×10^6 PBMCs to assess phenotype by flow cytometry (refer to Subheading 3.4), and use the remaining PBMCs for either NK cell purification or CD3 depletion.

3.1.2 RosetteSep™ Purification of NK Cells

This protocol is performed as per manufacturer's protocol with minor modifications.

1. Aspirate and discard the Ficoll-Paque layer above the RBCs after PBMC isolation (Subheading 3.1.1, **step 7**). Take care not to disturb the RBC layer, as the RBCs do not form a tight pellet.
2. Combine the RBCs into two 50 mL conical tubes and add wash buffer up to 50 mL mark. Spin at 400 *g* for 5 min.
3. Aspirate the supernatant and repeat the wash step two more times using 50 mL of wash buffer (*see* **Note 6**).
4. Resuspend RBCs in 50 mL wash buffer, mix well and take an aliquot of RBCs and dilute 1:10,000 times in wash buffer and count using hemacytometer.
5. Into the tube containing PBMCs, add a 100-fold excess of RBCs to that of viable PBMCs (100 RBCs: 1 viable PMBC) (*see* **Note 7**).
6. Spin the tube at 400 *g* for 5 min.
7. Aspirate the supernatant gently without disturbing the loose cell pellet, and resuspend the RBC–PBMC mix at 50×10^6 PBMCs per 1 mL of wash buffer (*see* **Note 8**).
8. To the RBC–PBMC cell mix add 1 μL RosetteSep™ Human NK cell Enrichment Cocktail per 1×10^6 PBMCs (*see* **Note 9**).
9. Mix well using a P-1000 pipette, and incubate at room temperature for 20 min. Mix contents gently by swirling the tube every 5 min.
10. After 20 min add equal volume of wash buffer, mix gently by pipetting up and down.
11. Add 15 mL Ficoll-Paque to a 50 mL conical tube and carefully layer the RBC–PBMC cell mix on top.
12. Spin the tube at 400 *g* for 20 min without brakes.
13. Carefully transfer the tube to biosafety cabinet without disturbing the NK cells at the interface. Aspirate the upper layer up to 20 mL mark and carefully recover the NK cell layer from the Ficoll-Paque interface using P-1000 pipette.
14. Transfer NK cells to a fresh 15 mL conical tube and add wash buffer to 10 mL mark.

15. Spin the tube at 400*g* for 5 min.
16. Aspirate supernatant and repeat wash step two more times.
17. Respsupend NK cells in 10 mL of NK cell medium, assess cell counts and viability using trypan blue exclusion method.
18. Set aside 1×10^6 NK cells to assess phenotype by flow cytometry (refer to Subheading 3.4).
19. The purified NK cells can be used directly for expansion by stimulation with K562 mbIL21 cell line or stored frozen (in freezing medium) until needed (*see* **Note 10**).

3.1.3 CD3 Depletion of PBMCs

This protocol describes CD3 depletion of PBMCs using Miltenyi's CD3 MicroBead system (*see* **Note 11**), as per manufacturer's protocol with minor modifications;

1. Transfer PBMCs obtained from Ficoll-Paque interface in a 50 mL sterile conical tube, and spin at 400*g* for 5 min. Resuspend up to 10×10^6 PBMCs in 80 μL of MACS buffer, and add 20 μL of CD3 MicroBeads per 80 μL of cells. Mix well by pipetting up and down using a P-1000 pipette.
2. Incubate the cells at 4 °C for 20 min.
3. During incubation, prepare the LD column: Place the LD column into the slot on the MidiMACS Separator magnet. Place a 5 mL FACS tube underneath the LD column and add 2 mL of MACS buffer to the column and allow to flow through the column under gravity. Discard the flow-through and place a fresh 15 mL sterile conical tube (collection tube) underneath the LD column.
4. After staining PBMCs with CD3 MicroBeads, add MACS buffer to 50 mL mark (*see* **Note 12**) and spin at 300*g* for 10 min, to remove unbound CD3 MicroBeads.
5. Aspirate the supernatant and repeat the wash step one more time using 10 mL of MACS buffer.
6. Aspirate supernatant and resuspend up to 100×10^6 PBMCs in 500 μL of MACS buffer.
7. Using a P-1000 pipette, add the CD3 MicroBead stained PBMCs to the LD column and allow the cells to pass through the column under gravity.
8. Once the 500 μL of cells completely pass into the column, wash the column with 1 mL of MACS buffer. Wait for the entire volume to pass into the column.
9. Repeat the wash step one more time, collecting the flow-through each time in the same collection tube.
10. The flow-through collected in the 15 mL conical tube contains the unlabeled, CD3 depleted PMBCs.

11. Spin the cells at 400 *g* for 5 min, aspirate and discard the supernatant.
12. Resuspend CD3 depleted PBMCs in 10 mL of NK cell medium.
13. Recover a 10 μL aliquot, dilute 10–20-folds with PBS and assess cell count and viability using trypan blue.
14. Set aside 1×10^6 PBMCs to assess phenotype by flow cytometry (refer to Subheading 3.4).
15. Use the CD3 depleted PBMCs for NK cell expansion by co-stimulation with irradiated K562 mbIL21 or store frozen until needed.

3.2 Culture and Irradiation of K562 mbIL21

The protocol for K562 mbIL21 irradiation is described here for a GammaCell 1000 Irradiator. Other methods such as X-ray irradiation can be used for this purpose. The user should refer to the Institutional guidelines and protocols, and obtain necessary training and certification before gaining access to an irradiator.

1. Maintain K562 mbIL21 cell line in RPMI complete medium and passage the cells by splitting 1:6 when the cell density reaches about 6×10^5 cells/mL.
2. For irradiation purpose, prepare a large culture of approximately 500×10^6 K562 mbIL21.
3. Spin the cells in 50 or 250 mL conical tubes.
4. Combine up to 100×10^6 cells per 50 mL conical tube or 500×10^6 cells in a 250 mL conical tube (*see* **Note 13**).
5. Irradiate cells in Gammacell irradiator: 10,000 cGy is equal to 100 Grays of irradiation. Set up 10,000 cGy exposure based on the display of Central dose for the day (the instrument displays the decaydose/minute for the day), calculate the amount of time required to expose cells to 10,000 cGy. Place the sample in the canister and place in the irradiation chamber, enter the calculated irradiation time and start the irradiator. If the maximum permissible duration of operation (generally about 20 min) is insufficient to achieve 10,000 cGy, repeat the irradiation process by calculating the time required to achieve the remaining amount of cGY.
6. Spin the irradiated cells at 400 *g* for 5 min.
7. Aspirate and discard supernatant and resuspend cells in freezing medium at a cell density of 20×10^6 cells per mL and freeze 1 mL per cryovial (*see* **Note 14**).

3.3 NK Cell Expansion

Our typical duration of NK cell expansion using this protocol is 21 days (3 stimulations), however shorter periods of expansions up to 14 days (2 stimulations) can be performed if sufficient numbers of NK cells are derived by day 14 (for the specific experimental need). Note that this platform can also support longer periods of expansion as previously reported [31].

3.3.1 Stimulation 1

1. On day 0, seed 5×10^6 CD3 depleted PBMCs or purified NK cells and 10×10^6 irradiated K562 mbIL21 (1:2 ratio) in a T75 flask (upright), resuspended in 40 mL of NK cell medium.
2. Perform a complete medium change on day 3—mix the cells gently to break up any cell aggregates and collect the single cell suspension (40 mL) in a 50 mL conical tube and spin at 400 *g* for 5 min.
3. Discard supernatant and resuspend the cell pellet in 20 mL of NK cell medium and transfer to the same T75 flask. Rinse the tube once with 20 mL of NK cell medium to completely recover the cells and transfer to the T75 flask.
4. On day 5 perform a second round of complete medium change (as described above) and distribute cells equally in two T75 flasks, with 40 mL of NK cell medium each.
5. On day 7, mix the cells thoroughly by pipetting them up and down using a 10 mL pipette. Collect three aliquots of 10 μL each, add equal volume of trypan blue and perform cell counts (in triplicates).
6. Set aside about 3×10^6 cells for phenotype analysis by flow cytometry (refer to Subheading 3.4), carry 5×10^6 cells forward for a second round of expansion by stimulation with K562mb.IL21. Freeze the remaining cells (from 3×10^6 to 50×10^6 cells per vial) in freezing medium, using controlled rate freezing container, for future use (*see* **Note 15**).

3.3.2 Stimulation 2

1. Seed 5×10^6 expanded cells from day 7 with 5×10^6 K562mb.Il21 (1:1 ratio) per T75 flask (upright) in 40 mL of NK cell medium (*see* **Note 16**).
2. On day 10, perform a complete medium change as described before and seed cells in two T75 flasks (upright) with 40 mL NK cell medium in each, or one T150 flask with 80 mL of NK cell medium.
3. On day 12, perform a complete medium change and split the cells in to a total of 4 flasks (incubated on its side) with 60 mL NK cell medium in each or into two T150 flasks with 120 mL (maximum volume) each (*see* **Note 17**).
4. At the end of stimulation 2—on day 14, mix the cells to break up any aggregates and combine cells into a single flask (or 500 mL conical centrifuge tube), mix well again and recover 3 aliquots of 10 μL each for cell counts.
5. Set aside, 8×10^6 cells for phenotype analysis by flow cytometry.
6. If the desired number of NK cells is achieved at this stage, freeze the cells in aliquots of 50×10^6 cells/vial or as desired, using a controlled rate freezer.

If an additional week of expansion is required to achieve desired numbers of NK cells, a third stimulation can be performed according to the protocol described in Subheading 3.3.2 using higher starting cell numbers ($20–50 \times 10^6$ NK cells) and scale up the culture volumes described above accordingly.

3.4 Phenotype Analysis and Calculating NK Cell Fold Expansion

3.4.1 Phenotype by Flow Cytometry

To determine the percentage of NK cells in PBMCs, CD3 depleted PBMCs and to assess the purity of RosetteSep purification of NK cells, perform a basic NK cell/T cell marker staining using CD3, CD56, CD16 and either NKG2D or NKp46 (Table 1, Tube 2). For expanded NK cells perform an extensive panel of staining as recommended in Table 1. Note that the recommended phenotype panel can be expanded further to include various chemokine receptors, intracellular markers such as granzyme B and perforin as well as cytokines (using either intracellular staining protocols, Bead-based immunoassay by flow cytometry per manufacturer's protocol or by ELISA based assays). Representative phenotype data for expanded NK cells is shown in Fig. 1.

1. Resuspend the PBMCs and purified cell populations (from Subheading 3.1) in blocking medium (at 5×10^5 cells/50 µL) and incubate at 4 °C for 30 min.
2. Transfer 50 µl of cells into a non-sterile "U" bottom 96-well plate for staining.

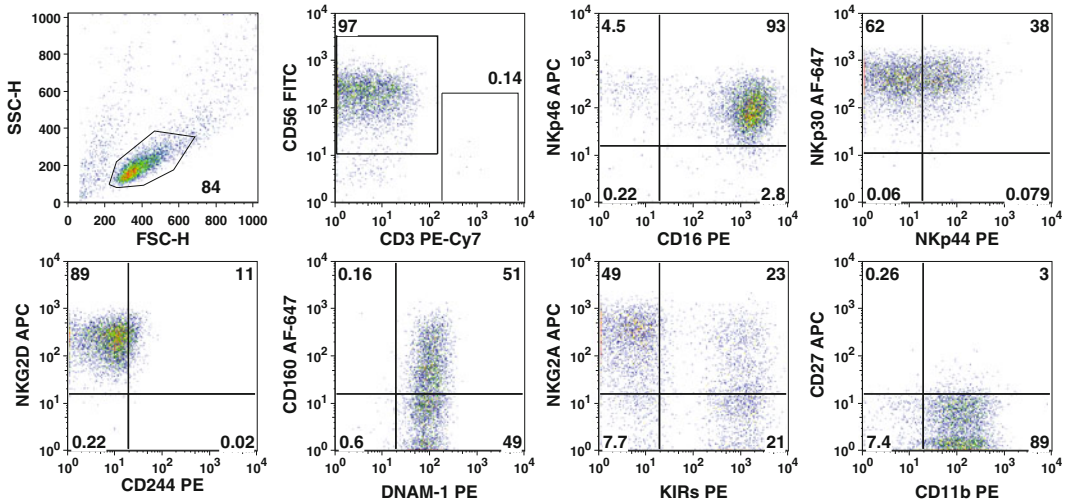


Fig. 1 Representative phenotype of expanded human NK cells on day 14. Expanded NK cells express various activating and inhibitory receptors. Note that ~100% of expanded NK cells express activating receptors NKG2D, DNAM-1, and NCRs (NKp46 and NKp30). Though the exact percentage and intensity of receptor expression is donor dependent, NK cells expanded from a majority of donors have fairly similar expression trends as shown here

Table 2
Recommendation for single color compensation and FMO controls

Single stain compensation controls[a]	
FITC	CD56 FITC
PE	CD16 PE
PE-Cy7	CD3 PE-Cy7
APC	NKG2D/NKp46 APC
Fluorescence minus one (FMO) controls[a]	
FMO 1	CD16 PE + CD3 PE-Cy7 + NKG2D/NKp46 APC
FMO 2	CD56 FITC + CD3 PE-Cy7 + NKG2D/NKp46 APC
FMO 3	CD56 FITC + CD16 PE + NKG2D/NKp46 APC
FMO 4	CD56 FITC + CD16 PE + CD3 PE-Cy7

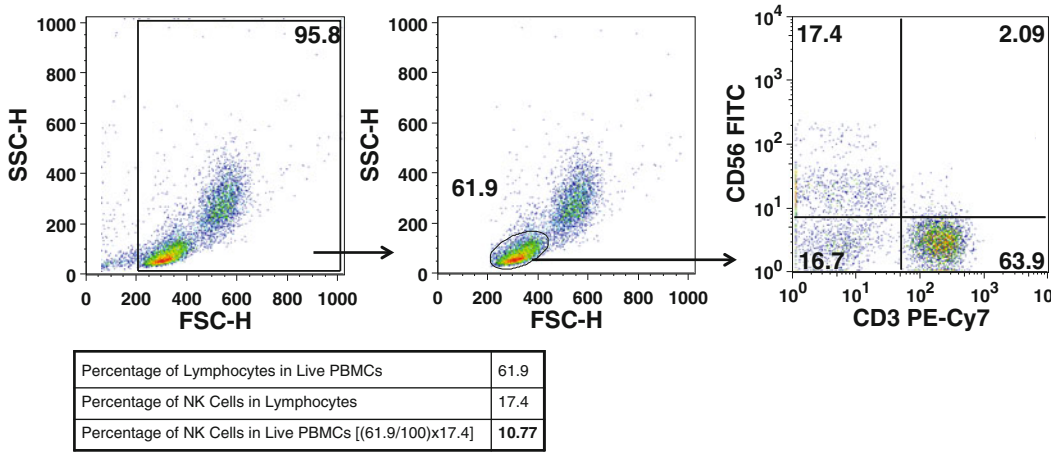
[a]For CD3 depleted primary or expanded NK cell population substitute CD3 PE-Cy7 staining control with CD56 PE-Cy7 or any other NK cell marker

3. To the 50 μL of cells, add antibodies as indicated in Table 1 (Tubes 1–2) and incubate at 4 °C for 30 min. Use PBMCs to stain for controls to optimize the flow cytometry PMT voltages—either single color compensation controls or FMO (fluorescence minus one) as per Table 2.
4. For the day 7 expanded cells perform compensation and phenotype staining as described above. For day 14 expanded cells perform a comprehensive staining (Tubes 1–12) and incubate at 4 °C for 30 min.
5. Add 150 μL of PBS to the cells and spin at 400*g* for 1 min to wash off excess antibody.
6. Discard the supernatant by quick and sharp inversion of the plate.
7. Repeat the wash step two more times.
8. Analyze the cells by flow cytometer immediately, alternatively, fix the cells in 200 μL of 1% formaldehyde (or 4% paraformaldehyde) and transfer to disposable FACS tubes. Fixed cells can be stored at 4 °C for a couple of days.

3.4.2 Calculating NK Cell Fold Expansion

If the NK cell expansion is initiated from RosetteSep™ purified NK cells, calculating the fold expansion of NK cells is straightforward. However, if PBMCs or CD3 depleted PBMCs are used for NK cell expansion the fold expansion will need to be calculated from cell count data and flow cytometry phenotype data as described below.

1. From the forward–side scatter plot of the flow cytometry data (acquired on days 0, 7, and 14), draw a gate around all viable cell populations (which include lymphocyte, monocyte, and granulocyte populations for cells on days 0). Open this cell population in a new dot plot and draw a gate around the lymphocyte population. Note the percentage of lymphocytes amongst the total viable PBMCs.



Percentage of Lymphocytes in Live PBMCs	61.9
Percentage of NK Cells in Lymphocytes	17.4
Percentage of NK Cells in Live PBMCs [(61.9/100)x17.4]	**10.77**

Fig. 2 Flow cytometry gating for calculating NK cell percentage in PBMCs or CD3 depleted PBMCs. The flow plots show the gating approach for accurately determining the percentage of NK cells. Draw a gate to exclude dead cells/debris in the forward–side scatter plot. Then plot the $CD3^{neg}CD56^{pos}$ population from the lymphocyte population. Calculate the percentage of NK cells in live total PBMCs (or CD3 depleted PBMCs), as shown here

2. Carry the lymphocyte population forward to a new dot plot and display the plot for CD3 vs CD56. Note the percentage of $CD3^{neg}CD56^{pos}$ NK cells amongst lymphocytes. Back calculate the percentage of NK cells amongst total viable PBMCs (Fig. 2) (*see* **Note 18**).
3. Input the calculated percentage of NK cells to calculate the actual number of NK cells present in PBMCs or CD3 depleted PBMCs used for expansion as well as the overall fold expansion of NK cells according to Table 3. If all the cells are not carried forward for expansion on Day 7 (and on day 14, if expansion is carried out for 21 days), present the data for overall NK cell expansion as "Inferred fold expansion" calculated based on observed fold expansion as shown in Table 3.

3.5 Assessment of NK Cell Cytotoxicity

Two approaches are widely used for determining NK cell function—degranulation assay and cytotoxicity assay. It is important to make a distinction between these assays. The degranulation assay uses the surface expression of CD107a on NK cell as a marker of cytolytic response by the NK cells against a target [40]. This method determines the percentage of NK cells that respond to a stimulus such as tumor target by releasing their cytolytic granules, however, does not give any information pertaining to the lysis of the target cells as a consequence of the release of lytic granules by NK cells. On the other hand, cytotoxicity assay provides assessment of percentage lysis of the tumor target by NK cells but does not give any information about the number/percentage of NK cells that react to a given tumor target. Overall, cytotoxicity assay provides data on the therapeutic efficacy of NK cells and thus is

Table 3
Recommendation for calculating NK cell fold expansion. Formulae are provided to create an Excel workbook calculator

	A	B	C	Notes
1	*Day 0*	Starting cell number for expansion	5,000,000	Input the number of cells used for expansion based on cell count
2		% NK cells	10.77	Calculated % from flow cytometry data
3		Number of NK cells used for expansion	538,500	=(C1/100)×C2
4	*Day 7*	Number/Yield of expanded cells	49,020,000	Input raw cell count
5		% NK cells	90	Calculated % from flow cytometry data
6		Number of NK cells in expanded population	44,118,000	=(C4/100)×C5
7		NK cell fold expansion (stimulation 1)	81.93	=C6/C3
8		Total cells carried forward for expansion	4,000,000	Based on cell count
9		NK cells carried forward for expansion	3,600,000	=(C8/100)×C5
10	*Day 14*	Number/Yield of expanded cells	220,400,000	Input raw cell count
11		% NK cells	97.6	Calculated % from flow cytometry data
12		Number of NK cells in expanded population	215,110,400	=(C10/100)×C11
13		NK cell fold expansion (stimulation 2)	59.8	=C12/C9
14		Inferred NK cell numbers	2,636,177,952	=C6×C13
		Overall inferred fold expansion of NK cells	*4895*	=C14/C3

used more often to assess NK cell function for adoptive immunotherapy applications. Numerous methods are available for determining the cytotoxicity of NK cells. Cytotoxicity assay using a nontoxic fluorescent intracellular dye Calcein AM—calcein release assay—is described below. Perform cytotoxicity assay using the classical NK cell target—K562 as control and any tumor cell line of choice based on the criteria of the study. Culture and maintain the tumor cell lines as per standard procedures followed in the laboratory or refer to the cell line source (such as ATCC) for guidelines on culture medium and passage. Maintain and culture K562 cell line in RPMI culture medium. Representative cytotoxicity data for different tumor cell lines is shown in Fig. 3.

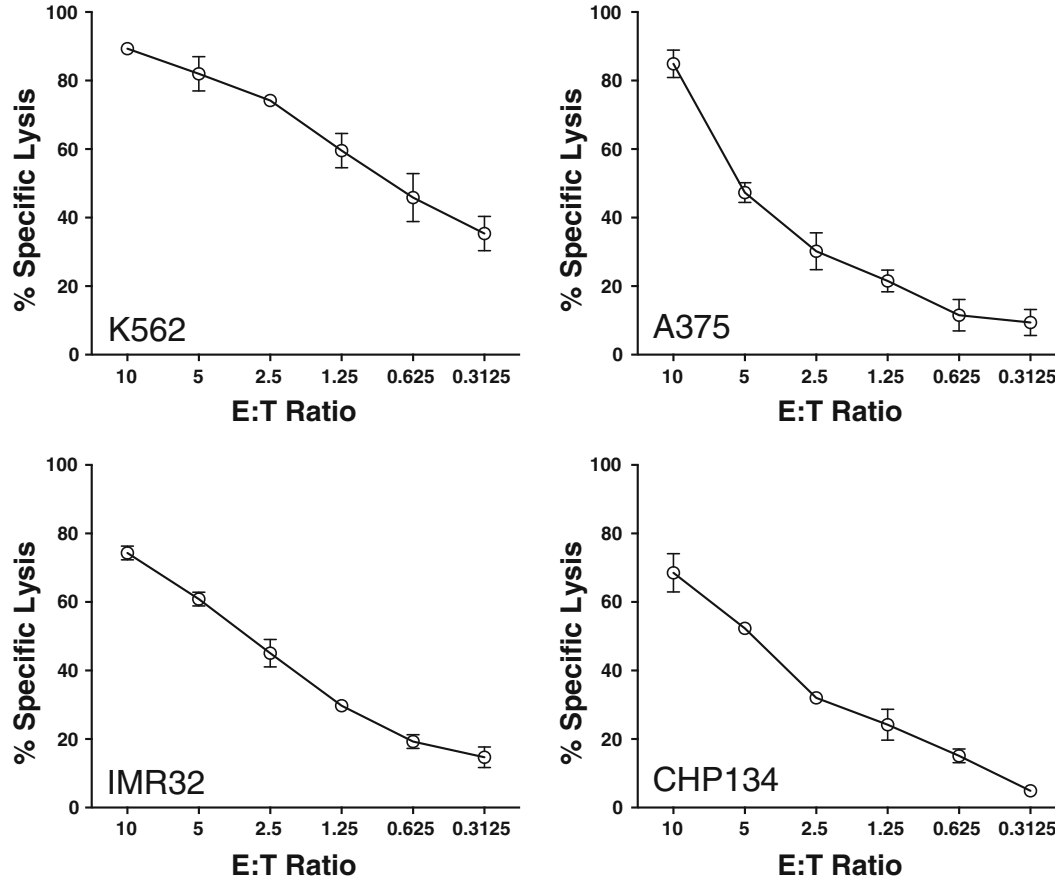


Fig. 3 Analysis of NK cell cytotoxicity using calcein release assay. Expanded NK cells are highly cytotoxic against diverse tumor targets. Data shows the cytotoxic potential of expanded human NK cells against—K562 (chronic myeloid leukemia cell line), A375 (malignant melanoma), IMR32 and CHP134 (neuroblastoma)—determined using calcein release assay. Partially reproduced (A375 and CHP134 data) from PloSOne "Denman CJ, Senyukov VV, Somanchi SS et al (2012) Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells. PLoS One 7: e30264"

1. Stain K562 and tumor cell line(s) of choice with Calcein AM—count and transfer 2×10^6 target cells into a 15 mL conical tube and spin at 400 *g* for 5 min.
2. Resuspend cell pellets at 1×10^6 cells/mL of complete culture medium.
3. Add 3 μL/mL of stock calcein (1 mg/mL) for a 333-fold dilution (this is optimized for K562, for other tumor cell lines titrate the Calcein AM between 250 and 500 fold for optimal results).
4. Incubate for 30 min at 37 °C in a CO_2 incubator, and vortex cells every 10 min to promote uniform staining.

5. Wash cells with 10 mL of RPMI complete medium to remove excess Calcein AM, spin at 400 *g* for 5 min.
6. Repeat the wash step two more times.
7. Resuspend calcein labeled target cells in 10 mL of RPMI complete medium and transfer to 50 mL tube.
8. Perform cell count and add RPMI complete medium to adjust the cell density to 1×10^5 cells/mL.
9. Count and resuspend NK cells at 1×10^6/mL in RPMI complete medium (*see* **Note 19**).
10. In a "U" bottom 96-well plate, seed 200 µL of NK cells per well in triplicates per target cell line (condition) used in the assay.
11. Add 100 µL of RPMI complete medium to the adjacent 5 columns of wells.
12. Mix the NK cells gently and perform a twofold serial dilution across the 5 columns of wells (transfer 100 µL of the cells from first column of wells). Change tips between each serial dilution.
13. Remove and discard the excess 100 µL of medium from the last (6th) column of wells (*see* **Note 20**).
14. Add 100 µL of RPMI complete medium to 6 wells for spontaneous release control.
15. Add 100 µL of 2% Triton X-100 to 6 wells for maximum release control (*see* **Note 21**).
16. Add 100 µL of calcein loaded K562 to wells containing NK cells and controls.
17. Mix gently to promote even mixing of cells and spin the plate at 100 *g* for 1 min to promote cell–cell contact.
18. Incubate the plate in a CO_2 incubator for 4 h at 37 °C.
19. After incubation, remove the plate and gently mix the contents, to promote even distribution of released calcein in the supernatant.
20. Spin the plate at 400 *g* for 1 min.
21. Carefully recover 100 µL of the supernatant and transfer into a clear flat bottom 96-well plate (or black walled clear bottom plates), maintain the same layout of samples in the analysis plate.
22. Read fluorescence intensity using a fluorescence spectrophotometer (excitation filter 485 nm: emission filter 530 nm).
23. Calculate percent specific lysis of NK cells in each group using the formula [(Test release – Spontaneous release)/(Maximum release – Spontaneous release)] × 100.

4 Notes

1. Higher dilution of the blood in PBS will improve recovery of PBMCs by preventing aggregation with RBCs.
2. A typical buffy coat sample (diluted with PBS) is loaded onto four 50 mL conical tubes. The conical tube can be held at a 45-degree angle to facilitate layering of blood without disturbing the Ficoll-Paque. A clear interface between blood and Ficoll-Paque is essential for good recovery of PBMCs. If the layer is disturbed, change to a different tube. Lower volume of blood (25–30 mL) can be added to each tube to avoid potential spillage during centrifugation.
3. Take care to not disturb the cells (white layer) at the interface while removing the top layer of plasma. Note that some RBC retention may occur in the PBMC interface if the cell density is very high.
4. The suction of standard pipette aids may be too high or abrupt and may cause recovery of some Ficoll-Paque layer, disturb the interface or cause back flow of cells into the tube. Use of P-1000 pipette gives greater control for recovering cells from the interface. Always position the tip of the pipette just above the cell layer to completely recover PBMCs and to avoid recovering too much of Ficoll-Paque layer. Combine PBMCs from multiple tubes into a single 50 mL tube at this stage.
5. If a lot of Ficoll-Paque is recovered along with the PBMC interface, centrifuge at 400 *g* for 10 min during the wash steps.
6. While aspirating the supernatant skim over the RBC layer to remove "white" granulocyte cell layer.
7. Either discard the remaining RBCs or save for later. To save, spin the RBCs at 400 *g* for 5 min, aspirate the supernatant and to the RBC pellet add an equal volume of Alsever's solution and store at 4 °C. These RBCs can be stored for up to 4 weeks and can be used for repurification of expanded NK cells if T cell contamination persists after expansion.
8. The volume of the RBC–PBMC cell pellet should be included in the final resuspension volume, e.g., for 500 million PBMCs (+100-fold excess RBCs), add wash buffer to 10 mL mark.
9. The RosetteSep™ Human NK cell Enrichment Cocktail contains tetrameric antibody complexes against CD3, CD4, CD36, CD66b, CD19, CD123 and against glycoprotein A for conjugation to RBCs, to deplete non-NK cells from PBMCs and allow for isolation of untouched NK cells.
10. NK cell expansion may be best from freshly isolated NK cells. Freezing can cause a loss of viability and depending on total NK cell recovery this may impact overall expansion.

11. Miltenyi's CD3 MicroBead system is available as research reagent or clinical reagent (CliniMACS® CD3 microbead). Other commercial reagents are also available for CD3 depletion of PBMCs for laboratory, preclinical, or clinical use of the cells.
12. If fewer than 300 million PBMCs are used for CD3 depletion, add 2 mL of MACS buffer per 10×10^6 PBMCs to wash.
13. The GammaCell 1000 (MS Nordion) irradiator can accommodate up to four 50 mL conical tubes or one 250 mL conical tube at one time.
14. Either freshly irradiated or irradiated and frozen K562 mbIL21 can be used for NK cell expansion without losing efficiency of stimulation.
15. If large numbers of NK cells are required from the expansion, e.g., to use in preclinical adoptive transfer studies, then carry forward all the cells from day 7 for a second stimulation.
16. If more than 5×10^6 cells are carried forward for a second round of expansion, then seed a maximum of 10×10^6 day 7 expanded cells + 10×10^6 K562mb.IL21cells per T75 flask in 40 mL of medium. Use multiple T75 flasks as needed.
17. The culture volume to be used (and thereby the number of flasks) will depend on the total cell numbers at each medium change. It is ideal to perform a cell count on days 10 and 12 to determine the total cell number. Keep the cell density under 5×10^5 cells/mL at the time of medium change on days 10 and 12.
18. Since all viable cells are included in the cell counts (including any monocytes and granulocytes), to account for NK cell numbers directly from CD3 vs CD56 plot (correlated to cell counts) will overestimate the starting NK cell percentage, thereby underestimating the fold expansion.
19. For cytotoxicity assay, NK cells can be used freshly from culture at the end of expansion. If frozen NK cells are used for the assay, thaw a vial of NK cells two days in advance to allow them to recover from freezing.
20. The wells will now contain NK cells ranging from the 100,000 cells per well in column 1 (per condition) to 3125 cells in the column 6. The effector–target (E:T) ratios achieved as per the described protocol are 10:1, 5:1, 2.5:1, 1.25:1, 0.625:1, 0.3125:1. If a higher or lower starting E:T ratio is desired, increase or decrease the initial NK cell density accordingly (e.g., 2×10^6 for 20:1 and 0.5×10^6 for 5:1 starting E:T ratio).
21. It is advisable to leave a row of blank wells between samples and spontaneous release control and maximum release control to prevent accidental cross contamination.

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Chapter 16

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

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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).

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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

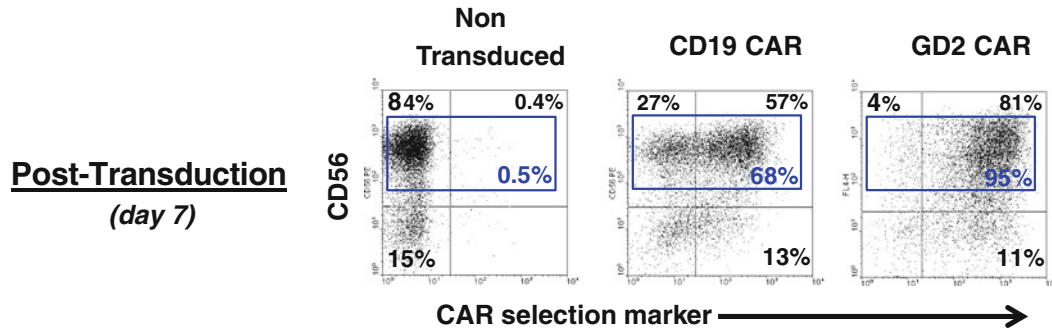

Fig. 1 Generation of expanded and activated NK cells transduced with CAR molecules. NK cells were transduced with retroviral vectors expressing CAR molecules against CD19 and GD2, respectively, with truncated surface molecules as selection markers. After a 3-day transduction as outlined in Subheading 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

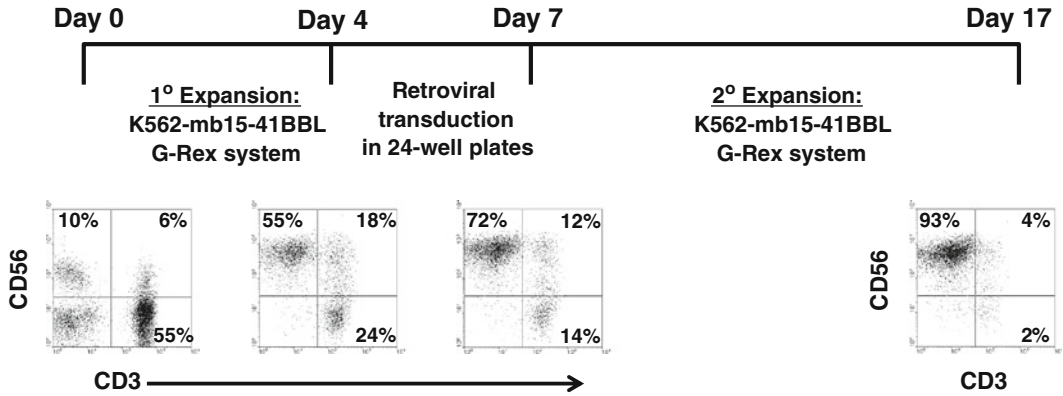

Fig. 2 NK cell expansion/transduction schema and phenotype analysis. PBMC obtained from normal donors were co-cultured in the presence of sub-lethally irradiated K562 mb15-41BBL and IL-2 in G-Rex flasks for 4 days and transduced with retroviral constructs expressing CARs for 3 additional days. Transduced 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.

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Chapter 17

Gene Modification of Human Natural Killer Cells Using a Retroviral Vector

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Abstract

As part of the innate immune system, natural killer (NK) cells are regarded as promising effector cells for adoptive cell therapy approaches to treat patients with cancer. In some cases, genetic modification of the NK cells may be considered but such manipulation has to be integrated into the expansion method to allow the generation of clinically relevant numbers of gene-modified NK cells. Therefore, an efficient gene transfer procedure is needed.

Our group developed a retrovirus-based transduction protocol capable of robust expansion of gene-modified NK cells with a high rate of transgene expression. Actively dividing cells is a prerequisite for efficient gene transfer when using a retroviral vector. In the procedure presented here, strong activation of the NK cells was provided by a combination of IL-15 and the K-562 feeder cells. Beside the interest in developing a simple procedure compliant with good manufacturing practice (GMP) for the production of therapeutic products, this approach also provides a valuable means of generating genetically modified primary NK cells for future preclinical studies.

Key words Natural killer cells, Retroviral vector, Gene transfer, Single transduction, K-562 feeder cells, NK cells expansion

1 Introduction

Natural killer (NK) cells represent a unique cell subset of the innate immune system broadly defined in humans as CD56+/CD3– lymphocytes. NK cells play a critical role in the early immune response with functions primarily regulated by inhibitory and activating receptors. Recognition of virus-infected cells or tumor cells involves natural cytotoxicity receptors (NCR) like NKp30, NKp44, and NKp46, the adhesion molecule DNAX accessory molecule-1 (DNAM-1), the Natural Killer Group 2, member D (NKG2D) receptor as well as 2B4 and TRAIL [1–3]. To date, most ligands recognized by NCRs are unknown with the exception of NKp30 and NKp44 [4, 5]. The stress-inducible

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proteins MICA/B and ULBPs are recognized by NKG2D while the Poliovirus-related proteins CD112 (Nectin-2) and CD155 (PVR) are recognized by DNAM-1. Finally, 2B4 and TRAIL recognize the molecule CD48 and the TRAIL receptors (TRAIL-RI and TRAIL-RII) respectively. Along with activating receptors, NK cells also express inhibitory receptors called killer-cell immunoglobulin-like receptors (KIRs) as well as the receptor complex CD94-NKG2A. The KIRs recognize groups of HLA-A, -B and -C alleles and the CD94-NKG2A complex recognizes HLA-E. Although KIR gene products are clonally distributed in the NK-cell repertoire, a large majority of NK cells express at least one KIR specific for a self MHC-class I allele. Signals received by the NK cells through both types of receptors (i.e. activating and inhibitory receptors) will determine the response against microorganisms or tumors. NK-cell cytotoxic response is mostly mediated by perforin/granzyme-induced apoptosis, and upon activation, NK cells will stimulate the inflammatory and adaptive immune response by secreting interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and granulocyte/macrophage colony-stimulating factor (GM-CSF) [1].

Because of their "built-in" antitumor activity, NK cells have been evaluated for the treatment of patients with hematological malignancies [6] as well as with solid tumors [7, 8] with some success giving promise as effector cells for adoptive cell therapeutics. However, in a complex tumor microenvironment where NK-cell activity is controlled by numerous events and with tumor cells developing evasion mechanisms to elude the NK-cell immune surveillance, strategies to overcome the immune modulation by the tumor microenvironment are needed [9]. Some options include genetically transforming the NK cells to render them resistant to the inhibitory factor TGF-β [10], redirecting NK-cell homing to different organs [11] or retargeting the NK cells to tumor-associated antigens such as CD19 or SLAMF7 [11]. Over the last couple of decades, retroviral gene transfer into hematopoietic stem cells and T cells has been successful using well-established procedures [12, 13]. However, the same is not true for NK cells and genetic modification of NK cells remains a challenge [11]. Our goal was to develop a methodology for the generation of clinical grade genetically modified NK cells under good manufacturing practice (GMP)-controlled conditions. The ideal specifications for the generation of such products would be a single round of retroviral transduction with the transduction step incorporated into the already established NK-cell activation and expansion steps. Further, the stipulations included a minimum of 50% transduction efficiency (i.e. transgene expression) without any detrimental effect on cell viability or function.

We are reporting here a simple GMP compliant retrovirus-based transduction procedure for NK cells that can be used for

the generation of NK-based therapeutic that meet the above listed specifications. Similar to T cells, NK cells are required to have active cell division to enable efficient gene transfer. To achieve an effective activation, NK cells are cultured for 24 h in the presence of IL-15 [14] and then stimulated with K-562 tumor cell line. The retroviral transduction is initiated at 96 h after NK-cell activation by the K-562 cell line using a previously described method for the transduction of T cells [15]. Because of the high rate of transduction obtained using the described procedure, post-transduction enrichment of genetically modified NK cells is not needed.

2 Materials

2.1 Separation of Mononuclear Cells and NK Cells Selection

1. Lymphoprep® (AXIS-SHIELD).
2. CliniMACS® Buffer: Add 20 mL of HSA (25%) to 1 L of CliniMACS® PBS/EDTA buffer (Miltenyi Biotec Inc.). Transfer the buffer into 250 mL conical tube.
3. CD56 MicroBeads (Miltenyi Biotec Inc.).
4. LS column (Miltenyi Biotec Inc.).
5. Turk's solution (EMD Millipore Corporation).

2.2 NK Cells Activation and Expansion

1. NK-Cell medium: Cellgro SCGM medium (Cellgenix) supplemented with 10% Heat-inactivated FBS (HyClone or equivalent) and 2 mM Glutamax (Gibco®).
2. IL-2 (Proleukine): Prepare working solution (200 IU/μL) in sterile water with 1% FBS and store in frozen aliquots at −80 °C.
3. IL-15 (R&D Systems): Prepare working solution (5 ng/μL) in sterile water with 1% FBS and store in frozen aliquots at −80 °C.
4. Chronic Myelogenous Leukemia cell line K-562 (ATCC) (*see* **Note 1**).
5. RPMI complete medium: RPMI with 10% Heat-inactivated FBS (HyClone or equivalent) and 2 mM Glutamax (Gibco®).
6. Trypan blue solution 0.4% (SIGMA).

2.3 NK-Cell Transduction

1. Generation and storage of retroviral supernatant (*see* **Note 2**).
2. RetroNectin® (CH-296) (TaKaRa Bio Inc.): Prepare working solution (7 μg/μL) in sterile water and store in frozen aliquots at −20 °C.
3. Non-tissue culture-treated 24-well plate (BD Falcon™ or equivalent).

3 Methods

Mononuclear cells (MNCs) can be isolated from various blood sources. The method presented in this chapter describes MNCs isolation from peripheral blood and cord blood using an identical approach. For convenience, we use the terminology "blood" to describe unseparated peripheral blood or cord blood samples.

3.1 Separation of Mononuclear Cells

1. Dilute the blood sample in CliniMACS® buffer with a 1:1 dilution for peripheral blood and 1:3 dilution for cord blood.
2. In a 50 mL conical tube, add 15 mL of Lymphoprep®. Prepare as many 50 mL tubes as needed knowing that 30 mL of diluted blood can be processed per tube.
3. On top of each tube containing the Lymphoprep®, carefully pipet down 30 mL of the diluted blood onto the Lymphoprep® keeping the interface blood–Lymphoprep® intact.
4. Centrifuge at $400 \times g$ for 30 min at room temperature (*see* **Note 3**).
5. Using a 5 mL pipette, carefully transfer the MNC layer into new 50 mL conical tube(s) containing 20 mL of CliniMACS® buffer. Fill the tube(s) with CliniMACS® buffer if needed.
6. Centrifuge at $400 \times g$ for 10 min, at room temperature.
7. Remove completely and discard the supernatant. Pool the cell pellets into one 50 mL conical tube.
8. Repeat the washing procedure with CliniMACS® buffer for two additional washes.
9. Resuspend the pellet in 5–10 mL of CliniMACS® buffer depending on the estimated total cell number obtained after MNCs isolation.
10. Count the cells in Turk's solution and calculate the total amount of MNCs.
11. A sample will be taken for cell count, and set aside 1×10^6 cells for phenotype analysis by flow cytometric analysis.

3.2 NK-Cell Selection (See Note 4)

1. Centrifuge the MNC suspension at $400 \times g$ for 10 min, at room temperature.
2. Remove completely and discard the supernatant.
3. Using CliniMACS® buffer, adjust the MNC cell suspension concentration to 125×10^6 MNCs per mL (equals 10×10^6 cells/80 μL of buffer per manufacturer's recommendations).
4. Add 250 μL of CD56 MicroBeads per 125×10^6 MNCs (equals 20 μL of CD56 MicroBeads/10×10^6 cells per manufacturer's recommendations).

5. Mix the MNC cell suspension and the CD56 MicroBeads well and incubate for 15 min in a 4 °C refrigerator.
6. Fill the 50 mL conical tube containing the mix of MNCs and CD56 MicroBeads with CliniMACS® buffer and centrifuge at $400 \times g$ for 10 min, room temperature.
7. During the wash, place a LS column in the magnetic field of the MACS Separator (magnet) and prepare the column by rinsing it with 3 mL of CliniMACS® buffer.
8. After wash, completely remove and discard the supernatant. Resuspend the CD56 MicroBeads-labeled MNCs up to 500 μL of CliniMACS® buffer per 10^8 MNCs.
9. Apply the MNCs cell suspension onto the LS column and collect the unlabeled cells into a 50 mL conical tube.
10. Wash the cells in the LD column three times with 3 mL of CliniMACS® buffer each time.
11. Remove the LS column containing the CD56-selected cells from the MACS separator magnet and place the column on a 15 mL conical tube.
12. Pipette 5 mL of CliniMACS® buffer into the column reservoir and apply the plunger immediately to flush out the CD56-positive cells (*see* **Note 5**).
13. Count the cells in Turk's solution and calculate the total amount of CD56+ cells.
14. A sample will be taken for cell count, and set aside 1×10^6 cells for phenotype analysis by flow cytometry analysis.

3.3 NK-Cell Activation

3.3.1 Day –1

1. 4×10^6 CD56-selected cells are cultured in a T25 flask in 8 mL of NK-Cell medium (cell concentration of 0.5×10^6 cells per mL) supplemented with IL-15 at 15 ng/mL and incubated for 24 h at 37 °C, 95 % humidity and 5 % CO_2 (*see* **Note 6**).
2. K-562 feeder cells culture will be split in three and fresh culture media added to ensure sufficient K-562 feeder cell number and adequate viability for the CD56+ cell activation (*see* **Note 7**).

3.3.2 Day 0

1. Collect K-562 feeder cells in one 50 mL conical tube, irradiated at 100 Gy and centrifuge at $400 \times g$ for 10 min.
2. Remove completely and discard the supernatant.
3. Fill the 50 mL tube containing the irradiated K-562 feeder cells with NK-Cell medium and repeat the wash step two more times.
4. Resuspend the cell pellet in 5–10 mL of NK-Cell medium depending on the estimated total K-562 feeder cell number.
5. Perform cell count and viability using Trypan Blue.

6. Collect the CD56-selected cells from the T25 flask (*see* **Note 8**).
7. Fill the 50 mL conical tube containing the CD56-selected cells with NK-Cell media and centrifuge at 400 × *g* for 10 min.
8. Remove completely and discard the supernatant.
9. Resuspend the CD56-selected cell pellet in 2 mL of NK-Cell medium.
10. Perform cell count and viability using Trypan Blue.
11. For cell activation, CD56-selected cells are co-cultured with irradiated K-562 feeder cells at a 1:2 ratio (i.e. 1 NK cell for 2 irradiated K-562 feeder cells). In one T25 (laid flat), add up to 3×10^6 CD56-selected cells and 6×10^6 irradiated K-562 feeder cells in 6 mL of NK-Cell medium supplemented with 200 IU/mL of IL-2 and incubated at 37 °C in 95% humidity and 5% CO_2.

