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Lentiviral Gene Transduction in Human and Mouse NK Cell Lines

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Abstract

Natural killer (NK) cells play a vital role in the control of cancer and microbial infections. A major hindrance in studying NK cells is the resistance of these cells to gene transfer. Considering over-expression and gene knockdown studies are crucial tools to study the biology of cells, technologies suitable for transferring genes into NK cells are invaluable. Among various technologies available for gene transfer, lentiviral-mediated transduction has been successful in introducing genes into NK cells. We have standardized methods of lentiviral infection in human and mouse NK cell lines. We obtain transduction efficiencies of 15% in the NK-92 cell line and 30–40% in LNK, YT, and DERL7 cell lines. This method allows efficient and stable introduction of genes and shRNAs into NK cell lines.

Keywords

Natural killer cells; lentivirus; transduction; flow cytometry; viral titration; human; mouse; NK-92; LNK

1. Introduction

Natural killer (NK) cells are large, granular lymphocytes which play a vital role in tumor immunosurveillance and combating microbial infections (1). These cells eliminate targets using multiple mechanisms and also recruit and amplify inflammatory responses. NK cells are able to lyse class I MHC-negative targets by releasing perforin, granzymes, and TNF ligands upon stimulation. These effector functions of NK cells are gradually acquired during their development and maturation. These cells are also known to recruit other immune cells to the sites of infection by releasing chemokines and cytokines. To understand the biology of NK cells and dissect specific gene functions, tools facilitating over-expression of genes or knockdown of genes by siRNA are invaluable. Resistance of NK cells to exogenous gene transfer is a major hindrance in understanding NK cell biology and the potential use of these cells in immunotherapy.

Human and mouse NK cells (primary cells and cell lines) are refractory to gene transfer. Methods like calcium phosphate precipitation (CaPO₄), liposome reagents, and electroporation techniques have resulted in very low rates of gene transfer(2). Nucleofection technology (Amaxa, Inc.) has been the most promising among nonviral technologies that has shown some success in gene transfer in NK cell lines (3, 4). Several viral-mediated gene transfer protocols have been reported, but these approaches have had only variable success (5–7). For example, gene introduction with vaccinia virus altered the phenotype of NK cells(6) while adenoviral vectors have shown to be ineffective because NK cells do not possess appropriate receptors to mediate attachment of the virus to the cell. In contrast,

Schroers et al. (8) showed that chimeric fiber-modified Ad5/F35 adenoviral vector efficiently infected primary human NK cells. Recently, ALAK (IL-2-activated NK) cells transduced by adenovirus have been used in tumor therapy in mice (9, 10). Use of retroviral transduction in primary NK cells has resulted in partial success, primarily because of the requirement of multiple rounds of infection to introduce genes (11). Among the viral-mediated gene transfer systems, lentivirus transductions have been the most efficient gene delivery systems available for hematopoietic cells and hard to transfect cells. Recently, Tran and Kung (7) demonstrated an average of 40% transduction efficiency with primary murine NK cells. Furthermore, they show that lentiviral transduction does not affect the viability, function, and phenotype of NK cells.

Lentiviral vectors (LV) are increasingly being utilized as a tool for introducing and obtaining stable expression of transgenes in both dividing and nondividing cells. Self-inactivating replication-incompetent lentiviral particles are generated by co-expressing the packaging elements of the virus along with the vector genome in the most commonly used virus-producing cell line, human embryonic kidney cell 293 or 293T (containing the SV40 large T antigen). The packaging elements of the HIV-1-based lentiviral vectors are from the HIV-1 genome. These HIV vector systems can be divided into three generations based on the progressive deletions of the packaging system from the parent vector. The first LV generation system contained all the HIV-1 genes except for the envelope in the parent vector. In the second generation, HIV genes including *vpu* and *nef* were deleted and the LV packing elements are provided as separate vectors. The third generation LV system offers maximum biosafety features by having the *gag*, *pol*, and *rev* genes provided as separate vectors. For this latest system, the envelope gene is from a heterologous virus, vesicular stomatitis virus (VSV), thus resulting in a pseudotyped virus with a broad host range.

