Methods in Molecular Biology™

VOLUME 121

Natural Killer Cell Protocols

Cellular and Molecular Methods

Edited by

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HUMANA PRESS

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Cloning Human Natural Killer Cells

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1. Introduction

Our understanding of the phenotypical and functional heterogeneity of human natural killer (NK) cells has greatly advanced over the past few years. This advancement has been greatly helped by the development of culture conditions for clonal proliferation of NK cells. Analysis of human NK cell clones has led to the original observation that different NK cell clones recognize distinct major histocompatibility complex (MHC) class I specificities. This has prompted the production of monoclonal antibodies directed to NK cell surface antigens clonally distributed, and, ultimately, the biochemical and molecular definition of the NK cell surface glycoproteins functioning as MHC class I receptors.

Here we describe a limiting dilution culture protocol that allows establishment of human NK cell clones from peripheral blood leukocytes with high efficiency.

2. Materials

1. RPMI 1640 (Gibco, cat. no. 31870-025).
2. RPMI + HEPES (25 m*M*): (Gibco, cat. no. 42401-018).
3. Lymphocyte separation medium (LSM): (ICN Biomedicals, cat. no. 50494/36427).
4. Human recombinant interleukin (IL)-2 Liquemin (25000 UI/5 mL, Roche).
5. RPMI 8866 cells (available from Dr. Bice Perussia, Jefferson Medical College, Kimmel Cancer Institute, BLSB 750, 233 S 10th Street, Philadelphia, PA 19107, USA).
6. Antibodies: Anti-human CD56 (Pharmingen, cat. no. 31661A, mouse IgG_1), anti-human CD3 (OKT3, mouse IgG_{2a}, ATCC, cat. no. CRL-8001), anti-mouse IgG_1-phycoerythrin (PE; SBA, cat. no. 1070-09), anti-mouse IgG_{2a}-fluorescein isothiocyanate (FITC; SBA, cat. no. 1080-02).
7. Phosphate-buffered saline (PBS).
8. PBS supplemented with 1% fetal calf serum (FCS).

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
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9. Complete medium (CM): RPMI 1640 medium (Gibco, cat. no. 31870-025) supplemented with 5% human serum (filtered through 0.8-μm filter unit, Nalgene, cat. no. 380-0080; not heat treated), 500U/mL of human recombinant IL2 (Roche), nonessential amino acids (from 100X stock; Gibco, cat. no. 11140-035), sodium pyruvate (from 100× stock; Gibco, cat. no. 11360-039), L-glutamine (from 100× stock; Glutamax I, Gibco, cat. no. 35050-038), kanamycin (100 μg/mL final from 100×; Gibco, cat. no. 15160-047), and 2-mercaptoethanol (5×10^{-5} *M* final from sterile stock). Do not add HEPES to CM. Filter through 0.22-μm filter unit.
10. Phytohemagglutinin (PHA) (Murex Diagnostics, HA16).
11. Freezing mix : 70% FCS, 10%DMSO, 20% RPMI-HEPES.
12. Cell sorter.
13. Gamma irradiation source.
14. 96-well plates, 24-well plates, 6-well plates, cryotubes. (No particular commercial source is required.)

3. Method

3.1. Preparation of NK Cells

Use sterile technique throughout the following procedures.

1. Collect 5 mL of blood from a blood donor with anticoagulants (heparin or EDTA) and dilute 1:1 with RPMI + HEPES.
2. In a 15-mL plastic tube gently lay 10 mL of diluted blood on 5 mL of LSM using a 5-mL wide mouth plastic pipet. Centrifuge for 30 min at 940*g* at room temperature with no brake. Red blood cells and granulocytes will sediment in the pellet, while peripheral blood mononuclear cells (PBMCs) will localize at the interface between LSM (below) and plasma (above).
3. Collect PBMCs at the interface with a pasteur pipet. Transfer PBMCs to a separate tube, wash them in RPMI + HEPES, and collect them by centrifuging for 15 min at 500*g*. Discard supernatant. Flick tube gently to resuspend pelleted cells.
4. Wash the pelleted PBMCs 2× with RPMI + HEPES. Collect by centrifuging for 10 min at 300*g*.
5. Resuspend the pellet of the heparinized blood cells in a 15-mL Falcon tube in 500 μL of ice-cold PBS–1% FCS containing anti-CD56 antibody (mouse IgG1, 10 μg/mL) and anti-CD3 antibody (mouse IgG_{2a}, 10 μg/mL). Incubate 30 min on ice.
6. Fill the tube of antibody-treated cells with 15 mL of ice-cold PBS–1% FCS and centrifuge at 300*g* for 10 min.
7. Wash 1× with ice-cold PBS–1% FCS by centrifuging for 10 min at 300*g*.
8. Resuspend the pellet of antibody-treated cells in 200 μL of PBS–1% FCS containing goat anti-mouse IgG_1-PE (1:100 dilution) and goat anti-mouse IgG_{2a}-FITC (1:50) and incubate on ice for 30 min.
9. Fill the tube of antibody-treated cells with 15 mL of ice-cold PBS and centrifuge at 300*g* for 10 min.
10. Wash 1× with ice cold PBS–1% FCS by centrifuging for 10 min at 300*g*.
11. Resuspend the stained cells in PBS with 1% FCS at a concentration of $3–5 \times 10^6$ cells/mL. Sort at least 10,000 of the $CD3^-CD56^+$ cells on a cell sorter.

12. Dilute 10,000 cells in 10 mL of CM. Perform progressive 10-fold limiting dilutions of these cells into CM until the cells have been diluted to 10 cells/mL (0.5 cells/50 μL) (**Note 1**). Prepare 50 mL of this final dilution per 10 × 96 U-bottom plates (5 mL/plate).

3.2. Preparation of Feeder Cells

Prepare these cells in parallel with NK cells.

1 To prepare allogeneic feeder cells, collect 50 mL of blood with anticoagulants from a different blood donor and dilute 1:3 with RPMI + HEPES.
2. Gently lay 30-mL aliquots of diluted blood on 15-mL LSM in a 50mL plastic tube and centrifuge for 30 min at 940*g* at room temperature with no brake.
3. Collect PBMCs at the interface between Ficoll and plasma with a pasteur pipet and wash in RPMI + HEPES by centrifuging for 15 min at 500*g*.
4. Wash 2× with RPMI + HEPES and collect cells by centrifuging for 10 min at 300*g*.
5. In parallel, wash 5×10^6 cultured RPMI 8866 cells twice with RPMI+HEPES (**Note 2**).
6. Irradiate 5×10^7 PBMC and 5×10^6 RPMI 8866 cells with 5000 Rads.
7. Wash the irradiated cells once with RPMI+HEPES by centrifuging at 300*g* for 10 min.
8. Resuspend the irradiated cells together in CM at concentrations of 1×10^6/mL of PBMC and 1×10^5/mL of RPMI 8866. Add 2 μg/mL of phytohemagglutinin (PHA) to these cells. This mixture is referred to as "restimulation mix" in subsequent procedures.

3.3. Plating and Growing NK Cell Clones

1. Mix 50 mL of NK cells and 50 mL of irradiated feeder cells in a flask and plate 100 μL/well in 96-well round bottom plates. Culture at 37°C in 5% CO_2.
2. Inspect culture clones for cell growth after 10–14 d (**Note 3**). Tranfer each well with clearly enlarged pellet when viewed from beneath to a single well of a 24-well plate and add 150 μL of CM. After about 3 d add an additional 250 μL of CM, and three days later, split to two wells. About 3 d later, transfer cells into one well of a 6-well plate. Expand as necessary when medium is turning yellow. Cells should be split when they reach a concentration of 1–2 × 10^6/mL. Usually, cells can be expanded up to 3–6 wells of a 6-well plate at 1–2 × 10^6/mL. Clone size ranges between ~10–40 million cells after 21–28 d of culture without restimulation.
3. Check NK cell surface phenotype of cloned cells by fluorescence-activated cell sorter (FACS) after staining with anti-CD3 and anti-CD56.

3.4. Maintenance of NK Cell Clones

1. Every 20–30 d NK cell clones slowly stop dividing. At this point they need to be restimulated with feeder cells.
2. Usually, we take aliquots of 3×10^5 NK cells/mL and mix them with 1 mL of restimulation mix prepared as described previously and plate them in one well of a 24-well plate.

3. Each well can be expanded into 3 wells of a 6-well plate containing about 3–6 million cells (**Notes 4–8**).

3.5. Storage of NK Cell Clones

1. Collect 10^6–10^7 cells and centrifuge for 5 min at 1200 rpm.
2. Discard supernatant, resuspend cells in 1 mL of freezing mix, and transfer to a cryotube.
3. Store cryotubes overnight at –80°C and then transfer the tubes to liquid N_2.

3.6. Plating and Growing NK Cell Bulk Cultures

1. For each 96-well round bottom plate mix 10 mL of the first dilution of NK cells with 10 mL of restimulation mix.
2. Plate the mix in 96-well round bottom plates at 200 µL/well.
3. Culture bulk NK cells for 5–8 d at 37°C in 5% CO_2.
4. Transfer 12 wells of the 96-well plate into one well of a 6-well plate and maintain by splitting to a new well of a 6-well plate every 2–4 d when medium begins to yellow. The cells grow best when kept at a concentration of around 1×10^6/mL (**Note 8**).

4. Notes

1. When performing serial dilution of NK cells, one should gently resuspend cells about 5× with pipettor to thoroughly distribute and dilute cells.
2. RPMI 8866 cells should be used when they are in exponential phase of growth.
3. The frequencies of cells capable of extensive proliferation under these culture conditions are typically 10–20/plate.
4. We have been able to grow cell clones up to 2 billion cells.
5. Individual clones can be analyzed for expression of killer cell Ig-like receptors (KIRs) and NKG2/CD94 receptors. The expression of these receptors is stable over years.
6. Individual clonal cultures can also be analyzed for their lytic activity against K562 target cells.
7. Transfection of clonal cultures by electroporation is virtually impossible. NK cell clones can be successfull transfected with vaccinia virus-based constructs.
8. One should routinely monitor bulk cultures for growth of cells expressing CD3 by flow cytofluorimetry to be sure that potentially contaminating T cells are not overgrowing NK cells.

Acknowledgment

The Basel Institute for Immunology was founded and is supported by Hoffmann-La Roche, CH-4002 Basel.

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Generation of Short-Term Murine Natural Killer Cell Clones to Analyze *Ly49* Gene Expression

Werner Held, Bente Lowin-Kropf, and David H. Raulet

1. Introduction

Natural killer (NK) cells express receptors specific for class I major histocompatibility complex (MHC) molecules. In the mouse, the class I specific receptors identified to date belong to the polymorphic Ly49 receptor family. Engagement of Ly49 receptors with their respective MHC ligands results in negative regulation of NK cell effector functions, consistent with a critical role of these receptors in "missing self" recognition. The Ly49 receptors analyzed so far are clonally distributed such that multiple distinct Ly49 receptors can be expressed by individual NK cells (for review *see* **refs. *1–3***). The finding that most NK cells that express the Ly49A receptor do so from a single *Ly49A* allele (whereby expression can occur from the maternal or the paternal chromosome) may thus reflect a putative receptor distribution process that restricts the number of Ly49 receptors expressed in a single NK cell ***(3–5)***.

Ly49 receptors are encoded by a small gene family that currently comprises nine members, denoted *Ly49A-I* (for review *see* **ref. *3***). The further and more detailed analysis of Ly49 receptor expression, however, is hampered owing to:

1. The lack of murine NK cell clones.
2. The limited number of monoclonal antibodies (mAbs) that recognize individual Ly49 receptors or alleles thereof.

We have thus developed and describe in detail below a procedure that allows the analysis by reverse transcription and polymerase chain reaction (RT-PCR) of the expression of Ly49 receptor genes in short-term clonal populations of mouse NK cells.

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
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2. Materials

1. Mice: C57BL/6J (B6), > 6 wk old.
2. Recombinant human interleukin-2 (rIL-2).
3. Cell culture medium: Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine and 4.5 g/L glucose (Gibco-BRL, Paisley, UK) supplemented with HEPES (10 m*M*), 2-mercaptoethanol (5×10^{-5} M), penicillin (50 µg/mL), streptomycin (50 µg/mL), neomycin (100 µg/mL) (all from Gibco-BRL) and 10% fetal calf serum (FCS).
4. ACK buffer: 0.16 *M* NH_4Cl, 0.1 m*M* Na_2EDTA, 0.01 *M* $KHCO_3$.
5. Nylon wool columns: Weigh out 0.6 g of nylon wool (type 200L, combed and scrubbed) (Robbins Scientific, Sunnyvale, CA). Fluff the nylon wool manually and package into a 10-mL syringe up to the 6-mL mark (i.e., 0.1 g/mL), wrap into tin foil, and autoclave. Such a column is good for one spleen (i.e., 10^8 cells).
6. Monoclonal antibodies (mAbs): anti-CD16/CD32 (2.4G2, anti-FcγII/III receptors) hybridoma supernatant to prevent nonspecific staining (available as FcBlock™ from Pharmingen, San Diego, CA), phycoerythrin (PE)-labeled anti-CD3 (145.2C11), fluoroisothiocyanate (FITC)-labeled anti-NK1.1 (PK136). Note that the NK1.1 antigen is expressed only in a few mouse strains including C57Bl/6 (*see* **Appendix**). The anti-DX5 antibody in conjunction with CD3 can be used to identify NK cells in all mouse strains. All mAbs are available from Pharmingen (San Diego, CA).
7. Plasticware: 96-Well U-bottom plates (such as Costar, cat. no. 3799, Cambridge, MA), tissue culture flasks (such as Falcon, cat. no. 3014, Becton Dickinson, Franklin Lakes, NJ).
8. Fluorescence activated cell sorter (such as FACStar^{plus} [Becton Dickinson, San Jose, CA]) equipped with a single cell deposition unit.
9. Total RNA isolation reagent (such as Trizol Reagent [Gibco-BRL]).
10. Oligo-dT (such as primer dT_{15}, Roche Molecular Biochemicals, cat. no. 814270, Mannheim, Germany).
11. RNase inhibitor (such as RNAguard, 33 U/µL, Pharmacia, cat. no. 27-0815-01, Uppsala, Sweden).
12. Reverse transcriptase and buffer (such as AMV RT, 20 U/µL, Roche Molecular Biochemicals #109 118).
13. Taq polymerase (such as AmpliTaq, 5 U/µL, Perkin Elmer, Emeryville, CA).
14. Thermocycler (such as Uno Thermoblock, Biometra, Tampa, FL).
15. Dideoxynucleotides (such as Roche Molecular Biochemicals).

3. Methods

3.1. Cell Culture and Sorting

Lymphokine-activated Killer cells (LAKs) are prepared following the method described by Karlhofer et al. ***(6)*** with modifications.

1. Warm culture medium to 37°C.
2. Attach a three-way stopcock and a $21^1/_2$-gage needle to a sterile nylon wool column. Add prewarmed medium to wet nylon wool. Close stopcock and remove air

bubbles by firmly tapping to the sides of the column. Run 10 mL of prewarmed medium through the column. Close stopcock and cover nylon wool with 1 mL of medium. Incubate 30 min at 37°C in CO_2 incubator.

3. Remove the spleen under sterile conditions. Prepare a single cell suspension by pressing the spleen through a steel mesh into a sterile Petri dish filled with 10 mL of medium. Transfer the cell suspension into a tube.
4. Leave for 2 min to sediment large debris.
5. Transfer the supernatant into a new tube and centrifuge for 5 min at 500*g*.
6. Remove the supernatant and lyse red blood cells by resuspending the cell pellet in 1 mL of ACK buffer, incubate for 1 min, and add 10 mL of medium.
7. Centrifuge for 5 min at 500*g*, then wash with 10 mL of medium.
8. Resuspend the cell pellet in 2 mL of prewarmed 37°C medium.
9. Drain equilibrated nylon wool column and apply spleen cell suspension.
10. Stop the flow when the suspension has completely entered the column, and add 1 mL of prewarmed medium to cover the nylon wool.
11. Incubate for 1 h at 37°C in a CO_2 incubator.
12. Elute nylon wool nonadherent cells with 7–10 mL of prewarmed medium (*see* **Note 1**). Centrifuge for 5 min at 500*g*.
13. Resuspend the cell pellet in 10 mL of medium containing rIL-2 at 250 ng/mL. Transfer to a small (25-cm^2) tissue culture flask and culture in a CO_2 incubator for 3 d.
14. Harvest LAKs. Adherent cells are detached by incubating for a few minutes with cold PBS containing 1.5 m*M* EDTA. Pool nonadherent and adherent cells.
15. Count viable cells, centrifuge for 5 min at 500*g*, and resuspend at 10^6 cells/25 µL of 2.4G2 hybridoma supernatant to block Fcγ receptors. Incubate for 20 min on ice.
16. Wash 1× with PBS containing 5% FCS.
17. Incubate the cell suspension with appropriate dilutions of PE-conjugated anti-CD3 plus FITC-labeled NK1.1 mAbs in PBS containing 5% FCS at 10^6 cells/25 µL.
18. Wash as above and resuspend at 2×10^6 cells/mL for single cell sorting.
19. Sort single $CD3^-$ $NK1.1^+$ blast cells (the latter is defined by an elevated forward and side scatter) (*see* **Fig. 1**) into wells of a round-bottom 96-well plate, which contain 200 µL of culture medium plus 250 ng/mL of rIL-2.
20. Wrap plates into tin foil and culture in a CO_2 incubator for 7 d (*see* **Note 2**).

3.2. RNA Isolation

The remainder of this procedure requires the usual precautions for work with RNA. The use of aerosol-resistant tips is recommended to prevent cross-contamination of the samples to be used later for PCR.

1. Visually inspect wells and mark those containing >10 cells (*see* **Note 3**).
2. From marked wells remove as much supernatant as possible without disturbing the cells.
3. Isolate total cellular RNA using the acid phenol method developed by Chomczynski and Sacchi *(7)*. Lyse the cells directly in the well by the addition of

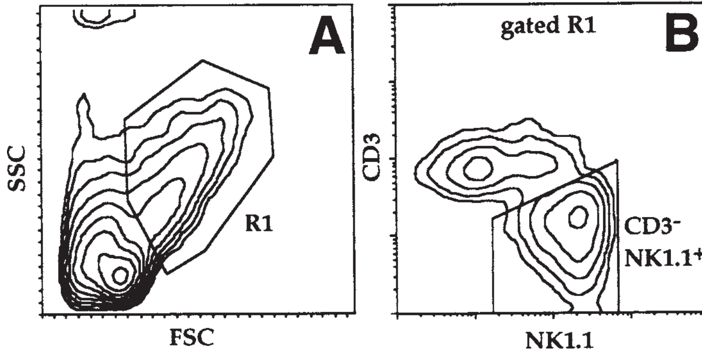

Fig. 1. Lymphokine-activated Killer cells used for NK cell cloning. Foreward (FSC) and side scatter gate (SSC) of d 3 lymphokine activated cells are shown in **(A)**. Cell surface expression of CD3 and NK1.1 is assessed in blast cells (R1) (identified based on an elevated FSC /SSC). To derive short term NK cell clones, a single $CD3^-NK1.1^+$ cell is deposited per microwell using a cell sorter equipped with a single cell deposition unit.

200 µL of Trizol reagent to which 10 µg/mL carrier tRNA has been added, mix well by pipetting up and down, and tranfer the lysate to a 1.5-mL Eppendorf tube. Incubate for 5 min at room temperature (*see* **Note 4**).

4. Add 40 µL of chloroform, shake by hand for 15 s, and incubate for 2–3 min at room temperature.
5. Centrifuge in a cooled (4°C) microfuge for 15 min at 12,000*g*.
6. Recover upper, aqueous phase (approx 60% of the total volume) and transfer to a new 1.5-mL Eppendorf tube.
7. Precipitate RNA by the addition of 100 µL of isopropanol, mix, and incubate at room temperature for 10 min.
8. Centrifuge in a cooled (4°C) microfuge for 10 min at 12,000*g*.
9. Wash the RNA pellet by adding 1 mL of 70% EtOH, mix and centrifuge in a cooled (4°C) microfuge for 5 min at 7500*g*.
10. Air-dry RNA pellet for 5–10 min.

3.3. Complementary DNA Preparation

1. Resuspend RNA pellet in a total of 7 µL of H_2O containing 0.3 µL of oligo-dT (150 µ*M*) as a primer.
2. Incubate for 5 min at 72°C.
3. Transfer directly on ice.
4. Add 13 µL of reverse transcriptase mix:

4.0 µL	5× concentrated reverse transcriptase buffer
5.0 µL	2 m*M* of each dATP, dCTP, dGTP, and dTTP
2.0 µL	0.1 m*M* DTT
1.1 µL	H_2O

0.6 µL RNase inhibitor
0.3 µL reverse transcriptase
total volume of 20 µL for cDNA preparation

5. Incubate for 1 h at 42°C, store at –20°C.

3.4. Polymerase Chain Reaction

1. Take 1 µL of the cDNA preparation for PCR.
2. Add 29 µL of PCR mix (*see* **Note 5**):
   0.6 µL of sense primer (10 m*M* stock)
   0.6 µL of antisense primer (10 m*M* stock)
   3 µL of 10 x PCR buffer containing 15 m*M* $MgCl_2$
   3 µL of 2 mM of each dATP, dCTP, dGTP, and dTTP
   0.15 µL of *Taq* polymerase
   total volume of 30 µL for PCR preparation.
3. The PCR is performed using the following conditions:
   Preheat PCR machine to 92°C, add samples, and leave at 92°C for 3 min, start cycles:
   92°C for 1 min, 55°C for 1 min, 72°C for 1 min
   40 cycles
   72°C for 5 min, then hold at 4°C.
4. One microliter of this PCR product (*see* **Note 6**) is used for reamplification using a set of nested PCR primers (*see* **Fig. 2**). Conditions for reamplification are the same as described previously except that the number of cycles is reduced to 20 (*see* **Note 7**).

3.5. Analysis of the PCR Product

1. One tenth (3 µL) of the second PCR product is run on an agarose gel to identify positive clones.
2. In the case of *Ly49A*, the presence of correct amplification product is verified by restriction enzyme digestions of one tenth (3 µL) of the second PCR product. Add 2 U of restriction enzyme plus the appropriate digestion buffer and bring volume to a total of 20 µL. Incubate at the appropriate temperature for 1 h (*see* **Note 8** and **Fig. 2**).
3. PCR and/or cleavage products are visualized under UV light following gel electrophoresis in the presence of ethidium bromide.

4. Notes

1. Nylon wool nonadherent cells are mostly T cells and NK cells with few B cells (<5% of total). Recovery is usually between 15 and 20×10^6 cells per B6 spleen.
2. Ly49 receptor expression is stable at least during the 7 d culture period used for expansion ***(5)***.
3. Approx 20–30% of the wells contain more than 10 cells.
4. The lysate can be stored at this stage at –80° C for at least a month.
5. Ly49-specific PCR primers:

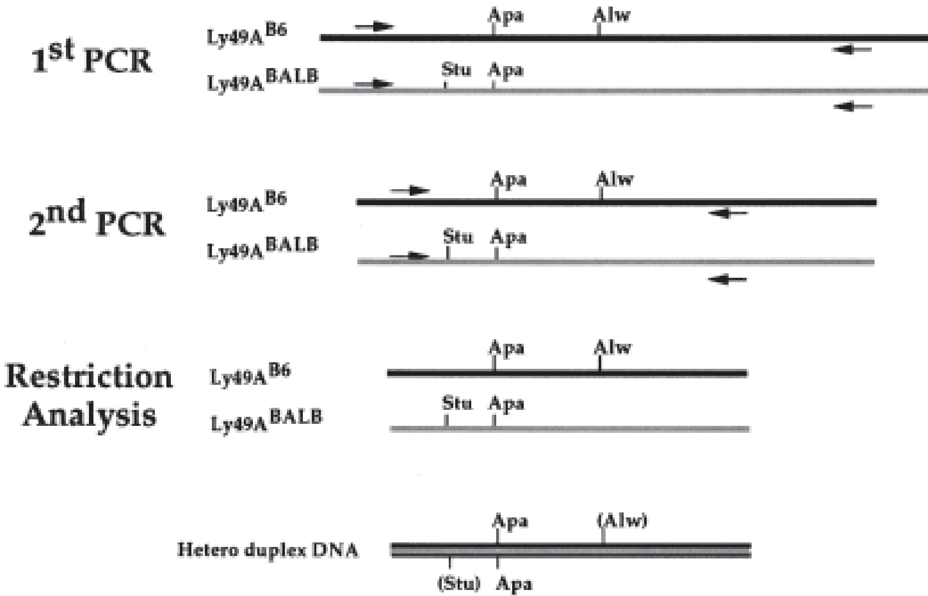

Fig. 2. Analysis of *Ly49A* gene expression in short-term NK cell clones. Complementary DNA templates derived from short-term NK cell clones are subjected to PCR amplification using *Ly49A*-specific primers (1st PCR). An aliquot of the first reaction is reamplified using an internal pair of *Ly49A*-specific primers (2nd PCR). The B6 or BALB/c origin, respectively, of the amplified product is determined using allele-specific restriction digests: Stu I specifically cleaves the BALB/c allele of *Ly49A* whereas Alw I specifically cleaves the B6 *Ly49A* allele. PCR amplification over the log phase may result in variable amounts of heteroduplex PCR products (one strand is of B6 and one strand is of BALB/c origin), which are resistant to allele-specific restriction digests.

Numbering of the primers is according to the Ly49 sequences published by Smith et al. ***(8)*** and denotes the most 5' base in the sense and the most 3' base in the antisense primer in the respective *Ly49* sequence. The *Ly49A*-specific primer pairs allow amplification of both the B6 and BALB allele of the *Ly49A* gene:

<u>Ly49A-specific</u>

1st round	52 sense:	5'-ACCAGAACCACTTCTTG$^{C}/_{A}$TAGC-3'
	976 antisense:	5'-CAAAACACTTGTTTGCAAGG-3'
2nd round	145 sense:	5'-GGAGGTCACTTATTCAATGG-3'
	535 antisense:	5'-CCATAGCAGAACCAGTATAC-3'

<u>Ly49C-specific</u>

1st round	99 sense:	5'-CTCCCACGATGAGTGAGCCA-3'
	827 antisense:	5'-GTAGGGAATATTACAGTCA-3'
2nd round	209 sense:	5'-GGCAACAGAAAGTGTTCAGC-3'
	680 antisense:	5'-AGACAATCCAATCCAGTAAT-3'

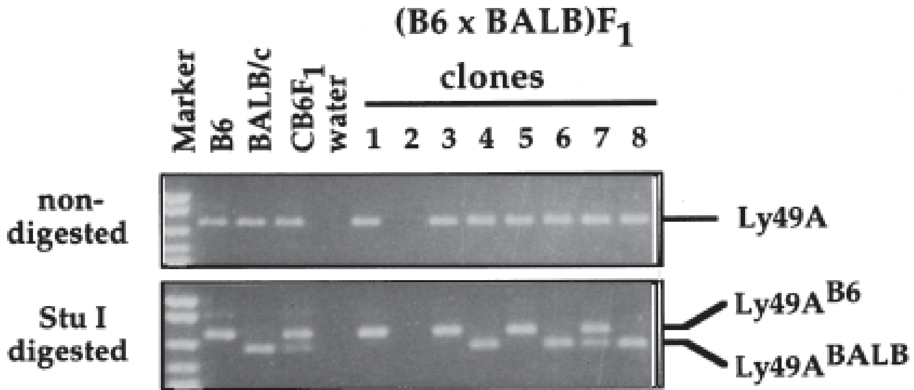

Fig. 3. *Ly49A* gene expression in Ly49A$^+$ short-term NK cell clones. Control amplifications include cDNA derived from B6, BALB/c, and (B6 × BALB/c)F_1 bulk NK cells RNA and no cDNA (water). No PCR product was obtained for clone no. 2. Whereas most clones express either the B6 or BALB/c *Ly49A* allele, some rare clones (such as no. 7) express both alleles. The relevant marker fragment sizes from the top are 622bp, 527bp, 404bp, and 309bp.

Ly49G2-specific

1st round	113 sense:	5'-CTCAACTGTGAGATTTCATG-3'
	814 antisense:	5'-TGTATGATTTATCACAGTCC-3'
2nd round	152 sense:	5'-CTAGTGAGGACTGAGGAGC-3'
	776 antisense:	5'-TTAGATAACGACATACATAA-3'

PCR primers to specifically detect the above and the remaining Ly49 family members were recently used by Toomey et al. ***(9)***.

6. A PCR product will be visible for many clones after gel electrophoresis in the presence of ethidium bromide after the first round of 40 cycles.
7. The number of cycles will have to be determined empirically for the particular primer pair used, as the reaction should be terminated as soon as there is enough PCR product for restriction digestion analysis and as long as the PCR reaction is in log phase. This is particularly important if two distinct target sequences are simultanously amplified (e.g., two alleles of the same *Ly49* gene). Amplification over log phase may result in the formation of heterodimeric double strands (one strand derived from each *Ly49* allele), which will be resistant to cleavage by allele specific restriction enzymes (*see* **Fig. 2**).
8. As an example, Alw I can be used to specifically cut the product of the B6 *Ly49A* allele, StuI specifically cleaves the product of the BALB *Ly49A* allele, whereas Apa I cleaves both *Ly49A* alleles (*see* **Figs. 2** and **3**) ***(4)***.

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3

Cloning and Culturing of Fetal Mouse Natural Killer Cells

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1. Introduction

The ability to study the properties and functions of individual cells is a major goal of cell biologists. Nowhere is this more true than in studies of the immune system, in which the complexity is such that results obtained at the population level often obscure critical aspects of the function and diversity of the component cells. The study of individual cells *per se* is still technically difficult and of necessity limited in scope, leading to the compromise in which populations of cells derived from a single parent cell (clones) are studied. Considerable valuable information can be obtained from even relatively small clones of limited life span (**ref. *1***, Chapter 2), but the ultimate aim is to produce clonal populations of cells that show indefinite growth and retain normal physiological properties, thereby permitting large-scale and long-term studies. The discovery of methods for cloning mouse and human T cells led directly to major advances in our understanding of the recognition mechanisms and functional capabilities of "individual" T cells. More recently, the development of procedures for the cloning of human NK cells was instrumental in the discovery of killer cell immunoglobulin-like inhibitory (KIR) receptors (***2,3***; and Chapter 1).

By contrast, for unknown reasons, it has proven exceedingly difficult to clone murine natural killer (NK) cells. In the early 1980s, following the discovery that interleukin-2 (IL-2) was not only a growth factor for T cells but also for NK cells ***(4)***, laboratories reported a number of reports of the cloning of cells with NK cell characteristics ***(5–8)***. However, the finding that many of the lines and clones obtained under these conditions expressed CD8 ***(7)***, coupled with the discovery that conventional T cells could acquire not only NK cell markers such as asialo-GM1 and NK1.1 ***(9,10)*** but also NK cell func-

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
Edited by: K. S. Campbell and M. Colonna © Humana Press Inc., Totowa, NJ

tion ***(9,11)***, led to doubts concerning the lineage of these clones. The subsequent development of monoclonal antibodies and DNA probes for studying T cell receptor gene expression showed directly that many, and by implication all, of these early NK clones were of T cell origin ***(12,13)***. Those clones that lacked CD4 and CD8, markers that were thought at the time to be expressed on all mature T cells, were, with the benefit of hindsight, presumably derived from γδ or $CD4^-CD8^-$ αβ T cells, a notion supported by the more recent finding that T cells of this type frequently express NK1.1 and other NK-related markers and display potent lytic activity against NK-sensitive targets ***(14)***. Indeed, in the human, most γδ T cells and clones express CD94 and/or p58/p70 inhibitory KIR ***(15)***. Paradoxically, therefore, by at least some criteria, γδ T cells qualify as NK cells.

This raises the critical issue of the exact nature of NK cells and their relationship to, and distinction from, T cells. Although it is clear that the vast majority of NK cells are thymus independent and lack expression and rearrangement of T cell receptor genes, growing evidence suggests that NK cells and T cells share a common precursor ***(16–18)***. Part between d 13 and 15 of development, prior to the onset of T cell receptor gene rearrangement, mouse fetal thymus contains a substantial population of cells that have NK cell characteristics and/or NK progenitor activity ***(18–21)***. The discovery of a method that allowed the rapid expansion and growth of these cells in vitro led to the first successful cloning of mouse NK cells ***(20)***. The same procedure was subsequently shown to allow the generation of long-lived NK cell lines from fetal liver ***(22)***.

That these lines and clones are indeed of an NK cell nature is shown by their close phenotypic and functional similarity to short-term cultured adult splenic NK cells ***(20,22)*** and by the absence of T cell receptor gene rearrangements (Shrestha, Petrie, and Brooks, unpublished observations). The only difference between these cells and adult splenic NK cells that has emerged so far is the frequent failure of fetal NK cells to express several members of the Ly49 family of inhibitory and activatory receptors ***(22–24)***. However, they do express high levels of one Ly49 family member, Ly49E, at least at the mRNA level ***(23)***, and show a limited ability to discriminate between class I-sufficient and class I-deficient targets ***(23)***. Indeed, when tested on a large panel of tumor target cells, their recognition capacity was found to be remarkably similar to that of adult NK cells. Most importantly, different clones of fetal NK cells displayed a similar broad specificity both to each other and to that of uncloned bulk populations of fetal or adult NK cells ***(24)***, suggesting that positive recognition of these target cells by NK cells is either dominated by a single NK cell receptor or that individual NK cells express multiple receptors. However, a third possibility needs to be considered, namely that fetal NK cell progenitors

undergo diversification during their development and growth in vitro. Evidence in support of this has come from the finding that individual fetal thymus NK cell progenitors invariably give rise to clones that contain subpopulations of cells that differ in their expression of a number of surface molecules putatively involved in intercellular recognition/costimulation/signal transduction including members of the Ly6 family, certain CD45 isoforms, CD8, and at least one member of the NKR-P1 family ***(24)***. Importantly, although most fetal NK clones fail to express Ly49A, C, D, G, and I, a few clones have been found that do express these molecules; in each case only a subpopulation of the cells present within the clone is positive ***(24)***. In addition, we have recently found that receptors for the non-classical class I molecule, Qa1, are also expressed in a mosaic manner within individual clones ***(25)***.

In this article we describe the methods we have used to generate NK cell clones from progenitor cells present in the fetal thymus. We have used essentially the same procedure to generate long-term lines ***(22)*** and clones (unpublished observations) from day 14 fetal liver. However, although the technical difficulties in obtaining cells from fetal thymus are substantial we prefer this source because clones derived from fetal thymus appear to be more vigorous than those obtained from fetal liver. Furthermore, the fact that the majority of individual d 14 thymocytes will grow and generate clones in the absence of feeder cells allows (1) early or immediate micromanipulation cloning, (2) direct observation of developing clones, and (3) the potential to study clones at various stages of development in the complete absence of any other cell type ***(20)***.

2. Materials

1. The culture medium used is high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with nonessential amino acids, mercaptoethanol, and fetal bovine serum (FBS). This is made up in-house using powdered and concentrated stocks and high-purity water (*see* **Note 1**). Specifically, high-glucose DMEM powdered medium (cat. no. 52100-039, Life Technologies, Paisley, UK) is dissolved initially in approx 5 L of Nanopure water (glass-distilled water passed through a Nanopure water purification apparatus made by Whatman, Maidstone, UK). Other high-purity water sources, such as MilliQ (Millipore, Bedford, MA) appear to be satisfactory. When completely dissolved, 200 mL of 100× nonessential amino acids (11140-035, Life Technologies) is added, followed by 2.75 g of sodium pyruvate (11840-048, Life Technologies) and 37 g of tissue culture grade sodium bicarbonate (11810-025, Life Technologies). When this has dissolved, 37 µL of neat 2-mercaptoethanol (Sigma, Poole, UK) is added (*see* **Note 2**), followed by slow addition of ~5 mL of HCl (specific gravity 1.18; the amount added should be such as to give a final pH of about 7.0 prior to filtration). The solution is diluted to 10 L with Nanopure water, and passed through a 0.22-µm filter into 500-mL glass bottles. It is stored at 4°C. Prior to use, FBS (*see* **Note 3**) is added to give 10% v/v (the complete culture medium is designated D10F). To minimize the likelihood of mycoplasma infection no antibiotics are used.

2. Medium for washing/manipulating cells is Hanks' balanced salt solution made up from powder (61200-093, Life Technologies) using Nanopure water and omitting bicarbonate. For washing cells, it is supplemented with 1% FBS (H1F); as a holding medium for cells or fetal thymuses it is supplemented with 10% FBS (H10F).
3. Recombinant human IL-2 is available from many commercial sources, but the large amounts needed for optimal growth of NK cells make it desirable to enter into some arrangement with the supplying company. Concentrated solutions of IL-2 must be stored in acid conditions. We routinely prepare stocks at 10^6 IU/mL in acetic acid/BSA (5 mg/mL of bovine serum albumin dissolved in 0.3% acetic acid in water). Such stacks are stable indefinitely at 4°C. They are diluted directly into D10F to give stable working solutions at 10^4 IU/mL. (*See* **Note 4** for discussion of unitage.)
4. Recombinant mouse IL-4 is also available from several commercial sources, although we have generally used IL4-containing supernatant from a cell line transfected with an expression vector for mouse IL-4. Stock solutions at 10^3 U/mL are prepared in H10F and these are stable indefinitely at 4°C. (*See* **Note 5** for discussion of unitage.)
5. Phorbol myristate acetate (PMA, P8139, Sigma) is purchased as a 1-mg vial and reconstituted in 1 mL of absolute ethanol. It is stored at –20°C in a 1.5-mL polypropylene reaction tube wrapped with sealing film to prevent evaporation. It is stable in this form for at least 1 yr. Working stock solutions at 1 µg/mL are made by diluting 2 µL of ethanol stock into 2 mL of H1F immediately before use (*see* **Note 6**).
6. Timed-mated mice can be purchased from some animal suppliers such as Bantin & Kingman, Hull, UK. Otherwise they can be set up in house by pairing individual male mice with one to three female mice overnight. Early in the morning female mice should be examined for vaginal plugs (sometimes these are not readily visible and can be detected only with a probe, e.g., a 200-µL pipet tip). Males, inseminated females, and noninseminated females should be separated, and the latter should not be remated for at least a week. The day of vaginal plug inspection is scored as d 0.
7. 96-Well flat-bottomed plates are required for cloning. We prefer half-area plates from Corning-Costar, High Wycombe, UK, cat. no. C6396.

3. Methods

3.1. Preparation of Thymocytes

1. At day 14 of gestation mice are killed and the pregnant uteri removed into a Petri dish.
2. The uteri and the individual placental membranes are opened with scissors to liberate the embryos. These are placed on filter paper circles that have been sterilized by autoclaving in Petri dishes.
3. They are placed under a dissecting microscope and thymus lobes excised using cataract knives. (*See* **Fig. 1** in Chapter 5, for localization of the fetal thymus.) This is a technically demanding procedure that is best learned by direct demon-

stration from an experienced worker. A more detailed description of the procedure can be found in **ref. *26***. The thymus lobes are collected into a small tube containing H10F which should be kept in ice throughout the dissection procedure.

4. The thymus lobes should be washed twice by allowing them to sediment in about 5 mL of H10F followed by careful removal of the supernatant. They should finally be resuspended in 0.5–1 mL of H10F and lobes plus medium placed in a Petri dish. Each individual lobe should then be teased apart using cataract knives under a dissecting microscope.
5. The cell suspension and macerated tissue is rinsed out of the Petri dish into a centrifuge tube with 5 mL of H1F and the tissue fragments are allowed to sediment for about 10 min.
6. The cell suspension is transferred to a fresh tube and centrifuged at 1000*g* for 5 min, then resuspended in 1–2 mL of D10F and counted. A typical yield is 5×10^4 cells per lobe.
7. Aliquots of $0.5–1 \times 10^6$ cells should be set up in the wells of 24-well plates in 2 mL of D10F containing 10 U/mL of recombinant mouse IL-4 and 10 ng/mL of PMA. Lower numbers of cells can be cultured if only a few lobes have been obtained.
8. After 24–48 h, by which time most of the original thymocytes will be actively proliferating, the cells should be cloned by either limiting dilution or micromanipulation.

3.2. Cloning by Limiting Dilution

1. Cells should be counted carefully on a hemacytometer. If the cells display any clumpiness they should be pipetted up and down in a fine Pasteur pipet or passed through a narrow-gage needle. It is important to ensure that the cells are entirely in single-cell suspension but excessive force should not be used, as this will damage the cells.
2. The cells are serially diluted to eventually give cell suspensions in the range of 1–10 cells/mL in D10F containing 10^4 IU/mL of recombinant human IL-2 and 10 ng/mL of PMA.
3. Aliquots of 100 µL are placed into 96-well flat-bottomed plates. The plates should be placed in a thoroughly humidified 37°C incubator in an atmosphere containing 10% CO_2 in air. It is best to leave the plates completely undisturbed at the back of the incubator for about 7 d.
4. Wells containing colonies are identified using an inverting microscope ideally fitted with an objective that allows the entire well to be seen in the field of vision. The cloning efficiencies are usually very high (30–100%). Only those plates in which the proportion of colonies (including those with very small colonies) is <10% should be used, thereby ensuring the probability of a given colony being derived from a single cell is >95% as determined by Poisson statistics.
5. Clones should be suspended using a pipettor, transferred to the wells of 24-well plates, and fed with 1 mL of D10F containing 10^4 IU/mL of IL-2 and 10 ng/mL of PMA. Most clones will grow rapidly and should be refed/subcultured with the same medium every 3–4 d.

6. A number of variations of the above cloning procedure have been used successfully (*see* **Note 7**).

3.3. Long-Term Maintenance of Clones

During the early stages of clonal development growth is very rapid, many clones showing doubling times of 12 h or less. After about 2 wk, growth slows, and by 4 wk, when clones have reached about 10^7 cells, growth usually becomes very slow. However, if clones are frequently refed and maintained at a density of about 5×10^5 cells/mL with occasional splitting into 2 or 3 wells most clones continue to grow, albeit slowly, and many eventually start to grow more rapidly. We have found that PMA together with low concentrations of IL-4 helps to promote growth/survival at this stage. Therefore, when clones have reached the point of very slow or static growth, they are refed 2–3 times per week by reducing the culture volume to about 1 mL and adding 1 mL of fresh medium containing 10^4 IU/mL of IL-2, 0.5 U/mL of IL-4, and 10 ng/mL of PMA. It should be noted, however, that IL4 can promote the transformation of NK cells into giant cells (*see* **Note 8**), so the dose of IL-4 needs to be low (0.5 U/mL), and when more rapid cell growth resumes it should be withdrawn completely. When cell growth has resumed it is also often found that PMA is no longer needed.

3.4. Freezing

Having derived clones it is advisable to freeze them for storage. This is best done either early on when clones are still growing reasonably fast or later when growth resumes. Aliquots containing $0.5–1 \times 10^6$ cells should be frozen in 0.5-mL volumes in screw-top vials.

1. Cells are centrifuged, resuspended in an appropriate volume of a freshly made mixture of 90% FBS and 10% dimethyl sulfoxide (DMSO), distributed into vials, and placed in a –80°C freezer in a cardboard freezer box containing dividers.
2. One day later, vials should be transferred to the vapor phase of a liquid nitrogen freezer.
3. To recover frozen cells, vials are thawed rapidly in a 37°C water bath and as soon as the contents have just melted the vials are placed in ice.
4. The cells are transferred to a centrifuge tube and gradually diluted to 5 mL with cold H1F over a period of about 5 min.
5. The cells are centrifuged, washed again with 5 mL of H1F, and resuspended in complete culture medium.

3.5. Mycoplasma Tests

Past experience has shown the importance of using mycoplasma-free cells for studies of NK function *(27)*. Because no feeder cells are needed for the growth of fetal NK cells, mycoplasma infection is not likely to occur provided

a rigorous aseptic technique is used, together with high-quality mycoplasma-tested 0.1-mm filtered FBS and no antibiotics. However, it is desirable to confirm the mycoplasma-free status of cells by direct testing. We have found the Chen technique ***(28)*** to be very simple and reliable. This requires a mycoplasma-free adherent indicator cell line—any line will do (we use the F10 variant of the mouse B16 melanoma or L cells).

1. Indicator cells, harvested by incubating rinsed cell monolayers with 0.5 m*M* EDTA in calcium/magnesium-free Dulbecco's phosphate-buffered saline at room temperature for a few minutes, are washed, counted, and diluted to 5×10^4/mL in D10F.
2. Aliquots of 5 mL are added to 60-mm bacteriological Petri dishes containing a sterile glass coverslip and 0.5 mL of fresh supernatant from test cultures. Negative controls receive 0.5 mL of the medium used for growing the test cells, and positive controls 0.5 mL of medium from a known mycoplasma-infected line (e.g., most lines of the CTLL2 IL-2-indicator cell are heavily infected).
3. The dishes are incubated at 37°C for 2–3 d.
4. The culture medium is poured off and about 3 mL of 95% ethanol added. After about 10 min this is poured off and replaced with a second aliquot of 95% ethanol. After a further 10 min the coverslips are removed and allowed to dry leaning against the lids of the corresponding dishes.
5. The dried coverslips are coded on the back with insoluble marker pen, and stained by laying the coverslips flat on the Petri dish lid, carefully loading them (so as to just cover the whole coverslip) with a solution of Hoechst 33258 (B2883, Sigma) at 2 µg/mL in water, freshly made from a stock solution of 1 mg/mL in water stored at 4°C.
6. After 5–10 min the staining solution is aspirated away and the coverslips are rinsed in a beaker of deionized water and left to dry.
7. They are mounted on glass slides, sealed with nail polish, and examined on a UV fluorescence microscope in a blind manner. Negative cultures will have staining only of the nuclei; positive cultures will show threadlike staining outside of the nuclei, especially along thin villous extensions from cells, and also in cell-free regions of the coverslip.
8. Infected cultures can be decontaminated by treatment with Mycoplasma Removal Agent (ICN, Costa Mesa, CA) according to the manufacturer's instructions.

4. Notes

1. Successful generation of NK cell clones is critically dependent on the medium used. During the development of the method we studied a range of different types of medium, and found surprising differences in their ability to support the growth of fetal NK cells. Media such as RPMI-1640 and Ham's F10 were unsatisfactory. Somewhat disturbingly, commercial 1× medium was often markedly inferior to the same medium made up in Nanopure water from powdered stocks obtained

from the same manufacturer. All glassware/plasticware used for making and storing medium should be scrupulously clean and should be sterilized in a hot air oven, not by autoclaving, as this can leave toxic deposits.

2. Contrary to general belief, 2-mercaptoethanol is stable at 4°C in serum-free solutions at 5×10^{-5} *M* for years, and does not need to be added freshly to medium just before use.
3. High quality FBS, preferably from suppliers who filter it through an 0.1-µm filter to remove any mycoplasma, should be used, such as from HyClone, Logan, UT or Sigma (cat. no. F7554). We have not noticed any difference between batches in their ability to support the growth of fetal NK cells, so batch testing is probably unnecessary. FBS should be purchased in frozen form and stored at –80°C until used. It should be thawed at room temperature, mixed well as soon as thawed, then kept at 4°C. It should not be heated to 56°C.
4. A critical factor in the generation of fetal NK cell clones is the use of adequate concentrations of IL-2. The functional IL-2 receptors on mouse NK cells are not saturated even when IL-2 is used at concentrations as high as 10^5 IU/mL ***(20,22)***, equivalent to about 1 µ*M* and well in excess of the amounts theoretically necessary to saturate all known forms of the IL-2 receptor. Use of IL-2 at 10^4 IU/mL is in practice adequate for the generation and maintenance of clones, but lower doses are not. This is still an extremely high concentration, roughly 100 times higher than needed for optimal growth of T cell clones. We determine IL-2 unitage using the standard CTLL2 bioassay ***(29)*** against the WHO international IL-2 standard (available from the National Institute for Biological Standards, South Mimms, UK or the Biological Response Modifiers Program, NCI, Frederick, MD). The very high levels of IL-2 needed for the growth of mouse NK cells suggest that it is not the natural growth factor for these cells. However, the problem is not due to the use of human IL-2 because recombinant mouse IL-2 is needed at similar doses. A possible candidate for the physiological ligand is IL-15. Mouse IL-15 does indeed readily support the growth of mouse NK cells, including fetal NK cells and clones (Brooks, unpublished observations). However, relatively high concentrations are still needed, and its current cost and limited availability make it an impractical alternative. Surprisingly, despite the apparent absence of IL-2Rα expression on fetal NK cells, as determined by immunofluorescence, mAbs specific for the IL-2Rα chain can inhibit the growth of fetal NK cells ***(20)***, suggesting that IL-2 promotes growth of these cells through conventional high-affinity IL-2 receptors and that the very high concentrations of IL-2 are needed to maximize the binding of IL-2 to the extremely small numbers of these receptors that are assembled, possibly transiently, on the surface of fetal NK cells.
5. A further critical component is mouse IL-4. Although fetal thymus NK cell progenitors will grow in IL-2 alone, they do so relatively slowly and do not form large clones. By contrast, day 14 fetal thymocytes exposed for even a few hours to IL-4 + PMA grow prolifically when transferred to high-dose IL-2 ***(20)***. In addition, low dose IL-4 in conjunction with PMA can promote the survival/proliferation of lines and clones during the quiescent phase. As with IL-2, the doses

of IL-4 used are important. The initial priming requires a "just saturating" dose. In our hands this is 10 U/mL, where a unit is defined as the amount of IL-4 that gives 50% maximal proliferation (compared with that obtained with saturating amounts of IL-4) of the CTLL2 line. However, the absence of an international mouse IL-4 standard makes this definition of a unit rather problematic. It is therefore recommended that the IL-4 preparation be titrated directly on fetal thymocytes in the presence of PMA, and the dose that is just sufficient to give maximal proliferation be used for priming. The dose that should be used for promoting survival of clones through the quiescent phase is 1/20th of this, i.e., in our hands 0.5 U/mL. Use of higher concentrations can cause NK cells to transform into giant cells *(see below)*.

6. PMA is highly unstable in aqueous solution, being degraded within a few hours. Therefore working aqueous stock solutions should always be made up just before use. The primary ethanol stock solution should be made at a sufficiently high concentration (e.g., 1 mg/mL) that the amount of ethanol added to cultures is below the level that interferes with cell growth. It should be noted that PMA is a toxic and potentially carcinogenic chemical and precautions should be taken to avoid spillage on the skin, etc. It should also be noted that PMA can inhibit the cytotoxic activity of NK cells and other cells. In our experience the extent to which PMA inhibits cytotoxicity is very variable, some lines and clones being profoundly inhibited, others not being inhibited at all. However, the effect is always reversible, and if lines or clones are placed in PMA-free medium high levels of cytolytic activity return within a few days. For those clones that are dependent on PMA for growth, withdrawal of PMA will of course lead to a slowing or cessation of growth, but this too can be reversed by returning cells to PMA-containing medium. Paradoxically, although PMA generally inhibits the expression of cytoxicity, it maintains the potential to display cytolytic activity at high levels. All clones tend to lose cytolytic activity during extended culture. However, if maintained with PMA loss of cytolytic activity occurs more slowly, albeit with the drawback that in order to reveal cytolytic activity, the PMA must be withdrawn.
7. Several variants of the cloning procedure have been used, often with similar success rates. For example, IL-4 priming and expansion in IL-2 can be achieved in a one-step procedure by culturing fetal thymocytes in D10F containing 10^4 IU/mL of IL-2, 10 U/mL of IL-4, and 10 ng/mL of PMA for 3–7 d followed by subsequent refeeding with medium lacking IL-4. In this way it is possible to clone fetal thymocytes directly ex vivo. However, there is a greater chance of their transforming into giant cells. Also, although we generally use PMA throughout the cloning procedure, it is actually essential only during the first few hours of priming. It is also necessary at late stages when clones grow very slowly. It is not essential during the period of rapid cell proliferation first in IL-4 and subsequently in IL-2. Finally, once clones (or lines) resume rapid growth following quiescence they can be cloned and recloned at will.
8. A frustrating problem associated with the growth of adult, but also to a lesser extent fetal, mouse NK cells is that there is a marked tendency for cells to trans-

form into giant granular cells that are clearly aberrant, generally lack cytotoxicity, and soon lose growth potential. The problem has been noticed by others ***(30)***. This transformation event occurs unpredictably, initially affecting just some replicate cultures of a clone or line. It appears to be more prevalent with cells from some strains (e.g., BALB/c) than others (C57BL/6). It is enhanced by both IL-4 and PMA, especially the former, necessitating the minimal essential use of this reagent. It is also enhanced by maintaining cells at too high a density, and especially if they are allowed to overgrow. Fortunately, with careful management most fetal lines and clones can be maintained free of these transformants, but some losses should be expected.

Acknowledgments

I would like to thank the Medical Research Council, UK and the University of Newcastle for their generous support of the work described here and of the subsequent studies that developed from it. I would also like to thank Andy Georgiou, whose enthusiasm and persistence played a vital role in the development of this technique.

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4

Techniques for Studying Development of Human Natural Killer Cells and T Cells

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1. Introduction

It is now commonly accepted that natural killer (NK) cells are closely related to T cells. Some severe combined immunodeficiency (SCID) patients have been described lacking T and NK cells, but having normal numbers of B and myeloid cells, suggesting a common origin of T and NK cells ***(1).*** Furthermore, T and NK cells share a number of phenotypic and functional characteristics, not present in B cells (reviewed in ***[2,3]***). In additional, both in humans ***(4)*** and mice ***(5)*** cells have been found with T and NK cell, but no B cell, progenitor activities

There may not be a major site for NK development comparable to the thymus for T cell development. NK cells may develop at several anatomical sites, including the thymus. This organ has been shown to contain bipotential T/NK progenitors, committed NK precursors, and mature NK cells ***(4,6,7)***. Moreover, the human fetal thymic microenvironment is permissive for NK development ***(8)***. However, because nude mice have normal numbers of NK cells and, in additional, NK cells are already present in the human fetal liver before formation of the thymic primordium ***(9)***, the thymus is dispensable for NK development. Most likely, bone marrow is a major site for NK cell development as studies from several laboratories have demonstrated that human NK cells ***(10–13)*** can differentiate from immature bone marrow progenitors in long-term bone marrow cultures. In addition, recent reports document that the fetal liver is a site for NK development both in the human ***(9,14)*** and the mouse ***(15)***.

Although little is known about the mechanisms that dictate NK development, it is, however, clear that cytokines play an important role in development

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
Edited by: K. S. Campbell and M. Colonna © Humana Press Inc., Totowa, NJ

of these cells as mice deficient for the common γ chain, shared by the IL-2, IL-4, IL-7, IL-9, and IL-15 receptors, lack NK cells. Strong perturbations of NK development were also observed in mice deficient for the IL-2Rβ chain. As this receptor is a component of the IL-2 and IL-15 receptors ***(16)***, either of these two factors may be important. IL-2-deficient mice have no NK activity, but this activity is inducible in these mice ***(17)***, indicating that IL-2 is dispensable. These findings together make it likely that IL-15 is important for development of NK cells. This notion is supported by the fact that IL-15 strongly promotes NK development from human ***(14,18)*** and murine progenitor cells ***(19,20)***.

Studies to the developmental relationship of NK and T cells have benefited from in vitro assays that have been developed in various laboratories. These assays now also permit studies to the mechanisms underlying differentiation of T and NK cells in which we make use of retrovirus-mediated gene transfer ***(21)***. Here we describe methods to study development of human NK cells from human progenitor cells using either IL-2 or IL-15 in combination with other cytokines. In addition, we describe a method to test T and NK cell development of $CD34^+$ cells simultaneously. These methods take advantage of the fact that human stem cells as well as committed progenitor cells express CD34. Using this marker one can select progenitor cells in a relatively straightforward way.

One should note that the NK differentiation assays described here do not give rise to cells expressing killer cell immunoglobulin-like receptors (KIRs), although CD94 expression is induced. This implies that in vitro systems, such as these, are not truly representative for all aspects of normal NK cell development. It will be interesting to further develop these assays in the future to determine under which in vitro conditions that the KIRs are induced.

2. Materials

2.1. Medium for Culture of Human $CD34^+$ Progenitor Cells

We use Yssel's medium ***(22)***. In our hands, this medium is superior to RPMI-1640 medium supplemented with either human or fetal calf serum. Yssel's medium is prepared as follows (*see* **Note 1**):

1. Dissolve in the following order in distilled water (suppliers of the ingredients and catalog numbers are indicated):
   a. Iscove's modified Dulbecco's medium (IMDM) (Gibco, Glasgow, Scotland, 074-2200). Instead of powdered medium, one can also use liquid IMDM (Gibco, 041-90560 M).
   b. $NaHCO_3$, 3.024 g/L (Merck, Darmstadt, Germany, 6329).
   c. Bovine serum albumin (BSA) (Sigma, St Louis, MO, A9647) to a final dilution of 0.25% (w/v).
   d. 2-Aminoethanol (Merck 102931), final concentration: 1.8 µg/L.
   e. Transferrin (Roche Molecular Biochemicals, Mannheim, Germany, 652-202) liquid, final concentration 40 µg/mL.

f. Insulin (Sigma, I 5500) to a final concentration of 5 μg/mL (dissolve insulin 10 mg/mL in 0.01 *N* HCL and add this to the medium).
g. Linoleic acid (Sigma, L 1376) to a final concentration of 2 μg/mL.
h. Oleic acid (Sigma, O 3879) to a final concentration of 2 μg/mL. (Stock linoleic and oleic acid should be stored at –20°C under nitrogen to prevent oxidation of unsaturated bonds. Therefore one should make glass ampoules containing 5 mg of linoleic and 5 mg of oleic acid. For preparation of the medium, dissolve the fatty acids in ethanol and add this mixture to the medium).
i. Antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin final concentrations; Roche Molecular Biochemicals, Mannheim, Germany).

2. Filter the medium (0.45-μm filter is sufficient), aliquot, and store at –20°C. The medium can be stored for up to 4 mo at –20°C. After thawing one should not keep the medium through more than 7 d at 4°C.
3. Although many cell types can be cultured in Yssel's medium, it is recommended to add 1–2% pooled human serum (collected from the blood of 6–19 healthy donors and decomplemented for 30 min at 56°C).

To save time in preparation of the medium, a 5× concentrated stock solution in IMDM of the **items c–i** can be made and stored at –20°C, which can be diluted to obtain 1× medium

2.2. Purification of CD34+ Cells

One can employ two methods to isolate CD34+ cells: One is to deplete the sample of interest (e.g., thymus, cord blood, bone marrow) of unwanted cells (i.e., mature T, B, NK and myeloid cells) followed by purification of wanted CD34+ cells by means of fluorescence-activated cell (FACS) sorting. For depletion procedures we use Dynabeads. Another method is to select the CD34+ cells with a monoclonal antibody (MAb) and colloidal superparamagnetic MACS. Necessary reagents are:

1. RPMI-1640 medium (Gibco-BRL Life Technologies Ltd., Paisley, Scotland).
2. Fetal calf serum (FCS; BioWhittaker, Verviers, Belgium).
3. DNase I (Sigma, St Louis, MO).
4. Ficoll (Lymphoprep, 1.077 g/mL, Nycomed Pharma, Oslo, Norway).
5. 50-mL tubes (Falcon plastics).
6. NH_4Cl lysis buffer for red blood cells: Mix 2.07 g of NH_4Cl, 5 mL of $KHCO_3$ stock (0.5 *M*), and 1 mL of Na_2EDTA-stock (250 m*M*). Fill up to 250 mL with distilled H_2O.
7. VarioMACS CD34 selection kit (Miltenyi Biotec Inc., Sunnyvale, CA). With this kit one can enrich for CD34+ cells following labeling with human IgG and modified CD34 (QBEND/10, mouse IgG) MAb and colloidal superparamagnetic MACS from Dynabeads (Dynal, Oslo, Norway). Antimouse Ig Dynabeads are also from Dynal.
8. FACStar Plus cell sorter, equipped with an argon laser emitting at 488 nm.

9. Percoll (Pharmacia, Lund, Sweden). To make 1.086 g/mL density Percoll solution, dilute 22.5 mL of stock Percoll with 2.5 mL of 10× concentrated PBS plus 15.9 mL of medium.

2.3. Antibodies for Purification of $CD34^+$ Cells and Analysis of Cells Developing from the $CD34^+$ Cells

It is recommended to collect hybridomas secreting those antibodies that are frequently used for depletion procedures from colleagues.

1. Hybridoma's secreting anti-glycophorin A (10F7 MN) and antibodies against CD3, CD4, CD8, and CD19 can be obtained from the American Type Culture Collection, ATCC, Rockville MD).
2. Anti-CD34-FITC (HPCA-2, Becton Dickinson & Co., San Jose, CA).
3. Anti-CD1a-phycoerythrin (PE) (T6-RD1, Coulter/Immunotech).
4. Antibodies to CD56 (FITC-NCAM 16.2, PE-Leu-19), CD19 (Leu-12), CD14 (Leu-M3), CD16 (Leu-11a), CD4 (Leu-3a), CD8 (Leu- 2a), and CD3 (Leu-4) are from Becton Dickinson.
5. Antibodies to TCRγδ and TCRαβ are from Coulter/Immunotech.
6. Antibodies against NKR-P1A (DX1, Pharmingen) and CD94 (Kp43, various suppliers).
7. Biotinylated anti-CD34 was obtained from Monosan (Sanbio, Uden, The Netherlands).
8. Streptavidin-CyChrome (CyCr) (PharMingen, San Diego, CA).
9. Most fluorescein isothiocyanate (FITC) and PE conjugated antibodies that we use for analysis of cell populations were purchased from Becton Dickinson (San José, CA, USA). Many companies offer antibodies conjugated with a third fluorochrome. In addition to Becton Dickinson, these are Caltag Laboratories (South San Francisco, CA) and Coulter/Immunotech (Luminy, Marseille, France). FITC-conjugated $F(ab')_2$ goat-antimouse IgG (H + L) is available for example from Zymed, San Francisco, CA.

2.4. Culturing of NK Cell Progenitors

1. 96-Well U-bottom plates (Costar, Cambridge, MA).
2. 2'-deoxyguanosine (2-dG; Sigma).
3. Gelfoam sponges, sterile (20–60 × 7 mm, Upjohn).
4. Isopore polycarbonate filters (0.8 µm, 13 mm diameter; Millipore, Bedford, MA 01730 USA, cat. no. ATTP 01300). Filters sterilized in advance by washing in 70% ethanol. Ethanol is removed by two additional washings in sterile culture medium in a 6-well plate.

2.5. Cytokines

Most cytokines can be purchased from companies like R & D (Minneapolis, MN) or Genzyme (Cambridge, MA).

1. Flt-3 ligand (the Flt-3L we use was a kind gift from Dr. M. G. Roncarolo, DNAX, Palo Alto, CA, but this cytokine can now be purchased from several companies).
2. Interleukin-7 (IL-7, our own source).