3.3.3 Day 3

1. Collect the CD56-selected cells co-cultured with irradiated K-562 feeder cells into one 50 mL conical tube and fill the tube with NK-Cell medium (*see* **Note 8**).
2. Centrifuge the cells at 400 × *g* for 10 min, at room temperature.
3. Remove completely and discard the supernatant. Resuspend the cell pellet in 5 mL of NK-Cell medium.
4. Perform cell count and viability using Trypan Blue.
5. In one T25 flask, add up to 3×10^6 CD56-activated cells in 6 mL of NK-Cell medium supplemented with 200 IU/mL of IL-2 and incubated at 37 °C, 95% humidity and 5% CO_2.
6. Prepare the Retronectin-coated Non-tissue culture-treated 24-well plate: Dilute the working solution of Retronectin to a final concentration of 7 μg/mL in PBS and add 1 mL of the diluted Retronectin to each well. Prepare as many well as needed.
7. Wrap the Retronectin-coated plate in Parafilm "M" and place the plate in a 4 °C refrigerator overnight (*see* **Note 9**).

3.4 NK-Cell Transduction

Diligently follow all regulations when handling retroviral vector and dispose waste materials accordingly. The focus of the chapter being the gene transfer into human NK cells; the production of the retroviral vector will not be discussed here.

3.4.1 Day 4

1. Remove Retronectin-coated plate from the 4 °C refrigerator and placed in the biosafety cabinet for 10 min.
2. Remove the Retronectin from the wells, and add 1 mL of pre-warmed NK-Cell medium per well.
3. Place the 24-well plate in the incubator for 30 min.

4. During the 30 min, remove the retroviral supernatant from the −80 °C and thaw it in a 37 °C water bath.
5. As soon as the retroviral supernatant is completely thaw, place the tube on ice for the duration of the transduction procedure (*see* **Note 10**).
6. Remove the Retronectin-coated plate from the incubator and aspirate out the NK-Cell medium from the wells.
7. Add 0.5 mL of retroviral supernatant and place the plate in the incubator at 37 °C, 95 % humidity and 5 % CO_2 for 30 min.
8. Remove the plate from the incubator, aspirate out the 0.5 mL of retroviral supernatant.
9. For a second time, add 0.5 mL of retroviral supernatant and place the plate in the incubator at 37 °C, 95 % humidity and 5 % CO_2 for 30 min.
10. During the second 30 min incubation of retroviral supernatant, collect the activated NK cells in a 50 mL conical tube and fill the tube with NK-Cell medium (*see* **Note 8**).
11. Centrifuge the cells at 400×*g* for 10 min, at room temperature.
12. Remove completely and discard the supernatant. Resuspend the cell pellet in 5 mL of NK-Cell media.
13. Perform cell count and viability using Trypan Blue.
14. Adjust the cell concentration to 300,000 NK cells per mL in NK-Cell medium and add IL-2 at 400 IU/mL.
15. After the last 30 min of incubation remove the retroviral supernatant from the wells and add 1 mL of NK cell suspension (i.e. 300,000 NK cells) per well.
16. Add 1 mL of retroviral supernatant per well. The final volume per well is now 2 mL and the final IL-2 concentration is 200 IU/mL (*see* **Note 11**).
17. Always keep some non-transduced NK cells in culture. Add 1 mL of NK cell suspension per well in a separate Non-tissue-treated 24-well plate and add 1 mL of NK-Cell medium plus IL-2 at the final concentration of 200 IU/mL.
18. Centrifuge the plates at 400×*g* for 1 min, at room temperature.
19. Incubate the plates containing the NK cells with and without retroviral supernatant at 37 °C in 95 % humidity and 5 % CO_2.

3.5 NK-Cell Expansion

3.5.1 Day 6

1. Remove 1 mL of supernatant from each well of the two cultures conditions (i.e. transduced and non-transduced) and replace with fresh NK-Cell media containing 400 IU/mL of IL-2.
2. Incubate the plates containing the NK cells with and without retroviral supernatant at 37 °C in 95 % humidity and 5 % CO_2.

3.5.2 Day 7

1. Collect the transduced and non-transduced NK cells in separate 50 mL conical tubes and fill the tubes with NK-Cell medium (*see* **Note 8**).
2. Centrifuge the cells at $400 \times g$ for 10 min, at room temperature.
3. Remove completely and discard the supernatant. Resuspend the cell pellets in 5 mL of NK-Cell medium.
4. From each culture, collect an aliquot of cells and perform cell count and viability using Trypan Blue.
5. Collect K-562 feeder cells in 50 mL conical tube, irradiate at 100 Gy and centrifuge at $400 \times g$ for 10 min.
6. Remove completely and discard the supernatant.
7. Pool the irradiated K-562 feeder cells in one 50 mL conical tube and fill with NK-Cell medium and repeat the wash step two more times.
8. Resuspend the cell pellet in 10–20 mL of NK-Cell medium depending on the estimated total K-562 feeder cell number.
9. Perform cell count and viability using Trypan Blue.
10. For NK cells expansion, co-culture transduced and non-transduced NK cells with irradiated K-562 feeder cells at a 1:2 ratio (i.e. 1 NK-Cell for 2 irradiated K-562 feeder cells). In one T75 flask (laid flat), add up to 6×10^6 NK cells and 12×10^6 irradiated K-562 feeder cells in 20 mL of NK-Cell medium supplemented with 200 IU/mL of IL-2 and incubated at 37 °C in 95 % humidity and 5 % CO_2.

3.5.3 Day 10

1. Collect transduced and non-transduced NK cells in separate 50 mL conical tubes and fill the tubes with NK-Cell medium (*see* **Note 8**).
2. Centrifuge the cells at $400 \times g$ for 10 min, at room temperature.
3. Remove completely and discard the supernatant. Resuspend the pooled cell pellets from each culture in 5–10 mL of NK-Cell media.
4. A sample will be taken for cell count and viability using Trypan Blue, and set aside 0.5×10^6 cells for analysis of transgene expression by flow cytometry analysis (if appropriate technic to detect the transgene) (Fig. 1).
5. In one T75 flask (laid flat), seed 6×10^6 NK cells in 20 mL of NK-Cell medium supplemented with 200 IU/mL of IL-2 and incubated at 37 °C in 95 % humidity and 5 % CO_2.

3.5.4 Day 12

1. Collect transduced and non-transduced NK cells in separate 50 mL conical tubes and fill the tubes with NK-Cell medium (*see* **Note 8**).

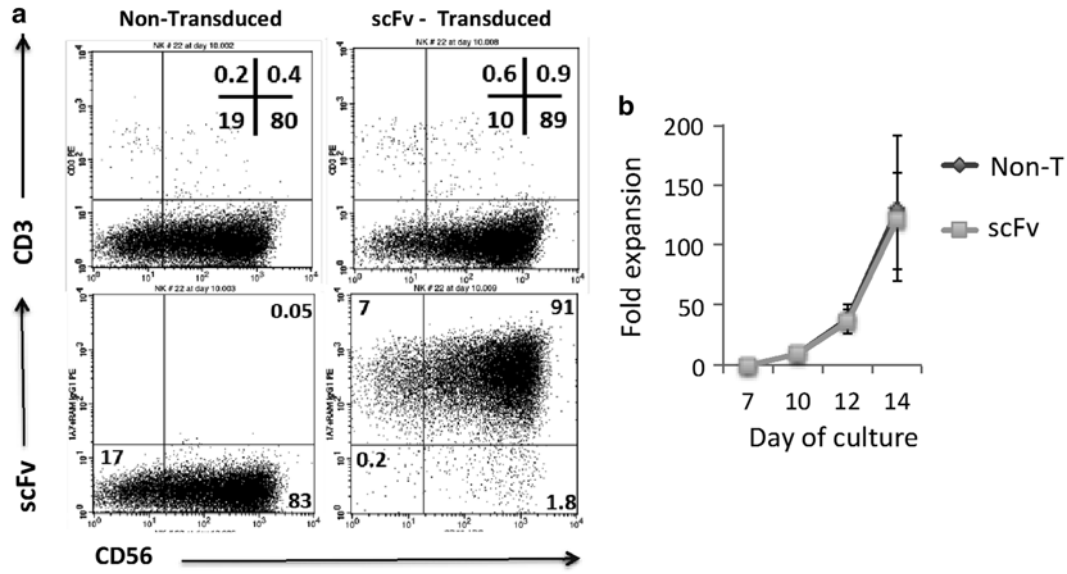

Fig. 1 A representative example of cord blood NK cells transduction and expansion. (**a**) CD56+ cells from a cord blood unit were transduced with an RD114-pseudotyped membrane retrovirus coding for a chimeric antigen receptor (scFv) specific for the ganglioside GD2. Flow cytometry analysis, performed at day 10 of the NK culture (6 days after transduction), shows a low contamination of CD3+ cells (~1 %) in both cultures with a transduction efficiency >90 %. (**b**) In this example, the transduction procedure does not affect the expansion of the NK cells ($n=5$) (*see* **Note 12**)

2. Centrifuge the cells at 400 × *g* for 10 min, at room temperature.
3. Remove completely and discard the supernatant. Resuspend the pooled cell pellets from each culture in 5–10 mL of NK-Cell media.
4. Perform cell count and viability using Trypan Blue, as well as for flow analysis to determine gene expression efficiency if appropriate as well as phenotypic characteristics of the genetically modified NK cells.
5. The transduced and non-transduced NK cells are now ready for use by the investigator to assess the effects of the gene of interest.

4 Notes

1. Parental K-562 cell line (i.e. unmodified cell line) as well as modified K-562 cell line [16] can be used for the activation and expansion of NK cells.
2. Feline endogenous virus RD114-pseudotyped SFG retroviral supernatant production and storage method has been previously described [15].

3. The centrifugation should be carried out in a swinging-bucket rotor centrifuge without brake and with a minimum acceleration.
4. Please refer to the manufacturer's instructions for the reagent requirements and positive selection column limitations. Although never tested in our laboratory, CD56+ cells can also be enriched using other commercially available systems.
5. The expected enrichment of the CD56+ cells is higher than 95 % from the MNCs fraction. However, and per the manufacturer recommendation, it is possible to increase the purity of the selected cells by running the CD56-labeled MNCs through a freshly prepared LS column following the described procedure.
6. We usually start with 4×10^6 CD56+ selected cells for our transduction experiment as the expansion process utilized in this procedure allow us to generate a large number of activated and transduced NK cells. The remaining CD56-selected cells are cryopreserved for further use. When using cryopreserved cells, the same procedure for NK cells transduction can be used with identical transduction efficiency results as for freshly isolated CD56+ cells. However, the cryopreservation and thawing procedure of CD56-selected cells will be responsible for a significant loss of viable cells (~50 % in some cases).
7. K-562 cells are fed with fresh media every 2 to 3 days. To feed the K-562 cells, the culture is split in three and fresh media is added. It is important to feed the K-562 cells at day −1 and day 6, the day before the initial activation of the CD56-selected cells and the expansion of the NK cells respectively.
8. It is not unusual to observe activated NK cells attached (loosely or strongly depending on the sample and the activation state of the NK cells) to the culture vessel. Therefore, collecting the NK cells from the culture vessel requires scraping and mixing well with NK-Cell media. Do not use trypsin as it is not needed to collect the NK cells from the culture vessel.
9. It is also possible to prepare a Retronectin-coated plate by incubating the plate for 4 h at 37 °C.
10. Do not leave the retroviral supernatant in the water bath for more than necessary as it might result in decrease in virus titer resulting in poor transduction efficiency.
11. For the transduction of NK cells, a total of 2 mL of retroviral supernatant is needed per well. Retroviral supernatant should be stored at −80 °C in aliquots of adequate volume to ensure the transduction of multiple wells.
12. Depending on the nature of the transgene engrafted, the expansion of the genetically modified NK cells may vary in comparison with the non-transduced NK cells.

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Chapter 18

Modification of Expanded NK Cells with Chimeric Antigen Receptor mRNA for Adoptive Cellular Therapy

Yaya Chu, Allyson Flower, and Mitchell S. Cairo

Abstract

NK cells are bone marrow-derived cytotoxic lymphocytes that play a major role in the rejection of tumors and cells infected by viruses. The regulation of NK activation vs inhibition is regulated by the expression of a variety of NK receptors (NKRs) and specific NKRs' ligands expressed on their targets. However, factors limiting NK therapy include small numbers of active NK cells in unexpanded peripheral blood and lack of specific tumor targeting. Chimeric antigen receptors (CAR) usually include a single-chain Fv variable fragment from a monoclonal antibody, a transmembrane hinge region, and a signaling domain such as CD28, CD3-zeta, 4-1BB (CD137), or 2B4 (CD244) endodimers. Redirecting NK cells with a CAR will circumvent the limitations of the lack of NK targeting specificity. This chapter focuses on the methods to expand human NK cells from peripheral blood by co-culturing with feeder cells and to modify the expanded NK cells efficiently with the in vitro transcribed CAR mRNA by electroporation and to test the functionality of the CAR-modified expanded NK cells for use in adoptive cellular immunotherapy.

Key words Chimeric antigen receptor, Natural killer cells, mRNA, Electroporation, Adoptive cell therapy

1 Introduction

Novel therapies that are both effective in eradicating resistant malignancy and associated with low toxicity are urgently needed to enhance the success of cancer therapy. Targeted immunotherapy is a strategy which augments the existing immune system to enhance tumor-specific cytotoxicity while escaping systemic toxicities associated with traditional cytotoxic chemotherapy. Essential to this approach is the identification of an ideal target cell surface antigen, which is highly expressed on the resistant tumor cells but minimally expressed in healthy tissue.

Natural killer (NK) cells are specialized lymphocytes which are an essential component of the innate immune system and play a critical role in tumor surveillance and rejection [1, 2]. NK cell function is regulated by a combination of activating and inhibitory

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signals including pro-inflammatory cytokines and NK cell surface receptor interaction with target cell ligands [3–5]. However, there are several limitations to NK cell-induced cytotoxicity including small numbers in circulating peripheral blood, lack of specificity for the tumor target and tumor-induced down-regulation of NK cell natural cytotoxicity receptors [5, 6]. Killer immunoglobulin-like receptors (KIRs) are a group of inhibitory NK cell receptors that bind to class I major histocompatibility complex (MHC)-determined human leukocyte antigen ligands. NK cell KIR recognition of self-class I MHC molecules mediates self-tolerance of NK cell activity. This interaction leads to suppression of NK cell cytotoxicity and contributes to immune escape [7]. The methods described here, including expansion of NK cells isolated from peripheral blood and incorporation of a tumor-specific CAR, detail a mechanism to overcome these limitations to NK cell cytotoxicity.

NK cell immunotherapeutic development has been limited by poor in vitro expansion and prohibitively short lifespan. Peripheral blood-derived NK (PBNK) cell expansion following peripheral blood mononuclear cell (PBMNC) exposure to inactivated, genetically engineered K562 artificial antigen-presenting cells (aAPC) expressing membrane bound interleukin (IL)-15 and 4-1BB ligand in the presence of IL2 produces a significant NK cell yield with more than 96% purity and high expression of activating receptors [8, 9]. Alternatively, K562-based aAPC expressing IL21 demonstrate similar phenotype and cytotoxicity, enhanced cytokine production, and significantly increased NK cell expansion and more sustained proliferation in comparison [10].

CAR-based immunotherapy creates a mechanism for directing the innate immune system to a specific cancer cell target. CARs are artificial receptors composed of an antigen-specific, monoclonal antibody-derived single chain variable fragment bound to an intracellular signaling domain [11]. Specifically directed CARs incorporated into T or NK cells create an opportunity for target-specific recognition to be combined with cellular activation. CAR T-cell therapy has shown promise in clinical trials but may be associated with severe complications including cytokine release syndrome and B-cell aplasia [12–15]. NK cell therapy has not been associated with these toxicities and has been demonstrated to induce a significant antitumor effect in the absence of graft-versus-host disease (GVHD) [16]. Our group has demonstrated that anti-CD20 CAR-modified NK cells had significantly increased cytotoxicity in vitro and reduced tumor burden and significantly prolonged xenografts survival in vivo against $CD20^{+}$ B-cell Non-Hodgkin Lymphoma (B-NHL) in comparison to CAR-negative controls [9].

Tumor-specific CAR mRNA is generated and incorporated into expanded NK cells using a nonviral electroporation-based transfection method. Subsequently, CAR expression is detected by flow cytometry using a fragment-specific antibody. When used for

the development of anti-CD20 CAR-modified NK cells, the electroporation-based transfection did not affect the expression of PBNK-activating receptors (CD16, CD69, NKG2D, CD244, NKp30, NKp44, NKp46) or inhibitory receptors (NKG2A, KIR2DS4, CD94, CD158a, CD158b, CD158e). Furthermore, in vitro cytotoxicity and intracellular interferon (IFN)-γ production were significantly enhanced by anti-CD20 CAR-modified NK cells against $CD20^+$ B-cell leukemia/lymphoma cell lines [9].

Once activated, NK cells function by direct cytotoxicity and by the production of activating cytokines that recruit the adaptive antitumor immune response. NK cell production of IFN-γ and TNF directly inhibit the growth and spread of tumors and recruit T cells and dendritic cells, which are the key components of adaptive antitumor immunity [17, 18]. CD107a as a marker of NK cell degranulation and direct cytotoxicity, IFN gamma expression as a marker of cytokine production, and standard europium release assay are used to measure NK cell activation and cytotoxicity in vitro [19].

The following is a detailed description of methods for development of CAR-modified PBNK cells and evaluation for enhanced cytotoxicity in vitro. Here, anti-CD20 CAR-modified expanded PBNK cells and their cytotoxicity against B-NHL cell lines are used as an example. However, this method is generally applicable to an alternative tumor cell target with an eligible cell surface antigen and reactive-modified CAR NK cell.

2 Materials

2.1 Expansion of NK Cells

2.1.1 Expansion of NK Cells by Co-culture with the Irradiated Feeder Cells

1. Leukocytes obtained after informed consent from healthy donors.
2. Peripheral blood mononuclear cells (PBMNC) obtained by Ficoll gradient (Amersham Biosciences) separation.
3. The K562-mb15-41BBL cell line or the K562-mb21-41BBL cell line [8] (*see* **Note 1**).
4. NK culture medium: RPMI-1640 (Life Technologies), +10% fetal bovine serum (FBS) and penicillin and streptomycin + 40 IU recombinant human interleukin-2 (IL-2) (Life Technologies).
5. RS 2000 X-ray Irradiator (Rad Source Technologies, Inc.).

2.1.2 NK Isolation

1. Cell separation buffer: Phosphate-buffered saline (PBS): pH 7.2, 0.5% BSA and 2 mM EDTA.
2. NK culture medium.
3. Human NK isolation kit (Miltenyi Biotec).
4. MidiMACS separator and LS column (Miltenyi Biotec).

2.2 Nucleofection of CAR mRNA

2.2.1 Generation of CAR mRNA

1. Vectors: pcDNA3 or pVAX1 (Life Technologies) containing a T7 promoter and an anti-CD20 CAR fragment (*see* **Note 2**).
2. Restriction Enzymes: XhoI or XbaI and digestion buffer (New England Biolabs).
3. Wizard® SV Gel and PCR Clean-Up System kit (Promega).
4. In vitro transcription kit (mMESSAGE mMACHINE T7 Ultra Kit, Life Technologies) containing nuclease-free water, T7 Enzyme Mix, 10×T7 Reaction Buffer, T7 2×NTP/ARCA, TURBO DNase, E-PAP, 25 mM $MnCl_2$, 10 mM ATP solution, 5×E-PAP buffer, and lithium chloride (LiCl).

2.2.2 Nucleofection

1. Nucleofector (Lonza).
2. CAR mRNA.
3. Nuclease-free water.
4. Amaxa® Human NK Cell Nucleofector® Kit (Lonza) containing human NK cell nucleofector solution, supplement, certified cuvettes, and plastic pipettes.
5. NK culture medium.

2.2.3 Flow Cytometric Analysis of CAR Expressing in NK Cells

1. Goat anti-mouse IgG, $F(ab')_2$ fragment-specific antibody conjugated with fluorescein isothiocyanate (FITC) (Jackson ImmunoResearch).
2. Staining buffer: PBS containing 0.1% (v/v) NaN_3 with 1% [w/v] bovine serum albumin.
3. MACSQuant Analyzer (Miltenyi Biotec).

2.3 Functional Analysis of CAR-Modified Expanded NK Cells In Vitro

2.3.1 In Vitro Cytotoxicity

1. Cell lines and tumor targets: The human cell lines Ramos, Daudi, RS4;11, U698M, and the T-cell ALL lines Jurkat purchased from American Type Culture Collection, ATCC; Raji, Rituximab-resistant Raji-2R and Raji-4RH cells provided by Matthew Barth, MD and Myron Czuczman, MD from Roswell Park Cancer Institute [20] (*see* **Note 3**).
2. Culture medium: RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and penicillin (100 IU/mL), streptomycin (Gibco).
3. DELFIA® EuTDA Cytotoxicity kit (Perkin Elmer) containing DELFIA BATDA reagent, 1 vial (50 μL), DELFIA Lysis Buffer, 1 vial (0.5 mL), DELFIA Europium solution, 1 bottle (200 mL) and 10 DELFIA microtitration plates (96-well).
4. A time-resolved fluorometer (Perkin Elmer).

2.3.2 Tumor Cell Recovery

1. Expanded PBNK cells (CAR^- exPBNK) and CAR mRNA-modified expanded PBNK cells (CAR^+ exPBNK).
2. Tumor target cells.

3. Culture medium: RPMI-1640 supplemented with 10% fetal bovine serum (Life Technologies).
4. CD19-Phycoerythrin (BD Biosciences) and 7-aminoactinomycin D (7-AAD) (BD Biosciences) (*see* **Note 4**).
5. BD FACScan (BD Biosciences).

2.3.3 Intracellular CD107a and IFN-γ Assays

1. Expanded PBNK cells (CAR$^-$exPBNK) and CAR mRNA-modified expanded PBNK cells.
2. Tumor target cells.
3. Culture medium: RPMI-1640 supplemented with 10% fetal bovine serum (Life Technologies).
4. Antibodies: Anti-CD107a-FITC, anti-CD56-PE, anti-INF-γ-PE, anti-CD56-PE-Cy5 (all from BD Biosciences).
5. BD Cytofix/Cytoperm™ Plus Fixation/Permeabilization (BD GolgiPlug™ protein transport inhibitor) kit (BD Biosciences) containing the Fixation/Permeabilization solution, BD Perm/Wash™ Buffer, and brefeldin A.
6. A FACScan flow cytometer (BD) or a MACSQuant Analyzer (Miltenyi Biotec).

3 Methods

3.1 Expansion of NK Cells

3.1.1 Expansion of NK Cells by Co-culture with the Irradiated Feeder Cells

1. Resuspend PBMNC in NK culture medium at a concentration of 1×10^6 cells/mL.
2. Irradiate the K562-mb15-41BBL cells at 100 Gy γ-irradiation.
3. Wash the irradiated K562-mb15-41BBL cells once and resuspend in NK culture medium at a concentration of 1×10^6 cells/mL.
4. Mix the same volume of the resuspended PBMNC and irradiated K562-mb15-41BBL cells in 50 mL conical tubes.
5. Transfer the mixed cells to 24-well plates at 2 mL mixed cells to each well (*see* **Note 5**).
6. Incubate at 37 °C in 5% CO_2.
7. Feed the cells every other day by removing half of the supernatant gently and adding the same volume of NK culture medium.
8. On day 7 and 14, count the cells and also monitor the NK expansion by flow cytometry (*see* **Note 6**).

3.1.2 NK Isolation

1. Collect the expanded cells after 14 days of co-culture with irradiated feeder cells in 50 mL conical tubes and determine the total cell number.
2. Centrifuge cell suspension at $300 \times g$ for 10 min. Aspirate the supernatant completely.

3. Resuspend cell pellet in 40 μL of cell separation buffer per 1×10^7 total cells.
4. Add 10 μL of NK Cell Biotin-Antibody Cocktail per 1×10^7 total cells (*see* **Note 7**).
5. Mix well and incubate for 10 min at 4 °C.
6. Add 30 μL of cell separation buffer per 1×10^7 total cells.
7. Add 20 μL of NK Cell MicroBead Cocktail per 1×10^7 total cells.
8. Mix well and refrigerate for an additional 15 min (4–8 °C).
9. Wash cells by adding 1–2 mL of cell separation buffer per 1×10^7 cells and centrifuge at $300 \times g$ for 10 min. Aspirate supernatant completely.
10. Resuspend up to 10^8 cells in 500 μL of cell separation buffer (*see* **Note 8**).
11. Place an LS column in a magnet field of a suitable separator and rinse the column with 3 mL of separation buffer.
12. Apply the cell suspension onto the column.
13. Collect unlabeled cells which pass through the column.
14. Wash the column with 3 mL of cell separation buffer twice and collect total effluent.
15. Centrifuge the effluent at $300 \times g$ for 10 min.
16. Resuspend the cells in NK culture medium at a concentration of 2.5×10^6 cells/mL.
17. Transfer the cells to tissue culture flasks.
18. Incubate at 37 °C in 5 % CO_2.
19. Every 2 days, remove half of the supernatant gently and add the same volume of NK culture medium (supplementing IL2 for the entire volume) until use (*see* **Note 9**).

3.2 Nucleofection of CAR mRNA

3.2.1 Production of CAR mRNA

1. Linearize the pcDNA3 or pVAX1 plasmid containing a T7 promoter site and the CAR with a restriction enzyme (*see* **Note 10**).
2. Purify the linearized plasmid with Wizard® SV Gel and PCR Clean-Up System kit (Promega).
3. Dissolve the purified plasmid in TE buffer at a concentration of 0.5–1 mg/mL.
4. Capped transcription reaction assembly for a 20 μL reaction: Mix 1 μg of the linearized plasmid, 10 μL of T7 2×NTP/ARCA, 2 μL of 10× T7 reaction buffer, 2 μL of T7 Enzyme mix, and nuclease-free water to total 20 μL in a RNase-free tube (*see* **Note 11**).
5. Incubate at 37 °C for 2 h (*see* **Note 12**).

6. Add 1 μL of TURBO DNase, mix and incubate at 37 °C for 15 min.
7. Poly (A) tailing procedure: Add 36 μL of nuclease-free water, 20 μL of 5×E-PAP buffer, 10 μL of 25 mM $MnCl_2$, 10 μL of ATP solution, and 4 μL of E-PAP to the 20 μL capped transcription reaction and mix well.
8. Incubate at 37 °C for 45 min.
9. Recovery of RNA: Add 50 μL of LiCl, mix well, and chill for ≥30 min at −20 °C.
10. Centrifuge at 12,000*g* for 15 min at 4 °C to pellet RNA.
11. Remove the supernatant.
12. Add 1 mL of 70% ethanol and invert the tube several times to mix.
13. Centrifuge at 12,000*g* for 15 min at 4 °C.
14. Remove the supernatant and air-dry the pellet for 10 min.
15. Dissolve the mRNA in nuclease-free water at a concentration of 1.0 mg/mL (*see* **Note 13**).
16. Store at −80 °C until use.

3.2.2 Nucleofection

1. For a single reaction, mix 82 μL of Nucleofector® Solution with 18 μL of supplement to make 100 μL of total electroporation buffer in biosafety cabinet.
2. Centrifuge 5–6 × 10^6 expanded purified NK cells at 200*g*, for 10 min, at room temperature (RT) (*see* **Note 14**).
3. Aspirate the supernatant and resuspend the cells in PBS.
4. Centrifuge at 200*g*, for 10 min, at RT.
5. Aspirate the supernatant and resuspend the cells in 100 μL of electroporation buffer.
6. Add CAR mRNA to NK cells in electroporation buffer at 80–100 μg/mL.
7. Transfer cell/mRNA suspension into the certified electroporation cuvette. Close the cuvette with the cap (*see* **Note 15**).
8. Select the Nucleofector® Program U-001 (*see* **Note 16**).
9. Insert the cuvette into the Nucleofector® and apply the selected program.
10. Return the cuvette to biosafety cabinet and using an aspirator add ~500 μL of the pre-warmed NK culture medium to the cuvette and gently transfer the sample into a 12-well plate (final volume of 4 mL NK medium per well/sample).
11. Incubate the cells in humidified 37 °C/5% CO_2 incubator until use.

3.2.3 Flow Cytometric Analysis of CAR Expressing in NK Cells

1. After 16 h of electroporation, transfer the nucleofected NK cells (approximately 2×10^5 cells) into a 5 mL polypropylene test tube.
2. Centrifuge the cell suspension for 3 min at $400 \times g$, discard supernatant and wash the cells in ice-cold PBS 3 times.
3. Resuspend the cells in 100 μL of staining buffer containing a goat anti-mouse IgG, $F(ab')_2$ fragment-specific antibody conjugated with FITC (1:100 dilution). Vortex the tubes prior to incubation.
4. Incubate for 30 min on ice.
5. Wash the cells twice with ice-cold PBS.
6. Discard the washing solution, resuspend the cells in 200 μL of staining buffer containing 20 μL 7-AAD. Analyze cells by flow cytometry.

Figure 1 provides the representative flow cytometric dot plot data of the CAR expression and duration in electroporated exPBNK cells.

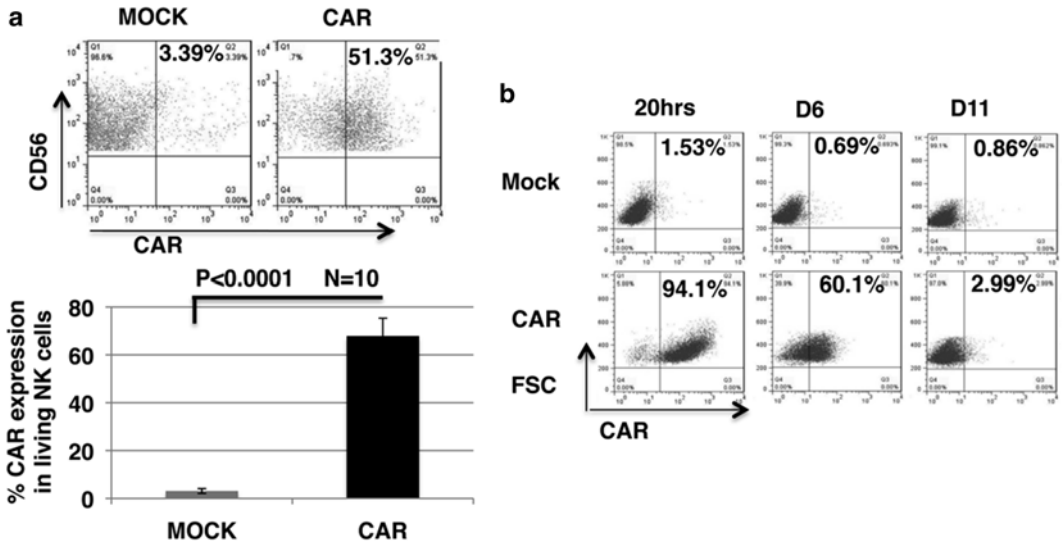

Fig. 1 Expression of anti-CD20 CAR in ex vivo-expanded PBNK cells by CAR mRNA nucleofection. (**a**) Anti-CD20 mRNA was nucleofected into expanded PBNK cells and the CAR expression was followed by flow cytometry. Flow cytometric density plots illustrate expression of the anti-CD20 CAR in one of the 10 donors and percentage of NK cells expressing anti-CD20 CAR as determined by flow cytometry 16 h after nucleofection ($p < 0.001$). Average values are reported as the mean ±SEM ($n = 10$). *P* value using unpaired Student's t-test was noted. (**b**) Anti-CD20 CAR expression was monitored by flow cytometry at the indicated times post nucleofection. Reproduced with permission from: Chu Y, Hochberg J, Yahr A et al (2015) Targeting $CD20^+$ aggressive B-cell non-Hodgkin lymphoma by anti-CD20 CAR mRNA-modified expanded natural killer cells in vitro and in NSG mice. Cancer Immunol Res 3: 333–344. doi:10.1158/2326-6066.CIR-14-0114

3.3 Functional Analysis of CAR-Modified Expanded NK Cells In Vitro

3.3.1 In Vitro Cytotoxicity

We here describe a procedure for a nonradioactive, time-resolved fluorescence, Europium TDA (EuTDA) cytotoxicity assay based on Perkin Elmer's well-known DELFIA technology. Figure 2 exemplifies the in vitro cytotoxicity of anti-CD20 CAR$^+$ exPBNK against CD20$^+$ B-NHL cells.

1. Warm the lysis buffer in a water bath (37 °C) just before use. Let the reagents reach room temperature before use. Check that the fluorescence-enhancing ligand is thoroughly thawed before use.

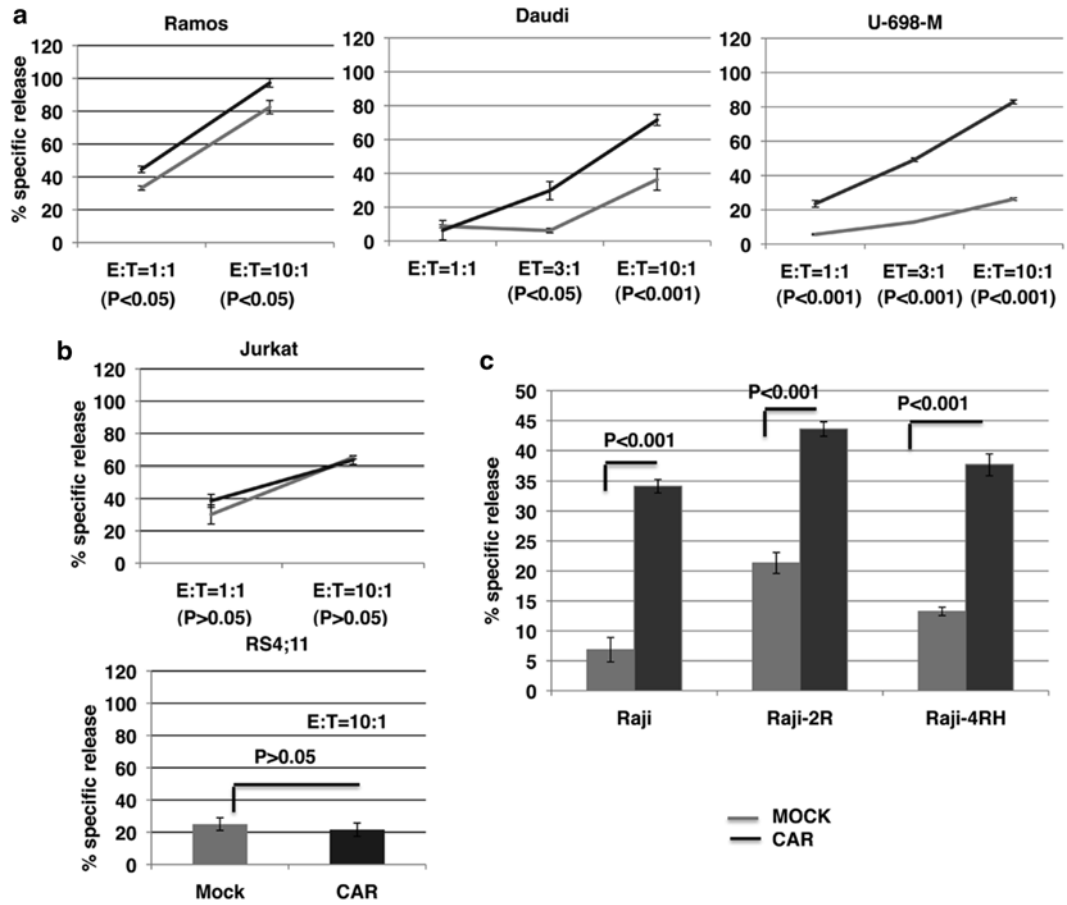

Fig. 2 Anti-CD20 CAR mRNA enhances expanded PBNK in vitro cytolytic activity against CD20$^+$ B-NHL cells. Expanded PBNK cells were electroporated in the absence (Mock, *gray color*) or in the presence of anti-CD20 CAR mRNA (CAR, *black color*). In vitro cytotoxicity was measured by standard europium release assay. (**a**) MOCK and CAR exPBNK cells were incubated with the BATDA-labeled CD20$^+$ NK sensitive Ramos (*top left*), CD20$^+$ NK-resistant Daudi (*top middle*), and U-698-M (*top right*) at the indicated E/T ratios. (**b**) MOCK and CAR exPBNK cells were incubated with the BATDA-labeled CD20$^-$ NK-sensitive Jurkat (*top*) and CD20$^-$ NK-resistant Rs4;11 (*bottom*) at the indicated E/T ratios as controls. (**c**) MOCK and CAR exPBNK cells were incubated with the BATDA-labeled CD20$^+$ rituximab-sensitive Raji and resistant Raji-2R and Raji-4RH at the E/T ratio = 10:1. Each data point represents the mean (±SEM, $n = 4$) percentage of specific europium release after culture. *P* values using unpaired Student's t-test were noted. Reproduced with permission from: Chu Y, Hochberg J, Yahr A et al (2015) Targeting CD20$^+$ aggressive B-cell non-Hodgkin lymphoma by anti-CD20 CAR mRNA-modified expanded natural killer cells in vitro and in NSG mice. Cancer Immunol Res 3: 333–344. doi:10.1158/2326-6066.CIR-14-0114

2. Wash the tumor target cells once with a PBS or NK culture medium.
3. Adjust the number of cells to about 1×10^6 cells/mL. Add 5 μL of the DELFIA® BATDA Labeling Reagent to 2–4 mL of cells in culture medium. Incubate for 20–30 min at 37 °C in a cell incubator (*see* **Note 17**).
4. Spin down the cells and resuspend in PBS.
5. Wash the cells 3–5 times. Resuspend the cell pellet carefully.
6. After the final wash, resuspend the pellet in culture medium and adjust to about 1×10^5 cells/mL.
7. Pipette 100 μL of loaded target cells (10,000 cells) per well, to a round-bottom sterile 96-well plate.
8. Resuspend NK cells in culture medium and add 100 μL CAR^+ or CAR^- exPBNK cells at varying effector:target ratio (from 1:1 to 10:1) (*see* **Note 18**).
9. Set up wells for detection of background, spontaneous release, and maximum release (*see* **Note 19**).
10. Incubate for 4 h in a humidified 5% CO_2 atmosphere at 37 °C.
11. Centrifuge for 5 min at $500 \times g$.
12. Transfer 20 μL of the supernatant to a flat-bottom plate.
13. Add 200 μL of Eu-solution.
14. Shake at 250 rpm using a plate shaker of at least 3 mm.
15. Measure fluorescence in a time-resolved fluorometer within 5 h (*see* **Note 20**).
16. Percentage of specific release was calculated as follows:

    % Specific release = 100 × (Experimental release – spontaneous release)/(Maximum release – spontaneous release)

    All tests were run in triplicate or quadruplicate.

3.3.2 Tumor Cell Recovery

To further examine whether tumor cells are killed and lysed by CAR^+ exPBNK cells, we here describe a procedure for tumor cell recovery assay.

1. Wash the tumor target cells with a PBS or culture medium 3 times.
2. After the final wash, resuspend the pellet in culture medium and adjust to about 1×10^5 cells/mL.
3. Similar to Subheading 3.3.1, add 100 μL of target cells (10,000 cells) per well to a round-bottom 96-well sterile plate.
4. Add 100 μL of CAR^+ or CAR^- exPBNK cells resuspended at 1×10^6 cells/mL (E:T 10:1) concentration.
5. To control wells (without exPBNK cells), add 100 μL of culture medium.

6. Incubate overnight in a humidified 5% CO_2 atmosphere at 37 °C.
7. Centrifuge the cell suspension for 3 min at 400 × *g*, discard supernatant and wash the cells once in ice-cold PBS for 3 times.
8. Resuspend the cells in 100 μL of staining buffer containing CD19-PE (BD Biosciences) (*see* **Note 4**).
9. Incubate for 30 min on ice.
10. Wash the cells twice with ice-cold PBS.
11. Discard the washing solution and resuspend the cells in 200 μL of staining buffer containing 7-AAD. Analyze cells by flow cytometry.

3.3.3 Intracellular CD107a and IFN-γ Assays

CD107a and IFN-γ produced by NK cells are functionally linked to their cytolytic activities. We here describe the procedure for examining intracellular CD107a and IFN-γ produced by CAR^+ and CAR^- exPBNK cells.

1. Spin down the CAR^+ and CAR^- exPBNK cells and resuspend in culture medium at 1×10^6 cells/mL.
2. Add 100 μL of the effector cell suspension per well to the round-bottom 96-well plate.
3. Spin down target cells and resuspend at 1×10^5 cells/mL in culture medium.
4. Pipette 100 μL of target cell suspension per well into the round-bottom 96-well plate to mix with the effector cells.
5. Add 20 μL anti-CD107a-FITC (BD Biosciences) to each well and incubate for 1 h at 37 °C.
6. After 1 h, add 20 μL of culture medium supplemented with BD GolgiPlug™ protein transport inhibitor containing brefeldin A (diluted 1:100) and BD GolgiStop™ protein transport inhibitor containing monensin (diluted 1:150) to each well, and return the cells to the incubator at 37 °C for 4–6 h (*see* **Note 21**).
7. After 4–6 h, centrifuge the cells at 450 × *g* for 3 min.
8. Resuspend the cells in 50 μL staining solution containing anti-CD56-PE-Cy5 and incubate samples in the dark for 30 min at 4 °C.
9. Centrifuge the cells at 450 *g* for 3 min.
10. Thoroughly resuspend cells in 100 μL of Fixation/Permeabilization solution per well for microwell plates and incubate for 20 min at 4 °C.
11. Wash cells two times in 1× BD Perm/Wash™ buffer (250 μL per well) and spin the cells at 450 *g* for 3 min (*see* **Note 22**).

12. Thoroughly resuspend fixed/permeabilized cells in 50 μL of BD Perm/Wash™ buffer containing a 20 μL of anti-INF-γPE. Incubate at 4 °C for 30 min in the dark.
13. Wash cells 2 times with 1× BD Perm/Wash™ buffer and resuspend in staining buffer for flow cytometric analysis.

4 Notes

1. K562-mbIL21-41BBL cells have recently been shown to support a mean 47,967-fold expansion of NK cells with a significant increase in telomere length in NK cells by Dean Lee's group [10]. We had successfully expanded NK cells with both feeder cells and had successfully modified both expanded NK cells with anti-CD20 CAR mRNA [9]. The methods and steps described in this chapter using K562-mbIL15-41BBL-expanded PBNK cells will apply for the K562-mbIL21-41BBL-expanded PBNK cells.
2. We used anti-CD20 CAR as an example to describe the methods and steps on how to electroporate the modified expanded NK cells with in vitro transcribed CAR mRNA. The methods can be adapted and applied on any other CAR to modify expanded NK cells with the CAR mRNA electroporation.
3. The tumor cells listed here are for cytolytic assays using anti-CD20 CAR-modified expanded NK cells, as described in our previous study [9]. If a different CAR is used to modify expanded NK cells, tumor cells expressing a specific antigen that can be recognized by that CAR should be chosen.
4. CD19-Phycoerythrin antibody was chosen to monitor total $CD20^{+}CD19^{+}$ tumor targets when using anti-CD20 CAR mRNA-modified exPBNK cells as we described [9]. If a different CAR is used to modify expanded NK cells, a different antibody that recognizes tumor cell-specific antigen should be chosen.
5. NK cells will expand and grow better when cultured at cell densities that allow cell–cell contact. We usually transfer 2 mL of the mixed diluted cells to a well in a 24-well plate at a final concentration of $1–1.5 \times 10^6$/mL for PBMC and 1×10^6/mL of K562-mb15-41BBL cells per well as previously reported by Dr. Dario Campana [8]. When using irradiated K562-mbIL21-41BBL cells as feeders, PBMC will be co-cultured with irradiated K562-mbIL21-41BBL cells in T-75 flasks at a ratio of 1:2 (PBMC:feeder) in NK cell medium at 2×10^5 PBMC/mL. Cultures will be refreshed with half-volume medium changes every 2 to 3 days, and re-stimulated with irradiated K562-mbIL21-41BBL cells at a ratio of 1:1 every 7 days [10].

6. After 5–7 days of co-culture, the cells should be monitored daily for changes in the medium pH and morphology. If the expansion goes well, you will be able to see the shape changes of the cells. NK cells will continue to expand in culture for 1 or 2 more weeks. The cells must be split if they are too crowded.
7. To isolate the untouched NK cells from the expansion mixture. Non-NK cells, i.e. T cells, B cells, stem cells, dendritic cells, monocytes, granulocytes and erythroid cells, are indirectly magnetically labeled by using a cocktail of biotin-conjugated antibodies and the NK Cell MicroBead Cocktail. Isolation of highly pure NK cells is achieved by depletion of magnetically labeled cells. The purity of NK cells after the isolation should be more than 95%.
8. For higher cell numbers, scale up buffer volume accordingly.
9. The isolated NK cells can be viable for around 2–3 weeks in NK culture medium. Then they will go through apoptosis and die.
10. The plasmid DNA should be relatively free of contaminating proteins and RNA and it must be linearized with a restriction enzyme downstream of the insert to be transcribed. It is generally worthwhile to examine the linearized template DNA by agarose gel electrophoresis to confirm that cleavage is complete because even a small amount of circular plasmid can affect the synthesis of the desired mRNA.
11. The following amounts are for a single 20 μL reaction when the RNA produced will be 300 bases to 5 kb in length. Reactions may be scaled up or down if desired. Vortex the 10× T7 Reaction Buffer and the T7 2× NTP/ARCA until they are completely in solution. Once thawed, store the ribonucleotides T7 (2× NTP/ARCA) on ice, but keep the 10× T7 Reaction Buffer at room temperature while assembling the reaction. The spermidine in the 10× T7 Reaction Buffer can coprecipitate the template DNA if the reaction is assembled on ice.
12. Typically, 80% yield is achieved after 1 h incubation. For maximum yield, 2 h incubation is recommended.
13. RNA concentrations and quality were measured using a Nanospectrophotometer (Thermo-Fisher) at 260 nm vs 280 nm. The yield of mRNA is expected to be about 30 μg for each reaction.
14. For freshly isolated exPBNK cells, no cultivation is required prior to Nucleofection®. For cryopreserved exPNK cells, we recommend incubating the thawed cells for overnight at 37 °C in NK medium before Nucleofection®.
15. The sample must cover the bottom of the cuvette without air bubbles.