The first successful report utilizing lentiviral vectors in NK cells described the transduction of primary murine NK cells (7). As the lentiviral transduction system allows efficient and stable integration of transgenes, NK cell lines can be utilized to develop in vitro models to study and further understand the biology of NK cells. Several human and mouse cell lines have been developed that can be used to study the function of NK cells. Among the human NK cell lines, NK-92 (12), YT (13), NKL (14), and DERL7(15) are the most commonly used. NK-92 was originally derived from a male non-Hodgkin's lymphoma patient (12). This IL-2-dependent NK cell line kills target cells and displays characteristics of activated NK cells. NK-92 produces copious amounts of IFN- γ upon stimulation by cytokines individually or in combination (16), making it an ideal model for studying gene regulation in NK cells. DERL7 (CD56⁺, CD3⁻, TCR $\gamma\delta$ ⁻) is a newly described cell line from a non-Hodgkin's lymphoma patient, which possesses both NK and T-cell surface markers (15). There is only one NK cell line derived from mice, designated LNK (CD132⁺, CD16⁺, CD3⁻, IgM⁻), which is an IL-2-dependent NK line derived from liver lymphocytes of BALB/c nude mice(17).

We have developed protocols which facilitate efficient and stable introduction of genes and short-hairpin RNA (shRNAs) into NK cell lines. The transduction efficiencies obtained are around 15% in NK-92 cells and 30–40% in LNK, YT, and DERL7 cells.

2. Materials

2.1. Plasmid Preparation

1. Plasmids: pLKO.1, pGIPZ, and pTRIPZ (Open Biosystems, www.openbiosystems.com); pMD2.G (Plasmid 12259; Addgene, www.addgene.org); and psPAX2 (Plasmid 12260; Addgene).
2. Bacterial strains: Vectors from Open Biosystems are generally transformed in *Escherichia coli* DH5 α . To prevent recombination of the plasmid with genomic DNA, use *E. coli* strains that lack the recombinase gene (*recA*). We recommend the use of commercially available electro- or chemical-competent *E. coli* strains like Stbl2 or Stbl4.
3. Luria–Bertani (LB) medium (1 L): Tryptone 10 g, yeast extract 5 g, and NaCl 10 g. Adjust volume to 1 L distilled water, sterilize by autoclaving, and store at room temperature (RT).
4. SOC medium (1 L): tryptone 20 g, yeast extract 5 g, 5 M NaCl 2 mL, 1 M KCl 2.5 mL, 1 M MgCl₂ 10 mL, 1 M MgSO₄ 10 mL, and 1 M glucose 20 mL. Adjust volume to 1 L distilled water, sterilize by autoclaving, and store at RT.
5. Antibiotics: Prepare ampicillin stock solution at 50 mg/mL in distilled water and store at –20°C.
6. Plasmid purification kits: Purchase mini- and maxi-prep kits from any commercial source. Endo-free maxi-prep plasmid purification kits are recommended to obtain transfection quality DNA free of endotoxin.

2.2. Transfection Reagents

1. 2 M calcium chloride (CaCl₂; Invitrogen, Walkersville, MD, USA).
2. 2× HEPES (2.5 M, Invitrogen).

2.3. Cell Lines and Culture Media

1. NK-92: This human NK cell line can be purchased from ATCC (American Type Culture Collection, Rockville, MD, USA; CRL-2047). NK-92 cells are cultured in RPMI 1640 medium (Cambrex, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, 50 µg/mL streptomycin, and 2 mM L-glutamine (PSG, Cambrex), recombinant human IL-2 (1000 IU/mL; Pepro-Tech, Rocky Hill, NJ), and recombinant human IL-15 (100 ng/mL; PeproTech).
2. DERL7 (CD56⁺, CD3[–], TCR $\gamma\delta$ [–]): This human cell line was kindly provided by Dr. L. Del Vecchio (A Cardarelli Hospital, Italy) and cultured in RPMI 1640 medium supplemented with 20% FBS, 100 IU/mL penicillin, 50 µg/mL streptomycin and 2 mM L-glutamine, recombinant human IL-2 (1000 U/mL), and stem cell factor (250 ng/mL; PeproTech).