3. IL-2 and IL-15 (A kind gift from the company Immunex, Seattle, WA. Also this cytokine can now be purchased).

3. Methods

3.1. Isolation of Progenitor Cells from Thymus (see Note 2)

1. Normal human thymocytes can be obtained from thymus fragments removed during corrective cardiac surgery of patients aged 1 mo to 2 yr. Thymic lobes are first cut into small pieces using a pair of surgical scissors and further gently minced using two scalpels in RPMI-1640 containing 2% (v/v) FCS and antibiotics (penicillin 500 IU/mL, streptomycin 100 μg/mL). DNase I (10 μg/mL) should be added to avoid large strings of DNA that come out of disrupted cells (*see* **Note 2**). It is recommended to perform dissection of the thymic material in glass Petri dishes.
2. Debris and large aggregates can be removed by letting the suspension settle for 10 min at 1*g* and carefully removing the supernatant with a pipet.
3. $CD34^+$ cells constitute <1% of the thymocyte suspension. It is therefore recommended to enrich these cells prior to purification. A simple way is to leave the thymocyte suspension overnight in the refrigerator before processing of the cells. Most small $CD4^+CD8^+$ thymocytes acquire a high density by the overnight storage at 4°C and can be removed by centrifugation over a Ficoll gradient. Thymocytes should be resuspended in RPMI and layered on top of Ficoll. We load a maximum number of 400×10^6 thymocytes on 15 mL of Ficoll (1.077 g/mL) in 50-mL Falcon tubes. The cells should be centrifuged for 30' at 900*g*.
4. Thymocytes recovered from the Ficoll interface should be washed twice (10 min at 400*g* in a standard table top centrifuge). One removes 80% of the thymocytes, mostly immature small cells, by this procedure and the remaining cell population is enriched both for mature $CD3^+$ and immature thymocytes. In typical samples one has 2–3% $CD34^+$ cells after ficolling.

3.1.1. Positive Enrichment Method

1. The ficolled cells are enriched for $CD34^+$ cells using a varioMACS CD34 separation kit (varioMACS, Miltenyi Biotec, Sunnyvale, CA). We use a modification of the manufacturer's instruction. Instead of 100 μL of reagents (A and B) per 10^8 cells, we use these amounts of reagents for 10^9 cells. Moreover incubation of the cells with the reagents is performed in Yssel's medium, rather than MACS buffer.
2. Prior to application on the column we wash the cells and resuspend them in MACS buffer.
3. Usually the enriched cells are 60–70% $CD34^+$ and need to be purified. This can be done by sorting on a FACS machine. To label the cells one can add as a rule of the thumb 100 ng to 1 μg of FITC or otherwise conjugated antibody to 10^6 cells. If one is interested in obtaining subpopulations of $CD34^+$ thymocytes, e.g., CD1a-$CD34^+$ T/NK progenitor cells or $CD1a^+CD34^+$ pre-T cells, the anti-CD34 labeled cells can be counterstained with relevant antibodies conjugated with a second fluorochrome. For example, for cell sorting of $CD34^+CD1a^-$ and $CD34^+CD1a^+$

subpopulations, cells are incubated with 1 μL/10^6 cells of anti-CD34-FITC and 0.5 μL/10^6 cells of anti-CD1a-PE at 4°C for 30 min.

4. Cells are isolated by flow cytometric activated cell sorting on a cell sorter. During the sorting procedure, cells should be kept at 4°C until use. All fractions used in subsequent experiments should be reanalyzed after sorting. Detailed procedures for flow cytometry analysis of cells can be found elsewhere ***(23)***.

3.1.2. Depletion Enrichment Method

Some precursor cells that express low levels of CD34 should be isolated using a depletion method to enrich for $CD34^+$ thymocytes.

1. The most convenient isolation method is to deplete for cells expressing CD4, CD8, and CD3. Because $CD3^-CD4^-CD8^-$ cells contain red blood cells, mature NK cells, and B cells, it is advised to include antibodies against CD19 (B cells), CD56 (NK cells), and glycophorin. To save antibodies, we first do a depletion of ficolled thymocytes with anti-CD8 and anti-CD4, followed by a second depletion using the other antibodies. The cells are labeled with the antibodies followed by removal of the labeled cells with magnetic Dynabeads according to the instructions of the manufacturer.
2. To check the quality of the remaining cell population, one can stain with FITC-conjugated $F(ab')_2$ goat-antimouse IgG (H + L) and analyze these with flow cytometry. It is, however, better to check purity with antibodies against the markers used for depletion. It is then important to remember that antibodies should be used against epitopes different from those recognized by the antibodies used for the depletion.

3.2. Isolation of $CD34^+$ Cells from Neonatal Cord Blood and Bone Marrow

1. Cord blood samples are collected in heparinized bottles.
2. The blood is diluted 1:5 with phosphate-buffered saline (PBS) and the mononuclear cells are isolated on a one-step Percoll gradient (1.086 g/mL). The Percoll is preferred above Ficoll (density 1.077 g/mL) because considerably more $CD34^+$ cells are recovered. Layer 20 mL of cell suspension in PBS on 10 mL of this Percoll solution and centrifuge 30 min at 900*g* in a table top centrifuge with the brake off.
3. A disadvantage is that more erythrocytes are present in these samples. These can be removed by lysis in NH_4Cl or can be depleted with anti-glycophorin antibody and magnetic beads. The latter treatment gives cleaner results. For NH_4Cl-mediated lysis, centrifuge the cells, aspirate all the wash fluid, resuspend in NH_4Cl lysis buffer, and put on ice for 20' followed by washing the cells three times. For antiglycophorin antibody treatment, we use 2 μL of 10F7MN antibody ascites to treat 100×10^6 cells in 1 mL on ice for 30 min. Wash the cells once by centrifugation at 400*g* and mix the labeled cells with Dynabeads coated with goat anti-mouse Ig at a ratio of two beads per cell. The cells tagged with the antibody

should then be removed with a magnet according to the manufacturer's instructions. Bone marrow samples are usually depleted of erythrocytes by centrifugation over a standard Ficoll gradient as described in **Subheading 3.1.**

4. Cord blood cells, recovered from the Percoll interface, and bone marrow cells can be further enriched for CD34$^+$ cells with the varioMACS CD34 selection kit according to the manufacturer's recommendations. The purity of these samples is already high (93% or more).
5. If one aims to obtain cells with a purity >99% or if one wishes to have subpopulations of CD34$^+$ cells (e.g., primitive CD34$^+$CD38$^-$ hematopoietic precursors or more mature CD34$^+$CD1a$^+$ precursors), one can counterstain the cells with the relevant FITC-, PE-, TRC-, CyChrome-, or PerCP-conjugated antibodies followed by FACS sorting (*see* **Subheading 3.1.1.** for details).

3.3. Differentiation of NK Cells in Medium Containing a Mixture of Cytokines

CD34$^+$ progenitor cells can differentiate into NK cells in a simple one dimensional culture system when cultured in the presence of IL-2 or IL-15 (*see* **Note 2**). The recovery of the cells in terms of numbers and viability is greatly enhanced when factors such as stem cell factor (SCF = c-kit ligand) or Flt-3 ligand (Flt-3L) are added to the cells. Furthermore, in our hands, IL-7 gives a further improvement of the cell yields of NK cells. Not all CD34$^+$ cell populations develop into NK cells in a culture system with IL-2/IL-15 SCF/Flt-3L and IL-7. In our hands optimal results with regard to cell recoveries are obtained with Flt-3L and IL-15, however, substituting IL-15 and Flt-3L with IL-2 and SCF, respectively, gives the same results with regard to NK differentiation. The most primitive hematopoietic cells, e.g., CD34$^+$CD38$^-$ cells from fetal liver, bone marrow, or neonatal cord blood, do not develop into NK cells in this cytokine mixture, but require additional support from stromal cells ***(26)*** *(see below)*. The cultures are set up as follows:

1. Ten thousand to 20,000 CD34$^+$ progenitor cells are cultured in 0.1 mL of complete medium in the presence of 25 U/mL of Flt-3 ligand, 10 ng/mL of IL-7, and 10 ng/mL of IL-15 (or 10 ng/mL of IL-2) in 96-well U-bottom plates. We prefer round bottomed plates over flat bottomed ones, because in our experience cell recoveries and viabilities are better in the former culture plates. Control cultures should be established without IL-15/IL-2.
2. The cultures are maintained in a humidified atmosphere at 37°C in 5% CO_2 for the indicated number of days. Usually NK development is completed between d 10 and d 14 (*see* **Note 3**).
3. At d 7 and 14, half of the culture medium is carefully removed and replaced by fresh medium containing the same concentration of cytokines.
4. At different time points cells should be counted and viability determined by trypan blue exclusion.

3.4. An Assay to Simultaneously Test T and NK Cell Development of CD34$^+$ Precursors Using Hybrid Human/Mouse Fetal Thymic Organ Cultures

The in vitro development of human T and NK cells from CD34$^+$ cells can be studied using the hybrid human/mouse fetal thymic organ culture (FTOC) in which human CD34$^+$ progenitor cells are cocultured with murine fetal thymuses *(27)*. This FTOC system supports development not only of T cells but also of NK cells and offers the opportunity to test simultaneously the capacity of a given precursor cell population to develop into T and NK cells. Interestingly, mature NK cells can be expanded from cells harvested from these FTOCs *(28)*. In contrast to IL-2/IL-15, the FTOC system supports NK development from primitive CD34$^+$CD38$^-$ into NK cells in accord with findings of Miller and collaborators, that NK-development of primitive CD34$^+$ progenitors requires a stromal support in addition to cytokines *(26)*. We perform this hybrid FTOC as follows.

3.4.1. Preparation of the Culture System

1. Each well of a 6-well plate is filled with 3 mL of culture medium supplemented with 1.35 m*M* of 2'-deoxyguanosine (2-dG), which is added to inhibit growth of endogenous mouse thymocytes. Culture medium consists of Yssel's medium with 5% FCS and 2% pooled normal human serum.
2. Gelfoam sponges are cut into three square pieces. Each piece is placed in one well and with the round side of a sterile tweezer the sponges are pressed to facilitate uptake of culture medium. Check for remaining air bubbles and remove these as well. It is also possible to avoid all this by just leaving the sponges in culture medium for 2–3 h before use.
3. One Isopore polycarbonate filter is placed on top of each gelfoam sponge. The system is now ready for use.

3.4.2. Isolation of Thymic Lobes

We make use of 14-d old fetal C57Bl/6 mice or of 15–16-d old fetal RAG-1-deficient mice. The advantage of using the latter embryo's is that the thymic lobes are somewhat bigger and therefore easier to handle. Although T cell development is impaired in RAG-1-deficient mice we have found that deoxyguanosine treatment is still necessary, as the presence of early mouse thymocytes present in thymuses of RAG-1-deficient mice negatively affects the expansion rate of human precursors added to these lobes.

1. Pregnant female mice are sacrificed by cervical dislocation.
2. The mice are cleaned with 70% ethanol and their abdomens are opened with a pair of scissors.
3. With a tweezer the string with fetuses is lifted from the abdomen and remaining sites of attachment are cut with a pair of scissors to totally release the string.

4. Each fetus is separately freed using a pair of scissors.
5. The fetus is placed on its back in a Petri dish and its head is cut off under a dissecting microscope with a tweezer, while another tweezer is used to keep the embryo in a stable position.
6. The first tweezer is then used to make a longitudinal incision of the sternum and each site of the sternum is pulled gently sideways to open the chest for inspection.
7. The two thymic lobes (at this age the lobes are still separated) are in proximity of each other situated on top of the heart (*see* **Fig. 1**, Chapter 5). The lobes can now be easily removed with a tweezer although care should be taken to not fracture the lobes by picking them up in the middle and squeezing them (*see* **Note 4**). The trick is to grip them at the base.
8. Each lobe is placed on top of the filter and routinely not more 12 lobes should be spread out on one filter.
9. The lobes are subsequently cultured for 5 d in an incubator at 37°C and 5% CO_2.

3.4.3. Removal of 2-Deoxyguanosine

1. After 5 d of culture to block development of endogenous hematopoietic cells, the thymic lobes are suspended in 2 m: of culture medium and washed for 3 h in the incubator to remove 2-dG.
2. The thymic lobes are now ready to reconstitute with the human precursor cells of interest. In case these precursor cells are not available at that time, the lobes can be placed back in the culture system as described in the absence of 2-dG. Although the thymuses are best used immediately after 2-dG treatment the lobes can be kept at least for 3 d until use.

3.4.4. Reconstitution of Thymic Lobes with Precursor Cells

1. Twenty-five microliters of culture medium containing purified human precursor cells are put in one well of a Terasaki plate.
2. One 2-dG-treated fetal mouse thymic lobe is added to each well with a tweezer. In general not more than 100,000 cells should be added to one fetal thymic lobe. We keep a minimum of 10,000 human precursor cells per lobe although successful FTOCs have been performed with 1000 cells. One has to take care that the lobe really sinks into the medium and does not stay on the surface of the drop.
3. After all the lobes are placed, put the lid on the plate and invert it (*see* **Note 5**). All the lobes should come down in the hanging drop. If, however, some lobes still adhere to the plastic, reinvert the plate, just pick up the lobe from the bottom, and while still in the well, release it again. All lobes now should come down if the plate is inverted. If the Terasaki plate is placed as such, unprotected, in an incubator evaporation of medium usually cannot be avoided. To prevent this from happening we first place the inverted Terasaki plate on top of a lid of a small Petri dish in a larger Petri dish to which we have added some distilled water to maintain a saturated humidified condition.
4. The lobes are cultured in the hanging drops for 2 d at 37°C in a 5% CO_2 atmosphere.

5. After this period the lobes are replaced in the filter/gelfoam culture system. To do this, reinvert the Terasaki plate and let the lobes sink to the bottom of well. Remove as much culture medium as possible with a Finnpipet, take out the lobes with a tweezer, and place them on a filter. It is recommended not to empty all wells at the same time before transferring the lobes because of the risk of drying of the lobes.
6. In our experience it is optimal if the lobes are cultured for an additional 3–4 wk in the case of reconstitution with early thymocytes and for 4–6 wk in case of reconstitution with extrathymic precursors (*see* **Note 6**). The culture medium needs to be refreshed every 2 wk.

3.4.5. Preparation of a Cell Suspension at the End of the Incubation Period

We use a procedure whereby mechanical force is used to disperse lobes into single cell suspensions.

1. One hundred microliters of medium should be put in the middle of one well of a 6-well plate and to this medium all mouse lobes of one experiment are added.
2. The lobes are now smashed with the flat side of the rubber end of a 1-mL syringe. Check the result of this procedure microscopically. Keep the cell suspension in the middle of the well.
3. Add some extra medium (200–300 mL) to prevent the well from drying out and repeat the procedure.
4. Add some extra medium and collect the medium with cells and rinse the well thoroughly to collect all of the remaining cells.
5. Vortex-mix the cell suspension, count the viable cells.

3.5. Labeling of Cells for Analysis of Differentiation by Flow Cytometry (see Note 6)

Staining of the cells should be done on the day of isolation. We routinely make use of a three-color FACS analysis to study the differentiation of human cells in the thymic lobes, as this offers so much more information than a one- or two-color analysis. Sometimes the relatively low number of cells harvested from the FTOC demands the use of as few cells per sample as possible. We have found that in principle 10,000 cells per sample still allows an analysis, although higher numbers are preferred. In this case, be sure to measure all the cells on the FACS. Of course the use of directly conjugated antibodies is the most feasible method, because relatively more cells are lost in the course of a multistep staining procedure. Although in principle control stainings should always be included, sometimes the yield of cells from the FTOC is sufficient for only one or two stainings. This is why it is recommended to carefully save the FACS parameters of an experiment that contained all the controls. In case of inadequate numbers one can roughly rely on parameters of a previous

experiment for a measurement. A very useful combination of antibodies to study human T cell development is obviously anti-CD3, anti-CD4, and anti-CD8. Because small numbers of γδ T cells also develop next to the majority of αβ T cells in the FTOC, one can also use antibodies against the αβ TCR or the γδ TCR instead of CD3. Early steps of T cell development can be monitored with combinations such as anti-CD1, anti-CD4, and anti-CD5 antibodies. If the FTOC is started with uncommitted precursors, a small percentage (usually between 1 and 4%) of NK cells also develop in the FTOC. To evaluate the numbers of NK cells in the FTOC a combination of anti-CD56 and anti-CD3 antibodies can be used.

1. The samples are first incubated for 10 min on ice with normal mouse serum Ig to prevent nonspecific binding of the MAbs, and then stained with fluorochrome-conjugated MAb.
2. Cells are stained for 30 min on ice with the indicated fluorochrome-labeled MAbs. We use the following FITC- or PE-coupled anti-mouse MAbs to analyze the differentiated NK cells: CD56 (FITC-NCAM 16.2, PE-Leu-19), CD19 (Leu-12), CD14 (Leu-M3), CD16 (Leu-11a), CD4 (Leu-3a), CD8 (Leu- 2a), and CD3 (Leu-4), biotinylated CD34, TCRγδ, TCRαβ, NKR-P1A (DX1) ***(24)***, and CD94 (Kp43) ***(25)***. When biotinylated MAbs are used, cells should be further incubated with Streptavidin-CyCr. Appropriate fluorochrome-conjugated, isotype-matched control Igs should be used in all experiments.
3. After staining, cells are washed and analyzed by flow cytometry ***(23)***.
4. Analyze samples by flow cytometry. We perform flow cytometry analyses with a FACScan.

4. Notes

1. Sometimes "self-made" Yssel's medium poorly supports growth of cell lines and leukocytes. The reasons can be manifold. Presumably the quality of the water and the ingredients are important. Be certain to use twice-distilled or equivalently pure water. It is therefore recommended to test each batch of medium for growth-supporting capacities before use in precious experiments.
2. $CD34^+$ cells from various origins are in general robust cells. They can easily sustain freezing in 10% dimethyl sulfoxide (DMSO) and storage in liquid nitrogen. One can quantitatively recover $CD34^+$ cells from frozen thymocytes, fetal liver, and cord blood cells. Also one can keep suspensions of thymocytes for 3 d at 4°C without affecting the quality of the, thereafter isolated, $CD34^+$ cells. One should not forget to add 10 µL of DNase I (Sigma) to the thymocyte suspension to reduce cell loss by aggregation with large strings of DNA from dead cells.
3. When one observes a quick yellowing of the medium and many more dead cells than usual, one should be aware of mycoplasma infections. It is recommended to regularly (i.e., at least once in 14 d) test all cell lines and media for the presence of mycoplasma.

4. Correct isolation of the thymic lobes requires practice. If preparation of the embryos is not done properly, tissue will be disrupted making it difficult to locate the lobes. In that case, it is advised to attempt to isolate intact lobes from another embryo rather than trying to pick out pieces of tissue resembling a lobe.
5. Sometimes there is overflow of one well into another during the process of inverting and reinverting the plates. Therefore it is advised to fill Terasaki plates with progenitor cells and thymic lobes in such a manner that different samples are separated by two rows of empty wells.
6. If one wants to analyze T/NK cell development at early time points one will find many mouse cells that are still present in the thymic lobes. Even after 4–5 wk mouse cells are still present. The human and mouse cells can be distinguished on basis of forward and side scatter profiles. Nonetheless it is recommended, particularly if one has limited experience with FTOCs, to counterstain samples with antihuman CD45 and to include controls with anti-mouse CD45. The reason is that some but not all antibodies against human antigens nonspecifically stick to mouse cells. One can perform a three-color analysis with, e.g., anti-CD45 FITC, and antibodies labeled with PE and TriColor.

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Murine Natural Killer Cell Cloning from Fetal Thymic Organ Cultures

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1. Introduction

The relationship of natural killer (NK) cells to T cells has been a longstanding enigma. It is well established that development of NK cells does not require a thymus (because they are present in athymic mice), recombination activating genes (because they are present in RAG-1 and RAG-2 knockout mice) or DNA-dependent protein kinase (because they are present in severe combined immunodeficiency [SCID] mice) ***(1,2)***. NK cells do not express CD3 or TCR on their surface and they are classically defined as $CD3^{-}CD56^{+}$ (in humans) or $CD3^{-}NK1.1^{+}$ (in mice) ***(3–5)***. However, there has been some suggestion that NK cells might share a common progenitor with T cells. This has been derived from the observations that some NK cells contain a truncated mRNA for TCR-β ***(4)***, that activated NK contain cytoplasmic CD3ε and that fetal NK cells also contain cytoplasmic CD3ε ***(3–7)***. Several studies demonstrated that fetal thymi contain progenitors that might develop into T cells or NK cells depending on whether they mature within a thymic or extrathymic microenvironment, respectively ***(7)***. The discovery of a novel lymphocyte subset that expresses markers for both T cells and NK cells, the so-called T/NK lymphocytes, raised further questions about the ontogenic relationship of NK to T cells ***(8,9)***. More recently, Sanchez et al. ***(7)*** showed that human fetal thymi contain a bipotential progenitor that could develop along either of the T or NK maturation pathways. This led us and others to determine whether NK cells can be demonstrated and cloned from fetal thymi ***(10,11)***. Surprisingly, we found that NK1.1 is among the earliest lymphohematopoietic genes to be transcribed as its mRNA is demonstrable in d 9 fetuses (exact organ distribution not yet determined) and in the earliest of thymic anlages (d 11

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
Edited by: K. S. Campbell and M. Colonna © Humana Press Inc., Totowa, NJ

of gestation) ***(11)***. Although T/NK cells can be demonstrated in fetal thymic organ cultures (FTOC) established from day 14 fetal thymi, the majority of NK1.1$^+$ cells obtained from fetal thymi appear to be the classical, CD3$^-$ NK1.1$^+$ cells ***(10,11)***.

The thymus gland is a rather intriguing organ. In the mouse, within the span of 20 d of gestation T cell progenitors are subjected to negative and positive selection, allowed to mature, and exported to the periphery ***(12)***. Because the outline of a thymic anlage is barely detectable before d 11 of gestation, all of these events take place within the span of 10 d at most. This cramped time frame makes it understandable that when studying fetal thymi, every minute counts. Indeed, it appears that the development and maturation of the various thymocyte subsets follow a scripted and highly regulated time frame. To ensure reproducibility, this fact has to be taken into consideration.

Thymic maturation is dependent on both the lymphocytic progenitors as well as the thymic stromal cells. Some lymphocyte subsets may not develop if removed from the thymic microenvironment or at least if removed from contact with the stromal or thymic dendritic cells. An excellent example of this is the many reports in the literature suggesting that if fetal thymocytes are cultured in single cell suspension, they may give rise to NK cells while fetal thymocytes cultured in fetal thymic organ cultures give rise to T lymphocytes ***(13,14)***. However, in most of these studies, the single cell suspensions were supplemented with interleukin-2 (IL-2) while the FTOC were not. It now appears that both the microenvironment as well as cytokine supplementation play important roles in the ultimate outcome of thymic development. The allure of FTOC is that one can examine the effect of various manipulations, such as the addition of cytokines or antibodies, on the development of the various thymocyte subsets. Moreover, the "nurturing" potential of the thymic stroma can be employed to examine the differentiation potential of a particular lymphocyte subset or even a single progenitor cell.

Most FTOC reported in the literature utilize d 14–16 fetal thymi, probably because these thymi are relatively easy to obtain at this stage of development and also because the major step of maturation, the appearance of CD4$^+$CD8$^+$ cells, does not occur until d 16–17 (depending on the mouse strain) of gestation. However, fetal thymi obtained at an earlier time point may be invaluable in examining early thymic progenitors that may not otherwise be demonstrable. For the generation of CD3$^-$NK1.1$^+$ clones from fetal thymi, the largest number of cells can be obtained from d 12 fetal thymi (*see* **Note 1**). Day 12 FTOC, cultured for 1 wk with or without IL-2 supplementation, contain as much as 20–70% NK1.1$^+$ cells, respectively. This percentage drops steadily by every succeeding day of gestation so that by d 17 of gestation, the majority of the NK1.1$^+$ cells (which now account for no more than 1–5%) are CD3$^+$. If one is

interested in cloning $CD3^+NK1.1^+$ fetal thymic cells, it appears that IL-12 supplementation favors the growth of these T/NK cells as opposed to the classical NK cells ***(11)***.

2. Materials

1. 35 × 10 mm sterile tissue culture Petri dishes (Falcon #3001).
2. Gelfoam ***sterile*** sponges (absorbable gelatin) 12 cm^2 × 7 mm (Upjohn NDC 0009-0315-03). Use by the expiration date printed on the package.
3. Nuclepore polycarbonate membrane filters 0.4 µm; 13 mm diameter (Corning/Costar #110407).
4. Tissue grinder (homogenizer) (Thomas Scientific, cat. no. 3431-D70).
5. FTOC media: Iscove's modified Dulbecco's medium (IMDM) (Gibco-BRL, cat. no. 430-2200), penicillin-streptomycin (final concentration 100 U/mL, and 100 mg/mL, respectively), L-glutamine (final concentration 2 m*M*), gentamicin (final concentration 50 µg/mL), 2-mercaptoethanol (2-ME) (final concentration 5×10^{-5} *M*), nonessential amino acids (100× stock diluted 1 mL/100 mL of media; Sigma, cat. no. M7145), and 5% fetal bovine serum. The above ingredients are filter sterilized (0.2 µm) before use, stored at 4°C and used within 30 d.
6. Thymic lobes (*see* **Note 2**): Obtaining fetal thymi can be greatly facilitated if one uses a magnifying microscope. A surgical microscope is ideal but expensive. A dissecting microscope with powerful objective lenses can be used as well. Jeweler's forceps and microsurgery scissors are essential for manipulating the fetal thymi. It is a good idea to practice first by obtaining the thymic lobes from neonatal mice and then from d 16 through d 19 embryos. These are relatively big thymi and one can develop an appreciation of the location and appearance of the fetal thymi. A d 16 fetal thymus is labeled in **Fig. 1**. Day 14–16 fetal thymi are easily recognizable and obtainable by picking up with surgical forceps. Day 12 and d 13 thymi, while readily recognizable, may be difficult to retrieve with a forceps and may be easier to obtain with a Pasteur pipet. Day 11 thymi are almost impossible to retrieve but one can obtain the supracardiac area and use in FTOC as the nonlymphoid components are generally necrotic by 1 wk of culture. It is important that the thymic lobes be bathed with the culture media as soon as they are obtained and should be established in FTOC no later than 1–2 h after acquisition.

3. Methods

The following procedure should be performed in a Tissue Culture laminar flow hood using sterile technique.

3.1. Preparation of Filters

1. Carefully separate the filters (white discs) from packing discs (blue discs).
2. Place the filters in Millipore water, and boil gently for 15 min. It is helpful to use a stir bar when boiling to keep filters from sticking together.
3. Gently (filters tear easily) remove the filters from water and lay flat on gauze or absorbent paper until dry. One may need to uncurl the filters.

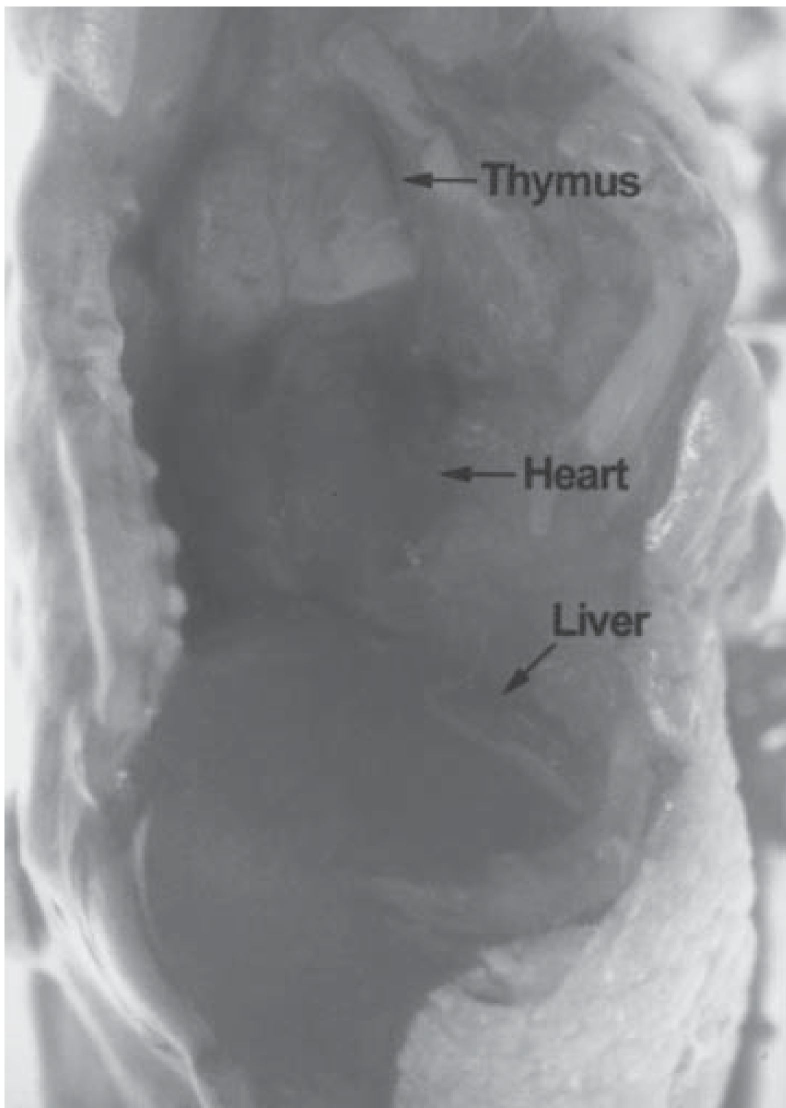

Fig. 1. The appearance of the thymus in a d 16 fetus.

4. Line a glass Petri dish with gauze and place filters on top of the gauze in a single layer. Place another piece of gauze on top to keep the filters in place. Place a glass lid on top of the dish. Sterilize by either autoclaving or ethylene oxide and use within 1 yr.

3.2. FTOC Preparation (see *Note 3*)

1. Cut Gelfoam sponges in half and place one piece of sponge in the bottom of a Petri dish. Add 2 mL of FTOC media, cover, and place in a 5% CO_2/37°C incu-

bator until the sponge is saturated with media. This could take up to 2 h. If in a rush, one can turn the sponge over after1 h to speed the saturation process. Dishes can also be prepared and left in an incubator for up to 24 h in advance.

2. After sponges are soaked, remove the dish from the incubator and return it to the tissue culture hood. Very carefully remove one prepared filter and place it on the sponge (SHINY SIDE UP). Now is a good time to add cytokines or antibodies if desired. If this is done, add to the remaining media in the dish: DO NOT put directly on the sponge. Fetal thymus lobes can now be placed on the filter. As a general rule of thumb, do not overcrowd the filter. Generally, four to six lobes per filter is an ideal number. Add two more mL of media to the dish and replace in incubator.
3. VERY IMPORTANT: Change media and additives every 3 d until the day of harvest. Usually pipet off 2 mL of old media and add 2 mL fresh. This should be done without disturbing the sponge and thymus.
4. Harvesting: Using small curved forceps, remove the sponge from the media and set in the lid of the dish, lift the filter off of the sponge, and gently scrape the thymic lobes off into the media in the bottom of the dish.
5. Transfer the thymi and media to a homogenizer (*see* **Note 4**). Slowly homogenize the tissue, pour the ground tissue into a test tube, and rinse the dish and grinder for maximum cell yield. Centrifuge, count, and use the cells (*see* **Note 5**).

3.3. FTOC Manipulation

FTOC do not need to be supplemented with any cytokines or growth factors to observe lymphocyte maturation. The advantage of FTOC is that one can examine the differentiation potential of lymphocyte progenitors at any one time point of gestation without worrying about the continually incoming stem cells. However, cytokines can be used to supplement FTOC and skew the differentiation pathway of various thymic subsets. The most commonly used cytokines and their concentrations are (*see* **Note 5**):

a. IL-2 (mouse or human recombinant IL-2 work) at 1000 IU/mL.
b. IL-4 (human does not work: use mouse) at 100 ng/mL.
c. IL-7 (human or mouse work) at 100 ng/mL.
d. IL-10 (mouse) at 1 ng/mL.
e. IL-12 (mouse) at 1 ng/mL.
f. Phorbol myristate acetate (PMA) at 1 ng/mL.
g. FLT-3 ligand at 1 ng/mL.
h. MIP-1α (mouse) at 1 ng/mL.

4. Notes

1. Time frame: The development and maturation of the various thymocyte subsets follow a scripted and highly regulated time frame. To ensure reproducibility, this fact has to be taken into consideration. For example, when we first started examining the development and maturation of NK cells in the fetal thymus, we

obtained rather confusing results, with some "d 12" thymi giving rise to NK cells and other "d 12" thymi giving rise to very few NK cells. We were able to resolve this confusion by applying strict time frames: an early dy 12 (morning) thymus may give different results from a late d 12 (afternoon) thymus ***(11)***. Perhaps the importance of each minute is best illustrated by our recently described phenomenon of a peak of NK1.1$^+$ cells (accounting for as much as 40% of fetal thymic lymphocytes) which is detectable only in a very narrow time frame of 12 h between d 15 and d 16 of gestation. Much confusion in the literature also arises from the lack of a uniform definition of what is d 0 of gestation, with some laboratories counting the day of the vaginal plug as d 0 while others count it as d 1. Some laboratories put male and female mice in a cage for one night, remove the male, and then consider that night as d 0 for any pregnant mice. It is therefore important to determine how gestational age was measured when reviewing the literature of fetal thymic development. In our laboratory, we usually count the day of the vaginal plug as d 0.

2. Alymphoid thymic lobes: Thymic lobes obtained from d 12–14 fetal thymi can be established as described above but with the addition of 2-deoxyguanosine (Sigma, cat. no. D0901) at 0.36 mg/mL final concentration. After 5 d, most of the lymphoid components should be dead and one should be left with mostly thymic stroma and some dendritic cells. It is a good idea to have a "sentry" dish set apart to determine that indeed all of the lymphoid progenitors are dead (determine by flow cytometry analysis of a single cell suspension of the sentry dish) before proceeding. Once satisfied that lymphoid progenitors are gone, one can add the lymphocyte of choice to the thymic lobes. This approach has been used to examine the developmental potential of CD4$^-$CD8$^-$ thymocytes obtained from adult thymi ***(15–19)***. There is no consensus on the best way to add these cells to the alymphoid thymic lobes. Some laboratories advocate using the "hanging drop" method whereby one cell (obtained by using the autocloner of a flow cytometry instrument), in a volume of 10 μL, is added to one thymic lobe placed in a Terasaki plate which is then turned upside down. This has been extremely disappointing in our hands. We find that just pipetting the cell, or cells, onto the surface of the thymic lobes (which have been treated with deoxyguanosine and not disturbed), in a volume not to exceed 25 mL, works just as well if not better. The trick is that the filter/sponge assembly should be washed several times to get rid of the deoxyguanosine and then adding enough media to barely cover the filter without covering the top of the thymic lobes (the lobes will get enough nutrition by capillary action). The cells added on top of the lobes will then be able to find their way into the thymic lobes.
3. Reaggregation method: FTOC are established as described above with deoxyguanosine. After the lymphoid cells are dead, the thymic lobes are harvested and treated with trypsin–versene mixture (BioWhittaker, cat. no. 17-161A) for 20 min. Trypsin activity is stopped by the addition of culture media. The cells are centrifuged and any remaining lymphoid cells removed by your depletion strategy of choice. Generally, sorting on flow cytometry is ideal. However,

paramagnetic bead depletion using CD45 antibody works just as well and is faster although the purity of the resulting population is reduced. Once the nonlymphoid cells are obtained they are mixed with the experimental lymphocytes (e.g., $CD4^-$ $CD8^-$ adult thymocytes, or $NK1.1^+$ cells) at a ratio of 0.5:1. The cells are centrifuged and the pellet resuspended in 5 mL and applied to the top of a fresh Gelfoam/filter assembly. The cells reconstitute themselves into what appears like an ersatz thymic lobe and differentiation does occur ***(17)***. Although more reproducible than the hanging-drop method, this approach is rather cumbersome and one is never sure whether one is biasing the results by the purity of the stromal and lymphoid cells utilized. We generally favor the layering method described previously.

4. FTOC harvesting: Many reports in the literature advocated enzymatic digestion of the thymic lobes after culture to obtain a single cell suspension. We found that such treatment resulted in the loss of several thymocyte subsets either by death or by stripping of the surface markers so as to render a wrong interpretation of the flow cytometry data. We find that homogenization of the thymic lobes is effective and feasible with no sacrifice of any subsets.
5. NK cell cloning: In order to clone NK cells from fetal thymi, it is imperative that d 12 fetuses be used because the NK progenitors quickly decline thereafter. We found that cloning NK cells from fetal thymi works best if they are allowed to mature in an intact thymic microenvironment; i.e., in FTOC first. Thus FTOC are set up and are bathed with IL-2 (10,000 IU/mL), FLT-3 ligand (1 ng/mL), MIP-1α (1 ng/mL) (References in the literature ***[20,21]*** suggest that recombinant mouse IL-15 works very well in expanding fetal NK cell clones. We have not been able to obtain this reagent and thus have no first hand experience with it.) Establish FTOC with the above additives for 1 wk after which the thymic lobes are harvested, rendered into single-cell suspension, and expanded further using the same culture medium and supplementation as described previously. After another 1 wk in culture, the cells are stained for NK1.1 (for those strains that express NK1.1) or DX5 ([Pharmingen, cat. no. 09944D for the fluorescein isothiocyanate [FITC]-labeled product] which is described as a pan NK marker for most strains, although some NK subsets may not stain with this antibody). The NK1.1+ cells are then sorted using an autocloner and depositing 0.3 cells/well. The wells are supplemented with the above medium and expanded.

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6

Generation of HLA Class I Transfected Target Cell Lines

Jenny E. Gumperz

1. Introduction

Normal human lymphoblastoid cell lines (LCLs) express six classical class I HLA genes from three gene loci, HLA-A, HLA-B, and HLA-C, which are located within the major histocompatibility complex (MHC). Because of the extensive allelic polymorphism of HLA class I genes, cell lines from heterozygous individuals can express up to six different class I alleles ***(1)***. Class I phenotypes, as well as other properties of different lymphoblastoid target cells, can therefore be very heterogeneous. Hence, in investigating the effects on and specificity of class I recognition by natural killer (NK) cells, it has proved useful to use transfected target cells that express only one class I allele, so that the effects of individual class I allotypes can be compared directly using the same target cell background.

Development of such target cells was made possible by the derivation of cell lines that have chromosomal deletions and mutations within the MHC, leading to defects in class I expression ***(2,3)***. Such cell lines which do not express endogenous class I alleles, can be transfected with DNA encoding a class I HLA gene in an expression vector, to generate target cell lines that express a single class I allele. Comparison of NK lysis of such transfected target cell lines with the original class I deficient line allowed the functional identification of class I specific inhibitory receptors on NK cells ***(4,5)***. Target cells expressing different class I alleles and mutated class I alleles have also been used to investigate the structural requirements for recognition by natural killer cell immunoglobulin-like receptors (KIRs) ***(6,7)***. Other analyses for which such transfected target cells can be useful include investigating KIR signaling pathways, studying the functional outcome of class I recognition in

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
Edited by: K. S. Campbell and M. Colonna © Humana Press Inc., Totowa, NJ

the presence of other signals to the NK cell, using double class I transfectants to study simultaneous recognition of different class I allotypes by different KIRs, the effects of class I recogniton on cytokine secretion and proliferation of NK cells, and the effects of class I expression on NK recognition of virally infected or tumor target cells.

This protocol describes transfection of lymphoblastoid (and similar) cell types with class I HLA genes by electroporation, followed by drug selection and growth in culture, then flow cytometric sorting, and initial analysis of gene expression. It applies to the generation of stable cDNA transfectants using an integrating vector, pSRα-neo ***(8,9)***, or to making genomic transfectants using an episomally replicating vector, pHEBo ***(10)***, and could be adapted for use with other vectors. Throughout the procedure, care should be taken to maintain sterility of the cells, media, and DNA samples. Factors influencing the success of transfection include the choice of host cell (*see* **Note 1**), the purity of the DNA used for transfection (*see* **Note 2**), the growth state of the cells prior to transfection, the buffer and electric field strength conditions used for electroporation, and the drug selection and culture conditions after transfection. Depending on the growth properties of the host cell, the process generally takes 1–2 mo to generate homogeneously expressing stable transfectants, and 2 wk to 1 mo for transient transfectants.

2. Materials

1. Qiagen midi- or maxiprep kit, for preparation of plasmid DNA (Qiagen, Chatsworth, CA).
2. Cell culture medium: The medium used to grow the cells is RPMI 1640 (containing L-glutamine and phenol red, Gibco-BRL, Grand Island, NY), with 10% heat inactivated bovine calf serum (BCS) by volume (Hyclone, Logan, UT), 100 U/mL penicillin/streptomycin (Gibco-BRL), and 2 m*M* L-glutamine (Gibco-BRL) in addition to that already in the RPMI medium. The bovine calf serum is heat inactivated by incubation at 56°C for 1 h. It is then divided into 50-mL aliquots and stored frozen at –20°C, and thawed just prior to use. Penicillin/streptomycin and L-glutamine 100X stock solutions are stored frozen at –20°C, and thawed just prior to use.
3. Transfection buffer: RPMI 1640 (Gibco-BRL).
4. Electroporation equipment: Bio-Rad gene pulser (Bio-Rad, Hercules, CA); 0.4 cm gapped electroporation cuvettes (Bio-Rad).
5. G418 (geneticin, Gibco/BRL), for use with the pSRα-neo expression vector. G418 is made up as a sterile stock solution of 10 mg/mL *active* G418, in 100 m*M* HEPES (base) and 150 m*M* NaCl. (Only a percentage of the powder supplied as G418 is active; this amount should be noted on the label of the vial, e.g., 714 µg/mg.) The stock solution is stored at 4°C for up to 6 mo.
6. Hygromycin B (Calbiochem, La Jolla, CA), for use with the pHEBo expression vector, is stored at 4°C, as a sterile stock solution at the concentration supplied by the manufacturer.

7. Drug selection medium: RPMI 1640 culture medium as described in **step 1**, with 20% fetal bovine serum (FBS, Hyclone), and G418 or hygromycin added at the appropriate concentration just before use.

3. Methods

3.1. Preparation of DNA for Transfection

1. The protocol for subcloning the class I gene into the expression vector will vary, depending on which vector is used, and the source of the DNA encoding the class I HLA gene chosen for transfection (*see* **Note 2**). For class I cDNAs it is generally best to use two different restriction sites for subcloning into the pSRα-neo expression vector, so that the gene is oriented properly with respect to the promoter, and to improve the efficiency of subcloning by preventing religation of vector ends without insert. (If only one restriction site is used for subcloning, the resulting constructs should be sequenced to determine the orientation of the class I gene.) For genomic sequences cloned into the pHEBo vector this is not necessary, as the promoter used for gene expression is the endogenous one contained within the class I DNA fragment. Choose restriction sites for cloning that are unique within the polylinker of the vector, and not present within the insert DNA fragment, or for which alternative enzymes that generate compatible ends can be used. Often it is useful to amplify by polymerase chain reaction (PCR) class I genes from their original cloning constructs with outside primers that encode restriction sites of choice, to generate fragments that can be easily subcloned into the expression vector. In such cases, the class I gene should be sequenced to ensure that coding mutations have not been introduced, as most polymerases used for PCR have a sufficiently high error rate that a large fraction of amplified sequences of the length of a class I cDNA (1.2 kb) will contain misincorporations.
2. Transform competent *E. coli* cells (prepared fresh or stored in frozen aliquots) with the vector containing the class I insert, and plate them on LB agar plates containing 50 μg/mL of ampicillin (*see* **Note 3**). Useful controls are bacteria transformed with: the uncut wild-type vector (positive control), the cut vector used for subcloning (vector religation and contamination with uncut vector control), and no vector (background control).
3. Pick resistant colonies and prepare minipreps of the plasmid DNA in LB medium containing 50 μg/mL ampicillin ***(11)***. Also prepare one or two minipreps from the uncut vector control plate.
4. Digest the miniprep DNA with a restriction enzyme that will linearize the plasmid, or with two enzymes to excise the class I gene. Determine which subclones contain insert of the appropriate size by agarose gel electrophoresis, and comparison to the similarly digested wild-type vector.
5. A minimum of 10–20 μg of purified plasmid DNA is required for each transfection. Prepare at least 50–100 μg of plasmid containing the class I DNA using Qiagen midi- or maxiprep columns. Also prepare wild-type vector plasmid for use as a control. After precipitating the column eluate with isopropanol according to the Qiagen protocol, resuspend the DNA in 0.5 mL of dH_2O, and transfer it

to a sterile 1.5-mL Eppendorf tube. Reprecipitate it to remove excess salt, by adding 1.0 mL of 95% ethanol, and 25 µL of sterile 3 *M* sodium acetate to reprecipitate the DNA. If there is no precipitate visible in the tube, add an additional 25 µL of sterile 3 *M* sodium acetate. Centrifuge the DNA pellet and wash it with 70% ethanol. Resuspend it in 200–300 µL of sterile dH_2O, and take the OD 260 and 280 nm of 5–10 µL diluted in 1.0 mL of dH_2O. The 260/280 ratio should be 1.3 or greater. Determine the concentration of DNA and adjust it to 0.5–1.0 mg/mL with sterile dH_2O.

*3.2. Preparation of Hematopoietic Host Cells for Transfection (*see ***Note 1****)*

1. 10–20 × 10^6 cells are needed for each transfection. Grow the cells for at least 1–2 wk prior to transfection in a humidified incubator at 37°C with 5–10% CO_2, and split them at approx a 1:3 to 1:5 ratio with fresh culture medium every 2–3 d to maintain high viability. By the time of transfection, the viability should be 80–90%, and the cells should be in log-phase growth. Two days prior to transfection feed the cells (rather than splitting them), by removing culture supernatant from the flask without disturbing the cells at the bottom of the flask, and replacing it with fresh medium. (To do this, the flasks should be kept standing upright instead of lying horizontally).
2. Susceptibility of different cell lines to selective drugs varies considerably, so the cells should be tested prior to transfection to determine the optimum drug concentration for selection. Place 0.5–1.0 × 10^6 viable cells per well, in a volume of 0.5–1.0 mL, into eight wells of a 24-well tissue culture plate. Add the selective drug from a stock solution to give a titration of final drug concentrations from 0 to several milligrams per milliliter (e.g., for G418, 0–4 mg/mL in 0.5 mg/mL increments is usually sufficient). Bring the volume of each well up to a total of 2.0 mL with culture medium. Incubate the plate in a humidified incubator at 37°C with 5–10% CO_2 for 7–10 d. Feed the cells every 1–2 d by carefully withdrawing 1.0 mL from the well, and replacing it with fresh medium of the appropriate drug concentration. Monitor the viability of the cells daily by examining them under a microscope. Note the lowest drug concentration that results in approx 99% cell death within 3–4 d, and total cell death within 7–10 d. Results of such testing for several different cell lines are given in **Table 1**.

3.3. Electroporation

1. Count the cells, and centrifuge enough to give 10–20 × 10^6 viable cells per transfection.
2. Pour off the supernatant, and resuspend the cells in a sufficient volume of RPMI 1640 for 0.8 mL of cell suspension per transfection.
3. Pipet 10–20 µg of the DNA solution in a volume of 10–40 µL into 0.4 cm gapped electroporation cuvettes, add 0.8 mL of cell suspension to each, and pipet up and down to mix. (Gapped electroporation cuvettes of 0.2 cm can also be used, but the electroporation conditions must be adjusted to give the appropriate electric field strength. *See below*). Include one sample with no DNA added as a negative

Table 1
Approximate G418 Concentrations for Selection of Various Hematopoietic Cell Lines

Cell line	[Active G418]
721.221	1.0–1.5 mg/mL
T2	1.0–1.5 mg/mL
C1R	1.0–1.5 mg/mL
Daudi	1.8–2.2 mg/mL
K562	1.5–2.0 mg/mL
P815	0.25–0.5 mg/mL

These concentrations are intended as guidelines only. Drug toxicity testing of the cells to be used for transfection as described in **Subheading 3.2., step 2** is recommended.

control, and one sample to which the wild-type vector DNA is added as a positive control to determine whether the transfection worked.

4. Cap the cuvettes and electroporate in a Bio-Rad gene pulser set at 250 mV and 960 μF, with the capacitance on "extend." Monitor the millisecond pulse decay time. The decay time depends mostly on the ionic strength and the volume of solution in the cuvette, and the concentration of cells in the suspension. Decay times of approx 8–20 ms have given good transfection results. Monitoring the decay time is also a good way to confirm that the electroporation conditions are consistent from one sample to the next, and one transfection to another.
5. Pipet the cells into T25 culture flasks containing 10 mL of culture medium supplemented with 10% FBS. The presence of cellular debris from cell lysis during the electroporation is normal. Incubate the cells in a humidified incubator for 48 h at 37°C with 5–10% CO_2.

3.4. Drug Selection and Culture of Resistant Cells

1. Examine the flasks of cells. The medium should look orange or yellow, and viable healthy-looking cells should be readily apparent by light microsopy. If the medium is still pink, let the cells incubate for an additional day. Count the viable cells in each flask. There should be about 40–70% of the starting cell number before electroporation.
2. Centrifuge the cells down and resuspend them in drug selection medium at a concentration of $1–2 \times 10^6$ cells/mL.
3. Pipet 2 mL per well into the wells of a 24-well tissue culture plate. Also fill two wells on each 24-well plate with 2 mL drug selection medium alone. This is useful for comparison with the cell-containing wells, because high concentrations of G418 make culture medium turn yellow, and hygromycin makes the

medium pink, which can make it difficult to visually monitor cell growth by changes in the the medium color. Incubate the cells in a humidified incubator at 37°C with 5–10% CO_2.

4. Carefully inspect the cells every day under a microscope, and feed them each day that large numbers of clearly viable healthy cells are visible and the medium is yellow. Carefully remove 1 mL of culture supernatant from the top of the wells without disturbing the cells, and replace it with fresh drug selection medium. (After being put in 24-well plates, the cells settle very thickly on the bottom of the wells, making it difficult to see individual cells. However, after 1 or 2 d of feeding, a space is usually cleared in the center of the well where individual cells can be clearly seen.) Generally, under appropriate drug selection concentrations, most of the cells appear dead (or small and dull but possibly still viable) after 3–5 d. As soon as this occurs, the cells should no longer be fed. At this time the medium is usually a yellowish orange.
5. Inspect the cells every 2–3 d under a microscope, to look for the appearance of viable cells. (Cells that are truly resistant to G418 appear completely viable and healthy, and grow at a normal rate, whereas cells that are Hygromycin B resistant can be less healthy-looking and grow somewhat slower). Do not feed the cells during this time. Overfeeding dilutes out necessary growth factors and will result in death of the transfected cells. For transfection with an integrating vector such as pSRα-neo, isolated viable cells should appear within 2–4 wk. For an episomally replicating vector such as pHEBo, viable resistant cells should be apparent within 1–2 wk.
6. Feed the cells with drug selection medium when the resistant cells have expanded and the medium is clearly yellow.
7. Allow the cells to grow until there is close to a monolayer of viable cells, then split them into two wells. When these cells have again expanded to a monolayer, split them into four wells. Next transfer three wells to a T25 culture flask. (Keep one well growing in the plate as a back-up). Maintain the cells in culture medium with the selective drug at the appropriate concentration. Split the cells at a ratio of 1:3 once or twice and freeze down several aliquots, so that an early stock of the transfectant is available, in case the population of class I expressing cells is later overgrown by nonexpressing cells. This is especially important for pHEBo transfectants which are not stable and can easily lose class I expression after extended growth in culture, even under continued drug selection.

3.5. Flow Cytometry

The pSRα-neo vector is an integrating vector, and thus generally produces stable class I transfectants. However, because drug selection selects only for integration of the G418 resistance gene, often a percentage of the resistant cells have lost or inactivated the class I gene upon integration. Therefore, it is necessary to sort the class I expressing cells to obtain a homogeneous population, and to prevent loss of class I-positive cells by overgrowth of nonexpressors. Sorting is also useful because the sorted cells can be grown without continued

G418 selection, allowing for a closer comparison with untransfected host cells. Repeated sorting can also be used to select incrementally for cells expressing higher levels of class I if initial expression levels appear low, although in general it is difficult to greatly increase class I expression levels by this method (*see* **Note 4**).

Sorting is not usually necessary for transfectants of the pHEBo vector, because this vector replicates primarily episomally, so the class I gene is not usually disrupted by transfection, and therefore all of the drug-resistant cells generally also express class I. However, pHEBo transfectants often have a broad range of class I expression levels, because the number of copies of the vector can vary significantly per cell. Also over time, as mentioned previously, a population of class I-negative cells can appear. Sorting can be used to eliminate this negative population or to generate a population of cells with a more uniform class I expression level.

1. Before sorting the cells, carry out a preliminary staining and flow cytometric analysis to determine the class I expression profile and expression level. Choose an appropriate anti-class I or anti-β_2m antibody for staining. Compare staining of the transfectants with that of the untransfected host cell. Using isotype matched negative control antibodies can be helpful, but is not absolutely necessary if the staining of the untransfected host cell is low. It is very helpful to include propidium iodide (PI) staining in the analysis, to facilitate gating out the dead cells, as there is often a large size range and significant numbers of granular cells that make accurately gating on live cells by forward and side scatter difficult.
2. Carefully gate out cells that stain brightly with PI, and analyse the class I staining of the transfectants as a histogram. If the transfection was successful, a bimodal distribution should be apparent, with anywhere from 1 to 99% of the cells expressing class I. For most transfectants, however, from 30 to 70% of the cells express class I at this stage. Sometimes a trimodal distribution is seen, with two distinct populations of positive cells expressing different levels of class I. In this case, cells can be sorted from either positive population to give transfectants homogeneously expressing different levels of class I.
3. Based on the viability and percentage of the cells that are class I positive, calculate the approximate cell number needed for sorting. Assume that the gate will be set to collect the brightest 30% of cells, and the cell sorter will only be able to collect one out of six gated cells. (Thus, if 40% of the cells express class I, 2.5×10^6 live unsorted cells will be needed to collect 5×10^4 sorted cells.) Grow the cells in culture until sufficient cells are available for sorting.
4. Stain the cells for sorting. Be sure that all reagents are sterile, and that the antibodies used for staining do not contain sodium azide. Keep the cells on ice in capped tubes.
5. Prepare sterile 96-well flat-bottomed tissue culture plates into which to sort the positive cells. For each transfectant, place 50–100 µL of FBS in two adjacent wells. If multiple sets of sorted cells are to be collected on the same plate, space

them out toward the corners of the plate to avoid cross contamination during sorting by splashing.

6. Carefully gate out dead cells, and set gates to select the brightest staining 20–50% of the positive cells. (It is usually better not to set a very small gate to select <5% of the positive population, as this may bias the sampling towards unusual cells. *See* **Note 4**.) Sort at least $5–10 \times 10^4$ positive cells (more for cells that do not grow well at low density) into each prepared well of the 96-well plate using sterile conditions. Take great care to ensure that all live cells from previous samples have been eliminated from the FACS machine before starting a new sample.
7. Gently spin the plate to draw the cells to the bottom of the well. Carefully remove 50–100 µL of the supernatant, and replace it with selection medium to give a final volume of approx 200 µL.
8. Incubate the cells in a humidified incubator at 37°C with 5–10% CO_2 until they have expanded to a monolayer. Split each well into two wells with selection medium. When the cells have again grown to a monolayer, transfer three wells to one well of a 24-well plate, keeping one well in the 96 well plate as a backup. Expand the cells as described previously, until they are growing well in T25 flasks. Freeze down several aliquots. The cells can now be grown in regular culture medium without drug selection, and should maintain stable class I expression.
9. Periodically monitor class I expression by flow cytometry.

3.6. Confirming the Identity of the Transfected Class I Allele

If multiple class I alleles have been transfected, it is advisable to confirm that the correct allele is expressed by each transfectant and that there has been no cross-contamination of the cultures. This can be done in two ways.

1. If class I monoclonal antibodies (mAbs) that are specific for individual transfected allotypes are available, the identity of the transfectants can be checked by flow cytometry.
2. If appropriate antibodies are not available, or if the results of the flow cytometric analysis are ambiguous, metabolic labeling followed by immunoprecipitation with a monomorphic class I antibody, neuraminidase treatment, and isoelectric focusing can be used to analyze the transfectants ***(12)***. For each transfectant, one band corresponding to β_2m and one band corresponding to the transfected class I heavy chain should be visible. (Exceptions to this are certain HLA-C allotypes that focus as multiple bands.) To facilitate identification of class I heavy chain bands, it is thus very helpful to compare the transfectants to a similar precipitate run on the same gel of the lymphoblastoid cell line from which the transfected class I allele was cloned. If this is not possible, the isoelectric point of the band can be estimated by comparison to standards and compared to the calculated value.

3.7. Double Transfectants

1. The lymphoblastoid cell lines C1R and 721.184 are class I deletion mutants that each express only one class I allele, Cw*0401, and Cw*0102 respectively. Thus

these cells can be used in the protocol described previously to generate transfectants expressing two different class I alleles.

2. Alternatively, stable class I transfectants made with the pSRα-neo vector that have been sorted and no longer need drug selection can be used as hosts to supertransfect class I genes encoded in the pHEBo vector.

4. Notes

1. The choice of "host" or "recipient" cell line will largely be determined by the type of experiment for which the transfectant is to be used. NK cells preferentially kill target cells of hematopoietic origin. Thus, these cells have been used most frequently as targets. The 721.221 lymphoblastoid cell line, which is Epstein–Barr virus transformed but not a tumor cell line, has been very useful because it completely lacks expression of endogenous class I alleles and it supports high levels of class I expression ***(2,3)***. Two similar lines 721.184 (from the same parental 721 cell line) and C1R (derived from a human plasma cell leukemia cell line), each express only one HLA-C allotype (*see* **Subheading 3.7., step 1**) ***(13–15)***. The T2 cell line is derived from a fusion of the 721.174 line with a T-cell line, and lacks the transporters associated with processing (TAP) proteins necessary for loading of most class I molecules with peptides in the ER ***(16)***. This cell is therefore useful for expressing class I molecules loaded with synthetic peptides ***(17)***, although it is not completely class I deficient and expresses substantial amounts of HLA-A*0201 and low levels of HLA-B*5101 at the cell surface. K562 is a myelomonocytic tumor cell line which is class I deficient ***(18)***. The reasons for the class I deficiency of this cell line are not well understood, but it can be transfected with class I genes and supports low to moderate levels of expression. Daudi is a Burkitt lymphoma cell line that lacks β_2m expression ***(19)***. This cell may thus be useful as a host cell that does not express any endogenous β_2m-associated molecules, and could be transfected with class I-β_2m fusion molecules ***(20)***. Finally, P815 is a murine mastocytoma cell line that is positive for FcRγRII and FcγRIII ***(21)***. It expresses H-2 but can be used for studies with human NK cells because the murine class I molecules are not recognized by human KIR. Because it expresses Fc receptors, the P815 cell line is particularly useful for redirected lysis experiments ***(22)***.
2. The vector chosen for transfection depends partly on the type of class I gene to be expressed. Genomic HLA-A and -B genes are expressed at very high levels with most vectors because they contain a powerful endogenous promoter. They are therefore suitable for use with the pHEBo vector which does not have a promoter. Expression of genomic HLA-C genes is usually not as high, apparently owing to elements within the 3' end of the gene that destabilize mRNA expression ***(23)***. High levels of expression can nevertheless be obtained if the host cell possesses multiple copies of the plasmid. However, because the pHEBo vector replicates as an episome, it can relatively easily be lost from the cell, making gene expression in this vector unstable. Another limiting factor in the use of genomic class I genes is that not many have been cloned. Therefore, class I cDNAs have been very

useful, as many different HLA-A, -B, and -C alleles have been cloned and sequenced ***(24)***. Class I cDNAs must be expressed in a vector that contains a promoter for gene expression. The pSRα promoter is a hybrid viral promoter that works well in host cells of hematopoeitic origin. Further, because the PSRα-neo vector is an integrating vector, transfectants can be generated that are stable without the need for continuous drug selection. Also, when expressed from cDNA the levels of HLA-A, -B, and -C are comparable ***(23)***. Finally, sensitivity of the host cells to the drug used for selection may influence the choice of vector used. The pHEBo vector encodes a resistance gene for Hygromycin B, whereas the PSRα-neo vector encodes a resistance gene for G418.

3. Most strains of *E. coli* used for transformation of the vector with the subcloned class I insert appear to produce DNA that can be used for transfection in to the Eukaryotic host cell. However, it is advisable to use a strain that is Rec A^-, and that does not have excessive carbohydrate content in its cell wall. DH5α has given good results. Methylation of the DNA by the bacterial host may in some cases also be a concern, as it may affect restriction sites or gene expression.
4. With transfection followed by selection of a small percentage of the cells, there is always a potential for selection and outgrowth of unintentionally mutated cells. Thus a possible artifact of this assay system is that the transfected cells may have unknown differences from the untransfected host cells. To minimize this possibility, it is best to freeze multiple aliquots of transfectants soon after transfection, and to maintain the transfected cells as lines rather than to clone them out. Testing multiple different transfectants of the same class I allele can also address this issue.

4.1. Troubleshooting

If drug-resistant cells do not grow out after transfection, assess the following points:

1. How are the transfection controls? The cells electroporated with no DNA should all be dead within 1 wk. If not, there is something wrong with the concentration or activity of the drug in the selection medium. If the cells transfected with vector alone are growing but those transfected with vector containing class I insert are not, there is a problem with the DNA construct. If no cells are growing, the problem is likely to be the transfection conditions.
   a. Repeat the transfection with cells that are highly viable and growing in log phase.
   b. Try using an alternative transfection buffer, "cytomix," that has been found to improve transfection results ***(25)***. (Note, however, that this buffer must be made up very carefully, with high-quality reagents.)
   c. Use conditioned medium during drug selection. A very common problem is that the host cells do not grow well at very low densities. Conditioned medium provides growth factors that facilitate growth of the initially small numbers of drug-resistant cells. Collect supernatant from untransfected host cells that are growing robustly. Filter it through a 0.2 mm filter and use it to dilute the

selection medium at a 1:1 ratio, then add the selective drug to the usual final concentration.

d. Purify a fresh batch of DNA, taking care not to overload the Qiagen column with bacterial culture medium. This is very important to obtain DNA that is free from protein and carbohydrate contamination.

e. Vary the electroporation conditions. This is best done as a titration of voltage and capacitance, with multiple samples, using a transient transfection system so that the results can be determined quickly.

Acknowledgments

J. G. is a Burroughs Wellcome fellow of the Life Sciences Research Fund.

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7

Murine Natural Killer Cells and Hybrid Resistance to Hemopoietic Cells In Vivo

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1. Introduction

The studies of hybrid resistance (HR) to murine bone marrow cell (BMC) grafts have been helpful in elucidating the biology and genetics of natural killer (NK) cell mediated recognition of incompatible cells ***(1)***. The discovery and claim for HR is contrary to the laws of transplantation genetics. Thus rejection of $H2^{b/b}$ BMC by $H2^{b/d}$ F1 hybrid mice did not fit with the known codominant inheritance of major histocompatibility complex (MHC) class I and II antigens. The development of the "missing self" hypothesis seems to explain most instances of HR; thus NK cells express a Ly49 family of receptors for "self" class I antigens. The Ly49 molecules and perhaps other receptors on NK cells receive "negative signals" from class I antigens ***(2)***. F1 $H2^{b/d}$ NK cells have two subpopulations of NK cells, those with receptors for $H2^{d}$ and those with receptors for $H2^{b}$. Transplantation of $H2^{b/b}$ BMC into these $H2^{b/d}$ hosts results in rejection because the subset of NK cells with receptors for $H2^{d}$ class I antigens fails to recognize $H2^{b/b}$ BMC as self. The results of BMC allografts also depend on a donor cell type, called the facilitating cell (FC) ***(3)***. FCs were detected when it was observed that BMCs depleted of T cells were hypersusceptible to rejection by irradiated mice; e.g., BMCs from mice with severe combined immunodeficiency (SCID) are strongly rejected ***(4)***. Addition of sources of T cells, e.g., thymocytes, can restore the FC and render SCID BMC grafts more like "normal" parental strain BMC grafts. Therefore two donor-type cells, stem cells and FCs, are potential targets for rejection by host effector cells. In most instances, the effector cells are NK cells, but sometimes $CD8^{+}$

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
Edited by: K. S. Campbell and M. Colonna © Humana Press Inc., Totowa, NJ

T cells reject either LNC or BMC allografts *(5)*. We present here our methods for studying murine BMC transplants in mice. Similar studies can also be done with grafts of spleen or lymph node cell (LNC) grafts that undergo graft-vs-host responses *(6)*. **Table 1** lists the outcome of BMC grafts in various donor–host combinations, almost always based on H2 type of donor. The NK gene complex on chromosome 6 has one or more genes that confer the relative ability of mice to reject H2 allogeneic or parental-strain BMC grafts *(7)*.