16. U-01 for Nucleofector® I Device, U-001 for Nucleofector® II Device.
17. **Labeling temperature:** 4–37 °C, use the temperature your cell line stands best. High temperature correlates with faster loading. **Labeling time:** 5–30 min, usually a very sensitive cell line should not be labeled longer than 5–10 min. Label until you get a maximum signal higher than 15,000–20,000, avoid loading too long. **Labeling concentrations:** Higher concentrations of BATDA result in higher signal until a plateau is reached.
18. Depending on the cell line, the optimal amount of target cells per well is normally in the range of 5000 to 10,000 cells. We usually use 10,000 target cells. The ligand should be used only in short-term assays and the incubation time should not exceed 4 h.
19. **Background:** An aliquot (taken immediately) from the diluted labeled target cell suspension is centrifuged. 100 μL of the supernatant is pipetted into the wells and 100 μL medium is added. 20 μL is transferred to the measuring plate and 200 μL of Eu solution is added. Shake the plate for 15 min and measure the fluorescence. **Spontaneous release:** Incubate the target cells (100 μL) with 100 μL of medium instead of effector cells. After centrifugation, transfer 20 μL of the supernatant to the flat-bottomed plate and add 200 μL Eu solution. Shake for 15 min and measure. Add probenecid (1–2 mmol/L) (Sigma P8761) into the wash solution if necessary to lower the spontaneous release. **Maximum release:** Incubate the target cells (100 μL) with 100 μL of medium supplemented with 10 μL of DELFIA lysis buffer or 100 μL of target cells in 100 μL of 2% Triton X-100 (1% final concentration of Triton X-100). After centrifugation, transfer 20 μL of the supernatant to the flat-bottomed plate and add 200 μL Eu solution. Shake for 15 min and measure.
20. Cytolytic activity can be evaluated using a time-resolved fluorometer using the vector (Perkin Elmer) or FilterMax F5 Multi-Mode Microplate Reader (Molecular Device).
21. We usually include fluorochrome-conjugated anti-CD107a mAb during the stimulation assay to enhance the sensitivity for detection of degranulating cells as previously described by Alter et al. [21]. To prevent degradation of internalized CD107a, monensin can be added to such assays [21]. Brefeldin A is added to prevent cytokine secretion [22].
22. Cell aggregation can be avoided by vortexing prior to the addition of the Fixation/Permeabilization solution. BD Perm/Wash™ buffer must be maintained in washing steps to keep cells permeabilized.

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Chapter 19

mRNA Transfection to Improve NK Cell Homing to Tumors

Emily R. Levy, Mattias Carlsten, and Richard W. Childs

Abstract

The ability of natural killer (NK) cells to mediate antitumor effects following adoptive transfer is dependent on their capacity to traffic to the microenvironment where tumors reside. Recent studies have shown that cytokine-activated and ex vivo-expanded NK cells lack or express at low levels homing receptors required to achieve tissue-specific tumor targeting by cells administered intravenously. In this chapter, we describe a method to enhance NK cell homing toward specific chemoattractants expressed in secondary lymphoid tissues through genetic modification of NK cells using mRNA electroporation. The method described here is scalable, cGMP-compliant, and offers a strategy to bolster the efficacy of adoptive NK cell immunotherapy for the treatment of hematological malignancies in the clinic.

Key words Natural killer cells, Cellular immunotherapy, Lymph node homing, Electroporation, Chemotaxis

1 Introduction

Natural killer (NK) cells are cytotoxic immune cells involved in tumor immune-surveillance [1]. Although NK cells have long been known to be capable of killing cancer cells independent of antigen recognition [1–3], the full therapeutic potential of NK cell-based immunotherapy has yet to be realized in the clinic.

As innate immune effectors, NK cells can kill their targets via several different pathways. NK cell degranulation triggered upon interaction with stressed cells with low or absent MHC class I expression leads to release of cytotoxic granules containing perforin and granzymes as well as cytokines, such as IFN-γ and TNF-α. Target cells can also be killed by NK cells via engagement of death receptor pathways such as FAS/FasL, TRAIL/TRAIL-R [2, 3]. Off-target toxicity profiles of adoptively transferred NK cells appear minimal as their relatively short lifespan in circulation avoids dangerous cytokine storms that occur commonly with many T-cell-based immunotherapies [3]. Research aimed at augmenting NK cell tumor killing has focused on efforts that promote their in vivo survival, homeostatic proliferation, and trafficking to tumor sites.

Srinivas S. Somanchi (ed.), *Natural Killer Cells: Methods and Protocols*, Methods in Molecular Biology, vol. 1441,
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These strategies, as well as combining NK cell therapy with a variety of drugs that bolster NK cell antitumor immunity by disrupting NK cell inhibitory receptor signaling or augmenting NK cell-mediated antibody-dependent cellular cytotoxicity (ADCC) or killing via TRAIL are all important developments in the emerging field of clinical NK cell immunotherapies [3, 4].

Despite these advances, recent data from animal models and humans have raised concerns regarding the ability of adoptively transferred ex vivo-expanded NK cells to home to the lymph nodes and bone marrow where hematological malignancies such as leukemia and lymphoma reside. Based on the above, investigators have now begun to explore a variety of novel strategies to manipulate the phenotype of NK cells and thus their function in vivo to optimize their ability to home to desired tumor targets [3–5]. The expression of chemokine receptors on NK cells may be critical to this process. NK cells expanded with genetically modified K562 cells or EBV-LCL contain predominantly CD56+/CD16+ NK cell populations that do not express CCR7, a chemokine receptor known to facilitate cellular homing to lymph nodes [6, 7]. 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 co-culturing with expanded NK cells. K562 cells expressing mbIL-21 and CCR7 (clone9. CCR7) rapidly transferred CCR7 to expanded NK following a brief 1 h co-culture, with up to 80% of NK cells acquiring CCR7 surface expression [8]. Although surface expression was transient, declining to baseline by 72 h, NK cells that became positive for CCR7 had improved NK cell migration toward the CCR7 ligands CCL19 and CCL21 in transwell migration experiments and had increased homing into the lymph nodes of mice.

Recently, we and others have shown that mRNA electroporation can be used to genetically modify the phenotype of NK cells to improve their homing [9]. The electroporation method calls for cells to be combined with the mRNA of a chosen protein in a small chamber where the cells receive small electric pulses that momentarily perturb the phospholipid bilayer of the cell membrane. The mRNA can then enter the cell and be transcribed for expression. We have found that mRNA electroporation using the cGMP-compliant MaxCyte system with unmodified mRNA coding for GFP and the cell surface marker CD34 results in rapid and highly efficient protein expression in NK cells without compromising their viability and cytotoxic function. NK cells electroporated with GFP mRNA rapidly became GFP-positive and remained fluorescent for more than two weeks. Following transfection of CD34 mRNA, nearly 100% of NK cells expressed CD34 that remained detectable on the cell surface for up to 5 days. Cell viability was not affected by transfection; with the exception of a slight reduction in

proliferative capacity compared to controls, no negative impacts of mRNA electroporation using the MaxCyte platform were observed [10]. Transfection of expanded NK cells did not alter expression of 20 cellular markers as assessed by flow cytometry, including activating and inhibitory NK cell receptors and death receptor ligands such as TRAIL. Further, electroporated NK cells maintained high cytotoxic function against K562 cells and other tumor cell lines.

Based on the findings by Somanchi et al., we have now utilized mRNA transfection with the MaxCyte system to genetically alter ex vivo expanded NK cells to express high surface levels of CCR7. Utilizing this approach, mRNA electroporated NK cells had substantially upregulated surface expression of CCR7. The level of CCR7 expression was dependent on the mRNA dose (Fig. 1a), peaked 8 h following transfection, and persisted for up to 48 h [10]. Importantly, CCR7 mRNA-electroporated NK cells showed marked enhanced in vitro migration capacity toward CCL19 and

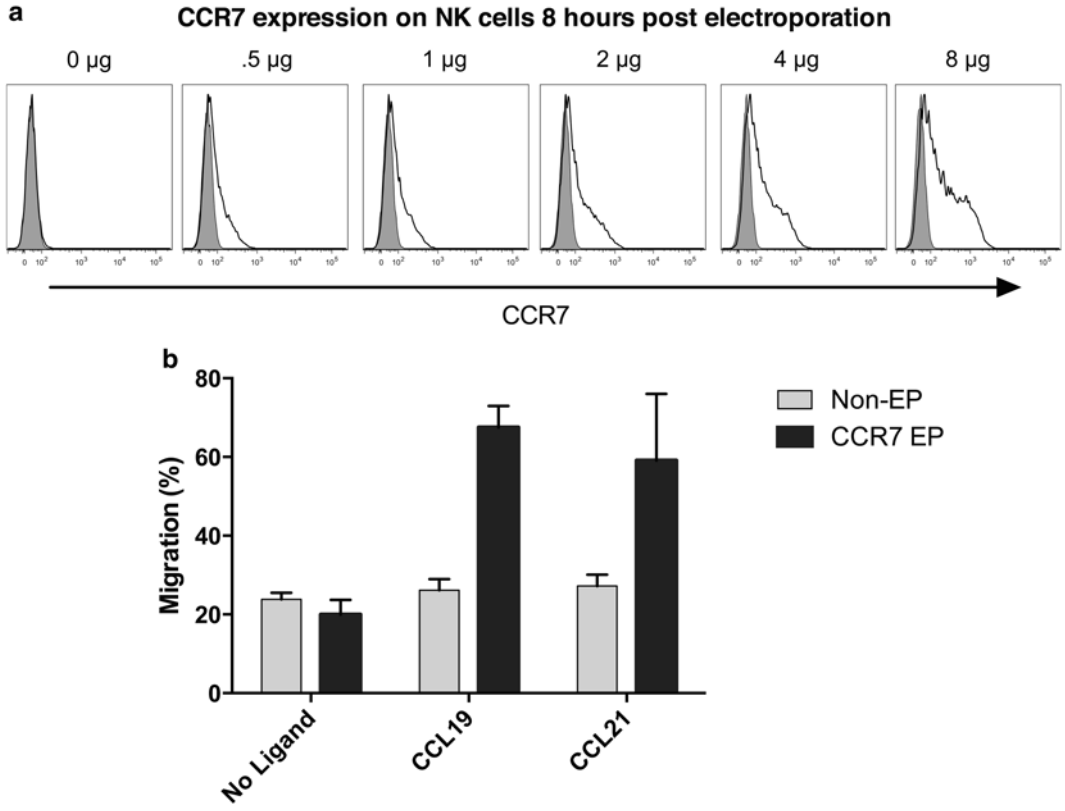

Fig. 1 Electroporation of in vitro-expanded human NK cells with CCR7 mRNA enhances surface expression of CCR7 resulting in a functional in vitro migration advantage toward the chemoattractant ligands CCL19 and CCL21. (**a**) CCR7 mRNA titration for NK cell electroporation: 0.5–8 µg mRNA per 1 million NK cells. Surface expression of CCR7 was analyzed 8 h after electroporation. (**b**) NK cell transwell in vitro migration toward 300 ng/mL of CCL19 and CCL21. Non-electroporated (Non-EP)-expanded NK cells were compared to expanded NK cells that had been electroporated with CCR7 mRNA 8 h previously (CCR7 EP)

CCL21, whereas non-electroporated NK cells remained incapable of migrating toward the ligand for this chemokine receptor (Fig. 1b). The availability of the cGMP-compliant platform described herein offers a method to efficiently genetically modify NK cells using a variety of different chemokine receptors which could improve NK tumor targeting in tissues such as the lymph node and bone marrow where hematological malignancies reside.

2 Materials

Prepare and store all reagents at room temperature (unless indicated otherwise).

2.1 Electroporation Components

1. Instrument: Maxcyte® GT transfection system (Maxctyte Inc.) with pre-loaded optimized cell transfection protocols (*see* **Note 1**).
2. HyClone Buffer: Proprietary Buffer (Thermoscientific).
3. NK cell culture medium: 89% X-Vivo 20 (Lonza), 10% Heat inactivated human AB serum, 1% 2 mM GlutaMAX-1, 500 IU/mL human IL-2. Store at 4 °C, aliquot and prepare at 37 °C for use.
4. mRNA (TriLink BioTechnologies). Store at −80 °C.
5. Sterile filtered 10×PBS (*see* **Note 2**).
6. Electroporation Cuvette (Maxcyte Inc.).
7. 48-well cell culture plate, growth area 0.95 cm^2.
8. Plastic cell culture flask (*see* **Note 3**).

2.2 Phenotype of Chemokine Receptor Expression Components

1. LSRFortessa Flow cytometer (BD biosciences) (*see* **Note 4**).
2. 96-well, round bottom, polypropylene tissue culture plate.
3. Commercially available fluorochrome-conjugated antibodies (*see* **Note 5**).
4. FACS buffer (89.5% PBS, 10% fetal bovine serum, 0.5% 0.5 M EDTA).
5. 1% paraformaldehyde solution in PBS.
6. Flowjo analysis software (Treestar Inc.).

2.3 In Vitro Migration Assay Components

1. Warm X-Vivo 20 (Lonza).
2. CCR7-specific Chemokine Ligands CCL19 and CCL21 (BioLegend).
3. Corning® Costar® Transwell® cell culture plates and inserts (Sigma-Aldrich), 5 μm pores.
4. 1.5 mL microcentrifuge tubes; 1 tube per migration well (Eppendorf).

5. CyQUANT® solution: CyQUANT® proliferation kit (Thermofisher Scientific)—Dilute the CyQUANT® lysis buffer 1:20 in ddH_2O and add CyQUANT® proliferation Dye 1:600. Prepare 350 μL of solution for each sample, plus 10% extra (e.g., for 10 samples you will need 3.9 mL of CyQUANT® solution: 3.69 mL ddH_2O, 195 μL lysis buffer, and 6.5 μL proliferation dye). 96-well flat bottom white assay plate (Costar).
6. Wallac 1420 Victor2 Luminescence plate reader (PerkinElmer).

3 Methods

The protocol below is optimized for NK cells isolated from CD3-depleted and CD56-selected PBMCs from healthy donors (department of transfusion medicine, NIH). NK cells are then co-cultured with an irradiated EBV-LCL transformed cell line and expanded in IL-2 containing medium for 14 days before use. The protocols below may need to be further optimized for freshly isolated NK cells and/or overnight cytokine-activated NK cells, or NK cells expanded using other means.

3.1 Electroporation of NK Cells

1. Turn on the Maxcyte® GT instrument and open the Maxcyte Electroporation program. Select the electroporation protocol NK2-OC.
2. Thaw mRNA and keep on ice.
3. Determine the number of cells needed for the study, and collect the desired number of cells from the culture flask into a 50 mL conical flask (*see* **Note 6**).
4. Centrifuge the sample at 311 *g* for 7 min.
5. Aspirate the supernatant completely.
6. Wash the sample with 5 mL HyClone buffer; centrifuge at 311 *g* for 7 min.
7. Determine the density and total volume of cell suspension needed for transfection (*see* **Note 7**). Carefully suspend the cells in HyClone buffer to achieve this volume.
8. Prepare a 1.5 mL Eppendorf tube with desired volume of mRNA (*see* **Note 8**). Place mRNA stock back on ice.
9. Add sterile 10×PBS to the aliquot of mRNA (*see* **Note 9**).
10. Without waiting, carefully add the cells to the mRNA/10×PBS mixture and mix carefully 3 times with the same pipette tip.
11. Transfer the contents of the Eppendorf to a sterile cuvette. Sweep each of the four corners of the chamber with cell suspension to make sure there are no air bubbles left in the cuvette.

12. Put the lid on the cuvette and plug the cuvette into the Maxcyte® GT.
13. Click "Start" and wait until the program indicates "complete."
14. Gingerly take the cuvette back to the biosafety cabinet and transfer all of the contents into the middle of one empty well of a flat bottom 48-well plate.
15. Incubate the plate at 37 °C, 6.5% CO_2 for 20 min (10–30 min).
16. While the cells are incubating, transfer warm NK cell medium into T75 or T25 culture flask. Optimal cell density is $0.5–2 \times 10^6$ cells/mL of NK cell medium.
17. Once the cells have had time to recover, place the 48-well plate in the biosafety cabinet. Using a sterile 1 mL pipette, carefully flush the well with cells with 300 μL of warm NK cell culture medium and transfer into a culture flask.

    Give the cells at least 1 h for recovery in culture before use (*see* **Note 10**).

3.2 mRNA Titration and Phenotype of Chemokine Receptor Expression

1. Electroporate samples with increasing amount of mRNA (e.g., 1, 2, 4, and 8 μg mRNA per million cells). Each separate titration sample will use a different volume of mRNA, therefore, use ddH2O to balance out the total volume of liquid in the sample (*see* **Note 11**).
2. Choose a time point or a scale of time for measuring the surface receptor expression for the protein of interest. For most receptors that we have analyzed, peak expression is within 24 h of electroporation. CCR7 has a peak expression 8 h post electroporation.
3. At the selected time point, flush the bottom of the cell culture flask using a sterile 5 mL serological pipette to create an even cell suspension. Draw up approximately 300 μL (the cone of the tip) and transfer directly into a well of an empty 96-well round bottom plate. Split the sample in half to create a well for isotype staining (stain approximately $0.1 \times 10^6–0.5 \times 10^6$ cells per sample).
4. Centrifuge the plate at 550*g* for 3 min. Toss the liquid from the plate into an appropriate discard container.
5. Suspend each sample in 50 μL of FACS buffer and add the appropriate amount of antibody master mix (*see* **Note 5**). There is also an option to add an FC receptor-blocking antibody before adding the master mix to minimize the nonspecific binding of the conjugated antibodies.
6. Incubate the samples in the dark, at 4 °C, for 15 min. Add 150 μL FACS buffer and gently suspend the cells. Centrifuge plate

at 550 *g* for 3 min. Toss the supernatant in an appropriate discard container.

7. Wash once more with 200 μL FACS buffer, centrifuge the plate at the same speed and time and toss the supernatant as previously outlined.
8. Resuspend the samples in 1% paraformaldehyde solution. Store samples in the fridge (4 °C) and protect from light exposure.
9. Repeat procedure for each time point.
10. Prior to running the samples on the flow cytometer, isolate the cell pellets and resuspend samples in 250 μL FACS buffer.
11. Analyze receptor expression by quantifying geometric MFI using the flowjo analysis software.

3.3 In Vitro Migration Assay

1. 8 h post electroporation, create an even suspension of cells by flushing the bottom of the culture flask using a 5 mL serological pipette. Collect the appropriate amount of cells for the assay (*see* **Note 12**).
2. Wash and resuspend the cells in plain X-Vivo 20 for a suspension of 0.5×10^6 cells/mL media. Let the cells rest in 37 °C until ready to use.
3. Dilute the chemokine ligand in warm plain X-Vivo 20 to the maximum desired ligand concentration. For CCL19 and CCL21, 300 ng/mL is sufficient for significant migration. The ligand solution can be further diluted out to serial concentrations.
4. Remove transwell inserts from the plate and transfer 600 μL of the chemokine solution into the appropriate wells. Carefully place the transwell inserts back into the wells.
5. Add 100 μL of the cell suspension on top of each transwell insert (5×10^4 NK cells total per well).
6. In three wells, designated maximum controls, add 5×10^4 NK cells directly into the wells without transwell inserts. These wells will be used to calculate the percentage migration.
7. Incubate for 2 h in 37 °C.
8. After assay is complete, carefully lift the transwell inserts out of the wells. Collect the media from each will into an individual 1.5 mL microcentrifuge tube. Wash the wells twice with 400 plain X-Vivo 20.
9. Spin tubes at 1200 *g* for 6 min.
10. Aspirate off all media. Isolated pellets can be stored at –80 °C until they are ready for analysis.
11. Suspend all samples in 350 μL CyQUANT® solution and plate triplicates in a 96-well flat bottom white assay plate (100 μL per well).

12. Protect the plate from light and place on ice until the sample is ready to be run.
13. Using a microplate reader, such as the Wallac 1420 Victor2, measure fluorescence of each well (485/535 nM for 1 s per well). Data can be exported and analyzed in Microsoft Excel.
14. Percent migration can be calculated by averaging the triplicate values, and dividing the average value for each sample by the maximum control (5×10^4 NK cells). Migration index can be calculated by dividing percent migration by the zero control.

4 Notes

1. We use the pre-loaded program NK-2 OC.
2. Use of 10×PBS to balance the osmolality of the mRNA suspension that will enter the cell sample.
3. The size of the flask can be determined by the cell number. Optimal cell density is $0.5–2 \times 10^6$ cells/mL of NK cell medium (e.g., 10×10^6 cells/5 mL in a standing T25 culture flask or 50×10^6 cells in 17 mL in a T75 standing culture flask).
4. We use the BD LSRFortessa flow cytometer with a 96-well plate loader attachment. This experiment may need to be further optimized when using a different flow cytometer and/or if 5 mL round bottom polypropylene tubes are used instead of plates.
5. To asses CCR7 expression we used an antibody cocktail consisting of 0.5 μL PeCy7 CD56 (BD Biosciences) per 50 μL FACS buffer, 2 μL CCR7 BV605 (Biolegend) per 50 μL FACS buffer, and 1 μL Aqua Live Dead marker (Life Technologies) per 50 μL FACS buffer. The isotype cocktail mix contains BV605 IgG2a (Biolegend) in lieu of CCR7. Additional cell markers conjugated to other fluorochromes can also be used for flow cytometry analysis.
6. This procedure usually leads to approximately 20–30% cell loss. A number of cells may be lost in the electroporation cuvette. Additional cells can be lost when transferring the sample from the 48-well plate into the final culture flask. Be mindful of the number of cells that you begin with.
7. The total suspension should be approximately $1–2 \times 10^6$ cells/10 μL suspension. The suspension includes cells in hyclone buffer, mRNA, and sterile 10×PBS. The total volume of the cell suspension is dependent on the size of the cuvette. OC-100 holds maximum volume of 100 μL of liquid, and the OC-400 holds a maximum volume of 400 μL of liquid (e.g., OC-100 will hold $10–20 \times 10^6$ cells in 100 μL suspension and

OC-400 will hold 40–80 × 10^6 cells in 400 μL). It is not necessary to fill the maximum volume of the cuvette. For example, 5 × 10^6 cells will be suspended in 50 μL in an OC-100 cuvette and 30 × 10^6 cells will be suspended in 300 μL using an OC-400 cuvette.

8. The desired amount of mRNA can be determined by titrating escalating mRNA doses with the cells to confer maximum protein expression (Fig. 1a). Our lab uses 4 μg/1 × 10^6 cells CCR7 mRNA. Concentration of stock mRNA can range from 0.5 to 3 mg/mL.
9. The volume of 10×PBS will be 1/10 of the total mRNA volume.
10. Each different type of mRNA will have different temporal expression. We have found that the peak surface expression of CCR7 is 8 h post electroporation.
11. The 8 μg sample will use 4 μL mRNA and the 2 μg sample will use 1 μL of mRNA (CCR7 mRNA stock from Trilink Biotechnologies is 2 mg/mL). Add 3 μL ddH2O to the 2 μg sample to compensate for this volume difference. The amount of 10×PBS will be consistent throughout all of the samples.
12. 5 × 10^4 cells will be loaded into each well. When planning for samples, be sure to include a negative control and a positive control for each electroporated cell sample. The zero control is 600 μL plain X-Vivo 20 loaded into the bottom chamber, 100 μL cells loaded into the top chamber, and the maximum control is 100 μL of cells loaded into 600 μL plain X-Vivo without the transwell membrane insert.

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Chapter 20

In Vitro Generation of Human NK Cells Expressing Chimeric Antigen Receptor Through Differentiation of Gene-Modified Hematopoietic Stem Cells

Emily Lowe, Laurel C. Truscott, and Satiro N. De Oliveira

Abstract

NK cells represent a very promising source for adoptive cellular approaches for cancer immunotherapy, and extensive research has been conducted, including clinical trials. Gene modification of NK cells can direct their specificity and enhance their function, but the efficiency of gene transfer techniques is very limited. Here we describe two protocols designed to generate mature human NK cells from gene-modified hematopoietic stem cells. These protocols use chimeric antigen receptor as the transgene, but could potentially be modified for the expression any particular transgene in human NK cells.

Key words Hematopoietic stem cells, Gene transfer, Lentiviral vector, OP9 cell line, NK cells

1 Introduction

Natural Killer (NK) cells are innate immune cells that mediate spontaneous cytotoxicity against tumor and virus-infected cells, and many clinical trials have attempted to harness their properties for cellular therapies [1–6]. Gene transfer technology can enhance the efficacy of such efforts by improving NK cell survival or function, or engineering antigen specificity [7–9].

Chimeric antigen receptors (CAR) are engineered fusion proteins that combine the antigen specificity of antigen-binding moieties of monoclonal antibodies and intracellular activation motifs capable to activate immune cells. Preliminary evidence suggests that NK cells with specificity directed by chimeric antigen receptors may have enhanced cytotoxicity [10–12].

Generation of mature NK cells from hematopoietic stem cells provides the opportunity of generation of younger NK cells and expansion of specific gene-modified clones starting from a smaller number of previously isolated and cryopreserved initial cells, with the added advantage of generation of multiple batches from the

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same donor [13–16]. In this chapter, we describe a protocol for NK cell differentiation from human hematopoietic stem cells (HSC) modified to express chimeric antigen receptors using co-culture with a feeder stroma of murine OP9-DL1 cells in presence of human recombinant cytokines [15]. Alternatively, we describe a feeder-free protocol for generation of gene-modified NK cells from human hematopoietic stem cells using insulin-like growth factor 1 (IGF-1) [17]. The transgene used in this protocol is a CD19-specific CAR, but the HSC could potentially receive any type of persistent gene modification for expression in differentiated NK cells.

2 Materials

2.1 Isolation and Cryopreservation of Umbilical Cord Blood HSC

1. Human primary cells from umbilical cord blood collected within 48 h for the CD34 isolation procedure (*see* **Note 1**).
2. Ficoll-Paque PLUS (GE Healthcare Life Sciences).
3. CD34 MicroBead Kit UltraPure (Miltenyi Biotec).
4. MidiMACS Starting Kit with LS columns (Miltenyi Biotec).
5. MACS BSA Stock Solution (Miltenyi Biotec).
6. autoMACS Rinsing Solution (Miltenyi Biotec).
7. Dulbecco's PBS (DPBS).
8. Freezing medium: 10% DMSO in heat-inactivated fetal bovine serum (Omega).

2.2 Lentiviral Transduction of HSC

1. Cryopreserved primary human CD34-positive hematopoietic stem cells (HSC) isolated from umbilical cord blood (*see* **Note 1**).
2. Transduction medium: Freshly prepared X-Vivo15 medium (Lonza), 50 ng/mL of recombinant human Stem Cell Factor (SCF) (R&D Systems), 50 ng/mL of recombinant human Flt-3 ligand (R&D Systems), and 50 ng/mL of recombinant human thrombopoietin (R&D Systems).
3. Fibronectin fragment CH-296 RetroNectin (Takara Shuzo Co.).
4. 48 well non-tissue culture-treated plate, sterile.
5. Third-generation lentiviral vector carrying second-generation chimeric antigen receptor construct (Fig. 1a) (*see* **Note 2**).

2.3 Differentiation of HSC to NK Cell Lineage

2.3.1 Co-culture with OP9-DL1 Stromal Cells

1. OP9 stromal cell line (ATCC) gene-modified to express Delta-like 1 (OP-DL1) (*see* **Note 3**).
2. Alpha-20 medium: alpha-Minimum Essential Medium (alpha-MEM) enriched with 20% of heat-inactivated fetal bovine serum (Omega), 2 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin.

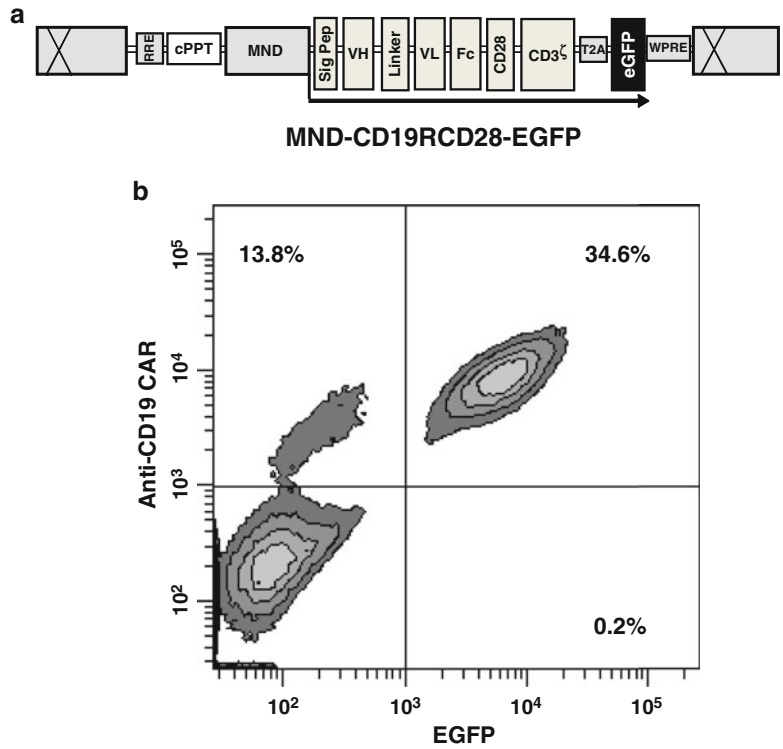

Fig. 1 Lentiviral vector for transduction of human HSC. (**a**) Diagram of third-generation lentiviral vector carrying anti-CD19 CAR and EGFP. (**b**) Co-expression of CAR and EGFP detected by flow cytometry in NK cells differentiated in vitro from human HSC, day 40 of culture. CAR expression was detected by using PE-Texas Red-conjugated polyclonal F(ab′)$_2$ fragment goat antihuman IgG1 Fcχ (Jackson ImmunoResearch Laboratories) [15]

3. NK differentiation medium: alpha-20 medium, enriched with 5 ng/mL of recombinant human SCF (R&D Systems), 5 ng/mL of recombinant human Flt-3 ligand (R&D Systems), 5 ng/mL of IL-7 (R&D Systems), and 10 ng/mL of IL-15 (R&D Systems).
4. 12 well tissue culture-treated plates, sterile.
5. T-75 and T-150 culture flasks.
6. 0.05% trypsin EDTA 1×.
7. Dulbecco's PBS (DPBS).
8. 70 μm cell strainer.

2.3.2 Feeder-Free Differentiation Protocol

1. AIM V CC medium: AIM V Complete medium (Life Technologies), 5% human AB serum (Corning), and 2 mM GlutaMAX (Life Technologies), freshly supplemented with 30 ng/mL of recombinant human cytokines SCF (R&D

Systems), 50 ng/mL of recombinant human Flt-3 ligand (R&D Systems), 50 ng/mL of recombinant human IL-15 (R&D Systems), and 100 ng/mL of recombinant human IGF-1 (R&D Systems).

2. Falcon 48, 12, and 6 well tissue culture-treated microplates, sterile.

3 Methods

3.1 Isolation and Cryopreservation of Umbilical Cord Blood HSC (See Note 1)

Isolation of CD34-positive cells from umbilical cord blood collected within 48 h will increase cell recovery. The cord blood unit can be kept at room temperature until isolation procedure. The isolation procedure should be conducted in a biosafety cabinet to ensure sterility and personal protection.

1. Dilute umbilical cord blood with equal volume of PBS.
2. Pipette 15 mL of Ficoll into a 50 mL conical tube.
3. Layer 35 mL of cord blood onto Ficoll.
4. Centrifuge at 400 × *g* for 30 min at 20 °C without brake.
5. Prepare isolation buffer by diluting MACS BSA Stock Solution into autoMACS Rinsing Solution according to the manufacturer's instructions, and keep it ice-cold.
6. Collect the buffy coat of all post-centrifugation Ficoll tubes with a sterile Pasteur pipette or a serological pipette into a new 50 mL conical tube.
7. Dilute such collected buffy coat with equal volume of PBS.
8. Centrifuge at 300 × *g* for 15 min at 4 °C, with brake on.
9. Remove supernatant.
10. Collect all cell pellets into one 50 mL conical tube and complete to 50 mL with ice-cold isolation buffer.
11. Count cells.
12. Centrifuge at 250 × *g* for 5 min at 4 °C, with brake on.
13. Remove supernatant and resuspend up to 1×10^8 cells in 300 μL of ice-cold isolation buffer.
14. Follow the manufacturer's instructions to use the reagents of the Miltenyi Biotec CD34 MicroBead Kit UltraPure and the MidiMACS Kit to perform immunomagnetic positive selection of CD34 HSC using the LS columns.
15. After completion of the isolation protocol, count the CD34-positive HSC and use immediately for transduction, or cryopreserve for posterior use. HSC cells kept in culture with human cytokines will undergo mostly onto myeloid lineage differentiation and lose expression of CD34.

3.2 Lentiviral Transduction of HSC

3.2.1 High-Titer Lentivirus for Modification of HSC

Our protocol for packaging and titer analysis of high-titer lentiviral vectors for gene modification of primary human cells is very extensive and has been published elsewhere [15, 18]. In brief, such vectors should have titer of at least 5×10^7–1×10^8 TU/mL to ensure very efficient transduction, what requires post-packaging procedures to obtain 100–1000-fold concentration. The vector used in this protocol is a third-generation replication-incompetent HIV-based lentivirus carrying a CD19-specific CAR and enhanced green fluorescent protein (EGFP) (Fig. 1) (*see* **Note 2**).

3.2.2 Lentiviral Transduction of HSC

Before an NK differentiation protocol is initiated, the investigators should evaluate in preliminary experiments the optimal transduction conditions using the available preparations of lentiviral vectors in human HSC [18] (*see* **Note 4**). Gene-modified HSC should be placed in NK differentiation culture conditions immediately after lentiviral transduction, and full expression of integrated vector copies will take place around 10 days after transduction. We routinely use overnight cytokine pre-stimulation of HSC in plates coated with fibronectin fragment CH-296 RetroNectin before treatment with the lentiviral vector [15, 18].

1. RetroNectin coating of transduction plate: fill the wells of a non-tissue culture-treated 48 well plate with 500 µL/well of RetroNectin 20 µg/mL in PBS and incubate for 2 h in room temperature; after aspirating the RetroNectin solution, fill the wells with 1 mL/well of blocking solution with 2% fetal bovine serum (FBS) in PBS and incubate for 30 min in room temperature; remove the blocking solution and wash twice with 1 mL/well of wash buffer with 0.025 M HEPES in PBS (*see* **Note 5**).
2. Resuspend human CD34-positive cells at 1×10^6 cells/mL in transduction medium.
3. Plate 400 µL/well in the RetroNectin coated 48 well transduction plate (from **step 1**).
4. Incubate the plate for 14 h in 5% CO_2 at 37 °C for pre-stimulation; the cells will attach to the bottom coated with RetroNectin.
5. After pre-stimulation, add the necessary volume of lentiviral vector to ensure transduction efficiency at a viral vector copy number of 1–3 copies/cell [15, 18] as determined in preliminary experiments using available vector preparations (*see* **Note 4**).
6. Incubate transduction plate with added lentiviral vectors for 24 h in 5% CO_2 at 37 °C before transferring HSC to differentiation cultures.

3.3 Culture and Passaging of OP9-DL1 Stromal Cells

It is fundamental to keep healthy, low-passage, stromal cells for co-culture with the primary human cells (*see* **Notes 3** and **6**).

1. Start OP9-DL1 stromal cell culture with 2×10^6–5×10^6 cells in a T-75 flask using alpha-20 culture medium without any cytokines (*see* **Note 7**).
2. To passage OP9-DL1, first carefully aspirate and remove the culture medium; rinse with 20 mL of PBS at room temperature, and completely remove PBS.
3. Add 12 mL of trypsin for a T-75 flask (or 30 mL for a T-150 flask) and incubate at 37 °C for 5–15 min until cell layer is dispersed.
4. Add at least the same volume of alpha-20 medium to resuspend cells in order to inactivate the trypsin.
5. Count the cells and add 2×10^6–5×10^6 cells to a T-75 flask, or 5×10^6–10×10^6 cells to a T-150 flask, for continued culture (*see* **Note 8**).
6. For the co-culture with human cells, plate 2×10^5–5×10^5 per well in a tissue culture-treated 12 well plate, in alpha-20 medium (*see* **Note 9**).

3.4 Differentiation of HSC to NK Cell Lineage

3.4.1 Differentiation by Co-culture with OP9-DL1 Stromal Cells

All co-cultures should be performed using freshly prepared NK differentiation medium from the first day.

1. Vigorously pipette the HSC in the transduction plate after adding 0.5–1 mL of NK differentiation medium per well, in order to detach all cells adherent to the bottom.
2. Count cells and adjust concentration to 5×10^5 cells/mL in NK differentiation medium.
3. Carefully remove the alpha-20 medium from the confluent OP-DL1 stroma cultured in the 12 well plates (*see* **Note 9**).
4. Seed the transduced HSC cells suspended in the NK differentiation medium by slow and gentle pipetting against the wall, at 3×10^5–5×10^5 cells per well.
5. Gently add freshly prepared NK differentiation medium by slow pipetting against the wall for a total volume of 2 mL per well.
6. Split HSC every 3–4 days; vigorously pipette using P-1000 to resuspend cells and detach stroma; remove stromal cells using a 70 μm cell strainer unto a 50 mL conical tube, then repeat **steps 2–5** in this section (*see* **Note 10**).
7. Maintain co-culture of transduced HSC with stromal cells for 35–40 days to obtain functional NK cells (*see* **Notes 11** and **12**) (Fig. 2a). CD56+ NK cells can be sorted via fluorescence activated cell sorting (FACS) or immunomagnetic bead selection for continued culture or functional assays such as cytotoxicity assessment.

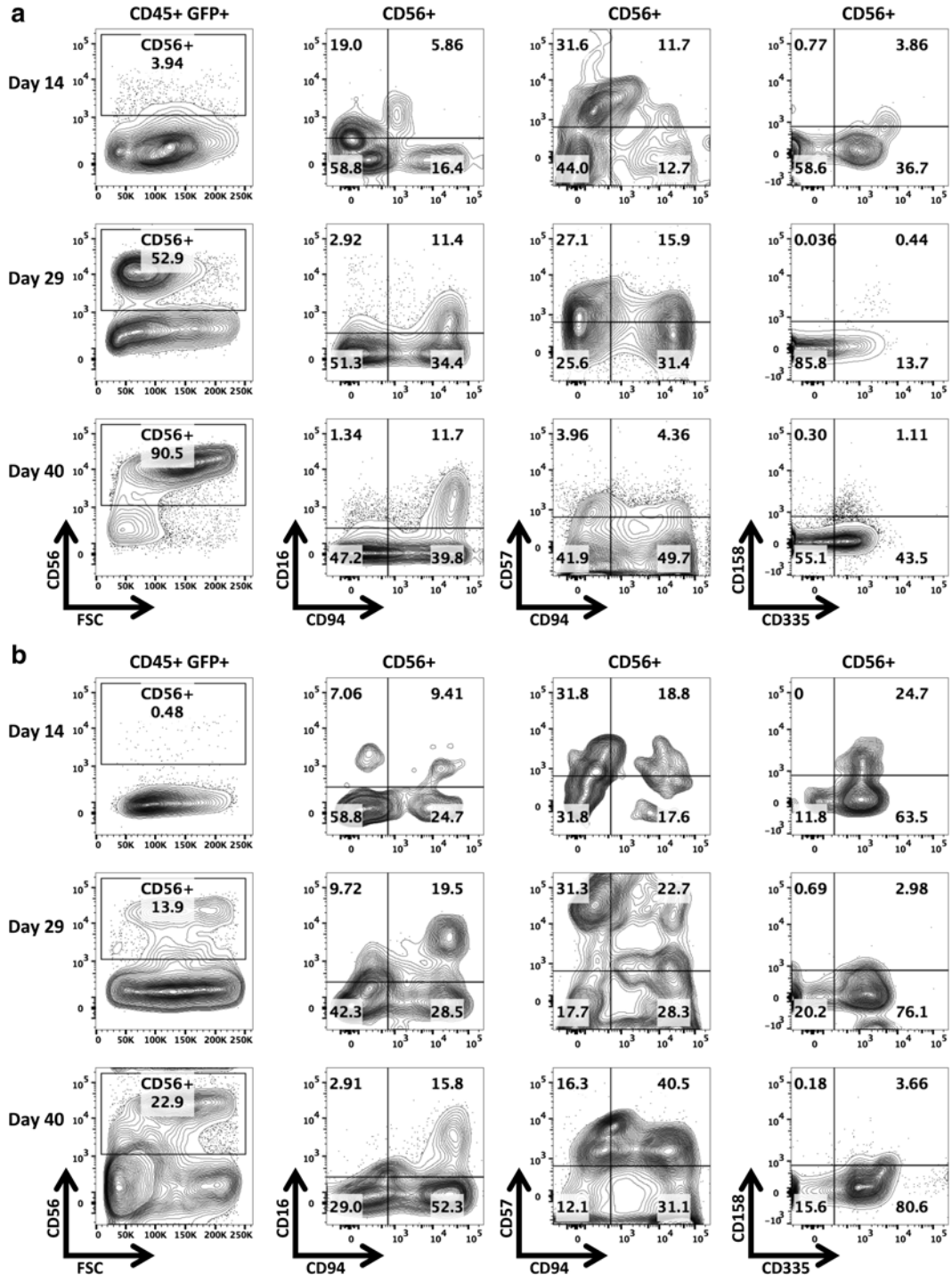

Fig. 2 Expression of NK-specific surface markers along progression of in vitro differentiation culture from gene-modified human HSC. (**a**) Co-culture with OP9-DL1 stromal cells. (**b**) Feeder-free culture. Representative plots were taken at days 14, 29, and 40 of both NK differentiation culture protocols. Gated human cells positive for EGFP were evaluated for the CD56, CD16, CD94, CD57, CD158, and CD335 surface markers

3.4.2 Feeder-Free Differentiation Culture (Alternate Protocol)

All media changes and splits should be performed using AIM V CC from the first day (*see* **Note 13**).

1. Vigorously pipette the HSC in the transduction plate after adding 0.5–1 mL of AIM V CC per well, in order to detach all cells adherent to the bottom.
2. Transfer cells to an appropriately sized conical tube. Centrifuge at 300 × *g* for 10 min. Remove supernatant and resuspend cell pellet by flicking the tube (*see* **Note 14**). Add 1 mL (or appropriate volume) of AIM V CC.
3. Count cells using a viability dye (e.g., trypan blue) and adjust viable cell concentration to 0.5×10^6 cells/mL in AIM V CC.
4. Plate cells into appropriately sized wells of tissue culture-treated microplates. For example, plate as many as 0.5 × cells in 1 mL of AIM V CC into one well of a 24 well plate, or up to 3×10^6 cells in 6 mL of AIM V CC into the well of a 6 well plate.
5. Cells are incubated at 37 °C in a humidified atmosphere with 5 % CO_2.
6. Differentiating cells will need to be split up to twice a week. Pipet to detach gently adherent cells and transfer half the media and cells to a neighboring well that has not been used or to a new equally sized plate. Replenish the media to the original volume in both wells with fresh AIM V CC (*see* **Note 15**).
7. In order to remove cellular debris, every 10–12 days the cells should be washed and completely replenished with fresh media and cytokines. Pipet to detach the slightly adherent cells, ignoring the strongly adherent cell populations. Transfer the non-adherent cells to conical tubes and centrifuge at 300 × *g* for 10 min. Remove supernatant and resuspend cell pellet in an appropriate volume of AIM V CC for counting cells. Count cells using a viability dye and adjust viable cell concentration to 1×10^6 cells/mL in AIM V CC. Plate cells into appropriately sized wells of Falcon tissue culture-treated microplates. For example, plate as many as 5×10^6 cells in 5 mL AIM V CC into one well of a 6 well plate (*see* **Note 15**).
8. CD56+ NK cells will begin to appear around Day 14, peak around Day 28, and begin to decline thereafter (Fig. 2b).
9. Between days 14 and 28, CD56+ NK cells can be sorted via fluorescence activated cell sorting (FACS) or immunomagnetic bead selection for continued culture or functional assays such as cytotoxicity assessment. Sorted NK cells can be cultured at 1×10^6 cells/mL, but will no longer appreciably expand, in AIM V medium (Life Technologies) supplemented with 5 % human AB serum (Corning) and 2 mM GlutaMAX

(Life Technologies) and 20 ng/mL of recombinant human IL-15 (R&D Systems). In our hands, sorted NK cells in this media remain cytotoxic for approximately 2 weeks longer.

4 Notes

1. The presented protocols can be applied to any source of human CD34-positive cells [14]. However, the final yield of differentiated NK cells is higher using fetal liver or umbilical cord blood, as opposed to bone marrow or mobilized peripheral stem cells [19].
2. The presence of a co-delivered marker, such as enhanced green fluorescent protein (EGFP) or a selection component, will allow enrichment for gene-modified NK cell precursors at any step of the differentiation protocol.
3. It is not clear if DL1 expression is necessary for successful generation of mature and functional NK cells. Preliminary evidence suggests that OP9-DL1 co-culture promotes faster differentiation and higher cell proliferation [13, 15, 20].
4. In clinically relevant experiments, the viral vector copy number per cell in HSC should be kept within 1–3 copies/cell, in order to avoid genotoxicity.
5. A plate coated with RetroNectin can be preserved at 4 °C for up to 7 days well filled with 1 mL of wash buffer.
6. In the co-culture protocol, we have used non-irradiated OP9-DL1 stromal cells.
7. Start OP9-DL1 stromal cell culture 1–2 weeks before HSC transduction, in order to have at least a full T-75 flask with confluent stroma before starting the protocol.
8. Passage OP9-DL1 cells cultured in flasks every 3–5 days and when no more confluent than 90% split cells at 1:5–1:10, i.e., using one-fifth to one-tenth of the volume of cell suspension from one flask to reseed a new flask. Fully confluent stroma decays in about 10–14 days. Timing of the passage of OP9-DL1 cells with the seeding of plates for the co-culture with HSC will optimize the protocol and avoid waste.
9. OP9-DL1 cells should be at 90% confluence before seeding the human cells, what usually takes 2–4 days after seeding on a tissue culture-treated 12 well plate.
10. Seed the HSC over fresh confluent OP9-DL1 stromal cells each time HSC are passaged. During the first 2 weeks, the human cells will proliferate faster and may require passaging more often.