3. LNK cell line: LNK is cultured in RPMI 1640 medium supplemented with 10% FBS, 100 IU/mL penicillin, 50 µg/mL streptomycin and 2 mM L-glutamine, 5% HEPES, 5% sodium pyruvate and 1 × beta-mercaptoethanol, and recombinant human IL-2 (1000 U/mL).
4. 293FT cell line (Invitrogen): This human embryonic kidney line transfected with the SV40 large T antigen is cultured in DMEM supplemented with 10% FBS, 100 IU/mL penicillin, 50 µg/mL streptomycin and 2 mM L-glutamine, 5% HEPES, 5% sodium pyruvate, and 1% gentamicin.
5. 3T3 cell line: This mouse fibroblast cell line is cultured in DMEM supplemented with 10% FBS, 100 IU/mL penicillin, 50 µg/mL streptomycin and 2 mM L-glutamine, 5% HEPES, and 5% sodium pyruvate.
6. Trypsin–EDTA: 2.5% trypsin and 0.5 M ethylenediamine tetraacetic acid (EDTA).
7. PBS–EDTA: Phosphate-buffered saline (pH 7.4) and 0.1 M EDTA.
8. Antibiotics: (a) Puromycin: prepare stock solution at 10 mg/mL in PBS and store at 20°C. (b) Geneticin (*G418*): prepare stock solution at 50– mg/mL in PBS and store at –20°C.
9. Polybrene (Sigma): Prepare stock solution at 8mg/mL in distilled water and filter sterilize. Aliquot (200µL) and store at –20°C.

2.4. Virus Production and Purification

1. 10 cm collagen-coated tissue culture grade plates (we have routinely utilized BD Bionocoat plates, but other brands may also be suitable)
2. Syringe-driven filter unit: Use low protein binding 0.45 µm pore size filters
3. 10 mL disposable syringe
4. 1.8 mL cryo-vials

3. Method

3.1. Preparation of Plasmids

1. Mix 100 ng of plasmid DNA with chemical-competent *E. coli* (DH5α, Top10, Stbl2, or Stbl4) in a 1.5 mL polypropylene tube and incubate on ice for a minimum of 30 min.
2. Heat shock the bacteria–plasmid mixture at 42°C for 30 s and immediately incubate on ice for 2 min.
3. Add 50 µL of SOC medium to the bacteria plasmid mixture and incubate in a shaker incubator (≈300 rpm) for 1 h.
4. Spread 100 µL of transformed bacteria onto LBA (LB-agar) containing ampicillin (100 µg/mL) and incubate overnight at 37°C bacterial incubator.

5. Pick at least 10 colonies from each plate and inoculate in 5 mL LB medium containing ampicillin (100 µg/mL) and incubate in a shaker incubator for 6 h.
6. Aliquot 3 mL of the bacteria from above (step 5) and extract plasmid using any commercially available mini-prep kit and verify plasmid by restriction enzyme digestion.
7. Aliquot 1 mL from step 5 and grow in 300 mL of LB medium (supplement with 100 µg/mL ampicillin) for 12–16 h at 37°C.
8. Centrifuge the bacteria at 6000×*g* for 15 min.
9. Extract plasmid using any commercial endotoxin-free maxi-prep kit.
10. Verify by restriction digestion and store plasmid at 4°C at a final concentration of 1 mg/mL in TE buffer.

3.2. Virus Production (see Note 1)

Day 1 (seeding 293FT cells)

1. Seed 10 mL of 2.5×10^5 /mL of 293FT cells in complete DMEM medium on a collagen-coated 10 cm plate and incubate overnight at 37°C in a CO₂ incubator (see Note 2).