2. Materials

2.1. Medium Preparations

1. Medium for cell transfer experiments: Use RPMI 1640 (or similar medium) to harvest, wash, and suspend cells for injection or infusion. Keep it chilled (4°C).
2. Elutriation medium: For 30× medium, add 30 g of EDTA, 30 g of dextrose, and 145 g of NaCl/L of sterile water. Stir overnight until complete solution occurs. Adjust pH to 7.2 and sterilize by filtration. To make diluted medium, add 66.6 mL of 30× medium and 33.3 mL of 30% bovine serum albumin (BSA) to 1900 mL of sterile water. Swirl until mixed and use at room temperature.
3. Tris ammonium chloride: 0.15 *M* or 8 g/L of NH_4Cl, 0.02 *M* or 3.16 g/L of Tris-HCl. Use this to lyse red blood cells.

2.2. Antibodies

The monoclonal antibodies (MAbs) used to deplete NK or other cells can be used as ammonium sulfate salt cuts (after dialysis). Alternatively, ascites fluid induced in SCID mice by implanting intraperitoneally with hybridomas provide potent antibody preparations. Recently, we have been purifying MAbs by column chromatography, using streptococcal proteins A or G. $F(ab')_2$ fragments are generated by pepsin digestion and column chromatography. All of these methods have been described. The amount of any one preparation to be used is determined by a dose–response in mice, testing for the ability to inhibit BMC allograft rejection. The usual dose range of purified MAb is 300–500 µg/mouse ip.

1. PK136 anti-NK1.1, a pan-NK receptor (American Type Culture Collection).
2. 5E6 anti-Ly49I/C, NK subset receptors ($ITIM^+$) for $H2^b$ (K^b) and other class I antigens.
3. 12A8, 4E5 anti-Ly49D, NK subset receptor ($ITIM^-$) for D^d, L^d, and D^{Sp2} (12A8 cross-reacts with Ly49A).
4. 4D11 anti-Ly49G2, NK subset receptor ($ITIM^+$) for D^d and L^d (gift from John Ortaldo, NCI, Frederick, MD).
5. GK1.5 (ATTC), YTS191.1.2 (Gift of Herman Waldmann) anti-CD4 T cell.
6. 2.43 (ATTC), YTS 169.4.2.1(Gift of Herman Waldmann) anti-CD8 T cell.
7. Rabbit anti-asialo GM1 serum (Wako Chemicals, Dallas, TX); this antiserum recognizes NK cells, certain $CD8^+$ T cells and certain activated macrophages.

Table 1
Relative Ability of Mice to Reject Allogeneic or Parental-Strain BMC Grafts

BMC Donor	Host strains		
H2[a]	"good responders"	"poor responders"	H2
b/b	B10.D2, NZB, B10.D2 × DBA/2	BALB/c, DBA/2	d/d
	B10.BR, C57BR, B10.BR × C3H	C3H, CBA	k/k
	B10.A, B10.A × A	A	a/a
	B10 × DBA/2, B10 × B10.D2, 129 × C3H	BALB/cxBALB.B	d/b
d/d	B6, B10, B10 × 129	129, BALB.B, D1.LP	b/b
	B10.BR	C3H, CBA	k/k
	NZB × NZW ($H2^{d/z}$)	B6 × DBA/2[b]	b/d
		BALB/cxBALB.B	d/b
k/k	NZB	B10.D2, DBA/2, BALB/c	d/d
	None	B10, B6, BALB.B, 129	b/b
	NZB × B6	B6 × DBA/2, B6 × BALB/c	b/d
b/b, D^d	B6, B10	129, BALB.B	b/b
[D8][c]	B10.BR	C3H, CBA	k/k

[a]NK subsets that reject $H2^{b/b}$ BMC include Ly49G2 and Ly49A; those that reject $H2^{d/d}$ BMC include Ly49C and Ly49I; and both $CD8^+$ T cells and NK cells reject $H2^{k/k}$ BMC.

[b]T-cell depleted $H2^{d/d}$ BMC are rejected ***(4)***.

[c]D^d is the target antigen of D8 BMC, recognized by host Ly49D receptor lacking immunoreceptor tyrosine-based inhibitory motifs (ITIM) ***(21)***.

2.3. Countercurrent Centrifugal Elutriation Equipment

A Beckman J-6M centrifuge equipped with a strobe light assembly, a JE-5.0 rotor with the 4 ml chamber (Beckman Instruments, Palo Alto, CA) provides the centrifugal force; a Masterflex L/S peristaltic pump with a digital variable-speed console drive (model 7523-209 Cole Palmer Instrument, Chicago, IL) and silicone tubing (ID 3/16 in. OD 5/16in. no. 95082-09 Cole Palmer) controls counterflow through the chamber.

2.4. Animals

2.4.1. Conventional vs Specific-Pathogen-Free (spf) Facilities

Mice can be housed in a conventional colony for bone marrow transplant studies. These mice survive lethal irradiation and marrow grafts for at least 7–10 d; if chimeras are generated, sepsis is prevented with acidified drinking water or antibiotics. Mice in the spf facility survive transplants very well. In

general, results are very similar in the two facilities; however, we did observe that perforin gene knockout mice lose the ability to reject BMC allografts if housed in a conventional facility.

2.4.2. Prevention of Sepsis in Irradiated Mice

Drinking water is treated with 1 *N* HCl to reduce the pH to 2.5, and the acidified water is given to mice 1 wk prior to irradiation and cell transfer. Alternatively, the following antibiotics are added: 250 mg of neomycin, 6.5 mg of polymyxin B, 50 mg of trimethoprin, and 250 mg sulfamethoxazole in a 500-mL volume of sterilized water. We never leave the mice on the same regimen more than 2 wk to prevent development of resistant organisms.

2.5. Other Materials

1. [^{125}]5-Iodo-2'-deoxyuridine (^{125}I-UDR) is purchased from Amersham (Chicago, IL) and is diluted to 300 µCi/mL with 0.03 *M* citric acid. The purity is about 90%.
2. 5-Fluoro-2'-deoxyuridine (FUdR, Sigma).

3. Methods

3.1. Preparation of Cells

3.1.1. Preparation of Bone Marrow Cells

1. Remove the spine, sacrum, sternum, and leg bones using a sterile technique. The yield will range from 10^8 to 3×10^8 nucleated cells, depending upon size and strain of mice.
2. Cut the bone into sizes of ~30 mm^3. Strip the muscles from the bones and place in mortar with 5 mL of medium. Gently crush the bones; add 20 mL of medium and allow the particulate matter to sink.
3. Take up fluid with a Pasteur or electronic pipet and filter through nylon bolt cloth or 200-mesh stainless steel gently into a 50-mL test tube. Too much force may contaminate the cell suspension with lipid and particulate matter.
4. Centrifuge (100*g* for 6 min) the cells and resuspend very gently, avoiding rapid pipetting or agitation to prevent cell death with DNA release ***(9)***. Filter the cells again and resuspend in medium. Count the cells with a Coulter electronic particle counter or a hemocytometer (count the Trypan blue excluding live cells). Keep on ice until used.

3.1.2. Preparation of Spleen Cells

1. Remove spleens with sterile technique. The yields will range from 20 (SCID mice) to 200 million cells.
2. Place spleens in a Petri dish with 4 mL of medium and gently crush the spleens, using the frosted ends of sterile microscope slides. Aspirate the suspension into a 3-mL syringe, using a 22-gage needle.
3. Perform **step 3** as described for BMC in **Subheading 3.1.1.**

4. Wash the cells as in **Subheading 3.1.1.** To lyse red blood cells (RBCs), resuspend the pellet in 5 mL of Tris NH_4Cl solution for 5 min, then wash and resuspend in 5 mL RPMI medium. Alternatively, resuspend the pellet in 0.5 mL of RPMI and add 2 mL of sterile distilled water to lyse RBC in less than 5 s if cells are to be cultured. RBC lysis is not necessary if the cells are to be infused into mice. Dilute in 30 mL of medium and refilter the cell suspension.
5. Wash the cells again, and refilter if debris remains. Count the cells as described previously.

3.1.3. Preparation of Thymus and Lymph Node Cells

1. Repeat **steps 1–3** in **Subheading 3.1.2.** with thymi. The yield of cells from a thymus is $1–2 \times 10^8$, and from cervical, mesenteric, axillary, and brachial nodes it is $3–6 \times 10^7$/mouse.
2. Wash the cells as described. Remove any fat that adheres to the side of the test tube with a cotton-tipped swab to avoid fat emboli. Resuspend the cells and filter them again. Count the cells as described above.

3.1.4. Counterflow Centrifugal Elutriation (CCE) of Marrow Cells (Fig. 1)

1. Setup: follow the manual for the instrument. Run about 250–300 mL of 70% ethanol through the system for sterilization at 40–50 mL/min while the centrifuge is off. Start the centrifuge when 100 mL of ethanol has gone through the system to remove air bubbles. Stop the pump and change to sterile distilled water (dH_2O). Wash the system with 250 mL of dH_2O to remove ethanol. While washing with dH_2O, turn on the balance and computer.
2. The pressure gauge should read ~0 psi before calibration. If it does not, turn off the centrifuge and increase the pump speed to 80 mL/min and restart the centrifuge. If the pressure is still too high, there may be a leak or air may be trapped in the chamber. Check the assembly and tilt rotor to eliminate any air.
3. Calibration: Albert Donnenberg kindly provided a computer program for gravimetric determination of the flow rate ***(10)***. A PM4800 Mettler balance interfaced with a 286 computer establishes a calibration curve to determine the corrected flow rates. Use computer commands to begin calibration; highest pump setting is 400 U = 160 mL/min). Set the pump for 100, 175, 250, 325, and 400 U in sequence; a data screen will follow. Then enter desired flow rates (1 U = 2.5 mL/min) and the speed to be used (3000 rpm).
4. Injection: Set pump to 40–50 mL/min and change tubing to elutriation medium for 2–3 min (check bubble trap—it should be half full). Set pump at 15 mL/min for BMC injection. Filter cells just prior to injection to avoid clumping. A maximum of 300 million cells at 20 million/mL are loaded rapidly (to minimize air intake) at a site between the pump and the centrifuge. This loads the BMC into the chamber that can be viewed through the window because the strobe light flashes 3000 times/min. A loading time of at least 30 min is recommended to allow time for optimal gradient development ***(11)***.
5. Collection: Turn off the computer, monitor, and balance. Run the pump at 15 mL/min until the cells leave the bubble trap (3–5 min). Set the pump to the

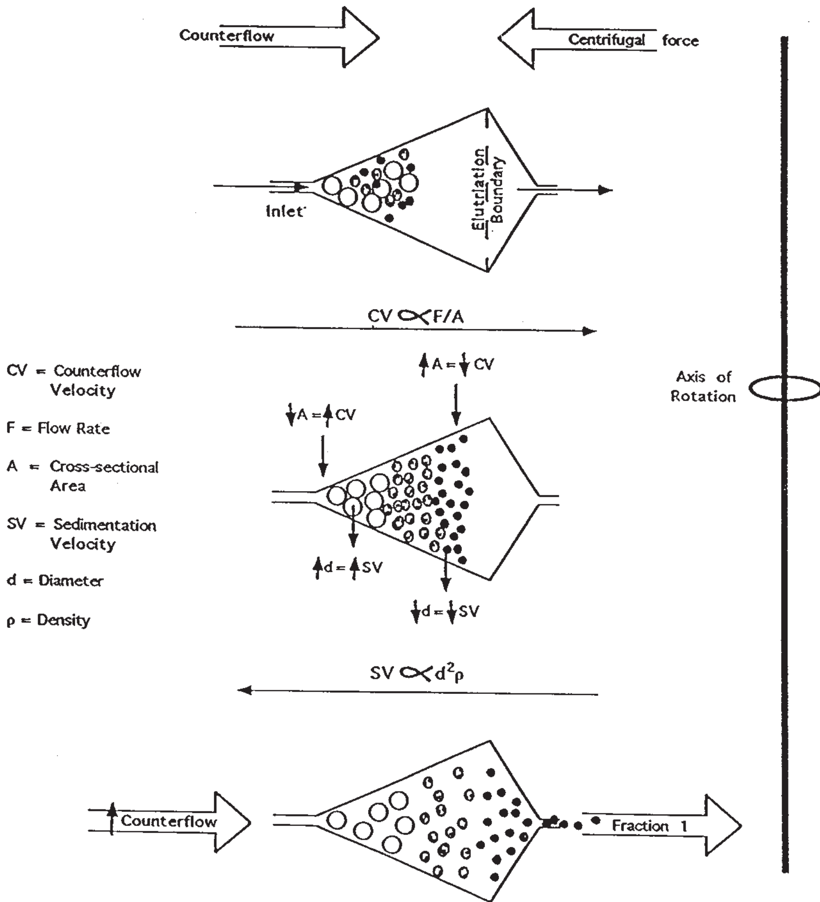

Fig. 1. Counterflow centrifugal elutriation. The two opposing forces are shown *above*. When the marrow cells are initially injected into the line, cells of different sizes enter the chamber. In the *center*, as the flow is held constant for about 30 minutes, the smaller cells move to the elutriation boundary and the large cells remain near the inlet (SV); the cells are aligned based primarily on size (d^2) but also by density (ρ). After the counterflow rate is increased, the smaller, more dense cells leave the boundary and become fraction 1. After 200 mL are collected of fraction 1 cells, the flow rate is increased again to begin collecting fraction 2 cells, and so on. The largest cells can be collected at the highest flow rate or the rotor is turned off so that the cells are removed from the chamber by flow only (rotor-off).

first corrected speed, increasing by 2–3 mL/min/min. Collect 200 mL into 50-mL conical tubes; continue to the next two or more fractions, and collect the last fraction after turning off the rotor. The larger stem cells are enriched in the rotor-off fraction and the small lymphocytes in the first two fractions. This CCE method

ideally separates larger stem cells from smaller lymphocyte-sized facilitating cells ***(12)***, however, success depends upon a number of factors (*see* **Note 1**).

3.2. Hemopoietic Transplantation and Its Evaluation

3.2.1. Irradiation, Cell Transfer, and Prevention of Sepsis

1. Place mice in a small animal irradiator (Gamma Cell 40, Atomic Energy Limited, Ottawa, Canada or Mark I, Shepherd & Assoc., San Fernando, CA) with a single (Mark I) or dual (Gamma Cell 40) ^{137}Cs source. Radiosensitive SCID mice receive 250 cGy while other mice receive 700–900 cGy total body irradiation.
2. One can infuse with hemopoietic cells within a few minutes to 1 d afterwards, usually about 2 h. Infuse inocula of cells in 0.5 mL of medium in a lateral tail vein. Warm mice under a strong incandescent lamp to dilate the tail veins; alternatively, rinse tails with hot water, using gauze. In most BMC inoculi, facilitating cells (FCs) exist and aid engraftment of donor stem cells in incompatible hosts. Elutriation separates stem cells from FCs, although a number of other methods can also be used, including cell sorting. Spleen, lymph node, and thymus cells can initiate graft-vs-host reactions in irradiated mice, and the IUdR uptake assay can be used to measure proliferation of the donor-derived cells. Radioresistant NK and/or $CD8^+$ T cells mediate rejection of the incompatible hematopoietic cells transferred ***(5)***.
3. Postirradiation sepsis is prevented by acidifying the water with HCl to pH 2.5 or by adding antibiotics (200 mg of neomycin, 52 mg of polymyxin B, 50 mg of trimethoprin, and 250 mg of sulfamethoxazole per 400 mL of drinking water) at least 1 wk before cell transfer. BMC transfers measure growth and differentiation of hematopoietic stem/progenitor cells.

3.2.2. IUDR to Measure Engraftment

1. Inject mice intraperitoneally with 10^{-7} *M* (26 µg) 5-fluoro-2'-deoxyuridine (FUdR) in a volume of 0.1 mL of medium, using a sterile solution of 26 mg/100 mL of RPMI 1640 medium, 30–90 min before injecting IUdR. FUdR inhibits thymidylate synthetase to decrease endogenous thymidine; this enhances IUdR incorporation into DNA ***(13)***.
2. Inject 0.3 µCi IUdR in 0.5 mL of medium intraperitoneally which is taken up by cells synthesizing DNA in the S-phase of the cell cycle. It is not necessary to give mice stable iodine in the drinking water beforehand to saturate the thyroid gland. The non-DNA radioactivity is excreted by 8 h, and therefore spleens taken out after this time (up to 30 h) can be directly counted for ^{125}I radioactivity; alternatively, spleens can be removed at least 2 h later and soaked in 70% ethanol for 1–3 d to elute non-DNA ^{125}I radioactivity.
3. The spleens, 5-µL aliquots of IUdR injection fluid (standards), and empty tubes (background) are counted. The mean background counts are subtracted from the standard and each spleen value. The formula for % IUdR uptake using cpm of ^{125}I is: [(experimental cpm) ÷ (mean standard cpm) × 0.9 (which is the fraction

purity of IUdR)] X 100. Radiation controls (RC) are mice irradiated but not infused with cells; if used, their mean cpm value is subtracted from each experimental value.

4. The arithmetic values are converted to $logarithmic_{10}$ values to stabilize the variances, because the SDs are linearly related to mean values. The geometric mean (95% confidence limits) values are calculated. Aberrant values are determined by the F-ratio method. The Student *t*-test and the Mann–Whitney U–test are used to compare two groups of 4–10 values, and the Newman–Keuls Multiple Comparison test is use to determine significantly different groups ($p < 0.05$) for experiments that have several groups of hosts for a given donor ***(13)***.

3.3. Optional Treatments Affecting Hybrid Resistance or Allogeneic Resistance to Engraftment

1. Depletion of all or most NK cells:
   a. Inject PK136 anti-NK1.1 MAbs (300–500 μg of purified antibody) i.p. 2 d before cell transfer.
   b. Inject 15–50 μL of rabbit anti-asialo GM1(Wako Chemicals. Ltd.) serum in 0.5 mL medium intraperitoneally between 2 d before and to the time of cell transfer; this reagent eliminates NK cells but also certain $CD8^+$ T cells.
   c. Inject cyclophosphamide (300 mg/kg body weight injected) i.p. 1–14 d prior to cell transfer. $CD8^+$ T cells capable of rejecting hematopoietic grafts are also affected ***(14)***.
   d. Inject 100 μCi ^{89}Sr i.p. 1 and 2 mo prior to cell transfer; alternatively, implant 15 mg β-estradiol in silastic tubing subcutaneously at least 6 wk prior to cell transfer. These treatments interfere with full maturation of NK cells ***(14)***.
2. Depletion of NK cell subsets: Inject SW5E6 anti-Ly49I/C, 12A8 or 4E5 anti-Ly49D, 4D11 anti-Ly49G2, or A1 anti-Ly49A MAbs intraperitoneally like PK136 as described previously. In certain cases, one can use $F(ab')_2$ MAbs to inhibit function of receptor without depleting cells (*see* **Note 2**).
3. Depletion of T cells: Inject 500 μg of purified anti-CD4 (GK1.5, YTS191.1.2) and/or anti-CD8 (YTS 169.4.2.1, 2.43) 6 and 4 d before cell transfer. Intravenous injection of MAbs is not necessary. Alternatively, use athymic nu/nu (nude) mice or mice with SCID.
4. Depletion of macrophages: Infuse silica particles (2.5 mg/0.5 mL of medium, sonicated) or carageenan (1 mg/0.5 mL, heated to dissolve) 1 d before cell transfer as described ***(13)***.
5. Perform FACS® analysis of spleen cells to confirm that the MAbs depleted the given cell type. It is essential to use MAbs directed against a different determinant to be sure that a cell type is missing; e.g., use anti-CD3 MAbs to ensure that anti-CD4 plus anti-CD8 eliminated most T cells. If one depletes NK cells with anti-NK1.1, use anti-DX5 or anti-2B4 pan-NK MAbs to determine whether or not the cells are missing. Test for depletion of $5E6(Ly49I/C)^+$ cells by staining for NK1.1 (should have about 50% depletion) or the Ly49C-specific NK2.1

4031170 MAb ***(15)***. Test for macrophage depletion by staining with F4/80 MAbs (Pharmingen).

6. Other methods that inhibit rejection of marrow allografts:
   a. Stimulate macrophages that are capable of inhibiting NK cell function ***(14)*** with 0.5 mg of heat-killed *C. parvum* (now *P. acnes*) infused intravenously 7–14 d prior to cell transfer; this coincides with peak phagocytic activity and enhanced hematopoiesis (*see* **Note 3**).
   b. Sublethally irradiate (400–600 cGy) mice 7–20 d prior to lethal irradiation and cell transfer ***(14)***. This treatment results in depletion of several immune cell types.
   c. Inject parental-strain spleen cells (10^7) intraperitoneally 4× at weekly intervals into F1 hybrid mice, the last time 7 d before cell transfer; specificity for donor type, e.g., $H2^{b/b}$, is obtained ***(1)***. The mechanism includes graft-vs-host induced suppression of NK cell function ***(16)***.
   d. Produce radiation BMC chimeras, using F1 hybrid BMC infused into irradiated parental-strain mice; this also produces specific loss of hybrid resistance ***(17)***. The mechanism has not been determined, but NK cell functions and numbers apparently are not altered ***(18)***.
7. Stimulation of allograft rejection:
   a. Inject 200 μg of polyinosinic–polycytidylic acid (poly I:C) intraperitoneally 1 d prior to irradiation and cell transfer; alternatively, inject 10^4 U of interferon-αβ intraperitoneally daily from d 0 to d 4 after cell transfer ***(19)***. Poly I:C stimulates macrophages to secrete interferon-αβ to activate NK cells.
   b. Infuse IL-2 propagated (host-type) NK cells (2×10^7/0.5 mL of medium) into hosts whose NK cells have been eliminated; inject 50,000 U of hrIL-2 intraperitoneally twice, at 12 and 36 h after donor cell transfer ***(20)***.

4. Notes

1. The success of counterflow centrifugal elutriation method for separating cells depends upon a number of conditions. The osmolality for mouse cells should be ~310 mOsm/mL, requiring the use of an osmometer. Because cells tend to aggregate at the inlet of the chamber, one should remove dead cells from the marrow cell suspension and use EDTA (3 m*M*) to inhibit platelet aggregation. One can omit EDTA if less cells, ~10^8, are loaded. One important step is to prevent cell death during manipulations. Even fast pipetting or resuspension can kill cells, liberating DNA into the medium ***(9)***. This causes clumping of cells in the chamber. This "piling up" of cells can lead to a retention of small cells in the chamber, which can "contaminate" the last two fractions of cells. The cells are exposed to much tubing throughout a run and it is helpful to replace the silicone tubing every one or two runs. A method to shorten the time it takes to establish a gradient in the chamber is to add cells closer to the centrifuge, using a syringe without its piston and a three-way stopcock so that one can pour the marrow cells into the line. In successful runs, the forward and side scatter of light determined by FACS® analysis will indicate that small cells predominate in fraction one and larger cells in the later fractions.

2. Modification of marrow grafts to control time that NK cells need to reject donor cells. This problem occurs when a reagent used to inhibit NK cells has a very short half-life, e.g., F(ab')$_2$ reagents, or when one is determining the time taken to reject a graft. For example, when using F(ab')$_2$ anti-Ly49D MAbs, we wanted to limit the time allowed for host B6 $H2^b$ NK cells to reject D8 ($H2^b$, D^d) BMC. To accomplish this, we administered the F(ab')$_2$ reagent on day 0 and infused anti-asialo GM1 serum intravenously on d 2 to immediately stop NK cell function by killing them ***(21,22)***. Thus the reagent only had to be active for 2 d.
3. Modification of marrow grafts to avoid problems with excessive endogenous proliferation in spleens of irradiated mice. This problem occurs if the animal has been treated with agents, e.g., heat-killed *C. parvum* organisms, that cause splenomegaly with increased hematopoiesis. In particular, the erythroid CFU-S are in cycle and resist lethal doses of irradiation, but transplantable CFU-S or stem cells remain radiosensitive. Perform two-step "stem cell rescue" experiments, in which the initial transplant is conducted as usual, but the isotope assay is replaced by harvesting the spleen cells of the hosts and making a cell suspension (after washing). The spleen cells (1/10 to 1/2 spleen equivalent) are infused into irradiated mice syngeneic with the original marrow donor and sensitized against cells of the original host. An ^{125}I-UdR assay is then performed 5 d after infusion of the spleen cells ***(13)***. For example, in a B6 to B6D2F1 transplant, prospective secondary B6 hosts are immunized with B6D2F1 spleen cells (10^7 spleen cells intraperitoneally 4–10 d before irradiation). The B6 stem cells generated in spleens of primary hosts, even if contaminated with host F1 stem cells, will be the only stem cells that will survive in the secondary host.

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8

Hybrid Resistance by Mouse NK Cells In Vitro

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1. Introduction

The study of hybrid resistance was greatly aided by the development of an in vitro assay by Chadwick and Miller ***(1)***. The assay allows one to test for the presence of negative or positive signaling in natural killer (NK) cells, and the ability of monoclonal antibodies (MAbs) or $F(ab')_2$ reagents to block those signals. There is already good evidence for negative signals between Ly49 NK cell receptors and their specific class I antigens, e.g., Ly49A and $H2\text{-}D^d$ ***(2)***. Thus, the inability of $H2^b$ Ly49A purified NK cells to kill $H2^d$ target cells can be reversed by adding MAb reagents that bind to either Ly49A or $H2\text{-}D^d$. We and others have extended these early findings to the Ly49C and I subsets of NK cells, and have recently detected positive signaling from D8 ($H2^b$, D^d transgene) to purified $H2^b$ $Ly49D^+$ NK cells that can be blocked by antibodies or their $F(ab')_2$ fragments to Ly49D. The assay can also be used to study signaling properties of fetal and neonatal NK cells lacking Ly49 receptors, which suggest that inhibitory non-Ly49 NK receptors are expressed on such NK cells and that these receptors can receive negative signals from class I molecules ***(3,4)***. The stimulation of bone marrow NK precursor cells with various cytokines, including IL-15, leads to generation of lytic $NK1.1^+Ly49^-$ cells whose function is similar to that of fetal NK cells, i.e., they can lyse YAC-1 tumor cells and class I deficient cells, but cannot lyse normal lymphoblasts and lack Ly49 receptors ***(5)***. These two types of in vitro systems allow the analysis of factors necessary for various stages in NK cell differentiation and positive and negative signaling to NK cells at different stages of maturation.

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
Edited by: K. S. Campbell and M. Colonna © Humana Press Inc., Totowa, NJ

2. Materials

2.1. Medium

1. 4+ DMEM: Dulbecco's minimum essential medium with 10% fetal calf serum (FCS), 100 U/mL of penicillin and streptomycin, 1 m*M* of sodium pyruvate, 2 m*M* of glutamine, 1× nonessential amino acids, 50 µg/mL of gentamycin, 2.5×10^{-5} *M* 2-mercaptoethanol (2-ME), and 1 µg/mL of indomethacin. Use this medium to grow fetal, neonatal and mature NK cells.
2. 4+ RPMI: RPMI-1640 medium with 10% FCS, 100 U/mL of streptomycin, 1 m*M* of sodium pyruvate, 2 m*M* of glutamine, 1× nonessential amino acids, 50 µg/mL of gentamycin, 5×10^{-5} M 2-ME, and 1 µg/mL of indomethacin. This medium is used to grow marrow progenitor cells and to generate T lymphoblasts from spleen cells stimulated with Concanavalin A (Con A).
3. Tris ammonium chloride: 0.15 *M* or 8 g/L of NH_4Cl, 0.02 *M* or 3.16 g/L of Tris HCl. Use this medium to lyse red blood cells (RBC).
4. Phosphate-buffered saline (PBS)–0.5% bovine serum albumin (BSA)/5 m*M* EDTA is used to wash cells after lysing RBCs prior to use of tissue culture medium, and for staining cells to purify NK progenitor and precursor cells.
5. PBS–2% FCS is used after lysis of RBCs in spleen cell suspensions for magnetic column cell separation procedures.

2.2. Cytokines

1. Recombinant human (rh) IL-2 (Chiron, Emeryville, CA). Use concentrations of 500 or 1000 U/mL for short-term spleen cell cultures for hybrid resistance. For long-term fetal liver NK cells, use 100 U/mL and for fetal thymus cells, use 5,000 U/mL.
2. For culturing marrow progenitor cells, use 20–25 ng/mL of rmIL-15 (gift from Dr. Tony Trout, Immunex R & D Corp., Seattle, WA), 20 ng/mL of IL-6 (Pharmingen), 0.5 ng/mL of IL-7 (PreProTec, Rocky Hill, NJ), 100 U/mL of mouse flt3 ligand (mflt3-L) (Gift from Donna Rennick, DNAX, Palo Alto, CA), and 50 ng/mL of rat stem cell factor (SCF) (c-kit-L, gift from Amgen, Thousand Oaks, CA) or 30 ng/mL of mSCF (Biosource Intl., San Francisco, CA).

2.3. Antibodies

1. TER119 MAb (PharMingen) reacts primarily with erythropoietic lineage cells.
2. Gr1(RB6-8C5) MAb (PharMingen) reacts primarily with myelopoietic cells.
3. CD22 (Cy34.1 MAb (PharMingen) reacts with B cells.
4. CD5 (53-7.3) MAb (PharMingen) reacts with all T cells and certain B cells.
5. B220 (RA3-6B2) MAb (PharMingen) reacts with B cells at all stages of differentiation, and other early lineage cells.
6. CD2 (RM2-5) MAb (PharMingen) reacts with all T cells and NK cells.
7. CD11b (M1/70) MAb (PharMingen) reacts with macrophages and some NK cells.
8. NK1.1 (PK136) MAb, a pan-NK receptor (ATCC or Pharmingen).
9. CD16 (2.4G2) MAb (PharMingen) reacts with anti-FcγRIII/II.
10. CD10 (2B8) MAb (PharMingen) reacts with c-kit on stem cells and other early lineage cells.

11. Sca2 (TSA-1, MTS35) MAb (PharMingen) reacts with certain early precursor/progenitor hematopoietic cells, as well as thymic epithelial, dendritic, and cortical lymphocytic cells, and bone marrow myeloid and B cells.
12. CD25/CD122 (PC61, 7D4, TM-β1) MAb (PharMingen) react with IL2 receptor α chain and IL-2/IL-15 receptor β chain.

2.3. Animals (see *Chapter 7* and *Notes 1* and *2*)

2.4. Other Materials

1. Streptavidin magnetic beads and Type CS Miltenyi depletion column (Miltenyi Biotec Inc, Auburn, CA).
2. Streptavidin-Red670
3. FACStar Plus® flow cytometer (Becton Dickinson, San Jose, CA) using Lysis II software.
4. NK cell enrichment cocktail (StemSep™, Vancouver, BC, Canada) that contains TER119, Gr1, CD22, and CD5 MAbs.
5. Anti-biotin tetramer (StemSep™).
6. Magnetic colloid (StemSep™).
7. StemSep™ column
8. Vario-Macs magnet (Miltenyi Biotec Inc, Auburn, CA).
9. Scintillation fluid (Budget-Solve™, Res. Prod. Intl., Mount Prospect, IL).

3. Methods

3.1. Culture of NK Cells

3.1.1. Adult NK Cells

1. Harvest fresh spleen cells. In some experiments, purify NK cells (magnetic and/or FACS® sorting; *see* **Notes 1** and **2**). In either case, wash cells twice in 4+ DMEM and resuspend in 3 mL of same at 3–5 × 10^6 cells/mL.
2. Add 15 µL 2ME (2.5 × 10^{-5} M) and 30 µL of rhIL-2 (1000 U/mL).
3. Plate cells (3–5 × 10^6/mL) 200 µL/well of a 96-well U-bottom plate; incubate in 10% CO_2, 37˚C for 1–6 d. For longer times, refeed with 2-ME and IL-2 at 3 d intervals (*see* **Notes 1** and **3**).

3.1.2. Fetal and Neonatal NK Cells

1. Remove thymus glands from d 14–15 fetuses, and express the cells using frosted ends of microscope slides into medium (4+ DMEM).
2. Remove livers of the same fetuses, and pipet the cells up and down in a pipet, using 4+ DMEM with 10% FCS; wash cells twice and resuspend in 4+ DMEM.
3. Culture fetal thymus cells (3–5 × 10^6/mL) in 4+ DMEM with 10 ng/mL phorbol myristate acetate (PMA) and 10 U/mL IL-4 for the first 3 d. This is not necessary for fetal liver cells (3–5 × 10^6/mL).
4. Subsequently add 5000 (thymus) or 100 (liver) U/mL of rhIL-2 and refeed every 4 d. These cells will remain alive and functional for several months or longer.

Split cells when necessary. Cultures can be started in 24-well plates and either maintained in such plates or transferred to 6-well plates.

3.2. Purification of Marrow NK Progenitor, Precursor, and Mature Cells

3.2.1. Purification of Marrow NK Progenitor Cells

1. Single-cell suspensions of bone marrow cells are made as described previously. Lyse RBCs with Tris ammonium chloride for 5 min, then wash cells twice with PBS–0.5% BSA/5 m*M* EDTA.
2. Stain cells on ice in PBS–0.5% BSA/5 m*M* EDTA at 3–4 × 10^7 cells/mL. For three-color sorting, incubate with anti-FcγRIII/II (2.4G2) to block Fc receptors for 20 min, and wash once.
3. Incubate cells with lineage specific biotinylated MAbs (PharMingen, San Diego, CA) to B220 (RA3-6B2), CD2 (RM2-5), Gr1 (RB6-8C5), CD11b (M1/70), and NK1.1 (PK136) for 15 min at 4˚C and wash twice.
4. Add streptavidin magnetic beads for 15 min at 4˚C, and wash once. Pass cells over a Type CS Miltenyi depletion column to remove lineage $(lin)^+$ cells according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA).
5. The flowthrough contains lin^- cells. Stain these cells with streptavidin-Red670, c-kit (2B8)-PE, and Sca2 (TSA)-FITC for 20 min, and wash twice.
6. Sort the $Lin^-Red670^-$, c-kit-PE^+, Sca2-$FITC^+$ cells of lymphoid and blast size, with a flow cytometer at 5 × 10^6/mL.
7. Infuse 1–3 × 10^3 purified cells intravenously into irradiated (8 Gy) syngeneic hosts or irradiated (1.8 Gy) severe combined immunodeficiency (SCID) mice.
8. Assay progenitor cell function by assessing: (a) NK cell numbers and phenotype, (b) ability of NK cells generated to lyse YAC-1 tumor cells, or (c) ability of NK cells generated to respond to IL-2 so as to become effector cells capable of lysing allogeneic or parental-strain T-cell blasts.

3.2.2. Purification of Marrow NK Precursor Cells

1. Same as **step 1** in **Subheading 3.3.1.**
2. Stain cells on ice in PBS–0.5% BSA-5 m*M* EDTA at 3–4 × 10^7 cells/mL. Incubate with anti-FcγRIII/II (2.4G2) to block Fc receptors for 20 min, and wash once.
3. Incubate cells from B6 SCID mice with MAbs (PharMingen) PE-NK1.1 and with FITC-IL2/IL-15Rβ (TM-β1), and wash twice.
4. Sort the NK1.1-PE^-, IL2/15Rb-$FITC^+$ cells of lymphoid and blast size, as described in **Subheading 3.1.1.**
5. Assay precursor cell function by the generation of mature NK cells, using cell sorter analysis and/or ability of cells to lyse ^{51}Cr-labeled YAC-1 or other tumor cells.

3.2.3. Purification of Mature NK Cells

1. Harvest cells from five spleens from medium into sterile distilled water (1:1 vol) to generate hypotonicity for 20 s to lyse RBCs; dilute with excess medium.

Alternatively, one can add Tris ammonium chloride to pelleted cells for 5 min to lyse RBC. In either case, wash twice with PBS/2% FCS (use throughout), removing the clot that does not decant with a pipet. Ammonium chloride can inhibit NK cell function ***(6)***, and is generally not recommended when obtaining mature NK cells (*see* **Note 3**). If cells are cultured for 24 or more hours after treatment with ammonium chloride, NK cell-mediated lytic ability recovers.

2. Resuspend cells at 4×10^7/mL; add 2.4G2 MAbs at 1:100 dilution to block Fc receptors; maintain at 4°C for 15 min, wash, and resuspend at 7×10^7/mL.
3. Add 1/100 dilution of NK cell enrichment cocktail that contains TER119, Gr1, CD22, and CD5 MAbs. Incubate at 4°C for 15 min, wash, and resuspend at 7×10^7/mL.
4. Add 1/10 dilution of anti-biotin tetramer and incubate at 4°C for 15 min.
5. Add 60 mL of magnetic colloid/mL of cells and incubate at 4°C for 15 min.
6. Place StemSep™ column in Vario-Macs magnet, attach three-way stop-cock to luer lock of column, and attach the blunt-end 22-gage needle to the stopcock directly below column.
7. Prime the column with PBS/2% FCS, using a syringe connected to the three-way stopcock.
8. Load the sample into the top of the column through a nylon mesh filter, and collect flow-through (total of 35 mL). Expect 20–60% recovery and 60% purity of NK cells.

3.3. Assay for Lysis of T Lymphoblasts and Tumor Cells

1. Prepare T lymphoblasts by culturing spleen cells, following lysis of RBC, in 4+ RPMI medium at $3–5 \times 10^6$/mL with 6 µg/mL Con A for 2 (1.5–3) d at 37°C in 5% CO_2 in air atmosphere.
2. Prepare tumor cells (YAC-1, P815, RMA, RMA-S, etc.) grown in 4+ RPMI medium at 37 °C in 5% CO_2 in air atmosphere by diluting them to $\sim 2 \times 10^5$/mL the night before the assay to stimulate log phase growth.
3. Label tumor cells or lymphoblasts by incubating 2×10^6 cells with 100–150 µCi ^{51}Cr-sodium chromate in RPMI 1640 medium for 90 min, as above. Alternatively, dilute to 5 mL with 4+ RPMI medium and incubate overnight in an upright T25 flask. In either case, wash the cells 3×, and resuspend in 300–500 µL. Shake/resuspend cells every 30 min. Incubate the cells in 5 mL of RPMI 1640/10% FCS for an extra 45 min to allow labeled but damaged cells to release ^{51}Cr. Wash the cells and adjust the concentration to $0.5–3 \times 10^3$ cells/100 µL (*see* **Note 3**).
4. Harvest effector NK cells into 4+ RPMI medium, wash, and adjust to concentrations ranging from $1–80 \times 10^3$/100 µL (E:T ratios of 1:1 to 80:1; *see* **Note 1**).
5. Plate 100 µL each of effectors and targets into wells of U-plates, spin the plates at 30–90*g* for 4 min, and incubate for 4 h at 37˚C in a 5% CO_2 in air atmosphere. Controls are spontaneous release (SR, targets without effectors) and maximal release (MR, targets lysed with 1% Triton X-100). Centrifuge the plates as described above.
6. Measure ^{51}Cr radioactivity of 100 µL of superantant of each well by adding the material to 2 mL of liquid scintillation fluid. Liquid scintillation counting is

superior to gamma counting because β rays comprise 93% of the ^{51}Cr radioactivity. The formula for % specific cytotoxicity using cpm of ^{51}Cr is: (Exp. – SR) ÷ (MR – SR) × 100.

4. Notes

1. A critical change in our approach led to an increase in success of the assays; the use of highly enriched, especially sorted, NK cells free of other cell types. The use of SCID mice lacking T cells is a big advantage in obtaining cells that are almost 100% NK cells. For populations containing LAK effectors, use E:T ratios of 100:1 or less, and for sorted NK cells, use E:T ratios of 25:1 or less.
2. There are strain differences in sources of effector cells, perhaps because the assay was developed using mice of the C57BL background. FVB and 129 mice serve as poor donors of NK precursor or mature cells, when the same methods are used. C57BL and their F1 hybrids, and BALB/c and their H2 congenics are good sources of cells for this assay.
3. Success in the in vitro assay for hybrid resistance depends upon a number of factors. These include excellent viability of target T lymphoblasts and effector NK cells, and numbers of target cells used per well. As mentioned previously, ammonium chloride may inhibit mature NK cell function ***(6)***, and could interfere with the response to IL-2. The lowest number of target cells that we can use is 500/well. This low number is not recommended for beginners unfamiliar with the method. This low number is important when the yield of sorted effector cells is limiting. The Chadwick and Miller protocol ***(1)*** should be adhered to for best results. Some variables that can be critical are 10% CO_2, length of 4 d, and use of A1 medium, although we have had success with DMEM. By 6 or more days, specificity of killing is often lost, in that syngeneic targets can be lysed. It is not essential to generate adherent lymphokine activated killer (ALAK) cells ***(7)***.

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9

Use of Activated Natural Killer Cells for Tumor Immunotherapy in Mouse and Human

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1. Introduction

Natural killer (NK) cells are a subset of lymphocytes with a distinct morphologic appearance (large granular lymphocytes [LGLs]) and the ability to spontaneously kill virally infected or tumor targets but to spare most normal cells ***(1)***. These effector cells are now known to be able to eliminate tumor cells by mechanisms involving either necrosis or apoptosis or both ***(2)***, and upon activation to produce and secrete a broad spectrum of cytokines ***(1)***. NK cells respond to a variety of biologic agents, including cytokines such as interleukin-2 (IL-2), IL-12 ,or interferons, by upregulation of cytolytic, secretory, and/or proliferative functions ***(1)***. They represent from 5% to 15% of peripheral blood lymphocytes in humans and account for a substantial but variable proportion of tissue-resident lymphocytes. In rodents, NK cells are mainly found in the spleen, liver, and lung tissues as well as in the blood ***(1)***. Phenotypic characteristics of NK cells are variable, depending on the state of their activation, but surface expression of CD16 (FcγRIII), CD56 (in humans), NKR-P1 (in rats), or NK1.1 (in mice) and the absence of the T-cell receptor complex on the cell surface have been accepted as the markers defining these effector cells.

In cancer-bearing hosts, NK cells have been considered to be the major component of antitumor immunity responsible for rapid elimination of blood-borne metastases ***(1)***. More recently, however, it has been realized that NK cells are also responsible for killing of tumor targets that have downregulated expression of major histocompatibility complex (MHC) class I molecules and are not recognized by tumor-specific T cells ***(1)***. A-NK cells are a subset of IL-2-activated NK cells described by us a few years ago ***(3)***. “A” stands for activation and adherence, both of which are necessary for isolation of these cells from human

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
Edited by: K. S. Campbell and M. Colonna © Humana Press Inc., Totowa, NJ

peripheral blood or rodent splenocytes. A-NK cells are powerful effector cells with attributes that facilitate antitumor activity in tissues. Thus, A-NK cells not only can kill all "NK-resistant" tumor targets and proliferate extensively in the presence of IL-2, giving rise to powerful non-MHC restricted effector cells, but they also produce a wide spectrum of cytokines and growth factors, readily migrate and enter into solid tissues, selectively localize to tumor metastases, and are effective in elimination of metastases and prolongation of survival in tumor-bearing hosts ***(1,3)***. These characteristics of human and rodent A-NK cells have been extensively investigated, because of the likely possibility that these effector cells can be useful in immunotherapy of cancer ***(1–3)***.

The potential of A-NK cells for elimination of established metastases in experimental animal models has been the main reason for developing at our institution a series of clinical protocols designed to test therapeutic effectiveness of these cells following their adoptive transfer to patients with cancer ***(4)***. In parallel with clinical studies, we have also developed syngeneic and xenogeneic tumor models of adoptive immunotherapy (AIT), to study the mechanisms responsible for the elimination of metastases by A-NK cells and to define the optimal strategies for the delivery and for sustaining in vivo antitumor activity of A-NK cells ***(5,6)***. To utilize A-NK cells more effectively for cancer therapy, a number of unanswered questions about their interactions with metastases, vascular endothelial cells, or extracellular matrix components and about their functions in the tumor microenvironment need to be addressed. Of the two types of animal tumor models we have utilized to gain better insights into *in situ* events, those established in syngeneic mice or rats allow for the use of immunocompetent hosts and for evaluation of antitumor effects mediated by the transferred vs endogenous immune cells. On the other hand, transfer of human A-NK cells to NK cell-depleted nude mice bearing established metastases of human carcinomas provides the means for examining in vivo antimetastatic potential of these cells in the absence of host T and NK cells. This allows for examination of therapeutic effects mediated only by transferred effector cells and may be advantageous in view of the possible interference by endogenous immune cells with antimetastatic functions of A-NK cells in vivo. Although each of the two experimental models has limitations, substantial benefits can be gained by formulating hypotheses that can be tested in both models and by judicious comparisons of results generated. The most urgently needed information that we expect to obtain from the models is related to the optimal dose, route, and frequency of A-NK cell administration and to the mechanisms responsible for upregulating A-NK cell survival as well as their antimetastatic activity *in situ*.

2. Materials

2.1. Animals

1. Nude mice: 6-wk-old BALB/c female nude mice are maintained under specific pathogen-free conditions in a laminar air flow room.
2. C57bl/6 mice: 8–12-wk-old C57bl/6 female mice are maintained under specified pathogen-free conditions.

2.2. A-NK Cells

1. Human effector cells are generated from NK cells isolated from normal human peripheral blood lymphocytes. Cultures of A-NK cells should contain >90% $CD3^{-}CD56^{+}$ cells, as determined by flow cytometry, and these cells should be pretested for antitumor activity in vitro.
2. Murine A-NK cells are generated from splenocytes. Cultures of A-NK cells should contain >90% $NK1.1^{+}$ cells and less than 5–8% $CD8^{+}$ cells, as determined by flow cytometry. The antitumor activity in vitro of these cells is best tested against YAC-1 and P815 tumor cells.

2.3. Tumor Cell Lines

1. The human HR cell line was established in our laboratory from a hepatic metastasis of gastric cancer ***(5)***. The cells grow as an adherent monolayer, and the line is passaged by mild trypsinization with 0.05% trypsin/EDTA solution (Gibco) followed by washing 3× in medium. Viability of cells should be >95%, as determined by a trypan blue dye exclusion test. The cells are resuspended in culture medium and plated at the cell density of 0.5×10^6/mL in T75 flasks. The cultures are periodically tested for mycoplasma, using a Gene Probe kit (Gene Probe, San Diego, CA).
2. The murine B16-F1 melanoma cells (ATCC no. CRL-6323) of C57BL/6 origin are maintained in culture medium and passaged by incubation of monolayers in the presence of 0.02% (w/v) EDTA for 10–15 min. at 37°C with gentle rotation of culture flasks. The detached cells are washed 2× in medium at room temperature, resuspended in culture medium, and adjusted to a cell density of 2.5×10^6 cells/mL for injection. Cell viability should be >95% based on a trypan blue dye exclusion test.

2.4. Solutions

1. Complete medium (CM) for culture of A-NK cells: RPMI 1640 supplemented with 10% (v/v) human AB serum or 10% (v/v) fetal calf serum (for human or murine cells, respectively) and 6000 IU of recombinant interleukin-2 (IL-2, Chiron, Emeryville, CA). Additional supplements include 2 m*M* of glutamine and antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin) for culture of human cells and 2-mercaptoethanol (5×10^{-5} *M*), 2 m*M* of glutamine, 10 mL/L of 100× nonessential amino acids, and antibiotics (1.6×10^5 U/L of penicillin and 0.8 g/L of streptomycin) for murine cells.

2. Medium for tumor cell lines: Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 2 m*M* of glutamine, and antibiotics *(see above)*. For culture of B16 melanoma cells, DMEM is preferable to RPMI, because tumor cells cultured in the former tend to induce a higher number of metastases per 10^5 injected cells than those cultured in the latter medium.
3. Ammonium chloride–potassium buffer for lysis of erythrocytes: 8.26 g of ammonium chloride, 1.0 g of potassium bicarbonate, and 0.037 g of EDTA per liter of water. Adjust to pH 7.4 with NaOH. Store at 4°C. Make fresh every week.
4. OCT medium (Miles Inc., Elkhart, IN).

2.5. Labels for A-NK Cells

1. Na ^{51}Cr (specific activity of 1 Ci/mL, Amersham or New England Nuclear).
2. 3,3'-Dioctadecyloxacarbocyanine perchlorate (Di-O) dye (Molecular Probes, Eugene, OR).
3. TRITC-rhodamine dye (Molecular Probes).

3. Methods

3.1. Generation of Peripheral Blood Mononuclear Cell (PBMC) Feeder Cells

Con A-stimulated allogeneic PBMC feeder cells are used for human A-NK cell cultures.

1. Obtain a leukapheresis product from a normal donor, separate PBMCs by Ficoll-Hypaque centrifugation, wash, and deplete of monocytes using adherence to plastic.
2. Culture the mononuclear cells at the density of $1–5 \times 10^6$ cells/mL in T-150 flasks, using RPMI culture medium supplemented with 10% human AB serum, Con A at 10 µg/mL, and 300 IU/mL of IL-2. Incubate the feeder cultures at 37°C in 5% CO_2 for 3–5 d.
3. Irradiate the feeder cells with 5000 rads and tested by Gram stain and for bacteriologic sterility (performed on supernatant of the culture) prior to addition to A-NK cell cultures.

3.2. Generation of Human A-NK Cells

Human NK cells are purified from PBMC by negative selection on antibody-coated magnetic beads.

1. Separate PBMCs from heparinized venous blood by Ficoll-Hypaque centrifugation, wash 3× in medium, and check cell viability by trypan blue exclusion. Remove B cells and monocytes using nylon wool columns ***(7)***.
2. Perform negative selection of NK cells by adding a cocktail (50 µL) of mouse monoclonal antibodies (anti-CD3, anti-CD5, anti-CD19 and anti-monocyte [63D3, ATCC no. HB44]) to 1×10^8 PBMCs. Incubate for 30 min on ice with frequent mixing. Using immunomagnetic beads coated with goat anti-mouse Ig (2 mL) and a powerful magnet, remove the antibody-positive PBMCs, leaving

behind NK cells. Adjust the ratio of beads to cells at 10:1 for optimal depletion of T cells, B cells, and monocytes.

3. Repeat **step 2**, but now use a bead-to-cell ratio of 30:1. Wash the negatively selected NK cells in medium and determine their viability by trypan blue exclusion.
4. To separate plastic-adherent from other NK cells, incubate purified NK cells at the density of 1×10^6/mL of CM containing IL-2 for 5 h (37°C, 5% CO_2) in T-162 culture flasks. This allows for adherence and capture of the activated subset of NK cells to the flat surface of each plastic flask.
5. Decant the nonadherent NK cells and wash the flask gently with prewarmed medium (37°C). Following the removal of nonadherent NK cells, supplement A-NK cell cultures with concanavalin A (ConA)-stimulated, irradiated allogeneic PBMCs feeder cells at 1×10^6 feeder cells/mL of culture.
6. Maintain the cultures at 37°C in an atmosphere of 5% CO_2 in air for 14 d and observe them daily. A-NK cells form clumps of floating cells. Maintain the cell concentration at $0.5–1.0 \times 10^6$/mL by adding CM to culture flasks.
7. Harvest the cells on d 14 of growth and evaluate the culture for purity and cytotoxicity against tumor cell targets.

3.3. Generation of Murine A-NK Cells

1. Murine A-NK cells are prepared from splenocytes ***(8)***. Harvest mouse spleens and prepare a single-cell suspension in medium.
2. Lyse erythrocytes by incubation with ammonium chloride–potassium buffer for 10 min at room temperature and wash the cells twice in medium.
3. Transfer the cells to T150 flasks and culture them at a density of $3–5 \times 10^6$/mL (37°C, 5% CO_2) in 50 mL of CM.
4. After 2–3 d of incubation, remove nonadherent cells and wash the flasks gently with prewarmed (37°C) CM to remove cells not firmly attached to plastic. Supplement cultures with fresh CM (50 mL) and incubate the cells for an additional 2–3 d.
5. After 5 d of culture, harvest the cells after a short treatment with 0.02% EDTA and wash them twice with medium before use.

3.4. Evaluation of A-NK Cell Antitumor Activity In Vitro

To ensure reproducibility of experiments performed with batches of effector cells generated at different times from different donors, establish criteria for acceptance or rejection of A-NK cell cultures for your laboratory.

1. Use the established criteria for evaluation of the cellular products generated at different times. For example, in our laboratory, cultures of human A-NK cells are used only for AIT experiments, provided they contain >90% $CD3^-CD56^+$ cells and that A-NK cells mediate >1200 lytic units (LUs) of activity against HR targets, as determined in 4-h ^{51}Cr-release assays performed under conditions described previously ***(9)***. The LU are defined as $LU_{20}/10^7$ effector cells, and the number of HR cell targets in each well is 1×10^3. It is important to establish a range of acceptable values, as effector cells generated from PBMC of various normal donors are likely to differ in potency of killing they mediate in vitro.

2. Murine A-NK cells should be used only if they contain >90% NK1.1$^+$ cells and less than 5–8% CD8$^+$ cells and if their cytotoxicity against YAC-1 and P815 targets at a 20:1 E:T ratio is at least 65% and 30%, respectively, as determined in 4-h ^{51}Cr-release assays as described previously ***(8)***.

3.5. Induction of Tumors/Metastases

3.5.1. Xenogeneic Nude Mouse Model

1. To facilitate establishment of hepatic metastases and to abolish endogenous NK cell activity in nude mice, treat the animals with cyclophosphamide (200 mg/kg) and anti-asialo GM1 antibody (0.2 mg/mouse) by intraperitoneal injections 1 d prior to tumor cell injection. Persistent lack of detectable NK activity is observed when splenocytes obtained from these mice are tested in 4-h ^{51}Cr-release cytotoxicity assays with K562 cells as targets.
2. To induce liver metastases, administer general anesthesia to mice by using methoxy-flurane inhalant. Position each mouse on the right side, and make a 0.5-cm long incision in the left subcoastal region. Expose the spleen, and slowly inject a 0.3-mL aliquot of HR cells ($0.5–1 \times 10^7$) resuspended in Hanks' balanced salt solution (HBSS) into the spleen, using a 27-gage needle. Arrest any bleeding from the injection site by the use of hydrogen peroxide. Close the opening in the skin with sterile clips. In some experiments, if the spleen is not needed for a subsequent intrasplenic injection of effector cells, it may be removed with forceps 10 min after tumor cell injection.
3. Once the tumor cells are injected, micrometastases are detectable in the liver by hematoxylin and eosin (H&E) staining of liver sections on d 4. By using HR cells transduced with the *LacZ* gene and X-Gal staining, it is possible to visualize and study hepatic micrometastases within 4 h of intrasplenic transfer of the tumor cells ***(10)***. Generally, macrometastases are not visible on H&E sections until d 14.
4. By d 20–30 after tumor cell injection, numerous metastases replacing most of the liver are present. The animals develop ascites and die between d 25 and 40. By histological examination on H&E sections of the liver, HR metastases appear to have poorly differentiated morphology, and no immune cells are visible at the border between the tumor nodules and normal liver tissue.

3.5.2. Syngeneic Rodent Models

A large variety of tumor cell lines are available for the induction of syngeneic tumors or tumor metastases in many different mouse or rat strains. Here, we describe our experience with the B16 melanoma syngeneic in C57BL/6 mice.

1. Pulmonary metastases: Place each mouse under a lamp to heat and expand the tail vein and inject at least 1×10^5 B16 melanoma cells intravenously to induce pulmonary metastases (*see* **Note 1**).
2. Extrapulmonary metastases: Following intravenous injection of B16 tumor cells into immunocompetent animals, extrapulmonary metastases are seldom seen. Liver metastases can be established by intrasplenic (*see* **Subheading 3.5.1.**) or

intraportal (*see* **Subheading 3.6.1.**) injection of tumor cells. Both procedures require general anesthesia and are fairly time consuming. To establish metastases in the liver as well as in many other organs (adrenal glands, bone marrow, meninges, kidney, and testes/ovaries) pretreat mice with an intraperitoneal injection of 200 mg/kg of cyclophosphamide (25 mg/mL) 24 h prior to intravenous tumor cell injection.

3. Subcutaneous or intravenous metastases are induced by injecting 2×10^5 B16 melanoma cells (1×10^6 cells/mL) subcutaneously or intraperitoneally, respectively.

3.6. Adoptive Transfer of A-NK Cells (see Note 2)

3.6.1. Routes of Injection

1. Intravenous injection: Immediately prior to injection, heat each mouse under a lamp to induce swelling of the lateral tail vein, which is difficult to locate at room temperature. Inject A-NK cells into the tail vein, using a 27-gage needle. You can safely inject up to 40×10^6 A-NK cells in 0.4 mL of medium over a period of 15–25 s (*see* **Note 3**).
2. Left ventricular injection: Injection of A-NK cells into the left ventricle of the heart will ensure a delivery of cells to all parts of the body proportional to the distribution of the cardiac output ***(11,12)***. Anesthetize mice with ethyl ether by inhalation, using a nose cone containing ethyl ether (*see* **Note 4**). A 27-gage needle connected to a plastic tube is used for delivery of A-NK cells. Insert the needle through the plastic tubing so that only 5.5 mm of the needle remains uncovered by the tubing. Cleanse the skin over the chest with 70% alcohol and perform a percutaneous cardiac puncture, with the needle 2 mm to the left of the sternum and approx 12 mm above the tip of the xiphoid process. The tip of the needle should be directed slightly in a caudal direction and angled about 25° relative to the sagittal plane. A total volume of 0.2–0.25 mL can safely be inoculated over a period of 20–25 s. Animals that have not recovered completely within the first 5 min after anesthesia should be excluded from the study.
3. Intraportal injection: To deliver A-NK cells directly to the liver, the cells can be injected into the portal vein (*see* **Note 5**). Anesthetize mice with Nembutal and open the anterior abdominal wall. Identify a branch of the portal vein and slowly inject 200 µL medium containing up to 20×10^6 A-NK cells, using a 30-gage needle. Terminate bleeding by light compression and close the abdominal wall with surgical clips.
4. Intraperitoneal and subcutaneous injections: A-NK cell delivery into the peritoneal cavity or the subcutaneous tissue is easily performed on animals slightly anesthetized by ether or CO_2 inhalation. Use 30-gage needles for this purpose. The subcutaneous injection can be delivered directly into or around tumors growing in the subcutaneous space. There is no limit to the number of A-NK cells that can be injected by these routes, but although the volume injected by the intraperitoneal route can be as high as 1 mL, no more than 100 µL should be injected subcutaneously, to prevent a diffuse and uncontrollable spread of the A-NK cells into the subcutaneous tissue.

3.6.2. Timing and Frequency of Adoptive Transfer of A-NK Cells

1. To treat established metastases, inject A-NK cells systemically or locoregionally after the time period necessary to establish metastases. This time period will vary for different animal models of metastasis. Remember that metastases are heterogeneous ***(13)*** and prior to therapy, try to determine parameters such as the size, extent of vascularization, level of expression of various extracellular matrix components (e.g., collagen, fibronectin, laminin) or infiltration of metastases by endogenous mononuclear cells. These parameters as well as the ratio of dense vs loose metastatic deposits ***(13)*** will determine the time and frequency of A-NK cell + IL-2 cycles selected for therapy.
2. Therapeutic endpoints of AIT (i.e., decrease in tumor burden or survival) may be profoundly influenced by the time of A-NK cell transfer relative to the induction of tumor or metastasis growth. Because we are most interested in effects of A-NK cells on the elimination of preformed metastases, we allow a period of time sufficient for metastasis formation in mice before therapy with A-NK cells. In the nude mouse liver metastasis model, 3-d metastases (small) or 7-d metastases (large) can be treated, albeit with quite different results, based on survival of animals treated with a single cycle of AIT ***(10)***. Although 3-d metastases are obviously easier to treat than 7-d metastases, the latter are of particular interest, because they more closely approximate the in vivo situation in cancer patients. This experimental design allows for the development of treatment protocols that will produce maximal therapeutic effects for small or large metastases. For example, if AIT is optimized in regard to A-NK cell entry and function within metastases in initial experiments, it may be then advisable to establish the number of AIT cycles that produce the highest number of cures in mice bearing established 3-d or 7-d liver metastases.

3.6.3. Support of Transferred A-NK Cells by Exogenous IL-2

1. When A-NK cells have been harvested and adjusted to the appropriate concentration, add recombinant or natural IL-2 to the cell suspension to a final concentration of 6000 IU/mL (*see* **Note 6**).
2. Immediately following injection of the A-NK cells, also inject IL-2 to keep the A-NK cells alive and optimally activated. Owing to the short half-life of IL-2, be prepared to give IL-2 injections often. Thus, inject at least 150,000–300,000 IU of IL-2 in 0.25–0.5 mL of medium intraperitoneally every 4 h. The same doses of IL-2 can be given subcutaneously, but the volume should be reduced to 100 µL per injection (*see* **Note 7**).

3.6.4. Use of Labeled A-NK Cells to Determine Their Localization In Vivo

For adoptive transfers, A-NK cells can be labeled with: (a) ^{51}Cr for short-term (<12 h) distribution studies; (b) rhodamine, for short-term (24 h) localization studies; and (c) Di-O dye, a lipophilic carbocyanine dye, for longer term localization studies.

1. To obtain ^{51}Cr-labeled A-NK cells, wash and pellet effector cells and incubate the pellet with Na ^{51}chromate (specific activity of 1 Ci/mL) for 45 min at 37°C. Following extensive washing, resuspend the labeled cells in medium at a concentration of 5×10^6/mL, and measure their radioactivity in a gamma counter to determine the total injected cpm prior to intravenous or intrasplenic transfer of 1×10^6 cells in a 0.2-mL aliquot of medium. At 30, 60, 120, or 240 min, sacrifice the animals, harvest various organs and determine radioactivity in each by performing counts in a gamma counter. Express the results as the percentage of total recovered cpm/organ. Perform this experiment with tumor-bearing as well as control animals, because the distribution of counts may be quite different in these animals.
2. To study localization of A-NK cells in tissues within 24 h after systemic or locoregional transfer, incubate them with TRITC-rhodamine solution (3 µg/mL of medium) for 1 h and check the cell suspension in a fluorescent microscope equipped with the appropriate filter (e.g., BP 546, FT 580, or LP 590) to make certain that all cells are uniformly labeled. Inject the cells systemically or locoregionally, as desired, to tumor- or metastasis-bearing mice. Sacrifice the mice and harvest the tissues at different times after the cell transfer (e.g., at 2, 6, 12, and 24 h). Embed the tissues in OCT medium, prepare 5-µm thick cryostat sections and examine them in a fluorescent or confocal microscope to estimate the numbers and localization in metastases of labeled effector cells.
3. To determine the extent of accumulation of effector cells in regressing metastases, label A-NK cells with a Di-O dye by suspending the cell pellet in the solution of Di-O (20 µg/mL of medium) for 5 min at room temperature. Wash the cells 3× and adjust the final volume to the concentration suitable for their injection into mice. Kill the mice at various times after therapy, remove the organs with metastases, embed them in OCT, and section in a cryostat to obtain 5-µm sections for examination in a fluorescent or confocal microscope.