11. In the OP9-DL1 co-culture protocol, differentiated NK cells will develop cytotoxicity against the stromal cells around day 35 of culture, as a surrogate of successful differentiation.
12. The use of a NK cell expansion protocol will increase the final yield of the cell generation when used in sequence to the protocols described in this chapter. Cells should be transferred to the expansion protocol at day 30 or later, when cells have higher expression of CD16 and other mature NK cell-specific markers.
13. In preparation of AIM V CC, human AB serum must be heat inactivated at 56 °C for 30 min and sterile filtered to remove excess lipids. Alternatively, the AIM V Complete medium can be sterile filtered prior to the addition of cytokines.
14. In order to resuspend the cell pellet, it is important to flick the tube to disperse the cells rather than to directly pipet the pellet, which can cause shearing and decrease viability of cells at this point.
15. As HSC differentiate, adherent cells such as endothelial cells and macrophages will become observable. NK and NK progenitors, however, are not strongly adherent; therefore, during splits, pipet to detach cells that are easily detachable and do not attempt to remove strongly adherent cells. If the well becomes overgrown with adherent cells, do not continue to use it for NK differentiation and expansion.

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Chapter 21

Engineering Receptor Expression on Natural Killer Cells Through Trogocytosis

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Abstract

Trogocytosis is a rapid contact-dependent process by which lymphocytes acquire membrane patches from the target cells ('donor' cells) with which they interact and this phenomenon has been shown to occur in various immune cells. The surface molecules acquired through trogocytosis are functionally incorporated in the 'acceptor' cells transiently. We had previously demonstrated that trogocytosis can be utilized in place of gene transfer to engineer surface receptor expression on NK cells for adoptive immunotherapy applications. In this chapter, we describe detailed protocol for trogocytosis—co-culture of NK cell with the donor cell line, phenotypic assessment of receptor uptake and persistence, and assessment of NK cell function (migration) following receptor acquisition.

Key words NK cells, Trogocytosis, CCR7, Adoptive immunotherapy

1 Introduction

Trogocytosis is a process of fast and cell-to-cell contact-dependent intercellular transfer of membrane patches. The phenomenon was initially described through transfer of ^{14}C labeled LPS from macrophages to lymphocytes [1]. Since then, trogocytosis has been shown to occur in many different immune cell types including T cells, B cells, dendritic cells, NK cells and macrophages/monocytes, and neutrophils [2–13] and even between tumor cells [14], both in vitro and in vivo. Although the biological purpose of trogocytosis remains unknown, it has been speculated to generate immune plasticity beyond genetic and epigenetic programming [15–18]. Additionally, the membrane-bound proteins acquired through trogocytosis have been shown to signal within 'acceptor' cells [19, 20], thus offering unique functionality to acceptor cells.

In the context of NK cells, trogocytosis was not only shown to generate suppressive NK cells through uptake of HLA-G1 [21], hyporesponsive NK cells through uptake of activating receptor ligands [22] and to cause fratricide of NK cells that acquire tumor

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derived ligands for NKG2D [23], but was also shown to enable their migration, through acquisition of CCR7 from dendritic cells [24] with potential consequence of abating graft-vs-host reaction.

Based on the ability of the process of trogocytosis to transfer intact surface molecules to NK cells and the functional impact they impart to NK cells, we had proposed the use of trogocytosis as a tool to engineer expression of membrane proteins on NK cells, in place of gene modification, for adoptive immunotherapy application and demonstrated the feasibility of the approach using in vivo model of lymph node homing [25]. More recently, this approach was also used to modify NK cells with chimeric antigen receptor (CAR) to target B-cell acute lymphoblastic leukemia [26].

In this chapter, a detailed protocol is described for efficient modification of NK cells through trogocytosis. This method can be used to assess the phenomenon of trogocytosis in NK cells or to modify NK cells with membrane-bound proteins of interest such as chemokine receptors or tumor targeting receptors such as CARs. Since trogocytosis involves transfer of membrane patches, this method can be used to simultaneously transfer multiple proteins or multicomponent protein complexes on to NK cells.

2 Materials

1. Human NK cells (activated/expanded NK cells or NK cell lines).
2. NK cell medium: RPMI 1640, 10% FBS, 1× GlutaMAX (2 mM), and 1× Pen/Strep. Freshly add 50 IU/ml of IL2 to medium just before use.
3. Ficoll-Paque Plus (GE Healthcare Life Sciences).
4. K562 parental cell line (K562-P).
5. K562 cell line gene modified with the desired chemokine receptor (CCR7 for the example given here) or protein of choice (K562-GM).
6. RPMI complete medium: RPMI 1640, 10% FBS, 1× Pen/Strep, 1× GlutaMAX.
7. Gammacell 1000 Irradiator.
8. Liquid nitrogen or dry ice/ethanol bath in a polyurethane ice bucket.
9. 37 °C water bath.
10. Trypan blue dye and hemocytometer.
11. 50 ml conical tubes.
12. 24 well plates (or T-75 flasks for larger co-culture setup).
13. PBS.

14. Acid wash buffer: Prepare citrate buffer containing 0.133 M citric acid and 0.066 M sodium phosphate dibasic, store at room temperature.
15. Stop buffer: Prepare RPMI 1640 containing 10% FBS and 10 mM HEPES, store at 4 °C.
16. Antibody cocktail: Prepare antibody cocktail with Anti-CD16 PE-Cy5, anti-CD56 PE-Cy5, or anti-NKG2D PE-Cy5 and antibody against the receptor of choice (e.g., CCR7) conjugated to FITC.
17. 1% formaldehyde in PBS.
18. CFSE or Calcein AM.
19. Serum-free RPMI medium: RPMI 1640, 1× GlutaMAX (2 mM), and 1× Pen/Strep.
20. Chemokines: Recombinant chemokines (CCL19 and CCL21 in the example given here) corresponding to the chemokine receptor of choice.
21. Corning 3 μM trans-well individual inserts (Sigma Aldrich).
22. FACS tubes.
23. Counting beads for flow cytometry (BD Biosciences).
24. Flow cytometer.
25. Software such as FlowJo or equivalent for analysis of flow cytometry data.

3 Methods

We recommend K562 cell line as the 'donor' cell for trogocytosis-mediated transfer of receptors to NK cells. Initially, gene modify K562 cells to express surface receptors (or ligands) of interest and create a stable cell line. Here, we describe the protocol using NK cells expanded on K562 mbIL21, for uptake of membrane receptors from K562 cell line gene modified to express the desired receptors—acceptor cell. Primary NK cells or NK cells activated with cytokines can be used as acceptor cells using this protocol.

3.1 Trogocytosis Co-culture

3.1.1 Preparing 'Acceptor' NK Cells

1. Thaw expanded NK cells and place in culture in NK cell medium at a cell density of 1×10^6–3×10^6 cells/ml, overnight to allow the cells to recover from thaw before performing the co-culture with donor cell line (*see* **Note 1**).
2. Next day, mix the NK cells and perform a cell count and test viability using trypan blue exclusion using a hemocytometer.
3. If the viability of NK cells is above 85%, proceed with the trogocytosis co-culture experiment.

4. If the viability of NK cells is below 85%, it is advisable to perform a Ficoll-Paque density centrifugation to eliminate dead cells (*see* **Note 2**).

3.1.2 Preparing 'Donor' Cells

For trogocytosis experiments, the donor cell line can be used fresh from culture (live cells) or irradiated (to prevent proliferation). For animal models of adoptive immunotherapy of NK cells modified by trogocytosis, it is advisable to irradiate the donor cell line, to prevent tumor growth in vivo. Alternatively, we had previously shown that the donor cells could be freeze/thaw lysed in a controlled manner [25] to facilitate removal of the majority of donor cells from the co-culture through Ficoll-Paque density centrifugation.

1. Maintain the gene-modified K562 (K562-GM) cell line and parental K562 (K562-P) in RPMI complete medium. It is important to periodically test for the expression of desired protein on these cells (*see* **Note 3**).
2. If irradiated donor cells are desired, collect up to 100×10^6 K562-GM cells in a 50 ml conical tube and irradiate to 100 Gy (10,000 rads) using a Gammacell irradiator (*see* **Note 4**) and freeze the irradiated K562 donor cells. In parallel, irradiate K562-P to serve as control.
3. Thaw a vial of donor cells the day of performing trogocytosis co-culture.
4. Wash three times with PBS, spinning each time at $400 \times g$ for 5 min and resuspend in NK cell medium.
5. If freeze/thaw lysed donor K562-GM cells are desired, then resuspend the irradiated cells (*see* **Note 5**) at 5×10^6 cells per ml of RPMI complete medium and freeze the medium by immersing the tube in liquid nitrogen (or dry ice-ethanol bath) for 2 min, immediately transfer the tube to 37 °C water bath, and keep in water bath until the contents completely thaw. In parallel, freeze/thaw lyse K562-P cell line to serve as a control.
6. Mix the cells gently and check for viability using trypan blue exclusion method. The goal is to have trypan blue positive cells that are structurally intact (round, and not disintegrated) (Fig. 1). If less than 5–10% cells are viable, then stop at this stage (*see* **Note 6**). If more cells are viable, then repeat the freeze/thaw process one more time, followed by viability check.
7. If the desirable extent of cell membrane damage is achieved, then wash the cells two times with fresh NK cell medium, spinning each time at $100 \times g$ for 10 min. Resuspend the pellet in 10 ml of NK cell medium.
8. Count the cells using trypan blue method (include the trypan blue positive and negative cells in the counts) in order to set up co-culture with NK cells.

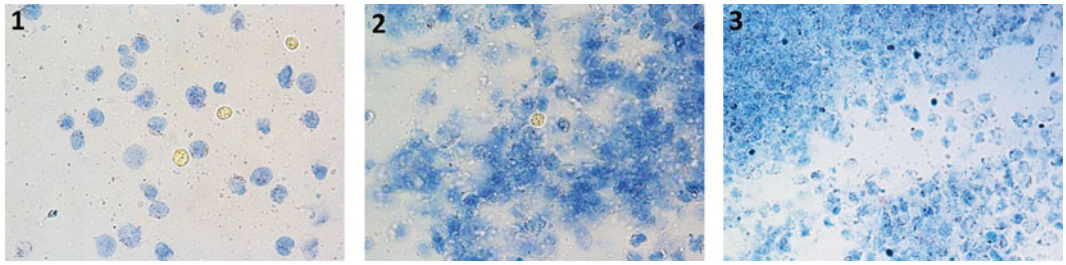

Fig. 1 Bright field image of freeze/thaw-treated K562 cells. The K562 cells were imaged after freeze/thaw treatment, the image shows impact of single cycle of freeze/thaw on K562 (*1*); here the cells appear structurally intact but are rendered trypan blue positive, also note the presence of few viable cells. Subsequent freeze/thaw cycles (*2* and *3*) cause excessive damage to cells leading to aggregation and are undesirable for co-culture. Reproduced from *Blood*, "Somanchi, S. S., Somanchi, A., Cooper, L. J., Dean A. Lee (2012) Engineering lymph node homing of ex vivo-expanded human natural killer cells via trogocytosis of the chemokine receptor CCR7. *Blood* **119**, 5164–5172"

3.1.3 Trogocytosis Co-culture Setup

1. Seed NK cells and K562-GM donor cells at a 1:1 E:T ratio (5×10^5 cells each) in a 24 well plate in a total volume of 2 ml NK cell medium per well. For a large batch of trogocytosis, seed the cells in a T75 flask, in a maximum of 20 ml NK cell medium. In parallel, set up co-culture with K562-P as control (*see* **Note 7**).
2. Spin the plate or flask (in flask holder adapter) at $100 \times g$ for 10 min without brake, to initiate cell-cell contact.
3. Incubate in a 5 % CO_2 incubator (at 37 °C) for 1 h.
4. After incubation, mix the contents to break up cell aggregates.
5. If freeze/thaw lysed cells are used as donor cells (K562-GM and K562-P), then load the cells on Ficoll-Paque and centrifuge as previously described (*see* **Note 2)**.

3.1.4 Acid Wash

It is important to perform an acid wash following trogocytosis, to dissociate NK-donor cell debris (receptors-ligand) interaction, in order to accurately measure the membrane uptake by NK cells, through trogocytosis.

1. Resuspend the NK cells recovered from Ficoll-Paque interface (NK+K562-GM; NK+K562-P) or NK cells co-cultured with irradiated donor cells, at a density of 5×10^6 cells/ml in acid wash buffer for 4 min at 20 °C, as previously described [20] in a 15–50 ml conical tube.
2. To stop the acid wash treatment, fill the conical tube to maximum volume with Stop buffer.
3. Spin the cells at $400 \times g$ for 5 min.
4. Wash the cells three more times using 10 ml of NK cell medium, spinning each time at $400 \times g$ for 5 min. Before the

last wash, split the cells in two aliquots, one (smaller aliquot) for flow cytometry and the second (larger aliquot) for the intended application—e.g., in vitro migration assay, in vivo adoptive transfer, etc.

3.2 Flow Cytometry

3.2.1 Data Acquisition

1. To analyze receptor acquisition by NK cells through trogocytosis, resuspend the acid-washed cell pellets (NK+K562-GM and NK+K562-P) in blocking buffer at a density of 5×10^5 cells/50 μl and incubate at 4 °C for 15 min. Split the NK+K562-GM co-cultured cells into two groups (5×10^5 cells each).
2. To group one, do not add any antibodies (unstained group).
3. To group two, add the staining antibody cocktail to the cells—CD16 (5 μl), CD56 (5 μl), or NKG2D (5 μl) plus antibody against receptor of choice (5 μl) (*see* **Notes 8** and **9**).
4. To NK+K562-P group, add the antibody cocktail as above. This group will serve as negative stain control for the acquired receptor.
5. Incubate at 4 °C in the dark for 30 min.
6. Wash the cells three times in PBS, spinning each time at $400 \times g$ for 5 min.
7. Fix the cells in 1 % formaldehyde.
8. Analyze the sample by flow cytometry and perform the data analysis on FlowJo or any flow cytometry software of preference.

3.2.2 Gating Scheme for Analysis

1. In FlowJo, or any other preferred software for analyzing the flow cytometry data, open the data for unstained control in a forward scatter (FSC) vs side scatter (SSC) plot.
2. Gate on the lymphocyte population, note that a larger granular cell population will be present representing K562 donor cells in co-cultures using irradiated donor cells. This population will be absent or significantly reduced if freeze/thaw lysed K562 donor cells are used for the co-culture (Fig. 2).
3. Select the gated lymphocytes and plot as CD16/CD56 (in this example, PE-Cy5) vs SSC-H. Then select the CD16/CD56 positive cell population and plot on CD16/CD56 vs receptor stain (in this example, CCR7 FITC) as shown in Fig. 3a, **and** draw the cutoff gate for the fluorescence channel pertaining to the acquired receptor (in this example, CCR7) based on the NK+K562-P control group.

This plot will show the percentage of NK cells that have acquired the receptor from donor cell line, and the overall fluorescence intensity of the acquired receptor on NK cells (amount of receptor acquired). The percentage of NK cells that are positive for the acquired receptor will be lower if freeze/thaw lysed donor cells are used, e.g., Figure 3b. This can be overcome by increasing the ratio of freeze/thaw lysed donor cells to NK cells in the co-culture.

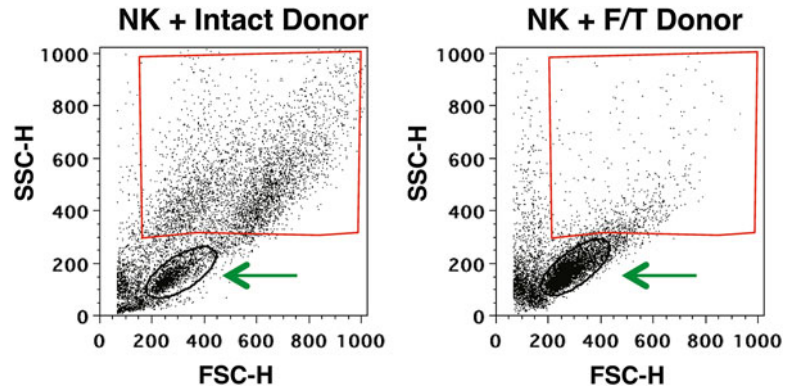

Fig. 2 Comparison of Forward and Side Scatter of NK cell co-culture with K562 'donor' cells. Flow plots show NK cells incubated with intact K562 cells (*left*) and NK cells co-cultured with freeze/thaw treated K562 after Ficoll-Paque separation (*right*). NK cells can be separated from the freeze/thaw treated K562 cells following Ficoll-Paque centrifugation (*red box*). Reproduced from *Blood*, "Somanchi, S. S., Somanchi, A., Cooper, L. J., Dean A. Lee (2012) Engineering lymph node homing of ex vivo-expanded human natural killer cells via trogocytosis of the chemokine receptor CCR7. *Blood* **119**, 5164–5172"

3.3 Assessing Function and Persistence of Acquired Receptors

In order to successfully apply the NK cells modified to express novel receptors through the process of trogocytosis, it is essential to understand if the acquired receptors are functionally incorporated in NK cells and the duration of persistence of these acquired receptors. We had shown previously that multiple receptors can be acquired simultaneously by NK cells from the donor cell, due to transfer of membrane patches from donor cell to the acceptor cell during the process of trogocytosis, and that different receptors/molecules persist for varying duration on the surface of NK cells [25]. Functional analysis of acquired receptors can be done either by assessing the intracellular signaling [20] or by assessing the desired functional outcome such as migration or enhanced cytotoxicity [25, 26]. Here we describe methods for assessing NK cell migration and persistence of acquired receptors.

3.3.1 In Vitro Migration Assay

Many methods are available to assess in vitro migration either using trans-well migration assay or real-time imaging of migration using micro-wells or flow-based micro-fabrications. Here we are describing the standard trans-well migration assay.

1. Stain NK cells with a fluorescent marker of choice, such as CFSE or Calcein AM (according to manufacturer's protocol), in order to distinguish from any contaminating K562-P/K562-GM at a later stage (while determining migration).
2. Set up the trogocytosis co-culture and prepare the NK cells for the migration assay as described in Subheadings 3.1.3 and

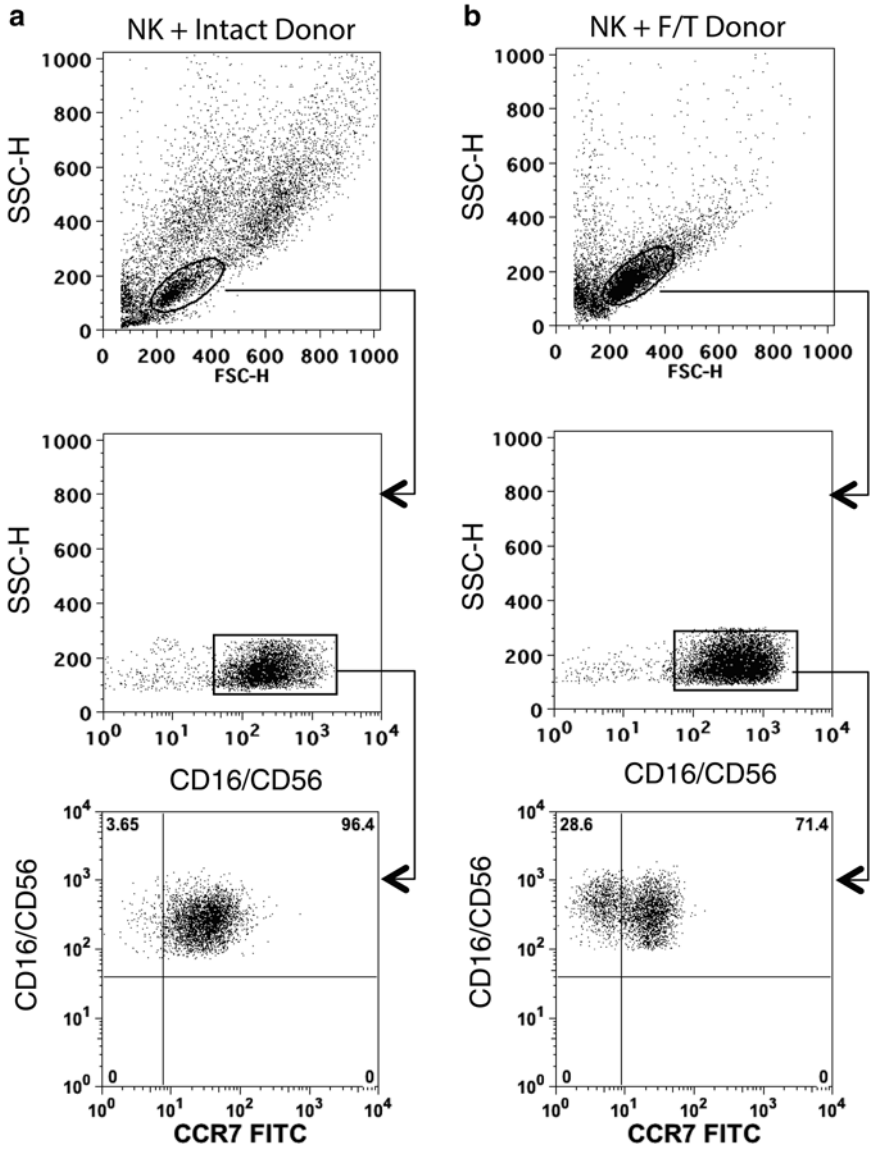

Fig. 3 Gating strategy to identify trogocytosis positive NK cells. The figure shows the gating strategy to positively identify NK cells and to assess the receptor acquisition by NK cells through trogocytosis. Note that here NK cells were stained with CD16 and CD56 and conjugated to the same fluorochrome to brightly stain NK cells to discriminate for autofluorescence of any contaminating K562. Reproduced from *Blood*, "Somanchi, S. S., Somanchi, A., Cooper, L. J., Dean A. Lee (2012) Engineering lymph node homing of ex vivo-expanded human natural killer cells via trogocytosis of the chemokine receptor CCR7. *Blood* **119**, 5164–5172"

3.1.4, and resuspend at a cell density of 1×10^6/ml using serum-free RPMI medium.

3. Place a 24 well plate in biosafety cabinet and add 1 ml of serum-free RPMI medium per well (lower chamber) with or without desired chemoattractant (*see* **Note 10**). Determine the

number of wells for the assay based on the number of conditions that are being tested. For recommended assay conditions, *see* **Note 11**. It is advisable to assay each condition in triplicates.

4. Place 3 μm trans-well inserts in each well and add 500 μl of NK cells per insert (5×10^5 cells per insert) (upper chamber) according to the conditions determined for the assay (e.g. *see* **Note 11**).
5. Place the lid on the 24 well plate and incubate at 37 °C in a CO_2 incubator for 6 h (longer incubation can be done up to 24 h depending on the assay needs).
6. Return the plate to biosafety cabinet and carefully remove the inserts.
7. Gently mix the contents of the lower chamber and recover into 5 ml FACS tubes to determine the cell counts (migration).
8. Add counting beads to the medium and run the sample on a Flow cytometer.
9. Set up data acquisition to count specific number of beads in all samples (e.g., 10,000 beads).
10. Analyze the data as number of NK cells per 10,000 beads in each sample to get comparative counts of NK cells present in each condition.
11. Plot the results as number of NK cells that have migrated in each condition normalized to the control (the most appropriate negative control for this comparison would be NK cells co-cultured with K562-P—Condition 5, per **Note 11**), or as percentage increase in migration of the test group compared to the control, in the presence of chemoattractant.

3.3.2 Persistence of Acquired Receptors

1. Set up the trogocytosis co-culture and prepare the NK cells as described in subheadings 3.1.3 and 3.1.4.
2. Take an aliquot of cells (time 0 h), wash twice in PBS, fix in 1% formaldehyde for 15 min at room temperature, and perform staining for analysis of receptor acquisition by flow cytometry as described in subheading 3.2.1 (**steps 2**–7). Store at 4 °C.
3. Return the remaining NK cells to culture in NK cell medium.
4. Take an aliquot of cells at various time points (*see* **Note 12**), wash two times in PBS, fix in 1% formaldehyde (*see* **Note 13**), then stain cells as before (if multiple time points are collected in a day, then store the stained cells at 4 °C until all the samples for the day are collected), and analyze the sample by flow cytometry (Fig. 4).

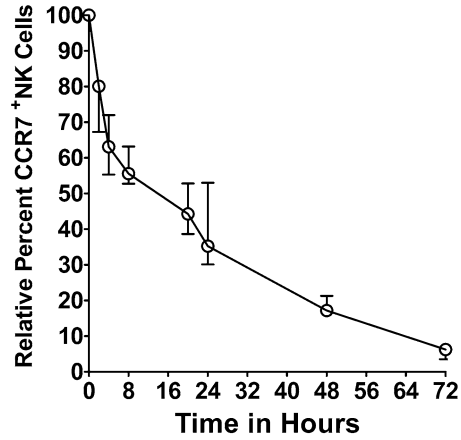

Fig. 4 Persistence of receptor acquired through trogocytosis. The acquired receptors are lost from NK cell surface over time. Here the median (± range) kinetics of persistence of acquired CCR7 is shown from three independent donor-derived NK cells. Reproduced from *Blood*, "Somanchi, S. S., Somanchi, A., Cooper, L. J., Dean A. Lee (2012) Engineering lymph node homing of ex vivo-expanded human natural killer cells via trogocytosis of the chemokine receptor CCR7. *Blood* **119**, 5164–5172"

4 Notes

1. For a large batch of NK cells, scale up the flask size accordingly. For primary NK cells, isolate NK cells by negative selection and either use it immediately for trogocytosis experiments or keep in culture overnight with minimal IL2 support (20–50 IU/ml) to help recover from the isolation process and to provide some activation. Freeze/thaw process will cause a loss of viability of primary NK cells and could diminish trogocytosis-mediated uptake of target cell membrane, hence should be avoided for primary NK cells.
2. Collect the NK cells and pass through 70 μm cell strainer to remove large aggregates of dead cells and carefully layer on Ficoll-Paque and spin at 400 × *g* for 20 min without brake. Collect the live NK cells from the interface and transfer to a fresh 15 ml conical tube and wash 3 times with PBS (10 ml). Resuspend the NK cells in NK cell medium and count the cell yield and viability as before.
3. Make a large cell bank of the K562 donor cell line and do not keep the cells in culture for long periods of time (more than 6 months), and periodically test the cell line for mycoplasma contamination.
4. Formal training and authorization to use the irradiator is mandated in most institutions, hence the detailed protocol for irra-

diation of donor cells is not provided here. Large stock of cells can be irradiated and frozen in aliquots.

5. We recommend use of irradiated K562 donor cells for freeze/thaw lysis for in vivo adoptive transfer applications. Since the freeze/thaw is done in a manner not to disintegrate all cells, the process retains 5–10% of viable cells, and in an in vivo application this viable cell contamination may lead to unintended tumor development.
6. Excessive freeze/thaw will disintegrate the cells and the generated membrane debris does not support trogocytosis-mediated uptake of membrane patches by NK cells.
7. We have previously observed a lower uptake of membrane patches by NK cells when we used freeze/thaw lysed donor cells. This could be either because of poor trogocytosis by NK cells from dead donor cells or because of disparity in E:T ratio created due to improper settlement of dead cells (floating) to the surface of the plate compared to live cells. Therefore it is advisable to use a higher ratio of donor cells to NK cells.
8. We had previously used CD16 and CD56 antibodies conjugated to same fluorochrome—PE-Cy5 to strongly label NK cells in order to distinguish them from the background autofluorescence of contaminating K562 cells, and used anti-CCR7 antibody conjugated to FITC [25].
9. Alternatively, pre-stain NK cells with fluorescent dyes (membrane or cytoplasmic dyes) to distinguish from K562. To assess membrane uptake by NK cells, K562 cells can be pre-stained with membrane dyes (with fluorescence compatibility with the dye used to stain NK cells). However, to specifically assess for the acquisition of the receptor of choice by the NK cells, it is advisable to stain with antibody against the receptor rather than staining the donor cell line with a membrane dye.
10. Since serum has chemoattractants, using serum-free medium in the migration assay will significantly reduce the background or nonspecific migration in the control group. Determine the concentration of the chemoattractant used in the assay either based on literature or by titration. We have previously used 300 ng/ml of CCL19/CCL21 in trans-well migration of NK cells.
11. With no chemoattractant in the lower chamber—Condition 1: NK cells without trogocytosis co-culture; Condition 2: NK cells co-cultured with K562-P; and Condition 3: NK cells co-cultured with K562-GM. (These conditions provide background/nonspecific migration of NK cells in the absence of any chemoattractant.)

    With appropriate chemoattractant in the lower chamber—Condition 4: NK cells without trogocytosis co-culture; Condition 5: NK cells co-cultured with K562-P; and Condition

6: NK cells co-cultured with K562-GM. (The conditions 4 and 5 provide background migration of NK cells in the presence of any chemoattractant and condition 6 provides specific migration in response to the chemoattractant.)

12. We have previously analyzed receptor persistence up to 72 h. Evaluate the duration of persistence until the NK cells completely lose all the acquired receptors from their surface.
13. Perform a trial staining using fixed K562-GM to ensure that fixation does not interfere with staining for the receptors. If the fixation interferes with staining, then skip the fixation step prior to staining.

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Chapter 22

Electroporation of siRNA to Silence Gene Expression in Primary NK Cells

Prasad V. Phatarpekar, Dean A. Lee, and Srinivas S. Somanchi

Abstract

Gene silencing through siRNA is an effective experimental tool to unravel molecular mechanisms involved in cellular processes. Here we describe a method to silence gene expression in primary human natural killer (NK) cells by transfecting ON-TARGETplus SMART pool siRNA using an electroporation-based method called Nucleofection®. The technique yields effective silencing of the target gene without any off-target effects.

Key words Human primary NK cell, siRNA, Nucleofection, Electroporation, ON-TARGET*plus*, SMART pool siRNA, Gene silencing

1 Introduction

Natural killer cells play a crucial role in the innate immune response against viral infections and tumors. Although these lymphocytes were identified in early 1970s [1, 2], we have gained significant insights into their complexity and regulation over the last decade. NK cells express a gamut of activating and inhibitory receptors that enable them to distinguish healthy cells from stressed, infected, or transformed cells. These germ line-encoded receptors allow NK cells to mount a rapid effector immune response without the need for prior antigen sensitization [2, 3]. In addition to killing the target cells, NK cells secrete the cytokines IFN-γ and TNF-α, which help promote innate and adaptive immune responses [4]. NK cells play a critical role in immune response, particularly against tumors, thus they have been the focus of considerable investigation to understand the complex signaling that governs their effector function.

Gene knockdown is a powerful tool to study the role of individual proteins in cellular function. The two standard approaches for gene knockdown are stable transduction with short hairpin RNA (shRNA) and transient transfection using small interfering

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RNAs (siRNA). Numerous genes involved in NK cell function have been examined by gene knockdown experiments using shRNA technology [5–8], including STAT3 [9] and PRDMI [10], in the context of NK cell malignancies. Most of these studies were conducted in NK cell lines to facilitate genetic manipulation because the primary human NK cells are relatively difficult to stably gene modify. Therefore siRNA, being small (double stranded RNA) molecule of about 20–25 base pairs in length, has been effectively used to transiently knockdown gene expression in primary NK cells as well as NK cell lines [11–18]. siRNA suppresses gene expression at the posttranscriptional level either by inducing degradation of mRNA or by blocking its translation into protein. Once inside the cell, siRNA associates with RNA-induced silencing complex (RISC), which with the help of the anti-sense strand targets the desired mRNA. Upon binding of the anti-sense strand to the complementary mRNA, RISC silences its expression either by cleaving the mRNA or by blocking its translation [19]. The effect of siRNA is transient owing to the limited number of molecules introduced into the cell during transfection (or electroporation), dilution of siRNA during cell division, and degradation by nucleases [20, 21].

In this chapter, we describe a method for siRNA-mediated knockdown of gene expression, using STAT3 as a model, in primary NK cells using ON-TARGET*plus* SMART pool siRNA. The ON-TARGET*plus* is a patented technique developed by Dharmacon (now GE Dharmacon) to chemically modify both sense and anti-sense strands of siRNA, thereby significantly reducing off-target effects [22]. A smart pool is a combination of four different ON-TARGET*plus* siRNAs that target different regions of the same mRNA to ensure effective silencing of the intended target [23]. The ON-TARGET*plus* siRNAs are introduced into the primary NK cells using electroporation. In our laboratory, this procedure yielded silencing of the expression of target gene in primary human NK cells without any off-target effect.

2 Materials

2.1 Isolation of NK Cells

1. Buffy coat (Regional Blood Bank).
2. 250 ml sterile bottle.
3. 50 ml and 15 ml conical tubes.
4. RPMI complete medium: RPMI 1640 supplemented with 10% FBS, 1× Pen/Strep, 1× GlutaMAX.
5. NK cell medium: RPMI 1640, 10% FBS, 1× Pen/Strep, 1× GlutaMAX and freshly supplemented with 50 IU/ml of IL2 (Proleukin, Novartis Vaccines and Diagnostics Inc) each time just prior to use.

6. Ficoll-Paque Plus (GE Healthcare Life Sciences).
7. PBS wash buffer: Freshly prepare 2% fetal bovine serum (FBS) in PBS.
8. RosetteSep™ Human NK cell Enrichment Cocktail (Stem Cell Technologies).
9. Red blood cell lysis buffer (Sigma Aldrich).

2.2 siRNA Electroporation

1. SMARTpool ON-TARGET*plus* STAT3 siRNA (GE Dharmacon).
2. ON-TARGET*plus* Non-targeting Control Pool (negative control) (GE Dharmacon).
3. 5× siRNA buffer (GE Dharmacon).
4. siRNA stock solution: Dilute the 5× siRNA buffer to 1× using RNAse-free water; spin the tube containing siRNA in benchtop centrifuge at maximum speed for 30 s; prepare siRNA stock solution of convenient concentration (100× stock based on the highest concentration to be used, if possible) by adding 1× siRNA buffer to the siRNA tube; and gently pipette the buffer up and down three to five times without creating any bubbles. Place the tube on a shaker for 30 min at room temperature to dissolve the siRNA in buffer. Spin in a benchtop centrifuge for 30 s. Aliquot the siRNA in appropriate volumes in sterile eppendorf tubes. Store the aliquots at –20 °C in a manual defrost or non-cycling freezer or alternatively in a –80 °C freezer (*see* **Note 1**).
5. Molecular grade RNase-free water.
6. Sterile eppendorf tubes.
7. Serum free medium: Prepare RPMI 1640 supplemented with 1× GlutaMAX.
8. 2× Medium: RPMI 1640 supplemented with 20% FBS, 2× Pen/Strep, and 1× GlutaMAX.
9. 6-well cell culture plate.
10. Humidified 37 °C/5% CO_2 incubator.
11. Amaxa Nucleofector™ Device (Lonza).
12. Nucleofector™ Kit for Human Natural Killer Cells (store at 4 °C) (Lonza).

2.3 RT-PCR

1. RNeasy plus mini kit (Qiagen).
2. OneStep RT-PCR kit (Qiagen).
3. PCR Thermal cycler.
4. PCR tubes.
5. Agarose.

6. GelRed (10,000×).
7. Electrophoresis power supply.
8. Gel tray.
9. Gel combs.
10. 100 bp DNA ladder.
11. Digital Gel Documentation System.

3 Methods

Depending on the aim of the study, either primary or activated/expanded NK cells can be used for gene silencing using siRNA (*see* **Note 2**). In this method, we are describing siRNA-mediated gene silencing of STAT3 in primary NK cells.

3.1 Isolation of Primary NK Cells

This protocol describes isolation of primary NK cells from peripheral blood PBMCs, using RosetteSep™ method. This is a negative selection method, where non-NK cells in the PBMCs are tethered to RBCs and cleared from NK cells via Ficoll-Paque centrifugation, through bi-specific Tetrameric antibody complexes (TAC). Please note that several other methods are available for negative selection of NK cells from different sources; the protocol described here is based on routine practice in our laboratory.

1. Transfer buffy coat sample to a 250 ml sterile bottle and add PBS to dilute the blood sample to 140 ml (*see* **Note 3**).
2. Add 15 ml of Ficoll-Paque to four 50 ml conical tubes.
3. Carefully layer 35 ml of diluted buffy coat sample on top of the Ficoll-Paque layer in each tube.
4. Gently, transfer the tubes to a centrifuge and spin cells at 400 × *g* for 20 min at room temperature, without brakes.
5. Discard the top 20–25 ml of plasma by aspiration.
6. Collect the PBMCs layer at the interface of Ficoll-Paque and plasma and transfer to a fresh 50 ml conical tube. Do not discard the tubes containing RBCs (*see* **Note 4**).
7. Add PBS to 50 ml mark and spin the tube at 400 × *g* for 10 min.
8. Discard the supernatant and repeat the wash step two more times.
9. Resuspend the PBMC cell pellet in 50 ml of RPMI complete medium and count the cell number and viability on hemocytometer using trypan blue.
10. Recover about 5×10^6 PBMCs for phenotype analysis. Use the remaining PBMCs to recover NK cells by RosetteSep™ method.
11. To isolate NK cells by RosetteSep™ method, combine PBMCs with RBCs at a 1:100 ratio (100-fold excess RBCs) in a 50 ml

conical tube. Spin the tube at 400 × *g* for 5 min and discard the supernatant.

12. Resuspend the PBMC:RBC cell mixture in PBS wash buffer at a cell density of 50×10^6 PBMCs per 1 ml.
13. For every 1×10^6 PBMCs in the mix, add 1 μl RosetteSep™ Human NK cell Enrichment Cocktail.
14. Mix well by pipetting up and down and incubate at room temperature for 20 min.
15. Mix contents to evenly resuspend cells every 5 min.
16. After incubation, add equal volume of PBS wash buffer and mix.
17. Layer the cells on to 15 ml of Ficoll-Paque in a 50 ml conical tube and spin at 400 × *g* for 20 min without brakes.
18. Aspirate the top 20–25 ml of the supernatant and carefully transfer the NK cell layer from the Ficoll-Paque interface to a 15 ml conical tube.
19. To wash, add 10 ml of PBS wash buffer to the NK cells, mix well, and spin at 400 × *g* for 5 min.
20. Aspirate the supernatant and repeat the wash step two more times.
21. Optional: If RBCs contaminate the NK cell preparation, perform RBC lysis. RBC lysis: Resuspend the cells in 10 ml of RBC lysis buffer and incubate on ice for 10 min, wash three times with PBS as before.
22. Resuspend the NK cell pellet in 10 ml of NK cell medium and assess cell counts and viability by trypan blue exclusion.
23. Set aside 1×10^6 NK cells to assess purity by flow cytometry (*see* **Note 5**).
24. Seed the remaining NK cells in T75 flask and add more NK cell medium to adjust the cell density to 0.5×10^6 cells/ml.
25. Incubate the NK cells in 5% CO_2 incubator at 37 °C overnight, before electroporation with siRNA.

3.2 Electroporation of NK Cells with siRNA

This protocol describes knockdown of STAT3 mRNA expression by nucleofecting STAT3 ON-TARGET*plus* SMART pool siRNA (GE Dharmacon) in primary human NK cells (*see* **Note 2**). The cells were nucleofected using Amaxa Nucleofector™ 2b device, which handles one sample per run. The consumables specific for the device, cuvettes and plastic pipettes, are included in the Amaxa® Human NK Cell Nucleofector® Kit.

1. Warm serum-free medium and 2× medium to 37 °C (*see* **Note 6**).
2. Add 1.9 ml of serum-free media per well (per condition of electroporation) in a 6 well plate and place it in the humidified 37 °C/5% CO_2 incubator. This pre-warmed media is to be used later to maintain Nucleofected NK cells (*see* **Note 7**).

3. Count viable NK cells using trypan blue exclusion method and recover 4 million viable NK cells per electroporation condition in a conical tube and spin at 100×*g* for 10 min (*see* **Notes 8** and **9**).
4. Discard the supernatant by aspiration, resuspend the cell pellet in pre-warmed serum-free medium, and centrifuge the cells at 100×*g* for 10 min (*see* **Note 10**).
5. While the cells are being centrifuged, bring the NK Cell Nucleofector® Solution and the supplement provided with the NK Cell Nucleofector® Kit to room temperature. Add the supplement to the Nucleofector® Solution. Also remove the siRNA aliquot from −20 °C and place it on ice to thaw.
6. After centrifugation, discard the supernatant completely without disturbing the NK pellet. Resuspend the pellet in 99 μl of working Nucleofector® Solution (*see* **Note 11**). If 100× working stock of the siRNA could not be prepared, then resuspend the NK cell pellet in appropriate volume of Nucleofector® Solution to attain a final volume of 100 μl (per condition of electroporation) after the addition of siRNA in the next step (*see* **Note 12**).
7. Combine NK cells resuspended in the working Nucleofector® Solution with siRNA (1 μl of 100× stock), mix gently (avoid bubbles), and transfer to electroporation cuvette. Transfer the reaction volume gently along the side of the cuvette to avoid bubbles and cap the cuvette.
8. Place the cuvette in the Nucleofector device, turn on the program X-01, and pulse the cells.
9. Gently transfer NK cells from the cuvette to the pre-warmed serum-free medium (*see* **Note 13**) in the 6 well plate (from **step 2**) using the plastic aspirator provided in the kit. Rinse the cuvette thoroughly but gently with the media from the same well to retrieve any remaining cells and transfer back in the well.
10. Incubate the cells in humidified 37 °C/5 % CO_2 incubator for 2 h.
11. After incubation, add 2 ml of 2× medium.
12. Also add 200 IU of IL2 to each well (for a final concentration of 50 IU of IL2/ml). Incubate in humidified 37 °C/5 % CO_2 incubator.
13. After 48 h, assess gene silencing by reverse transcription PCR (RT-PCR) (*see* **Note 14**).

3.3 Assessment of Gene Silencing

We assessed mRNA expression in primary NK cells electroporated with control and STAT3 siRNA by RT-PCR. RNA was isolated using RNeasy plus mini kit (Qiagen) as per manufacturer's protocol (*see* **Note 15**).

1. Measure RNA concentration using NanoDrop.
2. Prepare stock solutions with equal concentration of RNA for all the experimental treatments and use either immediately to set up RT-PCR or aliquot and store at −80 °C for later use.
3. Set up RT-PCRs for all the experimental treatments with gene-specific primers (Table 1) with equal amount of RNA (50–100 ng) using OneStep RT-PCR kit (Qiagen) as per manufacturer's instructions (*see* **Note 16**).
4. Prepare 1.2% agarose gel containing 1× GelRed.
5. Out of 50 μl RT-PCR reaction, use about 10–15 μl (same quantity for all the experimental treatments) for agarose gel electrophoresis.
6. Image the agarose gel using a Digital Gel Documentation System (Fig. 1) (*see* **Note 17**).

Table 1
List of RT-PCR primers

Gene	Primer sequence (5′ - 3′)	
STAT3	Forward	GGAGCTGGCTGACTGGAAGAG
	Reverse	CTCGATGCTCAGTCCTCGCTTG
STAT1	Forward	TCAGGCTCAGTCGGGGAATA
	Reverse	ATCACTTTTGTGTGCGTGCC
GAPDH	Forward	GGAGAAGGCTGGGGCTCATTTG
	Reverse	CTTCTGGGTGGCAGTGATGGC

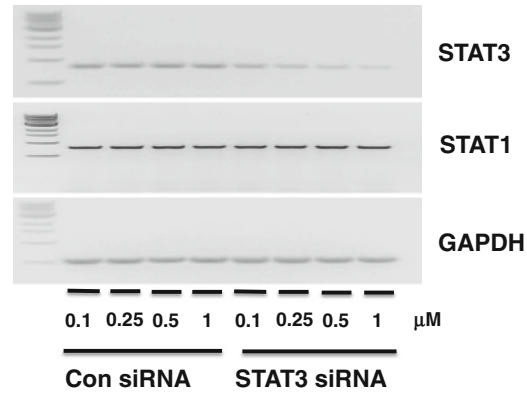

Fig. 1 Representative agarose gel image of siRNA-mediated silencing of STAT3. STAT3 ON-TARGET*plus* SMART pool siRNA electroporated in primary human NK cells using Amaxa Nucleofector™ mediated incremental decrease in STAT3 mRNA expression with increasing concentration of siRNA. There is no off-target effect as demonstrated using STAT1 as a control; GAPDH serves as the housekeeping gene

4 Notes

1. Open the 5× siRNA buffer and RNase-free water bottles and the siRNA tube in a cell culture hood. Preparation and addition of buffer to the tube, pipetting of the buffer, and aliquoting of siRNA should be carried out in the cell culture hood. Based on the experimental needs, prepare aliquots in as small a volume as possible, preferably in 0.5 ml eppendorf tubes, which thaw quickly on ice. Appropriately sized small aliquots are likely to be used up once thawed thereby preventing repeated freeze thaw cycles. Use of freshly thawed aliquot also ensures consistency in siRNA quality across experiments performed at different times.
2. We knocked down STAT3 expression in primary human NK cells to investigate the role of STAT3 in human NK cell functions. STAT3, a transcription factor, modulates expression of proteins through the transcriptional regulation of its target genes. Thus, STAT3 mediates its functions indirectly through other proteins. In order to study the role of STAT3 in cellular functions, its expression should be silenced before its transcriptional activity is stimulated. Due to this reason, we decided to use primary NK cells, in which STAT3 is more likely to be in a latent state, instead of activated or expanded NK cells with already activated STAT3. The functional properties of the protein of interest dictate the choice between primary and expanded or activated NK cells.
3. For buffy coat samples up to 70 ml volume, add PBS to dilute the sample to 140 ml. If the volume of buffy coat is more than 70 ml, then add equal volume of PBS to dilute the sample.
4. At this point, prepare the RBCs for NK cell purification by RosetteSep™ method, remove the Ficoll-Paque from the tubes and pool the RBCs into two 50 ml tubes, and then add PBS to 50 ml mark in each tube. Spin the RBCs at 400 × *g* for 10 min along with PBMCs (Subheading 3.1 **step 7**). Discard the supernatant. Note that the RBC pellet is fluid; so to minimize loss of RBCs, aspirate the supernatant carefully as you approach the RBC pellet. Repeat the wash steps two more times. Resuspend RBCs in each tube in 50 ml of PBS containing 2% FBS. Aliquot 10 μl of RBCs and dilute to 10 ml (10,000 fold dilution), and count the RBCs using a hemocytometer.
5. Stain the PBMCs (from Subheading 3.1, **step 10**) and NK cells with an antibody cocktail comprising CD3 (PeCy7), CD56 (FITC), CD16 (PE) and analyze by flow cytometry to determine the NK cell purity after RosetteSep™ separation.
6. These media could be made in bulk, aliquoted in appropriate volume, and stored at 4 °C.