Day 2 (calcium phosphate transfection)

2. The cells should be 60–80% confluent the following day (100% confluency must be avoided). Gently remove medium and replace with pre-warmed 3T3 complete DMEM medium and return the culture dish to the incubator for a minimum of 4 h.
3. Mix lentiviral vector, gag/pol (psPAX2), and VSVG (pMD2.G) DNAs in a ratio of 2:2:1. We use 21 µg of the parent lentiviral vector, 21 µg of psPAX2, and 10 µg of VSVG (see Note 3) in a 14 mL polypropylene tube (Falcon).
4. Add 36 µL of 2 M CaCl₂ solution and cell culture grade water to the plasmid mix to a final volume of 300 µL and vortex it for a second or gently flick the mixture.
5. It is necessary to next bubble air slowly into the mixture. We routinely do so with a Pasteur pipette. While the bubbling is ongoing, add 300 µL of 2 × HBSS into the tube in a drop-wise manner. This process should be slow and lasts 1–2 min.
6. Incubate the above mixture for 30 min at RT (see Note 4).
7. Add the mixture onto the 293FT plate drop-wise and mix the contents on the plate by gently swirling the plate. Return the plate to the CO₂ incubator at 37°C.
8. After 4–6 h, gently remove the medium from the plate and wash with 1× PBS. Add 10 mL of complete DMEM and incubate for 30–48 h at 37°C in a CO₂ incubator.

Day 3 (viral harvest)

9. Harvest the supernatant from the dish in a 15 mL capped polypropylene tube (Falcon). Centrifuge the tube at $360\times g$ for 5 min to pellet cellular debris.
10. Pass the supernatant through 0.45 μ M syringe-driven filter unit (see Note 5).
11. Aliquot the filtered supernatants into 1.8 mL cryo-vials and store them at -80°C .

3.3. Viral Titer

3.3.1. Biological Titering (see Note 6)—Day 1

1. The day before titering, plate 3T3 cells at a density of 2×10^5 cells/well of a 6-well plate and incubate in a CO_2 incubator overnight (see Note 7). Cell confluency should be approximately 30–50% the following day.

Day 2

2. Thaw the lentiviral sample (see Note 8) and prepare 10-fold serial dilutions in complete DMEM medium of the LV supernatant from 10^{-2} to 10^{-6} in sterile 5 mL polypropylene tubes (Falcon) with a minimal final volume of 2 mL for each dilution. Mix by gently pipetting the solution; DO NOT VORTEX.
3. Aspirate the medium from the 6-well overnight cultured plates and add 1 mL of the diluted virus-containing medium to their respective wells. Also include a control well.
4. Add polybrene to final concentration of 8 $\mu\text{g/mL}$ to each well and incubate overnight.

Day 3

5. Remove the infection medium from each well and replace with 2 mL of complete medium containing 10% FBS.

Day 4

6. Remove the medium from the well and replace with complete medium containing 2 $\mu\text{g/mL}$ puromycin to begin selecting transduced cells (see Note 9).

Days 5–13

7. Every 3–4 days, remove the medium and replace with fresh complete medium containing 2 $\mu\text{g/mL}$ puromycin

Day 14

8. After 10 days on selection medium, the control well should have no cells left and the sample well should have some discrete puromycin-resistant colonies observed.
9. Remove the medium and wash the wells with PBS twice.
10. Add 1 mL Crystal Violet solution (1% w/v in 10% ethanol) and incubate at room temperature for 10 min.
11. Remove crystal violet solution and wash the wells with PBS twice.