3.7. Assessment of Therapeutic Efficacy of A-NK Cells

The efficacy of AIT with A-NK cells + IL-2 is judged by the ability of these effector cells to prolong survival of animals with established metastases or to reduce the tumor burden.

3.7.1. Prolongation of Survival

1. To determine survival as an end point of therapy, select an animal model in which a tumor metastasizes aggressively, resulting in death of animals within a reasonable time period, e.g., 30–40 d ***(5,10)***.
2. Establish metastases in groups of 5 or 10 mice. Select systemic or locoregional therapy. At least four groups of animals will be necessary as follows: (a) control group not treated with any therapy; (b) control group sham-treated with HBSS; (b) experimental group treated with IL-2 alone; and (c) experimental group treated with a single cycle of A-NK cells and IL-2.
3. Determine the design of the experiment with respect to the age of established metastases (e.g., 3-d or 7-d liver metastases in the HR xenograft model), route of

effector cell delivery, the number of therapy cycles, and the route and dose of IL-2 to be injected to control and experimental animals. During the period of therapy and following it, observe the animals daily for signs of toxicity, discomfort, loss of activity, respiratory difficulties, or weight loss/gain.

4. To measure survival, maintain the animals until death or if they survive significantly longer than untreated controls, kill them on a predetermined date. Record the day of death and perform an autopsy to ascertain the cause of death or extent of remaining tumor, if any. Harvest the relevant organ and process it for microscopic examination. Examine multiple H&E stained sections (e.g., 30) from different areas of the organ to determine residual metastases.
5. Replicate each survival experiment at least four times with separate groups of control and experimental animals to obtain sufficiently large sample size for a meaningful statistical analysis. Compare survival times between treatment and control groups of by an appropriate statistic (e.g., the log-rank test) and check exact p values (using permutation or Monte Carlo methods) if the sample size of treatment groups is 5 or smaller.

3.7.2. Estimation of Total Tumor Burden

1. Subcutaneous and intraperitoneal tumors: To determine the size of solid tumors in the peritoneal cavity or those in the subcutaneous tissue, surgically remove the tumor and weigh it. Alternatively, estimate the volume of a subcutaneous tumor by measuring its three orthogonal diameters: length (L), width (W), and height (H). Assuming that the tumor is hemiellipsoid, its volume (Tv) is calculated as ***(14)***:

$$Tv = L \times W \times H \times 0.5236$$

   In case the intraperitoneal or subcutaneous tumor tissue cannot be removed by dissection, because it is too small or because of diffuse infiltration into the surrounding normal tissues, or in situations were the malignant tissue cannot be macroscopically dissociated from normal tissue, sections including all tissues suspected of being malignant must be evaluated microscopically as described below.
2. Estimation of total tumor burden of various organs: In cases of small or widespread, multiple metastases, it may not be possible to remove and weigh the tumor. Surgically remove the organ(s) containing the tumor or metastases and prepare sections for morphometric evaluation by light microscopy. The principle is basically the same for all types of tumors located in most organs. Below, we describe how the total volume of, e.g., tumor in the lungs can be estimated.
3. Cut cryostat sections from all lobes of the lungs in such a way that one section is sampled for, e.g., each 0.5 mm. The thickness of the sections is not critical but should be the same within each experiment and should not exceed 20 µm. As soon as tissue is identified in the section, sampling can commence. If the lungs are fixed and cut *in toto*, approx 30 sections will be sampled for each set of lungs.
4. Except for melanomas, which are easily recognized as black tissues owing to their content of melanin, stain the sections with hematoxylin or any other staining method that facilitates identification of the malignant tissue.

5. Measure the area of the tumor per section by viewing the tissue through a grid. Count every point in the grid covering tumor tissue. Because the total area of the tumor measured (A_t) divided by the total area of all the sections evaluated (A_s) is equal to the total volume of the tumor (V_t) divided by the volume of all the sections (V_s), the total volume of the tumor is:

$$V_t = (A_t/A_s) \times V_s$$

Because $A_s = n \times Y$ and $V_s = n \times d \times Y$, where n is the number of sections sampled, d is the distance between each section sampled, and Y is the area of the section, then:

$$V_t = (A_t/(n \times Y)) \times (n \times d \times Y) = A_t \times (d \times n \times Y/\, n \times Y) = d \times A_t$$

Knowing the area Q (in mm^2) represented by each grid-point, the tumor volume (in mm^3) is calculated as:

$$V_t = Q \times N \times d$$

where N is the total number of grid-points scored per each set of lungs (*see* **Note 8**).

*3.7.3. Number of Metastases on Organ Surfaces (see **Note 9**)*

1. Counting of melanotic metastases is fairly easy because of their black color, i.e., they are easily distinguishable from most normal tissues. Remove tumor-bearing organs and fix them in 4% (v/v) formaldehyde. Count the metastases under 2× or 4× power of magnification. Organs should be removed before the tumor nodules are so large that they become confluent, obscuring counting. Following injection of 1×10^6 B16 melanoma cells, lung metastases usually become confluent on d 14–16.
2. When counting nonpigmented tumors, the contrast between the malignant and the normal tissues has to be enhanced. For counting nodules on the lung surface, install 0.5 mL India ink into the trachea of collapsed lungs (i.e., lungs left *in situ*, but with opened thorax). Immediately after installation, remove the lungs and fix them in 4% formaldehyde. The tumor nodules appear as whitish dots on a black background.
3. When counting tumor nodules on the surface of the liver, inject 0.5 mL India ink intravenously into nembutal-anesthetized animals approx 5 min before they are killed. The tumor nodules appear as whitish dots on a black background.
4. Alternatively, remove the organ and place it in Bouin's solution for 24 h or longer. In most cases, the tumors will bleach more than the normal tissues, i.e., the tumor will appear whitish on a yellowish background (*see* **Note 10**).

4. Notes

1. During injection of tumor cells and A-NK cells, it is important to avoid sedimentation of the cells in the syringe. Therefore, the syringe should be filled with cells for only one animal at a time. In our hands, injection of 2×10^5 tumor cells results in the generation of a total of 10–50 metastases on the surface of the lungs. The metastatic lesions are easily recognized (as black dots on a pink background)

owing to their content of melanin. Injection of 1×10^6 cells generates more than 200–400 pulmonary metastases per lung. Injection of more cells is difficult and often leads to fatalities, probably owing to circulatory disturbances. The more cells are to be injected, the bigger the volume. When injecting 1×10^6 cells, we use a concentration of 2.5×10^6/mL (i.e., 400 µL per animal). The tumor nodules can be seen on the lung surface from d 3 by the aid of a dissection microscope. From d 7, the lesions are clearly macroscopically visible. Following injection of 1×10^6 cells, the animals become distressed by the tumor burden from approx d 17–19, which suggests that the experiment should be finished before that time.

2. A-NK cells can be delivered systemically or locoregionally. Intravenous injection is the easiest way to introduce these cells into the bloodstream. However, A-NK cells and other IL-2-activated effector cells circulate poorly, probably because of up-regulated expression of adhesion molecules and their rigidity ***(15)***, and it is often preferable to introduce them locoregionally, i.e., close to the tumor or directly into the tumor-bearing organ. For example, intrasplenic delivery of human A-NK cells in nude mice facilitates their rapid access to established liver metastases.
3. Injection of larger numbers of cells often results in fatalities, probably owing to circulatory distress.
4. To administer ethyl ether, use a spark-free fume hood. Left ventricular injections have the greatest chance of success if performed under ether anesthesia, probably owing to the release of catecholamines induced by the ether (ether tends to increase heart rate and blood pressure in contrast to most other anesthetics).
5. As an alternative to intraportal injection of tumor cells/A-NK cells, cells may be injected by the intrasplenic route, as described in **Subheading 3.5.1.**
6. Adoptive transfer of A-NK cells is always accompanied by systemic and/or locoregional delivery of IL-2. This practice is based on evidence that A-NK cells are dependent on IL-2 for their survival, tumor localization, and antitumor activities. It is important to ensure that IL-2 is available for A-NK cells at all times, from harvest until termination of the experiment. While performing in vivo work, it is necessary to remember that administration of exogenous IL-2 will induce activation of endogenous effector cells and create potential difficulties in interpretation of therapeutic results. In the xenograft nude mouse model this is avoided by twice weekly intraperitoneal injections of anti-asialo GM1 antibody (0.2 mg/mouse) during the period of therapy. This will eliminate endogenous (murine) NK cells without harming the adoptively transferred human A-NK cells.
7. IL-2 given at the dose of 150,000–300,000 IU every 4 h is usually well tolerated by the animals if given for 2–3 d. If higher doses are given or if the treatment is maintained for longer periods of time, serious side effects (such as capillary leak syndrome) might develop. At the first sign of discomfort (loss of activity, lowered food and water consumption, weight loss, respiratory distress), the IL-2 treatment should be stopped.
8. When estimating tumor burdens, the number of points in the grid and the number of sections that should be examined per organ depends on the size, number, and

distribution of the tumors. As a rule of thumb, the density of the grid should be high enough to ensure that the number of points scored per organ is at least 100.

9. As an alternative to the very laborious estimation of total tumor burden, numbers of metastases visible on organ surfaces can be counted. This method, however, gives only a partial picture of the tumor burden of the various organs and will not precisely reflect the real situation, particularly in cases where the treatment used is either highly efficient or inefficient against surface tumors.
10. When counting tumors on organ surfaces, more information is obtained if the sizes (diameters) of the individual lesions are also measured. Depending on the three-dimensional shape of the surface lesions, data can be calculated as "total area" (discoid or flat tumors) or "total volume" (spheric tumors) of surface tumors.

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Methods for In Vivo Analyses of Natural Killer (NK) Cells

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1. Introduction

Natural killer (NK) cells are a distinct compartment of lymphoid cells that have been shown to function as an effector cell population against virus infections, to provide a means of surveillance against tumor metastases, and, in some cases, to serve as an effector cell population for immunotherapy against established tumors. Since the initial observations suggesting that NK cells were representative of an "activity" expressed by some hematopoietic cells, as opposed to a defineable lineage of cells, an extraordinary increase in our capacity to identify, quantitate, and characterize these cells has occurred. This has been particularly apparent in the past several years, as numerous reagents for defining NK cell heterogeneity and function have been developed. There has also been a dramatic increase in the technology available for application of those reagents. NK cells have commonly been defined as a population of large granular lymphocytes (LGLs) that have a discrete phenotype, frequently defined as $CD3^-/TCR^-/CD16^+/CD56^+$, and that are capable of lysing certain tumor cells and virus-infected cells in the absence of any apparent, prior sensitization. NK cells also by definition lack expression of the TCR/CD3 complex and can be stimulated to lyse target cells utilizing surface receptors that do not detect specific antigen in the context of major histocompatibility complex (MHC) class I determinants. These findings initially led to their designation as mediators of non-MHC restricted cytotoxicity. It is now clear that MHC determinants are in fact recognized by NK cell surface receptors. Further, it has been determined that most such receptor–MHC interactions result in a turnoff of NK cells, although there are some exceptions in which receptor–MHC interactions activate NK cell lytic function (for recent reviews *see* **refs. *1*** and ***2***).

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
Edited by: K. S. Campbell and M. Colonna © Humana Press Inc., Totowa, NJ

In the same way that the functional definition of NK cells has required modification to accommodate new developments in our understanding of how these cells mediate lysis of target cells, it is also necessary to expand how NK cells are phenotypically defined as a result of the identification of additional cell surface molecules characteristic of these cells. At present, NK cells can be reasonably well defined phenotypically as $CD3^-/TCR^-/CD16^+/CD56^+/CD94^+/CD122^+/CD158^+/CD161^+$, with the additional consideration that some (e.g., CD158 and CD161) are actually representative of multigene families and that none, one, or multiple members may be expressed on a given NK cell. There are also some additional NK cell associated markers for which there are not currently CD assignments (e.g., Ly49s), as well as some that have CD assignments but are not frequently used in the phenotypic characterization of NK cells because of a wide cellular distribution (e.g., CD11a/CD18) ***(1,2)***. As is the case with other hematopoietic cells, there is not a uniquely discrete phenotypic marker that can identify a cell solely as an NK cell in humans, mice, or rats. It is also apparent that not all NK cells necessarily express all of the markers listed above. Given this situation, it is best to use multiple parameters for identifying a cell as belonging to the NK lineage. Because there is a close kinship of NK cells and T cells, approaches to NK cell identification should include a combination of defining expression of NK cell associated markers and a lack of expression of T cell markers. For instance, combining the determination of expression of CD16 and CD94, and lack of expression of CD3, would definitively indicate a cell as belonging to the NK lineage.

The functions of NK cells have also been more thoroughly documented in the past several years. Their cytolytic function involves the storage and exocytosis of granule associated mediators of necrotic cell death, such as cytolysin and granzymes. NK cells are also known to induce apoptotic death in target cells via Fas–FasL interactions. Further, their functions include production and secretion of numerous cytokines (e.g., interferon [IFN] or tumor necrosis factor [TNF]) (reviewed in **ref. *3***). These aspects of NK cell biology therefore represent a means of characterizing NK cells, and particularly for evaluation of the functional status of these cells.

During the past 10 yr, there have been numerous advances in the in vitro culture of heterogeneous populations of NK cells ***(4,5)*** and in the cloning of human ***(6,7)***, rat ***(8)***, and mouse ***(9,10)*** NK cells. Techniques for purifying populations of activated NK cells in numbers sufficient for adoptive transfer experiments have been developed and implemented in numerous laboratories ***(4,5,11–13)***. In fact, the development of reproducible means for identifying, quantifying, and characterizing such cells following transfer has proven to be more challenging than producing cells for these experiments. However, there are now a number of options available for investigating the trafficking and

localization of adoptively transferred NK cells that make use of radiolabels, membrane-associated fluorescent dyes, or congenic markers.

As there is clearly an increased level of knowledge regarding phenotypic characterization of NK cells, a far greater flexibility in approaching the identification, quantitation, and characterization of these cells in vivo is available. This has proved important, as some cell surface markers do not lend themselves to easy identification in fixed or frozen tissue samples (e.g., mouse NK1.1/NKR-P1C/Ly-55). However, other sets of reagents that work well in such samples are generally available that can be substituted. Advances in imaging technology available, such as multiparamter confocal microscopy, provide the necessary opportunities to utilize combinations of specific antibodies for definitive identification of cells using combinations of specific markers. Equally as important, this technology provides a means of determining relative levels of intensity of staining of a given set of markers. This can be important in phenotyping cells as some markers are differentially expressed on NK cells vs other cells (e.g., $CD56^{bright}$ vs $CD56^{dim}$ NK cells); and up- or down-regulation of markers (e.g., CD161) on NK cells can serve as an indicator of the state of activation of the cell ***(14)***. It is also possible to achieve multiparameter analyses using other imaging systems as well.

In this chapter, we discuss currently available approaches for identifying, quantitating, and characterizing NK cells in vivo, and particularly approaches for use in models utilizing established tumors. Further, we present approaches that may be useful in the examination of the presence, in solid tissue organs and tumors, of both endogenous populations of NK cells, and exogenous, adoptively transferred populations of NK cells.

2. Materials

1. Paraformaldehyde: Prepare 2% and 4% solutions in water. Purchased from Sigma.
2. 30% Sucrose solution: Prepare 30% solution in water. Purchased from Sigma.
3. *n*-Hexane: Obtained from Sigma. Prechill at –70°C.
4. Cryostat: The cryostat must have the capacity to cut 6–10 µm tissue slices. We use a Minotom cryostat (Damon/IEC, Needham Heights, MA).
5. Microscope slides: Polylysine-coated or other suitable slides (e.g., Superfrost; Fisher; Pittsburgh, PA) slides.
6. Buffer A: Phosphate-buffered saline (PBS; Gibco, Grand Island, NY) containing 0.5% BSA and 0.15% glycine, pH 7.4.
7. Airxol coverslip mounting solution (Air Products and Chemicals).
8. Proteinase K solution: Prepare a solution of 10 mg/mL in PBS (Gibco). Proteinase K can be purchased from Sigma.
9. TEA solutions: Dilute 10.72 mL of triethanolamine (TEA; 98%, Sigma) into 800 mL of water. Also prepare acetic anhydride/TEA solution by adding 625 mL of acetic anhydride (Sigma) to 200 mL of TEA solution.

10. RNA wash solution 1: 50% formamide/2X saline sodium citrate (SSC) (300 m*M* NaCl, 30 m*M* sodium citrate, pH 7.0).
11. NTB2 photographic emulsion (Eastman Kodak, Rochester, NY).
12. Genius buffers: Buffers I, II, and III (Roche Molecular Biochemicals, Indianapolis, IN).
13. NBT/BCIP solution: Prepare 0.5 g of nitroblue tretrazolium chloride (NBT) in 10 mL 70% dimethylformamide (stock A); Prepare 0.5 mg 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 10 mL of 100% dimethylformamide (stock B); Prepare 100 m*M* NaCl, 5 m*M* $MgCl_2$, 100 m*M* Tris-HCl, pH 9.5 (stock C); thoroughly mix 66 µL of stock A into 10 mL of stock C, then add 33 µL of stock B. Use within 30 min of preparation.
14. Conventional darkfield microscope (e.g., Nikon FXA).
15. Fluorescence microscope: Fluorescence microscope (e.g., Provis, Olympia; Valencia, PA) should have 40× or 60×, high numerical aperture, color corrected, oil immersion objective lenses and a high sensitivity, integrating three chip color camera (700 × 600 pixels) (e.g., Sony), and Corecco frame grabber board. We use the Optimas software package (Bioscan, Seattle, WA). For fluorescence visualization of TRITC, one must use filter combinations for rhodamine (e.g., BP 546, FT 580, and LP 590).
16. Confocal microscope: A confocal laser scanning microscope (e.g., Molecular Dynamics, Sunnyvale, CA or Leica, Deerfield, IL) with 60× and capacity for two-color image collection (514 nm laser, 535 nm primary beam splitter, 564 nm secondary beam splitter, 540 df30 nm final barrier to collect the fluorescein isothiocyanate [FITC] image and 590 nm final barrier to collect the Cy3 image). The confocal microscope that we use is able to scan at incremental pixel resolutions from 256 × 256 pixels to 1024 × 1024 pixels and incorporates the ImageSpace morphometric analysis engine (Molecular Dynamics).
17. Hank's balanced salt solution (HBSS; Gibco).
18. Hypotonic ammonium chloride buffer: Prepare 8.32 g of ammonium chloride, 0.84 g of Na HCO_3, and 43.2 mg of EDTA in 1000 mL sterile water.
19. Hypertonic citrate buffer: Prepare 7.014 g of sodium chloride and 4.412 g of sodium citrate in 1000 mL of sterile water.
20. Recombinant interleukin-2 (IL-2) (Cetus Oncology Corporation, Emeryville, CA).
21. 0.2% EDTA in PBS (filter sterilize).
22. Ficoll-Hypaque (e.g., Pharmacia, Piscataway, NJ).
23. Secondary monoclonal antibodies (MAb) coated magnetic beads (e.g., Advanced Magnetics; Boston, MA).
24. Culture Medium: Complete medium (CM) for the production of A-NK cells commonly consists of RPMI 1640 medium (Gibco) supplemented with 5–10% fetal bovine serum (FBS), 5×10^{-5} *M* 2-mercaptoethanol, 2 m*M* glutamine, 0.8 g/L streptomycin and 1.6×10^5 U/L penicillin, and 10 mL/L of 100× nonessential amino acids (all supplements from Gibco).
25. Gamma counter (e.g., LKB-Wallac 1280, Ultrogamma, adjusted to detect ^{125}I [20–48 keV]).

26. Preparation of tetramethylrhodamine isothiocyanate (TRITC): A stock solution of TRITC is prepared by adding 0.5 mL of acetone to approximately 0.5 g of TRITC (e.g., isomer R, T2018 from Sigma Fine Chemicals, St. Louis, MO). Five minutes later, PBS (Gibco) is added to achieve a final concentration of 30 μg/mL of TRITC. This stock solution of TRITC can be stored at –70°C in 1-mL aliquots for approx 1 yr. After thawing, TRITC should be used immediately, and should not be refrozen. Be aware that the toxicity, as well as the fluorescence intensity, of TRITC might differ from batch to batch. Each stock should therefore be titrated to find the highest possible concentration that will not harm or damage the NK cells with respect to function, e.g., IL-2-driven proliferation and cytolytic capacity.
27. Radionuclides: (^{125}I)-iododeoxyuridine (^{125}I-UdR) with a specific activity of 5 Ci/mg, is commercially available from numerous sources (e.g., New England Nuclear [NEN], Boston, MA). ^{35}S-labeled UTP (5 Ci/mg) is also available from NEN.
28. Monoclonal antibodies: Numerous MAbs recognizing NK cell associated markers have been reported. Any number of these are suitable for determination of the identity and purity of ex vivo cultured populations of NK cells. However, only a more limited number of these antibodies has proven suitable for use in the *in situ* identification of NK cells. Our experience is more related to the detection of rat NK cells, but mouse and human NK cells can also be detected in frozen or fixed sections of solid organs or tumors, as well. Rat NK cells are easily detected with anti-CD161A (e.g., MAb 3.2.3; Bioproducts for Science, Indianapolis, IN) or with anti-CD8a (e.g., MAb OX8; Bioproducts for Science) . However, in the case of anti-CD8a, other markers must also be used in conjunction, as CD8a is also expressed on T cells (e.g., CD8a$^+$/CD3$^-$ cells would be characterized as NK cells). In the mouse, the most commonly used marker for NK cells is NK1.1 (CD161C, NKR-P1C, Ly-55; MAb PK136; PharMingen, San Diego, CA). Although highly useful in many applications, this antibody has not proven suitable, in our hands, for detection of NK cells *in situ* in either frozen or fixed sections. It is, however, possible to use MAbs 2B4 or 3A4 (PharMingen; ***15,16***) in frozen sections (unpublished observations). For mouse and rat NK cells, it is also possible to use polyclonal anti-AsialoGM1 (AsGM1; Wako Chemicals USA,, Richmond, VA) in conjunction with T cell specific markers to identify NK cells *in situ* (e.g., AsGM1$^+$/CD3$^-$). In humans, a wide selection of MAb to NK cell associated markers are currently available. In our experience, anti-CD16 has proven unsuitable for use in frozen or fixed sections of solid organs. However, anti-CD56 (Becton Dickinson, San Jose, CA) can be used, with the exception of identification of NK cells in brain, as CD56 on NK cells is the 140-kDa isoform of neural cell adhesion molecule (NCAM) ***(17)***. It remains to be determined whether anti-CD94, -CD150, -CD158, or -CD161 specific MAbs will be useful for *in situ* analyses. Given the success in recent years in the generation of MAbs recognizing NK cell associated markers in mouse, rat, human, and other species as diverse as teleost fish and pigs, it is highly likely that a great number of the reagents will prove useful for the *in situ* detection of NK cells, but this awaits additional analyses. Alkaline phosphatase conjugated anti-digoxigenin antibodies are obtained from Roche.

3. Methods

3.1. Preparing Tissues to Analyze Endogenous Populations of NK Cells

In this section, we present methods for the identification, quantitation, and characterization of endogenous NK cells which have not been isolated, expanded ex vivo, and adoptively transferred. This does not present opportunities for discrete labeling of purified cells using radionuclides or free fluorescent dyes, but generally requires approaches involving the use of labeled antibodies or cDNA probes. However, a large number of reagents and technical applications are available that make this both easily accomplishable and experimentally meaningful.

3.1.1. Preparation of Tissue Sections for Light Microscopy

1. Organs and/or established tumors are fixed by perfusion with 4% paraformaldehyde introduced into the vascular system via a catheter inserted into major vessels (e.g., carotid, portal, etc.) or intraventricularly (e.g., right or left ventricle for perfusion of the pulmonary or bronchial arteries, respectively).
2. Following surgical removal, organs and/or established tumors are then fixed by immersion 4% paraformaldehyde for 4–18 h. Following fixation, organs are then placed in a 30% sucrose solution for 18–24 h.
3. At this time, the fixed organs are snap frozen in n-Hexane at –70°C, followed by cryostat sectioning at a thickness of 6–10 μm on a Minotom cryostat and mounted on poly-lysine coated or other suitable slides.

3.1.2. Immunocytochemical Labeling

1. Sections (6–10 μm sections on slides) are washed 3× in buffer A.
2. This is followed by a 30-min incubation with purified immunoglobulin or serum corresponding to the species of derivation of specific antibodies (50 μg/mL) at 25°C and three additional washes with buffer A. All the preceding steps are designed to ensure minimal nonspecific binding by the antibodies used for experimental purposes.
3. For direct immunofluoresence studies, directly labeled specific antibody sections are incubated for 30–60 min with a predetermined concentration (generally 1–5 μg/mL) of specific antibody(ies) followed by three washes with buffer A. For indirect immunofluorescence studies, sections are incubated for 30–60 min with primary antibody followed by three washes in buffer A, and a 30–60 min incubation with fluorochrome-labeled secondary antibody (1–2 μg/mL).
4. The sections are then washed 6× (5 min/wash) in buffer A, mounted in airxol, and coverslipped.
5. Slips should then be analyzed within 24–48 h of staining (*see* **Note 1**). When multiple specific antibodies are being used, directly labeled primary antibodies can be applied mixed. When multiple specific antibodies are being used in indirect immunofluorescence studies, if the primary antibodies are of different

isotypes, secondary isotype-specific antibodies may also be mixed prior to use. At present, fluorescence analyses can be accomplished with as many as four or five separate fluorochromes providing a breadth of opportunities for multiparameter analyses. In some instances, avidin-biotin labeling is also useful, and biotinylated antibodies are widely available, as is streptavidin conjugated with numerous fluorochromes (e.g., streptavidin-Cy3). Similarly, peroxidase-labeled antibodies may by used in some cases, but this generally precludes multiparameter analyses and also limits the opportunity to determine relative levels of staining of a given marker.

3.1.3. In Situ *Hybridization*

Numerous reports have supported the use of *in situ* hybridization both for the identification and quantitation of NK cells in tissues, and for the characterization of their function (e.g., production of TNF-α) ***(18–21)***.

1. Appropriate sequences for oligonucleotides for use as riboprobes can be accessed in related publications (e.g., ***17–20***). Both antisense and sense riboprobes can be made using digoxigenin-labeled or radiolabeled nucleotides.
2. Purified, linearized cDNA template is incubated in the presence of digoxigenin or ^{35}S-labeled UTP, unlabeled CTP, ATP, and GTP and the relevant polymerase for 2 h at 37°C.
3. The labeled RNA is precipitated in cold ethanol, dried, and resuspended in RNase-free water.
4. Hybridization is performed essentially as follows. Frozen sections are cut on a cryostat, fixed in 2% paraformaldehyde in PBS (10 min), permeabilized in 2% paraformaldehyde in PBS containing 0.1% Triton X-100 (10 min), washed twice in PBS, digested with Proteinase K (10 mg/mL, 5 min), washed in PBS containing 1% glycine, and acetylated by immersion in TEA solution for 5 min, followed by two 5-min immersions in acetic anhydride/TEA solution.
5. Sections are then dehydrated through a gradation of ethanol concentrations (30, 50, 70, 85, 95, 100, 100% in water) for 10–15 s each.
6. Following dehydration, the sections are hybridized overnight at 42°C in digoxigenin-labeled, specific riboprobe (controls are the sense strand of the probe and also include a "no probe" control).
7. Sections are then washed twice in 50% formamide/2× SSC (RNA wash solution 1) for 15 min at 50°C and nonspecific probe binding digested in RNAse for 30 min at 37°C.
8. Following further washes in RNA wash solution 1 and in 2× SSC, sections labeled with ^{35}S are dehydrated, dipped in NTB-2 photographic emulsion, dried, and stored prior to development.
9. Sections labeled with digoxygenin are washed in Genius buffer I (Roche), Genius buffer II, and incubated in alkaline phosphatase conjugated antibodies directed against digoxigenin for 1 h. Subsequently sections are washed in Genius buffer III, developed in NBT/BCIP solution overnight, washed in PBS, dehydrated, and mounted in permount. Observation can then be with undertaken with a conventional darkfield microscope.

3.2. Image Collection

Although others are available, the optical endpoints discussed in this section include light microscopic methods such as multicolor fluorescence microscopy, color image collection from brightfield microscopy, darkfield microscopic methods such as *in situ* hybridization using ^{35}S; and laser confocal microscopy.

3.2.1. Multicolor Fluorescence and Brightfield Light Microscopic Analyses

To perform these analyses, appropriately labeled sections are placed on a calibrated microscope stage, brought to focus, and using random number tables to generate stage axis positions, at least 10 random fields from each of five sections from each sample are located. Image fields are then collected directly, in random orientation at the microscope using 40× or 60×, high numerical aperture, color corrected, oil immersion objective lenses and a high sensitivity, integrating three chip color camera (700 × 600 pixels) (e.g., Sony), and Corecco frame grabber board. For fluorescent images, either multiple single-color cubes (blue, green, red, far red), in perfect registration, or multicolor cubes (green-red, blue-green-red) should be used. For peroxidase- or digoxigenin-labeled material, color images should be collected in brightfield.

Positive structures in a field can be selected by color and brightness threshold. The labeled components are then extracted and rendered to gray scale. If necessary, morphologic constraints such as cell shape or size can be placed on the extracted image, and the size and/or number of positively labelling structures quantified. If, for instance, a tumor profile is present within the image field, it will be delimited and the number of structures (e.g., labeled NK cells) per μm^2 calculated, relative to the number in the normal tissue. An important facet of such studies, apart from quantitation of structures within an organ or tumor relative to other tissues, is the potential for cell clustering and/or coclustering with vascular components. An algorithm can be used to determine clustering by measuring proximity of labeled structures to each other and generating a Q value based on the randomness of proximity. To examine coclustering, a value of cluster probability can be obtained for each component and correlated. If the distribution of the two markers is random, or if specific pairing is seen, this can be determined by software packages (e.g., Optimas) often used for such analyses.

Fluorescently labeled cells may be clearly defined within tissue sections; however, the use of immunoperoxidase does not allow such a clearly defined cellular demarcation. Although the structures of interest may be easily detected, discrete demarcation of label may on occasion prove difficult, owing to the diffusibility of colorimetric reaction product. Initially, it may be worthwhile to quantify structures as described previously, by delimiting an area of colored

reaction deposit. Thus, the area occupied by the NK cells can be expressed in relation to the total area of tumor or native tissue within the section area; or an area of labeled tumor (e.g., lacZ) can be expressed as a fraction of the total section area. These computer-aided methods allow rapid quantitation of large numbers of samples. However, if these methods prove insufficient owing to the potentially diffuse nature of the label, it is possible to employ classical manual and hence labor intensive, point counting stereologic approaches to quantify label structures within sectioned material.

3.2.2. Quantitative Autoradiography

For *in situ* hybridization analyses, sections labeled for the mRNA of interest are viewed optically in darkfield using "edge illuminated" darkfield microscopy (this allows the use of higher magnification objectives, with a more even illumination). Following labeling with radioactive probe, slides are dipped in photographic emulsion, and are developed after 4–8 d. "Hot spots" develop as dark spots/grains that are then collected digitally by computer (e.g., Sony 760 3 chip microscope (Tokyo, Japan); CCP/Optimas image processing software (Seattle, WA); CORECO frame grabbing board (Montreal, Canada)). Positively labeled cells may be imaged as clusters of silver grains on the autoradiograph. Thus, the number of positive cells can be clearly defined by a minimum cluster size, which allows automatic quantitation, with complete exclusion of background. Individually, the steps are: (1) image collection; (2) definition of minimum positive cluster size (this may be done on control specimens, or on the actual specimen being analyzed); (3) separation of clusters; (4) automatic rejection of all label clusters less than the minimum cluster size; and (5) quantification of the number of labeled cells. The shape and size of grains or clusters of grains in the autoradiograph can be subjectively defined with regard to representative size and shape of cells from the visual image, which then allows a computer generated tabulation of number of cells in a given field. One must be careful in setting the parameters of interpretation of what size/shape/color intensity should be used to define an NK cell.

3.2.3. Confocal Microscopy

Laser confocal microscopy (CLSM) offers several features not available when using traditional light microscopic, immunohistochemical techniques. Most importantly, the microfluorometric measurement of relative protein concentrations expressed by cells is possible. The confocal microscope optically sections cells, and hence offers the ability to collect images with a consistent vertical or *Z* section thickness. In our experience, consistent results are achieved using a section thickness of 0.5 μm, with a 60× objective lens. Using conventional fluorescence microscopy, the recovered epifluorescent signal comes

from the entire depth of the section, and as such, variations in section thickness or variations in cell size will cause significant variation in the recovered signal. Furthermore, it is not possible to ascertain whether the entire cell (approx 7 μm diameter) or only a fraction of the cell is contained within the sectioned profile. This will dramatically affect the detected signal, which will be additive in the Z plane by conventional microscopy. Also, the brightness of mercury arc lamps fluctuates, which will affect measurement of signal intensity. In CLSM, the stability of the laser illumination ensures consistent fluorochrome excitation, and hence sampling that will provide consistency in measurement of signal intensity.

Sections are best labeled with primary antibodies directly conjugated to fluorochromes (e.g., FITC or Cy3). To ensure maximal X-Y spatial resolution, sections should be scanned at 1024 × 1024 pixels, 60× objective lens, 0.2 μm pixel size, using two-color image collection (514 nm laser, 535 nm primary beam splitter, 564 nm secondary beam splitter, 540 df30 nm final barrier to collect the FITC image and 590 nm final barrier to collect the Cy3 image). The confocal microscope is able to scan at incremental pixel resolutions from 256 × 256 pixels to 1024 × 1024 pixels, with an optimal pixel resolution of 0.2 μm. Image planes throughout the depth of the specimen can then be collected at 0.5 mm intervals and the midplane section extracted. Prescans of sections labeled with control antibodies should be determined at background gray level which will allow a "0" value for gray scale to be assigned. Labeled cells are represented as clustered pixels with gray levels above background. Spatial localization of the labeled cells within the section may be determined automatically using the ImageSpace morphometric analysis engine which is central to the use of the CLSM. This will allow quantification of the spatial proximity of labeled cells. To quantify relative expression of markers on individual cells, labeling intensities for different markers may be expressed graphically as Fish-net plots ***(22)***, or quantitatively using automatic image processing. In this latter method, the fluorescence intensity of all pixels in individual cellular profiles collected by the confocal microscope image is reduced to a single mean gray scale value for each cell. This yields a quantitative estimate of single color label for all labeled cells within the scanned image. The same algorithm may be used for each of two colors in the same section, and single values for each color within each cell derived, combined, and analyzed using "colocalization" algorithms that tabulate labeling in a fashion similar to that seen in a two-dimensional flow cytometry plot.

3.3. Preparation of NK Cells for Adoptive Transfer

3.3.1. Mouse

Murine A-NK cells are prepared from splenocytes as previously described ***(4,23,24)***.

1. Mouse splenocytes are harvested by disaggregation of spleens into a single-cell suspension in isotonic medium such as HBSS.
2. Erythrocytes are removed by lysis using hypotonic ammonium chloride buffer (5 mL) treatment of pelleted cells for 5 min, at which time isotonicity is restored with hypertonic citrate buffer (5 mL).
3. Cells are then washed twice with HBSS, and resuspended in CM at a final concentration of 2×10^6 cells/mL.
4. The cells are then transferred in 50-mL aliquots into T150 flasks (Falcon Plastics, Oxnard, CA) and cultured at 37ϒC in air with 5% CO_2 tension. To generate purified cultures of NK cells, recombinant IL-2 (500–1000 U/mL) is added to each flask.
5. After 2–3 d of incubation, nonadherent cells are removed and the flasks are gently washed with prewarmed (37°C) CM to remove cells not firmly attached to the plastic. During this initial culture period NK cells firmly attach to the plastic surface, and they are then purified and expanded by eliminating nonadherent cells with gentle aggitation of flasks, and removal of nonadherent cells in the supernatant with two rounds of washing with warm CM. Fresh CM (50 mL) supplemented with rIL-2, is added and the cells are cultured for an additional 2–3 d.
6. After a total of 5–6 d of culture, cells are harvested after a short treatment with 0.02% EDTA in PBS, and washed twice with CM before use.

3.3.2. Rat

1. Splenocytes are harvested by disruption of spleens into a single cell suspension in isotonic medium.
2. Cells are then layered onto density gradient centrifugation medium with a specific gravity of 1.077 (e.g., Ficoll-Hypaque) and centrifuged at 400*g* for 15–30 min.
3. Lymphocytes are then harvested from the supernatant/Ficoll interface, washed 3× in CM.
4. Before culture, rat splenocytes are depleted of monocytes/macrophages by incubation on nylon wool columns for 60 min at 37°C (*see* **Note 2**). The non-adherent cells are washed out of the column with warm CM.
5. The cells are then counted and adjusted to 2×10^6/mL in CM and transferrred in 50-mL aliquots into T150 flasks and cultured at 37°C in air with 5% CO_2 tension.
6. To generate purified cultures of NK cells, recombinant rIL-2 (500–1000 U/mL) is added to each flask and the cells are cultured for 48 h. During this initial culture period NK cells firmly attach to the plastic surface, and they are then purified and expanded by eliminating nonadherent lymphoid cells with gentle agitation of flasks and removal of nonadherent cells in the supernatant with two rounds of washing with warm CM. Expansion of the adherent population of NK cells can then be induced during 48–72 additional hours of culture by addition of the conditioned medium from the initial period of culture or by addition of fresh CM containing rIL-2.

3.3.3. Human

1. Lymphocytes are harvested from heparinized peripheral blood (or leukopacks) by density gradient centrifugation as described previously.

2. Lymphocytes are then harvested from the supernatant/Ficoll interface, washed 3× in CM.
3. It is desirable to carry out an initial immunoselection step for depletion of other lineages of lymphocytes in which cells are coated with anti-CD3, -CD5, -CD19, and -CD14 and the then incubated with secondary MAb-coated magnetic beads as described ***(11,12,25)***. Lymphoid cells purified in this fashion generally are contaminated only by 2–5% of T and B cells or monocytes ***(11,12,25)***.
4. The highly purified NK cells are then counted, adjusted to 2×10^6/mL and incubated 6–14 d in CM with 22 n*M* rIL-2 on feeder layers of irradiated Concanavalin A-activated (2.5–5.0 µg/mL) allogeneic lymphocytes. Effector cells generated in this fashion are generally ≥98% $CD56^+/TCR^-$.

3.4. In Vivo Analyses of Exogenous, Adoptively Transferred Populations of NK Cells

The determination of the tissue distribution of exogenous NK cell populations is of special interest in studies of adoptive immunotherapy, where ex vivo activated NK cells are reinoculated into normal and cancer-bearing or infected hosts. The methods described in the following sections have been used successfully in the detection and quantification of the adoptively transferred cells in normal as well as neoplastic tissues ***(26–31)***. These approaches should also be applicable for the investigation of NK cell trafficking to sites of inflammation as well.

To identify the adoptively transferred cells in vivo, these cells must carry some kind of marker that allows for their detection and makes discrimination between exogenous and endogenous effector cells possible. The optimal cell label for studying cell traffic should fulfill each of the following criteria. First, the label should be nontoxic. Second, it should label all cells in a population to an equal extent. Third, as long as the cell is viable the label should remain tightly bound to it. Fourth, once the cell dies, the label should immediately dissociate from the cell to be excreted without reutilization by host cells. Finally, an assessment of the label in various tissues should be precise and reproducible. Unfortunately, none of the existing labels fulfills all of these criteria. Consequently, no matter which label is chosen, one or several of the requirements mentioned above will not be met. However, by carefully taking the strengths and weaknesses of a given label into consideration, and by employing two or more complementary labels, it is possible to obtain reasonably precise estimates of the in vivo traffic of adoptively transferred NK cells. Many different markers for the detection of cells in tissues are available such as radionuclides, fluorochromes, and various genetic markers (*see* **Note 3**). In this section, we will focus on the use of ^{125}I-UdR for determination of the distribution of populations of cells between organs and the fluorescent dye

rhodamine (TRITC) for the identification of individual NK cells in specified histological locations.

3.4.1. Labeling and Detection of ^{125}I-UdR-Labeled NK Cells

The advantages of labeling NK cells with a radionuclide before injection are that labeling as well as detection are easily performed. Gamma scintillation counting of organs removed from the recipient quickly provides a reliable picture of the distribution of the transferred cells. The disadvantage is that information on the single cell level, i.e., information of the exact location of the NK cells relative to malignant lesions, is not obtained. However, such assessments are possible, but require laborious autoradiographic analyses.

1. Following activation with IL-2 for several days, NK cells proliferate vigorously and will therefore incorporate most nucleotides added to the culture medium into their DNA, including ^{125}I-UdR ***(28)***. IL-2-activated NK cells are denoted A-NK cells in the remainder of this chapter.
2. 2–4 × 10^7 A-NK cells, stimulated with IL-2 for 2–5 d are incubated (37°C, 5% CO_2) for 18–22 h in 50 mL of CM containing 1–2 µCi of ^{125}IUdR. The cells are washed twice and are resuspended in RPMI 1640 (Gibco) to the appropriate concentration. In our experience using a gamma counter, this procedure results in 30–50 cpm/10^3 cells.
3. The labeled A-NK cells are injected by the selected route (usually intravenously). The inoculum should not exceed 300 µL. As many as 4 × 10^7 A-NK cells can be injected per animal, but 2–5 million cells are sufficient for a reliable detection of cells even in small organs containing only few of the injected cells. If 2 million cells are injected, 1% of the injected dose will be equal to 20,000 cells. This number of cells will create at least 300 cpm which is normally more than 10-fold above the background level.
4. At the beginning, at the midpoint, and at the end of the experiment, triplicate samples of cells of the same number as the amount injected into the animals are collected. 1 mL of CM with 1000 U/mL IL-2 is added per million cells in the cell samples which are incubated at 37°C (5% CO_2) until the animals are killed.
5. At various time points after injection of the ^{125}I-UdR-radiolabeled A-NK cells, at least five animals from each experimental group are killed and the organs of interest are removed and placed in 70% ethanol. The ethanol (at least 25 mL per organ) is changed every day for 3 d. This procedure removes all radioactivity not associated with intact cells ***(32,33)***. The cell samples are also placed in 70% ethanol, which is removed once a day by centrifugation of the cells. The cells are resuspended in fresh ethanol 3×, as are the collected organs.
6. The organs and cell samples are thereafter counted in a gamma counter adjusted to record ^{125}I. Results are expressed as percentage recovery of injected radioactivity, calculated as:

$$\text{\% recovery of injected dose} = \frac{\text{Radioactivity from } ^{125}\text{I in an organ}}{\text{Average radioactivity in the collected cell samples}}$$

3.4.2. Labeling and Detection of A-NK Cells Labeled with the Fluorescent Marker TRITC

TRITC was introduced by Butcher and Weissman ***(34,35)*** for the labeling and in vivo tracking of various lymphocyte populations. Labeling is fast and simple and does not require that the cells to be labeled are proliferating, in contrast to labeling with ^{125}I-UdR. Although TRITC-labeling is poorly suited for the analysis of the distribution of populations of cells between organs (owing to the heavy workload associated with production and analysis of sections of multiple organs), this method is very useful for the exact detection of individual A-NK cells in specified histological locations, e.g., metastases within organs or organ biopsies.

1. 3–6 × 10^8 A-NK cells are incubated with 15 μg TRITC in 50 mL of RPMI 1640 for 30 min at 37°C. After labeling, cells are washed twice and resuspended in RPMI 1640 to appropriate concentrations.
2. The labeled A-NK cells are injected by the selected route (usually intravenously). The inoculum should not exceed 300 μL. As many as 4 × 10^7 A-NK cells can be injected per animal, but at least 5 million cells should be injected to ensure a reliable detection of cells at locations containing only few of the injected cells.
3. At various times after injection of the TRITC-labeled A-NK cells, at least three to five animals from each experimental group are killed and the organs of interest are removed and placed in 4% formalin (*see* **Note 4**). After 16–48 h later, organs are placed in 30% sucrose for an additional 16–24 h. The organs are thereafter placed in tubes with ornithine carbamoyltransferse (OCT) compound and frozen by submerging into –70°C hexane. After 5 min, the samples are ready for cutting. Alternatively, the samples are placed in airtight containers where they can be stored for years at –70° to –130°C.
4. For identification of the A-NK cells, 8 μm cryo-sections are cut from the organs of interest. The sections are coverslipped using an aqueous mounting medium. The sections are examined at 10–100× using a fluorescence microscope with filter combinations for rhodamine (e.g., BP 546, FT 580, and LP 590). The TRITC-labeled cells will appear as bright red dots on a dark red background (*see* **Note 5**).
5. Quantitation of tumor infiltrating NK cells: Once a tumor has been identified (*see* **Note 6**), the size (mm^2) of the tumor should be measured using calipers. The number (*N*) of red dots within the tumor is counted. At least 20 tumor lesions from at least 10 different sections, randomly sampled from each organ, should be analyzed. For each animal, the average number *N* of tumor-infiltrating A-NK cells/mm^2 tumor tissue is calculated as using the following formula (*see* **Note 7**):

$$N = \frac{\hat{}\approx n}{\hat{}\approx \text{tumor size}}$$

4. Notes

1. Some fluorescent labels such as FITC or TRITC are photoquenched, and the fluorescent signal lessens in a reasonably short period of time. All slides should be

stored until use at 4°C in a darkened chamber. Newer fluorochromes such as Cy3 or Cy5 offer the advantage of higher quantum yield and markedly reduced photoquench. An additional problem in storing stained slides is a loss of the fluorochrome for labeled cells into surrounding cells, thus confounding results obtained.

2. While the production of mouse A-NK cells does not require depletion of monocytes/macrophages, this is an important step in the production of rat A-NK cells ***(5)***. Before culture in IL-2, rat splenocytes are depleted of monocytes/macrophages by incubation on nylon wool columns for 60 min at 37°C. The nonadherent cells are washed out of the column with warm RPMI 1640.
3. The many different techniques for labeling of lymphoid cells for studies of their in vivo migration and tissue distribution have been extensively reviewed ***(36)***. It is important to realize that the migration pattern of adoptively transferred effector cells drawn by these markers is not always the same, leading to very different interpretations as to the migratory capacity of the injected effector cells. In **Table 1**, the most important advantages and disadvantages of the methods most widely used are summarized.

Table 1

4. Due to leakage of TRITC from the labeled cells, identification of cells in tissue sections must be performed within the first 24–48 h after labeling. After 48 h the intensity of the TRITC-labeled cells will be too low to ensure reliable identification.
5. To best observe TRITC-labeled A-NK cells in tissue sections, look at the section shortly after cutting and coverslipping. As time passes, the TRITC bleaches and the background autofluorescence tends to increase. Thus, sections should not be older than 16–24 h when analyzed. Note also that TRITC does not survive dehydration in alcohol (as when making a paraffin section) or alcohol- and xylene-containing stains or mounting medias. TRITC-labeled cells can be found in sections of fresh frozen tissues, but the intensity is several fold lower than that of A-NK cells found in formalin fixed tissues.
6. The identification of tumors in the nonstained fixed tissue is easy in the case of melanomas which often produce enough melanin for proper identification of the malignant tissues ***(26)***. For amelanotic melanomas as well as most other tumor types, identification is much more complicated, especially if the tumors or tumor metastases are small. Some tumor types can be identified by fluorescence microscopy because their level of autofluorescence differs from that of the surrounding normal tissue. Sometimes it is helpful to stain the section with Hoechst 33342 (1 μg/mL in water; Molecular Probes, Eugene, OR) for 10 min before coverslipping. Most tumors reveal themselves by the shape and/or size of their nucleus, often very different from those of most normal tissue. To be sure that an area with TRITC-labeled A-NK cells represents a tumor tissue, a fluorescence micrograph of the TRITC-labeled cells should be taken. The coverslip is removed and the section is counterstained (e.g., with hematoxylin–eosin) and the exact same spot is identified under light microscopy ***(26)***.
7. The method for counting A-NK cells in tissue sections given in **Subheading 3.4.2.** does not take into account that many cells are cut and might be visible in more than one section ***(28,29,37)***. This method therefore tends to overestimate

Table 1
Cell Labels Frequently Used in Studies of the Tissue Distribution of Various Cell Types

Label	The labeling process	Time limit for detection	Assessment of label	Remarks
Radiolabels				
^{51}Cr	Easy (60 min)	12–24 h	Easy	High sp. release—nonspecific uptake in liver and spleen.
^{111}In	Easy (60 min)	12–24 h	Easy	High sp. release—nonspecific uptake in liver and spleen.
[^{3}H]Thymidine	Easy (1–12 h)	>24 h	Laborious	Reutilization by host cells has been reported.
[^{3}H]Uridine	Easy (1–12 h)	>24 h	Laborious	Reutilization by host cells has been reported.
^{125}I-UdR	Easy (1–12 h)	24–48 h	Easy	Labels dividing cells only.
Fluorochromes				
Rhodamine	Easy (60 min)	20–30 h	Easy–Laborious	Fading of fluorescence during microscopy.
FITC	Easy (60 min)	10–20 h	Easy–Laborious	Fading of fluorescence during microscopy.
H-33342	Easy (60 min)	20–30 h	Easy–Laborious	Fading of fluorescence during micros copy—some "spillover" staining of neighboring cells.
DiO/DiI	Easy (~ 12 h)	24–48 h	Easy–laborious	Dye accumulates in vesicles inside cell, making identification of individual cells difficult.
PK2/PKH26	Fairly easy (60 min)	24–48 h	Easy–laborious	Dye accumulates in vesicles inside cell, making identification of individual cells difficult.

Genetic markers				
Neomycin resistance	Complicated	Days–weeks	Very laborious	Exact enumeration of cells difficult—some cells might lose gene expression.
β-Galactosidase	Complicated	Days–weeks	Easy–laborious	Some cells might lose gene expression.
Congenic strains	Easy	Infinitely	Laborious	Only few models are available.

the exact number of A-NK cells in the tumor. For comparison of A-NK cell tumor infiltration between groups, this is of less importance. However, if the exact number is needed, corrections must be made to estimate the correct number of A-NK cells from the observed number. As a rule of thumb, multiplying the observed number of A-NK cells by 0.7 will provide a good estimate of the true number. A more precise method is to subtract the average number of A-NK cells/mm^2 tumor tissue found in 8-μm thick sections from that found in 16-μm sections. This will estimate the true number of A-NK cells in 8 μm sections. Alternatively, by using a microscope that allows for simultaneous viewing of two consecutive sections, the true number of A-NK cells are measured by counting only those A-NK cells observed in the first section that do not appear in the neighboring sections.

Acknowledgments

This work was funded in part by Grant CA68550 from the National Institutes of Health.

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11

Assay for the Transendothelial Migration of Human Natural Killer Cells

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1. Introduction

The natural killer (NK) cell has been shown to be important in mounting a host's initial response to many types of invaders, such as certain types of viruses, bacteria, and protozoa, and to be involved in the control of neoplastic growth and in allograft rejection ***(1,2)***. However, before any leukocyte can perform its function in any tissue, it must first traverse the barrier of endothelial cells lining the blood vessel wall. This chapter describes a method with which to study this transmigration and the surface molecules of the NK cell that might be involved. Unlike monocytes, neutrophils, or T cells, NK cells comprise a very small percentage of peripheral blood leukocytes, only 10% of the lymphocyte fraction. This makes it a challenge to measure their transmigration, as transmigration assays usually necessitate a large number of a pure population of cells. The method described here uses a population of cells enriched for, but not exclusively, NK cells, and measures the NK cell transmigration using phenotypic analysis of the starting and transmigrating populations. The technique involves culturing endothelial cells in tissue culture well inserts that contain pores through which the NK cells can fall after their transmigration across the endothelial cell monolayer. The NK cells are enriched using negative selection exclusively, so as not to activate them by engaging their cell surface molecules, which could potentially change their pattern and extent of transmigration. For these experiments, we use the operational definition of NK cells as $CD56^{+}$/$CD3^{-}$ cells ***(1,3)***.

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
Edited by: K. S. Campbell and M. Colonna © Humana Press Inc., Totowa, NJ

2. Materials

2.1. Endothelial Cell (EC) Culture and Preparation for Assay

1. Medium M199 (Life Technologies, Grand Island, NY).
2. Medium M199 with 20% human serum (HS), warmed to 37°C (20% HS/M199).
3. Corning-Costar (Cambridge, MA) 6.5 mm "Transwell Clear" culture inserts with 3-μm pores.
4. Fibronectin 50 μg/mL.
5. 24-well culture dishes (Corning).
6. Sterile forceps for handling inserts.
7. If desired, Interferon-γ (IFN-γ) (Roche Molecular Biochemicals, Indianapolis, IN), 200 U/mL in 20% HS/M199. Make this dilution immediately prior to use.

2.2. Enrichment of NK Cells from Peripheral Blood Mononuclear Cells (PBMC)

1. 50- and 15-mL conical tubes.
2. 180-mm and 100-mm Petri dishes.
3. RPMI medium (Life Technologies) chilled to 4°C.
4. 5% HS in RPMI (5% HS/RPMI) and 1% HS/RPMI at 4°C and 37°C.
5. Dulbecco's phosphate-buffered saline (DPBS, Life Technologies).
6. Rabbit-α-mouse IgG antibody, 30 μg/mL in DPBS.
7. The leukocyte fraction (buffy coat) from one unit of human blood.
8. Mouse monoclonal antibodies against CD14 and CD3 (and if possible CD19). These could be purified IgG or hybridoma supernatants (*see* **Subheading 3.2.**).
9. Refrigerated (4°C) centrifuge with the capacity to hold 100-mm Petri dishes.
10. Fluorescence activated cell scanner (e.g., FACScan™, Becton Dickinson, Mountain View, CA).
11. Fluorescein isothiocyanate (FITC)-conjugated α-mouse IgG antibody (DAKO, Carpenteria, CA).
12. Phycoerythrin (PE)-conjugated α-CD56 IgG antibody (Becton Dickinson, San Jose, CA).
13. Hemacytometer.
14. Ficoll/Paque (Pharmacia Biotech, Uppsala, Sweden).

2.3. Transmigration Assay

1. Confluent EC (resting or stimulated) in "Transwell Clear" inserts.
2. 1% HS/M199 at 4°C.
3. 5% HS/M199 at 37°C.
4. 24- and 96-well tissue culture dishes.
5. Hemacytometer.
6. 1- or 2-mL pipettes with 1/10 mL gradations.
7. Repeat pipettor to dispense 0.15 mL each time.
8. FITC conjugated α-CD45 antibody (Becton Dickinson, San Jose, CA), and PE conjugated α-CD56 antibody in one solution, each at a concentration of 5–10 μg/mL in PBS.
9. FACScan™ and tubes.

3. Methods

3.1. EC Preparation and Culture

In this procedure, ECs are cultured on filters with 3 µm pores, so that the NK cells will fall through after transmigration, and be available for counting and phenotype analysis. Pores measuring 3-µm are used because larger pores allow migration of ECs from the upper surface, clogging the potential route for the NK cells and creating a double monolayer of ECs. All steps should be performed under sterile conditions.

1. Harvest ECs from human umbilical veins and culture as previously described ***(4)***. Cells can be passaged once for expansion and then cultured for assay.
2. With sterile forceps, place 6.5-mm Transwell Clear inserts into wells of 24-well culture dishes. Add 80 µL of fibronectin at 50 µg/mL to each insert (the wells formed by the insert will be referred to as the "upper chambers"). Incubate for 15 min at room temperature and then aspirate the fibronectin. Add 0.5 mL of 20% HS/M199 to the lower chamber of each well. Allow the medium to pass up through the filter into the upper chamber. This will prevent trapping of air in the filter.
3. Harvest ECs as for any passage, and resuspend in 20% HS/M199 (37°C) to $\sim 2 \times 10^5$ cells/mL and add 0.15 mL of cell suspension to each inner chamber. The medium in the outer chambers should be at the same level as the medium in the inner chambers. If it is too low, add more as needed.
4. Culture ECs for 2–3 d. Check for confluence using an inverted phase-contrast microscope. The cells should appear as a cobblestone monolayer. It is important that the cells be confluent for a transmigration assay. The use of the "Transwell Clear" inserts is important for this reason, as, unlike other types of inserts, they allow observation of live monolayers using phase-contrast.
5. If desired, the monolayers may be stimulated with IFN–γ (or any cytokines of interest) once they reach confluence, which will cause the EC to enter into an activated state. The procedure for IFN–γ stimulation is as follows:
   a. Prepare wells of 24-well tissue culture dishes by placing 0.5 mL of 200 U/mL IFN–γ/20% HS M199 (37°C) in each well. Move the inserts from their original wells into these new wells. Gently aspirate the media from the inner chambers, being careful not to disturb the monolayers. Replace this media with 0.15 mL of the IFN–γ solution and culture for 3 d. At the end of this period, the ECs should appear elongated. It is important to manipulate the control, nonstimulated cells the same way, but omitting the IFN–γ.
   b. Before performing the assay, the IFN–γ must be washed away. Move the inserts to a 24-well plate containing fresh, warm M199. Gently aspirate the media from the inner chamber and gently add warm M199. Repeat.

3.2. Enrichment of NK Cells from PBMC

To perturb the NK cells as little a possible before the transmigration assay, a procedure using adherence and then negative selection to remove unwanted

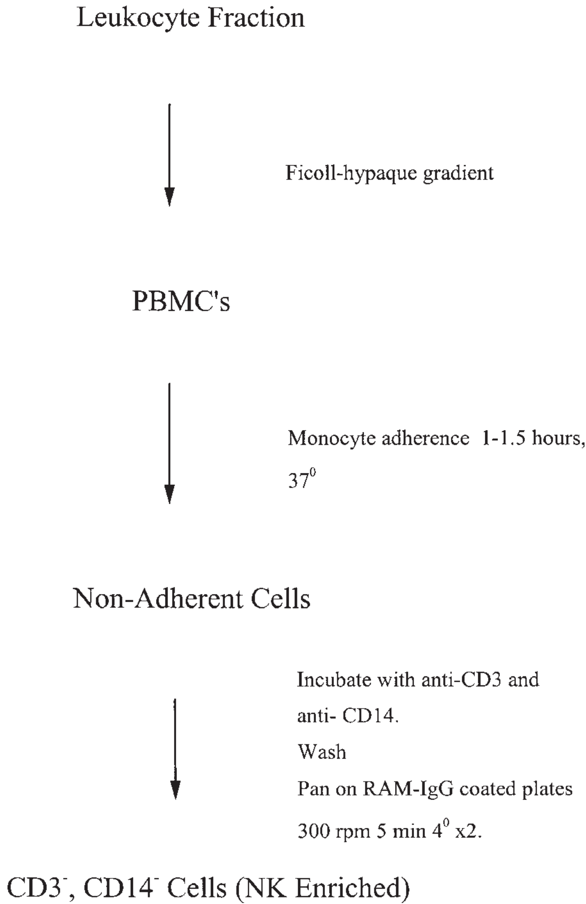

Fig. 1. Enrichment procedure for NK cells. NK cells are enriched from a population of peripheral blood mononuclear cells using a negative selection procedure to remove $CD14^+$ monocytes and $CD3^+$ T cells. The final population contains, on average, 50% NK cells.

cells is the best method for enriching PBMCs for NK cells (**Fig. 1**). The transmigration assay does not necessitate a pure population of NK cells (*see* **Subheading 3.3.**), but obviously, a high percentage of NK cells is preferable. Because a large number of NK cells are usually necessary for these assays and NK cells form only a small percentage of PBMCs, it is preferable to start with the PBMCs from the leukocyte fraction (buffy coat) of an entire unit of blood. Because of the number of cells involved, an ample source of antibody against CD14 (for monocytes), CD3 (for T cells), and if possible CD19 (for B cells) should be found. Hybridoma supernatants can be used for this procedure if

necessary. **This procedure must be performed with the utmost caution, as fresh leukocytes from as yet untested blood will be used.**

1. Petri dishes coated with rabbit-α-mouse (RAM) IgG antibody must be prepared. Add 10 mL of RAM-IgG at 30 μg/mL in DPBS to each of 10–15 100-mm Petri dishes (*see* **Note 1**). Incubate for 1 h at room temperature. Remove antibody solution (may be reused) and wash plates three times with DPBS. Store dishes at 4°C in DPBS until use.
2. Harvest PBMCs from the leukocyte fraction from one unit of blood using a Ficoll/Paque gradient: Prepare 50-mL conical tubes with 20 mL of Ficoll/Paque in each. Gently pipet the leukocyte fraction onto the Ficoll/Paque layer. Centrifuge at 2100 rpm for 20 min at room temperature with the brake off. Harvest the cloudy leukocyte layer from each tube and add these to tubes with fresh cold (4°C) RPMI. Centrifuge at 1200 rpm for 10 min at 4°C (brake on). Aspirate supernatant and resuspend and centrifuge cells again as described previously. After washing, resuspend the cells in warm (37°C) 5% HS/RPMI at 5×10^6/mL.
3. Add 1×10^8 cells (15 mL) to each 180-mm Petri dish. Incubate for 1 h at 37°C. Remove nonadherent cells and place into 50-mL conical tubes. Wash the surface of each dish with 5 mL of warm 1% HS/RPMI and add the washes to the original nonadherent cells. Repeat washes 2×. Count cells using a hemacytometer. Centrifuge the nonadherent cells at 1200 rpm for 10 min at 4°C. Aspirate supernatants.
4. Resuspend cells up to 1.5×10^7/mL in cold 5% HS/RPMI containing 20 mg/mL of purified α-CD14, α-CD3, and if available, α-CD19–IgG, or dilutions of hybridoma supernatants of these antibodies that will saturate the sites on all of the cells. Incubate on ice for 20 min.
5. Centrifuge cells as described in **step 3**. Aspirate antibody solution. Wash cells 2× with cold RPMI and resuspend to $2 – 3 \times 10^6$/mL in cold 5% HS/RPMI.
6. Aspirate the DPBS from two-thirds of the RAM–IgG-coated Petri dishes. Leave the rest of the dishes for **step 8** (*see* **Note 1**). Add 10 mL of cell suspension to each plate. Centrifuge and spin at 300 rpm for 5 min at 4°C with the brake off. Rotate plates 180° and spin again.
7. Remove nonadherent cells and place in 50-mL conical tubes. Gently wash the surface of the plates with cold 1% HS/RPMI, to remove remaining nonadherent cells, and place wash medium in the tubes. Repeat wash. Count the cells.
8. Centrifuge the cells and resuspend to $2 – 3 \times 10^6$ in cold 5% HS/RPMI. Repeat **steps 6** and **7** using fresh RAM-IgG-coated plates.
9. Resuspend final population of enriched cells in 10 mL of warm 5% HS/RPMI. Remove aliquots for counting and phenotyping and place the rest in a 37°C incubator until use (*see* **Note 2**).
10. Perform FACs analysis using FITC–RAM–IgG antibody (to detect remaining antibody-bound cells) and then α-CD56–PE antibody to determine the percent of NK cells in the population. If α-CD19 was not used, the NK cell percentage should be between 40% and 65% of the total (**Fig. 2**).