7. The media is used to resuspend NK cells after the electroporation. The volume of the media is set to 1.9 ml so that after the addition of 100 μl reaction volume, the total volume becomes 2 ml. This is estimated for 4×10^6 cells per reaction and to achieve 2×10^6/ml cell density during the 2 h incubation period after electroporation (*see* **steps 10** and **11** in Subheading 3). The volume can be adjusted based on the number of cells used per electroporation. Also, for lower volumes 12 or 24 well plates can be used.
8. Usually for pelleting, NK cells are centrifuged at $400 \times g$ for 5 min. However, to minimize stress on the cells before undergoing Nucleofection, we centrifuged at $100 \times g$.
9. We have electroporated 1–4 million NK cells per electroporation in our laboratory.
10. Cells were washed with serum-free medium to wash off residual serum and antibiotics. RNases present in the serum degrade siRNA [24] and electroporation makes the cells sensitive to antibiotics, which may affect cell viability.
11. Try to minimize the time NK cells remain suspended in the working Nucleofector® Solution (do not exceed 20 min) as longer storage in this solution reduces cell viability and electroporation efficiency.
12. To attain siRNA concentration of 100 nM in 100 μl Nucleofection® reaction, 10 μl of 1 μM siRNA stock solution should be added to the cell suspension. Thus, the cells should be resuspended in 90 μl of the working Nucleofector® Solution so that after the addition of siRNA the final reaction volume becomes 100 μl.
13. Immediately after electroporation, the cells are incubated in serum and antibiotic-free media to prevent degradation of siRNA by RNases present in the serum and to avoid any adverse effect of antibiotics on the freshly electroporated cells, which are sensitive to antibiotics.
14. Silencing of mRNA expression could be assessed as early as 24 h or even earlier (12–16 h).
15. RNA isolated from 0.5×10^6 primary NK cells provides enough RNA to set up multiple RT-PCR reactions.
16. Along with reactions with STAT3 specific primers, reactions were also set up with primers specific for GAPDH (housekeeping gene) and STAT1, another member of the STAT protein family to which STAT3 belongs, to assess off-target effects.
17. Estimate the extent of knockdown using ImageJ software normalizing the expression in control group to 100%. Alternatively, perform real-time PCR to obtain quantitative assessment of the gene knockdown. To quantitate the impact

of siRNA-mediated knockdown on protein expression, assess the protein level either by western blot or flow cytometry depending on protein localization (cytosolic or cell surface protein, respectively).

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Chapter 23

Mouse Xenograft Model for Intraperitoneal Administration of NK Cell Immunotherapy for Ovarian Cancer

David L. Hermanson, Laura Bendzick, and Dan S. Kaufman

Abstract

Natural killer (NK) cells are an attractive cell population for immunotherapy. Adoptive transfer of NK cells has been tested in multiple clinical trials including acute myeloid leukemia (AML) and ovarian cancer, although limitations do exist especially for treatment of solid tumors. In order to overcome these limitations, mouse xenograft models are needed for evaluation of various NK cell populations, as well as routes of NK cell administration. Here, we describe the methods used for the establishment of an intraperitoneal (ip) ovarian cancer mouse xenograft model with ip delivery of NK cells. This model has been successfully employed with multiple ovarian cell lines and could be applied to other tumor models where the tumor's primary location is in the peritoneal cavity. It is also compatible with multiple routes of NK cell administration. Bioluminescent imaging for monitoring tumor formation and response provides for easy visualization of NK cell tumor inhibition. This xenograft model is superior to other models because the tumor is implanted into the same physiological space where ovarian cancer is found, which allows for improved mimicking of actual disease.

Key words Natural killer cells, Mouse xenograft, Immunotherapy, Bioluminescent imaging, Ovarian cancer

1 Introduction

Natural killer (NK) cells are cytotoxic lymphocytes known to play an important role in the control of viral infections and various malignancies [1]. The adoptive transfer of haploidentical NK cells isolated from peripheral blood (PB-NK) can mediate dramatic anti-tumor effects against hematological malignancies, especially in the case of acute myeloid leukemia (AML) [2]. Furthermore, intravenous (iv) delivery of PB-NK cells has also been evaluated for the treatment of ovarian cancer and other solid tumors [3]. Additionally, the NK cell line, NK-92, has been used in clinical trials for advanced renal cell carcinoma and AML [4, 5]. These studies support the use of adoptively transferred NK cells for cancer immunotherapy.

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DOI 10.1007/978-1-4939-3684-7_23,

Currently, NK cells used for immunotherapy are isolated from an apheresis product followed by CD3 and CD19 depletion for the removal of T cells and B cells, respectively [2]. This procedure results in a heterogeneous cell population that typically consists of only approximately 30% NK cells [6]. Recently, new techniques have been defined for the ex vivo expansion and activation of PB-NK cells [7, 8]. Furthermore, methods for the generation of NK cells from either human embryonic stem cells (hESCs) or induced pluripotent stem cells (iPSCs) have also been developed [9–12]. These methods allow for the production of a >97% pure NK cell population that have the potential to become a standardized, off-the-shelf NK cell-based immunotherapy product.

In order to compare the various NK cell sources and routes of administration, we have developed a mouse xenograft model using bioluminescent imaging to monitor tumor growth (Fig. 1) [13]. Given that ovarian cancer is restricted to the peritoneal cavity, we have used this model to study the intraperitoneal (ip) delivery of NK cells rather than iv dosing [13]. This chapter will describe the methods used for the establishment of this mouse model for the study of ovarian cancer, as well as methods used for monitoring NK cell persistence.

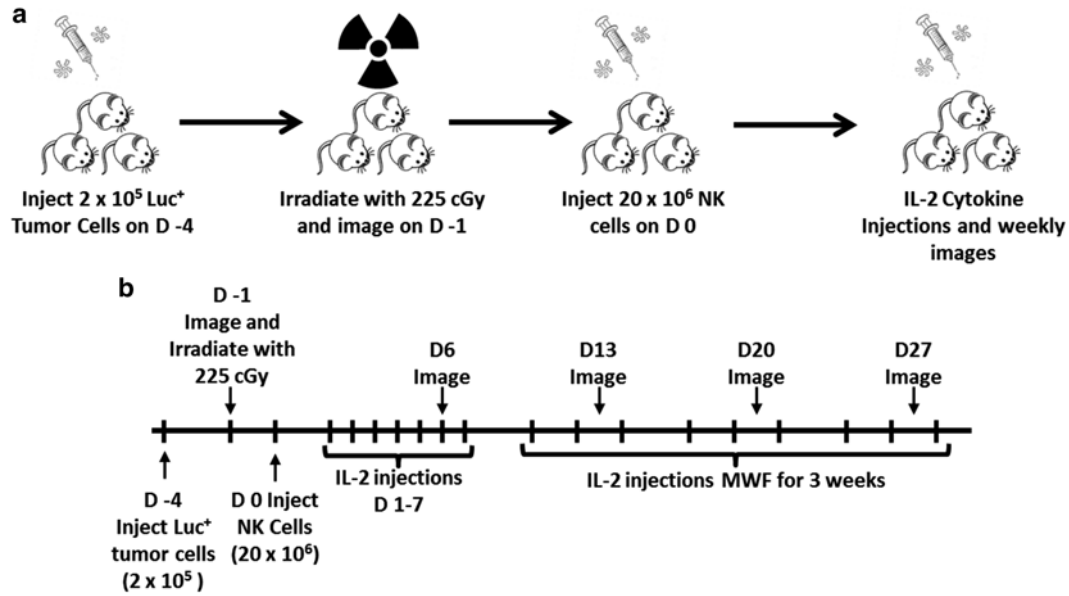

Fig. 1 Schematic diagram of the intraperitoneal xenograft mouse model. (**a**) Overview of the experimental outline including injection of luciferase (luc+) positive tumor cells, radiation of tumor-bearing mice with 225 cGy, injection of NK cells, and cytokine administration. (**b**) Timeline of injection schedule for cells and cytokines as well as imaging schedule

2 Materials

2.1 Cell Lines

1. MA148/GFP:Luc Cells. MA-148 cells were kindly provided by Sundaram Ramakrishnan (University of Minnesota). The insertion of the GFP:Luc construct has been previously reported [13]. These cells are maintained in RPMI-1640 plus L-glutamine, 10% FBS, and 1% Penicillin/Streptomycin.
2. PB-NK Cells. Cells were maintained in RPMI-1640, 10% FBS, 2 mM L-glutamine, 1% P/S, and 50 U/mL IL-2. The cells were stimulated with lethally irradiated membrane bound (mb) IL-21-expressing artificial antigen presenting cells (aAPCs) weekly, as has been previously reported [8, 9].

2.2 Mice

1. NOD.Cg-*Prkdcscid Il2rgtm1wjl*/SzJ mice, commonly known as NOD scid gamma-c or NSG mice, were obtained from The Jackson Laboratory. These mice lack mature T cells and B cells, have no functional NK cells, and have defective function of myeloid immune cells [14].

2.3 Injection of Tumor into Mice

1. 0.05% Trypsin-EDTA for harvesting tumor cells (Invitrogen).
2. Dulbecco's phosphate-buffered saline (DPBS) as tumor cell carrier (HyClone).
3. 1 cc U-100 Insulin Syringe 28G1/2.

2.4 Animal Imaging

1. D-Luciferin (Gold Biotechnology). Resuspend the 1 g of D-Luciferin in 40 mL DPBS and store at −80 °C in 0.5–1 mL aliquots. At this concentration, 100 μL is injected into each mouse.
2. IVIS Spectrum (Perkin-Elmer).
3. Living Image 4.0 for analysis of images (Perkin-Elmer).
4. X-Rad 320 Biological Irradiator (Precision X-ray).

2.5 Cytokines

1. Interleukin-2 (Proleukin (aldesleukin), Prometheus). Suspend 22 million units of IL-2 in 53 mL of DPBS. Store at −20 °C.

2.6 Analysis of NK Cell Survival

1. Heparin (1000 U/mL).
2. Ammonium Chloride (ACK) lysis buffer.
3. Blocking serum: DPBS supplemented with 5% human serum and 5% FBS and sterile filtered.
4. FACS buffer: 0.1% sodium azide and 2% FBS in DPBS.

3 Methods

3.1 Establishment of IP Tumor and Imaging

1. Passage tumor cells so that they are no more than 70–80% confluent on day of use. Typically we try and passage the cells 2 days prior to injection.

2. Four days prior to NK cell administration, harvest 2×10^5 luciferase-expressing MA148 cells per mouse (*see* **Note 1**) using 0.05 % trypsin for 5 min at 37 °C.
3. Centrifuge cells at $300 \times g$ for 5 min and resuspend in DPBS at a concentration of 1×10^6 cells/mL in a capped tube.
4. Inject 10–12-week-old female NSG mice (*see* **Note 2**) with 2×10^5 (*see* **Note 1**) luciferase-expressing tumor cells (200 μL). Injections should be given ip with a 1 cc U-100 insulin syringe 28G1/2 (*see* **Note 3**).
5. 72 h post-tumor injection (1 day prior to NK cell treatment). Image the mice using the IVIS Spectrum. Inject mice ip with 100 μL of D-Luciferin at a concentration of 25 mg/mL. Anesthetize the mice using Isoflurane (vaporizer setting at 2.5 % and a flow rate of 2.0 to anesthetize the mice and 0.5 L/min O_2 while imaging). Place mice in the IVIS spectrum imaging platform and acquire image with presets of Medium Binning, 30 s exposure, and F Stop of 1 (Fig. 2) (**Note 4**). Collect the data in Radiance. Acquire images from 3 to 5 min after administering D-Luciferin.
6. Analyze the data using Living Image by drawing an identical region of interest (roughly 3.6 cm wide and 4 cm tall) for each mouse in order to measure radiance (*see* **Note 5**).
7. Use the total flux (photons/s) to compare mice and assign into groups such that each group will have mice with similar total flux measurements (*see* **Note 6**).
8. Immediately following imaging, mice are irradiated using an X-Rad 320 Biological Irradiator set to 12.5 mA and 320KV to deliver 2.25 cGy (225 rads) per animal at a height of 50 cm (*see* **Note 7**).
9. Imaging is continued every 7 days to monitor tumor growth and effectiveness of the NK cell treatment.

3.2 Delivery of NK Cells and Cytokine Injections

1. On the day NK cells are to be given, count and determine the cell viability (*see* **Note 8**).

   Centrifuge ($300 \times g$ for 5 min) enough NK cells for a dose of 20×10^6 cells/mouse and wash once with DPBS. Centrifuge again and resuspend cells at 66.6×10^6 cells/mL in DPBS and place in a capped tube.
2. Inject 300 μL (20×10^6 cells) ip into each mouse using a 1 cc U-100 insulin syringe.

   28G1/2. Be sure to use a pipette to mix the cells immediately prior to loading each syringe (*see* **Note 3**).
3. The day following NK cell dosing, start the administration of cytokine injections (*see* **Note 9**). We give IL-2 at 5 μg/mouse/day (83,000 U/mouse/day) by injecting 200 μL of stock solution prepared as in the Subheading 2.

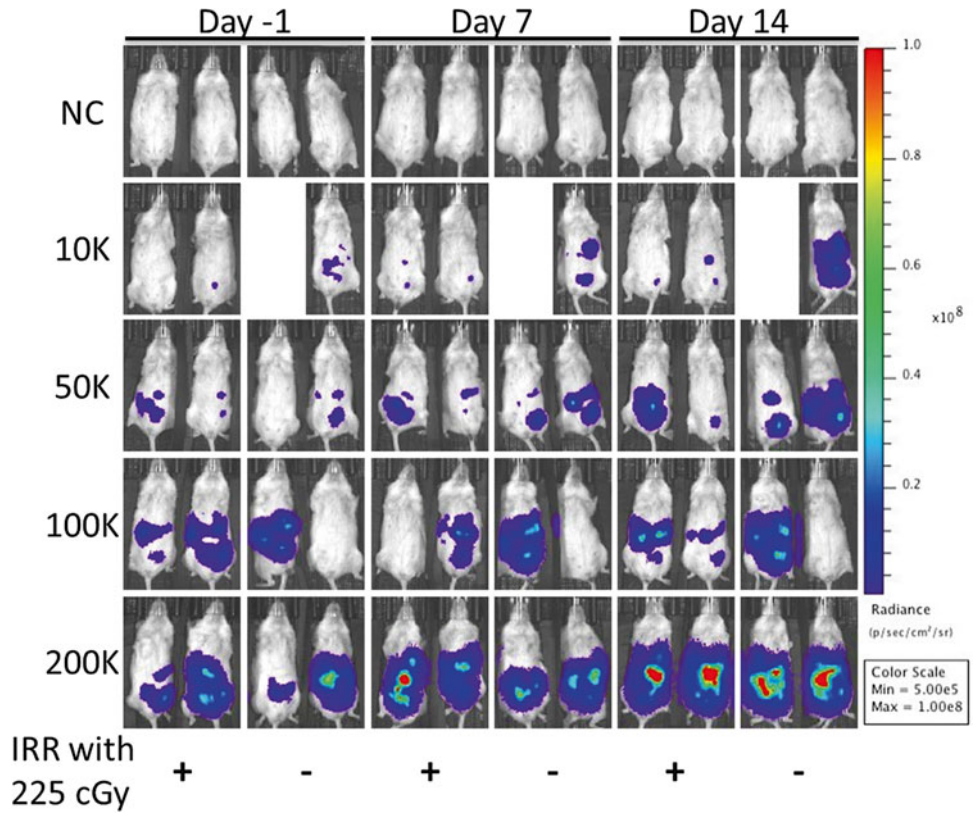

Fig. 2 Bioluminescence imaging of luciferase + tumor-bearing mice. Mice were injected ip with varying numbers of MA148/GFP:Luc$^+$ ovarian cancer cells on day 4. Mice were imaged starting on day 1 and weekly thereafter to monitor tumor formation and growth in order to determine the optimal number of cells for tumor formation. Two mice for each group received irradiation (IRR) (225 cGy) on day 1 while two mice were left untreated

4. Administer cytokine dosing as follows: every day for 7 days followed by three times a week (Monday, Wednesday, and Friday) for an additional 3 weeks (4 weeks total).

3.3 Monitoring NK Cell Engraftment and Survival in Blood

1. Draw blood from mice on both 7 and 21 days after giving them NK cells (*see* **Note 10**). Collect ~100 μL of blood through facial vein bleeding into 1.5 mL Eppendorf tubes containing 50 μL of heparin to prevent clotting.
2. Lyse red blood cells (RBCs) using ACK lysis buffer. Add 800 μL of ACK to the blood and incubate on ice for 5 min.
3. Centrifuge at 300 × *g* for 4 min and remove supernatant.
4. Repeat **steps 2** and **3** one more time.
5. Add 1 mL blocking serum and incubate on ice for 20 min.
6. Centrifuge at 300 × *g* for 4 min and remove supernatant.

7. Wash with FACS buffer once and stain with huCD45 and huCD56 for 20 min on ice. Other NK cell markers can be used if desired such as KIRs or NK cell markers NKp44 or NKp46.

3.4 Intraperitoneal Washes to Measure NK Cell Persistence

1. NK cell persistence within the peritoneal cavity can also be evaluated (*see* **Note 11**). Euthanize the mouse and carefully separate the skin from the peritoneal wall.
2. Once the skin is separated, make a small hole to allow access into the peritoneal cavity. This is best done by holding the peritoneum with tweezers while making a small cut with a scissors.
3. Use a glass pipette with a pipette bulb and carefully flush out the cavity with DPBS. Be careful not to puncture the liver with the pipette to avoid getting excess RBCs in the wash.
4. Repeat the washes with up to 10 mL of DPBS trying to rinse the entire cavity.
5. Keep the cells on ice until all of the samples are collected and then centrifuge the cells ($300 \times g$ for 4 min).
6. The washes should be clear unless there was ascites in the cavity. If there are any RBCs present, perform an RBC lysis as in Subheading 3.3.
7. Stain cells with anti-huCD45 and anti-huCD56 to identify NK cells. Other antibodies can be used as desired, as described in Subheading 3.3.

4 Notes

1. We have performed this with three different ovarian cell lines, MA148, A1847, and A2780 cells and in each case 2×10^5 cells resulted in consistent engraftment within 4 days (Fig. 2). If a different cell line is to be used, we recommend doing a dose response test by injecting different numbers of cells and imaging 3 days and 10 days later to ensure engraftment will occur. The ideal cell number is the fewest cells that will provide reliable engraftment. Too high of tumor burden at the beginning may lead to tumor growth faster than NK cell-mediated activity can be effective.
2. It is best to use mice aged 10–12 weeks, but we have been successful using mice as early as 8 weeks and as late as 14 weeks. Additionally, we have found that it is prudent to inject 5 extra mice at start of the studies, as there are often times a few mice where the tumor will not engraft.

3. Immediately prior to loading the syringe and injecting the mice, make sure to pipette the tumor cells gently to ensure they are well mixed. Each syringe can be used to inject 5 mice. Do not preload all of the syringes prior to injection, rather load one at a time and inject into mice immediately. We have found that a wider gauge needle can result in tract tumors subcutaneously and interfere with results.
4. These settings are recommended as a starting point. If the images become saturated, decrease the exposure time. We report our bioluminescence in total flux, which is independent of time. As the tumor grows, the images will remain saturated at even a 1 s exposure so increase the F stop on later days. Additionally, if imaging multiple mice at one time, place black dividing cards between the mice to avoid having the radiance from one mouse bleed over into nearby mice. This will not be immediately apparent, so don't assume the cards aren't necessary.
5. Make sure to draw the box large enough the first time to measure the entire abdominal area so as the tumors grow the entire luciferase positive area is still measured.
6. Negative controls typically are around $3–5 \times 10^5$ total flux. Mice with measured total flux less than 1×10^6 are typically discarded and removed from the experiment. Then, the remaining mice are ranked from highest to lowest total flux and each group is assigned mice in order.
7. This low level of irradiation will not significantly affect tumor burden.
8. If using an aAPC expansion system, try to time the administration of NK cells so that all of the aAPCs have had time to be removed from the culture to avoid affecting the cell counts. Typically, we stimulate our NK cells on Fridays and dose the mice on Wednesday after changing the media on Tuesday.
9. The administration of cytokines promotes NK cell engraftment and survival in vivo. We have found that IL-2 is equally effective as IL-15 in our model, but reports suggest IL-15 may be better in other models [15].
10. Blood draws have been performed at day 7 and 14, but we have noticed that it tends to be fairly hard on the mice in combination with IL-2 administration, so we have moved to bleeding on day 7 and 21.
11. NK cells can be found in the peritoneal cavity for up to 28 days.

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Chapter 24

Aerosol Delivery of Interleukin-2 in Combination with Adoptive Transfer of Natural Killer Cells for the Treatment of Lung Metastasis: Methodology and Effect

Simin Kiany and Nancy Gordon

Abstract

Natural killer (NK) cells are a subtype of lymphocytes with a major role as a host defense mechanism against tumor cells. Allogeneic NK cell therapy is being used as an alternative promising therapy for many different cancers. Interleukin-2 (IL-2) is a critical cytokine for NK cell proliferation, survival, and effector functions. Cytokine support is essential to activate, expand, and increase the life span of NK cells. Aerosol delivery of IL-2 in combination with adoptive transfer of NK cells offers a reasonable approach for the treatment of lung metastases as it avoids the deleterious side effects of systemic IL-2. Using a human OS mouse model, we demonstrated the efficacy of this approach. Combination therapy of aerosol IL-2 with NK cells resulted in a better therapeutic effect against OS lung metastases as compared with each therapy alone. Aerosol IL-2 selectively increased infiltration, retention, and proliferation of infused NK cells in the lung, and there was no local inflammation or toxicity in the lungs or any other organ. Our results demonstrate that delivery of IL-2 via the aerosol route offers a feasible and innovative approach to enhance the immunotherapeutic effect of NK cells against pulmonary metastases. In the following chapter, we describe the methodology and effect of this innovative therapeutic approach.

Key words Natural killer cell, Interleukin-2, Osteosarcoma

1 Introduction

Natural killer (NK) cells are a subset of lymphocytes that serve as the first-line immune defense mechanism against tumor cells. NK cell response occurs without initial pre-sensitization to tumor cells. Activation of NK cells by tumor cells triggers the release of perforin and granzyme leading to tumor cell lysis. In many cancer types, it has been reported that a higher number of intra-tumoral NK cells correlated with better prognosis [1–3]. Therefore, adoptive NK cell therapy is very appealing as an effective treatment for many types of cancers [4, 5].

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In our previous studies, using a human osteosarcoma (OS) mouse model, we demonstrated the efficacy of the combination therapy, NK cells plus aerosol human Interleukin-2 (IL-2) for the treatment of OS lung metastases [6, 7]. It is well known that IL-2 promotes proliferation, survival, and enhances NK cells effector functions. Ex vivo activated NK cells have short life spans and show poor tumor infiltration in vivo without cytokine support [8]. This problem can be circumvented by combining NK cell therapy with exogenous IL-2 infusions. However, systemic IL-2 administration induces life-threatening side effects, such as capillary leak syndrome, hypotension, and oliguria. These side effects are identified limitations [9, 10]. As previously demonstrated after aerosolized chemotherapy [11], direct administration of IL-2 to the lungs via inhalation offers several advantages over systemic delivery. These include the local delivery directly to the lungs and airways, administering a lower dose with potential fewer side effects and using a noninvasive delivery system ideal for repeated administration.

Using NK cells expanded on genetically engineered K562 cells as previously described [12, 13], we tested the efficacy of aerosol IL-2 alone, NK cell therapy alone, and combination aerosol IL-2 + NK cells in a human OS mouse model and demonstrated that aerosol IL-2 selectively increased infiltration, retention, and proliferation of infused NK cells in the lung without any toxicity. In addition, aerosol IL-2 enhanced the efficacy of NK cell therapy maybe due in part, to the higher local IL-2 concentration in the lungs [11, 14]. We also demonstrated that combining aerosol IL-2 with NK cell therapy resulted in a greater reduction in tumor burden as compared to either the NK cell therapy alone, the aerosol IL-2 alone or the aerosol PBS group (Figs. 1 and 2). Inhaled aerosol IL-2 offers an effective and feasible way to deliver a specific cytokine to the lungs and provides the basis for future combination therapies, in particular, for those tumors with metastatic predilection to the lung.

In this chapter, we provide a description of an OS mouse model [15] and outline the different methods and schedules for the NK cells and aerosolized IL2 administration as a methodology for preclinical adoptive immunotherapy studies whose main target are metastatic tumors to the lung.

2 Materials

1. Metastatic human LM7 osteosarcoma cell line (*see* **Note 1**).
2. Blood buffy coats from healthy donors.
3. Irradiated K562 (gamma irradiation at 100 Gy).
4. Complete cell culture medium: Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with nonessential

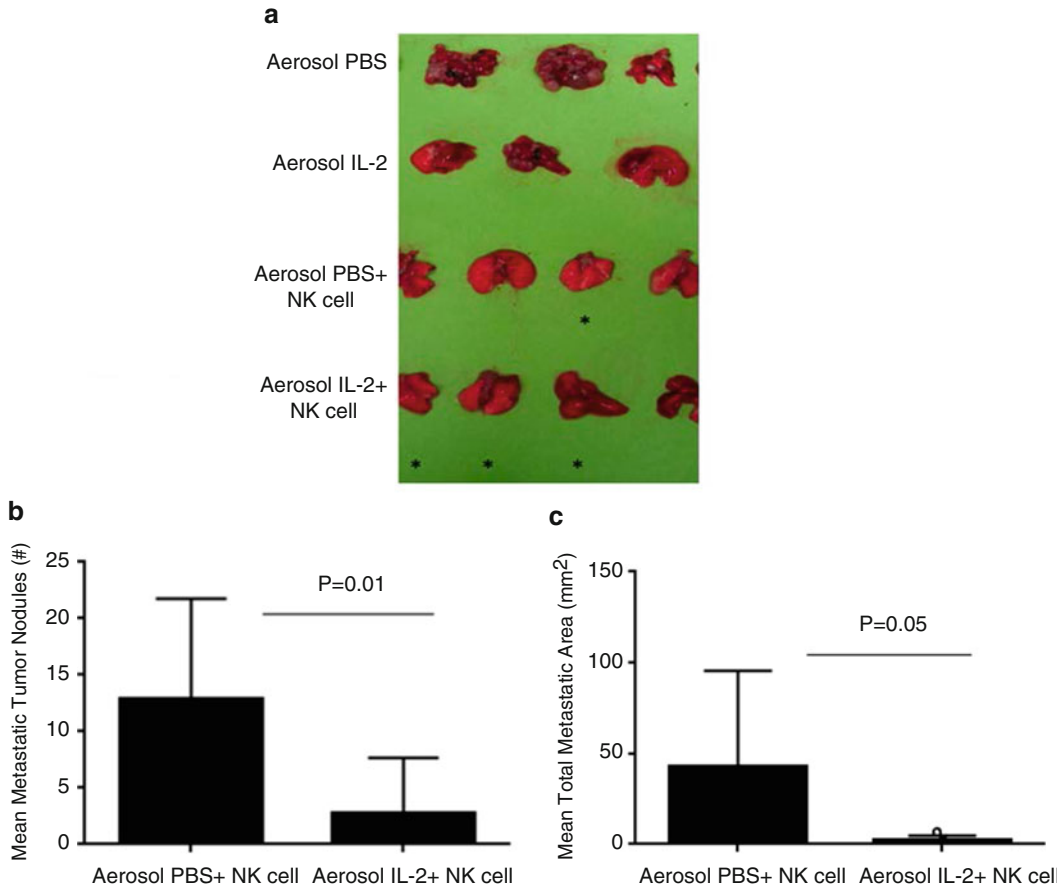

Fig. 1 Combination therapy with aerosol IL-2 and NK cells significantly decreases osteosarcoma lung metastasis from mice. Nude mice were injected intravenously with 3×10^6 LM7 cells. Mice were treated with aerosol PBS, aerosol IL-2, aerosol PBS plus NK cells, or aerosol IL-2 plus NK cells. Mice were sacrificed after 5 weeks of treatment. (**a**) Representative pictures of the lungs from the different groups. *Asterisks* represent "no visible nodules." (**b**) and (**c**) Mean number of lung nodules and total metastatic area demonstrates enhanced therapeutic efficacy of the aerosol IL-2 + NK cell combination therapy ($p = 0.01$ and $p = 0.05$ respectively). Partially reproduced from *Pediatric Blood and Cancer* "Guma, S. R., Lee, D. A., Yu, L., Gordon, N., Hughes, D., Stewart, J., Wang, W. L. and Kleinerman, E. S. (2014), Natural killer cell therapy and aerosol interleukin-2 for the treatment of osteosarcoma lung metastasis. Pediatr. Blood Cancer, 61: 618–626. doi: 10.1002/pbc.24801".

amino acids, 2 mM L-glutamine, 1 mmol/L sodium pyruvate, and 10% fetal bovine serum (FBS).

5. NK cell medium: RPMI 1640 medium (Cellgro/Mediatech) supplemented with 10% fetal bovine serum (Intergen), 2 mmol/L glutamine, and 1 mmol/L sodium pyruvate.
6. 1× phosphate-buffered saline (PBS).
7. Recombinant human IL-2 (*see* **Note 2**).
8. Ficoll-Paque (GE Healthcare Life sciences).

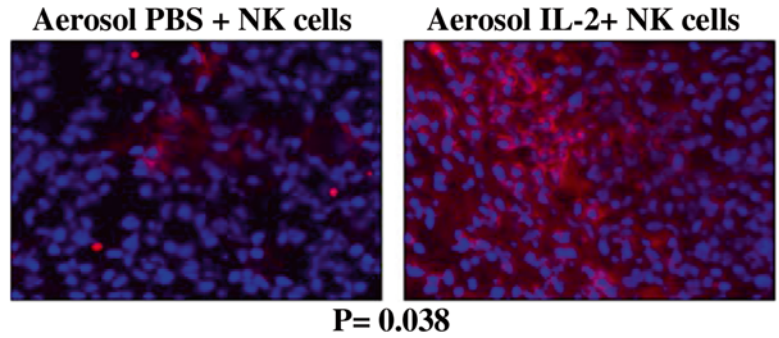

Fig. 2 Aerosol IL-2 increased the number of NK cells in the lung. Frozen sections of lungs were stained to demonstrate NK cells infiltration in the lungs. Anti-human NKG2D (*red*) and Hoechst 33258 (*blue*) were used to identified NK cells and cellular nuclei, respectively. Mean positive fluorescence in five random fields per section was calculated using SimplePCI software and $P < 0.05$ was considered significant. Reproduced from *Pediatric Blood and Cancer* "Guma, S. R., Lee, D. A., Yu, L., Gordon, N., Hughes, D., Stewart, J., Wang, W. L. and Kleinerman, E. S. (2014), Natural killer cell therapy and aerosol interleukin-2 for the treatment of osteosarcoma lung metastasis. Pediatr. Blood Cancer, 61: 618–626. doi: 10.1002/pbc.24801". (Copyright© 1999-2015 John Wiley and Sons, Inc. All rights Reserved)

9. RosetteSep Human NK Cell Enrichment Cocktail (Stem Cell Technologies).
10. OCT: Optimal cutting temperature compound (VWR Life Science, Seradigm).
11. CellTracker™ CM-Dil Dye (Molecular Probes): Prepare a stock solution of CM-Dil in DMSO at 1 mg/mL concentration.
12. 10% Buffered Formalin Acetate (Fisher Scientific).
13. Horse serum (Sigma).
14. Goat serum (Sigma).
15. Mouse anti-Human NKG2D (eBioscience).
16. Goat anti-mouse Alexa Fluor 546 (Molecular Probes).
17. Hoechst 33342 (Molecular Probes).
18. Fluoromount™ Aqueous Mounting Medium (Sigma).
19. IL-2 ELISA kit (eBioscience).
20. Animal: 4-week-old nu/nu mice (purchased from NCI) were used and kept under pathogen-free conditions in a laminar air flow room.
21. AeroTechII Nebulizer (CIS-USA).
22. Bird 3800 MicroBlender (Palm Springs).
23. Fluid, Absorbent, Fyrite CO_2 (Bacharach, Inc.).

3 Methods

3.1 NK Cells Activation and Expansion

Human NK cells were isolated from healthy donors' buffy coat and then were expanded in vitro for 3–4 weeks using recombinant human IL-2 and genetically engineered K562, as previously described [12, 13]. The brief methodology is as follows:

1. Add 4 μL of RosetteSep Human NK Cell Enrichment Cocktail to each 15 mL of buffy coat, mix well, and incubate at RT for 20 min.
2. Dilute the buffy coat with PBS+ 2% FBS at 1:1 ratio and over lay on 15 mL Ficoll-Paque. Centrifuge at $400 \times g$ for 20 min at RT with no brake.
3. Remove NK cells from the interface and wash them with PBS+ 2% FBS twice.
4. Seed isolated NK cells with irradiated K562 at a ratio of 1:2 in complete RPMI and add 50 IU/mL recombinant human IL-2 (*see* **Note 3**). Seed 15×10^6 NK cells with 30×10^6 irradiated K562 in 40 mL of RPMI.
5. On days three and five, replace half of the cultured media with fresh complete RPMI and add 50 IU/mL recombinant human IL-2 to the entire media. Keep the cell number equal to 1.5×10^6 NK cells/ mL media.
6. On day seven, restimulate NK cells with irradiated K562 at the ratio of 1:1 and resuspend in fresh complete RPMI plus 50 IU/mL recombinant human IL-2 at 1.3×10^6 NK cells/mL.
7. Three or four weeks after expansion, freeze NK cells in freezing medium (FBS+ 10% dimethyl sulfoxide, DMSO) at a concentration of 5×10^7 cells/ vial and keep them at −80 °C for further use (*see* **Note 4**).

3.2 Development of Osteosarcoma Tumor Model

1. Inject 3×10^6 LM7 cells suspended in 0.2 mL of PBS solution to 4-week-old nu/nu mouse intravenously (i.v.) (*see* **Note 5**).
2. Confirm presence of micrometastases at about 5–6 weeks by microscopic evaluation of hematoxylin-eosin (H&E) stained slides from lungs before starting treatment.
3. Consider 8–10 mice in each treatment group as follows: (1) Aerosol PBS, (2) Aerosol IL-2, (3) Aerosol PBS plus ex vivo expanded NK cells, and (4) Aerosol IL-2 plus ex vivo expanded NK cells (*see* **Note 6**).

3.3 NK Cell and Aerosolized IL2 Treatment

1. Inject 5×10^7 NK cells diluted in 0.2 mL PBS to each mouse intravenously twice a week (*see* Fig. 3) for 5 weeks, starting 1 day after the first aerosol IL-2 or PBS treatment (*see* **Note 7**).
2. Perform aerosol treatment every other day for 5 weeks as follows: add 10 mL PBS with or without recombinant human

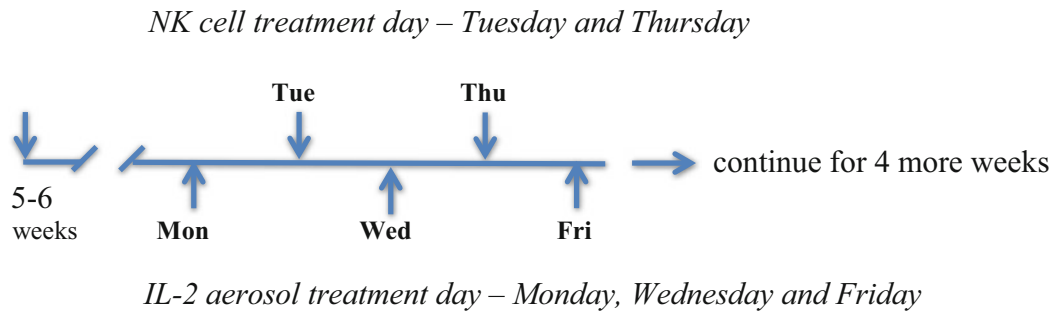

Fig. 3 Combination aerosol IL-2 + NK cell treatment schema

IL-2 (2000 U) to an AeroTechII nebulizer and expose mice to aerosol treatment in a sealed plastic cage for an hour each time (*see* **Note 8**). AeroTech II nebulizer should aerosolize IL-2 at a flow rate of 10 L of air per minute. The aerosol particles should be created by mixing air and CO_2 (5%) in a blender apparatus. Calibrate the system with a fluid fyrite to accurately determine the exact CO_2 amount in the mix.

3. Sacrifice mice after 5 weeks treatment. Expand lungs with OCT diluted with PBS at 1:1 ratio and then resect them.
4. Quantify and measure the number of tumor nodules. Prepare fresh frozen samples from the resected lungs and keep them at −80 °C for further use.

3.4 Detection of NK Cells in the Lung Upon Aerosol IL-2 Treatment

Below, we describe the methodology to detect the presence of NK cells in the lungs after aerosol IL-2 delivery. However, since every tumor differs in its behavior, it is recommended to test this methodology in animals with no tumors to assure investigators confidence of the technique and account for any discrepancy between the animals with no tumor vs. the animals with lung metastases.

3.4.1 Detection of NK Cells in the Lungs of Animals Without Lung Tumors Upon Aerosol IL-2 Treatment

1. On day 0—Expose nu/nu mice to aerosol IL-2 (2000 U) or PBS using the AeroTechII nebulizer, as described above, 24 h before NK cell injection.
2. On day 1—Prepare CM-Dil labeled NK cells as follows:
3. Dilute the CM-Dil dye stock solution in PBS at a concentration of 1 μM.
4. Resuspend NK cells in the working solution at 1×10^7 cells/mL and incubate cells for 5 min at 37 °C and then for 15 min at 4 °C (*see* **Note 9**).
5. Wash the cells by adding 8–10 mL of PBS and spin it down at $400 \times g$ for 5 min.
6. Repeat the wash step one more time.

7. Remove the PBS and resuspend labeled NK cell pellet in the appropriate volume of PBS to obtain a final concentration of 5×10^7 cells/0.2 mL of PBS per mouse.
8. Inject 5×10^7 CM-Dil-labeled NK cells i.v. per mouse.
9. Expose the mice to aerosolized IL-2 on the day of NK cell injection, and every other day for 1 week.
10. One, three, and seven days after NK cell injection, sacrifice mice and remove lungs, kidneys, heart, liver, and spleen (*see* **Note 10**).
11. Prepare frozen 5 μm sections of the lungs, spleen, liver, intestines, kidneys, and heart, acetone-fix for 10 min.
12. Rinse the slide with 1× PBS 3 min × 3.
13. Perform nuclear staining with Hoechst 33342 nucleic acid stain at 1:50,000 dilution (1 μL per 50 mL PBS) for 5–10 min.
14. Rinse the slide with 1×PBS 3 min × 3.
15. Before the tissue section becomes completely dry, mount the slides by adding two to three drops of mounting medium on the tissue section and gently put a coverslip.
16. Tissues from mice that have not received CM-Dil-labeled NK cells will serve as control.
17. Analyze slides under a fluorescent microscope.
18. Choose 3–5 random fields per slide for a minimum of three slides per tissues per group and quantify the number of NK cells in the lungs and other organs using a SimplePCI software.
19. Calculate the mean number of NK cells per organ per group and plot the values on a bar or dot plot graph.

3.4.2 Detection of NK Cells in the Lungs of Animals with Lung Tumors Upon Aerosol IL-2 Treatment

To investigate whether aerosol IL-2 treatment increased the number of NK cells in the OS lung nodules, the following steps should be followed:

1. Resect lungs from the different experimental groups described in Subheading 3.2.
2. Prepare 5 μm frozen sections.
3. Fix lung frozen sections in acetone for 10 min and incubate with protein block (1 % goat serum and 5 % horse serum diluted in 10 mL of PBS) for 30 min.
4. Rinse the slide with 1× PBS 3 min × 3.
5. Incubate with the anti-human NKG2D antibody at –4 °C overnight (*see* **Note 11**).
6. Rinse the slide with 1× PBS 3 min × 3.
7. Incubate with protein block for 30 min.

8. Rinse the slide with 1× PBS 3 min × 3.
9. Incubate with Alexa Fluor 546-labeled goat anti-mouse secondary antibody diluted at 1–10 μg/mL in PBS for 45 min to an hour.
10. Rinse the slide with 1× PBS 3 min × 3.
11. Stain nuclei using Hoechst 33342 diluted at 1:50,000 in PBS for 5–10 min.
12. Rinse the slide with 1× PBS 3 min × 3.
13. Mount samples by adding two to three drops of mounting medium on the tissue section and gently put a coverslip; read slides under a fluorescent microscope at 10×, 20×, and 40× magnifications.
14. Quantify the number of NK cells in the lungs and other organs using the SimplePCI software.

3.5 Methodology to Evaluate the Systemic Effects of Aerosol IL-2

Aerosol IL-2 systemic effect was evaluated both in nude and immunocompetent Balb/c mice.

1. Treat mice with aerosol IL-2 (2000 U) or aerosol PBS, every other day for 1 week and 1 month.
2. Sacrifice mice at the end of the treatment.
3. Remove lungs, kidneys, spleen, liver, intestines and heart and formalin-fix the tissues in 10% formalin for histological examination. H & E slides from all the paraffin embedded tissues should be analyzed for signs of toxicity due to the IL-2 therapy.
4. Draw blood samples from mice via retro-orbital bleeding or by intracardiac blood collection at the time of sacrifice, for complete blood count (CBC) and analysis of liver enzymes (ALT, AST, LDH) to evaluate the possible systemic effects caused by IL-2 inhalation. Perform liver enzyme tests using a specific kit and follow manufacturer's protocol (*see* **Note 12**).

3.6 Detection of Serum IL-2 Levels After Aerosol and Systemic IL-2 Delivery

To confirm the low to none systemic toxicity effects of aerosol IL-2 as compared to the systemic effects of the intraperitoneally (i.p.) delivered IL-2, serum levels of IL-2 are measured using an ELISA assay (*see* **Note 13**) as follows:

1. Collect serum from mice treated with aerosol IL-2 (2000 U), aerosol PBS, and IL-2 (20,000 U) i.p twice weekly for 2 and 5 weeks by retro-orbital bleeding.
2. Measure the serum levels of IL-2 using an IL-2 ELISA kit. Add 10 μL of each serum sample, in duplicate wells, to the ELISA plate and incubate at room temperature for 3 h.
3. Wash the plate three times with Wash Buffer (provided in the ELISA kit).

4. Add 100 μL of tetramethylbenzidine (TMB) substrate solution (provided in the ELISA kit) to all wells and incubate the plate at room temperature for 10 min.
5. Stop the colorimetric reaction by adding 100 μL of stop solution (provided in the ELISA kit) into each well and measure the absorbance values at 450 nm using a spectrophotometer.

4 Notes

1. We used the LM7 osteosarcoma cell line, which exclusively metastasizes to the lung. Do not split cells more than three to four times before injecting in mice. The cell line should be monitored to ensure that they are Mycoplasma negative. Any other lung metastatic cell lines may substitute the LM7 cells in similar studies.
2. We used recombinant human IL-2 for aerosol IL-2 administration from TECINTM Teceleukin, Bulk Ro 23-6019, National Cancer Institute, Frederick, MD. IL-2 was dissolved and diluted in PBS to achieve the desire concentration of 2000 U in 10 mL of PBS solution. Human IL-2 from Proleukin, Novartis, Inc., Basel, CH was used for NK cell expansion in vitro.
3. Reconstitute and dilute lyophilized IL-2 according to the manufacturer's instruction. IL-2 stock solution should be frozen in high concentrations, to prevent decomposition. Avoid repeated freeze-thaw cycles.
4. To account for variations in cytotoxicity and activity of NK cells, it is recommended that the expanded NK cells be examined prior to cryogenic preservation for expression of surface receptors such as NKG2D, CD16, and NKp46 by flow cytometry. Cell cytotoxicity may be evaluated by calcein release assay [12]. Briefly, label 10^6 target cells with 1–2 μM calcein-AM. Incubate for 1 h in cell culture incubator. Wash the target cells in RPMI media twice. Divide the target cells into three groups: (1) calcein-labeled target cells, which will reflect a measure of the spontaneous release; (2) calcein-labeled target cells treated with 2% tween 20, which will depict the maximum release; and (3) target cells co-cultured with effector NK cells. Seed the cells in a total volume of 200 μL in triplicates, in 96 well U-bottom plates at a ratio of 0.3125:1–1:10 effector to target cells, and incubate for 4 h. Transfer 100 μL of the media to a 96 well flat bottom plate, and measure calcein release, using a spectrophotometer at excitation and emission wavelengths of 485 nm and 530 nm, respectively. Calculate percent-specific lysis according to the formula [(test release – spontaneous release)/(maximum release – spontaneous release)] × 100.