12. Record the number of blue colonies observed in each of the wells with respect to the appropriate dilution.
13. Calculate the titer as transducing units (TU/mL) as follows:

$$\text{TU/mL} = (\text{number of discrete colonies/dilution factor})/\text{volume of infection medium used}.$$

3.3.2. qPCR-Based Titering (see Note 10)

14. Utilizing 150 μL of the viral supernatant, purify the viral RNA using the Nucleospin RNA Virus kit (Macherey-Nagel), according to the manufacturer's protocol. Elute the RNA from the column using 50 μL RNase-free water.
15. Remove contaminating vector DNA from the sample by treating the sample with DNase I (8 U/reaction) and incubating the tube at 37°C for 30 min followed by 5 min at 70°C.
16. Meanwhile, prepare 10-fold dilutions of the RNA Control template (Clontech, Mountain View, CA, USA) and your samples. You will use 2 μL for each reaction done in duplicates.
17. Prepare a Master Reaction mix containing 50nM of the forward and reverse primer, 1 $\times$ SYBR green, 1 $\times$ QTaQ DNA polymerase, 1 $\times$ reaction buffer, and 1 $\times$ qRT Mix (Clontech) with a final volume of 23 μL /sample. Add 2 μL of the diluted RNA control template and sample to the reaction.
18. Using a 7300 ABI Prism qPCR thermocycler, program the reaction as per manufacturer's protocol and run the reaction/analysis.
19. Determine the copy number based upon the raw copy number obtained from the qRT-PCR standard and factoring the dilutions and amount of sample used to obtain the copy number/mL. Utilize an infectivity coefficient (as determined by a biological titering assay) to relate the copies/mL to obtain the transforming units/mL.

3.4. Transduction of Human NK Cell Lines (NK-92 and DERL7)

Day 1

1. Plate the NK cell line in appropriate complete culture medium at a density under 1.0×10^6 cells/mL (see Note 11).

Day 2

2. Count the cells and resuspend in appropriate medium at 1.0×10^6 cells/mL.
3. Stimulate the lines with human IL-2 (100 U/mL) and human IL-12 (PeproTech; 100 ng/mL) for 2 h before transduction (see Note 12).
4. Following cytokine stimulation, readjust the concentration of the cells to 1.0×10^5 cells/mL using NK cell medium and plate 1 mL in each well of a 12-well plate.

5. Add viral supernatant for a multiplicity of infection between 20 and 100 of the titered virus to the medium-containing cells and polybrene (8 [H9262]g/mL; see Note 13) and incubate overnight at 37°C.
6. Alternatively, spinoculation by centrifugation of cells and virus-containing supernatant at $360\times g$ for 90 min at 32°C yields good transduction efficiency.

Day 3

7. Remove the medium by spinning the cells at $360 \times g$ for 5 min at RT and resuspend the cells in 0.5 mL of NK cell medium and 0.5 mL of conditioned NK cell medium (see Note 14).

Days 5–10 (selection of transgene-positive cells)

8. Cells can be selected by mammalian antibiotic resistance or YFP-positive cells depending on the marker used (see Fig. 14.1). We have titrated the required puromycin and hygromycin concentrations to be 2 [H9262]g/mL when using NK-92 or DERL7 cells (see Note 9).
9. Once selected for positive selection marker, choose up to five clones and check for knockdown (for shRNA) or transgene expression and select the clone with the desired expression level (see Note 15).

3.5. Transduction of Mouse NK Cell Line (LNK)

Day 1

1. Plate the LNK cell line in appropriate medium at a density under 1.0×10^5 cells/mL on a 6-well plate (see Note 16).

Day 2

2. Stimulate the lines with high dose of IL-2 (1000 U/mL) for 2 h before transduction.
3. Add viral supernatant for a multiplicity of infection between 20 and 100 of the titered virus to the medium-containing cells and polybrene (8 μ g/mL) and incubate overnight at 37°C.

Day 3

4. Remove the medium and add 0.5 mL of fresh NK cell medium and 0.5 mL of conditioned NK cell medium (see Note 14).

Days 5–10 (selection of transgene-positive cells)

5. Cells can be selected by mammalian antibiotic resistance or green fluorescent protein (GFP)-positive cells or other criteria depending on the marker used. We have titrated puromycin and hygromycin to be effective at killing non-transduced cells at a concentration of 2 μ g/mL (see Note 9).