Fig. 2

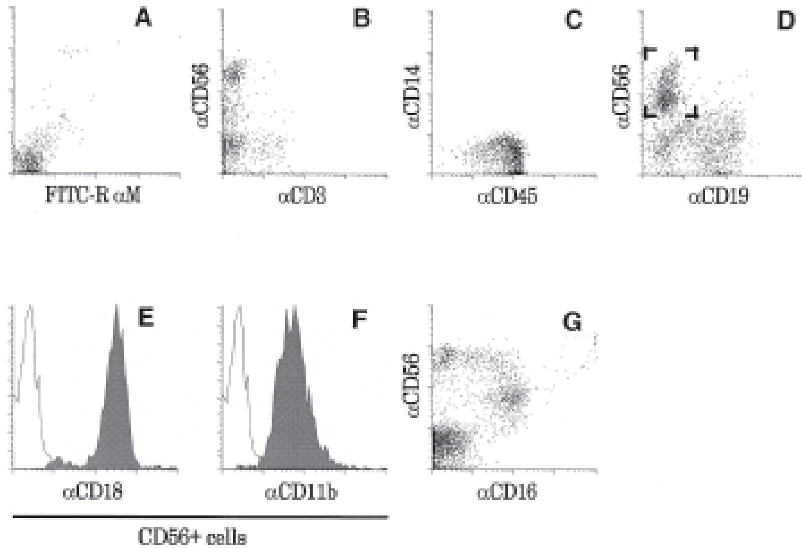

Fig. 2. Phenotype of NK-enriched cells (**ref. 5**). NK cells were enriched by negative selection from PBMC using plastic adherence and panning to remove $CD3^+$ and $CD14^+$ cells, as described. They were then stored at 37°C for 12 h. The enriched cells were stained with panels of mAbs to detect the presence of $CD56^+$ NK cells and other contaminating cells, and analyzed using a FACscan. In dot plots, the X-axis shows reactivity with FITC-labeled antibodies and the Y-axis shows reactivity with antibodies labeled with PE. All antibodies were pure IgG and were added at 5 µg/mL, except for hybridoma supernatants of anti-CD11b, added at a dilution of 1:2, and anti-CD3, added at 1:10. Anti-CD56, CD45, CD14, and CD16 were all conjugated to fluorochromes. Anti-CD3, CD19, CD18, and CD11b were all detected using FITC conjugated rabbit-antimouse-IgG (FITC-RaM [1:100]). **(A)** Lack of staining with FITC-RaM alone demonstrates that none of the antibody used in the panning step remained bound to the cells. **(B)** Very few $CD3^+$ cells remained (here, 6% of the total), and none of the $CD56^+$ cells were $CD3^+$. **(C)** Virtually no $CD14^+$ monocytes could be detected in the enriched population. **(D)** The major contaminating population was $CD19^+$ (B) cells, here, 40% of the total. (**E** and **F**) Data were gated on $CD56^+$ cells. All these cells showed positive staining for CD18 and CD11b (shaded histograms). **(G)** $CD56^+$ cells could be divided into $CD56^{bright}/CD16^{-\text{ or dim}}$ cells and $CD56^{dim}/CD16^+$ cells. (This figure reproduced by permission from **ref. *5***. Copyright 1996 by The American Society of Immunologists.)

3.3. Transmigration Assay (Fig. 3)

Fig. 3

1. Place Transwell inserts with confluent EC in new 24-well plates containing 0.5 mL of warm 5% HS/M199 per well. (If EC have been stimulated, wash them first. *See* **Subheading 3.1.**)

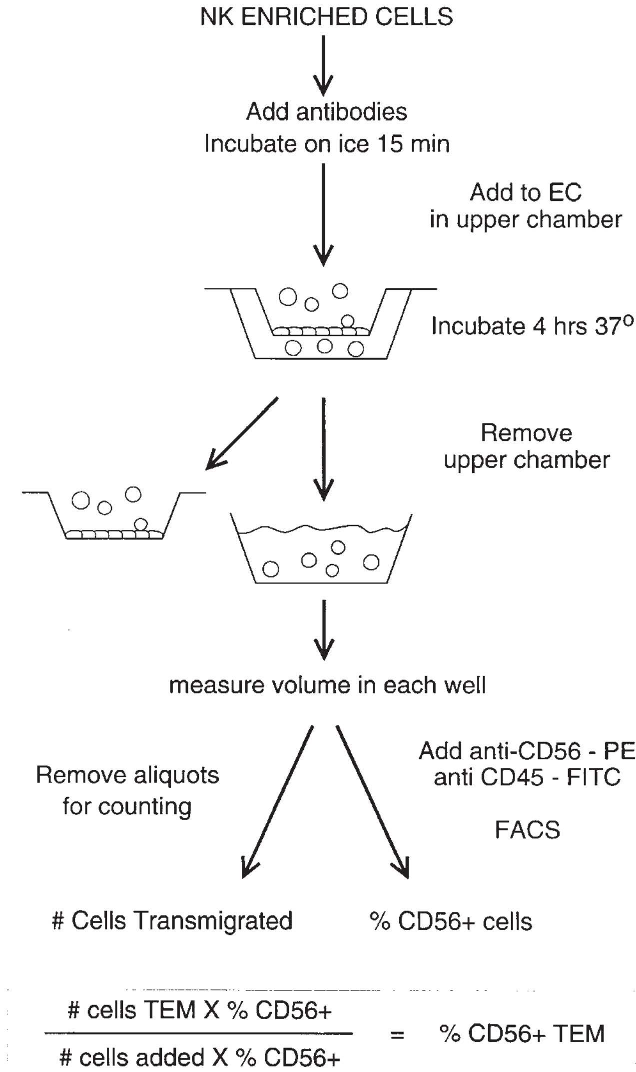

Fig. 3. Transmigration Assay. NK-enriched cells can be incubated with various antibodies and added to EC monolayers grown on Transwell inserts containing 3-µm pores. After allowing the cells to transmigrate into the lower chamber at 37°C, the upper chamber containing the nontransmigrated cells is removed, leaving only the transmigrated cells in the lower chamber. To determine the number of transmigrated cells in each case, the cells in each well are counted, and the percentage of NK cells in each case is determined by staining samples from each well with anti-CD56 and anti-CD45 monoclonal antibodies. The number of NK cells added to the wells is determined in the same fashion. This allows for the determination of the percentage of NK cells transmigrated in each well.

2. Resuspend NK-enriched cells and place in a 15-mL conical tube. Count the cells and divide them into the number of tubes corresponding to the number of variables you will be testing. Centrifuge these tubes at 1100 rpm for 5 min at 4°C. Resuspend cells to $0.7 - 2 \times 10^6$/mL (depending on the number of cells and variables that you have) in cold 5% HS/M199 +/– 25 µg/mL of blocking or control antibody, or any other agent you wish to assay. Incubate on ice for 15 min.
3. Gently aspirate media from the inner chambers of the EC cultures, and then add 0.15 mL of NK-enriched cells to each well. Assay each variable in triplicate, at the least. Incubate 37°C for 4 h, or as long as your assay indicates (*see* **Note 3**). Make sure that there is enough excess left from each tube for counting and FACS analysis.
4. Using a hemacytometer, count an aliquot of cells from each tube. Calculate the number of cells added to each well (no. of cells/mL × 0.15 mL).
5. Immunostain the remaining cells from each tube with PE-conjugated α-CD56 antibody, and use a FACScan to determine the percentage of NK cells in the original population. Then calculate the number of NK cells added to each well (*see* **step 8**). (If the assay is conducted immediately after the enrichment procedure, this FACS step is unnecessary. The original percentage of NK cells in the enriched population can be used for this calculation).
6. At the end of the incubation, remove the inserts from each well. (It might be necessary to pipet off media from underneath the insert and place it back in the well). Measure and record the exact volume of media in the bottom chamber of each well. Remove 0.15 mL from each well for counting. It is convienient to place these aliquots in separate wells of labeled 96-well plates. If you must wait to count the cells, the cells can be fixed with glutaraldehyde, but be sure to record the dilution factor caused by the fixation solution. Determine the number of cells transmigrated in each well (no. of cells/mL × no. of mL in lower chamber) (*see* **Note 4**).
7. Transfer the remaining cells to labeled FACScan tubes (two for each well), and centrifuge 1200 rpm for 5 min at 4°C. Aspirate media. To one tube from each well, add 5–10 µg/mL of α-CD56–PE + 5 – 10 mg/mL of α-CD45–FITC antibodies (used to correct for any debris in the sample) in cold 1% HS/M199. Use the other tube for an isotype-matched negative control monoclonal antibody. Incubate for 20 min on ice, centrifuge, wash 2× with PBS and resuspend for FACS analysis (*see* **Note 5**).
8. Calculate $CD56^+$ migration for each well as follows:

$$\frac{\%\ CD56^+ \text{ cells in added population} \times \text{no. of cells added}}{\%\ CD56^+ \text{ cells transmigrated} \times \text{no. of cells transmigrated}}$$

4. Notes

1. The number of RAM–IgG-coated dishes necessary will depend on the number of leukocytes harvested from the buffy coat, and the number of non-adherent leukocytes harvested in **Subheading 3.2., step 3**. The number of leukocytes obtained from a buffy coat can vary from 3×10^8 to 1×10^9. Usually, about one-third are

removed during the adherence step. Up to 3×10^7 cells can be panned on one RAM–gG-coated 100-mm plate (*see* **Subheading 3.2., steps 5** and **6**). The panning step is performed twice. Normally, the first panning leaves roughly 20% of the nonadherent starting population. The final yield is usually about 5% of the buffy coat leukocytes.

2. Because the enrichment procedure and transmigration assay are very lengthy, it may be necessary to store the cells overnight before performing the transmigration assay. The shorter the time the cells are stored, the closer they are to their original state in the blood, and the better their viability. As a rule, we never stored the enriched cells for more than 14 h.
3. Any type of time course may be used in the transmigration assay; however, in our experience, to get a significant, measurable number of cells transmigrating (~10%) it was necessary to allow the cells to transmigrate for 3.5–4 h *(5)*.
4. This procedure describes the harvesting of the transmigrated cells only. However, it is also possible to harvest the nonadherent cells from the inner chamber, and analyze those in a similar manner to the transmigrated cells.
5. The transmigration of the separate populations of $CD56^{high}$ and $CD56^{low}$ can also be determined from these data. This type of transmigration assay, in which the phenotype of the transmigrating cells is analyzed, can be used to measure any marker of interest on the starting and transmigrating populations, and can be used to measure transmigration of any cell type or mixture of cells.

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12

Methods of Analyzing Chromatin Changes Accompanying Apoptosis of Target Cells in Killer Cell Assays

Richard C. Duke

1. Introduction

Natural killer (NK) cells kill their targets by inducing two distinct modes of cell death—apoptosis and necrosis ***(1–4)***. The end result in both cases is target cell lysis, broadly defined as a loss of membrane integrity and most usually assessed by either chromium release or uptake of certain nonvital dyes such as eosin, trypan blue, or propidium iodide.

Apoptosis occurs under normal physiological conditions and requires the active participation of the cell itself—death occurs from within (reviewed in **ref.** ***5***; *see* also **Notes 1** and **2**). Apoptotic cell death can be induced by a variety of stimuli; however, NK cells are generally thought to use two mechanisms (*see* **Note 3** for further reference). First, NK cells can induce apoptosis through secretion of granzymes acting in concert with perforin ***(3,6)***. Second, NK cells can express a ligand for Fas (also called CD95 or Apo-1) allowing them to induce apoptosis in target cells that express the functional form of the receptor ***(7,8)***. Induction of apoptosis by both mechanisms involves activation of a family of proteases in the target cell termed caspases (*see* also Chapter 14). Caspases cleave a variety of cellular targets including nuclear and cytoskeletal components. The end result is that the cell undergoing apoptosis shows a progressive contraction of cell volume often accompanied by violent blebbing or zeiosis ***(5)***. The structural integrity of most cytoplasmic organelles is often preserved but dramatic changes occur in the nucleus. These changes involve condensation and fragmentation of chromatin into uniformly dense masses that can be readily detected microscopically using DNA-binding dyes (**Subhead-**

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
Edited by: K. S. Campbell and M. Colonna © Humana Press Inc., Totowa, NJ

ing 3.1.; *see* **Note 2** for further information regarding the importance of morphological assays). In many cells undergoing apoptosis, extensive double-stranded DNA cleavage also occurs although there are exceptions to this rule (*see* **Note 4**). The assays described in **Subheadings 3.2., 3.3.**, and **3.4.** take advantage of the fact that chromatin composed of fragmented DNA can be physically separated from intact chromatin. The TUNEL assay, described in **Subheading 3.5.**, detects double-stranded DNA cleavage at the single-cell level by using terminal deoxynucleotidal transferase (TdT) to add fluorescein isothiocyanate (FITC)-labeled dUTP at the cleavage site; positive cells are detected by microscopy or by flow cytometry. It is important to understand that a cell that has undergone apoptosis will eventually lyse. Temporally, therefore, one observes changes in nuclear structure that precede loss of membrane integrity; e.g., DNA fragmentation is detected prior to chromium release and chromatin condensation is observed microscopically before nonvital dyes are taken up ***(9,10)***.

In in vitro assays, it is easiest to consider that necrosis occurs when a cell lyses in the absence of demonstrable apoptotic changes. Necrosis results when a cell is unable to maintain homeostasis, leading to an influx of extracellular ions and water. Intracellular organelles, most notably the mitochondria, and the entire cell swell and eventually rupture. In high concentrations, purified perforin induces necrosis but not apoptosis ***(11)***. It has been shown that NK cells can induce cytolysis in the absence of apoptotic changes, strongly suggesting that perforin is the causative agent (***1,12***; *see* **Note 3** for further information). NK cells, as compared to cytotoxic T lymphocytes (CTLs), tend to be more likely to induce purely necrotic cell death because they express relatively higher amounts of perforin than do CTLs ***(13)***.

Because the mechanism of apoptosis is poorly understood at the present time, it is probably best to perform several assays to confirm an observation of apoptotic cell death (*see* **Note 2**). In addition, it is important to employ assays that detect cytolysis concomitantly with apoptosis assays to confirm that NK cell-mediated killing has occurred.

2. Materials

1. Tissue culture medium (TCM): In all instances, TCM refers to the culture medium which is typically employed in an investigator's laboratory to grow tumor cells and to culture primary lymphocytes.
2. Dye mixes for quantification of apoptotic cell death using fluorescent dyes and UV microscopy (**Subheading 3.1.**) (*see* **Note 5** regarding handling of these chemicals):
   a. Individual stock solutions of acridine orange (AO; Sigma Chemical, St. Louis, MO); ethidium bromide (EB, Sigma); propidium iodide (PI, Sigma); or

Hoechst 33342 (Hoe; Sigma) are prepared in deionized H_2O at a concentration of 1 mg/mL. Store stock solutions at 4°C for up to 12 mo and protected from light.

b. For use in assays, prepare working solutions of 100 µg/mL of AO + 100 µg/mL of EB (AO+EB) or 100 µg/mL of Hoe + 100 µg/mL of PI (Hoe + PI) in deionized H_2O. Working solutions can be used for up to 6 mo if stored at 4°C and protected from light.

3. Radiolabeled compounds for quantifying DNA fragmentation and lysis (**Subheadings 3.2.** and **3.3.**):
   a. 1 mCi/mL of iododeoxyuridine (^{125}IUdR) in aqueous solution (2000 Ci/mmol; ICN Pharmaceuticals, Costa Mesa, CA).
   b. 1 mCi/mL of [methyl-^{3}H]thymidine ([^{3}H]TdR) in aqueous solution (2.0 Ci/mmol; ICN).
   c. 5 mCi/mL of ^{51}Cr as Na_2CrO_4 in saline (200–900 Ci/g; ICN).
4. TTE solution for quantifying DNA fragmentation and lysis (**Subheading 3.2.**): 10 m*M* Tris HCl, 1 m*M* EDTA; 0.2% Triton X-100, pH 7.4 (store at 4°C).
5. Buffers and reagents for agarose gel electrophoresis (**Subheding 3.4.**):
   a. TE buffer: 10 m*M* Tris HCl; 1 m*M* EDTA, pH 7.4 (store at 4°C).
   b. TTE solution: TE buffer containing 0.2% Triton X-100 (store at 4°C).
   c. 5 m*M* NaCl (store at 4°C).
   d. Isopropanol (store at –20°C).
   e. 70% Ethanol (store at –20°C).
   f. 10X DNA loading buffer (store at RT): 20% Ficoll 400, 0.1 *M* EDTA, 1% sodium dodecylsulfate (SDS), 0.25% bromphenol blue, and 0.25% xylene cyanol FF.
   g. 10X TBE electrophoresis buffer stock solution (1 L): 108 g of Tris base, 55 g of boric acid, and 100 mL of 0.2 *M* EDTA, pH 8.0. Store at room temperature.
6. Buffers and reagents for TUNEL assay (**Subheading 3.5.**) (*see* **Note 6** regarding use of these reagents):
   a. TdT reaction buffer: 0.5 *M* cacodylic acid, sodium salt, pH 6.8, 1 m*M* $CoCl_2$, 0.5 m*M* dithiothreitol (DTT), 0.05% (w/v) bovine serum albumin (BSA), and 0.15 *M* NaCl. Store at room temperature or 4°C for several months.
   b. TdT/FITC–dUTP reaction mixture: TdT reaction buffer containing 1 µ*M* FITC–dUTP (fluorescein-12-dUTP, Roche Molecular Biochemicals, Indianapolis, IN) and 2–4 U TdT (terminal deoxynucleotidal transferase, Roche Molecular Biochemicals), prepared immediately before use.
   c. 4% Paraformaldehyde prepared in PBS.
7. Reagents for detecting Fas-mediated apoptosis (**Subheading 3.6.**):
   a. EGTA/$MgCl_2$ stock solution: 500 m*M* EGTA (tetrasodium EGTA, Sigma), 1 *M* $MgCl_2$, pH 7.0–7.5 (store at 4°C).
   b. Soluble human Fas–human F_c (Fas–F_c, Immunex, Seattle, WA) prepared at 1 mg/mL in ddH_2O (store at –20°C; avoid subjecting to multiple rounds of freezing and thawing).
   c. Anti-human Fas ligand antibody (Clone 4H9, Immunotech, Westbrook, ME) prepared at 1 mg/mL in ddH_2O (store at –20°C; avoid subjecting to multiple rounds of freezing and thawing).

d. PMA stock solution (1000X): 5 µg/mL PMA (phorbol 12-myristate 13-acetate, Sigma) dissolved in dimethyl sulfoxide (DMSO) (store at –20°C; avoid subjecting to multiple rounds of freezing and thawing).
e. Ionomycin stock solution (1000X): 500 µg/mL of Ionomycin (Sigma) dissolved in DMSO (store at –20°C; avoid subjecting to multiple rounds of freezing and thawing).

3. Methods

3.1. Quantification of Apoptotic Cell Death Using Fluorescent Dyes and UV Microscopy

In this protocol, fluorescent DNA-binding dyes are added to a mixture of effector and target cells and examined by fluorescence microscopy to visualize and count cells with aberrant chromatin distribution. Acridine orange and Hoechst 33342 are used to quantify how many cells have undergone apoptosis, but they cannot differentiate between intact (viable) and lysed (nonviable) cells. To do the latter, a mixture of acridine orange and ethidium bromide or Hoechst 33342 and propidium iodide is used.

Both live and dead cells take up acridine orange and Hoechst 33342, whereas only dead cells take up ethidium bromide and propidium iodide. AO intercalates into DNA, making it appear bright green, and binds to RNA in the cytoplasm, staining it red. Hoe binds only to DNA, making it appear bright blue. EB and PI intercalate into DNA, making it appear orange, but bind only weakly to RNA, which may appear slightly red. Thus a viable cell stained with AO + EB will have bright green chromatin and red cytoplasm, whereas a dead cell will have bright orange chromatin (EB overwhelms AO) and its cytoplasm, if it has any RNA remaining, will appear dark red. Similarly, a viable cell stained with Hoe + PI will have blue chromatin and no cytoplasmic staining, whereas a dead cell will have bright pink chromatin (Hoe and PI staining blend) and its cytoplasm, if it has any RNA remaining, will appear dark red.

It is extremely important to acquaint oneself with normal chromatin distribution in the target cell line being investigated. When stained with DNA-binding dyes, both live and dead nonapoptotic (normal) cell nuclei will have "structure;" variations in fluorescence intensity reflect the distribution of euchromatin and heterochromatin (*see* **Note 2**). Apoptotic nuclei, in contrast, have highly condensed chromatin that is uniformly fluorescent. This can take the form of crescents around the periphery of the nucleus, or the entire chromatin can be present as one or a group of featureless, bright spherical beads. In advanced apoptosis, the cell will have lost DNA or become fragmented into "apoptotic bodies" and overall brightness will be less than that of a normal cell.

This method can also be used to evaluate factors released by NK cells or isolated from lytic granules by incubating target cells with the material of interest prior to assessment of apoptosis.

Detection of Hoechst 33342 requires UV excitation wavelengths of less than 350 nm which may not be available on standard fluorescent microscopes.

3.1.1. Effector and Target Cells

In this protocol, it is important to be able to distinguish between effector and target cells by nuclear morphology. This is not as foreboding as might first be thought. Tumor cells are often used as targets in NK assays, and these have distinct nuclear morphologies compared to lymphocyte effector cell populations. In general, tumor cells, even those of hematopoietic origin, have much larger nuclei than normal lymphocytes and can be easily differentiated on this basis. Nonetheless, it is best to use highly purified effector cell populations and/or clones (*see* Chapter 1–5 and **ref. *8–10***) such that effector:target (E:T) ratios of less than 10:1, or preferably 1:1, are employed when utilizing this protocol.

Although this assay may appear too cumbersome for routine assays, it should not be overlooked as it provides a wealth of information. Target and effector cells can be coincubated for several days as there is no need to worry about spontaneous release of radioactive labels. This assay also provides information regarding the effect of targets on effector cell populations.

3.1.2. Assay

1. Using a minimum of 5×10^4 target cells per condition, incubate effector and target cells together as appropriate in TCM. Include the following controls: target cells without effectors and effector cells without targets. Targets and effectors can be incubated together in 96-well plates or tissue culture tubes.
2. At the end of the assay period, pellet cell mixtures by centrifugation (200*g*; 10 min) and carefully remove all but approx 25–30 µL of the supernatant.
3. Resuspend the cell pellet and transfer cell suspension to a 12 × 75-mm glass tube containing 2 µL of either AO + EB or Hoe + PI.
4. Place 10 µL of this mixture on a clean microscope slide and coverslip. Examine with a 40× to 60× objective using epiilumination and a filter combination suitable for observing fluorescein (in the case of AO + EB) or Dapi (in the case of Hoe + PI).
5. Count a minimum of 200 total target cells and record the number of each of the four cellular states: (a) live target cells with normal nuclei (LN: bright green [AO + EB] or blue [Hoe + PI] chromatin with organized structure); (b) live cells with apoptotic nuclei (LA: bright green [AO + EB] or blue [Hoe + PI] chromatin that is highly condensed or fragmented); (c) dead cells with normal nuclei (DN; bright orange [AO + EB] or pink [Hoe + PI] chromatin with organized structure); and (d) dead cells with apoptotic nuclei (DA; bright orange [AO + EB] or pink [Hoe + PI] chromatin that is highly condensed or fragmented).
6. Determine apoptotic index and percentage of necrotic cells using the following formulae:

$$\% \text{ apoptotic cells (apoptotic index)} = \frac{LA + DA}{LN + LA + DN + DA} \times 100$$

$$\% \text{ necrotic cells} = \frac{DN}{LN + LA + DN + DA} \times 100$$

$$\% \text{ dead cells} = \frac{DN + DA}{LN + LA + DN + DA} \times 100$$

3.2. Quantification of DNA Fragmentation and Lysis Using Cells Containing Radiolabeled DNA

The following protocol takes advantage of the size difference between fragmented DNA and intact chromatin to quantify target cell apoptosis. Target cell DNA is discriminated from effector cell DNA by metabolically labeling the target cell DNA with ^{125}IUdR prior to performing the assay.

In this protocol, fragmented DNA released from the nuclei of lysed cells is separated from intact chromatin by centrifugation. In brief, target and effector cells are incubated together for various times and the quantification assay is then performed in two steps. In the first step, intact target cells and large cell debris are pelleted by centrifugation. The supernatant, termed "S," is carefully withdrawn and saved. The "S" fraction contains fragmented DNA and cytoplasmic contents (including chromium) released from target cells that lysed during the assay period. Because apoptotic cells may not have lysed during the assay period, the cell pellet is treated with a hypotonic buffer containing EDTA and Triton which results in lysis of the cells and release of their nuclei. The lysate is then subjected to centrifugation, which results in sedimentation of intact nuclei and chromatin. The supernatant from the lysates, termed "T" for "top," contains fragmented DNA released from the nuclei of apoptotic cells as well as the remaining chromium. The pellet, termed "B" for "bottom," contains predominantly intact, chromosome-length chromatin. It should be noted that separation is not absolute as some fragmented DNA can be found in the intact fraction.

By utilizing cells which are doubly labeled with ^{125}IUdR and ^{51}Cr, simultaneous measurement of DNA fragmentation and lysis in the same target cell populations is possible.

3.2.1. DNA and Cytoplasmic Radiolabeling with ^{125}I-UdR and ^{51}Cr

1. Subculture target cells to be labeled at $1–4 \times 10^5$ cells/mL in TCM the day before the assay is to be performed. It is necessary that the cells be growing well (*see* **Note 7** for further information regarding DNA labeling).

2. Transfer cells to 17 × 100-mm polystyrene (Falcon) or 15-mL conical polypropylene tissue culture tubes and pellet by centrifugation (200*g*; 10 min). Use polypropylene tubes for target cells that are normally adherent.
3. Aspirate supernatant and resuspend in 200 µL TCM, so that concentration is 1–4 × 10^6 cells/mL.
4. Add 2 to 20 µCi (2–20 µL) of ^{125}I-UdR and incubate cells 90–120 min at 37°C. Add 100 µCi of ^{51}Cr during the last 60 min of the incubation period.
5. Wash cells 2× with 10 mL of TCM (prewarmed to 37°C). Resuspend cells in 10 mL of TCM and incubate for 30 min at 37°C. Pellet cells and resuspend in TCM at 1 × 10^5 cells/mL for assay.
6. Determine level of incorporation by measuring radioactivity in 10,000 target cells with a gamma counter. This procedure should yield an incorporation level of 0.5–3 cpm ^{125}I/cell and 0.05–0.5 cpm ^{51}Cr/cell. If lower levels of ^{125}I-UdR incorporation are observed, the cells may be infected with mycoplasma or may have not been subcultured at a low enough initial density. Use doubly labeled cells for experimentation only if the ratio of ^{125}I to ^{51}Cr is greater than 2:1.
7. *Note:* This assay was developed using a Beckman Gamma 5000 counter equipped with a ^{51}Cr Iso-Set Module and a Variable Discriminating Module. The ^{51}Cr module detects only ^{51}Cr and not ^{125}I. The variable module was calibrated so as to yield 100% of the radioactivity measured by the ^{51}Cr module as well as ^{125}I (windows: lower = 0 upper = 165). Thus the radioactivity present as ^{125}I in a mixed sample equals cpm (variable module) – cpm (^{51}Cr module).

3.2.2. Assay

1. Place 100 µL of the doubly-radiolabeled target cells (10^4) in a 1.5-mL microcentrifuge tube labeled "B" and gently mix with either 100 µL of TCM (for spontaneous release) or with various numbers of effector cells (10^4–10^6) in 100 µL of TCM (experimental).
2. Centrifuge tubes (200*g* for 10 min at 22°C) to establish cell-to-cell contact. Incubate for 1–16 h at 37°C. The incubation time will vary depending on the cells being assayed.
3. At the end of the desired incubation period, add 800 µL of ice-cold TCM to each microcentrifuge tube and pellet intact cells and large debris by centrifugation (200*g* for 10 min at 22°C).
4. Carefully withdraw culture supernatant, place in tube labeled "S," and set aside. The S fraction contains ^{51}Cr and ^{125}I-UdR-labeled DNA fragments released from target cells.
5. Lyse cell pellet in tube B by adding 1 mL of TTE and vortexing vigorously. Microcentrifuge (13,000*g*; for 10 min at 22°C) to separate intact chromatin from fragmented DNA. Carefully transfer the supernatant to a tube labeled "T." TTE lyses the cells releasing ^{125}I-UdR-labeled DNA fragments and the remaining ^{51}Cr.
6. Quantify the amounts of ^{125}I and ^{51}Cr present in the S, T, and B fractions with a gamma counter.
7. Calculate percentage fragmented DNA (as cpm ^{125}I) for spontaneous and experimental conditions using the following formula:

$$\% \text{ fragmented DNA} = \frac{S + T}{S + T + B} \times 100$$

where S + T is the ^{125}I radioactivity (cpm) present as unsedimented chromatin in the S and T fractions, and S + T + B is the ^{125}I in S, T, and B fractions. Spontaneous DNA fragmentation should not exceed 30% (*see* **Note 7**).

8. Calculate % lysis (as cpm ^{51}Cr release) for spontaneous and experimental conditions using the following formula:

$$\% \text{ lysis} = \frac{S}{S + T} \times 100$$

where S is the radioactivity (cpm) present as ^{51}Cr in the S fraction, and S + T is the radioactivity present as ^{51}Cr in the S and T fractions.

9. Calculate percent specific fragmented DNA or lysis using the following formula:

$$\% \text{ specific fragmented DNA or lysis} = \frac{expt - spont}{100 - spont} \times 100$$

where *expt* is the experimental value (with NK cells) and *spont* is the spontaneous value (with TCM alone) for percent fragmented DNA or lysis as calculated in **steps 7** and **8**.

10. By utilizing cells that are labeled with ^{125}I-UdR or [^{3}H]TdR, but not with ^{51}Cr, it is possible to use this assay to quantify DNA fragmentation alone.
11. Spontaneous DNA fragmentation should not exceed 30% (*see* **Note 7**).

3.3. Quantification of DNA Fragmentation Using the [^{3}H]TdR Release Assay or JAM Test

In this protocol, as first described by Matzinger ***(15)***, target cells labeled in their DNA with [^{3}H]TdR are harvested after the coincubation period with NK cells onto a fiberglass filtermat using a semiautomated cell harvester. DNA that has been fragmented passes through the filtermat whereas intact, chromosome-length, DNA does not. The level of target cell DNA fragmentation is therefore inversely proportional to the amount of [^{3}H]TdR retained on the filtermat.

3.3.1. DNA Radiolabeling with [^{3}H]TdR

1. Subculture target cells to be labeled at 1–4 × 10^5 cells/mL in TCM (in tissue culture flasks, multiwell plates, or Petri dishes). It is necessary only that the cells be growing well (*see* **Note 7** for further information regarding DNA labeling).
2. Add 0.5–2 µCi/ml [^{3}H]TdR to cells and incubate for 12–18 h at 37°C.
3. Wash cells once with TCM to eliminate unincorporated [^{3}H]TdR (prewarmed to 37°C).

4. Resuspend cells in TCM at 1×10^4 to 1×10^5 cells/mL for assay (*see* **Subheading 3.3.2.**).
5. Determine level of incorporation by measuring radioactivity in 10,000 target cells with a beta scintillation counter. This procedure should yield an incorporation level of 0.2–3 cpm [^{3}H]/cell. If lower levels of [^{3}H]TdR incorporation are observed, the cells may be infected with mycoplasma or may have not been subcultured at a low enough initial density.

3.3.2. Assay

1. Place 100 µL of [^{3}H]TdR-labeled target cells (10^3–10^4) in individual wells of 96-well, U-, or V-bottomed microtiter plates that contain various concentrations of effector cells (10^4–10^6) in 100 µL of TCM. Three wells containing target cells alone are used for the determination of spontaneous DNA fragmentation.
2. Centrifuge the plates (200*g*; 10 min; 22°C) to establish cell-to-cell contact. Incubate for 1–16 h at 37°C. The incubation time will vary depending on the cells being assayed.
3. At the end of the desired incubation period, the plates are harvested using a semiautomated cell harvester and the counts retained on the filtermat are quantified with a beta scintillation counter in the same manner as for proliferation assays employing [^{3}H]TdR.
4. Percent specific DNA fragmentation is calculated using the following formula:

$$\% \text{ specific fragmented DNA or lysis} = \frac{spont - expt}{spont} \times 100$$

where *spont* is the [^{3}H] radioactivity (cpm) retained on the filtermat from target cells incubated without NK cells (spontaneous) and *expt* is the [^{3}H] radioactivity (cpm) retained on the filtermat from target cells incubated with NK cells (experimental).

3.4. Qualitative Analysis of Internucleosomal DNA Fragmentation by Agarose Gel Electrophoresis

In this protocol, fragmented target cell DNA is isolated, concentrated, and analyzed by agarose gel electrophoresis. This procedure demonstrates the internucleosomal DNA cleavage associated with apoptosis ***(9,14)***, but is not quantitative owing to limitations inherent in DNA recovery and solubilization. Therefore, this method is used to confirm an observation of apoptosis made with the quantitative assays.

Target cells labeled in their DNA with ^{125}I-UdR or [^{3}H]TdR, *but not* labeled cytoplasmically with ^{51}Cr, are incubated with NK cells in microcentrifuge tubes as described in **Subheading 3.2.2.** In general, 1×10^4 ^{125}I-UdR-labeled or 1×10^5 [^{3}H]TdR-labeled targets should be used per condition to insure that sufficient radioactivity is present to detect target cell-derived DNA fragments following electrophoresis.

3.4.1. Assay

1. Place 100 µL of radiolabeled target cells in a 1.5-mL microcentrifuge tube labeled "B" and gently mix with either 100 µL of TCM (for spontaneous release) or with various numbers of effector cells (10^4–10^7) in 100 µL TCM (experimental).
2. Centrifuge tubes (200*g*; 10 min; 22°C) to establish cell-to-cell contact. Incubate for 1–16 h at 37°C. The incubation time will vary depending on the cells being assayed.
3. At the end of the desired incubation period, add 300 µL of ice-cold PBS to each microcentrifuge tube and pellet intact cells and large debris by centrifugation (200*g*; 10 min; 22°C).
4. Carefully withdraw culture supernatant, place in tube labeled "S," and set aside. The S fraction contains radiolabeled DNA fragments released from target cells.
5. Lyse cell pellet in B tube by adding 0.5 mL of TTE and vortexing vigorously. Microcentrifuge (13,000*g*; 10 min; 22°C) to separate intact chromatin from fragmented DNA. Carefully transfer the supernatant to a tube labeled "T." TTE lyses the cells releasing radiolabeled DNA fragments.
6. Add 0.5 m: of TE to the pelleted nuclei and large chromatin in tube B.
7. Add 0.1 mL of ice-cold 5 *M* NaCl to the approx 0.5-mL solution in tubes S, T, and B. Vortex vigorously and add 0.7 mL of ice-cold isopropanol to each tube. Vortex vigorously and place at –20°C overnight to precipitate DNA.
8. Microcentrifuge 10 min at maximum speed, 4°C. Carefully remove supernatant by rapidly inverting tube or by aspirating with a disposable pipet tip attached to a vacuum line. With tube upside down, use a cotton-tipped swab to remove any drops adhering to walls of tube, taking care not to disturb the DNA pellet. The precipitated DNA and some of the salt form a loose pellet that may be virtually invisible.
9. Half-fill tubes with ice-cold 70% ethanol and microcentrifuge 10 min at maximum speed, 4°C. Carefully remove and discard supernatant as described in **step 3**.
10. Stand tube in an inverted position over absorbent paper to allow as much remaining supernatant as possible to drain away. After 30 min, place tube upright, and allow to air dry 3–4 h.
11. Add 20–50 µL of TE buffer to DNA pellets and incubate for 24–72 h at 37°C. The time required to solubilize the DNA depends on the amount and average size of the DNA present in the sample. Because this protocol is for a qualitative assessment of apoptosis, analysis of the T fractions is of greatest interest. The DNA in the T fractions is small and can be readily solubilized in TE buffer such that it is ready to apply to the agarose gel within 24 h.
12. Add 10X loading buffer to 1X final concentration. Heat 10 min at 65°C. Immediately apply 10–20 µL of sample to well of a 1% agarose gel and electrophorese using a standard TBE buffer until bromphenol blue dye has migrated to approx 2 cm from end of gel.
13. Visualize radiolabeled DNA by autoradiography or by using a phosphorimager.
14. This protocol is simple and provides reasonably good results. However, the DNA is not thoroughly cleaned of proteins, salts, and detergents. Thus a more thorough DNA isolation method may be required if the results obtained are not satisfactory.

3.5. Flow Cytometric Quantification of Apoptosis Using TUNEL Staining

Terminal deoxynucleotidal transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) is a method for detecting apoptotic cells that exhibit double-stranded DNA fragmentation ***(16,17)***. This protocol outlines a method for quantifying TUNEL-positive cells by flow cytometric analysis. In addition, it is compatible with simultaneous multicolor cell surface staining allowing quantification of both target and NK cell death by performing TUNEL analysis immediately following cell surface staining. *See* **Note 6** for further information regarding the TUNEL assay.

1. Incubate a minimum of 5×10^5 target cells with an appropriate number of effector cells in a 12×75-mm polypropylene tissue culture tube in a total volume of 1 mL TCM.
2. At the end of the incubation period, fill the tube with ice-cold PBS and centrifuge (200*g*; 10 min; 4°C) to pellet cells. Discard supernatant.
3. Add 1 mL of ice-cold 4% paraformaldehyde (prepared in PBS) and tap gently to mix (*see* **Notes 8–11** for further information regarding fixation and permeabilization).
4. Incubate 15 min on ice and transfer to a 1.5-mL microcentrifuge tube.
5. Pellet cells by centrifugation (400*g*; 5 min; 4°C), discard supernatant and wash once with 1.5 mL of PBS.
6. Add 200 µL PBS, tap gently to resuspend.
7. Add 1 mL of ice cold 70% ethanol pipetting gently to mix (**see Notes 8** and **11**).
8. Place at –20°C for at least 3 d (*see* **Notes 9–11**).
9. Pellet cells by centrifugation (600*g*; 5 min; 4°C), discard supernatant and wash twice with 1.5 mL of PBS. Be certain that cells are being pelleted efficiently as fixed cells are more difficult to pellet.
10. Remove as much of the supernatant as possible and discard.
11. Add 0.5 mL of TdT reaction buffer, pellet cells, and remove as much supernatant as possible by inverting the tube and touching the lip to a cotton-tipped swab after decanting.
12. Add 50 µL of the TdT/FITC–dUTP reaction mixture. Protect cells from light from this step forward.
13. Incubate for 60 min at 37°C.
14. Add 400 µL of PBS containing 0.1% BSA. Pellet cells by centrifugation (600*g*; 5 min; 4°C).
15. Wash once more with 1.5 mL of PBS, resuspend cells in 0.5 mL of PBS
16. Perform flow cytometric analysis, gating cells on a control sample consisting of target cells incubated alone and treated as described in **steps 1–15** but omitting the TdT from the 50-µL reaction mixture in **step 12**.
17. For multicolor analysis to detect target or effector cells separately, perform immunofluorescence cell surface staining prior to the TUNEL procedure and resuspending cells in PBS. It is important to use PE- or Texas red-, but not

allopyocyanin-, conjugated antibodies for cell surface staining as these fluorochromes are not destroyed by the fixation steps used in the TUNEL procedure.

18. A commercial kit can be used in place of this protocol and following the manufacturer's instructions; however, purchasing the individual reagents is much more economical.

3.6. Distinguishing Fas-Mediated from Granule Exocytosis-Mediated Target Cell Apoptosis

NK cells predominantly make use of granule exocytosis to kill their targets; however, it has recently been reported that NK cells can express Fas ligand and kill targets expressing the Fas receptor (**Ref.** *7,8*; *see* also **Note 3**). It is important to note that some target cells that are sensitive to NK cell-mediated killing, such as YAC-1, are capable of being killed via Fas receptor whereas others, such as K562, are not. Thus it is possible to discriminate between Fas-mediated and granule exocytosis-mediated target cells apoptosis on this basis alone.

With target cells that express the Fas receptor, the contribution of Fas-mediated apoptosis to cytolysis can be determined in three ways. First, the assay can be performed in the presence of EGTA. EGTA chelates extracellular calcium which is required for granule exocytosis- but not Fas-mediated apoptosis ***(18)***. Second, Fas-mediated apoptosis can be blocked by soluble Fas-Fc ***(18)***. Finally, Fas-mediated apoptosis can be blocked with anti-Fas ligand antibody ***(19)***.

When performing assays in the presence of EGTA, it is critical to understand that extracellular calcium is required not only for granule exocytosis but also for receptor-mediated activation of the NK cell to express Fas ligand ***(20)***. To overcome this potential underestimation of NK cell activity, effector cell populations can be treated with PMA plus Ionomycin prior to addition to the assay. Pretreatment with PMA + Ionomycin results in expression of Fas ligand by lymphocytes that can then kill target cells expressing Fas receptor in the presence of EGTA.

The strategies for discriminating between Fas- and granule exocytosis-mediated apoptosis can be applied to any of the protocols described above in **Subheadings 3.1.–3.5.**

3.6.1. Using PMA and Ionomycin to Stimulate NK Cell Effector Populations to Express Fas Ligand

This subprotocol describes a method for treating NK cell effector populations with PMA and Ionomycin to induce expression of Fas ligand prior to coincubation with target cells.

1. Pellet NK cell effectors and resuspend in 2 mL of TCM in 12 × 75 mm polypropylene tissue culture tube.
2. Add 2 μL of 1000× PMA stock solution (final conc. = 5 ng/mL). Vortex thoroughly to mix. PMA is only slightly soluble in aqueous solutions so immediate vortexing is required.

3. Add 2 µL of 100× Ionomycin stock solution (final conc. = 500 ng/mL). Vortex thoroughly to mix. Ionomycin is only slightly soluble in aqueous solutions so immediate vortexing is required.
4. Incubate for 2–3 h at 37°C.
5. Wash cells twice with TCM and resuspend in TCM at the appropriate concentration for use in cytotoxicity and/or apoptosis assays as described in **Subheadings 3.1.–3.5.**

3.6.2. Using EGTA to Inhibit Granule Exocytosis-Mediated Apoptosis

1. Resuspend target cells at the appropriate concentration for use in cytotoxicity and/or apoptosis assays as described in **Subheadings 3.1.–3.5.** in TCM alone or in TCM containing EGTA and $MgCl_2$ such that the final concentration of EGTA and $MgCl_2$ present in the assay is 5 m*M* and 10 m*M*, respectively.
2. TCM containing EGTA and $MgCl_2$ is prepared fresh using the EGTA/$MgCl_2$ stock solution described in **Subheading 2.**
3. Comparing results obtained with assays run in the presence (granule exocytosis-mediated and Fas-mediated apoptosis) vs absence (Fas-mediated apoptosis only) of extracellular calcium gives an estimation of the relative contribution of each pathway to the cytolytic potential of a given NK cell effector population.

3.6.3. Using Soluble Fas–F_c and Anti-Fas Ligand Antibody to Inhibit Fas-Mediated Apoptosis

Soluble Fas-F_c and anti-Fas ligand antibody are added to assays at a final concentration of 10 µg/mL and 5 µg/mL to block Fas-mediated apoptosis.

4. Notes

1. **Figure 1** shows a schematic representation of how NK cell-induced target cell apoptosis is thought to proceed. Despite an enormous amount of progress in the last few years, the mechanism of apoptosis remains poorly understood at this time, even in systems that have been extensively studied. As discussed in greater detail below, it is impossible to predict whether DNA fragmentation in a given cell type undergoing apoptosis will involve double-stranded cleavage, single-stranded nicking, or any fragmentation. Similarly, not all cells contain the same types of caspases and not all caspases are activated by different inducers in the same cell type. Therefore, when examining NK cell-mediated killing involving a cell type or effector cell population or molecule that has not been reported, an empirical approach must be undertaken.
2. It is important to understand that apoptosis was first—and probably is still best—described morphologically. This point cannot be stressed enough to investigators who are new to the field and who may consider morphological studies too subjective and/or cumbersome. Our own experience indicates that apoptosis assays based on biochemical changes or flow cytometric analyses should always be backed up with morphological studies. It is extremely important to acquaint oneself with normal chromatin distribution in the cell line being investigated; differ-

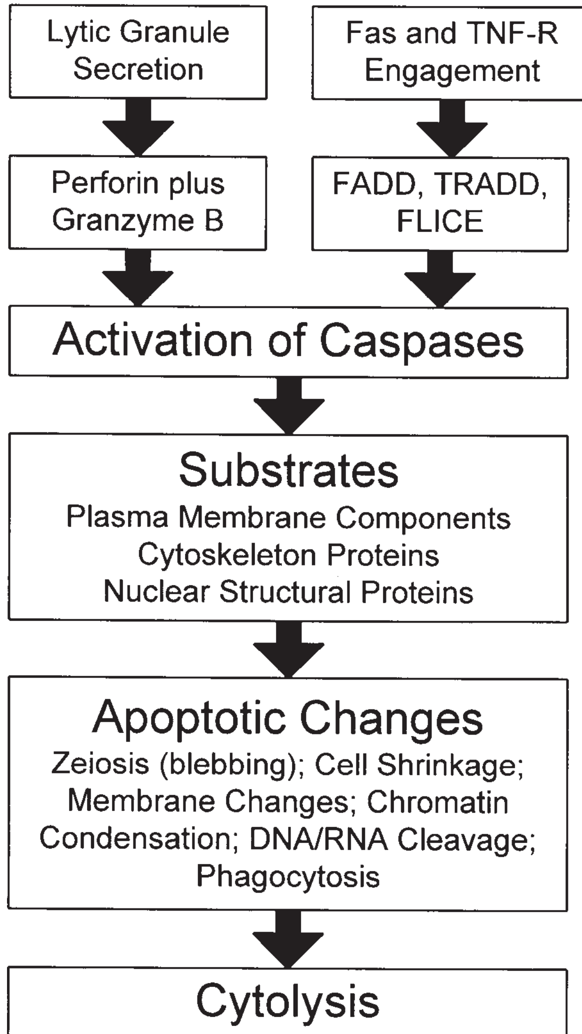

Fig. 1. Schematic representation of NK cell-induced apoptosis. In brief, NK cells can use two mechanisms to induce apoptosis in their targets. Mechanism 1 involves secretion of lytic granules; apoptosis is induced by granzymes in combination with perforin. Granule-mediated exocytosis requires engagement of the NK cell receptor and extracellular calcium is required for granule secretion as well as perforin polymerization. Mechanism 2 involves expression of Fas ligand by the NK cell which can induce apoptosis in target cells expressing functional Fas receptor. Expression of Fas ligand requires engagement of the NK cell receptor and extracellular calcium; however, once Fas ligand is expressed, induction of apoptosis proceeds in the absence of extracellular calcium. Both mechanisms lead to activation of caspases and cleavage of substrates resulting in biochemical changes in the target cell encompassing apoptotic cell death.

ent cell types may present strikingly different patterns of "normal" chromatin organization. Similarly, it is a good idea to obtain experience in recognizing apoptotic nuclei in the cells under investigation. A convenient way to induce apoptosis in most cell types is to treat them with 20–100 μ*M* valinomycin (Sigma) for 1–24 h ***(10,21)***. Valinomycin is a potassium ionophore that induces apoptosis rapidly in most cultured cell lines and is useful as a positive control. It is prepared as a 5 m*M* stock solution in EtOH which can be stored indefinitely at –20°C or –70°C. It is important to vortex samples thoroughly after addition of valinomycin and to not add the compound in such a way that the final concentration of EtOH in the cell cultures exceeds 2% (v/v). **Figure 2** shows the typical change in nuclear chromatin morphology that accompanies apoptosis induced by valinomycin. As a positive control for necrosis, cells can be heated to 43–45°C for 2 h.

3. NK cells can use two separate mechanisms to induce apoptosis. The first, and predominant, mechanism involves secretion of lytic granules that contain perforin and a variety of enzymes, the most important of which appears to be granzyme B (*see* also Chapter 13). Granzyme B is a potent activator of many of the caspases that have been identified in apoptosis. In purified form, the ability of granzyme B to induce apoptosis requires sublethal concentrations of perforin ***(6)***. Thus it is important to understand that some NK cell populations may induce necrosis before granzyme B is able to induce apoptosis, particularly those effector cell populations that express high amounts of perforin. This could explain the conflicting reports concerning the ability of NK cells, or lytic granules derived from these cells, to induce apoptosis vs necrosis ***(1,2,22)***. The second mechanism involves expression of Fas ligand, and perhaps tumor necrosis factor (TNF), by activated NK cells. This pathway is probably of little consequence in the killing of targets directly recognized by NK cells as it requires much longer to initiate than the perforin/granzyme B pathway. However, it could play a role in killing bystander cells that express functional Fas receptor and should be considered. Potential targets for bystander killing by NK cells in vivo could be activated T and B cells, macrophages, and certain subsets of bone marrow progenitors ***(23,26)***.
4. Although the demonstration of extensive DNA fragmentation and a nucleosome ladder have been used as absolute evidence for apoptosis, this conclusion is not warranted as there are now numerous examples of cells that do not degrade their DNA to oligonucleosomes during apoptosis. For example, some cells undergo random double-stranded DNA cleavage only every 50–300 kilobases; this event may actually precede internucleosomal cleavage in most cell types undergoing apoptosis. Nevertheless, the large fragments cannot be detected in conventional sedimentation or agarose gel electrophoresis assays but instead are observed using pulsed-field electrophoresis or by neutral sucrose-gradient density centrifugation ***(27)***. Once again, it is important to note that atypical chromatin cleavage was observed using regimens that induced typical apoptosis in related cells; the changes were not observed if the cells were killed via necrosis. Cells that are necrotic may also undergo a minor amount of DNA fragmentation although this is typically not internucleosomal in nature.

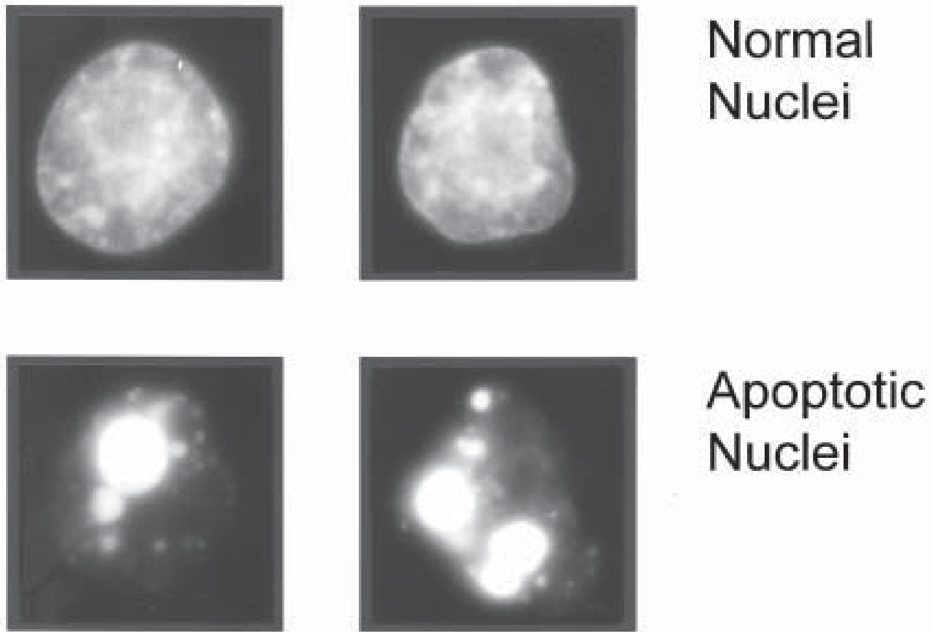

Fig. 2. Nuclear changes typical of apoptotic cell death. Murine leukemia cells were incubated alone (*top panels*) or with 50 µm valinomycin (*bottom panels*) for 2 h at 37°C. Cells were stained with Hoechst 33342 and observed by UV microscopy. Normal nuclei have "structure;" variations in fluorescent intensity reflect the distribution of euchromatin and heterochromatin. Apoptotic nuclei, in contrast, have highly condensed chromatin that is uniformly fluorescent. This can take the form of crescents around the periphery of the nucleus, or the entire chromatin can be present as one or a group of featureless, bright spherical beads.

5. CAUTION: Acridine orange, ethidium bromide, and propidium iodide have been found by the Ames test to be highly mutagenic and should be handled with care.
6. One practical drawback of the TUNEL technique is that it is much more expensive to perform than other methods (e.g., staining with AO + EB costs approx $0.10 per sample vs as much as $8.00 per sample using TUNEL). It is highly recommended that TdT as well as FITC-dUTP be titrated by the investigator to avoid using them in excessively high concentrations. The wash step with TdT buffer (**Subheading 3.5., step 11**) optimizes conditions for TdT and is critical for achieving good end labeling with dUTP and TdT at low concentrations.
7. Radiolabeling DNA may lead to high (>30%) values for spontaneous DNA fragmentation which may not be associated with apoptotic morphology. If the cells being tested are infected with mycoplasma, the mycoplasma may incorporate the radiolabel, which will appear in the S or T fractions during quantification, lead-

ing to an apparently high spontaneous DNA fragmentation even though the cells appear healthy. In general, high levels of spontaneous DNA fragmentation suggest that the cells are being adversely affected. This finding should not be ignored and adequate measures should be taken to obtain lower values of spontaneous fragmentation and apoptosis.

8. The final ethanol concentration used to permeabilize the cells should be 60–70% as higher concentrations will cause excessive shrinkage and difficulties in obtaining a cell pellet as well as acquiring data in the flow cytometer.
9. For optimal results it is recommended that the cells be stored in 70% ethanol for at least 3 d (**Subheading 3.5., step 8**) before continuing with end-labeling.
10. In some cells reversing **steps 3–8** such that cells are fixed/permeabilized with 70% ethanol prior to fixation with paraformaldehyde may provide better results.
11. To avoid excessive clumping of cells, it is recommended that paraformaldehyde and ethanol be added slowly as the cells are gently mixed.

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13

Methods for Detecting Lytic Granules in Natural Killer Cells

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1. Introduction

Natural killer (NK) cells are able to identify and destroy target cells with absent or altered major histocomaptibility complex (MHC) class I, thereby mediating "natural cytotoxicity." They also have the capacity to kill antibody-coated target cells by antibody-dependent cellular cytotoxicity (ADCC). The proteins that mediate these killing processes are stored in specialised secretory granules in the NK cells. When the NK cell recognizes its target these granules polarize toward the point of membrane contact and secrete their contents into the space between the cells. The major components of these granules are known (reviewed in **ref.** ***1***) and one of these proteins, perforin, inserts into the target cell membrane polymerizing to form a pore through which other proteins (granzymes) can pass and initiate cell death through the apoptotic pathway. Perforin alone can lyse the target cells by the formation of membrane pores although this process is relatively slow and it is likely that the granzymes are critical in effecting rapid cell death in vivo.

Experimentally the detection of lytic granules is useful in several ways: (1) as markers of an active NK cell population in vivo; (2) for monitoring the ability of NK cells to destroy targets, and (3) for visualizing the action of NK cells during target cell destruction (*see* **Notes 1** and **5**). Most of these techniques can also be used to detect lytic granules in cytotoxic T lymphocytes (CTLs) as it is only the recognition of targets that differs between NK cells and CTLs, and the method of target destruction is identical for both.

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
Edited by: K. S. Campbell and M. Colonna © Humana Press Inc., Totowa, NJ

The following sections describe the various ways for detecting lytic granules both before and after secretion from the NK cell. Several of these techniques rely on the use of antibodies that are available. Because the lytic granules of NK and CTL cells are also their lysosomes (reviewed in **ref. *2***), it is possible to detect these organelles using markers or assays that recognize lysosomal hydrolases. The use of suitable lysosomal markers is also described.

2. Materials

2.1. Immunofluoresence

1. Poly-L-lysine (Sigma).
2. Phosphate-buffered saline (PBS) containing 1% fetal calf serum (FCS).
3. Mounting medium (e.g., 10% PBS/90% glycerol).
4. 2.5% DABCO (1,4-diazabicyclo[2.2.2]octane; Sigma).

2.2. Enzymatic Assays for Granzymes and Perforin

1. *N*-α-carbobenzoxy-L-Lys-thiobenzyl ester (BLT; Sigma).
2. *tert*-Butyloxycarbonyl-Ala-Ala-Asp-thiobenzyl ester (Enzyme Systems Products).
3. Lysis buffer: 50 m*M* Tris-HCl, pH 8.0; 1% Triton X-100; 1 *M* NaCl (*see* **Note 4**).
4. Substrate buffer: 0.1 *M* HEPES, pH 7.0; 0.3 *M* NaCl; 1 m*M* EDTA; 0.1 m*M* substrate.
5. 0.11 m*M* 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB; Sigma).
6. Microplate reader (Molecular Devices).
7. Alsever's solution: 0.11 *M* dextrose, 0.072 *M* NaCl, 0.027 *M* sodium citrate.
8. Gelatine veronal buffered saline (GVB; tablets available from Sigma).
9. Melittin (Sigma)

2.3. Detecting Lytic Granule Secretion

1. Serum-free medium (such as AIM V medium from Gibco).
2. Substrate buffer: 34 mg of *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide (Sigma) in 20 mL of 50 m*M* sodium citrate, pH 4.8; 0.2% Triton X-100. This buffer can be made without Triton X-100 and stored indefinitely at –20°C.
3. Stop buffer (for 500 mL): 33 m*M* glycine (4.99 g), 83 m*M* sodium carbonate (4.4 g), 67 m*M* NaCl (1.96 g), adjusted to pH 10.7.

2.4. Killing of Target Cells

1. ^{51}Cr (Amersham, UK).
2. 96-Well cell harvester and reader (Pharmacia).
3. [^{3}H]Thymidine (Amersham, UK).

3. Methods

3.1. Immunofluoresence

Lytic granules within NK cells can be identified by using immunofluorescence and antibodies against granule proteins. In addition, polarization of the

lytic granules following recognition of a target cell can be visualized by immunofluorescence following conjugate formation. This section outlines protocols for the detection of lytic granules within cells.

3.1.1. Staining the Lytic Granules

Lytic granules can be detected with antibodies against perforin, the granzymes, lysosomal hydrolases (e.g., cathepsins), or lysosomal membrane proteins (e.g., lamps). Antibodies are available that react against the human and mouse antigens ***(3)***. An alternative for other species is to use biotinylated soybean trypsin inhibitor (SBTI) to recognize trypsin-like serine proteases as this is not species-specific and works well in conjunction with an avidin-fluorescein ligand.

1. Precoat a glass slide or coverslip with poly-L-lysine at room temperature for 15 min.
2. Rinse the slide with distilled water.
3. After washing cells with PBS, allow cells to settle on slide for about 5 min at 37°C.
4. Fix cells on ice for 5 min using methanol that has been prechilled at –20°C (*see* **Note 2**).
5. Transfer slide or coverslip into PBS containing 1% FCS. The slide can now be stored in PBS/1% FCS at 4°C until ready for staining.
6. Add antibody or antibodies for staining, diluted to the optimal concentration in PBS/1% FCS and incubate in a humidified chamber for 30 min or longer at room temperature. For 13-mm coverslips, 100 µL of antibody can be placed on a piece of parafilm, and the coverslip can be placed inverted over the antibody with a pair of fine tweezers.
7. Wash away any unbound antibody with PBS/1% FCS. For coverslips this can be done in a 6-cm dish with three sequential 5-min washes with PBS/1% FCS.
8. Add the second, conjugated antibody (e.g., fluorescein isothiocyanate [FITC]-goat antimouse IgG), diluted appropriately in PBS/1% FCS and incubate for 30 min in a humidified chamber at room temperature (*see* **Note 6**).
9. Wash as before and mount the coverslip inverted on a slide with a mounting medium containing an antifade agent such as 2.5% DABCO.

3.1.2. Visualizing the Polarization of Granules

The lytic granules polarize rapidly in response to recognition of a target cell. By allowing conjugates to form for 15–20 min prior to analysis more than 50% of the conjugates are observed to have lytic granules polarized toward the site of membrane contact. This section describes a method to visualize this polarization of granules.

1. Precoat a glass slide with poly-L-lysine for 15 min at room temperature.
2. Rinse the slide with distilled water.
3. Mix NK cells and targets in a 1:1 ratio in media at approx 10^6 cells/mL, and place on the poly-L-lysine-coated slide at 37°C in a humidified chamber for 15–20 min. This allows the cells time to conjugate and adhere to the glass slide.

4. Fix cells immediately as outlined in **Subheading 2.1.1.** Following fixation, slides can be stored in PBS/1% FCS at 4°C until ready for staining.
5. Proceed with staining as outlined in **Subheading 2.1.1.**

3.2. Enzymatic Assays for Granzymes and Perforin

The lytic granules contain proteins whose activities are readily detectable using appropriate assays. For example, the granzymes can be detected by cleavage of synthetic substrates, and perforin can be assayed by its ability to bind and insert into red blood cell membranes and thereby release hemoglobin. This section outlines methods for the detection of the lytic granule proteins.

3.2.1. Enzymatic Detection of Granzymes A and B

Granzymes A and B are serine proteases; thus their presence can be detected by hydrolysis of specific substrates. For granzyme A, which has substrate specificity similar to trypsin (i.e., cleavage after Arg or Lys residues), the specific substrate is *N*-α-carbobenzoxy-L-Lys-thiobenzyl ester (BLT). For granzyme B, which has Asp-ase activity (i.e., cleavage after Asp residues) the substrate is *tert*-butyloxycarbonyl-Ala-Ala-Asp-thiobenzyl ester.

1. Pellet cells and wash twice in serum-free medium or PBS.
2. Prepare a cell lysate as follows: Lyse cells at 10^7 cells/mL in lysis buffer by adding lysis buffer to cell pellet, and vortex-mixing and leave on ice for 30 min.
3. Spin out cell debris at 13,000 rpm in a microfuge at 4°C and store the lysate at –20°C. The protein concentration of the cell lysate can be determined using the Bio-Rad protein assay to calculate specific activities for the enzyme assay.
4. To assay for granzyme activity, dilute 50 µg of cellular protein to 20 µL total volume (diluted with lysis buffer) in a 96-well plate. Add 180 µL of substrate buffer. Color development owing to nonspecific indicator reduction can be determined by performing identical assays in the absence of substrate. Negative controls should be prepared by the addition of 20 µL of cell lysis buffer to the same reactions including substrate and inhibitor.
5. Incubate at 37°C, reading the maximal rate of change (V_{max}) of absorbance at 405 nm over 30 min on a microplate reader.
6. Calculate the specific activity of granzymes A or B where one unit is defined as the amount of enzyme activity required to produce a change in absorbance at 405 nm of 1 OD at 37°C. If granzyme B is difficult to detect, add more substrate to the assay buffer and repeat the assay.

3.2.2. Hemolytic Assay for Perforin

Perforin alone is able to introduce pores into the membranes of target cells. This assay measures the release of hemoglobin from lysed red blood cells incubated with a solution containing perforin. Note that different species of blood are differentially sensitive to different species of perforin, so initially a variety

of blood types should be tested to determine the most sensitive blood type for the perforin being used.