5. We used the metastatic human LM7 OS cell line. To avoid the antigenic reaction caused by the human OS cells, nude mice were chosen for these studies. Any other lung metastatic cell lines are suitable for these studies if used with the appropriate mouse model.
6. Researchers can determine the number of mice per group based on statistical considerations.
7. Culture the newly thawed NK cells in fresh RPMI+50 IU/mL of human IL-2 for 1–2 days to allow for the recovery and activation of NK cells prior to the in vivo therapy experiments.
8. After completion of aerosol delivery to the animals, the sealed plastic cage should be opened and left inside the hood until all the fumes are dissipated to decrease environmental contamination.
9. For this experiment, consider a minimum of three mice per group and stain 5×10^7 NK cells for injection/mouse.
10. The choice of time point at which the mice will be sacrificed may be determined by the researcher, and varies with the type of study and animal model.
11. It is recommended that the antibody be titrated accordingly.
12. Our results showed no evidence of inflammation or toxicity in the spleen, liver, heart, intestines or kidneys at 1 week or 1 month after aerosol IL-2 treatment. Liver enzymes and CBC tests were also normal [6].
13. The aerosol IL-2 dose we used in our animal studies was 2000 U administered twice weekly for 2 and 5 weeks. The aerosol dose was ten times lower than the systemic IL-2 dose of 20,000 U which was intraperitoneally (i.p.) delivered. Two and five weeks after aerosol IL-2 administration, serum levels of IL-2 significantly increased when compared to the aerosol PBS treatment group. However, these levels were still significantly lower than those of the i.p. IL-2 treatment group after a single i.p. dose administration [6].

Acknowledgments

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Chapter 25

Noninvasive Imaging of Natural Killer Cell-Mediated Apoptosis in a Mouse Tumor Model

Thoudam Debraj Singh, Jaetae Lee, and Yong Hyun Jeon

Abstract

Natural killer (NK) cells are cytotoxic lymphocytes that induce apoptosis in cancer cells infected with viruses and bacteria through a caspase-3-dependent pathway. Effective NK cell-based immunotherapy requires highly sensitive imaging tools for in vivo monitoring of the dynamic events involved in apoptosis. Here, we describe a noninvasive bioluminescence imaging approach to determine the antitumor effects of NK cell-based therapy by serial imaging of caspase-3-dependent apoptosis in a mouse model of human glioma.

Key words Natural killer cells, Caspase-3 biosensor, Apoptosis, Immunotherapy, Reporter gene, *Renilla* luciferase

1 Introduction

Natural killer (NK) cells are cytotoxic lymphocytes that are the critical effectors of innate immunity [1, 2]. The killing effect of NK cells is regulated by activating receptors that identify ligands on target cancer cells or by inhibitory receptors that recognize major histocompatibility complex class I molecules on cancer cells. NK cells induce caspase-3-dependent apoptosis of cancer cells by exocytosis of cytotoxic granules containing perforins and granzymes and by aggregation of proteins belonging to tumor necrosis factor (TNF) superfamily (FasL/TNF) and their corresponding ligands (Fas/TNFR) [3–5].

The therapeutic effect of NK cell-based immunotherapy depends on the ability of these cells to maintain a balance between their activating and inhibitory receptors and their susceptibility to cancer cells [5–7]. Although many studies have examined the efficacy of NK cells in tumor immunotherapy, their distinct clinical benefits have been not demonstrated because of several factors such as alteration of their functions and unfavorable tumor microenvironments [8]. Another problem is the lack of reliable tools for the in vivo monitoring of apoptosis induced by NK cell therapy in a cancer model. Therefore,

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effective, practical, and noninvasive imaging tools are required to evaluate the efficacy of NK cell-based therapy, to monitor the progression of apoptosis in tumors accurately, and to understand the mechanisms underlying the failure of NK cell therapy.

A specialized molecular imaging system that can demonstrate the dynamic processes involved in apoptosis will provide a comprehensive understanding of apoptosis-related biochemical cascades. This system will also facilitate the evaluation of various important factors for NK cell-based therapy, such as dose, frequency, and injection route. In a recent study, apoptosis induced in a human glioma model by chemotherapeutic drugs was successfully monitored by performing noninvasive imaging with a reporter of caspase-3 proteolysis [9]. This apoptosis biosensor contained split luciferase domains fused with interacting peptides pepA and pepB and an intervening caspase-3 cleavage motif DEVD. Upon induction of apoptosis, this biosensor was proteolytically cleaved by caspase-3 at the DEVD motif. The cleavage induced the reconstitution of the luciferase activity through an interaction between NLuc and CLuc (Fig. 1). This system enabled real-time monitoring of the progression of apoptosis in living subjects in a repetitive and noninvasive manner. These advantages of the apoptosis biosensor suggest that it can be used to monitor the killing activity of NK cells both in vitro and in vivo.

In this chapter, we have emphasized on the application of this caspase-3 biosensor for monitoring the effects of NK cell-based immunotherapy in vivo. For this, we established human glioma cells expressing the caspase-3 biosensor as a surrogate marker for caspase-3 activation and *Renilla* luciferase (Rluc) as a surrogate marker for cell viability. Human NK92 cells were used as effector cells for NK cell immunotherapy. Activation of apoptosis by NK cell therapy was serially monitored in a glioma model by using the caspase-3 biosensor, and its therapeutic outcomes were evaluated using Rluc.

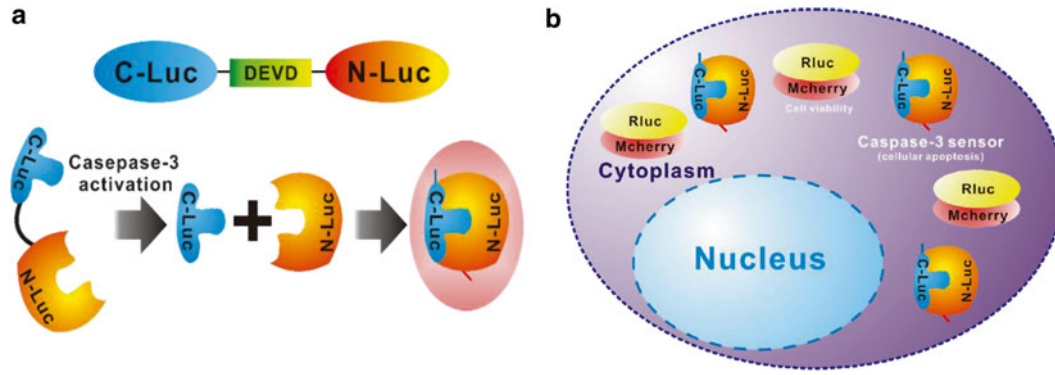

Fig. 1 Schematic representation of (**a**) Caspase-3 sensor (**b**) Stable glioma cancer D54 cells coexpressing caspase-3 sensor, R*luc*, and mCherry genes

2 Materials

2.1 Cell Lines and Culture Medium

1. Human IL-2-dependent NK92 cells line (ATCC).
2. Tumor cell line of choice: For the purposes of this chapter, we are describing the method using human glioma cell line D54 (provided by Dr. Alnawaz Rehemtulla, University of Michigan).
3. NK cell culture medium: Alpha-minimum essential medium without ribonucleosides and deoxyribonucleosides (HyClone), 2 mM L-glutamine (Sigma), 1.5 g/L sodium bicarbonate (Sigma), 0.2 mM inositol (Sigma), 0.1 mM 2-mercaptoethanol (Invitrogen), 0.02 mM folic acid (Sigma), 12.5% horse serum (HyClone), 12.5% fetal bovine serum (HyClone), 1% antibiotics (penicillin/streptomycin; Gibco), 1000 IU/mL IL-2 (Roche).
4. Commercial NK cell medium (optional): (ALyS505NK-EX) supplemented with 10% human serum, 1000 IU/mL IL-2, and 1% penicillin/streptomycin (this media can also be used instead of NK cell culture medium Subheading 2.1, **item 3**).
5. D54 cell culture medium: RPMI 1640 medium (HyClone), 10% FBS (HyClone), 1× Penicillin/Streptomycin (Gibco), 1× GlutaMAX (Gibco).
6. 96-Well black plates with a clear bottom (Corning).
7. T-75 Flasks (BD Falcon).
8. 6-Well plates (Corning).

2.2 Establishment of Caspase-3 Biosensor and Rluc Cell Line

1. Geneticin (G418; Gibco).
2. Puromycin (Sigma).
3. X-tremeGENE 9 transfection reagent (Roche).
4. Lentiviral vector coexpressing genes encoding Rluc, mCherry, and puromycin under the control of CMV promoter (GeneCopoeia).
5. Caspase-3 Biosensor plasmid [9] was provided by Dr. Alnawaz Rehemtulla (University of Michigan). This caspase-3 sensor is constructed into the vector of pEF that includes the neomycin resistance gene. Thus, it can be selected with neomycin (G418) after transfection of caspase-3 biosensor.
6. FACSAria II (BD Biosciences).

2.3 In Vivo Experiments: Mice, Imaging Reagents, and Instruments

1. Six-week-old pathogen-free female BALB/c nude mice.
2. D-Luciferin (DV Medical Research Innovations): Prepare fresh stock solution of 30 mg/mL D-luciferin in DPBS. Filter the solution using a 0.2-μm filter.

3. Coelenterazine h: For in vitro experiment, dilute commercial RediJect Coelenterazine h (120 μg/mL stock concentration; PerkinElmer) in respective culture media to 1 μg/mL. For in vivo experiment, dilute commercial Coelenterazine h (NanoLight Technology) in absolute ethanol (Sigma) to prepare 2 mg/mL stock solution.
4. GloSensor™ reagent (Promega): Prepare HEPES buffer (10 mM) in deionized water; adjusted pH to 7.5 by using KOH. Add 817 μL HEPES buffer to a reagent vial containing 25 mg of GloSensor™ reagent.
5. Isoflurane: Keep at room temperature, away from light.
6. IVIS Lumina III (PerkinElmer).

3 Methods

3.1 NK92 Cell Culture

1. Seed NK92 cells at a density of 1×10^5 cells/mL and culture in a T-75 flask containing NK cell medium.
2. To passage NK92 cell, collect a large, healthy colony of cells (as observed under a microscope) transfer to a conical tube and resuspend in 5–10 mL fresh NK cell medium to obtain a single cell suspension (*see* **Note 1**).
3. Centrifuge the cells at $300 \times g$ (*see* **Note 2**).
4. Reseed the cells in a T-75 flask in NK cell medium at a density of 1×10^5 cells/mL for subculture.

3.2 Expression of Caspase-3 Biosensor, Rluc, mCherry in Tumor Cells

Different cancer cell lines can be transfected with the caspase-3 sensor construct using X-tremeGENE 9 transfection reagent, and stable clones can be selected with geneticin (Gibco) as described below. The established stable cell lines expressing the caspase-3 sensor should be transducted with lenti-Rluc-mCherry vector and selected for stable clones by FACS sorting to get tumor cells coexpressing caspase-3 sensor, Rluc-mCherry. But for the purposes of this chapter, we are describing this method using D54 cell line. It should be noted that Caspase-3 biosensor can also be applicable to another human cancer cell lines. In our experiences, we successfully monitored the caspase-3 activation in thyroid cancer, colon cancer, and breast cancer cells with caspase-3 biosensor after treatment of Natural killer cells such as NK92 (IL-2 independent), NK92MI (IL-2 independent), and primary human NK cells derived from peripheral blood.

1. Transfect 5×10^5 cells D54 glioma cells in a 6-well plate with the caspase-3 biosensor construct using X-tremeGENE 9 transfection reagent as per manufacturer's protocol (*see* **Note 3**).

2. Culture the transfected D54 cells in RPMI complete medium supplemented with geneticin for 2 weeks to select for stable expression of caspase-3 biosensor (*see* **Note 4**).
3. Transduce (5 MOI) lentiviral vector coexpressing Rluc, mCherry, and puromycin genes to 1×10^6 cells D54 caspase-3 biosensor stable cells in presence 10 μg/mL polybrene. Replace with RPMI complete media after 24 h containing puromycin.
4. Culture transduced cells in RPMI culture medium supplemented with puromycin for 2 weeks to select for stable expression of Rluc and mCherry genes (*see* **Note 5**).
5. Sort using the FACSAria II cell sorter to isolate a pure population of cells coexpressing Rluc and mCherry (*see* **Note 6**).
6. Maintain established cell line stably coexpressing the caspase-3 biosensor, Rluc, and mCherry (labeled in this case as D54/CR) in culture under geneticin and puromycin selection (Fig. 1).

3.3 In Vitro Bioluminescence Imaging

In order to validate engineered reporter cells as a caspase-3 biosensor, it is essential to perform an in vitro assay in live cells (without cell lysis) for detection of caspase-3 activation and viability upon treatment of NK92 cell. This assay will provide a readout for a time- and dose-dependent increase in bioluminescence activity of the caspase-3 biosensor, which will indicate activation of apoptotic pathway in the tumor cells following NK cell mediated lysis.

1. Seed the gene-modified tumor cell line—D54/CR—cells at a density of 1×10^4 cells in 100 μL/well in a 96-well black plate with a clear bottom.
2. After 24 h, replace old culture medium with 100 μL NK cell medium supplemented with 1 % GloSensor™ and equilibrate for at least 1–2 h until a steady-state basal bioluminescence signal is obtained at 37 °C in a 5 % CO_2 atmosphere. Measure the basal luciferase activity in D54/CR cells using IVIS Lumina III (*see* **Note 7**).
3. Add 10 μL NK92 cells at different target to effector ratios (1:1, 1:2.5, 1:5, and 1:10) in 100 μL of NK cell medium (99 μL) supplemented with 1 % GloSensor™ (1 μL) and incubated at 37 °C in a 5 % CO_2 atmosphere, and measure the bioluminescence activity of the caspase-3 biosensor after 2, 4, and 6 h of incubation, using IVIS Lumina III (Fig. 2) (*see* **Note 8**).
4. Measure the luciferase activity of Rluc by adding 1 μg/mL Coelenterazine h and determine viability of tumor cells—D54/CR—cells using IVIS Lumina III.

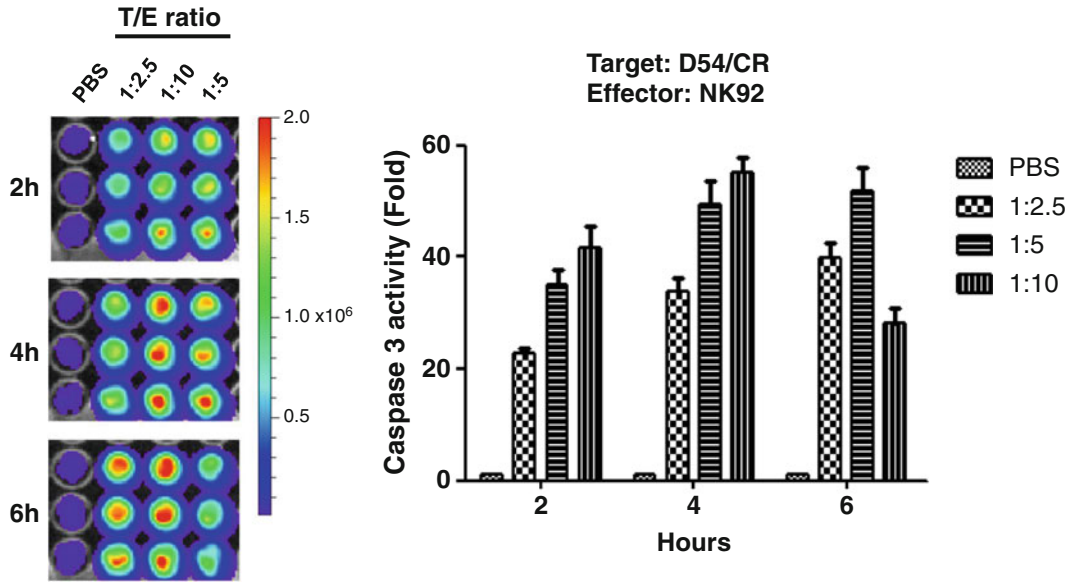

Fig. 2 In vitro monitoring of caspase-3 activity after NK92 treatment in D54/CR cells

3.4 Noninvasive In Vivo Imaging of Caspase-3 Activation

The in vivo imaging study is described to demonstrate the feasibility of caspase-3 biosensor for evaluation of caspase-3-dependent apoptosis event noninvasively and repetitively in living mice with D54/CR tumor. The dosages of NK cells to be used in mouse model need to be titrated when using a different cell line.

1. Inject 5×10^6 D54/CR cells subcutaneously into the right hind flank region of mice ($n = 30$ mice) (*see* **Note 9**).
2. Monitor the mice for tumor formation by inspection and palpation. Normally, the tumors are detectable within 2–3 weeks (*see* **Note 10**).
3. Determine basal bioluminescence activity of the caspase-3 biosensor and Rluc D54/CR tumors after 3 weeks.
4. Randomized into three groups ($n = 10$ mice/group) with equivalent Rluc mean bioluminescence value.
5. *Group 1*: Administer 100 μL PBS (control group) via tail vein.
6. *Group 2*: Administer 2×10^6 NK92 cells resuspended in 100 μL of PBS via the tail vein (*see* **Note 11**).
7. *Group 3*: Administer 1×10^7 NK92 cells resuspended in 100 μL of PBS via the tail vein.
8. For in vivo BLI, administer each tumor-bearing mouse with a single dose of the substrate of each reporter protein as follows: (1) To determine viability of tumor cells through the luciferase activity of Rluc: Anesthetize the mice and then inject 2 mg/kg of Coelenterazine h carefully injected through the tail vein.

Place the mice in the imaging chamber of IVIS Lumina III immediately after injecting the substrates, and acquire bioluminescent images. (2) To determine the bioluminescence activity of the caspase-3 biosensor: Inject 150 mg/kg of D-luciferin intraperitoneal, and anesthetize the mice using 1%–2% isoflurane/air mixture within 5 min after injecting D-luciferin. Obtain bioluminescent images of mice using IVIS Lumina III.

9. BLI with the caspase-3 biosensor and Rluc was performed using IVIS Lumina III at designated times points (12, 24, and 48 h) after treatment with NK92 cells (*see* **Note 12**).
10. Acquire the images of caspase-3 activation using the sequence imaging acquisition mode (Fig. 3a, black arrow) and by changing respective parameters such as field of view (FOV), image acquisition time (from second to minute), and delay time. Same FOV should be used at each imaging time point (Fig. 4, blue arrow) because different FOVs cannot be used to compare the changes in bioluminescence activity at different time points (*see* **Note 13**). Analyze Region of interest (ROI) on the mice images to determine the peak activity of the caspase-3 biosensor and Rluc, using Living Image software. Representative

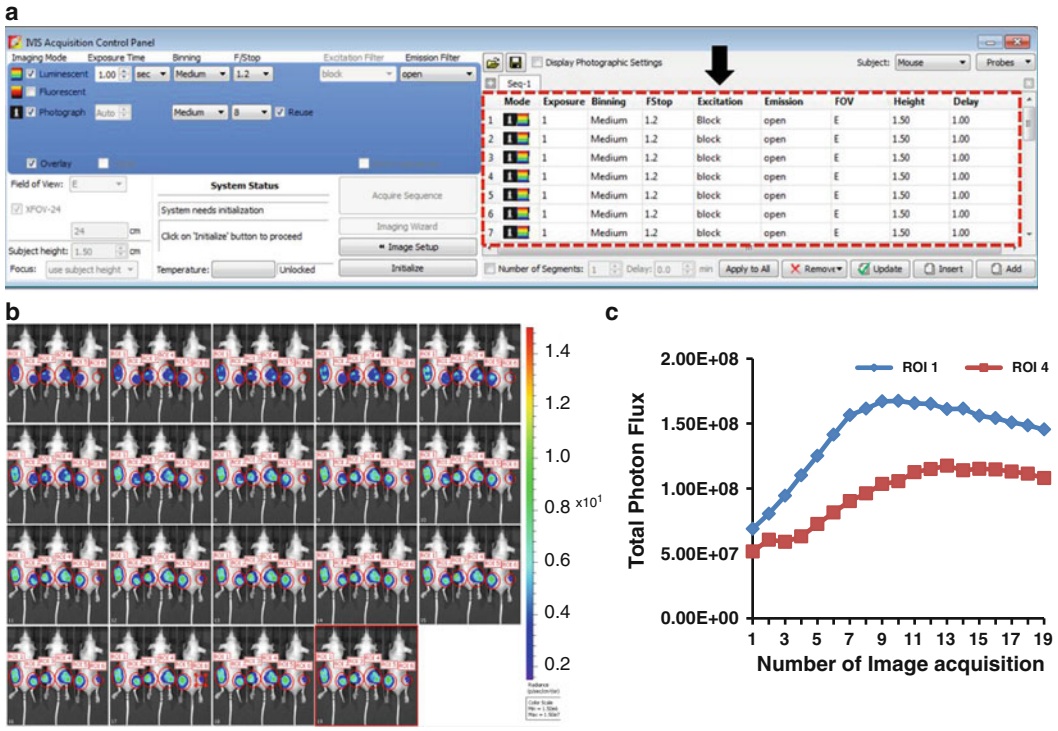

Fig. 3 (**a**) Schematic image for visualization of caspase-3 activation by sequential imaging acquisition mode, (**b**) Representative data of sequential imaging acquisition and (**c**) ROI analysis

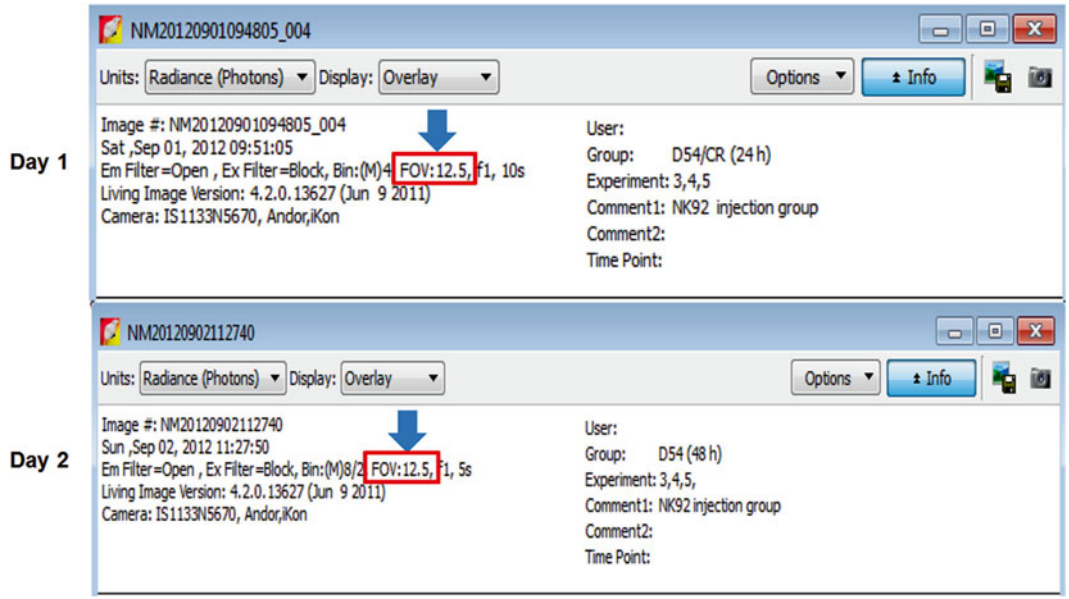

Fig. 4 Template showing sequential imaging acquisition parameters

Table 1
Representative example of calculation of relative caspase-3 activity

	Day 0	Day 1	Day 2	Day 3
Caspase-3 activity (photon flux)	6.84×10^7	2.08×10^8	3.62×10^8	2.33×10^8
Rluc activity (photon flux)	1.07×10^8	9.07×10^7	8.05×10^7	6.08×10^7
Relative Caspase-3 activity (Caspase-3 activity/Rluc activity)	0.638	2.294	4.495	3.837

sequential images obtained for caspase-3 biosensor, and a graph depicting 2 ROIs from these sequential images are shown in Fig. 3b, c.

11. Finally, calculate the relative increase in the bioluminescence activity of the caspase-3 biosensor by dividing the bioluminescence activity of the caspase-3 biosensor (cellular apoptosis) with the bioluminescence activity of Rluc (cell viability) at each time point (*see* Table 1).

4 Notes

1. The condition of NK92 cells was examined carefully. If a colony was small, the cells were incubated for an additional day to obtain larger colonies.

2. Viability of cells was >80% (determined by trypan blue dye exclusion test). After centrifugation, the conditioned medium of NK92 cells was retained and was used for culturing fresh NK92 cells to enhance their proliferation.
3. Prepare transfection complex by incubating 2 μg plasmid DNA and 6 μL transfection reagent in 100 μL of opti-MEM medium for 15 min at room temperature. Transfect D54 cells by adding 100 μL transfection complex in each well containing 2 mL complete media. Replace the media after 24 h. However, titration of DNA and transfection reagent is necessary to get maximum transfection efficiency in different cell lines.
4. Optimal lowest concentration (kill curve) of geneticin to kill 100% nontransfected cells was determined by treating D54 cells plated in 6-well plates with different concentrations of geneticin ranging from 100 to 1000 μg/mL. We used 200–400 μg/mL geneticin for selecting stable clones expressing the caspase-3 biosensor.
5. The optimal lowest concentration (kill curve) of puromycin to kill 100% nontransfected cells was determined by treating D54 cells plated in 6-well plates with different concentrations of puromycin ranging from 1 to 10 ng/mL. We used 6 ng/mL puromycin for selecting stable clones.
6. The lentiviral vector used in this study contains the internal ribosome entry site (IRES) flanked by two multiple cloning sites under the control CMV (cytomegalovirus) promoter. Rluc and mCherry genes located in upstream and downstream of IRES, respectively. Thus, mCherry fluorescent protein can be used for surrogate for Rluc genes and we can expect that all mCherry positive cells are also Rluc positive cells as Rluc and mCherry are expressed in a single construct. We measure the Rluc activity from live cells plated in 96-well black plate with clear bottom in the presences of its substrate without cell lysis using a Luminometer or IVIS machine.
7. The use of the 1% GloSensor™ reagent allowed the serial monitoring of apoptosis events in intact cells expressing the caspase-3 biosensor for at least 7 days without adding D-luciferin at each time point.
8. For negative control, add 5 μL PBS in 100 μL of NK cell medium (99 μL) supplemented with 1% GloSensor™ (1 μL) in first column of D54/CR cells.
9. The number of mice to be used in the study should be determined based on statistical considerations for each experiment.
10. Since the duration of tumor formation may vary for different cell lines, monitoring of tumor growth is necessary for at least twice a week using optical imaging or palpation and inspection.

11. PBS or cell suspension containing 5×10^6 NK92 cells was carefully injected into the tail vein of tumor-bearing mice (100 or 200 μL of NK cell suspension is usually used for in vivo therapy. Please note that NK cells should be injected slowly because rapid injection of NK cells may result in the death of mice).
12. Rluc signal decreased rapidly within 1 min after injecting its substrate. Therefore, BLI by using Rluc should be carried out before performing BLI for the caspase-3 biosensor.
13. Sequential images of BLI of the caspase-3 biosensor with 5 or 10 s (image acquisition time) were captured for 30–40 min at 1 min interval (delay time). Bioluminescence activity of the caspase-3 biosensor peaked within 30 min after injecting its substrate and remained at that level for approximately 0.5–1 h.

Acknowledgments

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Chapter 26

Noninvasive In Vivo Fluorescence Imaging of NK Cells in Preclinical Models of Adoptive Immunotherapy

Srinivas S. Somanchi

Abstract

Preclinical animal models play a vital role in developing novel adoptive immunotherapies for cancer. In these in vivo models, it is essential to track the adoptively transferred cells to understand their tissue localization (biodistribution) in order to correlate with observed therapeutic outcomes as well as to develop novel approaches to promote homing to tumors or organs of interest. This chapter describes a simple and quick method for fluorescence labeling and in vivo imaging of adoptively transferred NK cells in small animal models.

Key words NK cells, Adoptive immunotherapy, Fluorescence imaging, Near infrared dye, DiR

1 Introduction

Preclinical animal tumor models play an important role in evaluating adoptive immunotherapy for cancer. Noninvasive bioluminescence imaging of tumor xenografts in vivo, using tumor cells gene modified to express firefly or Renilla luciferase gene, has dramatically enabled the tracking of tumor progression and regression in response to therapy in real time. Likewise, noninvasive imaging of adoptively transferred NK cells is essential to understand their biodistribution, persistence and specific homing to target organs or tumors so that novel strategies can be developed to promote localization of adoptively transferred cells to the tumor or to organs of interest—sites of tumor metastasis. Although developing a similar approach for NK cells by engineering them to express fluorescence proteins, to complement the bioluminescence tumor imaging is an appealing option, most gene transfer methods yield low efficiency of NK cell gene modification. Additionally, the fluorescence proteins currently available lack sufficient fluorescence signal strength for effective detection in vivo. Therefore, fluorescent dyes have emerged as appealing alternatives as they afford a convenient and rapid means to label cells for in vivo imaging

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following adoptive transfer, particularly dyes that are fluorescent at the near-infrared (NIF) spectrum, as they have little interference from autofluorescence from tissue or fur of the animals. Although imaging fluorescently labeled cells is not a clinically viable option, it has been demonstrated to be an excellent choice for small animal models.

Cytoplasmic and lipophilic dyes have been extensively reported in literature for in vivo imaging of cells [1]. The cytoplasmic dyes are potentially more toxic to the cells as shown with CFSE, [2] and while CFSE is an excellent candidate for flow cytometry based assessment of cell division ex vivo, its fluorescence overlaps with tissue autofluorescence and makes the specific signal weak over tissue depth for in vivo imaging. In contrast, lipophilic dyes such as PKH26 have been widely used for tracking lymphocytes [3], and hematopoietic stem and progenitor cells [4]. However, the newer carbocyanine lipophilic dyes, such as DiIC18(7) or 1,1′-dioctadecyltetramethyl indotricarbocyanine iodide (DiR) have improved properties for in vivo imaging. The near infrared emission spectrum of dyes like DiR (780 nm) is a distinct advantage as there is little florescence interference due to autofluorescence from tissue or fur of the animals and the near infrared wavelength has high tissue penetrance [5]. DiR dye has been extensively used in diverse applications of in vivo imaging such as tracking hematopoietic stem cell homing [6], T cells adoptive immunotherapy [7], murine embryonic stem cells [8], murine macrophages [9], to study inducible pluripotent stem cell-derived neural stem cells as delivery vehicles for cancer gene therapy [10], murine myeloid derived suppressor cell (MDSC) [11], homing and uptake of nanovectors (grapefruit derived nanovectors GNVs) by tumor cells for drug/gene delivery [12] and trafficking of cytotoxic T lymphocytes and cytokine activated killer cells in adoptive immunotherapy model of gastric carcinoma [13]. Additionally, DiR dye is high photostable and photoconvertible, making it amenable for tracking single cells using intravital microscopy as shown with hematopoietic cells [14].

However, it is important to assess the merits of any method for a given application. For example, DiR dye may be an excellent choice for monitoring trafficking of infused NK cells over short periods of time but may not be suitable for long-term imaging of NK cell persistence, because—firstly, when the infused NK cells undergo cell division the membrane incorporated dye will get progressively diluted resulting in decrease in fluorescence signal over time, secondly, the dye would persist in the membranes of dead cells resulting in continued DiR fluorescence signal that does not correlate with the presence of viable NK cells and thirdly there is a potential for "microenvironment contamination" of DiR dye as reported in literature [15]. Therefore, in vivo fluorescence imaging data should be corroborated with flow cytometry analysis of

isolated cells or with immunohistochemistry of tissue sections using NK cell markers. Additionally, the migration of NK cells to major organs or larger tumor masses may be easier to image in a noninvasive manner, but homing to smaller localized regions such as lymph nodes [16] or tumor micrometastases may require imaging of the resected tissue. In this chapter, the methods for staining expanded human NK cells with DiR dye and noninvasive in vivo imaging of labeled NK cells are described.

2 Materials

1. Human NK cells (primary NK cells, activated/expanded NK cells or NK cell lines).
2. Ficoll-Paque Plus (GE Healthcare Life Sciences).
3. 50 ml conical tubes.
4. T75 culture flask.
5. NK cell medium: RPMI 1640, 10% FBS, 1× GlutaMAX (2 mM) and 1× Pen/Strep. Freshly add 50 IU/ml of IL2 to medium just before use.
6. DiIC18(7) or 1,1′-dioctadecyltetramethyl indotricarbocyanine iodide (DiR) (PerkinElmer): Prepare a stock solution of 50 mg/ml in ethanol (by dissolving 25 mg of the dye in 500 μl of ethanol) and store at 4 °C.
7. 2× DiR Staining medium: Just prior to staining NK cells, add DiR stock solution to NK cell medium at 1:500 dilution to make 2× DiR staining medium (100 μg/ml).
8. 70 μm cell strainer.
9. Mice (strain of choice) (*see* **Note 1**).
10. Alfalfa free diet or purified diet [17].
11. Isoflurane.
12. 1 cc syringes with 28–30 gauge needle.
13. Imaging: IVIS Spectrum preclinical in vivo imaging systems (Caliper Life Sciences): with 710 nm (excitation): 760 nm (emission) filter set; isoflurane vaporizer and oxygen supply.
14. Living Image Software (PerkinElmer).

3 Methods

The methodology described in this chapter is optimized for human peripheral blood NK cells, expanded for 3 weeks on K562 mbIL21. This method can be adopted for use with primary human NK cells, human NK cell lines, NK cells activated by cytokines or other

expansion platforms, as well as NK cells from animal sources (such as nonhuman primates and mice), but it may be essential to optimize the dye concentration and NK cell density to achieve maximal staining without affecting the NK cell viability.

3.1 Staining NK Cells with DiR

1. To use frozen stock of NK cells for this protocol thaw and place NK cells in culture at a cell density of 1×10^6/ml in a T75 flask (up to 40 ml, upright) in NK cell medium, the day before staining with DiR dye.
2. Count and assess the viability of NK cells by trypan blue exclusion.
3. If the viability of the NK cells is above 85% transfer desired number of NK cells (*see* **Note 2**), for staining with DiR dye, to 50 ml conical tube and spin at $400 \times g$ for 5 min (If the viability of NK cells is below 85% *see* **Note 3**).
4. Gently resuspend the NK cell pellet at 4×10^6 cells/ml using NK cell medium (2× cell density). To the resuspended NK cells, add equal volume of 2× DiR staining medium to achieve a final cell density of 2×10^6 cells/ml and dye concentration of 50 μg/ml (*see* **Note 4**).
5. Incubate the cells at 37°C for 20 min. Mix the contents every 5 min by gently inverting the tube.
6. Spin the cells at $200 \times g$ for 10 min (*see* **Note 5**).
7. Aspirate the supernatant and gently resuspend the cell pellet in 50 ml of NK cell medium and spin at $200 \times g$ for 10 min.
8. Repeat the wash step one more time.
9. Resuspend the NK cells in 50 ml of NK cell medium and pass through a 70 μm cell strainer and count the cells in a hemocytometer and assess viability using trypan blue exclusion (*see* **Note 6**).
10. An aliquot of cells from each batch of staining can be used to assess impact of DiR staining on NK cell cytolytic function compared to unstained NK cells using cytotoxicity assay of choice (*see* **Note 7**) (Fig. 1).
11. Spin the number of NK cells required for adoptive transfer experiment in a fresh conical tube at $400 \times g$ for 5 min, and resuspend in desired volume of medium for injecting in mice (no more than 200 μl via i.v per mouse) (*see* **Note 8**).

3.2 Infusion of DiR Stained NK Cells

For adoptive transfer experiments, determine the choice of mouse strain depending on experimental needs. Note that the DiR dye fluoresces at near-infrared spectrum, and thus there is minimal autofluorescence from the organs or fur of mice that would interfere with the specific fluorescence signal. However, to avoid fluorescence interference due to diet (in the gut), it is recommended

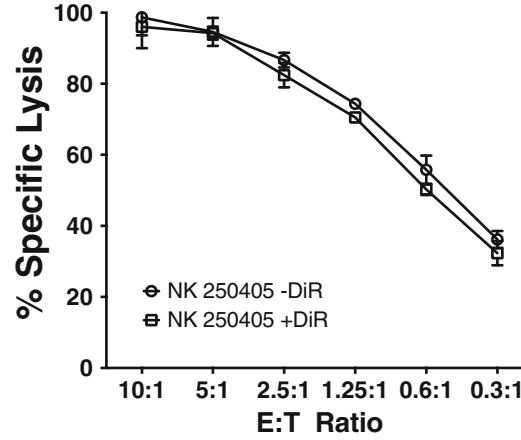

Fig. 1 Assessing impact of DiR labeling on NK cell cytolytic function. Cytotoxicity of expanded NK cells with and without DiR labeling is compared against K562 cell line by calcein release assay. Note that the optimized labeling procedure described in the methods does not significant impact cytolytic function of NK cells

to feed the animal alfalfa free diet or purified diet [17] starting at least 1 week prior to imaging experiments.

1. Design the mouse experiments with appropriate number of groups based on experimental needs (*see* **Note 9**). Determine the number of mice per group based on statistical considerations for a given experiment.
2. Inject DiR stained NK cells into mice via preferred route of administration (e.g., intravenous or intraperitoneal) based on the experiment needs (*see* **Note 8**).
3. Image the mice to assess biodistribution and homing of adoptively transferred NK cells immediately after NK cell administration and every day (or as desired, up to 1 week, *see* **Note 10**).

3.3 Fluorescence In Vivo Imaging

The method below describes whole animal imaging for visualizing DiR labeled NK cells in vivo. However, to assess homing of NK cells to smaller localized regions such as lymph nodes or smaller tumor masses, it may be essential to sacrifice the mice and resect the tissue of choice for imaging by IVIS Spectrum or by other methods such as flow cytometry of isolated cells or immunohistochemistry of the tissue sections. In such cases perform the euthanasia and tissue resection as per institutional regulations.

1. First, turn the IVIS Spectrum imaging system "On" and open the Living image software. Click on 'Initialize' icon to prepare for imaging.
2. While the instrument is initializing, anesthetize mice with isoflurane using isoflurane vaporizer: Fill the vaporizer with iso-

flurane, close the chamber and turn on the gas supply and flowmeter. Set the oxygen flow rate between 500 and 1000 ml per minute and isoflurane between 3 and 5%. Then place the mice in the anesthetizing (induction) chamber (*see* **Note 11**).

3. Once the mice are motionless, turn 'off' the gas supply to induction chamber and turn 'on' the gas supply (O_2:isoflurane mix) to imaging stage of IVIS Spectrum and transfer the mice to the imaging stage. Place the mice in nose-cones connected to the gas supply.
4. On the IVIS acquisition control panel of the Living Image software, check the boxes next to photograph and fluorescence and select auto-exposure (*see* **Note 12**) (alternatively, manually adjust binning, f-stop and duration of exposure by trial, avoiding saturation of signal (*see* **Note 13**)).
5. Select 710 nm and 760 nm (excitation–emission) filter set.
6. Select field of view from A to D depending on the number of mice on the imaging stage.
7. Acquire images and save files in Living Image format. The photograph and fluorescence images are saved together and can be displayed as an overlaid image.
8. Analyze the images by importing them into Living Image analysis software. Analyze and report data as Flux (photons/second) per region of interest (ROI), which compensates for differences between individual image acquisition settings (such as binning, f-stop and duration of exposure) across various time points of the experiment (Figs. 2 and 3).

4 Notes

1. In vivo fluorescence imaging using DiR can be performed with most strains of mice. The mouse strain should be chosen based on experimental need and since the DiR dye fluoresces at near-infrared spectrum (748 nm Ex–780 nm Em) there is negligible autofluorescence from tissue or from the fur of the mice.
2. The number of NK cells to be stained with DiR will depend on the experimental need. We normally inject 10×10^6 NK cells per mouse in our adoptive transfer experiments.
3. If the viability of the NK cells is low, it is essential to perform a Ficoll-Paque density centrifugation to remove nonviable cells and debris. Layer the NK cells on 15 ml of Ficoll-Paque in a 50 ml conical tube and spin at $400 \times g$ for 20 min without brakes. Recover the viable NK cells from the interface of Ficoll-Paque and medium, and transfer to a fresh 15 ml conical tube. To wash cells, add fresh NK cell medium to fill the tube and

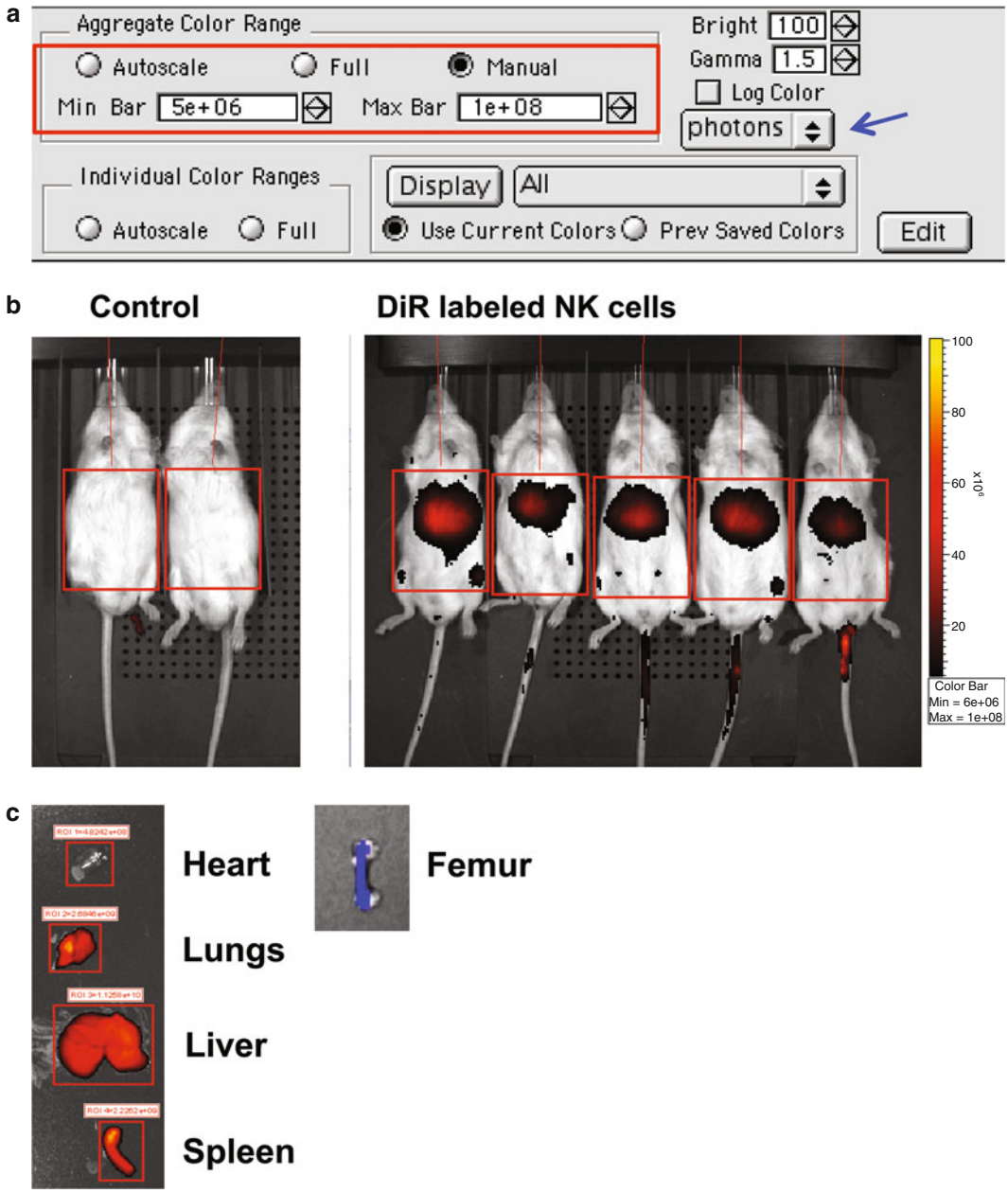

Fig. 2 Fluorescence in vivo imaging of DiR labeled NK cell following adoptive transfer in mice. (**a**) Open the image files as a group in Living Image software, and change the output to "Photons" (*Blue arrow*), select the "aggregated color range" (scale) to Manual (*red box*) and enter the minimum and maximum "color bar" values to display desired level of color without saturation (if the images are analyzed individually, enter the same minimum and maximum values for all images, to display them in the same scale). (**b**) The fluorescence images were acquired 24 h after NK cell infusion. The noninvasive in vivo image is a merger of the acquired photograph and fluorescence images and shows fluorescence signal predominantly in the liver (**c**) Imaging of the resected organs shows that the DiR fluorescence is present in lungs, liver, and spleen indicating migration of NK cells to these organs

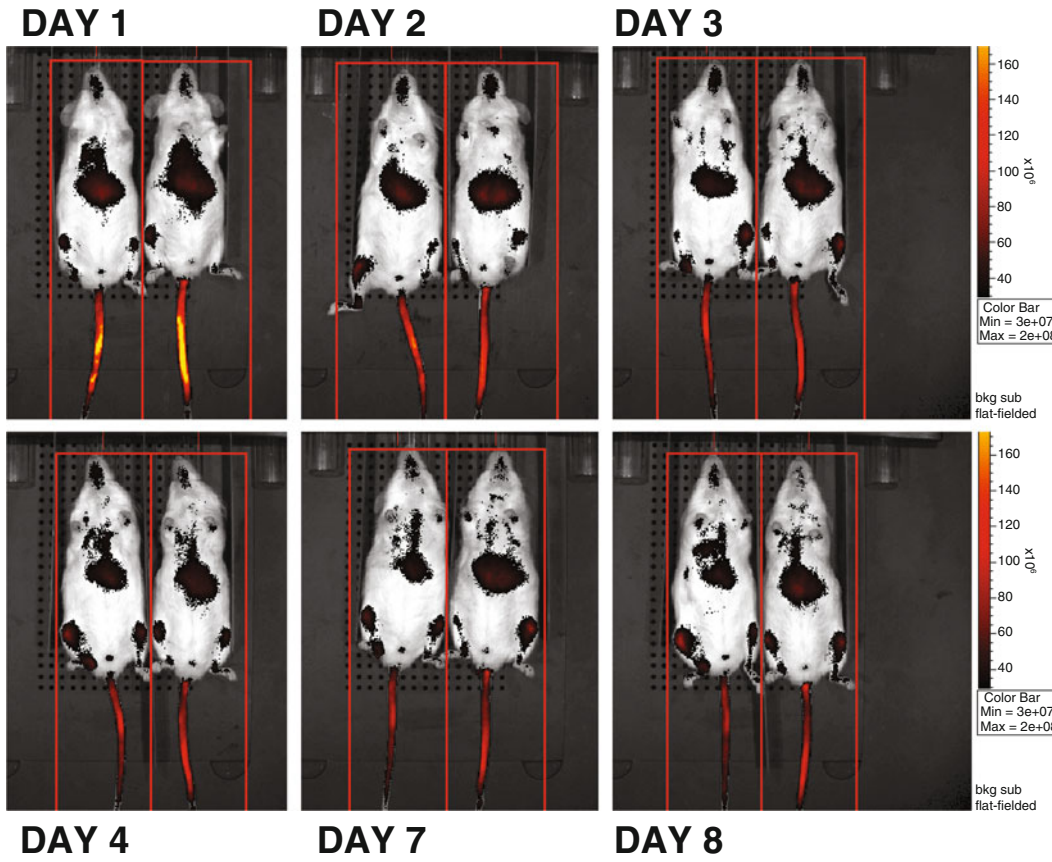

Fig. 3 Following adoptive transfer of 10×10^6 DiR labeled expanded human NK cells the mice were imaged daily for 8 days. The whole body imaging shows an increase in fluorescence observed from the hind limbs over time, indicating potential bone marrow homing of adoptively transferred NK cells

spin at $400 \times g$ for 5 min. Repeat the wash step a second time. Resuspend NK cells in NK cell medium and count the cells and test viability by trypan blue exclusion.