6. Once selected for the positive selection marker, select up to five clones and check for knockdown (for shRNA) or trans-gene expression and select the clone with desired expression level (see Note 15).

4. Notes

1. You must obtain approval from your institutional biosafety committee (IBC) before producing and/or utilizing lentivirus in the laboratory. All the vectors and the packaging systems should be listed in the IBC. Safety hoods, incubators, and centrifuges used for lentivirus work should be clearly indicated in the laboratory.
2. Refer to the manual from the commercial supplier for subculturing conditions for 293FT cells. Always use low passage number of 293FT cells for virus production. 293FT with high passage number will result in significantly lower viral titers. Do not allow the cell line to become over-confluent at any time.
3. The ratio and amount of plasmids are variable and should be optimized based on the titers obtained.
4. The mixture will form fine precipitates. However, if they form large precipitates, disperse the precipitates using a Pasteur pipette.
5. You could further concentrate the virus by ultracentrifugation to get a higher titer of virus (18).
6. The method for titering will depend upon the marker available within the LV system utilized. Biological titering will only be useful for LV systems with a selectable marker such as puromycin or blasticidin. If a reporter gene (e.g., green fluorescent protein or DsRed) is present, a flow cytometry-based method is needed. Titering, using qPCR-based methods, will work on all systems regardless of the selection or reporter gene present, but only provides the number of copies of genes present in the sample, and does not reflect the biological infectivity of the lentivirus. Therefore, if qPCR-based methods are utilized, an infectivity coefficient needs to be determined with the addition of a biological titering assay to obtain a ratio between the qPCR titer and the biological titer.
7. Various cell lines may be used for titering the lentivirus such as HeLa (human cervical cancer; ATCC CCL-2) or HT1080 (human fibrosarcoma; ATCC CCL-121). These cells are easy to work with, grow attached to the plate/flask, and rapidly divide. It is generally not recommended to use the packaging cell line (i.e., 293T cells) for titering, as carryover plasmid from the transfection may result in an overestimate of the titer obtained.
8. Lentiviruses should not be thawed more than three times. Viral titers are known to decrease with each thaw.
9. The actual concentration for puromycin selection will need to be determined for the different cell lines used as the sensitivity of alternate cell lines to puromycin may differ from that reported here.

10. This protocol shown below is based upon Clontech's protocol for determining the lentivirus titer via RNA isolation. There are also other qPCR-based methods that can be done by isolating DNA and/or RNA at various steps through virus production and using gene-specific primers for the lentiviral vectors such as LV1, LV2, GAG, etc. (18, 19).
11. NK cells grow well at a density of $0.5\text{--}1.0 \times 10^6$ cells/mL. In our experience, we see that they do not transduce well if they are plated at a lower or higher density.
12. Stimulation of DERL7 cell line with human IL-2 (100 U/mL) and stem cell factor (250 ng/mL) results in better transduction efficiencies.
13. Retronectin can be used as an alternative to polybrene as human NK cells express VLA-4 and VLA-5, cell surface molecules which mediate adhesion to fibronectin (20).
14. Conditioned NK cell medium is filter-sterilized medium from log-phase culture of NK-92 or DERL-7 cell lines. This medium provides additional factors for the growth and survival of infected NK cells.
15. Expression or knockdown of the gene can be assayed by western blot or real-time PCR.
16. LNK cells are adherent cells and can be dislodged from the culture dish by washing once with PBS and then incubating the cells in PBS containing 1 mM EDTA for 5 min at 37°C in a CO₂ incubator. The cells should then be washed with PBS to remove EDTA from the cells.

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Fig. 14.1.

Flow cytometric analyses of yellow fluorescent protein (YFP)-positive cells in NK-92 and DERL7 cells post-lentiviral transduction compared to controls.