1. Once gathered, blood cells (sheep, rabbit, guinea pig, human, or other) should be stored in Alsever's solution at 4°C.
2. On the day of the assay, pellet blood in centrifuge. Wash blood with GVB until supernatant appears clear.
3. Make the red blood cell solution for the assay by adding a certain percentage of packed cell volume to GVB. For example, for a 1% red blood cell solution, add 100 µL of packed cells to 10 mL of GVB. The percentage of red blood cells to be used must be determined empirically. Generally, if the sample of perforin to be used in the assay is expected to be quite active then a higher percentage of red blood cells can be used in the assay. To increase sensitivity, decrease the concentration of red blood cells used.
4. The solution must contain $CaCl_2$ for perforin to be active. Add $CaCl_2$ to the blood cells to a final concentration of 5 m*M*. Note that if there is EDTA in the fraction/sample to be analyzed then additional $CaCl_2$ must be added to account for this (e.g., a final concentration of 10 m*M* instead of 5 m*M*). Also note that detergent in the perforin fraction/sample will disturb the assay!
5. Place an aliquot (10–50 µL) of the fraction/sample of interest into a round-bottom 96-well plate. Use melittin (5 µL) as a positive control, and an equivalent volume of an appropriate buffer alone as a negative control.
6. Add 200 mL of red blood cell solution to each well, and incubate for 20 min at 37°C.
7. Centrifuge plate at 1800 rpm at 4°C for 3 min.
8. Transfer 100 µL of supernatant to a 96-well flat-bottom plate.
9. Read absorbance at 540 nm if a high concentration of blood cells was used or 415 nm if a lower concentration of blood cells was used.
10. Calculate % lysis as follows:

$$\% \text{ lysis} = 100 \times (OD_{sample} - OD_{spontaneous})/(OD_{melittin} - OD_{spontaneous})$$

11. To determine hemolytic units, perform the assay using a dilution series of the fraction/sample and then plot % lysis vs volume of sample used. Calculate the volume of sample required to induce 50% lysis. Total hemolytic units are the total number of units in the sample (i.e., total volume of sample divided by the volume required to induce 50% lysis).

3.3. Detecting Lytic Granule Secretion

Because lytic granules are also the lysosomes of NK cells, then lytic granule secretion can be detected by measuring either the release of cytotoxic components or the release of lysosomal hydrolases. Both enzymatic assays and biological assays can be used to detect the release of lytic granule components, as outlined below.

3.3.1. Enzymatic Assays to Measure Lytic Granule Secretion

Lytic granule secretion can be triggered either by incubation of NK cells with appropriate target cells or by crosslinking of receptors that trigger

degranulation (e.g., CD16) ***(4)***. Incubation with targets is exactly the same as the incubation set up for killing assays (*see below*) but using unlabeled target cells and assaying the supernatant for enzymatic activities for granzymes A, B, or β-hexosaminidase. Although both granzymes A and B could be used as markers for lytic granule secretion, the protocol below uses β-hexosaminidase, which we find to be the most sensitive and quantitative assay for degranulation.

This method uses crosslinking of a cell surface receptor on the NK cell in order to trigger degranulation.

1. Using a 96 well round-bottomed plate add 100 μL of CD16 antibody at 1 μg/mL in PBS to each well.
2. Incubate for 1 h at room temperature or overnight at 4°C to coat the wells with antibody.
3. Block nonspecific binding with media containing >1% FCS for 1 h at room temperature.
4. Add 100 μL of cells to each well of this "incubation" plate at a concentration of 5×10^6/mL (i.e., 5×10^5 cells per well) in serum-free medium (such as AIM V medium from Gibco) (*see* **Note 4**). Use triplicate samples. As controls plate identical samples into uncoated wells. In addition, in a separate "assay" plate (uncoated) add 100 μL of cells adding Triton X-100 to a final concentration of 0.1%, mix by pipeting, and leave on ice.
5. Meanwhile transfer the incubation plate to 37°C for 15 min.
6. Centrifuge the plate at 1000 rpm for 5 min at 4°C and place on ice.
7. Transfer 20 μL of supernatant from each sample into the assay plate on ice. Be careful not to remove any cells from all samples except those intended for measuring total enzyme activity. To these samples resuspend the cells, add Triton X-100 to 0.1%, mix by pipeting, and remove 20-μL aliquots for measuring total enzyme activity.
8. Assay β-hexosaminidase activity in both supernatants and lysates by adding 20 μL of substrate to each well.
9. Incubate at 37°C for 1 h.
10. Add 200 μL of stop buffer to each well and read absorbance at 415 nm.
11. Calculate % total hexosaminidase release as follows:

$$\% \text{ release} = 100 \times (OD_{supernatant})/OD_{lysate})$$

It is often useful to establish the time course of release by incubating the cells on the antibody-coated wells for different time points between 5 min and 2 h.

3.4. Killing of Target Cells

3.4.1. Chromium Release

Cytotoxicity of NK cells can be measured by assaying the release of ^{51}Cr from labeled target cells. ^{51}Cr is thought to bind to cytoplasmic proteins of the cell. Following NK cell attack on the target cell these proteins are released to

the surrounding media, providing a marker for the cytoplasmic events of apoptosis.

To test ADCC, target cells can be coated with an antibody that recognizes a receptor on the NK cell that triggers degranulation when crosslinked (e.g., CD16), provided that the target cells express an Fc receptor. Alternatively, target cells lacking the MHC class I molecule can be used to test natural cytotoxicity.

1. Label appropriate target cells with ^{51}Cr as follows: Wash with PBS and pellet 10^6 target cells (this is sufficient for 100 assays).
2. Add 100 µL of ^{51}Cr to pelleted cells and allow to label at 37°C for a minimum of 1 h.
3. Wash cells three times with PBS/1% FCS.
4. Resuspend labeled targets in 10 mL RPMI (final concentration 10^5 cells/mL). At this point, an appropriate antibody can be added to the target cells at twice the working concentration.
5. Wash effector cells and resuspend in RPMI at an appropriate dilution. Usually a range of effector-to-target ratios will be used, so a number of dilutions will be required.
6. Combine 100 µL of labeled target cells (i.e., 10^4 cells/well) with 100 µL of effector cells in a 96-well round-bottom plate. All samples should be plated in triplicate. As a control for spontaneous release, combine 100 µL labeled target cells with 100 µL of RPMI. As a measure of total release combine 100 µL labeled target cells with 100 µL of 1% Triton X-100 in PBS. Incubate at 37°C for 4 h.
7. Pellet cells by centrifugation at 1000 rpm for 10 min at 4°C.
8. Transfer 100 µL of supernatant to a tube for counting.
9. Count ^{51}Cr release using a gamma counter.
10. Calculate % lysis using the following formula: % lysis = 100 × (experimental counts-spontaneous counts)/(total counts-spontaneous counts).

3.4.2. Killing of Target Cells—[^{3}H]Thymidine Release

A second method of measuring cytotoxicity is to measure the release of [^{3}H]thymidine from labeled target cells incubated with NK cells. Rather than a measure of the cytoplasmic events of apoptosis, this is thought to represent the nuclear events of apoptosis and is therefore a complementary technique to the ^{51}Cr release assay. Again, target cells can either be treated with an antibody that induces degranulation in NK cells, or target cells lacking MHC class I molecules can be used. This assay is vastly simplified ***(5)*** by the availability of a 96-well cell harvester and reader in which case the total number of cpm remaining in the cells after killing is measured and release is calculated as the amount remaining in the cells after killing as a percentage of the total cpm incorporated at the beginning of the assay (*see* **Note 3**).

1. The day before the assay check that the target cells are growing well and are not too dense, to allow efficient incorporation of the radioactive thymidine into cel-

lular DNA. A suitable concentration for cells would be 3×10^4 cells/mL but optimal labeling conditions should be established for each target cell line.

2. On the day of the assay, label cells in a final concentration of 5 µCi/mL of [^{3}H]thymidine for 4 h at 37°C. Wash target cells three times in medium and count the cells using trypan blue to check for > 90% viability. Resuspend at a concentration of 5×10^4 cells/mL in medium. An appropriate antibody can be added at this point at twice the working concentration.
3. Wash effector cells and resuspend at 5×10^6 in medium. Serial dilutions can be made to provide at least four effector-to-target ratios.
4. Combine 100 µL of labeled target cells (i.e., 5×10^3 cells/assay) with 100 µL of effector cells in a 96-well plate. All samples should be performed in triplicate. As controls for both spontaneous and total release, combine 100 µL of targets with 100 µL of medium.
5. Incubate at 37°C for 2–4 h. This assay is significantly more sensitive than the chromium assay and normally only half the incubation time used for the chromium assay is required to see the same results.
6. Harvest the plate and obtain the total cpm left in the cells after killing, calculating the percentage DNA degradation as $Z - X/ Z \times 100\%$ where Z is the total cpm in the target cells alone and X is the number of cpm after incubation with NK cells. Ideally Z should be measured from samples taken at both the beginning and end of the assays to ensure that spontaneous lysis is not occurring.
7. Results can then be expressed graphically plotting effector-to-target ratio on a log scale on the *x*-axis and % DNA released on the *y*-axis.

4. Notes

1. Selecting the best method for detecting lytic granules will vary according to the source of NK cells. Directly ex vivo NK cells may not express such high levels of the lytic proteins perforin and granzymes A and B as cells that have been stimulated in vitro for some time. In this case it may be easier to detect granules by virtue of their lysosomal contents, using either antibodies against lysosomal markers or enzyme assays for lysosomal hydrolases. In addition some cell lines may not express all of the lytic markers (e.g., the human NK cell line YT does not express granzyme B ***[6]***).
2. When visualizing lytic granules using antibodies it may be worth testing other fixation conditions as some antibodies work better after defined fixation conditions.
3. The thymidine release assay for killing can be sensitive to the number of cells used. Too many cells per well reduces the efficiency of washing during harvesting and reduces the apparent release of DNA observed. Similarly, although the plates can be frozen before harvesting this can also lead to clumping of the cells which reduces the apparent DNA release.
4. For the assay for β-hexosaminidase Tris was inhibitory to the assay, whereas for the granzyme assays Tris is required. It is important to use serum-free medium when detecting the release of lysosomal and granzyme substrates from NK cells as serum can often cleave the substrates alone.

5. To verify the presence of the marker of choice it may be useful to carry out a Western blot of a cell lysate made with 2×10^7 cells /mL. This allows a single lysate to be rapidly screened for protein expression of several markers and confirms the lack of cross-reactivity by providing the molecular weight of the protein.
6. Because NK cells express the FcR then care must be taken to ensure antibody specificity during staining. It is always important to include a control with the second stage antibody alone.

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14

Fluorogenic Substrates as Detectors of Caspase Activity During Natural Killer Cell-Induced Apoptosis

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1. Introduction

Natural killer (NK) cells mediate target cell lysis by two independent pathways, one involving exocytosis of preformed granules (which contain perforin and granzymes A and B), the other requiring ligation of CD95 on target cells with CD95 (APO-1/Fas) ligand on the effector cell ***(1–3)***. Both processes lead to target cell apoptosis and lysis ***(3)***. Perforin and granzymes A (and B) have been implicated as main contributors to membrane and/or nuclear disintegration of target cells by the secretory pathway ***(3)***, whereas granzyme B and CD95 induce early DNA fragmentation by activation of multiple cysteine proteases of the caspase cascade, including caspase-8 (FLICE) ***(4)*** and caspase-3 (CPP32) ***(5)***. The exact mechanisms governing NK-induced death are far more complicated than delineated herein, and the in vivo relevance of their different components require further study. The roles of KIR and class I major histocompatibility complex (MHC) in the effector/target interaction are described in other chapters of this book. Caspases (*C*ysteine-*Asp*art-*ases*) are key effector molecules involved in programmed cell death (PCD), although some of them can also participate in other physiological processes such as activation of proinflammatory cytokines. Since the discovery of the prototype death protease *Ced-3* in *Caenorhabditis elegans* ***(6)*** more than 15 mammalian and invertebrate caspases have been described ***(7)***. They can be divided into different subclasses, based on structural similarities in either prodomains, or their catalytic subunits. Some of these structural similarities correlate with specificity, as discussed later (*see*

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
Edited by: K. S. Campbell and M. Colonna © Humana Press Inc., Totowa, NJ

Table 1
Cleavage Sites of Some Known Caspase Substrates

	Cleavage site		
Enzyme	P_4 $\mathbf{P_1}$	P_0	Substrate
Caspase-1	YVA**D**	G	optimized substrate
	FEA**D**	G	pro-IL-1ß
	FED**D**	G	pro-ICE (casp.-1)
	IET**D**	S	pro-CPP32 (casp.-3)
	YVP**D**	S	p110 PITSLRE kinase α2-1
Caspase-3	DEV**D**	G	PARP
	DEP**D**	S	SREBP-1
	YVP**D**	S	p110 PITSLRE kinase α2-1
CED-3	DQM**D**	G	baculovirus p 35
	LEA**D**	S	ICE-rel-III
	DNR**D**	G	CED-3
	DMQ**D**	N	PKC-δ
	DEA**D**	G	Rb
Unknown	VEI**D**	N	lamin B
	ELP**D**	G	actin

The P_1 aspartate is indicated in bold. Consensus between caspase-3 and Ced-3 substrates in P_4 position is underlined.

It is also advisable to consult Thornbery et al., 1997 ***(14)***, for additional information concerning substrate sequence specificity for different caspases.

Table 1). All caspases, however, are specific for aspartic acid in the P_1 position of their substrates. Caspases exist as latent zymogens (**Fig. 1**) that contain an N-terminal precursor domain followed by the region that forms the two subunits of the catalytic domain after proteolytic processing. The core of the catalytic center of these enzymes is formed by a conserved amino acid sequence: QACXG (for caspase-8 X = Q; for most of all other known caspases X = R). The cysteine at the core of this peptide directly participates in catalysis and defines these proteases as cysteine proteases. The proforms of caspases are activated by proteolytic cleavage at specific aspartic residues. Generally, an initial cleavage event occurs that separates the C-terminal "short" subunit of the protease from the rest of the molecule, allowing assembly of an active protease that autocatalytically cleaves off its N-terminal prodomain to generate the mature active enzyme. Once activated, many caspases can propagate proteolytic activation of other family members by processing their proforms

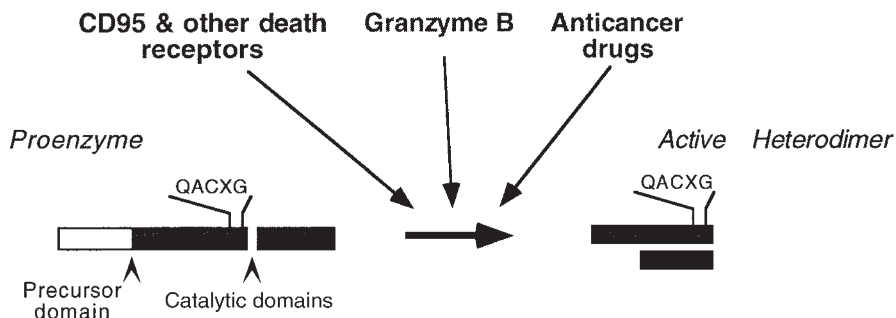

Fig. 1. Schematic representation of caspase activation. Caspases exist as inactive pro-enzymes. Proteolytic (auto)activation leads to formation of an active hetero-tetramer containing two smaller and two larger subunits, one of each derived from a common proenzyme polypeptide. For clarity, only the hetero-dimer derived from a single proenzyme is indicated. See text for more details.

through cleavage at specific aspartic acid residues. A family of cellular inhibitors of caspases (IAPs) has been identified ***(8–10)***. In addition, some viruses are known to produce caspase inhibitory proteins such as CrmA of pox viruses and p35 of the insect baculoviruses. Several synthetic peptidyl inhibitors of caspases have been designed by taking advantage of the known specificity of caspases for certain substrates (*see* **Table 1**). Usually, in such an inhibitor, the P_1 aspartate is modified by chloro-, fluoromethyl-ketone (cmk-, fmk-) or an aldehyde group. The aldehyde-based compounds are reversible inhibitors, whereas the cmk-, fmk-based reagents form covalent interactions with the active-site cysteine of caspases and thus are irreversible inhibitors. Tetra-peptides may be better inhibitors in vitro, but they usually penetrate poorly through cellular membranes. Therefore they are more useful for caspase blockage in cellular extracts. For inhibition of caspases in intact cells tri-peptide compounds, especially benzyl-oxyl-carbonyl-Val-Ala-Asp-CH_2F (zVAD-fmk; obtained by Enzyme Systems Products, Dublin, CA), were proven to be very effective. Thus, this broad-range caspase inhibitor is very useful as a control and reference substance for caspase research.

In this chapter we describe fluorometric methods of detection of caspases in cells as well as cellular extracts. The method is based on the increase of fluorescence of $(aspartyl)_2$-Rhodamine 110 (D_2Rhodamine 110) upon removal of aspartyl groups from the substrate (*see* **Note 1** for more information). Liberation of each NH_2- group (previously occupied by aspartate) increases the fluorescence intensity of Rhodamine 110 by 100-fold. Theoretically the signal from cells carrying active caspases should shift four decades on the logarithmic scale. In reality, not all substrate that entered the cell is processed and, in addi-

tion, the signal is partially absorbed by cytoplasmic content. Therefore, the increase of signal intensity is usually significantly lower (1–1.5 on a log scale). The hypo-osmotic condition used in the protocol facilitates substrate loading onto cells and β-mercaptoethanol or dithiothreitol (DTT) protects the cysteine residue in the active center of the caspases from oxidation. Please note that Rhodamine 110 has quite a wide fluorescence spectrum, and the signal collected in the FL-2 channel is only about 30% weaker than in the FL-1 channel (which is usually used to detect the dye). Therefore, only two-color fluorescence should be performed with the second signal being collected in the FL-3 channel (i.e., cy5 surface staining or PI, 7-AAD).

2. Materials

1. D_2Rhodamine 110 (50 m*M*): Stock solution is stable for at least 1 mo at –20°C or for 1 yr at –70°C. A stock solution is prepared by diluting 63 mg of powdered D_2Rhodamine 110 in 1 mL of dimethyl sulfoxide (DMSO) before 1 mL of absolute ethanol is added; the stock solution is then 50 m*M*. The dye dissolves very well in DMSO and the addition of ethanol keeps the stock liquid even at low temperatures, saving time upon stock usage. Because the dye is not very stable in H_2O, be careful not to contaminate the stock solution with it. Higher concentrated stock solutions, i.e., 100–200 m*M*, are also easily achievable. D_2Rhodamine 110 can be synthesized, i.e., by Enzyme Systems Products (Dublin, CA).
2. β-mercaptoethanol (100 m*M*): Dissolve 347µL of absolute β-ME in 50 mL of H_2O. The solution is stable at 4°C for at least 3 mo, protect from light.
3. Phosphate-buffered saline (PBS): Dissolve in 900 mL of H_2O, 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4. Adjust pH to 7.3 and the volume to 1 L, and store at 4°C (you will need it cold).
4. dH_2O: Deionized H_2O, store at room temperature.
5. FACS® Brand Lysing Solution (Becton Dickinson, San Jose, CA; cat. no. 92-0002).
6. Flow cytometer (i.e., FACScan, Becton Dickinson) equipped with blue-light laser (488 nm) and compatible plasticware, (i.e., FALCON no. 2058).
7. Centrifuge compatible with plasticware for flow cytometer.
8. Pipetting equipment.

The materials and equipment listed below are required only for measurement of caspase activity in cell extracts (**Subheading 3.3.**).

9. Lysis buffer: 50 m*M* HEPES, pH 7.3, 1 % Triton X-100, and 10 m*M* DTT. The buffer without DTT can be stored at room temperature, DTT should be added directly before use.
10. Caspase buffer: 50 m*M* HEPES, 100 m*M* NaCl, 0.1% CHAPS (3-[cyclohexylamino]-1-propanesulfonic acid), 10 m*M* DTT, and 10% sucrose. Prepare freshly before use.
11. Bench-top centrifuge.

12. Spectrofluorometer (*see* **Note 2**).
13. Water bath.

3. Methods

3.1. Detection of Caspase Activity in Peripheral Blood Mononuclear Cells (PBMC) from Whole Blood

1. Prepare 1 m*M* solution of D_2Rhodamine 110 in H_2O, from the stock solution.
2. Take 200 µL of whole blood (heparinized) (*see* **Notes 3–6**).
3. Add β-mercaptoethanol to final concentration 10 m*M* (22 µL of 100 m*M* stock solution).
4. Add D_2Rhodamine 110 to a final concentration of 100 µ*M* (24 µL of 1 m*M* stock solution).
5. Add 250 µL of dH_2O (osmotic loading).
6. Incubate for 8–10 min at 37°C.
7. Stop the reaction by pipetting 2 mL of cold PBS (≤4°C) (A smaller amount of cold PBS [i.e., 0.5 or 1 mL] can be added if this simplifies the following staining procedures.) From now on cells have to be stored on ice.
8. Perform the additional individual staining procedures.
9. At the end of staining resuspend blood cells in about 50 µL of cold PBS and perform erythrolysis by addition of 2 mL of Becton Dickinson lysis solution according to the manufacturer's instructions (application of a smaller amount of lysis solution, i.e., 1–1.5 mL causes incomplete lysis of erythrocytes, but can be used at times when this simplifies sample handling).
10. Measure samples by flow cytometry (i.e., use FL-1 channel when FACScan is employed).

IMPORTANT: **The samples have to be measured within 3 h.**

3.2. Detection of Caspase Activity in Isolated PBMC and Other Cells as Well as Cell Lines

1. Dilute an aliquot of D_2Rhodamine 110 to 1 m*M* with H_2O.
2. Prepare 10^5–10^6 cells in 420 µL of culture medium (*see* **Notes 3**, **4**, **6–10**) for additional information).
3. Add β-mercaptoethanol to a final concentration of 10 m*M* (50 µL of 100 m*M* stock solution).
4. Add D_2Dhodamine 110 to a final concentration of 60 µ*M* (30 µL of 1 m*M* stock solution).
5. Add 750 µL of dH_2O (osmotic loading).
6. Incubate for 8–10 min at 37°C.
7. Stop the reaction by pipetting 3 mL cold PBS (≤4°C). Keep cells on ice and proceed with additional staining steps i.e. for detection of specific cell subsets if desired.
8. Measure samples by flow cytometry (i.e., use FL-1 channel when FACScan is employed).

IMPORTANT: **The samples have to be measured within 3 h.**

3.3. Detection of Caspase Activity in Cell Extracts

1. Prepare ~10^6 cells per sample. Perform desired experimental procedures, (*see* **Notes 1**, **3**, **10**, and **11** for additional information).
2. At the end of an experiment pellet cells in eppendorf tubes (ETs) by centrifugation 300 g (~1000 rpm) at 4°C for 10 min in a benchtop centrifuge.
3. Discard supernatant, briefly dry ETs by inverting on a paper towel (if necessary remove the rest of liquid with a pipet or a piece of Whatman paper).
4. Lyse cells in 100 µL of lysis buffer for 10 min on ice, vortex samples vigorously about every two minutes.
5. Centrifuge extracts at 12,000*g* for 20 min at 4 °C and transfer supernatant into fresh ETs. Measure protein concentration (i.e., by Bio-Rad protein assay according to the manufacturer's instruction), and adjust samples to same concentration with the lysis buffer, transfer 30 µL of extract into fresh ET and dilute it 1:10 with caspase assay buffer.
6. Add the dye (D_2Rhodamine 110) to a final concentration of 10 µ*M* (3 µL of 1 m*M* solution) and incubate for 20 min at 30 °C.
7. In the meantime switch on the spectrofluorometer (the lamp needs usually 5–15 min to warm-up).
8. Terminate the reaction by placing the tubes on ice.
9. Transfer probes into 500-µL quartz cuvets (glass cuvets can be used if excitation wavelength is 488 nm or longer), and measure in spectrofluorometer (excitation: 488 nm; emission: 550 nm), if 1 mL cuvets need to be used, samples can be diluted with 300 µL of cold H_2O or PBS (600 µL of total volume is usually sufficient to fill 1-mL cuvet).

Blank cuvet should be filled with 30 µL lysis buffer, 270 µL caspase buffer, and 3 µL of 1 m*M* D_2Rhodamine 110, (do not forget to dilute the blank in case the experimental samples are diluted to a total volume of 600 µL).

4. Notes

1. D_2Rhodamine 110 can not distinguish between different caspases, if necessary DABCYL-xxxxxxx-EDANS -based substrates can be used where the caspase-specific sequence is placed between DABCYL and EDANS (i.e. DABCYL-DEVDAPK-EDANS for caspase-3 or DABCYL-YVADAPK-EDANS for caspase-1. For the design of new substrates the authors recommend recent publications ***(11, 12, 13, 14)***. 7-amino-4-trifluoromethyl-coumarin (AFC) -based substrates have also been used successfully for detection of caspase activity in cell extracts ***(10)***, and the appropriate kit can be obtained, i.e.. from Clontech (Palo Alto CA).
2. The authors used a Shimadzu RF-510 spectrofluorometer (Shimadzu, Japan) equipped with an UV lamp.
3. It is advisable to stain each sample in duplicate and calculate the mean of two values obtained for the same sample.
4. Since the FSC/SSC signal will change slightly due to osmotic loading, for identification of specific blood cell subpopulations it is advisable to prestain a given cell population with antibody against a specific surface marker.

5. We have usually used 200 µL of blood per sample, but staining is also possible with 50 µL of blood. One can then either reduce the amount of used reagents or dilute the whole sample with PBS (room temp.) and follow the above protocol.
6. Inconsistency in your results can often be caused by carry-over of the dye on the external wall of the pipet tip.
7. To discriminate between effector and target cells: (i) FSC/SSC criteria can be used (which is quite difficult and not very precise); (ii) if cells differ significantly in their DNA content, Propidium Iodide or 7-amino-actinomycin-D (7-AAD) staining can be used; (iii) prestaining with antibody against a specific surface marker for one of two cell populations can be used if it is sufficiently stable (please note that dead cells may stain nonspecifically so target cells should be stained).
8. When staining adherent cells, it is better to prestain them for caspase activity in, i.e., twelve-well plate or a Petri dish, then scrape them off and transfer them to FACS-compatible tubes.
9. Staining of adherent cells is often less successful since detaching may cause spontaneous activation of caspases owing to accompanying damage.
10. For best results start the staining procedure about 1 h before typical apoptotic morphology is observed.
11. Some cell lines express large quantities of caspases, which can be activated partially during lysis. If the signal from experimental samples is very high as compared to the blank cuvet, but no difference between, i.e., control and samples exposed to dye is observed, it is recommended to, (i) shorten the incubation step at 30°C to 10 min; (ii) repeat the entire experiment by using ~2×10^5 cells; (iii) harvest cells earlier.

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15

Techniques For Studying Murine Natural Killer Cells in Defense Against Viral Infection

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1. Introduction

Natural killer (NK) cells constitute an important frontline defense against a range of viruses including herpesviruses, human immunodeficiency virus type 1, and hepatitis viruses ***(1)***. NK cell deficiencies can lead to serious, life-threatening virus infections, with herpesviruses being of particular importance ***(2)***. In recent years, considerable knowledge has been gained on a range of aspects of NK cell biology, such as the nature and role of receptors expressed by NK cells; NK cell responsiveness to cytokines; and the cytokines they produce. Many of these studies have made use of animal models. Mouse models have been useful for studying a number of aspects of virus–NK cell interactions, including the cytokine response following virus infection ***(3)***; defining host loci controlling NK cell function following virus infection, notably *Cmv1* and *Rmp1* ***(4,5)***; and the role of virus-encoded proteins in immune evasion from NK cell surveillance ***(6)***. Murine cytomegalovirus (MCMV), which a member of the herpesvirus family, and ectromelia virus (EV), a poxvirus, serve as important model systems for ongoing research in these areas. Much remains to be learned about how NK cells contribute to the control of virus infections.

In this chapter we describe methods used in our laboratories to evaluate the role of NK cells in response to MCMV and EV infections. Areas covered include propagation and quantification of virus stocks; infection of mice; methods for depleting NK cells in vivo; methods for quantifying NK cell activity on tumor targets; and monitoring of NK cells by flow cytometry.

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
Edited by: K. S. Campbell and M. Colonna © Humana Press Inc., Totowa, NJ

2. Materials

1. Mice for use in infection experiments are specific-pathogen-free animals in which NK cell activation is basal. Mice are also sex- and age-matched, and are normally used between 8–12 wk of age. Mouse strains most commonly used for studies on NK cells in the virus infection models described in this chapter are BALB/c and C57BL/6J (B6). In both the MCMV and EV models, the BALB/c and B6 strains are susceptible and resistant, respectively. The B6 strain expresses the NK1.1 alloantigen and its use permits in vivo depletion of NK cells using the anti-NK1.1 monoclonal antibody PK136. We have recently developed the BALB.B6-*Cmv1r* congenic mouse strain which has the *Cmv1r* resistance allelle and also expresses the NK1.1 antigen on the BALB/c background ***(7)***.
2. Medium for growing mouse embryo fibroblasts (MEFs): The basal medium used for growing MEFs is minimum essential medium (MEM, GibcoBRL, no. 41500-091, Grand Island, NY) made up at 333 milliosmolar. To 950 mL of de-ionized, distilled water (ddH_2O) is added MEM powder for 1 L, 10 mL of a 50× gentamycin/penicillin stock solution (gentamycin, 4.0 mg/mL; penicillin, 10,000 IU/mL), and 2.2 g of sodium bicarbonate. The pH adjusted to 7.2 with concentrated HCl. Double-strength MEM is made up as described previously except that 475 mL of ddH_2O is added. All media and solutions are sterilized through 0.22-µm membrane filters.
3. Mouse osmolality phosphate-buffered saline (PBS) (MOBS, 333 milliosmolar): This is used for washing MEFs and for injection of virus stocks. It is prepared by adding 0.2 g of potassium chloride, 0.2 g of potassium dihydrogen phosphate, 9.83 g of sodium chloride, and 1.15 g of disodium orthophosphate dihydrate per liter of ddH_2O. The pH is adjusted to 7.2.
4. Trypsin for dispersion of embryonic tissue and passaging MEFs: Make trypsin (DIFCO trypsin 1:250) up at 5% w/v in ddH_2O to give a 20× stock solution. Stir gently for a few hours at 4°C. Pellet insoluble material by centrifuging in a refrigerated centrifuge (10 min, 2000*g*). Collect supernatant and filter sterilize. Prepare 5-mL aliquots and store at –20°C.
5. Medium for growing BS-C-1 cells: Prepare MEM as described in **step 2**. The complete medium should contain the following; 10% fetal calf serum (FCS), 2 m*M* L-glutamine, penicillin (100 U/mL), streptomycin (100 mg/mL), and 1 m*M* HEPES.
6. Medium for growing CV-1 and YAC-1 cells: Prepare MEM as described in **step 1**. It should be supplemented with 5% FCS, 2 m*M* L-glutamine, penicillin (100 U/mL), streptomycin (100 µg/mL), and 1 m*M* HEPES.
7. Methylcellulose: To 250 mL of ddH_2O in a 500-mL Schott bottle add 8.75 g methyl cellulose powder (4000 centipoise, Fisher Scientific Company, Pittsburgh, PA). Do not shake or mix at this stage. Tighten lids to fingertightness and then autoclave (15 psi, 20 min). The lids are then firmly tightened and when cool enough to touch the bottles are periodically shaken until they have cooled to room temperature. When cool the methylcellulose should be quite viscous. At least one day prior to use in plaque assays add 250 mL of double-strength MEM prewarmed to 37°C to 250 mL of prewarmed methylcellulose solution. Then add

10 mL newborn calf serum (NCS) to give 2% v/v NCS. The final concentration of methylcellulose is 1.75%. For the enumeration of EV plaques on BS-C-1 cell monolayers, the semiviscous overlay contains methylcellulose at a final concentration of 0.7% in MEM containing 5% FCS, 2 m*M* L-glutamine, penicillin (100 U/mL), and streptomycin (100 µg/mL).

8. Methylene blue–formalin: Add 900 mL of ddH_2O to 5 g of methylene blue powder. This is mixed on a magnetic stirrer for several hours. The solution is then passed through Whatman filter paper to exclude undissolved dye and 100 mL of formaldehyde solution is added. The final concentrations of the methylene blue and formalin are 0.5% w/v and 10% v/v, respectively. The stain is stable indefinitely.
9. Crystal violet–formalin–ethanol: Add 200 mL ethanol and 100 mL of formaldehyde solution to 2.5 g of crystal violet powder in a 2-L Schott bottle, swirl to dissolve the stain, and then add 800 mL of ddH_2O to bring the total volume to 1 L. Mix on a magnetic stirrer for 2 h. Remove undissolved stain by passing through Whatman paper. The stain is stable indefinitely.
10. ACK lysing buffer (red cell lysis buffer): Add 800 mL of ddH_2O to 8.29 g of ammonium chloride (NH_4Cl; 0.15 *M*), 1 g of potassium hydrogen carbonate ($KHCO_3$; 1.0 *M*), 3.72 mg of disodium EDTA (Na_2EDTA; 0.01 *M*), and dissolve thoroughly. Adjust pH to 7.2 with 1 *N* of HCl. Add ddH_2O to 1 L, filter sterilize, and store at 4°C.
11. Tris-HCl: 1 m*M* and 10 m*M* solutions, pH 9.0, sterile.
12. Sucrose solutions: Prepare 36% (w/v) sucrose solution in 10 m*M* Tris-HCl, pH 9.0. Also required are 40%, 36%, 32%, 28%, and 24% (w/v) sucrose solutions in 1 m*M* Tris-HCl, pH 9.0, sterile.
13. Antibodies and flow cytometry reagents: anti-NK1.1 MAb (PK136, murine IgG2a; ATCC no. HB-191), rabbit anti-asialo GM_1 antibody (Wako Pure Chemical Industries, Osaka, Japan), fluorescein isothiocyanate (FITC)-conjugated anti-NK1.1 (clone PK136), anti-CD4 (clone GK1.5), anti-CD8 (clone 53.6.7), anti-CD45R/B220 (clone RA3-6B2), anti-CD11b (clone M1/70), and anti-Ly-6G (clone RB6-8C5) (all available from Pharmingen, San Diego, CA), and streptavidin–FITC conjugate (Amersham International, UK).
14. Cell lines: BS-C-1, (American Type Culture Collection [ATCC] no. CCL-26); CV-1, (ATCC no. CCL-70); and YAC-1, (ATCC no. TIB-160); and M2-10B4, (ATCC no. CRL-1972).
15. Viruses: MCMV (strains K181; or Smith, ATCC no. VR-1399); and EV (Moscow strain; ATCC no. VR-1374).
16. Radioisotopes: Sodium chromate (^{51}Cr) solution (no. CJS.1P, Amersham).
17. Other materials and equipment: 630 cm^2 glass roller bottle; 7 mL glass homogenizers (Pyrex); 850 cm^2 roller bottles (Corning Costar, Corning, NY); cup sonicator (Branson Sonic Power, Danbury, CT); SW-27 centrifuge tubes and rotors for ultracentrifugation (Beckman); Eppendorf Safe-Lock tubes (Hamburg, Germany); Polytron homogenizer (ProScientifiC, Monroe, CT); 96-well LumaPlates and Packard TopCount gamma counter (Packard); 5-mL Falcon tubes (Falcon no. 2054, Becton Dickonson Labware, NJ); and FACscan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

3. Methods

3.1. Preparation of Mouse Embryo Fibroblasts

Embryos are obtained from mice (outbred Swiss or BALB/c) at 15–17 d of pregnancy. Approximately 5–10 pregnant mice are used depending on the batch size of MEFs required. All procedures and dissections are carried out in a laminar flow hood.

1. The pregnant mice are killed by cervical dislocation or by CO_2 inhalation and swabbed with 70% v/v ethanol. The uterus is aseptically removed and transferred into sterile Petri dishes containing MOBS. The embryos are then dissected out of the embryonic sacs and the heads severed. Following dissection to remove limbs and viscera the remaining embryonic tissue is then transferred to a fresh deep Petri dish containing a small volume of fresh MOBS (< 5 mL).
2. The embryos are then finely minced with sterile scissors. Additional MOBS is added to the dish and the tissue fragments allowed to settle. The supernatant is then carefully removed and discarded. The tissue fragments are then aseptically transferred to a sterile 500-mL Schott bottle containing a magnetic flea sterilized with 70% ethanol.
3. Add 10 mL of prewarmed working strength trypsin solution (0.25% w/v; made up in MOBS) per embryo. Incubate on a magnetic stirrer for 1 h in a 37°C room.
4. Add an equal volume of MEM containing 10% NCS and then strain the cell suspension through a sterile metal tea strainer to remove the cell clumps and debris.
5. Centrifuge the cell suspension for 10 min at 500*g*. Discard the supernatant and resuspend the cells in approx 2 mL of MEM containing 10% NCS per embryo. Seed the cells at approx one embryo/80 cm^2 flask or three embryos/630 cm^2 glass roller bottle (Wheaton). Add MEM containing 10% NCS to give 20 mL/flask and 80 mL/roller bottle. For the roller bottles gently bubble through CO_2 (10% CO_2 in medical air) and seal with parafilm.
6. Incubate at 37°C with the roller bottles rotating at approximately 1 rpm. The flasks are incubated in a CO_2 incubator. After 24 h replace the old medium. Incubate the cells until they are 100% confluent (approx 3–4 d).
7. Discard the medium and wash the cells with prewarmed MOBS. Then add 0.25% w/v trypsin in MOBS (1 mL per flask or 5 mL per roller bottle). Incubate for 3–5 min to detach the cells. Rinse out cells with MEM containing 10% NCS. Pellet cells in 50-mL conical tubes for 10 min at 500*g*. The cells are then resuspended into sterile, ice-chilled MEM containing 20% NCS and 10% dimethyl sulfoxide at rates of 2 mL/flask and 12 mL/roller bottle. One-milliliter aliquots are then stored in cryotubes in the gas phase of liquid nitrogen.

3.2. Virus Stocks

3.2.1. Preparation of Tissue Culture Stock of MCMV

MCMV is passaged in MEF monolayers to generate tissue culture-derived stocks (*see* **Notes 2** and **3**). Large stocks (>100 mL) can be produced in roller

bottles, although smaller stocks can be grown in tissue culture flasks. In the description that follows, amounts for the infection of MEFs in roller bottles are described; the amounts for single 80 cm^2 flasks are expressed in parentheses. MEF monoloyers are grown to confluence in MEM containing 10% NCS; cells are infected and thereafter maintained in MEM containing 2% NCS.

1. Thaw vials of MEFs rapidly at 37°C and slowly dilute the cells 10-fold in warm medium. Pellet the cells by centrifugation at 500*g* and resuspend in medium. Seed at least 2×10^7 cells in 100 mL medium per roller bottle (10^6 cells in 25 mL of medium). Gently bubble through $C0_2$ (10% $C0_2$ in medical air), seal, and incubate at 37°C for 3–4 d, or until fibroblasts reach 75–100% confluency. Do not allow the monolayers to remain confluent for an extended period prior to infection (*see* **Note 4**).
2. Gently wash the monolayers with 25 mL (5 mL) of warm MOBS to remove excess serum. Infect monolayers at a multiplicity of infection (m.o.i.) of 0.01–0.05 in 15 mL (3 mL) of medium containing 2% NCS. For example, assuming there are 10^8 (10^7) cells in each roller bottle (flask), between 10^6–5×10^6 (10^5–5×10^5) plaque-forming units (PFUs) of MCMV is required. Incubate for 1 h at 37°C. Top up with 85 mL (22 mL) of medium, gas with CO_2, and incubate at 37°C for approx 3 d, or until at least 90% of the cells exhibit cytopathic effect (CPE), characterized as rounding and swelling of cells and eventually extensive detachment.
3. Pour off the supernatant and remove cell debris by centrifugation at 2000*g*. Dispense fluid in aliquots (generally 0.5–3 mL) and store at –70°C until required (*see* **Note 5**). Virus can be quantified by plaque assay on MEFs (described below). Titers of 10^6 PFU/mL are routinely achieved.

3.2.2. Preparation of Salivary Gland Stock of MCMV

In preparing virulent salivary gland stocks of MCMV, the best approach is to first prepare a master stock, from which submaster and working stocks can be generated. This strategy is preferable to multiple rounds of passage in vivo for which there is a risk in ultimately generating virus stocks with altered virulence characteristics. Wild-type MCMV (strains K181; or Smith, ATCC VR-1399) is first detected in the salivary glands at around 7 d postinfection (p.i.) and virus titers peak between 17 and 21 d p.i. All stocks are produced in 21-d-old female mice—either inbred or outbred mice may be used. Master stocks can be prepared in as few as 10 mice; working stocks are usually prepared from at least 100 infected mice. Unlike tissue culture-derived MCMV, salivary gland MCMV is unstable at room temperature and titres diminish within a few hours. Stability is improved by working with the virus at 4°C in the presence of serum (e.g., 0.05% v/v NCS).

1. To prepare a master stock, infect mice with 10^4–10^5 PFU of tissue culture MCMV by the intraperitoneal route. Harvest salivary glands (described below) after 17–21 d.

2. Submaster and working stocks are generated by the intraperitoneal inoculation of 10^3–5×10^3 PFUs of the salivary gland master stock. The virus inoculum is diluted in cold MOBS containing 0.05% NCS. Salivary glands are harvested after 17–21 d.
3. To harvest salivary glands, mice are killed by CO_2 inhalation and the head, throat, and chest swabbed with 70% v/v ethanol. A vertical midline incision is made from the jawline to the sternum and the skin pulled aside to expose the salivary glands, which comprise the submaxillary, sublingual, and parotid glands (*see* Plate 45, p. 55, in **ref. *8***). The entire glands are excised and placed in a preweighed bottle containing cold MEM + 2% NCS (medium). All subsequent manipulations are performed at 4°C. The pooled glands are weighed, removed from the medium and minced briefly with curved scissors. A 20% w/v homogenate is made in cold medium, using sterile 70-mL glass homogenizers (Pyrex). The glands can be homogenized in batches of 10 and then pooled. The homogenate is clarified by centrifugation (2000*g*, 20 min) and the supernatant harvested (avoiding the upper fatty layer) and dispensed into 100–200-µL aliquots. Although salivary gland MCMV virus stocks may be stored for several days at –70°C, we have found that they lose titer after several weeks (*see* **Note 5**). Thus, we recommend long-term storage in liquid nitrogen, where the virus is stable for several years.

3.2.3. Plaque Assay for Quantifying MCMV

1. Seed 24-well trays with MEFs (*see* **Note 3**) at a concentration of 2×10^5 cells/well in 1 mL of MEM containing 10% NCS, and incubate for 24–40 h at 37°C/5%$C0_2$.
2. Rapidly thaw the samples of MCMV to be titerd in a 37°C water bath and perform serial dilutions in MEM + 2% NCS. For convenience when titrating a large number of samples, fourfold or 10-fold dilutions can be performed in 96-well trays using a multiple pipettor. It is important to change pipet tips between each dilution. All dilutions are performed in duplicate and generally at least six serial dilutions are made per sample. Given the instability of MCMV derived from organ homogenates, it is advisable to titrate large numbers of samples in batches.
3. Carefully remove medium from MEF monolayers with an unplugged Pasteur pipet attached to a suction device and add 200 µL of diluted virus. Care must be taken to prevent the monolayers from drying out.
4. Incubate the trays for 1 h at 37°C/5%$C0_2$. Remove the virus inoculum (*see* **Note 6**) and add 1 mL medium containing 1.75% methylcellulose. Incubate for a further 4–5 d, at which time plaque development is visible. Add 1 mL of methylene blue–formalin solution and leave at room temperature overnight.
5. Under a running tap, gently rinse out the methylene blue–formalin/methylcellulose medium and allow trays to dry inverted for several hours. Plaques can then be counted using a stereo microscope. MCMV plaques typically show an area of clearing in the monolayer with darkly stained, enlarged cells around the edges (**Fig. 1**).
6. The MCMV titer is calculated as follows: PFU/mL = no. plaques × dilution factor × 5. If titrating individual organs, the MCMV titer per organ can be calculated, taking into account the % homogenate made (*see below*).

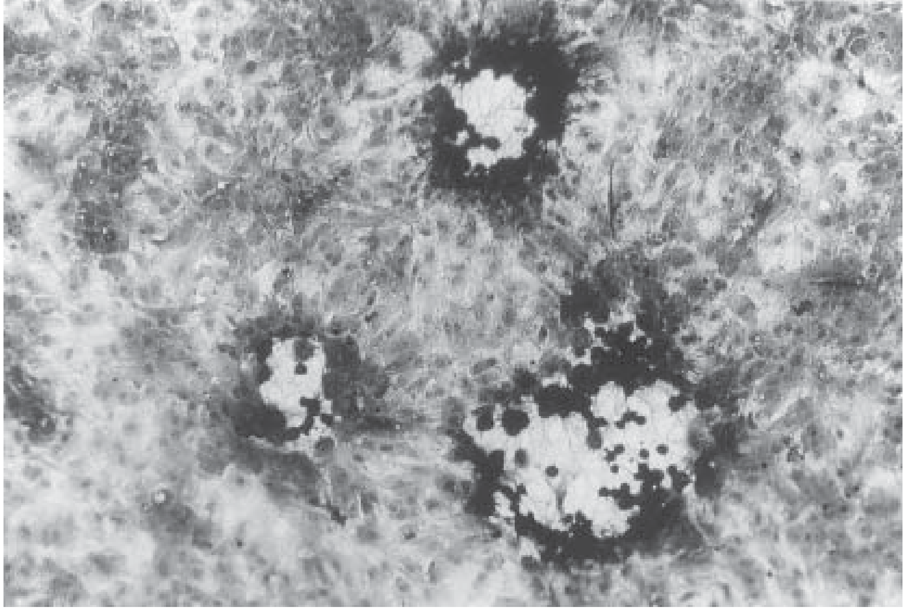

Fig. 1. MCMV plaques on mouse embryo fibroblast monolayer after 4 d. Stained with methylene blue/formalin.

3.2.4. Preparation and Purification of EV Stock

For the preparation of EV stocks, the procedure using CV-1 cells is described here although BS-C-1 cells may also be used with comparable virus yields. The advantage of using CV-1 cells is that they grow faster than BS-C-1 cells. It may be convenient to grow cells initially in 175 cm^2 flasks before transferring them to roller bottles. Virus yield is improved when the cells are in a log phase of growth and if an m.o.i. of 0.025 is used (*see* **Note 8**).

1. Grow CV-1 cells in three 850 cm^2 roller bottles to 90% confluency. This may take about three days if cells from one 175 cm^2 flask are seeded into one roller bottle. Maintain cultures on rollers in a 37°C warm room or an incubator maintained at 37°C.
2. Prepare the virus inoculum as follows once the inoculum dose for each roller bottle has been calculated based on the recommended m.o.i. For example, at 90% confluency, one 850 cm^2 roller bottle should yield approx 10^8 CV-1 cells and the virus dose for each roller bottle should be 2.5×10^6 PFU. The crude virus used for infecting cells is first dispersed by sonication or by trypsinization. If using a cup sonicator, sonicate the inoculum (in 2 mL of MEM containing 2.5% FCS in a 10-mL capped tube) at 100 W for 3×10 s bursts with a 10-s pause between every burst. Adjust the total volume of the inoculum to 7.5 mL with MEM containing

2.5% FCS. If using a probe sonicator (probe sterilized with 70% ethanol), sonicate the inoculum (in 2 mL of PBS in a 10-mL capped tube) at 80 W for 3 × 10 s with a 10-s pause between every step. If a sonicator is not available, then mix an equal volume of the EV stock and 0.25 mg/mL of trypsin and vortex vigorously prior to incubation for 30 min in a 37°C water bath. Vortex-mix the mixture at 10 min intervals and finally dilute the trypsinized virus in complete MEM containing 2.5% FCS to a final volume of 7.5 mL.

3. Remove the growth medium in the roller bottles by aspiration and add the virus inoculum (7.5 mL/roller bottle). Holding the roller bottle with both hands, gently rock the bottles while turning before placing them on rollers in a warm room or an incubator maintained at 37°C. After 2 h, add 150 mL of complete MEM containing 5% FCS and incubate for 2–3 d. Examine the monolayers for plaque formation at d 2 and 3 postinfection. The cells should be harvested when about 60–70% of the cells appear infected. Incubation for longer periods may result in reduced virus yields especially when cells detach from the surface.
4. Using a disposable sterile cell scraper, detach the cells from the roller bottle wall into the medium and collect the cell suspension into sterile screw cap conical tubes. Centrifuge the cell suspension for 5 min at 1800*g* at 4°C. Discard supernatant after disinfection with sodium hypochloride. The virus-infected cell pellet may be used as a source of crude virus stock. The crude virus is purified using zonal sucrose gradient centrifugation.
5. Resuspend cell pellets from three roller bottles in 14 mL of 10 m*M* of Tris-HCl, pH 9.0, and keep samples on ice throughout the entire procedure. Lyse the cell suspension using a tight-fitting, sterile glass Dounce homogenizer with about 40 strokes. Centrifuge for 5 min at 300*g* at 4°C to remove nuclei (pellet) and save the supernatant (lysate). Resuspend the pellet in 3–5 mL of 10 m*M* of Tris-HCl and centrifuge as described previously. Save the lysate from this step and pool with lysate from the previous step into a 50-mL conical tube.
6. Sonicate the lysate using a sterile probe sonicator at 100 W for 4 × 20 s with a 20-s pause between every sonication step. The tube containing the lysate must be kept on ice throughout the entire procedure.
7. The sonicated lysate is then layered onto a cushion of 17 mL of 36% sucrose (made up in 10 m*M* Tris-HCl, pH 9.0) in a sterile SW-27 centrifuge tube (Beckman). Centrifuge for 80 min at 32,900*g* in an SW-27 rotor at 4°C. Discard the supernatant and resuspend the pellet in 1 mL of 1 m*M* Tris-HCl, pH 9.0, and sonicate once for 20 s in a cup sonicator as described previously before overlaying onto a sucrose gradient (*see below*).
8. One day before use, prepare a sterile 24–40% continuous sucrose gradient in a sterile SW-27 centrifuge tube by layering 6.8 mL of each of the following sucrose solutions (in 1 m*M* Tris-HCl, pH 9.0) in the tube: 40%, 36%, 32%, 28%, and 24%. Allow the gradient to sit overnight at 4°C before use.
9. Carefully overlay the sucrose gradient with the sonicated virus pellet (contained in 1 m*M* of Tris-HCl) from **step 7** and centrifuge for 50 min at 26,000*g* (SW-27 rotor; Beckman) at 4°C. The virus will appear as a milky band. Collect the virus band carefully into a sterile SW-27 tube and fill the tube with 1 m*M* Tris-HCl.

10. Centrifuge the tube for 60 min at 32,900*g* at 4ºC. Discard the supernatant, and add 1 mL of 1 mM Tris-HCl, pH 9.0, to the pellet; sonicate as described previously in a cup sonicator, divide into 50-µL aliquots; and freeze at –70°C (*see* **Note 10**). Use one of these aliquots to determine the virus titer (*see below*).

3.2.5. Plaque Assay for Quantifying Ectromelia Virus

EV infectivity is quantified using BS-C-1 cell monolayers (CV-1 cells may also be used but the plaques tend to be smaller).

1. One day before the plaque assay, seed 3×10^5 BS-C-1 cells/2 mL MEM containing 10% FCS in wells of 6-well cluster plates. Rock the plates very gently to ensure that the cells are seeded evenly.
2. Sonicate the purified virus at 80 W for 20 s in a cup sonicator. If the titre of a crude virus stock is to be determined, sonicate/trypsinize for longer as described above in **Subheading 3.2.4.** Make eight 10-fold serial dilutions of the virus stocks (using a separate pipet tip each time) in cold PBS containing 1 m*M* HEPES and 1% FCS. To quantify EV titers in organ tissues, *see* **Subheading 3.3.2.**
3. Remove medium from monolayers of BS-C-1 cells (leave enough medium in the wells, i.e., approx 200 µL to prevent the monolayers from drying out in the biohazard hood). Add 100 µL of each dilution to individual wells of BS-C-1 cell monolayers and incubate in a CO_2 incubator at 37°C for 1 h.
4. Overlay monolayers with 2 mL of MEM containing 0.7% methylcellulose and 5% FCS and incubate in a CO_2 incubator at 35°C (*see* **Note 9**). After 3–4 d when plaques have formed, aspirate the medium containing methylcellulose (*see* **Note 6**), add 0.5 mL of the crystal violet stain, aspirate after 10 min, and allow monolayers to dry.
5. Count the number of plaques and calculate the titre as follows:
   virus titer (PFUs/mL) = number of plaques × dilution factor × 10. When calculating the titers in mouse organs, the % homogenate made must be taken into account and the titer is expressed as PFUs/g tissue.

3.3. Virus Infection of Mice

3.3.1. MCMV

We have used the intraperitoneal route of inoculation to establish an MCMV infection in mice, although footpad and intranasal routes have been used successfully by other groups. During acute infection, the spleen and liver are the main visceral organs that sustain a high virus load in the immunocompetent animal, although later in infection high titers are observed in the salivary glands. The antiviral actions of NK cells are mainly exhibited within the first 5 d following infection, although they do contribute to limiting virus replication in the salivary glands later in infection ***(9)***. Age, mouse strain, and virus dose each affect the titers recovered from these organs and the efficacy of NK cell-mediated clearance ***(10,11)***.

1. Mice are infected by the intraperitoneal route with 2×10^4 PFUs salivary gland-derived MCMV diluted in MOBS + 0.5% NCS (*see* **Note 1**). For comparative studies (e.g., effects of anti-NK treatments) at least four mice per group are used.
2. Mice are killed by CO_2 inhalation and the spleens and livers removed aseptically. Salivary glands are generally removed at later stages of infection, after d 7 p.i. Organs are transferred into individual, preweighed bottles containing approx 5 mL of cold MEM + 2% NCS.
3. All the following manipulations are performed at 4°C. After weighing, the organs are transferred to glass grinders and 10–20% w/v homogenates made using MEM + 2% NCS.
4. The homogenates are clarified by centrifugation at 2000*g* for 20 min and duplicate 200-µL aliquots are stored at –70°C until quantification of virus by plaque assay.

3.3.2. Ectromelia Virus

For the EV model, both footpad and intravenous routes of inoculation are generally used. We generally use the Moscow strain of EV (ATCC VR-1374) and the intravenous route of inoculation for studies on NK cells. As for MCMV, EV seeds and replicates to high titers in the spleen and liver as well as several other organs in immunocompetent mice during the acute phase of infection. In C57BL/6 mice infected with EV, splenic NK cell activity is augmented and is usually between three- and 81-fold higher (depending on the time after infection) than the background activity of splenic NK cells from uninfected mice. The peak cytolytic activity is measurable 3 d after intravenous infection and generally drops to background levels by d 6 when the virus-specific CTL activity peaks. Thus, in the EV model, the antiviral activity of NK cells is demonstrable during the first 4 d after infection.

1. Mice are inoculated by the intravenous route with 10^3 PFUs of purified EV contained in 200 µL of PBS (*see* **Note 7**). We generally use four or five mice per group for comparative studies.
2. Mice are killed by CO_2 euthanasia or by cervical dislocation and the spleens and livers (and other organs such as lung, thymus, ovaries, etc. if required) removed aseptically into individual, preweighed Eppendorf Safe-Lock tubes on ice.
3. Obtain the weight of the organ (by subtracting the weight of the empty tube from that of the tube and organ). Add 1 mL of ice-cold PBS containing 1 m*M* of HEPES and 1% FCS. The tissue is homogenized in the 2-mL Eppendorf Safe-Lock tube. Sit the tube on ice and homogenize for 1 min or longer if required using a Polytron homogenizer in a biohazard hood.
4. Centrifuge tubes at 3000 rpm in a bench-top Eppendorf centrifuge at 4°C for 1 min to remove tissue debris in the homogenate. Transfer supernatants to fresh tubes.
5. Sonicate the homogenate (supernatant) as described in **step 2**, **Subheading 3.2.4.** and make six 10-fold serial dilutions in PBS containing 1 m*M* HEPES and 1% FCS. Continue with plaque assay as described in **Subheading 3.2.5.**

3.4. Depletion of NK Cells In Vivo by Pretreatment with PK136 or Anti-Asialo GM1

To assess the role of NK cells in mediating host resistance against virus infection the NK cell response can be modulated by various pretreatments. These can include NK cell depletions using specific antibody preparations (*see* **Note 11**); modulation using activatory or inhibitory cytokines, modulation using neutralizing antibodies to activatory or inhibitory cytokines, and use of drugs that modify or affect NK cell function ***(3–5,12)***. In this subheading we will describe the protocols we use for depleting NK cells in vivo.

1. Groups of mice to be treated are given i.p. inoculations of 0.1 mL of 100 µg of rabbit anti-asialo GM1 antibody or 0.1 mL of 100–200 µg of the anti-NK1.1 MAb PK136. Groups of mice also receive isotype-matched irrelevant antibody controls or MOBS alone.
2. Two days later the mice are retreated with the same dilution of antibody and then infected by the intraperitoneal route with MCMV or the intravenous route with EV.

3.5. Assay for NK Cell Activity

We generally use YAC-1 lymphoma targets to measure the cytolytic activity of NK cells. The assay is carried out in triplicate using various effector:target ratios where the number of ^{51}Cr-labeled targets is kept constant at 2×10^4 cells per well of U-bottom 96-well cluster plates. The use of cells in a log growth phase results in low spontaneous ^{51}Cr release. MEM containing 1% FCS and 2 m*M* L-glutamine, penicillin (100 U/mL), streptomycin (100 µg/mL), and 1 m*M* HEPES is used for the NK assay.

1. Label YAC-1 cells with ^{51}Cr (sodium chromate [^{51}Cr] solution; approx 50 µCi for 2×10^6 cells in a final volume of 200 µL) for 1 h at 37°C in a humidified atmosphere containing 5% CO_2 in air.
2. Wash target cells three times in MEM and resuspend at a concentration of 2×10^5 cells/mL for use. Perform the final wash just prior to addition of the targets to the effector cells.
3. Obtain single cell suspensions of spleens from uninfected and virus-infected mice. Pellet the spleen cells by centrifugation and lyse the erythrocytes with ACK lysis buffer (approx 1 mL of ACK lysis buffer for every 0.2-mL cell pellet).
4. Wash splenocytes 3 times in MEM. Resuspend cells in MEM and obtain a cell count for each population. Adjust the concentration to 2×10^7 cells/mL.
5. Add 100 µL of the spleen cell suspension into triplicate wells (100 mL = 2×10^6 cells) of 96-well U-bottom cluster plates. To the next three wells containing 100 µL of MEM, add 50 µL of cells. Then perform another two sequential threefold serial dilutions across the plate for each population (transferring 50 µL of diluted cells into 100 µL of MEM). Remove 50 µL from the final set of triplicate wells in the dilution series so that the residual volume is 100 µL. Once the targets (2×10^4 cells/well) have been added this will yield effector:target ratios of

100:1; 33:1; 11:1, and 3:1. The spontaneous release value is obtained by culturing the same number of ^{51}C-labeled cells (2×10^4 cells/well) in MEM alone and the maximal release value is similarly obtained by culturing these cells with 1% Triton-X 100. The final volume in all wells should be 200 µL. Incubate the plates for 4 h at 37°C in a humidified incubator containing 5% CO_2 in air.

6. Centrifuge plates at 500*g* for 1 min. Carefully remove 25 µL of medium from each well using a multichannel pipettor without disturbing the cells in the bottom of the wells and add into the corresponding wells of 96-well LumaPlate. Allow to dry and count the radioactivity in a Packard TopCount gamma counter. Calculate the specific lysis using the formula:

$$\% \text{ specific lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100$$

3.6. Monitoring of NK Cells by Flow Cytometry

NK cells are not only activated but they also proliferate following infection with viruses such as MCMV or EV. Hence the frequency as well as absolute numbers of this leukocyte subset also increases. These increases as well as the absence of NK cells following in vivo elimination with antibodies can be monitored by flow cytometry. This is generally done in parallel with the ^{51}Cr release assay to monitor the lytic activity (as described in **Subheading 3.6.**). We use the following fluorochrome-conjugated monoclonal antibody (MAb) for single-color analysis: FITC conjugated anti-NK1.1 (clone PK136), anti-CD4 (clone GK1.5), anti-CD8 (clone 53.6.7), anti-CD45R/B220 (clone RA3-6B2), anti-CD11b (clone M1/70), and anti-Ly-6G (clone RB6-8C5). We also use biotinylated anti-asialo GM1 which is detected with streptavidin–FITC conjugate. Inclusion of markers for T cells, B cells, macrophages, and granulocytes is necessary to assess the effect of NK cell depletion in vivo on these other leukocyte subsets. The MAb conjugates must be pretitrated before use for flow cytometry. Splenocytes from untreated or NK cell depleting antibody-treated uninfected and virus-infected mice are used.

1. Obtain a single cell suspension from spleens, lyse the red cells, wash 3× in PBS (1% FCS/0.1% sodium azide) by centrifugation at 500*g* and resuspend at a 2×10^7 cells/mL.
2. Add 10 mL of the pretitrated MAb conjugates to 50 mL of the splenocyte suspension from **step 1** (i.e., 1×10^6 cells) in 5-mL Falcon tubes for 20 min at 4°C.
3. Wash 2× in PBS (1% FCS/0.1% sodium azide) at 4°C in a refrigerated centrifuge, and resuspended in 300 µL of PBS (1% FCS/0.1% sodium azide).
4. For those samples in which a secondary streptavidin–FITC antibody is used for detection, wash as in **step 3** and resuspend cells in 50 µL of PBS. Add 10 mL of diluted, pretritrated streptavidin-FITC and incubate at 4°C for 20 min. Wash 3× and resuspend cells in 300 µL of ice-cold PBS (1% FCS/0.1% sodium azide).

5. For each sample, 20,000 events are collected and analyzed on a FACscan flow cytometer.

4. Notes

1. We house MCMV-infected mice in a designated virus room. In our hands we have found no serological evidence for spread of MCMV from cages of infected mice housed side-by-side to cages of sentinel mice. However, this is based on studies using the K181 MCMV strain, and we have no direct data on the Smith strain.
2. Either salivary gland MCMV or tissue culture MCMV can be used as the inoculum for propagation of MCMV tissue culture stocks. However, if the former is used, the inoculum must be removed after the 1-h adsorption step, as the cellular debris is detrimental to MEF on prolonged culture.
3. We routinely titrate MCMV on MEF. The NIH-3T3 cell line can be used, although in our hands we have found that MCMV does not plaque as well on 3T3 cells. A recent report using the M2-10B4 mouse bone marrow stromal cell line (ATCC no. CRL-1972) has indicated that these cells are comparable to MEF for MCMV propagation and quantification by plaque assay ***(13)***.
4. The virus titre of MCMV tissue culture stocks is greatly influenced by the general health of the MEFs. Cells that are overgrown prior to infection or are maintained for long periods in medium that is too alkaline or acidic produce poor titres.
5. MCMV is highly unstable at temperatures around –20°C. Virus stocks must not be stored at this temperature. Tissue culture stocks of MCMV can be stored at –70°C or in liquid nitrogen. Salivary gland stocks are best stored in liquid nitrogen for long-term storage. Frozen vials of MCMV must be rapidly thawed. However, upon thawing, tissue culture MCMV is stable for several hours at room temperature.
6. When aspirating tissue culture media containing viruses from tissue cultures (relevant to a range of protocols in **Subheading 3.2.**), use an in-line trap in the vacuum line to hold the aspirated material until disinfection with sodium hypochloride or by autoclaving. We carry out all virological procedures in laminar flow hoods and tend to passage cell lines at times other than when viruses are been handled. The UV lamps in the hoods can be turned on between virological and tissue culture work to minimize risks of contaminating cell lines.
7. EV-infected mice are also housed in a designated virus room, in isolator cages and preferably in a negative pressure room. The Moscow strain of EV is highly virulent and can wipe out susceptible mouse colonies if one is not careful. We have not had a single outbreak of EV infection of sentinel or other mice housed in the same room as EV-infected mice. We find that good animal husbandry practice is critical when working with EV.
8. EV stocks may also be prepared from spleens or livers of EV-infected susceptible BALB/c mice (*see* **Subheading 3.2.4.**). A 20% homogenate may be used. However, we find that tissue culture grown virus is ideal as high titered stocks can be prepared and virus purification is possible.