4. Anticipate some cell death during staining. So stain more cells (about 1.5×) than needed for the experiment.
5. After staining with DiR dye the NK cell pellet will appear blue.
6. Although after DiR staining the cell pellet appears blue, the live cells do not appear blue under bright-field microscopy. Hence it is possible to distinguish trypan blue positive cells and assess viability by trypan blue exclusion method.
7. Various methods are available for determining the cytotoxicity of NK cells such as Chromium-51 release assay ("gold standard"), nonradioactive green fluorescence dye—Calcein release assay, flow cytometry-based assays, and bioluminescence imaging. We routinely perform calcein release assay for determining cytolytic potential of expanded NK cells (Fig. 1) [18].

8. We routinely inject 10×10^6 cells per mouse in 100–200 μl of injection volume. The cell dose and volume can be altered based on individual experimental need. The cells can be resuspended in serum-free culture medium or in PBS/saline. When resuspending in PBS/saline ensure that the cells are healthy and in suspension (no visible aggregates) prior to i.v. administration.
9. In addition to test groups, include an untreated control group and a carrier control group (PBS or medium control).
10. This method is ideal for short term imaging, rather than assessment of long-term persistence of adoptively transferred cells because (1) the dye can get diluted upon cell division leading to lower signal strength and/or (2) membranes from dead NK cells will continue to provide fluorescent signal.
11. Standard operating procedures for anesthetizing mice and using IVIS Spectrum would be available at institutional vivarium cores and formal training would be essential before experimental work can be conducted.
12. If mice are to be imaged for adoptively transferred (fluorescent) NK cells in conjuncture with bioluminescent tumor xenograft, check the "luminescence" box.
13. In our experience f-stop of 2, small binning and exposure time of 1–5 s is sufficient to acquire fluorescent image. A detailed stepwise manual to navigate the acquisition software is available from the manufacturer.

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Chapter 27

In Vivo ^{19}F-Magnetic Resonance Imaging of Adoptively Transferred NK Cells

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Abstract

In order to assess the biodistribution, homing, and persistence of adoptively transferred natural killer (NK) cell immunotherapies, there is a need for imaging methodology suitable for use in preclinical studies with relevance to clinical translation. Amongst the available approaches, ^{19}F-MRI is very appealing for in vivo imaging due to the absence of background signal, enabling clear detection of ^{19}F labeled cells in vivo. Here we describe a methodology for in vivo imaging of adoptively transferred NK cells labeled with ^{19}F nanoemulsion, using clinically translatable technology of ^{19}F/^{1}H magnetic resonance imaging.

Key words NK cells, Adoptive immunotherapy, In vivo imaging, ^{19}F, Magnetic resonance imaging

1 Introduction

The preclinical and clinical development of pharmacologic cancer therapeutics generally requires in vivo measures of biodistribution and pharmacokinetics/dynamics. For cancer immunotherapy with adoptively transferred effector cells, the parameters of persistence, homing, trafficking, and proliferation constitute the analogous measures of immunokinetics. Blood sampling with cell quantitation by flow cytometry is the most commonly used method to assess persistence and proliferation, but ignores tissue localization and may under or overrepresent what is actually occurring at the tumor site. This is particularly true for tumors that are not readily accessible for repeated tissue sampling, such as brain tumors.

We developed a robust system for expanding NK cells from peripheral blood using a genetically engineered feeder cell K562 Cl9.mbIL21 [1, 2], and are now using this approach to generate NK cells for clinical trials of adoptive immunotherapy in leukemias, solid tumors, and brain tumors. Radiologic methods of localizing and quantifying infused cells in vivo include nonclinical methods such as

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fluorescent proteins [3] or small-molecule labels [4–7] and bioluminescence (e.g., firefly luciferase/luciferin) [8], and clinically applicable methods such as radioactive labeling for PET imaging using ^{111}Indium [9–11], ^{18}FDG [12, 13] or thymidine kinase and ^{18}F-FEAU [14], and magnetic resonance imaging (MRI) using iron oxide [15–18]. For imaging NK cells during NK cell adoptive immunotherapy, the PET approaches are toxic to NK cells (^{111}Indium) or require gene modification (thymidine kinase) that has proven difficult in these cells, and difficulty in loading iron oxide nanoparticles may yield insufficient contrast-to-noise for imaging a low concentration of these small cells using traditional MRI. Ideally, methods that can be adapted for both preclinical and clinical studies are needed. With a drive to develop hotspot imaging with heteronuclear MRI contrast agent, ^{19}F has emerged as a promising imaging target. Although ^{19}F/^{1}H imaging was first described in 1977 [19], this method has gained attention for tracking and imaging cells in recent years [20–24]. The main advantage of ^{19}F as contrast agent for MRI imaging is the absence of background signal (noise) in tissue, which enables clear visualization of the labeled cells. ^{19}F perfluorocarbon nano-emulsions have been used for in vivo tracking of immune cells such as DCs [22, 25] and T cells [26, 27]. The main innovation of the nano-emulsion, in addition to improving imaging sensitivity, is that it eliminates the need for transfection to label cells [28] in favor of simple co-incubation. This makes the method significantly more appealing for NK cell applications.

Here we describe the preclinical proof-of-principle methodology for ^{19}F labeling and in vivo imaging of expanded NK cells by ^{19}F MRI in a mouse model of intracranial NK cell adoptive immunotherapy. For the methods described here, we established an orthotopic model of brain tumor in mice, using cranial guide screw. The cranial guide screw method has been effectively used for establishing xenograft models for a number of brain tumor types [29–31]. For additional visual guidance on the guide screw method for intracranial xenografts, refer to the video article in the *Journal of Visual Experiments* [32].

2 Materials

1. NK cells (*see* **Note 1**).
2. NK cell medium: RPMI 1640, 10% FBS, 1× GlutaMAX, 1× Pen/Strep. Add 50 IU/ml of IL2 to medium just before using for NK cell culture.
3. Tissue culture flasks (T25, T75).
4. Ficoll-Paque Plus (GE Healthcare Life sciences).
5. DAOY (Medulloblastoma cell line) ATCC.
6. MEM complete medium: Supplement MEM medium with 10% FBS, 2× L-glutamine, 0.075% sodium bicarbonate, 1 mM sodium pyruvate, 1× antibiotic–antimycotic, and 1× MEM nonessential amino acids.

7. Cell Sense ^{19}F reagent: CS-ATM-DM Green (Celsense Inc) (*see* **Note 2**).
8. 2× Lysis buffer: 2% Triton X-100 in D_2O (Deuterium Oxide; Sigma).
9. Reference standard: 0.1% TFA (Trifluoroacetic acid; Sigma) in D_2O.
10. Deuterium oxide (Sigma).
11. 5 mm NMR tubes (Sigma).
12. Bruker 300 MHz DPX NMR spectrometer.
13. 15 ml and 50 ml sterile conical tubes.
14. K562 cell line (ATCC).
15. Complete culture medium: RPMI 1640, 10% FBS, 1× GlutaMAX, 1× Pen/Strep.
16. Calcein-AM (1 mg/ml stock) (Life Technologies).
17. 2% Triton X-100: Prepare a 2% Triton X-100 solution in complete culture medium and store at 4 °C.
18. 4–8-week-old NOD *scid* gamma (NSG) Mice (Jackson Laboratories).
19. SD-80 screwdriver—Part: 26030 (Plastics One, Inc).
20. 3 M® tissue adhesive.
21. Drill Bit #56 (High Speed Steel)—Part: D#56 (Plastics One, Inc).
22. DH-1 Pin Vise Starrett (0.030–0.062)—Part: 50600 (Plastics One, Inc).
23. SU1 Guide Screw W-0.5 mm hole; Nylon—Part: C212GN (Plastics One, Inc).
24. Dummy (stylet) 0.018 in-0.45 mm; Nylon—Part: C212SDN (Plastics One, Inc).
25. 7T Biospec Small Animal MRI system with BG6 gradients and a ^{1}H MRI volume coil with 35 mm inner diameter (ID) (Bruker Biospin Corp., Billerica, MA).
26. Quadrature ^{19}F volume coil with 35 mm ID (Rapid MR International LLC, Columbus, OH).
27. Spherical NMR Bulb (529-A, Wilmad-Labglass, Vineland, NJ).

3 Methods

3.1 Establishing Murine Intracranial Tumor Model

3.1.1 Placing Guide Screws in Cranium

1. The number of mice to be used for any experiment should be determined based upon statistical criteria.
2. Anesthetize the mice using 3–4% inhalant isoflurane (*see* **Note 3**).
3. Shave the scalps of mice from the nape of the neck to between the eyes using an electric shaver, clean the surgical area once with a sterile betadine swab followed by wiping one time with a sterile 70% isopropyl alcohol pad.

4. With a sterile scalpel make an incision of approximately 0.5 inch in caudal to cranial direction, over the site of guide screw placement (*see* **Note 4**).
5. Carefully drill a hole in the skull at the site of guide screw placement using DH-1 Pin Vise Starrett hand drill and drill bit.
6. Secure the nylon guide screw using a screwdriver until the guide screw is flush with the surface of the skull. It is important to stop rotating the screwdriver once the screw has met the resistance of the surface of the skull in order to prevent stripping or collapse of the burr hole.
7. Then, place the stylet in the shaft of the guide screw to close the opening.
8. Close the incision using forceps and seal the wound with 3 M® tissue adhesive, covering the guide screw with the scalp.
9. Allow the animals to rest for 7 days after surgery.

3.1.2 Intracranial Injection of Tumor Cells

Culture and maintain the tumor cell line of choice in appropriate culture medium in sterile culture condition and periodically test for mycoplasma. Use tumor cell lines expressing bioluminescence or fluorescence markers for convenience of imaging the tumor during the experiments. The protocol is described here for DAOY medulloblastoma cell line. If a different cell line is used, an initial tumor cell dose titration is recommended for establishing the tumor model, as different cell lines have different in vivo growth kinetics.

1. Culture and maintain DAOY cells in MEM complete medium. Passage the cells at 80% confluency at a split ratio of 1:6.
2. On the day of injection (day 7 after guide screw placement), trypsinize tumor cells and wash twice in PBS. Resuspend cells in PBS and pass through 70 μm nylon mesh to remove cell aggregates, and perform cell counts.
3. Recover desired number of cells for injection into mice into a fresh tube (*see* **Note 5**), spin at $400 \times g$ for 5 min and resuspend the pellet at 10×10^6 cells/ml in PBS so that 5 μl would contain 50,000 cells for intracranial injection.
4. To inject the tumor cells, anesthetize and prepare mice for surgery as described in Subheading 3.1.1, **step 2**.
5. Use a sterile scalpel to make an incision directly over the guide screw of enough length to expose the guide screw.
6. Remove the stylet.
7. Use a blunt-tipped Hamilton syringe and stereotactic device to inject up to 5 μL total volume of tumor cells at a steady rate of 0.5 μL/min. It is important that the injected cells are placed at least one millimeter beyond the end of the guide screw.

8. Once the entire volume has been delivered, rest the syringe in position for 1 min before removal in order to allow all injected cells to settle.
9. Remove the Hamilton syringe and replace the stylet. Close the incision using forceps and seal the wound with 3 M® tissue adhesive, covering the guide screw with the scalp.
10. Allow the tumor to establish for about 7 days (*see* **Note 6**), and randomly separate the mice into control and treatment groups.

3.2 In Vivo Infusion of ^{19}F Labeled NK Cells

3.2.1 Labeling NK Cells with ^{19}F

1. One day before NK cell infusion to mice, thaw a vial of expanded NK cells in a 37 °C water bath and transfer cells to a 15 ml conical tube containing 10 ml of pre-warmed NK cell medium. Spin the cells at 400 × *g* for 5 min. (If using NK cells that are in culture, start the process from **step 5**).
2. Remove the supernatant and resuspend cells in 10 ml of NK cell medium. Count and check for viability of NK cells using trypan blue exclusion method.
3. Transfer NK cells to an appropriate culture flask (*see* **Note 7**) and add NK cell medium to adjust cell density to 1×10^6/ml (*see* **Note 8**).
4. Incubate the cells overnight in CO_2 incubator at 37 °C.
5. Next day, prior to ^{19}F labeling, count the NK cells and check for viability by trypan blue method.
6. If the viability of NK cells is above 90%, proceed to **step 14**.
7. If the viability of NK cells is below 90%, perform a Ficoll-Paque density centrifugation to remove nonviable cells and debris.
8. In a 50 ml conical tube add 15 ml of Ficoll-Paque and carefully layer up to 35 ml of NK cells on the Ficoll-Paque layer (for smaller NK cell culture volumes use a 15 ml conical tube to maximize recovery).
9. Spin at 400 × *g* for 20 min without brakes.
10. Recover the NK cells from the Ficoll–medium interface and transfer to a fresh 15 ml conical tube.
11. To wash cells, add PBS to fill the tube and spin at 400 × *g* for 5 min.
12. Repeat the wash two more times.
13. Resuspend NK cells in NK cell medium, count and check viability by trypan blue exclusion.
14. Recover the desired number of NK cells (*see* **Note 9**) for labeling with ^{19}F, spin at 400 × *g* for 5 min.
15. Discard supernatant and resuspend at 3×10^6/ml using NK cell medium (*see* **Note 10**).
16. Seed cells in appropriate culture plate based on cell volume (*see* **Note 11**).

17. Add Cell Sense ^{19}F reagent (CS-ATM DM Green) to the cells at 5 mg/ml (*see* **Note 12**) and mix gently and thoroughly using a P-1000 pipette.
18. Incubate the cells overnight (16–18 h) at 37 °C in a CO_2 incubator.
19. On the day of infusion, mix and transfer the NK cells to a 15 ml conical tube, rinse the wells thoroughly with NK cell medium and transfer to conical tube to completely recover the NK cells.
20. To wash off excess ^{19}F, fill the tube with NK cell medium and spin at 400 × *g* for 5 min.
21. Repeat the wash **step 2** more times.
22. An aliquot of the ^{19}F labeled NK cells can be used to determine the number of fluorine atoms incorporated per cell by NMR (*see* Subheading 3.4) and to determine the effect of ^{19}F labeling on NK cell cytolytic function (*see* Subheading 3.5).

3.2.2 Intracranial NK Cell Infusion

1. Resuspend the ^{19}F labeled NK cell pellet at 1×10^6 cells/3 μl of PBS for intracranial injection into mice.
2. Shave the scalps of mice and clean scalp with sterile betadine swab followed by 70% isopropyl alcohol.
3. Make an incision using sterile scalpel to expose the guide screw.
4. Infuse ^{19}F labeled NK cells intratumorally using the **steps 5–8** described in Subheading 3.1.2.
5. Rest the mice for 2 h to recover from the surgery.
6. Image mice to detect the infused ^{19}F labeled NK cell by MRI.

3.3 In Vivo MRI Imaging of ^{19}F Labeled NK Cells

Magnetic resonance imaging (MRI) is a well-known diagnostic modality that provides good image resolution and exquisite soft-tissue contrast. MRI is based on measurement of the spatial distribution of hydrogen nuclei. Sensitivity is high because ^{1}H is the most abundant nucleus in vivo and because its nuclear magnetic resonance (NMR) signal strength is highest of all stable isotopes. ^{19}F has the second highest relative NMR signal strength, and low abundance in normal tissue ensures little background signal against which ^{19}F-labeled cells must be detected. This procedure outlines a method that can be used for coregistered imaging of labeled cells with anatomic reference using traditional MRI.

1. Anesthetize the mouse that is to be imaged (*see* **Note 13**) and place on an imaging sled that includes a method for delivering anesthesia (if an inhalable anesthetic is used) while maintaining body temperature.
2. Attach sensors for remote monitoring of physiological status (*see* **Note 14**). Make sure that the region of the body that will be scanned is immobilized to minimize MRI artifacts that are

caused by motion. Place an external reference containing a small amount of ^{19}F (Cell Sense reagent) near the target imaging site, in a small NMR bulb.

3. Place the mouse at isocenter inside the MRI magnet, at the center of the imaging gradients and within the RF coil(s) (*see* **Note 15**).
4. Acquire localizing scans to confirm that the animal is positioned correctly. A 3-plane fast spin-echo (FSE) sequence (TE_{eff} = 46 ms, TR = 2 s, ETL = 8, 128 × 128 image matrix over 45 mm × 45 mm field of view), for example, allows visualization of animal position after; *see* Fig. 1). Adjust as necessary to center the target anatomy within the imaging system.
5. Once position is confirmed, make sure that the RF coil is tuned and matched. Then adjust the center frequency, shims, and calibrate the RF excitation power for the MRI scanner. Many of these optimizations are performed automatically during prescan.
6. Acquire anatomic reference images using traditional ^{1}H MRI. The exact configuration of sequences that highlights target anatomy will depend on the anatomic region of interest and the field strength for the scanner. For visualizing NK cells within the murine brain, a single 3D T2-weighted fast spin-echo imaging sequence (TE_{eff} = 72 ms, TR = 1500 ms, ETL = 16, 128 × 128 × 32 image matrix covering a 3 cm × 3 cm × 3 cm field-of-view) provided excellent anatomic context with approximately 6.5 min scan time.
7. Without moving the animal, slide the ^{1}H MRI RF coil out of the magnet and replace it with the ^{19}F volume coil. Change the reference frequency of the MRI scanner to correspond with ^{19}F, and confirm that the coil is tuned and matched. Set the excitation calibration (*see* **Note 16**). Fine-tune the ^{19}F reference frequency by running a quick pulse-acquire spectroscopy sequence (90 excitation, TR = 1000 ms, 2048 point readout

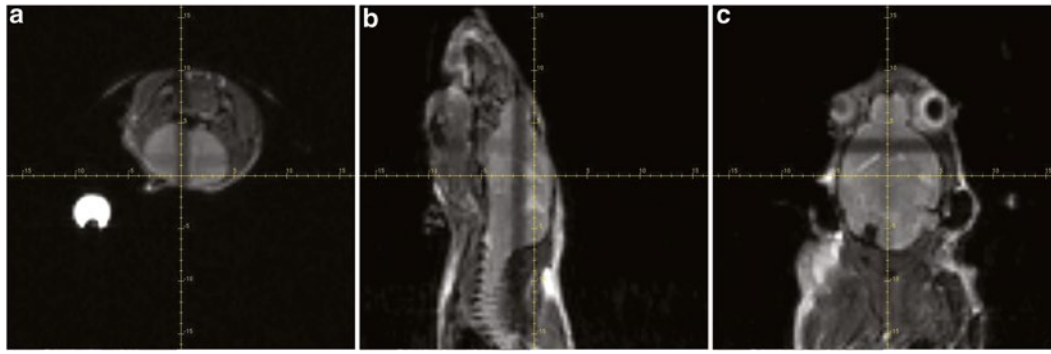

Fig. 1 Representative localizer scans. Interleaved (**a**) axial, (**b**) sagittal, and (**c**) coronal images show that target anatomy (brain) is positioned correctly within the imaging system

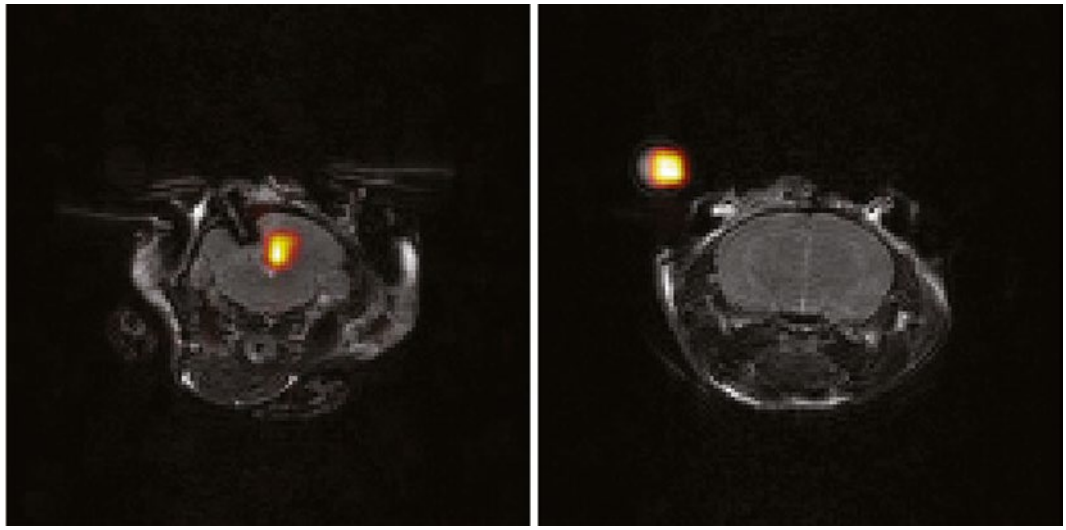

Fig. 2 Representative overlay of ^{19}F images on T_2-weighted anatomic references. *Left* image shows intracranial signal after injection of ^{19}F-labeled NK cells. At *right*, agreement between proton and ^{19}F images in external reference demonstrates good co-registration

over 25 kHz bandwidth, approximately 10 averages to ensure sufficient signal-to-noise to measure the resonance frequency of the reagent in tissue and/or within the external NMR bulb).

8. Acquire ^{19}F images that are co-registered with anatomic reference images using the 3D fast spin-echo sequence, tailored for ^{19}F (TE_{eff} = 71.5 ms, TR = 700 ms, ETL = 24, 80 signal averages, 32 × 32 × 32 matrix size covering 3 cm × 3 cm × 3 cm field-of-view) (*see* **Note 17**). Make sure that the slice prescription and imaging geometry match the anatomic references.
9. Overlay ^{19}F images (**step 8**) on the anatomic reference images (**step 6**) using image-processing software such as Matlab (Mathworks) or Photoshop (Adobe Systems Inc.) *see* Fig. 2.

3.4 Determining ^{19}F Labeling of NK Cells

The NK cells labeled by the dual-mode imaging reagent CS-ATM DM Green described in Subheading 3.2.1 can be assessed for ^{19}F labeling qualitatively by flow cytometry and quantitatively by nuclear magnetic resonance (NMR).

1. To qualitatively assess labeling by flow cytometry, recover about 5×10^5 unlabeled NK (as control) and ^{19}F labeled NK cells in NK cell medium into separate FACS tubes. Run the samples on flow cytometer in the FITC channel (*see* **Note 18**) (Fig. 3a).
2. To determine the number of fluorine atoms incorporated per cell by NMR, collect 3×10^6 ^{19}F labeled NK cells in a 15 ml conical tube add PBS to fill the tube.
3. Spin cells at 400 × *g* for 5 min.

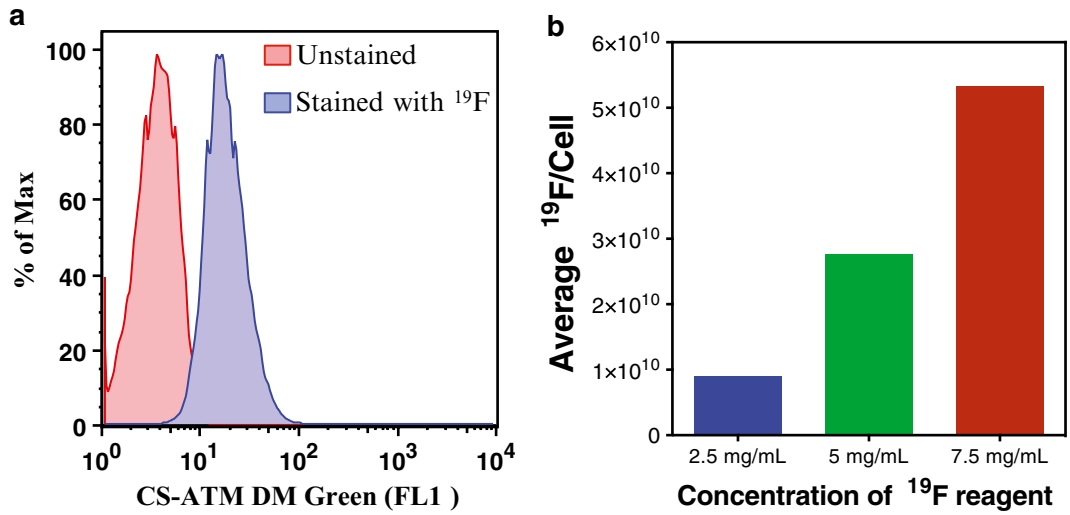

Fig. 3 Determining ^{19}F labeling of NK cells. (**a**) Qualitative assessment of CS-ATM DM green (^{19}F) labeling of NK cells by flow cytometry compared to unstained control NK cells. (**b**) Quantitative analysis of ^{19}F labeling of NK cells, showing the number of fluorine atoms/NK cell after overnight incubation with CS-ATM DM Green

4. Aspirate the supernatant and repeat wash step four more times for a total of five washes.
5. Aspirate the PBS as completely as possible taking care not to lose NK cells.
6. To the NK cell pellet add 100 µl of 2× lysis buffer, and mix well using P-200 pipette to promote complete lysis of cells.
7. To the lysate, add 200 µl of D_2O.
8. Prior to running the sample on NMR, add 200 µl of 0.1 % TFA as an internal reference standard for fluorine, and mix thoroughly using a P-200 pipette.
9. Transfer the sample to 5 mm NMR tubes and acquire ^{19}F NMR spectrum (*see* **Note 19**).
10. Calculate the number of fluorine atoms per cell using the following formula: (Fig. 3b).

$$^{19}\mathrm{F}_{\mathrm{atoms/cell}} = 3I_s M_r N_a / I_r N_c$$

I_s = Integrated area of major peak of the cell pellet
M_r = Moles of TFA reference (1.75×10^{-6} moles for 200 µl of 0.1 % TFA)
N_a = Avogadro's number
I_r = Integrated area under TFA reference peak
N_c = number of cells in the pellet

3.5 Effect of ^{19}F Labeling on NK Cell Function

It is essential to understand the effect of ^{19}F labeling on NK cells cytolytic function, as this could impact their therapeutic potential. Numerous methods can be used to determine NK cell cytotoxicity

such as Chromium-51 release assay, LDH release assay, nonradioactive Europium release and flow cytometry based methods, to name a few. This protocol describes calcein release assay for quantitating cytotoxicity of NK cell with and without ^{19}F labeling using K562 as the target tumor cell line.

1. Maintain K562 cell line in complete culture medium and passage at 1:6 split ratio when the cell density exceeds 0.6×10^6 cells/ml.
2. To stain K562 with calcein, count and transfer 2×10^6 K562 cells (*see* **Note 20**) into a 15 ml conical tube and spin at $400 \times g$ for 5 min. Resuspend cell pellet in 2 ml of fresh complete culture medium (cell density of 1×10^6/ml).
3. Add stock calcein (1 mg/ml) to the cells at 1:333 fold dilution (3 μl/ml). Incubate for 30 min at 37 °C in a CO_2 incubator, and vortex cells every 5 min.
4. Wash the cells three times with 10 ml of complete culture medium to remove excess calcein-AM, spin at $400 \times g$ for 5 min. Resuspend calcein labeled K562 in 10 ml medium and transfer to a 50 ml conical tube, perform a cell count and add medium to adjust cell density to 1×10^5 cells/ml.
5. Count and resuspend NK cells with and without ^{19}F labeling in complete culture medium at 1×10^6 cells/ml (*see* **Note 21**).
6. Seed 200 μl of NK cells per well in 3 wells, in a "U" bottom 96-well plate (e.g., unlabeled NK cells in A1, B1, C1, and ^{19}F labeled NK cells in A7, B7, C7).
7. Add 100 μl of complete culture medium to wells A2–C6 and A8–C12.
8. Perform a twofold serial dilution of NK cells (by transferring 100 μl of NK cells serially across columns) (*see* **Note 22**).
9. Add 100 μl of complete culture medium to 6 wells (e.g., E1–E6) for spontaneous release control.
10. Add 100 μl of 2% Triton X -100 to 6 wells (e.g., G1–G6) for maximum release control (*see* **Note 23**).
11. Add 100 μl of calcein loaded K562 to the wells containing NK cells and the spontaneous control and maximum release control wells. Mix gently.
12. Spin the plate at $100 \times g$ for 1 min.
13. Incubate the plate for 4 h at 37 °C in a CO_2 incubator.
14. After 4 h, remove the plate, gently mix the contents of each well, in order to evenly distribute the released calcein using a P-200 pipette and spin the plate at $400 \times g$ for 1 min.
15. Carefully aspirate 100 μl of the supernatant using P-200 pipette and transfer into a clear flat bottom 96-well plate (*see* **Note 23**).

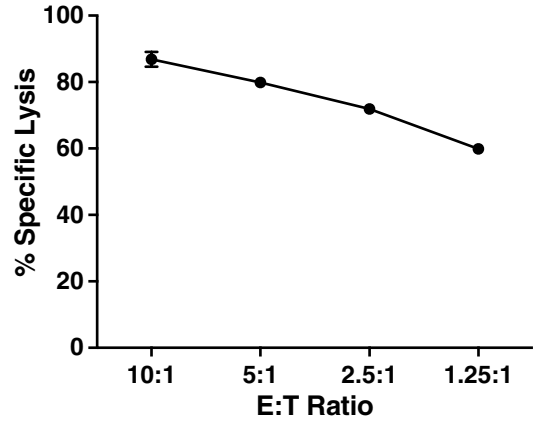

Fig. 4 Representative NK cell cytotoxicity assay. Cytotoxicity of NK cells labeled with 7.5 mg/ml of ^{19}F, note that the NK cells continue to be highly cytotoxic against K562 cell line after labeling with ^{19}F (CS-ATM DM Green) reagent

16. Read fluorescence intensity using a fluorescence spectrophotometer (excitation filter 485 nm: emission filter 530 nm).
17. Calculate percent specific lysis of NK cells using the formula [(Test release—Spontaneous release)/(Maximum release—Spontaneous release)] × 100 (*see* **Note 24**) (Fig. 4).

4 Notes

1. NK cell lines, primary or expanded human NK cells can be used for this study depending on the user's interest or research goal. The protocol described here is for expanded NK cells. If primary NK cells or NK cell lines are used for experiments, optimize ^{19}F labeling as per *see* **Note 10**.
2. Dual mode MRI/Optical reagent is used for this protocol. This reagent contains ^{19}F as well as fluorescein within the emulsion to facilitate qualitative assessment of cell labeling by flow cytometry as well as quantitative assessment of ^{19}F labeling (number of fluorine atoms per cell) by fluorine Nuclear Magnetic Resonance (NMR).
3. Animals should be continually monitored for anesthetic depth with toe or tail pinch throughout the duration of anesthesia and surgical procedures.
4. Surgical coordinates used for various locations in the mouse brain:

   Cerebellum: 2 mm posterior to lambdoid suture, 2 mm lateral of midline, 2–3 mm below skull surface

   Cerebrum: 2 mm anterior to lambdoid suture, 2 mm lateral of midline, 2–3 mm below skull surface

4th Ventricle: 1.5 mm posterior to lambdoid suture, 0.5 mm lateral of midline, 4 mm below skull surface

Brainstem/Pons: 1.5 mm posterior to lambdoid suture, 0.5 mm lateral of midline, 5 mm below skull surface

5. It is strongly suggested that a tumor growth curve is established prior to any therapeutic experiment for each separate cell line and at various numbers of injected cells. This will assist in determining the number of cells to be injected for the calculated experiment timeline and tumor burden.

   For the experiment detailed in this chapter, 50,000 DAOY medulloblastoma cells were injected because these cells establish tumors and progress extremely quickly. However, in cell lines that take significantly longer to establish tumors, cell numbers will be much higher based on established growth curves.

6. Criteria for tumor establishment can be measured in a number of ways. If the tumor cells injected are not labeled for fluorescence or luminescence (e.g., firefly luciferase) imaging, magnetic resonance imaging can be used to monitor tumor establishment. However, whether or not imaging is done to observe tumor establishment, mice should be monitored for neurological, behavioral, and overall health effects of tumor burden. Tumors are allowed to grow until tumor burden is clinically visible. Mice are then euthanized and the brain can be collected for histopathological analyses.

7. Use T25 flask for culture volume from 5 to 10 ml, and for volume between 10 and 40 ml, use a T75 flask. Over 40 ml of culture volume use multiple flasks. Incubate the flasks upright in the CO_2 incubator.

8. We expand NK cells in the presence of 50 IU/ml of IL2; therefore, we supplement the medium with 50 IU/ml of IL2 to provide the cytokine support to NK cells after thawing. If a different amount of IL2 was used for activating or expanding NK cells prior to freezing them, then use the same concentration of IL2 in the medium after thawing.

9. Always stain more cells than absolutely needed for injection/experiments as loss of cells is expected during wash steps or due to loss of viability in culture.

10. We tested various cell densities and reagent concentration to optimize ^{19}F labeling of the cells in the minimum possible volume (that did not affect the NK cell viability and labeling efficiency). If primary NK cells or NK cell lines are used, we recommend optimizing the ^{19}F labeling initially with cell numbers ranging from 1×10^6/ml to 5×10^6/ml.

11. For staining NK cells with ^{19}F reagent, we normally seed up to 1 ml of cells/well of 24-well plate; 2 ml of cells/well of 12-well plate; and 3 ml/well of 6-well plate. For volume more than 3 ml use multiple wells of a 6-well plate.

12. We performed labeling optimization (at 3×10^6 cells/ml) using ^{19}F at concentrations of 0, 2.5, 5, and 7.5 mg/ml for NK cells expanded on K562 Cl9.mbIL21 feeder cell platform. The ^{19}F labeling of these cells saturated at 5 mg/ml as analyzed by NMR, using this protocol. Similar optimization should be performed if primary NK cells, NK cell activated and expanded by other methods or NK cell lines are used for ^{19}F labeling studies.
13. Inhalable anesthetics such as isoflurane are convenient because they allow remote adjustment, without having to interrupt the imaging protocol or move the animal. Unfortunately, isoflurane contains ^{19}F, which will concentrate in tissue to give a competing signal. Injectable anesthetics do not add a ^{19}F signal, but dosing must be carefully administered to ensure that the animal is sedated long enough for the imaging measurement.
14. Sensors may include bellows for monitoring respiratory rate, electrocardiogram (ECG) for monitoring heart rate, and/or a thermocouple to monitor body temperature. All of these must be MRI compatible. Physiological monitoring systems for animal models are commercially available, such as the Model 1030 Monitoring & Gating System from Small Animal Instruments, Inc.
15. The sensitivity of ^{19}F scans needs to be enhanced as much as possible by using good quality coils for signal excitation and detection. There is a wide range of possible coil configurations, including the use of large "volume" coils that give relatively uniform sensitivity, small "surface" coils that provide high sensitivity over a small region, and arrays of surface coils that provide good sensitivity over an extended range. Multinuclear measurements can be particularly complex, since coils are needed that are sensitive to the resonance frequencies of both ^{1}H, for anatomic reference using traditional MRI, and ^{19}F for cell tracking. The approach described in this work utilizes volume coils for both nuclear targets. The two volume coils have the same dimensions and are simply exchanged in order to swap between imaging targets.
16. The ^{19}F signal is generally not strong enough to permit use of automatic prescan adjustments for calibration of the excitation power. Calibration measurements can be performed prior to imaging using a ^{19}F phantom, or if a reference standard is present during the imaging measurement, signal may be strong enough for manual calibration. A simple pulse-acquire MR spectroscopy sequence can be used for this purpose. The repetition time for the sequence should be set at greater than several times the characteristic spin–lattice (T_1) relaxation time of the ^{19}F agent at the field strength for the MRI system. Then, the excitation power for the pulse-acquire sequence can be set very low and then increased until the observed signal reaches a maximum, which corresponds to a 90° excitation. A slightly more accurate approach would involve several measurements

ranging from low power to approximately 180° excitation (when the signal vanishes) and fitting observations to the expected sinusoidal signal response curve. Calibration may need to be adjusted if coil loading changes substantially between measurement conditions.

17. The relaxation characteristics of the ^{19}F agent should be measured prior to imaging. The spin–lattice (T_1) and spin–spin (T_2) relaxation times will vary with the field strength of the MRI magnet. There are a number of methods that can be used for making these measurements. We used a saturation-recovery imaging sequence to measure the T_1 of CelSense at 7 T, and a Carr–Purcell–Meiboom–Gill (CPMG) spin-echo sequence to measure T_2. With $T_1 \cong 550$ ms and $T_2 \cong 240$ ms, efficient sequences with a long echo train and relatively short repetition times (TR) can be used for imaging with good sensitivity.
18. Select live cell population in forward and side scatter plot and display this population on FITC channel of the flow cytometer. The fluorescence method is strictly a qualitative assessment of labeling and is not a substitute for quantitative estimation of ^{19}F labeling by NMR analysis, which can provide number of fluorine atoms incorporated per cell, which could subsequently be used to calculate the number of NK cells at a given location based on ^{19}F signal strength in vivo.
19. Since most institutions offer NMR as a core service, to acquire and analyze ^{19}F spectrum by NMR, we recommend the users to refer to their Institutional core resources. The NMR should be equipped with probe for observing ^{19}F.
20. The recommended number of K562 cells is for one full 96-well plate cytotoxicity assay. Since 10,000 target cells are seeded per well—a minimum of 1×10^6 cells are needed for a full 96-well plate, including +4 for pipetting errors; however, 2×10^6 cells are recommended here as a twofold excess to accommodate for cell loss during wash steps and for accommodating multichannel pipetting of target cells from a media basin.
21. Note that the target cells are resuspended at 1×10^5 cells/ml and NK cells are resuspended at 1×10^6 cells/ml, this recommendation is for setting up the cytotoxicity assay at effector to target (E:T) ratios starting at 10:1. If primary NK cells or NK cell lines are used, the E:T ratio can be changed as desired, which will then dictate the density for resuspending NK cells (e.g., 2×10^6 NK cells/ml for a 20:1 E:T ratio). For the recommended assay 600,000 NK cells will be used per cytotoxicity condition; therefore, about 650,000 NK cells will be needed per condition to accommodate for errors.

22. After serial dilution the wells A6, B6, C6 and A12, B12, C12 will have 200 μl of cells. Discard 100 μl of cells from these wells.
23. A blank row is recommended between test and controls to prevent accidental spill over during setup and fluorescence bleed-over during plate reading.
24. A well optimized ^{19}F labeling of NK cells should have sufficient fluorine atoms to provide strong signal in MRI for in vivo imaging of NK cells, with minimal impact on NK cell cytolytic function.

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Chapter 28

Generation of BiKEs and TriKEs to Improve NK Cell-Mediated Targeting of Tumor Cells

Martin Felices, Todd R. Lenvik, Zachary B. Davis, Jeffrey S. Miller, and Daniel A. Vallera

Abstract

Cancer immunotherapies have gained significant momentum over the past decade, particularly with the advent of checkpoint inhibitors and CAR T-cells. While the latter personalized targeted immunotherapy has revolutionized the field, a need for off-the-shelf therapies remains. The ability of NK cells to quickly lyse antibody-coated tumors and potently secrete cytokines without prior priming has made NK cells ideal candidates for antigen-specific immunotherapy. NK cells have been targeted to tumors through two main strategies: mono-specific antibodies and bi/tri-specific antibodies. Mono-specific antibodies drive NK cell antibody-dependent cell-mediated cytotoxicity (ADCC) of tumor cells. Bi/tri-specific antibodies drive re-directed lysis of tumor cells through binding of a tumor antigen and direct binding and crosslinking of the CD16 receptor on NK cells, thus bypassing the need for binding of the Fc portion of mono-specific antibodies. This chapter focuses on the generation of bi- and tri-specific killer engagers (BiKEs and TriKEs) meant to target NK cells to tumors. BiKEs and TriKEs are smaller molecules composed of 2–3 variable portions of antibodies with different specificities, and represent a novel and more versatile strategy compared to traditional bi- and tri-specific antibody platforms.

Key words BiKE, TriKE, Bi-specific, Tri-specific, Targeted immunotherapy, NK, Natural killer, ADCC, Redirected lysis

1 Overview

Targeted cancer immunotherapies are currently a subject of great clinical interest and potential [1]. While a great deal of interest has recently been placed upon generation of chimeric antigen receptor (CAR) expressing T cells from monoclonal antibodies shown to target human malignancies [2], and even more recently upon generation of CAR-expressing natural killer (NK) cells [3, 4], these approaches require a personalized approach that is expensive, time consuming, and difficult to apply on a large scale. There is a clear

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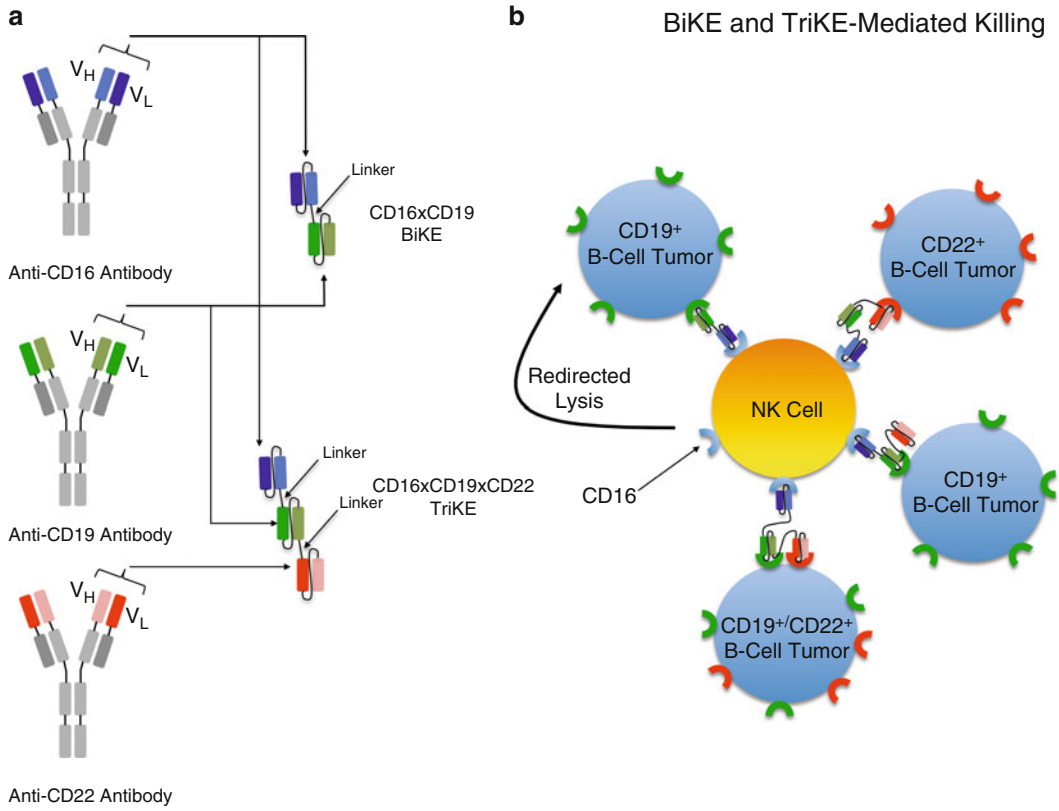

Fig. 1 Structure and function of BiKEs and TriKEs. (**a**) BiKEs and TriKEs are constructed from a single heavy (V_H) and light (V_L) chain of the variable region of each antibody of interest. V_H and V_L domains are joined by a short flexible polypeptide linker to prevent dissociation. Shown here is a BiKE constructed from the variable regions of anti-CD16 and anti-CD19 and a TriKE constructed from the variable regions of anti-CD16, anti-CD19, and anti-CD22. (**b**) BiKE and TriKE binding to NK cells and their targets result in the formation of an immunological synapse and triggers NK killing of the target cell through activation of the low affinity Fc receptor, CD16, on NK cells. The CD16 × CD19 × CD22 TriKE can recognize targets expressing CD19 (*green receptors*), CD22 (*red receptors*) or both receptors simultaneously allowing for more versatile target recognition than the CD16 × CD19 BiKE

need for targeted off-the-shelf therapies that augment the current monoclonal antibody approach. This chapter focuses on generation of bi- and tri-specific killer engagers (BiKEs and TriKEs) meant to target NK cells to the tumor synapse and induce their activation at that site (Fig. 1). Unlike full-length bi- and tri-specific antibodies, BiKEs and TriKEs are small molecules containing two (BiKE) or three (TriKE) single chain variable fragments (scFv) from antibodies of different specificity.

1.1 Mediation of NK Cell Function Through CD16 and ADCC

NK cells are ideal candidates for immune cell-targeted therapies because they do not require prior sensitization to lyse tumor targets and to release pro-inflammatory cytokines, are not HLA-restricted, and can mediate graft-versus-leukemia (or tumor) without inducing graft-versus-host disease [5, 6]. Although NK cells possess a variety of activating receptors and can mediate

function in several different ways, their role in antibody-dependent cell-mediated cytotoxicity (ADCC) is of particular relevance in this chapter. ADCC is mediated by CD16 (FcγRIII), the low affinity receptor for IgG Fc [7]. Two isoforms of CD16 exist in humans, CD16A and CD16B [8]. CD16A is expressed in NK cells, macrophages, and placental trophoblasts as a polypeptide-anchored transmembrane protein while CD16B is expressed in neutrophils in a GPI-anchored form [9–12]. Although the extracellular portion of CD16A and CD16B share a high level of homology (95–97%), CD16A can trigger killing of tumor targets and cytokine production while CD16B cannot [9, 13–15].