9. EV plaque assays are incubated at 34–35°C as this helps slow down BS-C-1 cell growth and facilitates plaque formation. At 37°C, the cells sometimes come off over the 4-d incubation period.
10. Unlike MCMV, EV is very stable and may be stored at –70°C for very long periods.
11. Although both anti-asialo GM1 antibody and the MAb PK136 can be used to deplete NK cells in vivo, a recent comparison of these reagents showed the anti-asialo GM1 antibody also affects activated cytotoxic T cells if given after virus infection. Thus, the authors concluded the anti-NK1.1 MAb allows for the best differentiation of activated CTL and NK cells in vivo ***(14)***. In our experience, we have found that almost 50% of $CD8^+$ T lymphocytes in EV-infected C57BL/6 mice express asialoGM1. If these mice are treated with anti-asialo GM1, NK cells as well as about 50% of $CD8^+$ T lymphocytes are eliminated. Futhermore, using flow cytometry, we have found that whereas approx 4–5% of the splenocytes from naive C57BL/6 mice express the NK1.1 antigen (consistent with the known frequency of NK cells in the spleen), almost 14% of splenocytes express asialo GM1. Using two-color analysis, we have found that $CD4^+$, $CD8^+$, and $CD11b^+$ cells (macrophages and some granulocytes) also express asialoGM1. It should be noted that only certain strains express the NK1.1 antigen as listed in **Appendix 2**, and hence the anti-NK1.1 MAb can only be used in these strains.

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Assays for Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) and Reverse ADCC (Redirected Cytotoxicity) in Human Natural Killer Cells

Bice Perussia and Matthew J. Loza

1. Introduction

Crosslinking of molecules involved in the recognition of target cells elicits natural killer (NK) cell cytotoxicity, inducing either release of preformed cytotoxic mediators via granule exocytosis, or (enhanced) expression of ligands for target cell membrane-associated molecules that transduce apoptotic signals, e.g., Fas Ligand (FasL) or TRAIL (reviewed in refs. ***1,2***). Target cell recognition by NK cells occurs via either of two ways: (1) receptors capable of binding molecules on the target cells that include, but are not limited to, adhesion molecules and the identities of which are incompletely defined (reviewed in **ref. *3***); and (2) receptors for the Fc fragment of IgG (FcγRIIIA, CD16) (***4***, reviewed in **ref. *5***). The latter bind immunecomplexed IgG and therefore target cells that, not recognized spontaneously by NK cells, are sensitized with anti-target cell antibodies (Abs) of the IgG class. The two types of cytotoxicity elicited are referred to as "spontaneous" and "Ab-dependent" cell-mediated cytotoxicity (ADCC).

Although the physiological significance of ADCC remains elusive, it can be used by peripheral NK cells to lyse tumor-derived (or other) cells targeted for sensitization using tumor-specific IgG or bifunctional monoclonal antibodies (MAbs) that bridge target and effector NK cells reacting, respectively, via their antigen-specific $F(ab')_2$ with either only target cells (IgG, the Fc of which can then bind FcγRIIIA), or both the target cells and a cytotoxicity-triggering molecule on the effector NK cells (bifunctional Ab), as reported for tumor immunotherapy ***(6,7)***. (*See* the schematics in **Fig. 1**.) In addition, participation of NK

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
Edited by: K. S. Campbell and M. Colonna © Humana Press Inc., Totowa, NJ

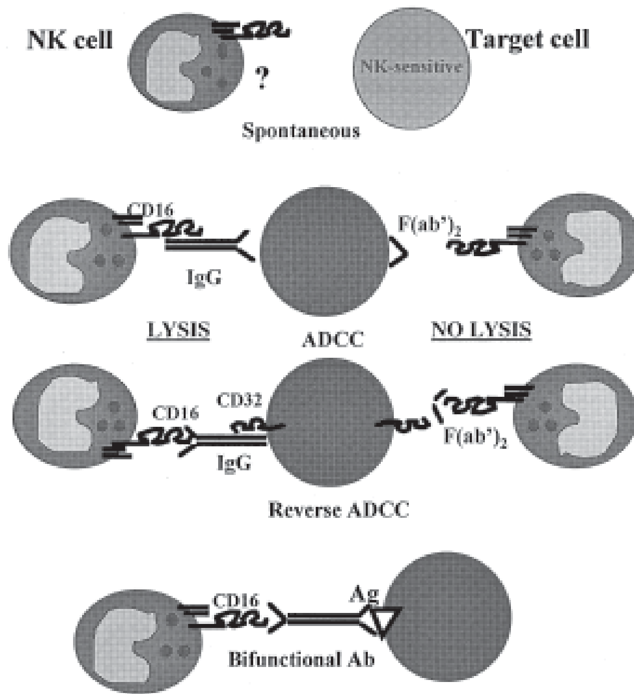

Fig. 1. NK cell-mediated ADCC, redirected cytotoxicity, and cytotoxicity induced by bifunctional Ab: schematics of their rationale, compared with spontaneous cytotoxicity. A darker color of the target cell indicates low or null sensitivity to NK cell-mediated spontaneous cytotoxicity.

cells in pathogenesis of autoimmune diseases possibly via ADCC of auto-Ab-coated target cells, or activation of other NK cell functions following FcγRIIIA triggering *(8)*, cannot be excluded.

Based on these principles as well is the so-called "reverse ADCC" (**Fig. 1**), also referred to as "redirected cytotoxicity." This is mediated upon stimulation of FcγRIIIA or other activating molecules on NK cells with anti-NK cell-specific IgG MAb. In this case the $F(ab')_2$ portion of the Ab is presented to the NK cells immobilized after binding of its Fc fragment to FcγRII/CD32 on target cells otherwise insensitive to spontaneous killing by NK cells. This type of cytotoxicity is elicited, experimentally, to define the role of specific NK cell membrane molecules in transducing signals that activate or inhibit NK cell functions (e.g., **refs.** ***9*** and ***10***). Whether it can be elicited also in vivo is yet to be defined. A similar mechanism may participate to activate NK cells in pathological conditions in which anti-FcγRIIIA MAbs are used for therapy, e.g., tumors ***(11)*** or purpura thrombocytopenica ***(12)***, and it might function in conditions in which anti-NK cell IgG Abs are produced in vivo ***(13)***.

The standard assay to analyze these three types of cytotoxicity is based on determination of ^{51}Cr release from metabolically labeled NK-insensitive target cells in a given period of time after their incubation in the presence of defined numbers of effector cells capable of binding the target cells in the presence of the appropriate Ab (**Fig. 2**). The cell-membrane-permeable Na_2CrO_4 is used for labeling because it binds cytoplasmic proteins and, although spontaneously released from the cells with slow kinetics, is released massively following target cell necrosis. This, resulting in cell membrane disruption, allows extracellular release of cytoplasmic proteins and of the bound $[^{51}Cr]Na_2CrO_4$. Necrosis also follows apoptosis, and this assay does not distinguish between the two forms of cell death. Mature peripheral blood NK cells mediate apoptosis-induced target cell death primarily via FasL-target cell Fas interaction: definition of its role in the observed target cell lysis requires the use of blocking anti-Fas MAb.

The high sensitivity of the ^{51}Cr release-based technique for ADCC and redirected cytotoxicity is indicated by the fact that significant target cell lysis is detected using effector cells that express the relevant surface antigen at levels undetectable by immunofluorescence. Other assays can also be used that involve nonradioactive labels or are based on induced release of specific enzymes. Kits for these are commercially available, and the only difference with ^{51}Cr release assays is the compound(s) used to label the target cells. These assays are not discussed here because either they are not sufficiently standardized for use with NK cells, or they need dedicated and costly instrumentation likely to prevent their widespread use. However, all suggestions given here are valid also when using labels different from $[^{51}Cr]Na_2CrO_4$.

2. Materials

1. Culture medium: RPMI-1640, supplemented with 10% heat-inactivated (45 min, 56°C) fetal bovine serum (FBS), 2 m*M* glutamine (complete medium), and, if desired, antibiotics, such as 0.5 U/mL penicillin and/or 0.5 mg/mL streptomycin. The complete medium is stored at 4°C; it needs to be replenished of glutamine if used later than 1 mo after preparation, given the short half-life of glutamine even at 4°C.
2. Ficoll-Na metrizoate density gradient (1.077 g/mL), such as Ficoll/Hypaque (Pharmacia, Uppsala, Sweden) or Lymphoprep (Sigma Chemical Co., St. Louis, MO) for peripheral blood lymphocyte (PBL) separation. It is stored at 4°C in the dark.
3. Phosphate-buffered saline (PBS): 12 m*M* Na_2HPO_4; 12 m*M* NaH_2PO_4; pH 7.2; 0.15 *M* NaCl buffer, with 5% FBS supplement.
4. Polyclonal sera: Rabbit sera are preferred to other species, but also mouse or rat sera can be used. Use of goat sera is not advisable, given the weak binding of goat IgG to FcγR. Antisera are produced using the target cells of interest as immunogen and are heat-inactivated (45 min, 56°C) before use. They are used for ADCC and are stored in aliquots frozen at –20°C for an indefinite time.

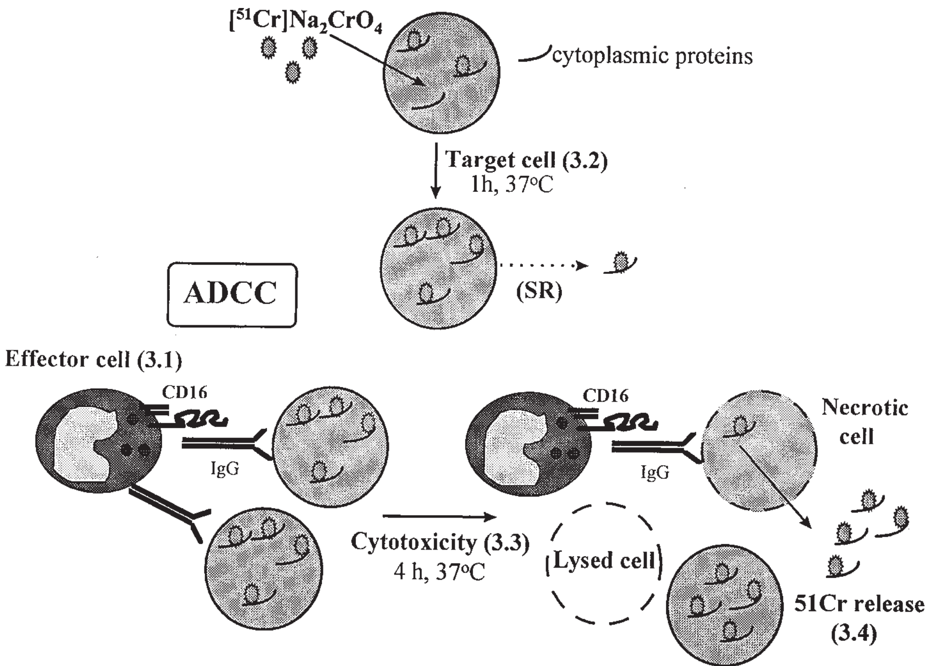

Fig. 2. ^{51}Cr release assay for NK cell-mediated ADCC. Numbers indicate the corresponding steps discussed in the text.

5. Murine IgG MAbs reacting with human NK cells (e.g., CD16, CD2, and receptors activating or inhibiting NK cell functions) or with target cells (e.g., anti-HLA-class I or class II, nonpolymorphic determinants). Some of the B-cell hybrids producing these MAbs are available from the American Type Culture Collection (ATCC, Rockville, MD); all other ones reported in the literature are expected to be available from individual investigators. The hybrid cells producing MAbs are cultured or injected into mice to produce supernatants or ascites to be used as such or from which to purify IgG and their $F(ab')_2$ fragments. Purified IgG, but not $F(ab')_2$ fragments, are also commercially available. (*See* **Appendix** for a list of some MAb to NK cells).
6. Target cells insensitive to spontaneous cytotoxicity (*see* **Note 1**): Murine mastocytoma P815-X2 (FcγRII/III$^+$) or P815-y (FcγRII/III$^-$), murine leukemia L1210 (both DBA/2 origin); human target cells expressing FcγRIIA (either constitutively or upon transfection) and with little or no sensitivity to spontaneous cytotoxicity (e.g., the monocytic THP-1 cell line [***14***]). Other target cell lines, including the major histocompatibility complex (MHC) class I antigen negative 721.221 and C1R (***9***), transfected or not with different MHC (HLA) haplotypes, the rhabdomyosarcoma-derived RDMC ***(15)***, and any desired suspension culture or adherent cell line can be used, as appropriate. The parental cell lines are available from ATCC. Transfected cell lines can be provided by individual investigators.

7. [^{51}Cr]Na_2CrO_4 (200–900 Ci/g in saline): store, lead-protected, at 4°C. Its $t_{1/2}$ is 28 d. It is advisable to use the radionucleide within two $t_{1/2}$ (~ 2 mo), adjusting the amount used based on actual radioactivity levels at any given time. Later use requires larger volumes and this may hamper labeling because of adverse effects on the cells of the resulting high salt concentrations.
8. Triton X-100 solution: Make 1% solution in H_2O (with a pinch of any blue dye added, e.g., crystal violet). This is used to determine maximum release (MR) of ^{51}Cr from the target cells. Other reagents can be used; e.g., H_2O, 0.1 *M*, HCl, 0.2 % sodium dodecyl sulfate (SDS). All are stored indefinitely at room temperature. Adding the dye has only practical reasons: to facilitate remembering this essential control, and to facilitate maintaining the right sequence of the samples to be counted (*see* **Subheading 3.3.2., step 7**).

3. Methods

3.1. Effector Cells

A previous publication in this same series ***(16)*** deals with the detailed methods for the purification of human PBLs and NK cells (*see* **Notes 2 and 3**).

To quantitate and interpret the results correctly, a series of effector cell numbers has to be used. Serial dilutions of effector cells are made, in triplicate, either in the same 96-well round-bottom microtiter plate in which the cytotoxicity assay is to be performed, or in tubes. A total of 600 or 450 µL of the first cell concentration are needed if 1:2 or 1:3 dilutions are made, respectively (e.g., 5×10^6 cells/mL of RPMI-10% FBS, if using 10^4 target cells and starting at a 50:1 effector-to-target cell [E:T] ratio) (*see* **Note 4**).

3.2. Target Cell Labeling

All procedures need to be performed under the conditions required for the use of radioactive material. The reader is expected to be familiar with these and to follow them; this topic is not addressed.

3.2.1. Cells Growing in Suspension

Depending on the cell line, and/or on personal preference, either a 1-h (a) or an overnight (12–14 h) incubation (b) can be used for [^{51}Cr]Na_2CrO_4 labeling (*see* **Notes 5–7**).

1a. 1-h labeling: Centrifuge the needed number of target cells (10^6/plate if using 10^4 target cell/well) (~ 180 × g, 5 min), discard the supernatant, resuspend the pellet in the small volume of medium remaining in the tube, add 50–100 µCi of [^{51}Cr]Na_2CrO_4/10^6 cells, and incubate at 37°C (water bath) for 45 min.

1b. Overnight labeling: Resuspend a number of target cells smaller (~ 2/3) than needed at ~ 5×10^5/mL complete medium, add the required amount of [^{51}Cr]Na_2CrO_4 (as in **step 1a**), and culture the cells overnight in a 37°C incubator, 5% CO_2, 95% humidified atmosphere. The day after, centrifuge the cells (~ 180*g*, 5 min).

2. Fill up the tube with complete medium and centrifuge (~ 180*g*, 5 min).
3. Resuspend the cells in ~10 mL of complete medium, wash (centrifuge at ~ 180*g* for 5 min at room temperature), and repeat a third time (total of three washes) (*see* **Note 8**).
4. Count the cells, and resuspend them at 10^5/mL of complete medium containing a predetermined concentration of the polyclonal anti-target cell antiserum (ADCC) or the MAb anti-effector cells (redirected cytotoxicity) (*see* **Notes 2, 9**, and **10**).

3.2.2. Adherent Target Cells

1. Collect (by trypsinization) and wash the desired number of target cells.
2. Resuspend the cells ($1–2 \times 10^5$/mL of complete medium, depending on the number needed to obtain a confluent monolayer in 96-well flat-bottom microtiter plates after overnight incubation), and add 50–100 μCi of $[^{51}Cr]Na_2CrO_4/10^6$ cells.
3. Distribute 100 μL of target cell suspension in each well of a 96-well flat-bottom microtiter plate (triplicate wells), and incubate overnight in a 5% CO_2, 95% humidified atmosphere incubator at 37°C.
4. After incubation, aspirate the medium and wash each well (200 mL of PBS, 5% heat-inactivated FBS) 3× at room temperature.
5. Add 100 μL of complete medium, containing the predetermined concentration of the polyclonal anti-target cell serum (ADCC, or the anti-effector cell MAb (redirected cytotoxicity) (*see* **Notes 9** and **10**), to each well.

3.3. Cytotoxicity Assay

3.3.1. Suspension Target Cells

1. Plate the effector cells (at the first concentration) in the first three wells of a 96-well round-bottom microtiter plate; make the desired dilutions (100-μL triplicate aliquots as in **Subheading 3.1.**).
2. Add 100 μL of radiolabeled target cell suspension to the wells already containing 100 μL of effector cells and to six additional wells.
3. Add 100 μL of complete medium (spontaneous release [SR]) to three of the six additional wells, and 100 μL of Triton X-100 solution to the remaining three wells (maximum release [MR]).
4. Centrifuge the plate(s): ~ 100*g* for 1 min at room temperature.
5. Incubate 4 h (5% CO_2, 95% humidified atmosphere incubator at 37°C).
6. Centrifuge the plate(s): ~ 200*g* for 2 min at 4°C.
7. Carefully collect 100 μL of cell-free supernatant (with a multichannel pipet), and transfer to small vials to fit the available gamma counter's holders.
8. Count (1 min/sample) in a gamma counter.

3.3.2. Adherent Target Cells

Some adherent cell lines, e.g., RDMC ***(15)***, can be labeled as indicated in **Subheading 3.2.1**. (cell lines growing in suspension) immediately after

trypsinization, and used as nonadherent. After trypsinization, some other cell lines either (rarely) lose the antigen recognized by the polyclonal antiserum, or, more frequently, tend to clump upon centrifugation, making it very difficult to maintain them in single cell suspension. In this case, the following procedure is recommended.

1. Add 100 µL of each of the serial effector cell dilutions (previously made in tubes) to triplicate wells (96-well flat-bottom microtiter plates) containing the target cells (prepared as described in **Subheading 3.2.2.**).
2. Add 100 µL of complete medium to six additional wells containing target cells (SR).
3. Proceed as in **Subheading 3.3.1.; steps 3–6**.
4. Add 100 µL of Triton X-100 solution to all wells.
5. Incubate for 1 h as in **Subheading 3.3.1.; step 4**.
6. Again collect 100 µL of supernatant and place in new small vials.
7. Place in a gamma counter and count the first (pink) and second (blue) supernatants sequentially; count for 1 min/sample.

3.4. Determination of Specific ^{51}Cr Release Assay and Calculation of Lytic Units

3.4.1. Cytotoxicity Assays Against Target Cells in Suspension

The percent specific ^{51}Cr release is calculated as (cpm E minus cpm SR)/(cpm MR minus cpm SR) × 100; percent SR = cpm SR/cpm MR; cpm, counts per minute; E, experimental sample; SR, supernatant from target cells with complete medium added; MR, supernatant from target cells with Triton X-100 solution added. Each value is calculated as the mean of triplicates (*see* **Notes 11–13**).

3.4.2. Cytotoxicity Assays Against Adherent Target Cells

To account for the possibility that different wells contain slightly different numbers of target cells, MR is evaluated in each well (this is the reason for lysing the target cells in all wells after the first 4-h incubation). The average ^{51}Cr release values are calculated, and the average SR is then subtracted from them. Calculations need to take into consideration that in the MR cpm are also included from experimental release of the supernatant harvested before target cell lysis. Therefore: % SR = $[A/(B \text{ minus } 1/2\ A)]_{SR} \times 100$; % specific ^{51}Cr release is $[A/(B - 1/2\ A)]_E$ minus % SR. A and B, cpm in the first (released during the 4 h assay) and second (Triton X-100-induced release) supernatants collected from each well, respectively; SR and E, cpm in the SR and experimental samples, respectively (*see* **Notes 11–13**).

3.4.3. Lytic Units

One lytic unit (LU) is defined as the number of effector cells that kill a predetermined percentage of target cells during the incubation time. It can be

calculated, based on linear regression analysis (Van Krogh equation) of the cytotoxicity curve *(17)*, for any desired number of effector cells. For meaningful calculation, the (fixed) percentage of lysed target cells taken as reference is chosen from those (usually the mean one) that fell within the linear portion of the cytotoxicity curve with any particular cell line.

4. Notes

4.1. General Considerations

Most of the following Notes, with the exception of those specifically related to Ab concentrations, apply also to spontaneous cytotoxicity assays.

1. If target cells are sensitive also to spontaneous cytotoxicity, care must be taken to exclude that lysis occurs, in part or completely, independently from FcγRIIIA (ADCC) or other receptors when examining the effect of triggering molecules with modulating activity on NK cell functions (redirected cytotoxicity). **Note 12** discusses this issue in detail.
2. ADCC is also mediated by monocytes (via binding of immune complexed target cells to FcγRI and IIA), macrophages (via FcγRI, FcγRIIA, and FcγRIIIA) ***(18,19)***, and polymorphonuclear granulocytes. Among the latter, both neutrophils (PMNs), the majority of which are lost from, and basophils, enriched in the mononuclear cell layer after discontinuous density gradient centrifugation on Ficoll/Hypaque, express FcγRIIA or FcγRIIIB. FcγRI is expressed on PMN stimulated with interferon (IFN)-γ ***(19)***. Also, several antigens are shared by the distinct cell types, e.g., FcγRIIIA on NK cells and macrophages ***(18)***, and some adhesion molecules, e.g., CD11/CD18, on NK cells and PMNs ***(20)***. Using leukocytes or mononuclear cells, even if after adherence, the participation of these cell types to ADCC or to redirected cytotoxicity using MAb to shared surface antigens cannot be excluded. However, NK cell dependence of the cytotoxicity observed can be defined based on either/both accurate preparation of the effector cells (e.g., following controlled depletion of specific cell types) and the use of a concentration of the anti-target or -effector cells Ab which, optimal to elicit NK cell cytotoxicity, is suboptimal or inefficient to elicit PMN and monocyte/macrophage cytotoxicity. Usually the low Ab concentrations optimal to elicit NK cell-mediated ADCC or redirected cytotoxicity do not allow detecting that mediated by the other cell types. Provided the Abs are titrated using NK cells, it can be reasonably assumed that these are the effector cells responsible for the detected ^{51}Cr release using any mononuclear peripheral blood cell preparation.

4.2. Use of PBL Preparations Treated in Some Way

3. If PBLs are pretreated with cytokines, it is sufficient to centrifuge the cells, after treatment, and resuspend them at the desired concentration in fresh complete medium.
4. Total PBL can be used, in the majority of cases, at an initial 50:1 E:T ratio, with 1:2 dilutions. Other PBL preparations (e.g., PBL stimulated with cytokines, puri-

fied or cultured NK cells, and NK cell clones) have to be used at a lower (frequently much lower, i.e., 1:1) initial E:T ratio, and possibly using 1:3 dilutions to ensure obtaining a complete cytotoxicity spectrum. The optimal conditions to be used can only be tested experimentally.

4.3. Low ^{51}Cr Incorporation by the Target Cell

5. When unusual target cells are used (e.g., particularly, primary cells), it is advisable to check their ability to incorporate (and release) ^{51}Cr. For this, count cpm in duplicate target cell aliquots identical to the one that will be used per well in the cytotoxicity assay. To check the releasable ^{51}Cr, mix labeled target cells and Triton X-100 solution as for the assay. Centrifuge after a 1-h incubation at 37°C, and count cpm in a volume of supernatant identical to that to be collected in the assay. In most cases, Triton X-100 solution will induce release of about 90% of the incorporated radioactivity.
6. Test different incubation times and cell concentrations for labeling.
7. When using primary cells, their constitutive inability to incorporate ^{51}Cr may be overcome by stimulating them to proliferate. For example, use 5-d phytohemagglutinin (PHA)-activated, instead of fresh, PBLs.

4.4. High Spontaneous Release

8. There are primarily three reasons for high (>15 %) spontaneous release:
   a. Contamination. Short-term (4h) ^{51}Cr release assays do not need to be performed under sterile conditions, provided clean medium and cells are used. However, if the stocks of either the effector or the target cells are contaminated, bacterial contamination may develop and result in toxicity to, and maximal lysis of, the target cells. Check carefully all cells to be used for lack of contamination, and use medium with antibiotics added.
   b. Incorrect collection of the supernatants. This can also result in high (>10 %) standard deviation (SD) of the triplicate cpm values. Two possibilities account for this:
      i. Collection of target cells that have not been lysed. This may depend on inaccurate centrifugation following which some target cells are still in suspension. Centrifuge longer (4 min is enough). This is preferable to higher centrifugation speed to avoid risking mistreatment-dependent target cell lysis.
      ii. Collection of pelleted, not lysed, target cells. Pay more attention when dipping the tip for collection. Keeping the tip against the wall of the well at a ~ 45° angle, and practice collection with cells detectable by eye (e.g., erythrocytes) helps. If still unable to correctly collect the supernatants, possible solutions are: (1) collect a smaller volume (~ 80 instead of 100 μL). This, however, can be done only if enough cpm are released in this volume, or (2) resort to commercially available (and costly) collection machines.
   c. Characteristics of the target cells. This may depend on:
      i. Target cells not in optimal conditions at the time of labeling. For optimal labeling and behavior, the target cells need to be in their proliferative phase

when labeled. When in doubt, split a well growing culture 1:2 the day before labeling. Target cell cultures containing up to 15 % dead cells can be used for labeling. It is preferable, however, to deplete dead cells by centrifugation on Ficoll/Hypaque (700*g* for 10 min, followed by three washes with complete medium). Cell cultures containing <80 % viable target cells should not be used, given (very likely) unacceptable (> 20 %) SR.

ii. Intrinsic characteristics of the target cells. Usually these target cells release ^{51}Cr with faster kinetics in the first hour of incubation. The high SR may then be decreased by incubating the ^{51}Cr-labeled target cells (suspended in ~ 10 mL of complete medium after the third wash) in a 37°C water bath for 1 h. After this time, centrifuge the cells (~ 180*g* for 5 min at room temperature), resuspend them at the desired concentration, and use them immediately. If SR remains high, the only solution is to change target cells.

4.5. Choice of the Ab

9. *Choice of the Ab for* ***ADCC***: Although variably effective to mediate ADCC ***(21)***, none of the available tested MAbs is optimal for sensitization of target cells for ADCC. Theoretically, however, MAbs of the IgG3 isotype would be expected to be appropriate (reviewed in **ref.** ***22***). Polyclonal (rabbit) sera are preferable. As a rule of thumb, 10^{-3}–10^{-4} dilutions are usually appropriate.
10. *Choice of the Ab for* ***redirected cytotoxicity***: Murine IgG1, 2a, and 2b, once complexed with antigens (i.e., NK cells), all bind murine or human FcγRIIA and can be used. Supernatants are the most reliable source of MAbs for redirected cytotoxicity because they contain only the MAb of interest (they are usually optimal at 1:2–1:8 dilutions). Purified IgG have the same advantage, but large amounts of supernatants or ascites, and additional work, are needed for their purification. It is thus suggested that preliminary experiments are performed with supernatants. If positive results are obtained, these are confirmed using purified IgG (optimal concentrations usually 0.2–2 μg/mL). At least two MAbs reacting with distinct epitopes on the same surface molecule should be used, if possible, and controls performed with $F(ab')_2$ of the same Ab (expected not to modulate NK cell activity). Ascites is the last choice, because of the possibility that they contain factors (proteins, something else?) that may activate or inhibit either spontaneous or Ab-dependent cytotoxicity. **Reference** ***23*** details the techniques of purification of murine IgG and their fragments.

4.6. Suboptimal Killing

11. There may be several reasons for observing suboptimal killing:
    a. Low effector cell number: increase the first E:T ratio used. However, never exceed 10^6 cells/well (i.e., 100:1, when using a standard number of 10^4 effector cells): the geometry of the well is such not to allow optimal contact between the cells when using larger cell numbers. If higher E:T is still desired, decrease, if possible, the number of target cells (never < 5×10^3/well; otherwise the number of cpm may be too low for meaningful interpretation of the results).

b. Suboptimal concentration of the polyclonal anti-target cell serum (ADCC) or of the MAb anti-effector cells (redirected cytotoxicity). These may be either in excess (prozone effect, possibly depending on agglutination of the cells and inadequate interaction with the effector cells) or in deficiency (concentration too low to allow appropriate crosslinking of the receptor). Each batch of Abs to be used needs to be tested for optimal sensitization in preliminary experiments using PBL as effector cells. The concentrations defined in this way are usually appropriate for assays with any other NK cell population. In rare cases it may be better to sensitize the cells using a higher Ab concentration, and washing the excess Ab before the assay (centrifuge the cells and resuspend them in complete medium at the desired cell concentration, without additional washing). This is valid only in the case of ADCC; target cells cannot be washed after incubation with the anti-effector cell Ab because the affinity of FcγRII for monomeric, non antigen-complexed, IgG is too low to allow the Ab to remain bound.

c. Number of target cells lower than theoretical. If plating target cells requires a long time (e.g., if the assays involve numerous plates), care must be taken to maintain the cells in an homogeneous single cell suspension. Keep mixing the stock target cell suspension from time to time while plating.

4.7. Cytotoxicity Remains at Plateau Levels at All E:T Ratios Used

12. Possible causes of unusually excessive cytotoxicity and their cures include:
    a. Once contamination is excluded (*see **Note 8***), the most likely cause is target cell sensitivity to "spontaneous cytotoxicity." Always run a control with nonsensitized target cells, and in the presence of an irrelevant antitarget MAb, e.g., CD56, or of $F(ab')_2$ fragments of the experimental MAb (for redirected cytotoxicity). If assurance of lack of spontaneous cytotoxicity is needed, and the target cells available are sensitive to spontaneous cytotoxicity, the only possibility is to use trypsin-treated effector cells. Trypsinization abolishes spontaneous cytotoxicity without cleaving, or inhibiting the function of, FcγRIIIA on NK cells ***(24,25)***. For trypsinization: (1) resuspend the effector cell preparation at 5×10^6 cells/mL of RPMI without FBS and with added trypsin (0.5 mg/mL), and DNase (50–100 µg/mL) to prevent cell loss in the DNA released by dying cells; (2) incubate the cells for 20 min in a 37°C water bath; (3) stop the reaction by adding ice-cold RPMI-10% FBS; (4) wash once with complete medium (~180*g* for 5 min); (5) resuspend the cells at the desired concentration in complete medium. For redirected cytotoxicity assays, preliminary experiments need to be performed to ensure that the antigen recognized by the desired MAb is trypsin resistant and remains functional after trypsinization. Tests in our laboratory indicate that CD16 and the p58/p50 killer cell immunoglobin-like receptors (KIR) on NK cells are trypsin resistant, whereas CD2, CD56, CD94, CD161, and both KIR p70 and p140 receptors are trypsin sensitive.
    b. High cytotoxic activity of the effector cells. Decrease the number of the effector cells; however, in some cases, the target cells cannot be used. For

example, whereas commonly used murine target cell lines (P815 and L1210) are insensitive to spontaneous cytotoxicity by fresh PBL, or by PBL stimulated for 18 h with several cytokines (Interleukin–2 [IL-2], IL-12, IL-15, and, likely, IL-18, alone or in combination), the same cell lines are sensitive to "spontaneous" cytotoxicity mediated by NK cells from short-term in vitro cultures or by NK cell clones (cultured for longer time periods with high IL-2 doses). The only (common) human cell line almost completely insensitive to spontaneous cytotoxicity by any NK cell preparation or clones is, in our experience, the monocytic THP-1 cell line ***(14)***.

4.8. Bad Triplicates (>5–10% SD Between Triplicates)

13. Bad triplicates can be due to:
    a. Lack of maintaining homogeneous single cell suspensions (either/both effector or/and target cells) (*see* **Note 12**) or contamination (*see* **Note 8**).
    b. Inaccurate plating or collection of supernatants. For both plating and collection, be careful and precise (it is advisable not to push all the cell suspension, or supernatants, out of the tip, to avoid forming bubbles that prevent precision: it is better to plate the same, lesser, volume everywhere than to have different volumes because of air bubble formation). For collection of supernatants, *see* **Note 8**. In any event, the best idea is to practice with soapy water!

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Multiple Color Immunofluorescence for Cytokine Detection at the Single Cell Level

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1. Introduction

Upon proper stimulation (e.g., crosslinking FcγRIIIA [CD16], interaction with antibody [Ab]-nonsensitized target cells, cytokines), mature natural killer (NK) cells produce and release cytokines involved in the modulation of immune responses (reviewed in **refs.** ***1,2***), including interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), GM-CSF, interleukin–5 (IL-5), and IL-10 ***(3–7)***. Cytokine production in vitro is synergistically induced by combinations of these factors and by nonspecific stimuli, such as phorbol diesters and Ca^{2+} ionophores ***(5,8)***.

Cytokines are easily detected in the supernatants from NK, and other, cells using enzyme-linked immunosorbent and radioimmunoassays (ELISA and RIA, respectively) ***(9,10)***. These are practical assays to determine whether cytokines are produced and, if so, to quantitate them at a population level. Their limitation is their inability to distinguish whether only subsets or all cells within a given population are responsible for cytokine production, and to exclude the participation of contaminating cells in it. Precise identification of the cells responsible for cytokine production with these techniques requires using mostly homogeneous cell populations, with the consequent caveat related to the possibility that negative results following stimulation (e.g., IFN-γ production by fresh T or NK cells in response to IL-2 or IL-12) may simply depend on absence of accessory cells from maximally purified cell populations ***(11)***. Issues related to quantitative analysis of cytokine production at the single-cell level also remain unsolved even after resorting to the use (at times impractical) of clonal NK cell populations.

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
Edited by: K. S. Campbell and M. Colonna © Humana Press Inc., Totowa, NJ

Several methods to analyze cytokine production at the single-cell level exist ***(12)***. These have been instrumental in defining the frequency of cytokine-producing cells ***(13)***, and the existence of the $CD4^+$ T helper cell Th1 and Th2 subsets ***(14,15)***, and will likely be so for the definition of analogous subsets possibly present among NK cells ***(16)*** and other leukocyte types (e.g., dendritic cells, monocytes). Two methods are available for such an analysis: detection of intracellularly accumulated cytokines using multiple-color immunofluorescence (IF) and detection of secreted cytokines in enzyme-linked immunosorbent spot assay (ELISPOT).

Intracellular staining allows defining both the cytokines produced by single cells in a mixed population and the phenotype of the producing cells based on expression of lineage-specific differentiation surface antigens simultaneously detectable by IF ***(14,16)***.This also overcomes possible complications related to the need, as discussed previously ***(11)***, for accessory cells to induce optimal cytokine production under certain types of stimulation. Aside from this practical advantage, more detailed information is obtained from the analysis of intracellular cytokines (i.e., number of producing cells, overlapping or distinction of cell populations producing distinct cytokines, levels of cytokine production by different cells and/or upon different stimulation). From a conceptual point of view, the limitation of this technique relates to the lack of information on the actual secretion of the cytokines of interest that, although likely, cannot be proven without additional assays (e.g., RIA). The technical limitations are those related to the availability of the appropriate reagents and equipment (a flow cytofluorimeter for simultaneous detection of at least two fluorochromes) and the need to perform multiple-color IF with optimal combinations of fluorochrome-labeled reagents that are not interfering with each other. Most of the needed reagents are commercially available, but the experiments need to be carefully planned to take advantage of the best combinations of them; for this reason, it is suggested that an expert in the technical aspects of flow cytofluorimetry is consulted for optimal planning. The most standard protocol for this technique, schematically shown in **Figs. 1** and **2**, involves: (1) stimulation of the cells with the appropriate stimuli, (2) induced accumulation of the produced cytokines in the cytoplasm (Golgi and endoplasmic reticulum) using brefeldin A (BFA), (3) surface phenotyping of live cells to allow identifying those of interest in a positive or negative fashion, (4) fixation of the cells to prevent leakage of the cytokines during the subsequent (5) permeabilization step (needed to allow their detection) and cytokine staining, and finally (6) flow cytofluorimetric analysis.

The alternative ELISPOT assay has the advantage of allowing detection of numbers of individual cytokine-producing cells even smaller than that detectable with certainty via intracellular staining in IF, in which anything below 1%

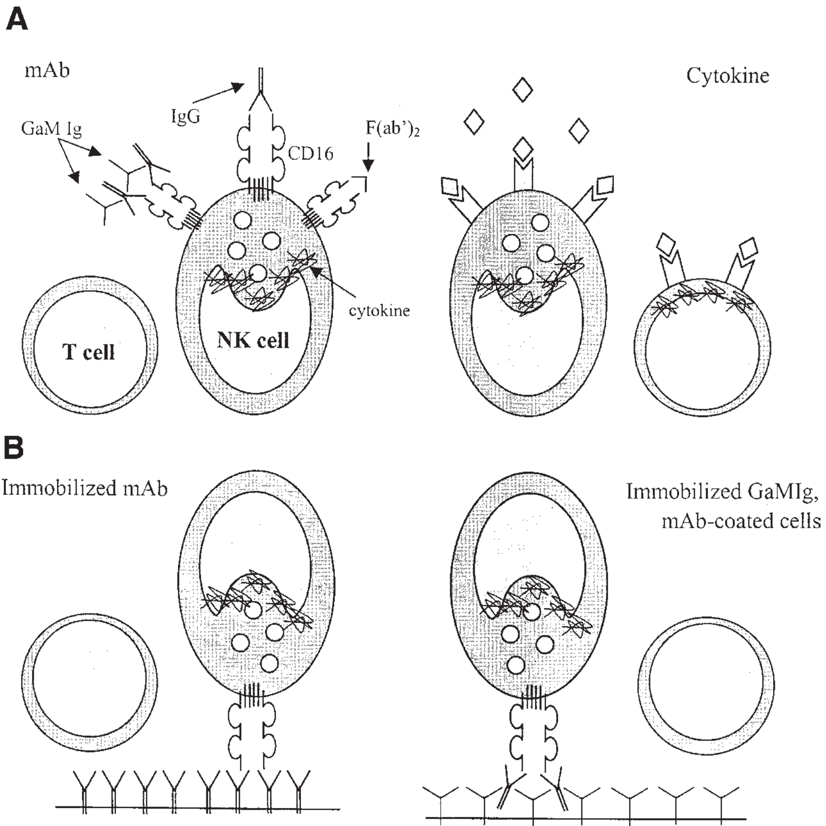

Fig. 1.

may be questionable. Also, this assay avoids the use of BFA (which may be toxic, depending on the cell type and incubation time used), and, most importantly, provides information on the actual secretion of the cytokine in the exam. Finally, combined with RIA or ELISA performed on supernatants from purified cell populations, the amount of cytokine produced per cell can also be quantitated exactly. However, the limitations of the ELISPOT assay are those related to the use of a purified cell population. Standard protocols for this assay (*see* **refs. *17–20*** for details) involve: (1) stimulation of the cells in wells coated with Ab to the cytokine(s) of interest; (2) capture of the cytokine released during stimulation by the plate-bound Ab in the vicinity of the cell; (3) elimination of the cells; (4) detection of the immobilized cytokine with a second Ab to the cytokine of interest either directly conjugated to alkaline phosphatase or horseradish peroxidase or indirectly detected by an additional labeled secondary Ab, visualized following enzymatic reaction with the appropriate substrate; and (5)

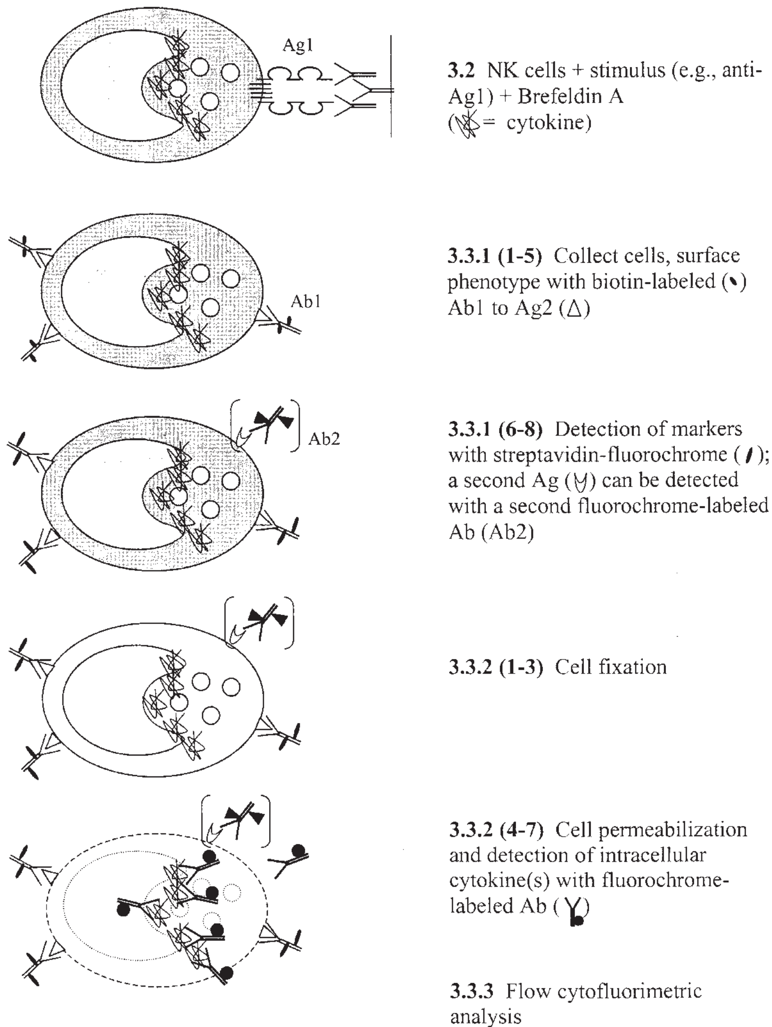

Fig. 2.

count of the colored spots visible where the cytokines are located and each corresponding to a previously present producer cell.

The choice between the two assays is based, as always, on the specific goals of the experiments performed, the information desired, and practical considerations. In this chapter, only intracellular cytokine detection by multiple-color IF is detailed.

2. Materials

1. Culture medium: RPMI-1640, supplemented with 10% heat-inactivated (45 min, 56°C) fetal bovine serum (FBS), 2 m*M* glutamine (complete medium), and,

optionally, antibiotics, such as 50 U/mL of penicillin and/or 50 μg/mL of streptomycin. It is stored at 4°C; glutamine needs to be replenished if the medium is stored for longer than 1 mo after preparation.

2. Ficoll-Na metrizoate density gradient (1.007 g/mL), such as Ficoll/Hypaque, Lymphoprep (Pharmacia, Uppsala, Sweden) or Hystopaque-1077 (Sigma Chemical, St. Louis, MO) for peripheral blood lymphocyte (PBL) separation. It is stored in the dark at 4°C.
3. Phosphate-buffered saline (PBS): 12 m*M* Na_2HPO_4; 12 m*M* NaH_2PO_4, 150 m*M* NaCl, pH 7.2.
4. Incubation buffer for surface phenotyping by IF: PBS with added 5% (v/v) human plasma and 0.02% (w/v) NaN_3. It is stored at 4°C. Human plasma is used to prevent IgG binding to monocytes possibly present in the cell population to be analyzed; it serves to reduce the background nonspecific IF.
5. Washing buffer for surface phenotyping by IF: PBS with added 0.1% (w/v) gelatin and 0.02% NaN_3. It is stored at 4°C.
6. Stimulators: Those used more frequently are:
   a. Phorbol diesters (e.g., phorbol myristate acetate [PMA]) and Ca^{2+} ionophore (e.g., A23187). Aliquots of their concentrated stock solutions in complete medium can be stored indefinitely at –20°C.
   b. Cytokines (e.g., IL-2, IL-12, IL-15, IL-18). These are usually used in recombinant form. They are stored in aliquots at –80°C, as a sterile concentrated solution in complete medium.
   c. Anti-NK cell monoclonal Abs (*see* **step 9**).
7. BFA (Sigma): Stock solution 0.5–1 mg/mL of complete medium. Aliquots can be stored at –20°C for longer than 1 yr.
8. Goat IgG anti-mouse Ig (GaMIg). Provided no contamination occurs, aliquots of this can be stored for years as a concentrated (~1–5 mg/mL) sterile solution in PBS at 4°C. (*see* **Note 1**).
9. MAb reacting with human NK cells (e.g., CD16 and other receptors activating or inhibiting NK cell functions). B cell hybrids producing some of these MAb are available from the American Type Culture Collection (ATCC, Rockville, MD); others, described in the literature, are expected to be available from individual investigators. These hybrid cell lines are either grown in culture or injected into mice to produce supernatants and ascites, respectively. These can be used for further purification of IgG and their $F(ab')_2$ fragments ***(21)***. Purified MAbs, but not their $F(ab')_2$ fragments, are also commercially available. (*See* **Appendix** for a list of MAbs reacting with NK cells, and **Note 2**).
10. Biotin- or fluorochrome-labeled MAbs to NK (e.g., CD56, CD161), non-NK (e.g., CD3 anti-T, CD21 anti-B, CD14 anti-monocyte), and other pan-leukocyte cell surface markers (e.g., major histocompatibility complex [MHC] class I antigens, nonpolymorphic determinant, or β_2 microglobulin) to be used for compensation controls (*see* **Subheading 3.3.3.**). These reagents, some of which are commercially available, are stored at 4°C in the dark for longer than 1 yr.
11. Fluorochrome (e.g., fluorescein isothiocyanate [FITC], phycoerythrin [PE], RED670 [R670])-conjugated streptavidin and anti-cytokine MAb (for intracellu-

lar staining; e.g., anti-IFN-γ). These are commercially available. They are stored as indicated above for any other Ab reagents (*see* **Note 3**).

12. Fixation and permeabilization reagents: The following methods have been optimized for using the Fix & Perm Cell Permeabilization Kit (Caltag Laboratories, Burlingame, CA). Other commercial sources may be appropriate (*see* **Note 4**).
13. Buffers for immobilization of Abs to plastics: 50 m*M* Tris, pH 9.5, or carbonate buffer, 0.1 *M* $Na_2CO_3/NaHCO_3$, pH 9.5. These are stored indefinitely at room temperature.
14. Trypsin: 5-10 mg/mL of stock solution in serum-free medium. Store in aliquots at –80°C. Defrost the needed aliquot(s) immediately before use and discard any that are left over.
15. DNase: 0.5–1 mg/mL of stock solution in serum-free medium. Store and use as for trypsin described previously. For practicality, the solutions of trypsin and DNase can be combined, each at the indicated concentration. These are used to detach cells adherent to plastic-immobilized Ab.
16. Formaldehyde: 1% solution (w/v) in PBS. It is better to prepare immediately before use.

3. Methods

3.1. Preparation of Reagents

3.1.1. Effector Cells

Freshly isolated PBL, total lymphocyte populations or purified NK cells from short-term PBL cultures ***(22)***, and NK cell clones can be used in the following procedures. Detailed protocols for the purification of PBL and NK cells have been published in a previous issue of this series ***(23)***, those for selection and growth of NK cell clones are reported in Chapters 1–5 in this volume (*see* **Note 5**). Methods of stimulation other than those described below, although very useful and appropriate when other functional outcomes are studied or different means of cytokine detection (e.g., RIA or ELISA) are used, are not recommended, as the reagents used may interfere with the flow cytometric analysis (among the nonrecommended methods are, e.g., crosslinking of receptors with MAb immobilized on Sepharose or other beads, or IgG Ab-coated erythrocytes).

3.1.2. Plastic-Immobilized Ab

The desired Abs are immobilized to polystyrene tissue culture nontreated flat-bottom plates, or similar dishes, either directly or via a secondary Ab reacting with mouse Ig (e.g., GaMIg) (**Fig. 1**). These are used (*see* **Subheading 3.2.**) in the stimulation of cytokine production. (*See* **Note 6**).

1. Dilute the desired Ab to ~10 μg/mL in either Tris or carbonate buffer (*see* **Note 7**).
2. To the wells of a plate add an appropriate volume of the Ab solution to cover completely and in excess, the bottom of the plate/well. To two additional wells

add the same volume of either the buffer used to dilute the Ab, or an irrelevant Ab in the same buffer (for negative controls). The volumes of Ab solution sufficient to coat different size wells are as follows: 50 µL, 500 µL, and 1.5 mL for 96-, 24-, and 6-well flat-bottom plates, respectively (*see* **Note 8**).
3. Incubate the plates at least 30 min at room temperature (Tris buffer), or 18 h at 4°C (carbonate buffer) (*see* **Note 7**).
4. Wash the wells 3× with PBS (aspirate the buffer and replace it with a volume of PBS sufficient to fill the well).
5. Add the same volume of complete medium (with 10% FBS) to all wells; incubate for 30 min at room temperature to saturate any protein-free site on the plate, and then replace it with new complete medium (*see* **Note 9**).

3.2. Induction of Cytokine Production

Several methods of cell stimulation (**Subheading 3.2.2.**, **steps 1a** and **1b**) can be used depending on the planned experiment (*see* **Fig. 1**).

3.2.1. Plating of Cells

1. Resuspend the cells (5×10^6/mL) in complete medium.
2. Add the desired number of cells to the appropriate wells, round or flat bottom depending on the type of stimulation (*see* **Subheadings 3.2.2.** and **3.2.3.**). The number of cells to be used per stimulation depends on the number of experimental points to be analyzed. Generally, use $1–5 \times 10^5$ cells per sample to be analyzed by flow cytofluorimetry (*see* **Note 10**). Cells are also added to two additional wells for negative (no stimulus) and positive (maximal nonspecific stimulus) controls. To obtain an adequate monolayer on flat-bottom Ab-coated plates, the maximum numbers of cells to be added/well are no more than: ~10^5 cells, 50–100 µL; 10^6 cells, 0.5–1 mL; and 3×10^6 cells, 1.5-3 mL in 96-, 24-, and 6-well plates, respectively (*see* **Note 11**).

3.2.2. Cell Stimulation with Reagents in Suspension

1. Add the stimuli at the final desired concentration, or an equivalent volume of complete medium (negative control), to the cells plated in round-bottom tissue culture-treated plates or in tissue culture-treated tubes of the appropriate size. A 2.5 and 10×10^6 maximum cell number are cultured, under optimal conditions, in 5- and 10-mL tubes, respectively. The following are the suggested final concentrations for maximal (plateau) stimulation of cytokine production by NK cells with the most common reagents used individually:
   a. 1×10^{-9} M phorbol diester PMA and 0.1 mg/mL of A23187 (Ca^{2+} ionophore), (used also as positive control, i.e., maximal stimulation) (*see* **Note 12**).
   b. Cytokines: 50–100 U/mL of IL-2; 2–5 ng/mL of IL-12; 10 ng/mL of IL-15.
   c. MAb: e.g., anti-CD16, purified Ig or their $F(ab')_2$ fragments (~5–10 mg/mL), incubated in combination with or without GaMIg (concentration fivefold that of the primary MAb) to crosslink the surface molecule of interest (*see* **Note 13**).

2. After stimulation, transfer the cells from the wells to tubes, wash them once with PBS (centrifuge ~180g, 5 min, room temperature), and resuspend them in complete medium. If the cells have been stimulated in tubes, just add PBS. In either case, continue from **Subheading 3.3.**

3.2.3. Cell Stimulation with Plastic-Immobilized Anti-Receptor MAb

For stimulation with immobilized antibodies, add the appropriate number of cells (5×10^6/mL) to plates precoated with the desired Ab (*see* plates preparation in **Subheading 3.1.2.**) and to two additional wells for positive and negative controls. When plates coated directly with the MAb of interest are used, controls are cells added to wells not coated with Ab in the same plate. When GaMIg-coated plates are used, controls are cells sensitized with the desired MAb, or the irrelevant MAb for the control samples, immediately before adding them to the plate. For this:

1. To the cells (10^7/mL of incubation buffer for IF) add the MAb directed to the receptor to be crosslinked at the appropriate, predetermined, saturating concentration.
2. Incubate the cells for 30 min on ice.
3. Wash the cells 2–3× with ice-cold PBS. (For each wash: add PBS, centrifuge at 170g, 5 min, 4°C, discard the supernatant, resuspend the cells in PBS).
4. Resuspend the cells (concentration indicated previously) in complete medium and add them to the plates.
5. After adding the desired stimulus, as indicated in **Subheading 3.2.2., steps 1a** and **1b**, add Brefeldin A (BFA) (5–10 µg/mL) to each well (*see* **Note 14**).
6. Incubate the cells (plates) in a humidified 37°C incubator 5% CO_2 in air (*see* **Note 15**) for 3–6 h (*see* **Note 16**).
7. After stimulation, collect the nonadherent cells (if any) from the plate, vigorously washing the dish/well with PBS using a Pasteur pipet. Transfer them to a tube.
8. If all or a significant percentage of cells remains on the plate (*see* **Note 17**), detach them with trypsin (*see* **Note 18**) as follows:
   a. Wash the well once with PBS and transfer the collected cells in a tube.
   b. Centrifuge the cells (180 g, 5 min, room temperature).
   c. Resuspend the cells in about half the original volume RPMI-1640 (without FBS) with added 1 mg/mL of trypsin and 50 mg/mL of DNase. This serves to digest DNA released from possibly dying cells, thus preventing loss of viable cells trapped in it.
   d. Transfer the cells back to the same well from which they were collected. This is needed to ensure identical treatment of all the cells from the same experimental sample.
   e. Incubate the cells 20 min at 37°C.
   f. Stop the reaction by adding an equal volume of ice-cold complete medium to each sample.
   g. Transfer the cells (now in suspension) to a new tube.

h. Wash each well 2–3× with complete medium to remove all the cells, and combine these with those collected in **step g**.
i. Wash the cells once with PBS (centrifuge ~180g, 5 min), and resuspend them in complete medium.

3.3. Detection of Cytokine Producing Cells

3.3.1. Surface Phenotyping (Direct Immunofluorescence)

1. Resuspend the collected cells in incubation buffer for IF (50 µL of cell suspension, containing the number of cells needed per experimental point to be analyzed) (*see* **Fig. 2** and **Note 10**).
2. Add 50 µL of the cell suspension to an appropriate number of wells of a 96-well round-bottom microtiter plate. The number of wells needed is based on the number of experimental points to be analyzed (*see* **Note 10**).
3. Add 20 µL of the desired experimental MAb (diluted at the desired concentration in washing buffer for IF). The MAb are either fluorochrome- or biotin-labeled (appropriate, saturating, concentration to be used is to be determined in preliminary experiments) (*see* **Note 19**). Add also the MAb for compensation control, or irrelevant MAb for negative control (*see* **Note 20**).
4. Incubate the cells, 20 or 30 min, at room temperature or 4°C, respectively (*see* **Note 21**).
5. Wash the cells twice: Each time add to the wells 200 µL of washing buffer for IF, centrifuge at 720*g* for 2 s at 4°C (*see* **Note 22**), aspirate, and discard the cell-free supernatant.
6. Resuspend the cells by flicking the plates carefully, to avoid interwell jumps, in the small volume of PBS left in the wells after the last wash.
7. Add 20 µL of the appropriate, experimentally predetermined, dilution of the secondary reagent to detect the primary Ab used (e.g., fluorochrome-labeled streptavidin). If additional surface markers are required to be detected, add 20 µL of the fluorochrome-labeled MAb, or irrelevant labeled MAb for negative control (as before; the appropriate concentrations have to be predetermined experimentally).
8. Incubate the cells as in **step 4**.
9. Wash the cells 3× as in **step 5**, but using PBS with no additive.

3.3.2. Intracellular Cytokine Detection

1. Resuspend the cells in 50 µL of fixation reagent.
2. Incubate the cells at room temperature for 10 min (modifications of volume used and incubation time for optimal fixation can be made according to the manufacturer's specifications).
3. Wash the cells twice with PBS (centrifuge the plates at 180*g*; carefully aspirate the supernatant so as not to disturb the cell pellet, which is frequently film-like. For this, it is helpful to hold the plate at a ~ 90° angle while aspirating the supernatant slowly from the top of the well (*see* **Note 23**).

4. Resuspend the cells in 50 µL of permeabilization reagent in which the appropriate, predetermined, concentration of the fluorochrome-labeled MAb to the cytokine(s) of interest has been diluted (*see* **Notes 4** and **24**).
5. Incubate the cells for 30 min at 4°C (*see* **step 2**).
6. Wash the cells 3× with PBS (*see* **step 3**).
7. Resuspend the cells in 1% formaldehyde in PBS (~ 70 µL, or the volume required for flow cytofluorimetric analysis).
8. Store the samples, if not analyzed immediately, in the dark (wrap the plate with aluminum foil) at 4°C for up to 1 wk.

3.3.3. Flow Cytometric Analysis

1. Analyze the samples in a flow cytofluorimeter. Either the total population of cells, or NK cells only (gated on the basis of their reactivity or lack of reactivity with Ab to differentiation surface antigens expressed, respectively, on NK or other non-NK cells) can be analyzed. (*see* **Note 25**).
2. Record both the percentage of positive cells and the mean fluorescence intensity of the antigen analyzed.
3. The values of the above parameters obtained following the experimental stimulation can/should be compared to those obtained following maximal nonspecific stimulation (e.g., PMA and ionophore).

4. Notes

4.1. Materials and Reagents

1. Although theoretically IgG from any species can be used, the preferred species is goat (GaMIg), because of the very low, if any, binding affinity of goat IgG for FcγR on human leukocytes. It is preferable to use anti-Ig $F(ab')_2$ (those anti-Ig heavy chain specific Ig), which crossreact with any Ig class and isotype.
   Most anti-mouse Ig crossreact with rat Ig, and can be used to detect MAbs of both species. Possible crossreactivity of the Ab with human Ig may introduce a confounding variable related to reactivity with B cell sIg or cell-bound Ig. To prevent this, the serum/Ig should be adsorbed on human Ig, and, best but not essential, affinity-purified on mouse Ig columns ***(22)***. Human Ig-adsorbed and affinity-purified Abs are also available commercially.
2. In planning experiments, the reactivity of FcγRIIIA (CD16) on NK cells with the Fc portion of the MAb used for stimulation or analysis must be considered. Ab-coated plates and the MAb used to stimulate/detect NK cells may elicit signaling through the FcR of NK cells. Control irrelevant, isotype-matched MAb should be used in parallel with the antibody used for crosslinking. $F(ab')_2$ fragments of the MAb are useful to ensure unintentional FcγR-induced activation.
3. The Ab required to detect a cytokine of interest may vary, depending on the assay. A MAb used in ELISA or RIA may not be appropriate for intracellular cytokine staining. The MAb used for this must efficiently bind the cytokine after cell fixation (which likely alters the form of the cytokine). To ensure that the proper Abs are used, these should be tested on cells known to produce the cytokine of

interest upon stimulation. Purified MAbs can be labeled with biotin or conjugated to FITC, according to standard procedures. Directly labeled MAbs, including PE- and R670-conjugates, are also commercially available. Although theoretically anti-cytokine MAbs can be fluorochrome-labeled in house, the best are those commercially available. However, in our experience, each batch must be tested (in a standard control assay) before use. In our experience, commercially available batches that do not work properly exist, but are usually replaced by the manufacturer, if convincing proof is provided.

4. Although classical permeabilization an/or fixation reagents may be used that can be made in house (e.g., saponin, digitonin), it is our experience that the most appropriate ones are those commercially available. Unfortunately, their composition is neither indicated nor given by the manufacturer on request.
5. It is not necessary to purify NK cells, although T and other cells present in the cultures may also produce the same cytokine. To ensure analyzing NK cells, homogeneous NK cell populations can be used (purified from the cultures) or, very simply, cells other than NK are excluded from the flow cytometric analysis. This is accomplished by gating the cells expressing NK cell-specific markers or those not expressing markers, present on other cell types, detected by the appropriate MAbs. However, the desired MAbs may not always be available for this. This point is discussed in detail in the Notes in **Subheading 4.3.**
6. GaMIg-coated plates are preferable to those with directly bound MAb. Standardization of the adherence procedure is easier and more reproducible, and the GaMIg does not suffer from the inter-MAb variability in their plastic-binding efficiency.
7. Depending on the antibody, higher or lower concentrations (in this order of magnitude) may be used. Either buffer can be used; however, carbonate buffer gives more reproducible results with most plate types. Different plate brands, different batches of the same brand, and different plates in the same batch may vary significantly in the efficiency to bind proteins. The only solution for negative results is to precheck the batch of plates in use; in this case, change them. Always record the batch number for reference.
8. Tissue culture nontreated plates are highly hydrophobic, and any aqueous solution tends to be repelled and accumulate in a corner. This can result in uneven coating, and consequently inadequate cell stimulation. Check carefully each well after adding the Ab solution: if necessary, add more volume and/or spread the solution with the tip of the pipet. Check again after 15–20 min and, if needed, spread again.
9. Ab-coated plates can be stored at 4°C for extended periods of time (months) before use, provided care is taken to prevent bacterial or fungal contamination. They are better stored with the Ab solution in them, but may be stored at this point, too. Care should be taken to prevent evaporation of the Ab solution or the media. For this, store the plates tightly wrapped with Parafilm.

4.2. Optimization of the Stimulation

10. Enough cells for at least two sets of staining are required per experimental stimulation. The first is needed for negative control, background staining, using irrelevant MAb. The second one includes the samples in which surface phenotype

and intracellular cytokine(s) are to be analyzed simultaneously. For example, to detect three cytokines in a specific cell subset, enough cells are needed for three samples: one is used as negative control, a second is used for simultaneous analysis of one surface marker and two cytokines, and the third for that of two additional surface markers and a different cytokine. Also, there may be significant cell loss during the staining process, with consequent difficulties in data interpretation. This may be especially important if the stimulation used induces significant cell apoptosis. To overcome this, use more cells per analysis.