In human NK cells, CD16 is mostly expressed in the CD56dim subset, although populations of CD56bright CD16+ NK cells have been observed after transplant [16, 17]. Engagement of CD16 through encounter with the Fc portion of antibodies or direct crosslinking by anti-CD16 antibody results in signals through the immunoreceptor tyrosine-based activation motif (ITAM) of the associated FcεRIγ and CD3ζ chain subunits, leading to cytokine and cytotoxic responses [18–20]. Unlike other activating receptors present in human NK cells, CD16 can robustly mediate activation without the need for co-engagement of other receptors [21]. These signaling properties allow for NK CD16-mediated targeting of antibody-coated cells in natural settings of viral infection, autoimmunity and the onset of some forms of tumors [22–24]. The latter has been exploited in the clinic by generating monoclonal antibodies (mAbs) targeting specific tumor antigens to drive ADCC against those tumors [25–29].

1.2 Potential Advantages of BiKEs and TriKEs Over Conventional Antibodies

Driving ADCC through mAbs has resulted in significant clinical success. Specific targeting of tumors with BiKEs and TriKEs has the potential to build upon this success and improve efficacy. Binding affinity appears to be an important component of ADCC. This impression is supported by increased rituximab-driven cytotoxicity of B cell tumors mediated by NK cells containing the CD16A-158VV or VF allotypes which, when compared to the CD16A-158FF allotype, display decreased affinity for the Fc portions of antibodies [30]. Therefore, BiKEs and TriKEs might improve NK cell function by generating a stronger interaction through binding with anti-CD16 than that produced by binding of CD16 to the natural Fc portion of antibodies. This increase in affinity and cytotoxicity was demonstrated in a study comparing natural binding of CD16 to the Fc portion of an anti-HER2 antibody versus binding of CD16 through an anti-HER2 x anti-CD16 bi-specific antibody. Data showed a 3.4 fold increase in affinity in the bi-specific antibody versus binding of the native anti-HER2 Fc [31]. The efficacy of therapeutic mAbs in vivo, in contrast to their high ADCC efficacy in vitro, is further attenuated by the presence of physiologic serum IgG levels in plasma. In the in vivo setting, ADCC potency is diminished by saturation of CD16 receptors,

thus competing for binding with the therapeutic mAb [32]. Such competition for binding of the Fc portion of therapeutic antibodies requires high serum levels of the mAb to be sustained over several months of treatment in order to achieve in vivo efficacy [33, 34]. BiKEs and TriKEs bypass this obstacle by binding the CD16 receptor directly. An additional benefit that BiKEs and TriKEs may have over mAbs is superior biodistribution as a consequence of their smaller size, particularly in the treatment of solid tumors [35–37]. In addition to these advantages, BiKEs and TriKEs are non-immunogenic, have quick clearance properties and can be engineered quickly to target known tumor antigens. These attributes make them an ideal pharmaceutical platform for potentiated NK cell-based immunotherapies.

1.3 Bi- and Tri-specific Reagents Targeting NK Cells and Tumors

Over the past two decades multivalent antibodies, and more recently BiKEs and TriKEs, have been used to target tumor antigens and CD16 on NK cells [38]. While approaches for assembly of multivalent antibodies and the current methodology for BiKE and TriKE engineering has evolved, the function of these reagents remains unchanged. Bi-specific and Tri-specific reagents have been generated to engage CD16 on the NK cell and the following tumor antigens: CD20 and CD19 on B cell non-Hodgkin's lymphomas [39–47], CD19 and CD33 on mixed lineage leukemia [48], CD33 or CD33 and CD123 on acute myelogenous leukemia (AML) [49–51], HLA Class II on lymphoma [52], CD30 on Hodgkin's disease [53–62], EGF-R on EGF-R+ tumors [63, 64], HER2/neu on metastatic breast cancer and other HER2 expressing tumors [31, 65–71], and MOV19 on ovarian cancer [72]. Our group has contributed to the field through generation and testing of BiKEs and TriKEs that target CD16 and CD19/CD22 on B cell non-Hodgkin's lymphomas [73], CD33 on AML [74] and MDS/MDSCs [75], and EpCAM on prostate, breast, colon, head, and neck carcinomas [76]. Activation through the BiKEs and TriKEs elicited potent cytotoxicity and cytokine secretion. In the case of the CD16×CD19×CD22 TriKE, the CD107a response to primary CLL and ALL exceeded that of rituximab. The CD16×CD33 BiKE was capable of overcoming HLA-mediated inhibition with primary refractory AML blasts and restored function of NK cells from MDS patients. Encouraged by their translational potential, we are currently producing some versions of these reagents for clinical use. Basic reagent production methods are described in the next section.

2 Methodology

2.1 Selection of Variable Fragment Source and Linkers

BiKE design is a complex process. This section provides an overview of the entire methodology (summarized in Fig. 2). Once a target of interest has been defined, the first step in the design of BiKEs requires selection of a source for the variable fragments.

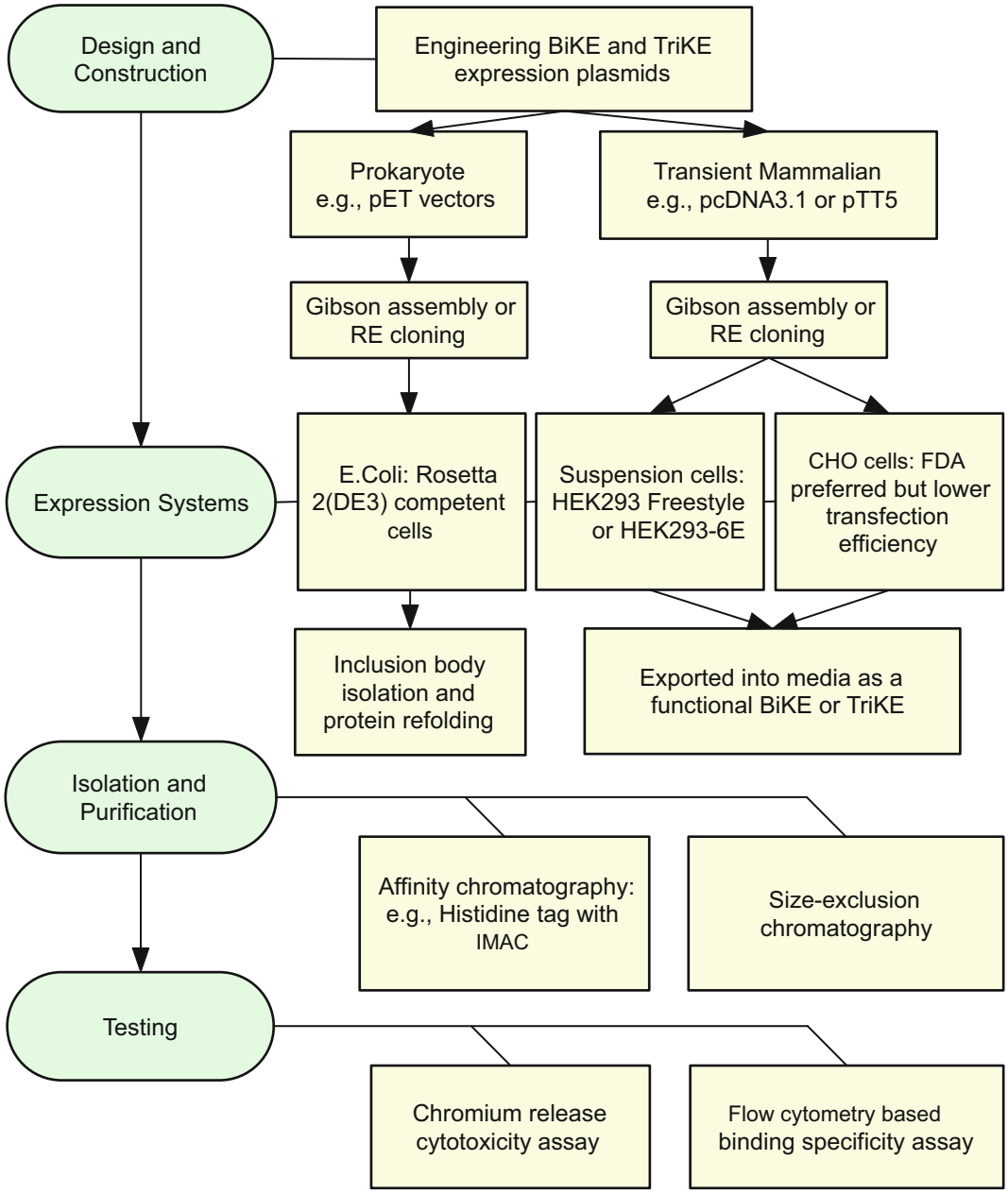

Fig. 2 Workflow for generation of BiKEs and TriKEs. In the left (*green*) are the four steps necessary for generation and validation of the BiKE/TriKE constructs. In the right (*yellow*) are possible options for each of the steps. CHO: Chinese hamster ovary cells. IMAC: immobilized metal affinity chromatography. RE: restriction enzyme

Sequences for relevant fragments can be obtained from published work, hybridomas, B cells from immunized animals, phage display and other such display technology. For bacterial expression systems, phage display is ideal because the constructs are selected in bacteria, essentially pre-screening their function in the system of expression. The next step involves selection of a proper linker. BiKEs combine two different antigen-binding sites with a short flexible linker. The antigen-binding domains are single-chain variable fragments (scFv), which consist of heavy and light variable domains, also fused with a flexible linker (V_H-linker-V_L) [35]. The main linker design is important to the function of the BiKE by allowing separation of the functional domains as well as providing flexibility to bind the two (or three in the case of the TriKE) epitopes on the different targeted cells [77]. The (SGGG)4 linker is one of the first flexible linkers used in the construction of single-chain variable fragments (scFv) [78]. Another commonly used linker, the 218s linker (GSTSGSGKPGSGEGSTKG), is reported to improve proteolytic stability and reduce aggregation [79]. To reduce immunogenicity, our group utilized an HMA linker (PSGQAGAAASESLFVSNHAY) between the antiCD16 and the tumor antigen scFv [76].

2.2 Selection of Vector and Expression System

Once the components of the BiKE have been determined, selection of an appropriate vector for expression follows. We and other investigators focus on plasmid expression systems in bacteria and mammalian cells to create BiKEs, but there are other less utilized expression systems, such as lentivirus or sleeping beauty, which will not be discussed in this section. For bacterial expression systems, the pET vector is the system most commonly used in conjunction with the Rosetta 2(DE3) host cells (Novagen). The Rosetta 2(DE3) cells contain an IPTG- inducible T7 RNA polymerase, which is compatible with the pET vectors. Another feature of this strain is that it has been engineered to express a "universal" set of transfer RNAs as a way to mitigate the need for codon optimization. For transient mammalian expression systems, the pTT5 vector can be utilized in conjunction with the HEK293-E6 suspension cells or the pcDNA3.1 system can be used with the HEK293 Freestyle cells (Invitrogen). Reported yields have been higher in the HEK293-E6 system [80]. These cells express a truncated variant of the Epstein Barr virus (EBV) for which pTT5 vector contains the short EBV oriP for episomal replication. These two systems display advantages in yields and ease of use but a number of other systems utilizing different vectors can also be applied [80, 81].

2.3 Cloning the BiKE/TriKE Components into Expression Vector

Upon selection of a vector, one can begin cloning the BiKE components into the vector backbone. Significant advances have been made in the recombination technique. While there are several ways to clone DNA fragments into the vector backbone, we and others

favor Gibson assembly because it is cost and time efficient [82]. Gibson assembly utilizes in vitro homologous recombination through insertion of a DNA fragment into a vector, where insertion is directed by homologous regions that are present at the end of the insert DNA and the linearized vector DNA [83]. An advantage of Gibson assembly over standard restriction cloning is that it requires little to no restriction enzyme utilization and multiple pieces can be cloned in one reaction. With the advent of this method, together with recent access to inexpensive high-fidelity synthetic DNA, it is now possible to construct BiKE expression plasmids in a few days of labor.

2.4 Expression and Isolation of the BiKEs/TriKEs

Following preparation, the BiKE expression vector can then be chemically transduced into E. coli or transfected into mammalian cells through lipid or chemical means and, to a lesser extent, through electroporation. The advantage of E. coli versus the mammalian system is that it allows for quick, easy, robust, and inexpensive expression of the BiKEs [84]. An important difference between the bacterial and mammalian systems is that in the mammalian system, fully functional proteins are secreted and can be harvested from the supernatant. A disadvantage of bacteria is that most recombinant proteins are found in an insoluble form, termed an inclusion body [85]. To resolve this problem, lysis of the bacteria and isolation of the inclusion bodies through centrifugation followed by solubilization with strong denaturing reagents is required. The protein then must be refolded. Refolding is carried out at low protein concentrations. Conditions for refolding of the recombinant protein must be optimized (i.e., pH, ionic strength, temperature, and redox environment). The protein can then be isolated through size exclusion chromatography or through the use of an affinity tag, such as histidine-tags [85, 86]. As discussed, both systems have advantages and disadvantages. While the bacterial system is quick, easy, and robust, the mammalian system does not require re-folding and can be utilized to generate smaller amounts of functional protein quickly for initial screening. Another consideration possibly favoring the mammalian approach is that most therapeutic recombinant proteins gaining FDA approval are made in Chinese hamster ovary (CHO) cells [87].

2.5 Testing the BiKEs and TriKEs

Flow cytometry is used to evaluate binding of the constructs to their respective targets. Prior to incorporation into the full bi- or tri-specific constructs containing the anti-CD16 variable portion and the linker/s, individual variable portions containing a His-tag or similar small tag are incubated with cells expressing the antigen of interest or cells expressing an irrelevant antigen, to evaluate non-specific binding. A biotinylated anti-His antibody is then used to recognize the His-tag on the variable portion, followed by addition of fluorescently labeled streptavidin to attain fluorescent

conjugation. To ensure that the variable fragment is binding to the desired antigen, binding is then compared to fluorescently labeled commercial antibodies to the antigen of choice. Alternatively, the variable portion can be biotinylated or fluorescently labeled directly. However, this approach may increase risk of altering binding to the antigen. If the variable construct is designed from a known antibody for which fluorescently labeled forms already exist, the construct can be tested in a competition assay. In such assays, increasing concentrations of construct are bound to the cells expressing the specific antigen prior to addition of the known fluorescently labeled antibody. Specific binding is then measured by a decrease in binding of the fluorescently labeled full antibody form, indicating binding of the variable fragment to the antigen.

Once specific binding has been confirmed, the variable portion is incorporated into a full BiKE or TriKE construct and the functional activity of the construct is evaluated by two different methods. First, the ability of NK cells to degranulate in response to targets coated in the construct is assessed by a redirected lysis assay. Peripheral blood mononuclear cells (PBMCs) or purified NK cells are co-cultured with targets at a range of effector to target (E:T) ratios (1:1 to 20:1) in the presence or absence of a saturating concentration of the BiKE/TriKE of interest. Higher ratios are required for PMBCs when compared to purified NK cells. Effectors, targets, and constructs are incubated together for several hours (usually 4) and then surface LAMP-1 (CD107a), used to evaluate degranulation, and intracellular IFN-γ and TNF-α, used to evaluate cytokine secretion, are assessed on the NK cells by flow cytometry. Irrelevant targets are used as a negative control in this assay, while full length antibodies that direct ADCC towards the antigen of choice are used as a positive/comparative control.

While this assay determines the level to which NK cells are activated, it does not reflect the level of target cell killing in response to the NK cell activation. To evaluate target cell killing a cytotoxicity assay, such as a chromium release assay, is performed. In this assay, target cells are labeled with radioactive Chromium-51 (^{51}Cr) prior to co-culture with PBMCs or purified NK cells and the BiKE/TriKE. E:T ratios in this assay range from 20:1 to 0.625:1. Wells containing targets without NK cells are plated for use as maximum (10% SDS mediated lysis) and minimum (no treatment) release groups. These groups are used for the calculation of percent targets killed. During the incubation, as target cells are killed they release ^{51}Cr into the supernatant while the targets that remain alive keep the ^{51}Cr sequestered inside the cell. ^{51}Cr release is then assessed on a gamma counter and the percent of targets killed is calculated. Controls similar to those mentioned in the flow-based assay are also included. Once the specificity and efficacy of the BiKEs/TriKEs has been determined, the constructs can now be tested with clinical samples and/or in more complex in vivo killing assays utilizing NSG mice, engrafted xenogeneic tumors, and transferred human NK cells.

3 Future Directions

Although current BiKE and TriKE constructs display great translational potential, efforts are currently underway to further improve their efficacy. One obstacle that could limit the efficacy of BiKEs and TriKEs, as well as all other antibody therapy mediated through NK cells, is CD16 expression. NK cell-mediated ADCC by therapeutic antibodies depends on ligation of CD16, on the NK cell, with the Fc portion of the antibody [88]. BiKEs and TriKEs, as well as other formats of bi- and tri-specific antibodies, mediate redirected lysis of the target and NK cell function through direct binding and crosslinking of the CD16 receptor. This bears relevance because CD16 is rapidly clipped from the surface of NK cells activated through CD16 by matrix metalloproteinases (MMPs), in particular ADAM-17 [89–92]. Activation through cytokines can also result in the clipping of CD16 [93]. Loss of surface CD16 expression on activated NK likely results in a diminished capacity to mediate subsequent rounds of ADCC. To address this concern, we and others are currently evaluating MMP-specific inhibitors as a means to prevent CD16 clipping during NK cell activation [94, 95]. We have demonstrated that inhibition of ADAM-17 results in superior function post CD16 crosslinking and can potentiate rituximab-mediated responses in vitro. We have also shown that ADAM-17 inhibition can enhance BiKE mediated killing against myeloid targets in vitro [74]. These results indicate that co-treatment with ADAM-17 inhibitor may be a good strategy to enhance BiKE/TriKE function in the clinic.

A different approach to circumvent the CD16 problem is to target other receptors on the NK cells with the BiKEs and TriKEs. CD16 was originally selected owing to its ability to potently activate NK cells and overcome inhibitory signaling [21]. This was highlighted in the BiKE system showing that the CD16×CD33 BiKE could overcome HLA-mediated inhibition in primary AML blasts and could restore NK cell function from MDS patients, whose natural cytotoxicity is thought to be impaired [74, 75]. However, co-engagement of other receptors, particularly NKG2D and 2B4, has been shown to induce activation similar to that provided by CD16 alone [21]. There is also potential for TriKEs engaging CD16, a tumor antigen, and another NK cell activating or co-stimulatory receptor. For instance, co-engagement of CD16 with DNAM-1, CD2, or 2B4 was shown to potentiate function in NK cells from MDS patients [75]. Co-administration of cytokines may also enhance BiKE mediated NK cell function. Several cytokines, including IL-15, IL-2, IL-21, and IL-12 have prominent roles in NK cell development, proliferation, survival, and/or activation. Encouraged by these attributes, trials are underway to implement them in the clinic [96]. Besides the aforementioned

attributes, some of these cytokines have been shown to also potentiate ADCC, making them an interesting co-therapeutic approach.

While personalized CAR-T cell therapies have recently enjoyed a great deal of clinical success [2], there remains a clear need for off-the shelf reagents that enhance targeting of the immune system to tumor antigens. Directing targeting of NK cells is a compelling therapeutic approach on the basis of their ability to quickly kill tumors and secrete cytokines without prior priming [5]. BiKEs and TriKEs are an important conduit for achieving this since they are relatively easy to produce, drive potent NK cell activation through CD16 crosslinking, and can be utilized to target almost any tumor antigen for which an antibody has been designed. This is true regardless of whether the antibody displays activating properties because the activation is driven through the CD16 scFv. To date our group has primarily focused on non-Hodgkin's lymphoma (through CD19 and CD22), AML and MDS (through CD33), and breast, colon, and lung carcinomas (through EpCAM) [73–76]. Notably, there are an abundance of promising tumor antigens for which therapeutic antibodies have been designed that could be incorporated into BiKE and TriKE platforms [37]. These include CD30 (Hodgkin's lymphoma), CD52 (CLL), CEA (breast, colon, and lung), gpA33 (colorectal), CAIX (renal cell), Mucins (breast, colon, lung, and ovarian), PSMA (prostate), VEGFR (epithelium-derived solid tumors), VEGF and Integrins αVβ3 and α5β1 (tumor vasculature), EGFR (breast, lung, colon, glioma, and head and neck), and ERBB2 and ERBB3 (breast, lung, colon, ovarian, and prostate). This list, by no means comprehensive, enumerates several important hematological and solid tumors that potentially could be targeted through the powerful BiKE platform.

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Chapter 29

Regulatory Considerations for NK Cells Used in Human Immunotherapy Applications

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Abstract

Translating cellular therapy from the laboratory to the clinic is a complicated process that involves scale-up of procedures to generate clinically relevant cell numbers, adaptation to reagents and equipment that are qualified for human use, establishing parameters of safety for reagents and equipment that are not already qualified for human use, codifying these processes into standards of practice and rules of conduct, and obtaining approval from regulatory bodies based on those codified standards and rules. As the laws and regulations that apply to cellular therapy will vary by time and geography, this chapter reviews some common key principles for the manufacturing of NK cells for human use that will need to be considered within the constraints of local policies and regulations.

Key words NK cell adoptive immunotherapy, Clinical trials, Release criteria, Good manufacturing practice, Certificate of analysis

1 Introduction

The clinical application of cellular therapies has almost exclusively been the domain of academic research institutions. Pharmaceutical companies have rarely, until recently, acted on the commercial potential of these therapies because they differ greatly from and are more complex than the industry's experience in manufacturing, packaging, distribution, and delivery. In addition, cellular therapies do not generally fit the standard early-phase trial models of determining pharmacokinetics, pharmacodynamics, and dose-dependent toxicity and efficacy. Quality assurance and quality control (QA/QC) measures also differ greatly with respect to assessing the purity and potency of individual product lots.

Nonetheless, the basic principles of manufacturing products of high quality, purity, and strength still apply for maintaining reproducible safety and potency for human use. The Food and Drug Administration (FDA) [1] and the European Medicines Agency (EMA) [2] provide regulations for Current Good Manufacturing

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Practice (cGMP) that apply to US and European products, respectively, and may differ on important points as described below. The FDA rules governing GMP are codified as federal regulations under Section 21, (i.e., 21 CFR 211). GMP regulations are harmonized by the EMA under guidelines (e.g., EU GMP Guide Part I) that support processes that meet harmonized standards (e.g., ISO 9001), which then may be adopted as law by each country. Major differences in the two guidelines still exist, and efforts to further harmonize them are ongoing. The World Health Organization (WHO) has also published its own GMP requirements [3].

For Phase I trials, the Investigational New Drug (IND) application that is submitted to the FDA must contain sufficient information on the facilities, processes, equipment, and reagents used in the manufacturing process to ensure safety, identity, strength, quality, and purity of the investigational product. As stated in the *Guidance for Industry: CGMP for Phase 1 Investigational Drugs*, "Adherence to CGMP during manufacture of phase 1 investigational drugs occurs mostly through well-defined, written procedures, adequately controlled equipment and manufacturing environment, and accurately and consistently recorded data from manufacturing (including testing)." [4] These elements are codified in the IND application [5] through a detailed Chemistry, Manufacturing, and Control (CMC) document that describes in detail how the product will be manufactured in order to meet the regulations described above. This chapter will discuss specific elements in the clinical-grade manufacturing of NK cells as they relate to these guidelines, with emphasis on FDA regulations and guidance. A full list of documents providing guidance from the FDA on cell and gene therapy products is available at http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/default.htm.

2 Chemistry, Manufacturing, and Control (CMC)

The CMC document lists all of the reagents, processes, and equipment that will be used to manufacture the NK cell product, and describes the location and qualifications of the laboratory in which the manufacturing is performed. Processes need to be described in sufficient detail to convince the regulatory reviewer that a product of proper identification, quality, purity, and strength is being manufactured. The content required in a CMC varies based on the trial, but the CMC for a Phase I trial will be evaluated mainly from the point of *risk* to the patient.

Elements of the CMC for manufacturing NK cells should include [5–7]:

1. Source of, and method of acquiring, starting material for NK cells
2. Donor testing to establish infectious disease safety
3. Cell isolation and processing procedures
4. Reagents used
5. Final formulation
6. Testing to be performed
7. Release criteria to be met (Certificate of Analysis)
8. Product labeling and tracking
9. Stability monitoring plan
10. Manufacturing facility details

3 Standard Operating Procedures (SOPs)

Standard operating procedures provide detailed information on every aspect of cell manufacturing sufficient to ensure that technicians independently performing the procedure will experience the same outcome, and therefor well-written SOPs are essential to ensuring product consistency. Where additional detail is needed to ensure standardized procedures, the CMC may refer to SOPs, but they are not usually included as part of the IND application. This enables uniform practice for procedures that are common to multiple clinical products, and allows procedures to be modified when necessary to improve outcomes or reproducibility without having to modify the CMC of multiple protocols.

Certificate of Analysis (CofA)—The CofA clearly defines the results of final product testing.

Release Criteria—The release criteria are the minimum criteria that must be met in each of the parameters defined by the CofA in order for a product to be released for use in the clinical trial.

4 Specific Issues in Manufacturing NK Cells that Affect Product Safety

4.1 The Use of Products Not Approved for Use in Humans

It is relatively straightforward to use products that are already approved for use in humans in the manufacture of new products for use in humans. A copy of the product insert is typically sufficient documentation to establish safety. However, the manufacture of NK cells for clinical trials will almost certainly employ reagents or equipment that do not have such approvals. For example, CliniMACS columns and CliniMACS CD34 Reagent have FDA approval, but the XS columns for small-scale depletion and the CD3 Reagent do not. The CliniMACS reagents may be included in the CMC with a simple description and a copy of the product

insert, but the XS columns and CD3 Reagent require a letter of cross-reference to the Drug Master File from the company. Some products, such as media or cytokines, are not approved for human use but a CofA for each production lot is available from the manufacturer to demonstrate safety for use as a reagent. Some reagents will not have either a master file or CofA available, such as "homegrown" reagents, which require additional explanation in the CMC and independent testing to generate a CofA for each lot of the reagent produced during the course of the study, demonstrating that it meets the release criteria established in the CMC.

4.1.1 Qualification of Source Leukocytes

In the USA, cells of human origin used for adoptive cell therapy must be obtained from individuals that meet donation criteria as established by 21 CFR 1271 subpart C. Screening and testing measures must be established to ensure safety from relevant communicable diseases. It should be noted that unlike bone marrow or peripheral blood stem cells, donor testing must be within 7 days of cell collection for therapeutic lymphocytes.

4.1.2 Qualification of Feeder Cell Lines

Feeder cell lines are an example of reagents that require developing a CofA for release testing as part of the manufacturing process. Many of the current methods for ex vivo expansion or activation of NK cells for clinical use rely on feeder cells such as primary cell lines derived from peripheral blood [8] or umbilical cord [9], EBV-immortalized lymphoblastoid cell lines [10], cancer cell lines [11, 12], or genetically modified cancer cell lines [13, 14]. These feeder cells represent "reagents" in the NK cell manufacturing process that must be qualified as safe for human use [15]. For example, the leukemia cell line K562 was genetically modified with cytokines and costimulatory molecules to create the Clone9.mbIL21 cell line used in our clinical trials. The genetic modification of this cell line was described in the CMC with regard to transposon plasmid structure and endotoxin content, restriction fragment length, transposable element sequence, qPCR for integration copy number, karyotype and STR fingerprinting for cell line identity, and absence of bacteria, mycoplasma, or virus contamination (Fig. 1).

The most difficult and costly assays for cell line qualification are those required to determine absence of viral contamination. Contract research organizations (CRO) that specialize in these assays not only perform the assays at contracted rates but can provide valuable consultation services for determining which assays to perform. We typically perform preliminary bacterial and mycoplasma testing prior to accepting the cell line into the GMP facility. A master cell bank (MCB) is then generated, cryopreserved, and quarantined. Preliminary sterility testing is again performed in-house, and then aliquots of the MCB are sent for full and final testing by the CRO. Additional working cell banks (WCB) can be derived from the fully qualified MCB and release-tested with a more limited CofA [7].

MASTER CELL BANK – CERTIFICATE OF ANALYSIS

Cell Line: CJLCKT64.86.41BBL.CD19.MBIL21 (Clone 9.MBIL21)
Cell Concentration at Freezing: 2 x 10^7/mL 1 mL/vial
Expiration Date:

TEST	LABORATORY	SPECIFICATION	RESULT
In-House Testing			
Endotoxin LAL (Endosafe)	MDACC CTL QC Lab	≤ 5.0 EU/mL	
Viability at Freezing By Trypan Blue Exclusion	MDACC CTL QC lab	>70%	
Sterility BACTEC – Aerobic BACTEC - Anaerobic	MDACC Microbiology Laboratory	Negative	
Tests Sent to Reference Laboratories			
Replication Competent Lentivirus (RCL) Assay	Indiana University Vector Production Facility	Negative	
Mycoplasma by PCR	CAGT QC Laboratory	Negative	
510836.BSV Sterility Test Using a Two Media Direct Inoculation Method for Bulk or Final Product per Current USP, 21 CFR, European Pharmacopoeia (EP) and/or Japanese Pharmacopoeia (JP)	BioReliance	Negative	
003800.BSV In Vitro Assay for Viral Contaminants, 28 days	BioReliance	Negative	
005002.BSV In Vivo Assay for Viral Contaminants	BioReliance	Negative	
013013GMP.BUK Quantitative TEM for detection of Viruses, Fungi, Yeasts, Bacteria and mycoplasmas (200 cell profiles)	BioReliance	Negative	
032900.BSV 9CFR In Vitro Bovine Virus Assay	BioReliance	Negative	
102062GMP.BSV Test for Presence of Agar-Cultivable and Non Agar-Cultivable Mycoplasma (USP,EP, 1993 PTC)	BioReliance	Negative	

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Page 1 of 2

Fig. 1 Certificate of Analysis for feeder cell line K562 Clone9.mbIL21

MASTER CELL BANK – CERTIFICATE OF ANALYSIS

Cell Line: CJLCKT64.86.41BBL.CD19.MBIL21 (Clone 9.MBIL21)
Cell Concentration at Freezing: 2×10^7/mL 1 mL/vial
Expiration Date:

TEST	LABORATORY	SPECIFICATION	RESULT
032900.BSV In Vitro Assay for the Presence of Bovine Viruses, According to 9 CFR Requirements	BioReliance	Negative	
105130.BSV Product Enhanced Reverse Transcriptase (PERT) Assay for the Detection of Retrovirus in Biological Samples	BioReliance	Negative	
105252.BSV Qualitative Real-Time PCR Assay for the Detection of Bovine Polyoma Virus (BPyV)	BioReliance	Negative	
300100GMP.BSV Detection of 14 Viruses by Real-Time Polymerase Chain Reaction Assays (Human Panel 1)	BioReliance	Negative	
380801.BSV Cell Culture Identification and Characterization Using Isoenzyme analysis	BioReliance	Negative	
510021.BSV Test for Bacteriostatic/ Fungistatic Activity of a Test Article Using Direct Inoculation Method	BioReliance	Negative	

Comments: _____

_____

_____

Approvals:

Laboratory Director: Date:

Quality Assurance Coordinator Date:

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Page 2 of 2

Fig. 1 (continued)

4.2 Cell Culture

4.2.1 Media

Cell culture media is not intended for human use, so a CofA for the media must be available demonstrating sterility and minimal endotoxin content. If the media contains additives derived from animals, mycoplasma testing must also be available.

4.2.2 Serum vs. Serum-Free

Fetal bovine serum (FBS) is commonly used as a supplement for cell culture media, providing a wide array of known and unknown factors essential for cell growth and survival. In 1997 the WHO advised that bovine-derived additives be avoided in pharmaceutical manufacturing and in products administered to patients because of the risk of transmissible spongiform encephalopathy (TSE) [16]. The EMA [17] and the FDA have also issued guidance for avoiding the use of animal products to reduce TSE risk. However, it should be noted that bovine serum is listed as Category IV for TSE (containing no detectable infectivity), and FBS for human use should additionally be prepared from geographic regions in which TSE risk is low. To date, there has been one case of bovine spongiform encephalopathy (BSE) confirmed in the US, in 2003 [18]. FBS also carries risk for xenogeneic immune reactions [19], and other factors provide additional motivation to finding alternatives to FBS in cell culture for clinical applications, such as uncontrollable variations in composition, limited availability, and cost.

Human serum may replace the use of FBS in some circumstances, particularly when autologous serum is available. For applications in which the need is solely to provide protein for product stability, purified human serum albumin may be used to further decrease the risks described above. Despite these concerns, the use of FBS for expanding NK cells remains in common use because of unparalleled support of growth and survival. Residual FBS should be removed by thorough washing of NK cells prior to formulation of the final infusion product.

4.2.3 Antibiotics

The use of antibiotics should be avoided as much as possible during all phases of NK cell manufacturing. Antibiotics may suppress nascent bacterial contamination without completely eliminating the contamination, resulting in infectious agents that may bloom after infusion despite passing release criteria. The use of antibiotics masks poor aseptic technique and has been associated with increased mycoplasma contamination [20]. If the product cannot be manufactured without the use of antibiotics, a validated removal system should be employed prior to the final product formulation, and an assay for residual content may be required as part of the release testing.

4.2.4 Closed Systems

Aseptic technique and cross-contamination are greatly improved by the use of closed culture systems. While not required, their use in early-phase trials will provide validation and experience for later phase trials in which larger culture volumes and cell numbers are too cumbersome and risky for open systems. Closed systems often

introduce greater losses to the manufacturing process, making them impractical for procedures involving small volumes and/or cell numbers—e.g., initial processing of small-volume draws of peripheral blood.

4.2.5 Final Formulation

The final formulation of an NK cell product must be in an infusion solution acceptable for human use. Sterile, non-pyrogenic, neutral-buffered isotonic saline solutions commonly used in clinical settings that may be adapted for this purpose include Plasma-Lyte A, lactated ringers (LR) solution, and Buminate 5% [21]. There is at least one phosphate-buffered saline solution made expressly for this purpose (Miltenyi CliniMACS PBS-EDTA Buffer). To avoid cell clumping and thereby improve stability, 0.5% HSA may be included in the cell suspension buffer.

4.2.6 Cryopreservation

It has long been recognized that cryopreservation has an adverse effect on NK cell function [22], and this has been confirmed in recent clinical trials [21]. Cryopreservation media used for freezing cord blood or peripheral blood mononuclear cells for future use in stem cell transplantation typically uses HSA 5% in place of serum to enable direct infusion of the thawed product without washing. Inclusion of serum in cryopreservation media provides superior results compared to HSA, but requires washing the cells prior to infusion and this additional manipulation also bears a cost of cell loss, time, and the need for additional sterility testing after the procedure, in addition to the safety issues mentioned above.

Dimethyl sulfoxide (DMSO) is the most common cryoprotectant used in freezing media, typically at a concentration of 10%. DMSO in limited amounts may be directly infused to patients after thawing, obviating the need for post-thaw washing. Our institutional practice guidelines recommend limiting the quantity of infused DMSO to 1 mL/kg. An infusion bag of 100 mL with 10% DMSO would approach recommended limit for a child weighing 10 kg. Adjusting the cryopreservation volume or washing the product to remove the DMSO prior to infusion may be necessary in clinical trials treating small children or infusing large volumes of cryopreserved cells.

4.3 Release Criteria

Release criteria are the results of final product testing that must be achieved in order to allow the product to be released for use in the clinical trial. Release testing provides the last line of quality assurance for product safety and efficacy, demonstrating that adherence to the CMC and SOP procedures results in a product that meets minimum specifications. The specific testing utilized for release criteria should ensure a product that is as safe as possible with the testing modalities available and in a timeframe that is practical for the clinical setting (*see* **Note 1**). The criteria should be as stringent as possible but not more stringent than can be routinely achievable by the manufacturing method being used (*see* **Note 2**, Fig. 2).

IND/IDE Certificate of Analysis

Protocol Title:	A Phase I/II Clinical Trial Testing the Safety and Feasibility of IL-21-Expanded Natural Killer Cells for the Induction of Relapsed/Refractory Acute Myeloid Leukemia		
Principle Investigator	Dean A. Lee, M.D., Ph.D.	Protocol#: 2012-0079	IND/IDE #: 15367
Co-Principle Investigator		Date:	
Quality Assurance			
Laboratory Director			

Product Information: NK Cells Expanded with K562 Clone 9.mbIL21

Unique Unit Identification (BMT #): _____

Recipient Name: _____ MDACC #: _____

Released Criteria (Cryopreservation):

Test:	Specifications:	Results
Sterility	☐ No growth at 14 days ☐ Negative to date	
Endotoxin (LAL)	< 5EU/Kg	
Viability (7AAD)	≥ 70%	
$CD3^+$ Cells	< 1 x 10^5 $CD3^+$/kg	
Immunophenotyping	≥ 80% $CD56^+CD3^-$	
	< 1% $CD3^+$	
	< 1% $CD32^+$ of viable cells	
	< 5% $CD19^+$	

Released Criteria (Post-Wash):

Gram stain (Final Product)	No organisms seen	
Visual Inspection (Final Product)	No evidence of contamination	
NK Cell Dose ($CD56^+CD3^-$) (Final Product)	Select required dose: ☐ Dose 1: 1 x 10^6 $CD56^+CD3^-$/kg ☐ Dose 2: 3 x 10^6 $CD56^+CD3^-$/kg ☐ Dose 3: 1 x 10^7 $CD56^+CD3^-$/kg ☐ Dose 4: 3 x 10^7 $CD56^+CD3^-$/kg ☐ Dose 5: 1 x 10^8 $CD56^+CD3^-$/kg ☐ Dose 6: 3 x 10^8 $CD56^+CD3^-$/kg	

(continued on next page)

Fig. 2 Certificate of Analysis for the final NK cell product as used in protocol 2012-0079 (NCT01787474)

IND/IDE Certificate of Analysis

Review of Final Product for Transplant Suitability:

Product meets or exceeds all criteria (check one): ❒ Yes ❒ No (Explain if no checked. Laboratory & Medical Director approval needed)

_____

_____

_____

Released By: _____ Date: _____
Quality Assurance Coordinator (or designee)

If applicable:

Comments: _____

_____

_____

Laboratory Director: _____ Date: _____

Laboratory Medical Director: _____ Date: _____

Fig. 2 (continued)

4.3.1 Identity/Purity: Inclusion of Desired Cells

Assays to establish the identity and purity are generally met simultaneously for NK cell products through flow cytometry. Identity (e.g., are these cells really NK cells?) and purity (e.g., how many of these cells are NK cells?) may be established through positive identification of both the intended agent and/or identification of known contaminating agents.

NK cells are most often defined as $CD3^{neg}CD56^{pos}$ lymphocytes, but may also be defined as $CD3^{neg}CD16/56^{pos}$ or $CD3^{neg}NKp46^{pos}$. Under some circumstances it may be desirable to mandate that a product contain a minimum content of a specific NK cell subset (e.g., $KIR2DL1^{pos}$).

4.3.2 Identity/Purity: Exclusion of Undesirable Cells

T Cells—Many NK cell expansion methods also expand T cells. In the autologous settings contaminating T cells may be beneficial and may not cause harm, but they still increase media utilization during cell culture, decrease the purity of the product, and may decrease the potency of the product. T cells of donor origin also may lead to increased toxicity by causing graft-vs-host disease (GvHD). Our release criteria mandate <5 % CD3+ lymphocytes for autologous products, but specify a maximum of 1 % and 10^5/kg for allogeneic products. This is based on a historic threshold of 10^5/kg T cells for causing GvHD in haploidentical transplants and haploidentical donor lymphocyte infusions [23].

B Cells—B cells of donor origin infused into immunocompromised patients may be a source of EBV-driven lymphoproliferative disease. B cell content may be controlled by additional processing to deplete CD19+ cells [24].

Feeder Cells—Residual contaminating feeder cells should be assessed, particularly when tumor cell lines are being used. A unique signature distinguishing the tumor cells from cultured cells should be developed. Flow cytometry-based methods allow for exclusion of dead cells from the analysis, which may complicate interpretation of PCR-based studies. For genetically modified K562 used in propagation of NK cells (Activating and Propagating Cells (AaPC)), we assess surface expression of the endogenous gene CD32 and the transgene CD19 to distinguish AaPC from T cells and NK cells, as donor monocytes and B cells are not found in these cultures.

Dead Cells—Viability is an indirect measure of product quality, stability, purity, and potency. Viability of fresh products should be at least 95 % unless the products have been heavily manipulated. Cryopreserved NK cells often demonstrate viability of >90 % immediately after thawing, but cells continue to die over the next 12–24 h, often declining to 70 % or less. In most cases, cells are prepared for infusion as soon as possible after thawing, so viability assessments are likely overestimated when performed at these early time points.

4.3.3 Sterility Testing

Viral—Viral testing of donor leukocytes, feeder cells, and serum is described above. As these are the only sources of viral contamination, further testing for viral contamination of the final product is not required.

Bacterial/Fungal—Assessment of bacterial or fungal contamination is composed of successively more sensitive but also lengthier assays. Visual inspection demonstrating cloudy or discolored media is immediate and may be used at any time. Gram stain and endotoxin may be performed within an hour or less and are complimentary. Gram stain may reveal organisms that do not produce endotoxin, but endotoxin is more a more sensitive measure when present. Full-length cultures (typically 14 days) are the most sensitive, but because of the long timeframe before final results are available it may not be practical in some settings for use as a release criterion (*see* **Note 1**).

Mycoplasma and related organisms—Cell cultures may become contaminated with common mollicutes (bacteria lacking a cell wall which survive as obligate intracellular parasites) such as Mycoplasma and Ureaplasma. In addition to an infection risk (particularly for immunocompromised patients), we have identified mycoplasma contamination as a major cause of poor NK cell proliferation during ex vivo expansion. The luminometric assays are based on pathogen-specific enzymes and will detect most mollicutes except for Ureaplasma (e.g., MycoAlert (Lonza), MycoProbe (R&D)). They can be performed quickly for same-day test results. PCR-based assays will also detect genus such as Ureaplasma that are not typically identified by enzyme-based methods, but generally take a longer time for results to be available (e.g., MycoSensor (Agilent), Universal Mycoplasma Detection Kit (ATCC)). Our standard is to provide final release testing on all cryopreserved products using the PCR-based assay, with the luminometric assay used for release testing of fresh products or cryopreserved products that are scheduled to be administered before PCR assay results are available.

4.3.4 Potency

Potency is defined as "the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result." (21 CFR 600.3(s) [25]. Potency assays measure the relative biologic function of a product, and most often, the biologic function of relevance to NK cells is cytotoxicity. Quantitative assays are preferred, but qualitative biologic assays may also be used [7]. Assays should be chosen for ease of use in the GMP environment, rapid return of results, and accuracy, sensitivity, specificity and reproducibility. Unfortunately, the most common biologic assays used for measuring NK cell cytotoxicity (Chromium- or calcein-release against K562 targets) is relatively specific but is neither accurate (killing of

K562 may be incomplete [26] and is unlikely to predict killing of the patient's tumor), sensitive (a tenfold increase in NK cells typically correlates to a 20% increase in killing), nor reproducible (high day-to-day variability).

Alternative measures may provide more detailed assessment of NK cell potency (e.g., single-cell analysis [27]), but may be too complicated for the GMP environment. Other alternatives include measurement of degranulation or cytokine production in response to an activating signal. We developed a rapid assay that solves some of the accuracy and reproducibility problems of chromium- and caclein-release assays and is simple to use in the GMP environment [26].

In addition to a variety of possible readouts for potency, each of these assays may be used with a variety of activating sources. K562 and 721.221 are often used as cell line targets specific to NK cell function because their lack of MHC expression does not support antigen-specific T cell activation. However, this results in a bias toward missing-ligand effects, which may not be present in vivo in the patient. Alternatives include patient-specific targets (applicable primarily to leukemias), testing across engineered target panels [28], or target-independent approaches such as plate-bound ligands or chemical activators (e.g., PMA/ionomycin).

Fortunately, a validated measure of product potency is not required for Phase I trials, but should be in development during Phase II trials and is required for Phase III. Future work will be needed to identify potency assays that the NK cell community can agree upon.

5 Notes

1. The stringency for release criteria may vary depending on the practical ability to complete the testing. For instance, mycoplasma testing can be performed with a rapid, less-sensitive enzymatic assay or a slower, more sensitive PCR-based assay. A cryopreserved product may be stored as a long as necessary to allow time for results to be obtained using the best test available, and therefor it is appropriate to specify the PCR-based test for the release criteria of these products. However, when performing release testing for fresh cellular products that often need to be infused within hours of final preparation in order to maintain maximum potency, the QA/QC personnel may not be able to complete the PCR-based assay in a time-frame compatible with administering the product within its shelf-life. In such cases, release criteria may be established on the basis of a rapid screening test, provided the protocol incorporates a reasonable action plan for patient safety to be followed in the event of a positive result from the more definitive test (e.g.,

evaluate the patient for signs of infection and initiate a macrolide antibiotic). This also applies to gram stain and endotoxin as the bacterial testing for release criteria while awaiting final culture results.

2. The more stringent the release criteria, the more likely the findings of the clinical trial will be based on safety and efficacy of the cells being tested. However, high stringency may not be achievable or may come at a manufacturing cost that is not sustainable. For instance, early clinical trials of NK cells utilized steady-state leukapheresis products from which T cells were removed by CD3 immunomagnetic negative selection. This resulted in products for which NK cell purity was relatively low at ~30%. High purity could be obtained by instituting a second step of CD56 immunomagnetic positive selection. However, this increased cost and resulted in large cell losses such that adequate doses were often not achieved. An appropriate balance of purity and feasibility must be sought.

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INDEX

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