11. Several reasons can account for suboptimal stimulation in Ab-coated plates: (a) suboptimal coating (titrate the MAb concentration carefully); (b) uneven coating (*see* **Note 8**); (c) excessive cell number, resulting in cells accumulating on the bottom of the well in several layers, only the bottom one of which is in contact with the Ab. Also, to enhance cytokine production when using anti-NK cell MAb as stimulus it may be helpful to add low concentrations of any NK-activating cytokine (e.g., IL-2, IL-12) with which the experimental stimulus synergizes ***(5,8,24)***. This is then to be taken into consideration for a correct interpretation of the results.
12. The concentrations (in these orders of magnitude) may differ depending on the cell population to be stimulated. If excessive cell death is observed, decrease the concentration of both reagents. In addition, detection of surface molecules (e.g., CD56) that are not down-modulated by PMA may be suboptimal after this treatment. In this case, titrate the MAb to the surface marker of interest using PMA-treated cells.
13. GaMIg can be added, if useful, to increase stimulation as from preliminary experiments. It is not necessary to wash the excess primary MAb, but the GaMIg concentration needs to be in at least fivefold excess. This is to overcome the excess cell-free MAbs that can compete with the cell-bound one for GaMIg binding, and make the stimulation suboptimal.
14. BFA prevents the release of cytokines from the cells, resulting in their accumulation in the endoplasmic recticulum and Golgi apparatus. When longer stimulation times are needed, it may be beneficial to add 5 μg/mL BFA, either midway through or at the end of the stimulation to ensure that cytokines are not exocytosed. BFA can be substituted by monensin, or other agents with similar activity.
15. Although 5% CO_2 is appropriate, in the case of suboptimal NK cell response, consider increasing the CO_2 to 8%.
16. The time of stimulation needs to be optimized. Shorter stimulation times are inefficient; longer ones may be detrimental, depending on whether or not they induce high degree of cell death. For example, the nonspecific PMA + A23187 stimulation, and specific CD16 crosslinking result in significant cell death by 6-h culture. It is our experience that the percentage of NK cells in which significant levels of intracellular IFN-γ is detectable after PMA + A23187 stimulation is >75–80% after a 3-h stimulation but significantly lower, and more inconsistent, after 6 h. Additional factors such as kinetics of transcription/trans-

lation of the cytokines of interest contribute to the choice of the incubation time optimal for stimulation.

4.3. Surface Phenotyping to Identify Cytokine Producing Cells

17. Always look at the plates under the microscope after stimulation. Do not assume all cells will be in suspension: you may end up with no cells to be analyzed. Depending on the receptor being crosslinked and the duration of stimulation, cells may be either detached or firmly attached to the MAb-coated plate after stimulation. The behavior of the different molecules upon crosslinking is better tested in preliminary experiments. For example, CD16 is rapidly internalized upon crosslinking ***(25,26)*** and down-modulated upon PMA stimulation ***(27)***, (reviewed in **ref.** ***28***): its disappearance from the cell membrane within 30 min results in release of the cells from the anti-CD16-coated plate by the end of a 3-h stimulation. The p70 killer cell Ig-like receptor (KIR), instead, is not down-modulated upon crosslinking, and cells adhering to anti-p70-coated plates are firmly adherent after the same incubation time. Tests in our laboratory have shown that CD161 and CD56 are not down-regulated after PMA + A23187 stimulation.
18. Trypsin releases the cells from the plate, but it may also cleave surface markers of interest, thus preventing the possibility to identify the cells expressing these markers using reagents directed to them. CD16 ***(29)*** and the p58/p50 KIRs are trypsin resistant whereas CD2, CD56, CD94, CD161 ***(30)***, and both p70 and p140 KIRs are trypsin sensitive.
19. The concentration of MAb to be used should be determined in preliminary titration tests: for best results, choose the lowest (saturating) concentration that stains the cells at plateau levels. In mixed cell populations (e.g., PBL or short-term NK cell cultures), detection of a lineage-specific surface marker can distinguish the different cell types producing the cytokines of interest. CD56 is a convenient marker for NK cell detection, given its resistance to the most commonly used stimuli. Other surface markers can be used to discriminate subsets of NK cells in purified populations. Alternatively, MAbs to surface markers on cells other than NK can be used. The choice of surface markers may be limited (*see* **Notes 16** and **17**).
20. Compensation controls are necessary to properly calibrate the flow cytometer to differentiate between the fluorochromes used for Ab detection. Both a negative and a positive control are needed with, respectively, the fluorochrome alone and a fluorochrome-labeled anti-pan leukocyte MAb alone. Staining the cells with a biotin-labeled Ab to a surface Ag expressed at high density, followed by a streptavidin-fluorochrome conjugate is the method described here. The intensity of staining for compensation should be equal to or greater than that of the positive staining sets. Biotin-labeled anti-MHC class I Ag MAb is convenient for compensation controls. If only one fluorochrome is used, no compensation controls are necessary. Negative controls need to be performed to distinguish between specific and nonspecific fluorescence. Irrelevant MAbs are used to determine the background fluorescence, which results from a combination of the nonspecific binding of the MAb and the autofluorescence of the cells analyzed.

21. Incubation at 4°C may be necessary to reduce background staining, especially if the surface marker of interest is expressed at low density on the cell surface.
22. If significant cell loss occurs during the washing procedure, slower centrifugation speed for longer time may be necessary. Loss depends on increased cell fragility as a result of stimulation or trypsinization.

4.4. Optimization of the Intracellular Cytokine Detection

23. Cell loss is most likely to occur during these and subsequent washes. Fixed cells tend to form a thin film, which is easily lost if washed without proper care. The slower centrifuge speed is necessary to prevent the damaging/clumping of the cells, especially after permeabilization.
24. The fluorochrome-labeled anti-cytokine MAb has to be titrated preliminarily using cells stimulated to produce the cytokine of interest. For this, nonspecific stimulation (PMA + Ca^{2+} ionophore) is best to induce high levels of cytokine production (*see also* **Note 12**). Choose the lowest saturating concentration of Ab that stains the cells at plateau levels. Background IF after fixation may increase (although this is usually not a major problem working with NK cells). Unacceptable background is usually taken care of adding 5% (v:v) rabbit serum to the buffers used for the incubation and washing of the permeabilized, fixed cells.

4.5. Flow Cytofluorimetric Analysis

25. It is essential that calibration/setup of the flow cytometer is carried out for each condition (nonstimulated or stimulated cells). Using the negative control sample, adjust forward and side scatter to appropriately display the cells on scale and to allow for the desired gating to fluorescence displays. For optimal results, autofluorescence (negative control) background should be included within the first decade of a 3–4-decade logarithmic scale. Cells in each experimental (Ab-treated) sample are then gated on the same population as that of the control to confirm that the Ab^+ population is reacting, yet on scale (*see* **Notes 19** and **24**). In the case of multifluorochrome-treated samples, compensation controls should then be run; this is to correct, electronically, for the detection of emission of a given fluorochrome by the detector for a different one due to intrinsic spectral overlap of the dyes (*see* **Note 20**). Cells in each positively single-fluorochrome-stained compensation control should be run and gated to produce a two-color dot plot. Compensation should be adjusted such that the Ab^+ population for each fluorochrome is parallel to the appropriate axis and in a straight line. Improper compensation could yield false multifluorescent populations detectable even in single-stained samples, and especially confusing or artefactual results in multistained samples. Experimental samples are then run and data collected, fine-tuning compensation if necessary. (Consult **refs. *31*** and ***32*** for better understanding the principles and applications of flow cytofluorimetric techniques).

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18

Generation of Antibodies to Cell Surface Markers on Mature Natural Killer Cells

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1. Introduction

A limitation in the ability to analyze the function of the natural killer cell Ly49 and other receptors is the lack of antibodies to most of them. Even when monoclonal antibodies (MAbs) have been generated, they do not always react with a given receptor from all strains of mice. For example, 5E6 binds to Ly49C and/or I from B6, C3H, and NZB strains, but fails to react with any NK cells of 129, C57BR, C58, or FVB mice. Besides Ly49 receptors, NKR-P1 receptor gene products (NKR-P1B) also contain an (immunoreceptor tyrosine-based inhibiting motif (ITIM) in the cytoplasmic domain ***(1)***. In the human system, the NKG2 family of receptor molecules with or without ITIMs associate as heterodimers with CD94 molecules ***(2)***, and are therefore candidates for murine non-Ly49 receptors for class I antigens. Molecules similar to human killer cell immunoglobulin-like receptors (KIRs) have not been cloned in mice. Other receptors receive activating or positive signals and therefore may have important NK cell functions ***(3,4)***. We describe here our approaches to develop anti-NK cell antibodies to study their receptors.

2. Materials

2.1. Research Animals

New Zealand rabbits are purchased from a local rabbitry and are maintained in separate cages. F344 rats were purchased from Taconic Farms (Germantown,

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
Edited by: K. S. Campbell and M. Colonna © Humana Press Inc., Totowa, NJ

NY). Mice of various strains were bred in the Microbiology Colony of this University and are described in Chapter 7.

2.2. Medium and Solutions

1. Enzyme-linked immunosorbent assay (ELISA) coating buffer: 1.59 g of Na_2CO_4, 2.93 g of $NaHCO_3$, 0.2 g of NaN_3 in 1000 mL dH_2O, pH 9.6.
2. Tris-buffered saline (TBS): 10 m*M* Tris-HCl (1.21 g/L), 150 m*M* NaCl (8.77 g/L), pH 8.0.
3. TBST: TBS with 0.05% Tween-20.
4. ELISA blocking buffer: 5% Casein hydrolysate in TBST.
5. Live cell ELISA medium: DMEM medium with 2.5% fetal calf serum (FCS).
6. HAT selection medium for selection of hybridomas: RPMI 1640 medium with 20% FCS, 10% NCTClog, 2 m*M* glutamine, 1× OAA-BP1 Coxaloacetic acid-bovine pancreas insulin, and HAT supplement (13.6 μg/mL hypoxanthine, 0.16 μg/mL of aminopterin, and 3.8 μg/mL of thymidine).
7. Dulbecco's modified Eagle's medium + (DMEM+) (SP2/0 hybridoma cells growth medium): DMEM with 2 m*M* L-glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin and 20% heat-inactivated FCS.
8. Peptide coupling buffer: 50 m*M* Tris-HCl, 5 m*M* EDTA-Na, pH 8.5.
9. Cysteine solution: 0.05 *M* cysteine in water.
10. 1 *M* NaCl solution.
11. 1 *M* Tris-HCl solution pH 9.5.
12. Antibody elution buffer: 100 m*M* Glycine, pH 2.5–3.0.
13. PBS-1% BSA: Phosphate-buffered saline (PBS) solution containing 1% bovine serum albumin (BSA).

2.3. Tissue Culture and FACS® Analysis Materials and Methods (see Chapters 7 and 8)

2.4. Other Materials

1. Keyhole-limpet hemocyanin (KLH; Pierce).
2. M-Maleimidobenzoyl-N-hydroxysuccimide (MBS; Pierce)
3. 96-Well flat and U-bottomed microtiter plates (Corning, NY).
4. *p*-Nitrophenyl phosphate disodium solution: Add 1 Sigma 104® tablet in 5 mL of 10% diethanolamine buffer, pH 9.8.
5. Goat antirabbit Ig-alkaline phosphatase conjugate.
6. 96-Well flexible flat-bottomed plate (Falcon no. 3912).
7. Histopaque (Sigma, St. Louis, MO).
8. Polyethylene glycol (PEG) 1500 (Boehringer Mannheim Biochemicals): Prepare as 50% solution in water.
9. Methylcellulose cloning medium (StemSep, Vancouver, BC).
10. Sulfolink gel support columns (Pierce, Rockford, IL).

3. Methods

3.1. Production of Abs Against Murine NK Cell Surface Markers in Mice and Rats

3.1.1. Immunogens

Use one of the following or combinations as desired:

1. Cells: Use PK136 anti-NK1.1 MAbs to purify NK cells by cell sorter *(5)* or by magnetic bead sorting as described in **Chapter 8**.
2. Peptides: Synthesize peptides that represent portions of the extracellular domains of NK receptor molecules, e.g., Ly49I, which differ slightly from other Ly49 molecules. Add a cysteine to the NH-terminus to allow coupling with KLH. Conjugate peptides with KLH, using MBS at pH 7.3 for 3 h to form an immunogen after dialysis.
3. DNA: Insert cDNA coding for Ly 49, 2B4, or other NK receptor molecules into an expression vector, CMVI (gift from David Russell of this University), which uses the early promoter and intron A of cytomegalovirus (CMV).

3.1.2. Immunization

Use one of the following techniques:

1. Inject NK cells (~10^6) free of FCS intrasplenically into H2-identical mice, e.g., B6 to 129 strain (to avoid generating mainly Abs to major histocompatibility complex [MHC] antigens), or into rats (*see* **Note 1**). This is repeated at 1–2 mo in mice and monthly in rats. Once the serum shows a high titer of reactivity by the screening assays (*see below*), the animals receive a final intrasplenic injection (*see* **Note 2**). The spleen is harvested 3 d later to fuse B cells with myeloma cells to generate hybridomas.
2. Inject peptide (100 µg) immunogens subcutaneously. with complete Freund's adjuvant (CFA), and later inject peptide with incomplete Freund's adjuvant (IFA) at biweekly intervals. Serum is obtained to detect Abs reactive against the relevant antigen. Mice and rats require at least five injections.
3. Inject cDNA (100 µg/6–8-wk-old mouse; 200 µg/10–12-wk-old rat) i.m. or s.c. at 2-wk intervals; mice or rats can be injected intrasplenically as described previously for NK cells. The final immunization is performed after a titer of 1:10,000 by ELISA is reached, and the spleens are harvested within 7 d to make fusion partners with SP2/0 myeloma cells (*see* **Note 2**).

3.1.3. ELISA Assay for Screening Peptide Antigens

1. Add the peptide at 5 µg/mL in ELISA coating buffer to wells of microtiter U-plates (150 µL volumes; *see* **Note 3**). Allow binding to occur overnight at 4°C.
2. Wash the wells 3× and add ELISA blocking buffer and incubate for 1 h at 37°C.
3. Add 1:10 to 1:10,000 dilutions of serum in TBST (150 µL) and incubate for 2 h at 37°C.

4. Wash 3× with TBST, add secondary antibodies, e.g., goat antirabbit Ig-alkaline phosphatase conjugate at 1:1000, and incubate at 37°C for 1 h.
5. Wash thrice and add substrate (P-nitrophenyl phosphate disodium solution) for 30 min at 37°C.
6. Read values at 409 nm in a UV plate reader.

3.1.4. ELISA Assay for Screening on Viable Cells (see Note 4).

1. Cells to be tested, e.g., BW5147 lymphoma cells expressing Ly49 receptor genes, are suspended in 4 + RPMI medium at 5×10^6/mL and are plated (100 µL) into wells of a 96-well flexible flat-bottomed plate ***(6)***.
2. Add primary antibody from hybridoma culture or serum, diluted with RPMI 1640 medium/2.5% FCS (use throughout), and incubate for 30 min at 4°C.
3. Centrifuge the plate at 850*g* for 3 min, and wash twice.
4. Add 100 µL of secondary antibody (goat antimouse, antirat, or antirabbit Ig labeled with alkaline phosphatase, usually 1:1000 dilution), and incubate for 30 min at 4°C.
5. Centrifuge the plate at 850*g* for 3 min, and then wash twice with TBS to try to completely free the plate of phosphate.
6. After the final wash, add 100 µL of P-nitrophenyl phosphate disodium substrate solution and incubate at 37°C for 30 min.
7. Read values at 409 nm in a UV plate reader.

3.1.5. Screening by FACS® Analysis

1. Use lymphoma cells transfected or not with the genes for NK receptor molecules, e.g., BW5147-Ly49I^{B6} or -Ly49C^{BALB} cells. Incubate cells at 4°C for 20 min with hybridoma supernatants undiluted or sera diluted to 1:10 to 1:1000.
2. Wash in PBS-1% BSA.
3. Add the secondary fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat Ig antibody and incubate for 20 min at 4°C, and wash again.
4. Screen the appropriate sera or supernatant fluids first by ELISA and then by FACS® analysis (*see* **Note 4**).

3.1.6. Production of Hybridoma MAbs

Fuse spleen cells of immunized mice or rats with HAT-sensitive SP2/O myeloma cells that cannot secrete Ig unless fused with B cells, using the following recent modification of standard methods ***(7)***.

1. Purify viable spleen cells by centrifugation over Histopaque. Collect the cells at the interface and wash 3×.
2. Mix spleen cells and BALB/c SP2/0 myeloma cells grown in DMEM+ at a 5:1 ratio and centrifuge (5–10 min, 200–400*g*).
3. Remove the supernatant completely to avoid diluting the PEG. Break the pellet gently by tapping the bottom of the conical tube. Place in a 37°C water bath.
4. Add 1 mL of 50% PEG 1500, continually stirring with the pipet tip; continue for 1–2 min.

5. Add 1 mL of medium without FCS prewarmed to 37°C to the fusion mixture, and stir continually for 1 min.
6. Add 3 mL of warmed medium and stir for another 3 min.
7. Finally add 10 mL of warmed medium. Centrifuge the cells and discard the supernatant.
8. Resuspend the cells in HAT selection medium and seed cells into 96-well microtiter plates by limiting dilution.
9. Screen supernatants of wells containing successfully fused cells by ELISA (*see* **Note 4**).
10. Transfer cells of positive well to 24-well plates and retest supernatants. Use limiting dilution or methylcellulose cloning medium (StemSep, Vancouver) to clone hybridomas.
11. Grow these cloned cells and test their supernatants; expand the cell numbers to grow cells (10^7) in SCID mice 7 d after 0.5 mL pristane i.p. and with 15–20 µL rabbit anti-asialo GMI serum i.p. produce MAbs.
12. Cryopreserve (–80°C or liquid nitogen) positive hybridomas.

3.2. Production of Rabbit Polyclonal Monospecific Abs Against NK Cell Receptors

3.2.1. Immunizations

1. Immunize New Zealand rabbits with KLH-peptide conjugates (100 µg of peptide) with (CFA) and inject peptide with (IFA) at biweekly intervals. Rabbits usually require three injections. If immunizing with cDNA, inject the cDNA (500–1000 µg/4 pound 10–12-wk-old rabbit) i.m. or s.c. at 2-wk intervals.
2. Bleed the rabbits and test various dilutions of serum for reactivity to antigens, e.g., Ly49I– vs Ly49C– derived peptides, using an ELISA method. Rabbits are bled (~30 mL) every 2 wk.

3.2.2. Affinity Purification of Antibodies

Although polyclonal rabbit antisera can be used directly for staining, immunoprecipitation, ELISA, and Western blotting, such sera are not optimal for blocking experiments in the in vitro assay, FACS® sorting, or depletion of specific NK cell subsets in vivo. Affinity purification is accomplished by passing the polyclonal antisera over peptide affinity matrix columns as follows:

1. Couple peptides with terminal cysteine residues to Sulfolink gel support columns by incubating 1 mg of peptide (resuspended in 2–3 mL of peptide coupling buffer) per 1 mL of gel matrix.
2. Mix the columns at room tempersture for 15 min followed by a 30-min incubation at room temperature without mixing.
3. Compare the A_{280} of the wash material with that of the starting material to determine coupling efficiency.
4. Block nonspecific binding sites on the affinity matrix by adding a 0.05 *M* cysteine solution to the column and incubate as described for the peptide binding.

5. Wash the column 4× with 4 mL each of wash buffer (1 *M* NaCl).
6. Add antiserum to the column for affinity purification for 1 h at room temperature with gentle mixing.
7. Wash the column with 17 mL of PBS.
8. Elute affinity-purified antibodies by adding 8 mL of glycine buffer. Collect 1-mL fractions and neutralize with 50 µL of 1 *M* Tris pH 9.5. Monitor elution by reading A_{280} of collected fractions.
9. Pool fractions containing peak values of eluted antibody, and dialyze. Purified antibodies can be used directly or after conjugation with fluorochromes.

For those antisera to peptides that share crossreactive epitopes such as those for Ly49C and Ly49I, the crossreactive antibodies can be removed by using peptide columns. For example, antisera to Ly49I peptide can be adsorbed to an Ly49C peptide column and the eluate, which contains anti-Ly49I antibodies, can then be passed over an Ly49I peptide column for affinity purification, as described previously. In addition, the crossreactive antibodies can be obtained by elution from the Ly49C peptide column, as described previously.

4. Notes

1. A limitation in using normal cells expressing NK receptors, as immunogens is the relativley low expression of Ly49 and other receptors per cell.
2. The cDNA for NK receptor genes can be used to generate MAbs; our own experience suggests that intrasplenic injection is far superior to intramuscular injection to initiate the immune response.
3. The source of microtiter plates is important for the ELISA; after testing four separate U-plates, the product of the Corning company proved to be the best for this purpose.
4. The adage “you get what you screen for” in hybridoma methodology has validity. If the goal is to obtain MAbs that can be used to stain cells for FACS® analysis or sorting, it is logical to use that method for screening. We have opted to use the ELISA screening to save time and costs of FACS® analysis. This has led to several cases of hybridoma supernatant fluids positive by cellular ELISA but negative by FACS® analysis.

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19

Human Single-Chain Fv Fragments Specific for Natural Killer Cell Receptors from Phage Display Libraries

Barbara Uchanska-Ziegler, Qiao Xugang, Armin Volz, Ahuva Nissim, and Andreas Ziegler

1. Introduction

The exquisite specificity of monoclonal antibodies (MAbs) has made them extremely versatile tools for scientific, diagnostic, and therapeutic purposes. However, their production is time consuming and requires the immunization of animals and the subsequent use of rodent cells. These procedures can be circumvented by the application of in vitro-selected single-chain Fv (scFv) fragments, a method increasingly used to obtain reagents principally suitable for recognizing any desired antigen (reviewed in **refs. *1–4***).

scFv fragments consist of the variable regions of the light (V_L) and heavy (V_H) chains of an antibody molecule, connected by a suitable linker. In principle, there are two ways to produce scFv fragments, either by genetic engineering of existing MAbs ***(5)*** or by selection from libraries of human or animal origin containing antibody fragments generated by cloning repertoires of V-genes ***(6)***. These constructs can be connected with the gene for the minor coat protein (pIII) of filamentous bacteriophage ***(7)*** and displayed by this phage as fusion protein ***(8)***. The phage particle exhibits antibody-like reactivity and it carries the DNA sequence for the scFv fragment that it displays. Specific phages can be selected from large repertoires (up to 10^{12}) on any desired antigen, and used as culture supernatant with antibody-like specificity. Alternatively, scFv fragments may be expressed in a different strain of bacteria as soluble proteins, without pIII polypeptide and phage particle attached ***(9)***.

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
Edited by: K. S. Campbell and M. Colonna © Humana Press Inc., Totowa, NJ

Two principal types of libraries can be employed for scFv generation: one type of library is derived from lymphocytes of immunized animals or human donors, and it contains V_H and V_L chains that were rearranged in vivo and paired at random in vitro (reviewed in **refs. *10–15***). However, one disadvantage of these libraries is the fact that they are directed toward a single or a limited number of antigens, and they have to be newly constructed every time when a novel, unrelated antigen is employed. Potentially more versatile are "naive" libraries constructed either from V genes generated in vivo in unimmunized donors ***(16)*** or entirely in vitro ***(9,17)***. Such "synthetic" libraries usually contain rearranged V_H and V_L chain genes, generated by means of polymerase chain reaction (PCR) amplification from μ, κ, and λ mRNA or by using V_H and V_L chain gene segments generated in vivo and encoding part or all of each CDR3 loop by inserting random sequences.

Phage display of antibody fragments created by cloning of immunoglobulin V-genes is a rapidly developing method for the generation of antibody-like proteins. However, despite the availability of phage display libraries with high complexity ($>10^9$) and a considerable collection of scFv fragments specific not only for different small molecules such as haptens but also for complex human intracellular and cell surface molecules, scFv reagents have not yet replaced MAbs. The main reason for this is the affinity of scFv fragments toward their targets, which is generally lower than that of MAbs. Although procedures have been devised to overcome these problems (affinity maturation in vitro), the immune system appears *a priori* better suited to deal with the complexity of antigenic determinants. Nevertheless, useful scFv fragments have been produced in just 1–2 wk, and we will describe a route to obtain such reagents against NK cell receptors.

The aim of this chapter is to provide a detailed, practical instruction that will enable scientists with some experience in molecular biology to select antigen-specific scFv fragments from a phage display library, using killer cell immunoglobulin-like receptors (KIRs) of the p58 family (NKAT2) and of the p70 family (NKAT4), respectively, as immunogens. Notes about potential pitfalls are included as well.

2. Materials

2.1. Chemicals, Solutions, and Media

Unless otherwise stated, chemicals were purchased from ICN Pharmaceuticals, (Costa Mesa, CA), Merck (Darmstadt, Germany), Serva (Heidelberg, Germany), and Sigma (München, Germany); restriction enzymes and antibiotics from Boehringer Mannheim GmbH (Mannheim, Germany), MBI Fermentas (St. Leon-Rot, Germany), and Stratagene Cloning System (La Jolla, CA); primary and secondary antibodies from Bio-Rad (Hercules, CA), Caltag Labo-

ratories (Burlingame, CA), Coulter-Immunotech (Hamburg, Germany), Pharmacia Biotech (Uppsala, Sweden) or Sigma (München, Germany). Bacto-agar, bacto-tryptone and yeast extract were from Difco Laboratories (Detroit, MI).

All chemicals should be of molecular biology grade and all solutions should either be autoclaved or sterilized by passing through a 0.45-mm disposable filter.

2.1.1. Culture Media for Growing Bacteria

1. 2× TY: Dissolve 16 g of bacto-tryptone, 10 g of yeast extract, and 5 g of NaCl in 1 *l* of double- distilled (dd)H_2O; adjust pH to 7.0.
2. Ampicillin (1000×): 100 mg/mL in ddH_20, filter sterilize, and keep at –20°C.
3. Kanamycin (300×): 75 mg/mL in ddH_2O, filter sterilize, and keep at –20°C.
4. 20% (w/v) glucose in ddH_2O, filter sterilize.
5. 2× TY/A/K: 2× TY supplemented with 100 µg/mL of ampicillin and 25 mg/mL of kanamycin.
6. 2× TY/Amp/Gluc: 2× TY containing 100 µg/mL of ampicillin and 1% glucose (add from separate 20% stock).
7. 2× TY/Amp/0.1 Gluc: 2× TY supplemented with 100 µg/mL of ampicillin and 1% glucose (add from separate 20% stock).
8. 2× TY/Gcl: 2× TY containing 15% glycerol, for storing bacteria at –70°C.
9. TYE: Dissolve 15 g of bacto-agar, 8 g of NaCl, 10 g of bacto-tryptone, 5 g of yeast extract in 1L of ddH_2O. Before use, add 100 mg/mL of ampicillin (at 50° of C).
10. TYE/Amp/Gluc: TYE containing 100 mg/mL of ampicillin and 1% glucose.
11. IPTG (1000×): 100 m*M* Isopropyl-β-D-thiogalactopyranoside in ddH_2O, filter sterilize, and keep in aliquots at –20°C.
12. H-top agar: 10 g of tryptone, 8 g of NaCl, 7 g of agar in 1 L of ddH_2O. Autoclave for 15 min, cool to 50°C, divide into 100-mL bottles, reautoclave, cool, and store at room temperature. Before use, melt the agar in a microwave oven, and use at 45°C.

2.1.2. Reagents and Solutions for Phage Selection

1. 50 m*M* Sodium hydrogen carbonate, pH 9.6.
2. Phosphate-buffered saline (PBS): Dissolve 5.84 g of NaCl, 4.72 g of Na_2HPO_4, and 2.64 g of $NaH_2PO_4 \cdot 2H_2O$ in 1 L of ddH_2O, and adjust pH to 7.2 with NaOH.
3. PBS/Tween-20: PBS containing 0.1% Tween-20 (v/v).
4. MPBS: 2% (w/v) Marvel (dried skimmed milk powder) in PBS.
5. Triethylamine: 100 m*M* in ddH_2O; dissolve 700 µL of triethylamine (7.18 *M*) in 50 mL of water for instant use.
6. 1 *M* Tris-HCl, pH 7.4 (neutralization buffer).
7. PEG/NaCl: 20% (w/v) Polyethylene glycol 6000, 2.5 *M* NaCl.
8. TE (Tris-EDTA): 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 7.4.

2.1.3. Analysis of Phage Population by PCR and Restriction Digest

1. 7.5% Acrylamide Gel Solution: standard gel system to resolve smaller DNA fragments (e.g., 50-2000 bp). Mix thoroughly 3.75 mL of 40% acrylamide (38 g of

acrylamide, 2 g of N,N´-methylenebisacrylamide, add H_2O to 100 mL), 3 mL 10× TBE (90 m*M* Tris base, 90 m*M* boric acid, 1 m*M* EDTA), 0.2 mL of 10% APS (0.1 g ammonium persulfate in ddH_2O to 1.0 mL; use freshly prepared solution), 0.02 mL of TEMED and 23.25 mL of H_2O. Pour the mixture between glass plates and insert comb. After the gel has polymerized, remove the comb and rinse wells with 1× TBE buffer to remove unpolymerized acrylamide.
2. Gel loading solution: 15% Ficoll; 0.25% bromophenol blue (BPB). Mix digested PCR products 1:1 with gel loading solution. Load 5 mL/well and run the gel at about 40 mA.
3. Ethidium bromide. (**Ethidium bromide is highly carcinogenic; handle with extreme care and wear gloves.**): Prepare 0.5 mg/mL of 1000× stock solution in H_2O. **Protect from light**. Working solution: 5 µg/mL for DNA staining. Stain your gel for 15 min, rinse with water, and visualize the DNA using a UV transilluminator.
4. DNA molecular weight standard: 100 bp ladder from Gibco-BRL Life Technologies (Karlsruhe, Germany).

2.1.4. Reagents and Solutions for Enzyme-Linked Immunosorbent Assay (ELISA)

1. 50 m*M* Sodium hydrogen carbonate, pH 9.6.
2. MPBS: *see* **Subheading 2.1.2.** Alternatively: 3% (w/v) bovine serum albumin (BSA) in PBS; use MPBS for blocking nonspecific binding sites during phage-ELISA; however, 3% BSA/PBS seems to be better for ELISA with scFv fragments.
3. PBS: *see* **Subheading 2.1.2.**
4. PBS/Tween 20: *see* **Subheading 2.1.2.**
5. TMB (3,3',5,5'-Tetramethylbenzidine) tablets or substance. (**Highly carcinogenic; wear gloves**): Dissolve tablets according to the manufacturer's instruction. Alternatively, dissolve 100 µg/mL of TMB in 100 m*M* sodium acetate, pH 6.0, and add 10 µL of 30% hydrogen peroxide.
6. 30% Hydrogen peroxide.
7. 1 *M* Sulfuric acid.

2.2. Primers, Enzymes, PCR

The following primers are needed:

1. LMB3: 5'-CAGGAAACAGCTATGAC-3' (upstream from pelB leader sequence in pHEN1 vector).
2. PCR-H-Link: 5'-ACCGCCAGAGCCACCTCCGCC-3' (upstream, position 430–450, of linker sequence in pHEN1 vector).
3. For nonradioactive sequencing, the "Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza dGTP," available from Amersham, Life Science (Buckinghamshire, UK) was used and the following IRD-800 labeled primers were employed (MWG-Biotech, Ebersberg, Germany):
   M13 universal, 18mer: 5'-TGTAAAACGACGGCCAGT-3'
   M13 reverse, 18mer: 5'-CAGGAAACAGCTATGACC-3'

5. Restriction enzyme: Mva I [10 U/μL] (MBI Fermentas) which recognizes specifically CC(A,T)GG sequences.

The conditions for the digestion were as follows (all reagents are delivered with the enzyme):

5 μL of PCR product
1.5 μL of 10× incubation buffer
0.07 μL of 10% BSA
0.25 μL (2.5 U) of Mva I
8.18 μL of ddH_2O
Incubate for 60 min at 37°C; use at once or store at –20°C.

The PCR reaction mix (PCR- Kit; USB, Cleveland, OH) was composed as shown in **Tables 1** and **2**.

2.3. Antibodies

1. Myc1-9E10: MAb recognizing human *p62c-myc* gene product and specific for the C-terminal peptide EQKLISEED; available from the European Collection of Animal Cell Cultures, (ECACC), Centre for Applied Microbiology and Research, Porton Down, Salisbury, SP4 0JG, England.
2. HP-3E4: MAb recognizing human p58 NK cell receptors, preferentially NKAT1. It crossreacts also with NKAT2 and other KIRs (*see* **Appendix 1**) (kindly provided by Dr. M. Colonna).
3. 5.133: MAb recognizing human p70 NK cell receptors, NKAT3 and NKAT4 (*see* **Appendix 1**); (kindly provided by Dr. M. Colonna).
4. GL 183: MAb recognizing the p58 NKAT2 and other KIRs (*see* **Apppendix 1**), available from Coulter-Immunotech.
5. Horseradish peroxidase (HRP)-conjugated donkey anti-sheep immunoglobulin (whole molecule) available from Sigma.
6. Sheep anti-M13 antiserum (also HRP-conjugated) available from Pharmacia Biotech.
7. HRP-conjugated goat anti-mouse IgG (H + L chain specific): available from Bio-Rad.
8. FITC-conjugated goat anti-mouse IgG (g + L chain specific); available from Caltag.
9. Goat anti-human IgG (Fc specific) available from Caltag.

2.4. Bacterial Vector

The pHEN1 ***(18)*** bacterial expression vector contains the gene for gIIIp. This vector codes in addition for a C-terminal sequence of the expressed scFv protein that can be recognized by the MAb Myc1-9E10.

2.5. Synthetic Library, Bacterial Strains, and Helper Phages

The "Synthetic scFv Library (no. 1)" can be purchased from the Cambridge Centre for Protein Engineering, Medical Research Council Centre, Hills Road,

Table 1
Table Title

Components	Volume (µL)	Concentration of stock solution	Final concentration
10× PCR buffer	3	10 m*M* Tris-HCl, pH 8.3	1 m*M*
		50 m*M* KCl	5 m*M*
		0.1% gelatin	0.01%
$MgCl_2$	1.8	25 m*M*	1.5 m*M*
dNTP	0.6	10 m*M* each dATP,	Each 0.2 m*M*
			dCPT, dGPT, dTTP
LMB3[a]	3	10 µ*M*	1 µ*M*
H-Link[a]	3	10 µ*M*	1 µ*M*
Taq polymerase	0.2	5 U/µL	1 U
Template[b]	5		
Sterile ddH_2O to	30		

[a]Because all scFv fragments from the phage library employed here contain identical light chains, only heavy chain specific PCR primers are applied.

[b]It is not required that the DNA used for the PCR reaction is perfectly clean. Lysates of bacteria can be used as a good source of DNA as well: Either dissolve one bacterial colony in 40 µL of ddH_20 and boil for 10 min and use 5 µL of lysate for the PCR reaction or, even simpler, touch the bacterial colony with a toothpick and transfer bacteria directly to 3 µL of PCR buffer, mix well, and perform the first denaturation step twice (*see* **Table 2**).

Table 2
PCR Reaction Conditions

Step	Temperature (°C)	Time (min)	Number of cycles
Denaturation	95	5	1×
Denaturation	95	1	5×
Anneal	58	1	
Extension	72	1	
Denaturation	95	1	30×
Anneal	55	1	
Extension	72	1	
Extension	72	10	1×

Cambridge, CB2 2QH, England. Contact Ms. Fiona Sait by letter, Fax 44-1223-402140, or by e-mail: fs1@mrc-lmb.cam.ac.uk.

This library consists of : one tube of phage-infected bacterial library stock; one tube of TG1 cells; one tube of HB2151 cells, two tubes of positive controls (1× TG1 infected with anti-NIP clone and 1× TG1 infected with anti-MBP

clone, both selected from this library); and one tube of the negative control (TG1 infected with pHEN1 only).

1. *E. coli* TG1 strain (K12*, Δ(lac-proAB), supE, thi, hsdD5/F'traD36, proA+B, lacIq, lac ZDM15*) ***(19)***. T-phage-resistant suppressor strain of TG1 is used for propagation of phage particles. This bacterial strain prevents recognition of the amber stop codon located behind the scFv gene and synthesizes a fusion protein consisting of an scFv fragment and phage protein gIIIp.
2. *E. coli* HB2151 strain (K12, *ara, Δ(lac-proAB), thi/F' proA+B, lacIq lacZDM15*) ***(20)***. This is a nonsuppressor strain used for the expression of scFv fragments. This strain recognizes the amber stop codon located behind the scFv gene, resulting in translation termination and production of soluble scFv fragments.
3. Helper phage: M13 K07 is required for the rescue of phagemid libraries; it can be purchased from Pharmacia Biotech. Alternatively, phage VCS M13 is offered by Stratagene Cloning Systems.

2.6. Antigen

The purified, soluble forms of NKAT1, NKAT2, NKAT3, and NKAT4 proteins are composed of the KIR extracellular domains fused to the human IgG1-C_H2 and-C_H3 constant regions. These were obtained from Dr. M. Colonna ***(21)***. The fusion proteins were purified by affinity chromatography with protein A and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Two KIRs were used as selection targets: NKAT2-IgG1, with two Ig-SF domains and NKAT4-IgG1, with three Ig-SF domains. All four soluble KIR proteins, human pooled IgG, as well as different unrelated proteins were used in screening either in phage-ELISA or scFv ELISA . The Jurkat cell line and Jurkat cells transfected with NKAT1, NKAT2, and NKAT4 ***(22)*** were used for the detection of cell surface expressed KIRs by monoclonal scFv fragments using a FACScan flow cytometer (Becton-Dickinson, Oxnard, CA).

2.7. Plastic Material

1. Nunc Maxisorb immuno test tubes and the Nunc Bio-Assay dish are available from Nunc GmbH (Wiesbaden, Germany).
2. 96-Well flat-bottomed ELISA assay plates, e.g., Falcon MicroTest III flexible plates, are available from Becton Dickinson Labware or Nunc GmbH.
3. 96-Well tissue culture treated plates, flat-bottomed (any labware supplier).
4. 96-Well fluid transfer device (optional).

During the entire procedure, use disposable plasticware and polypropylene tubes.

2.8. Laboratory Equipment

1. Bacterial shaker with thermostat (30°C and 37°C).
2. Laboratory thermostat (37°C).

3. Clean bench.
4. Water bath.
5. Under-and-over turntable.
6. High-speed centrifuge (4°C).
7. Normal laboratory centrifuge with thermostat (4°C), swingout rotor, and microtiter plate-holder.
8. Microcentrifuge.
9. ELISA reader.
10. Gel electrophoresis and Western blot equipment.
11. Microtiter plate shaker.
12. Microtiter plate washer (optional).
13. Freezers (4°C, –20°C, –70°C).
14. Spectrophotometer.
15. UV transilluminator.
16. UV Microscope or flow cytometer.
17. Glass flasks for growing bacteria (0.5 L; 1 L; 2 L)
18. Set of adjustable pipets (0.1–5mL; 0.5–10 µL; 10–200 µL; 100–1000 µL).
19. Glass spreader.
20. Disposable filters (0.2 µm or 0.45 µm).

3. Methods

In this chapter we describe the use of a human synthetic phage-antibody display library, here referred to as "Synthetic scFv Library (no. 1)" for the production of monoclonal scFv fragments. This library represents >10^8 clones constructed of in vitro rearranged human V_H genes with a random 4–12 amino acid residue long V_H-CDR3, derived from a bank of 50 cloned V_H gene segments, and paired with only a single Vl3 light chain gene segment. For details regarding the construction of this library *see* Nissim et al. ***(17)***. To obtain specific scFv bearing particles (**Fig. 1**), phage clones of this library are usually subjected to three to five rounds of selection. After each round of selection, the TG1 suppressor strain of *Escherichia coli* (*E. coli*) is infected with the antigen-bound phage population. The resulting phages are again bound to the antigen for the next round of selection. After the last round of selection, this phage population can be tested by a variety of assays, including ELISA, for reactivity with the antigen, and the complexity of the population can be assessed by restriction analysis of the phage DNA. Different clones of enriched positive phages can then be tested for crossreactivity with different antigens, probed with V_H family-specific oligonucleotides for CDR3 length or sequenced. For the production of soluble scFv fragments, phage particles with binding activity obtained from single TG1 colonies are used for the infection of HB2151, a nonsuppressor strain of *E. coli*, and soluble scFv fragments are rescued either from the bacterial supernatant, inclusion bodies, or the periplasma. Because

the scFv fragments may have different affinities toward the antigen, the assays mentioned previously will have to be repeated.

Before you start to work with the library, make sure that you prepare all media, chemicals, and equipment. As a first step, prepare a large quantity of helper phage, as for every round of selection you will need them. Start growing bacteria and helper phages on the same day. Plan to do this 3 d before you start the selection. Be prepared to invest 9–10 d for the whole selection procedure.

3.1. Growing E. coli for Helper Phage

1. Pick up from a minimal media plate one or two colonies of *E. coli* TG1 or other bacteria recommended for your helper phage.
2. Transfer each into 5 mL of 2× TY medium and grow shaking overnight at 37°C.
3. Dilute the overnight cultures 1:100 into fresh 2× TY medium.
4. Grow shaking at 37°C until the OD at 600 nm (OD_{600}) reaches 0.4–0.5.
5. The bacterial culture is now in log phase, ideal for the infection with phages. Take the best growing bacteria for the infection.

3.2. Production of Helper Phage

1. Grow separately *E. coli* TG1 to an OD_{600} of 0.2. Keep on ice until infection, but not longer then 30 min.
2. During this time (**step 1**) prepare 100-fold serial dilution of M13 K07 helper phage (from undiluted to 10^6 dilution).
3. For every dilution, infect 200 µL of TG1 (from **step 1**) with 10 µL of helper phage, leave for about 10 min at room temperature and transfer to 37°C water bath for 30 min; **do not shake.**
4. During this time, melt H-top agar in a microwave oven; cool down to 45°C.
5. Prepare enough Petri dishes with warm TYE (without ampicillin).
6. Add TG1/phage to 3 mL of H-top agar (the temperature should not exceed 42°C) and pour onto TYE.
7. When solidified, transfer plates to incubator at 37°C and grow overnight.
8. From a plate containing separate plaques, pick a small plaque and transfer into 3–5 mL of exponentially growing TG1 cells (from **Subheading 3.1.**). Grow with shaking at 37°C for 2 h.
9. Dilute this culture further with 500 mL of 2× TY (use 1–2 L flask) and incubate with shaking at 37°C for 1 h.
10. Add kanamycin to a final concentration of 50 µg/mL (do not use glucose) and grow overnight (about 10–12 h) with shaking at 37°C, or better at 30°C.
11. Centrifuge down bacteria at 10,800*g* (4°C) for 15 min. Save supernatant containing phages and discard bacterial pellet.
12. Concentrate phages by adding PEG/NaCl to the supernatant at a ratio (v/v) 1:4 (100 mLPEG/NaCl + 400 mL of supernatant), mix well, and leave for a minimum of 30 min to 1 h on ice. Centrifuge the mixture for 30 min at 10,800*g* (4°C). Aspirate the supernatant.

13. Resuspend the pellet in 8 mL of TE and add 2 mL of PEG/NaCl; leave on ice for 20 min.
14. Centrifuge at 3300*g* for 30 min (4°C) and remove supernatant with rests of PEG/NaCl.
15. Resuspend pellet in 5 mL of PBS, centrifuge at 11,600*g* (4°C) using a microcentrifuge, and remove bacterial debris.
16. Dissolve pellet in 2 mL of PBS and filter phage stock using a 0.45-mm sterile filter.
17. Titer the stock using 10-fold dilutions. It is recommended that the helper phage stock should be about $1 \times 10^{12} – 1 \times 10^{13}$ plague forming unit (P.F.U.)/mL. For short-term storage, leave the helper phage stock at 4°C. For longer storage intervals, dilute phage in PBS/15% glycerol and store at –20°C or better at –70°C.

3.3. Growing the Library and a Secondary Library Stock

Because phages are not particularly stable if kept for longer periods at –70°C, the library is generally stored at this temperature as bacteria harboring phagemid. The library is very valuable, so it is recommended to grow a secondary bacterial stock of it after arrival in your laboratory. For this purpose, one can either use the whole volume of the library stock at once or part of it. We used both strategies, but for the newcomers to this field it is advisable to start with a small aliquot of the library, e.g., 50 µL, that contains about 5×10^8 bacterial clones.

3.3.1. Growing the Library for Selection

1. Inoculate 50 µL of the library stock into 50 mL 2× TY supplemented just before use with 100 µg/mL ampicillin and 1% glucose. (If you decide to use the whole library stock [500 µL], add the library to 500 mL of culture medium.)
2. Grow shaking at 37°C until OD_{600} is 0.5–0.6 (it takes 1–2 h).
3. Infect 10 mL of this culture with about 5×10^{10} helper phage followed by incubation in a water bath at 37°C without shaking for 30 min (**the remaining 40 mL of bacterial culture are used to prepare a secondary stock of the library**; *see* **Subheading 3.3.2.**).
4. Centrifuge at 3300 *g* at 4°C for 10 min, remove supernatant,and resuspend the pellet gently in prewarmed 300 mL of 2× TY/A/K (no glucose).
5. Shake at 30°C overnight.
6. Centrifuge the overnight culture at 10,800*g*, 4°C for 15 min. Transfer supernatant containing pHEN1 phagemid particles to a new tube.
7. Add PEG/NaCl according to **step 15** of **Subheading 3.2.** Mix well and leave at least for 1 h on ice.
8. Centrifuge at 10,800*g*, 4°C for 30 min. Discard supernatant containing PEG/NaCl, resuspend the pellet in 40 mL of water, and add 8 mL of PEG/NaCl. Mix and leave for at least 20 min on ice.
9. Centrifuge at 3300*g*, 4°C for 30 min, and aspirate supernatant. It is advisable at this point to recentrifuge the pellet briefly in order to remove any remainders of PEG/NaCl completely.

10. Resuspend the pellet in 2 mL of PBS and centrifuge at 3300*g* (4°C) for 10 min to remove bacterial debris (pellet), and titer the phage stock.
11. This pHEN1 phage stock should yield 1–5 × 10^{13} transforming units (TU) phage. Short term storage of the stock should be at 4°C, for long-term in PBS/15% glycerol at –70°C.

3.3.2. Preparation of the Secondary Library Stock

1. Grow the remaining 40 mL of bacterial culture for further 2 h with shaking at 37°C.
2. Centrifuge the cells at 10,800*g,* 4°C for 10 min.
3. Resuspend the cells in a small volume of 2× TY (about 1–2 mL).
4. Take a large, air-dry Nunc Bio-Assay dish, pour TYE/Amp/Gluc, and spread the cells on it.
5. Grow the culture overnight at 37°C.
6. Add to the dish 1–2 mL of 2× TY/15% glycerol, scrape the bacteria with a glass spreader, mix well, and divide into 50-μL portions containing about 1 × 10^8 bacteria each and store at –70°C.

3.4. First Round of Selection

Positive phages from the library can be selected using different antigens, methodologies and selection conditions (for review *see* **ref. *23***). The method detailed here is performed with immunotubes coated with soluble antigen. To obtain phages specific for the antigen that do not exhibit crossreactivity with similar antigen(s), it is advisable, for each round of selection, to preselect the library with the crossreactive molecule. In case of the protocol described here, this would be human IgG, as both NKAT2 and NKAT4 are employed as fusion constructs of a human γ1 Fc-fragment and the extracellular portion of the KIR protein.

Take care during the whole selection procedure to keep immunotubes moist. Each washing step with immunotubes is performed by pouring buffer in and immediately out.

1. One day before selection will start, coat immunotubes overnight at room temperature (or 4°C, depending on the sensitivity of the protein) with 4 mL of the antigen dissolved in sodium hydrogen carbonate, pH 9.6. We employed one tube with 800 μg/mL of human IgG, and another with 10 μg/mL of either NKAT2-Ig-Fc or NKAT4-Ig-Fc. Do not forget to cover the tubes. Also at the same time, start to grow TG1 cells that will be used the next day for the propagation of selected phage. Proceed as described in **Subheading 3.1.**
2. Next day, wash tubes 3× with PBS and block nonspecific binding sites by incubation for 2 h at 37°C with 2% MPBS. Fill the whole tube with this solution and cover it.
3. Wash tubes 3× with PBS and preselect 5 × 10^{12} tu pHEN1 phage from **Subheading 3.3.1.** in 4 mL of 2% MPBS on a tube coated with human IgG. Cover the tube and incubate for 2 h at room temperature using an under-and-over turntable.

4. Transfer unbound phage into the tube coated with KIR-IgG fusion protein, add 100 μg/mL of human IgG, cover, and incubate as follows: 30 min with continuous rotation, afterwards without rotation for at least 90 min, both at room temperature.
5. Supernatant containing unbound phage may be either discarded or used for the selection with a different antigen.
6. Wash tubes extensively (10×) with PBS/Tween-20 followed by 10× with PBS only (all detergent rests have to be removed), and put the tube upside down to remove the excess of PBS.
7. Elute bound phage as follows: add to the tube 1 mL of 100 m*M* triethylamine, cover, and incubate for 10 min at room temperature using a rotating turntable. During this time prepare a new tube with 0.5 mL of 1*M* Tris-HCl, pH 7.4.
8. Transfer the eluted phage into the new tube. For quick neutralization of phage particles remaining in the immunotube, add another 200 μL of 1*M* Tris-HCl, pH 7.4, to this tube. At this point, you can store both tubes at 4°C for a short time or proceed further.
9. Take 9 mL of exponentially growing TG1 (*see* **step 1** of this protocol) and infect with 1 mL of eluted phage. Add an additional 4 mL of TG1 cells to the immunotube. This allows the recovery of phage particles that have not been eluted during **step 7**. Incubate both cultures at 37°C without shaking (water bath) for 30 min.
10. Pool both cultures. Save 100 μL of infected TG1 cells which will be used (**step 11**) for titration of the phage. Centrifuge the remaining cells at 3300*g* for 10 min at 4°C, resuspend in 1 mL of TY, plate on a large Nunc Bio-Assay dish prefilled with TYE/Amp/Gluc, and incubate overnight at 30°C.
11. Use 100 mL of infected TG1 cells for five 100-fold dilutions in TY medium. Plate 50 μL of each dilution ($10^1 – 10^6$) on Petri dishes as described previously. Grow until colonies are visible (at least overnight) and count. You should get a titer of at least 10^4 for the first round.

3.5. The Second and Further Rounds of Selection

1. Next day, add 2 mL of 2× TY/Glc to the Nunc Bio-Assay dish, loosen the cells, and mix well. Prepare 50-μL portions of scraped TG1 bacteria; with one of these inoculate the culture medium (**step 2**) and store the rest at –70°C.
2. Inoculate 50 mL of 2× TY/Amp/Gluc with 50 μL of TG1 cells from **step 1** and grow with shaking at 37°C until OD_{600} reaches 0.5 (about 2 h). **Perform all further steps of the selection as described previously, starting from step 3 of Subheading 3.3.1. onwards until step 11 of Subheading 3.4. Repeat the selection for an additional 3 to 4×.**
3. After each round of selection, do not forget to infect TG1 cells with the selected phage for titer estimation (*see* **Subheading 3.4.**). Elevation of phage TU (up to 10^8) is the first indication that the selection is working. To assess the reactivity of selected phage, perform phage-ELISA (*see* **Subheading 3.8.**) or DNA analysis of the phage population (*see* **Subheading 2.2.** as well as **Figs. 1** and **2**).

3.6. Expression of Soluble scFv Fragments

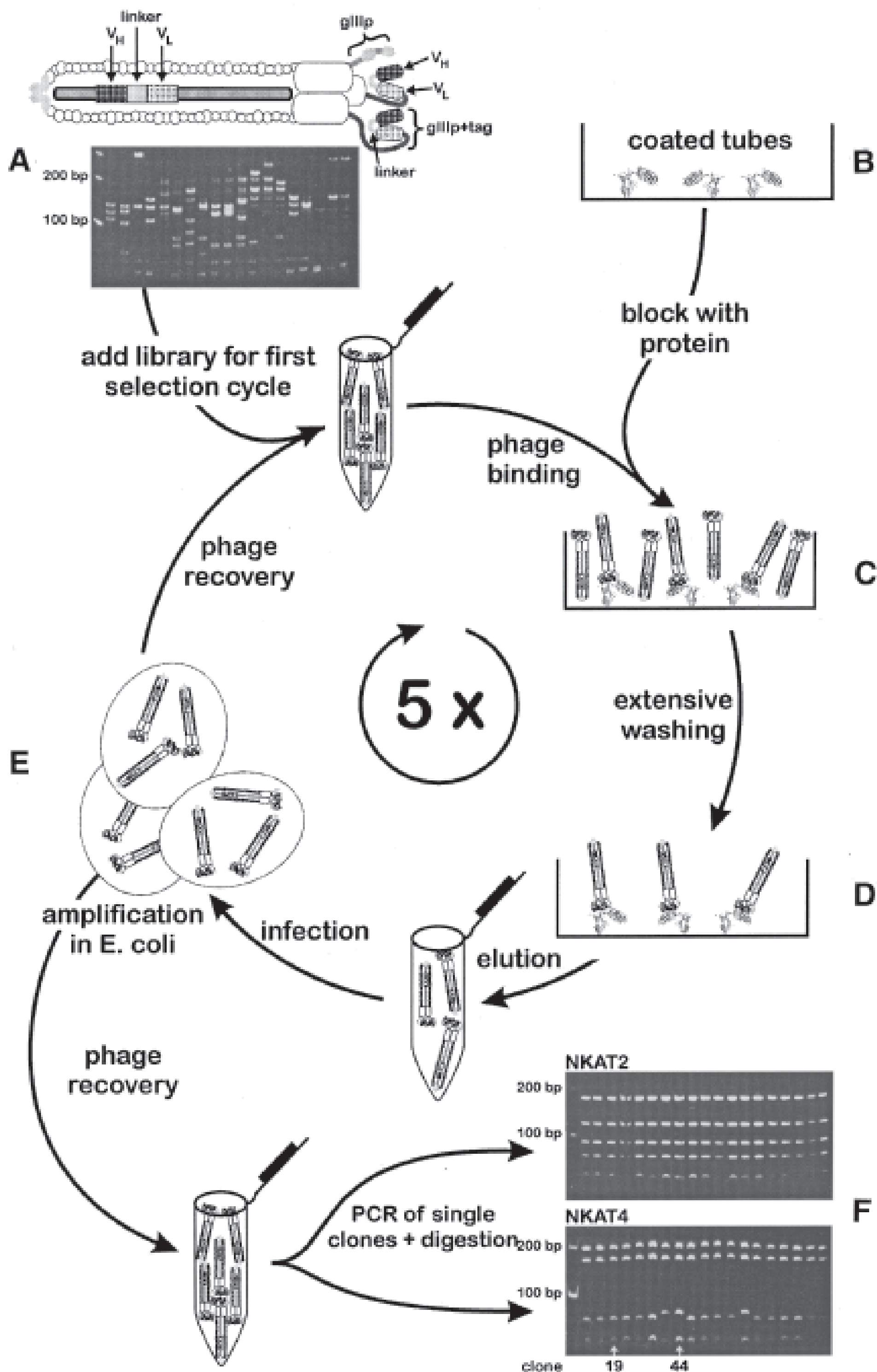
For the production of soluble scFv fragments, the nonsuppressor strain HB2151 of *E. coli* is infected with the eluted phage obtained after the last round of selection. After titration, a single, infected bacterial colony is induced with IPTG. IPTG is a potent inducer for the scFv construct within the pHEN1 vector and leads to expression of soluble scFv fragments. To obtain efficient induction, the glucose concentration of the medium has to be lowered (*see* **step 4**) for the last incubation before induction. The synthetic scFv Library (no. 1) yields soluble scFv fragments that carry a c-myc tag (*see* **Subheading 2.4.**). This property can be exploited for the detection and purification of scFv fragments using a MAb.

1. Infect 200 µL exponentially growing *E. coli* HB2151 (OD_{600} of 0.4) with 10 µL (about $10^5 – 10^6$ TU) of eluted phage.
2. Incubate for 30 min without shaking (water bath) and plate 50 µL of undiluted, 50 µL of a 1:10^2 dilution, 50 µL of a 1:10^4 dilution, and 50 µL of a 1:10^6 dilution on TYE/Amp/Gluc. Incubate overnight at 37°C.
3. Transfer individual colonies into single wells of 96-well plates containing 100 µL 2× TY/Amp/Gluc per well. Shake overnight at 37°C.
4. Prepare a duplicate of this plate by transferring a small aliquot (about 2 µL) into a new 96-well plate containing 200 µL of fresh 2× TY/Amp/0.1 Gluc per well. (The rest can be used to make a stock of the first plate, by adding glycerol to a final concentration of 15% and storing at –70°C.) Incubate a new plate, shaking at 37°C until OD_{600} is 0.9 (about 3 h).
5. Add to each well 25 µL of 2× TY/Amp supplemented with 9 m*M* IPTG (final concentration 1 m*M* IPTG). Incubate further at 30°C overnight.
6. Centrifuge the plate at 1800*g* for 10 min (do not use the break of the centrifuge to keep bacteria at the bottom of the well) and use 100 µL of the supernatant for ELISA.

3.7. Screening by ELISA

ELISA is probably the simplest method for testing the activity of phage populations or clones and soluble scFv fragments against soluble antigens that can be immobilized. For these assays, 96-well microtiter plates are employed. The best results are obtained when flexible plates are used.

1. Coat a 96-well plate with 100 µL/well of antigen. Take care to apply the antigen in the same buffer and at the same concentration used for selection. Leave at room temperature overnight.
2. Wash plate 3× with PBS and add 200 µL/well of 2% MPBS for phage ELISA or 3% BSA/PBS for scFv ELISA. Incubate for 2 h at 37°C.
3. Discard the solution; wash 3× with PBS.
4. Add 10 µL/well PEG-precipitated phage (about 10^{10} TU) and 90 µL of 2% MPBS or 100 µL of scFv supernatant. Incubate for 90 min at room temperature.



linker
V_H
V_L
gIIIp
V_H
V_L
gIIIp+tag
linker
A
200 bp
100 bp
coated tubes
B
block with protein
add library for first selection cycle
phage binding
C
phage recovery
5 x
extensive washing
E
D
amplification in E. coli
infection
elution
phage recovery
NKAT2
200 bp
100 bp
PCR of single clones + digestion
F
NKAT4
200 bp
100 bp
clone
19
44

5. Discard the solution and wash 3× with PBS/Tween-20 followed by three washes with PBS.
6. Add 100 μL of HRP-conjugated anti-M13 serum (appropriate dilution 1:1000 to 1:5000 in 2% MPBS or 3% BSA/PBS), incubate at room temperature for 90 min. To detect scFv fragments, the MAb Myc1-9E10 can be employed, followed by the addition of conjugated anti-mouse IgG.
7. Wash 5× with PBS/Tween-20.
8. Prepare 50 mL of TMB in a tube covered with aluminum foil, followed by addition of 10 uL of 30% hydrogen peroxide immediately before use.
9. Add 100 μL/well of this solution and leave at room temperature in the dark for 10 min. A positive reaction is indicated by the development of blue color.
10. Stop the reaction by adding 50 μL/well of 1 *M* sulfuric acid. Now the color should turn yellow.
11. Read OD_{650} and OD_{450}. To obtain the result, subtract the OD_{650} value from the OD_{450} (This is carried out automatically by modern ELISA readers).

Typical ELISA results are shown in **Fig. 2a** and **b**. While the enrichment of the specifically reacting phage is given in **Fig. 2a**, **Fig. 2b** shows an ELISA of NKAT4-selected scFv fragments with different KIR molecules and human IgG or IgG-Fc. **Figure 2c** depicts the reactivity of an scFv fragment against NKAT2-positive transfectants in flow cytometry, using Myc1-9E10 and a fluorochrome-conjugated anti-mouse IgG.

3.8. Analysis of Phage by PCR and Sequencing

The complexity of the phage population before, during, and after selection as well as that of clones can best be assessed by gel electrophoresis of PCR products (*see* **Fig. 1**).This type of analysis as well as sequencing of individual

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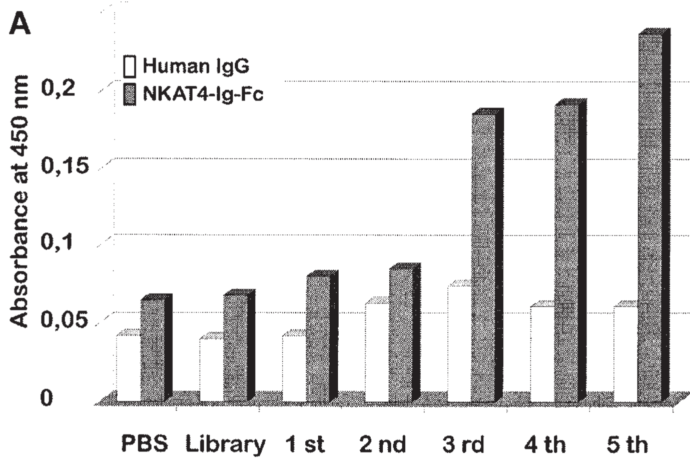
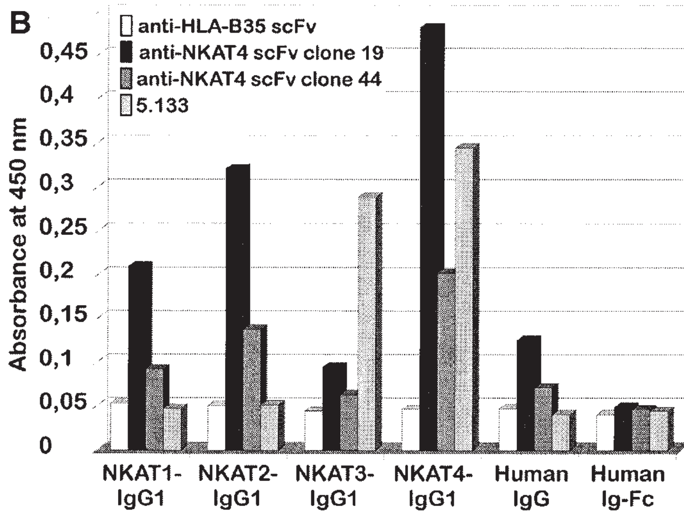
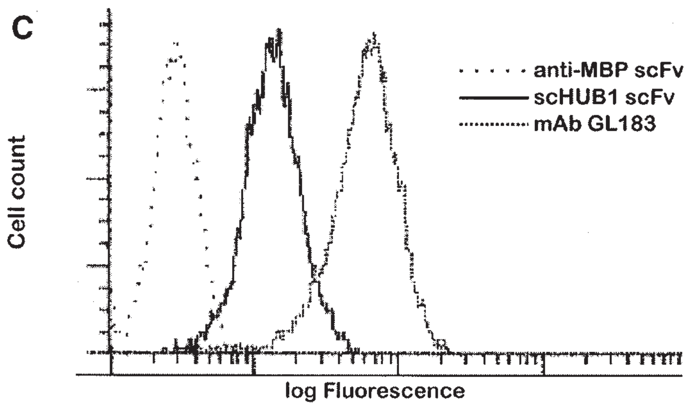
Fig. 1. Essentials of the phage selection procedure. **(A)** A filamentous phage is depicted that contains DNA with the information for specific V_H and V_L fragments, connected by a linker, and carries scFv-gIIIp fusion proteins with a tag on its surface. Analysis of individual phage by gel electrophoresis reveals extensive heterogeneity of the starting population (library stock). **(B)** Tubes are coated with the antigen (a KIR2D protein is given as an example). **(C)** The phage population is added to the coated tube. **(D)** After incubation and washing, antigen-binding phage will still stick to the tube. **(E)** These phage particles are eluted, and the population is amplified in *E. coli*. After recovery of the bound phage, the selection cycle is repeated until a phage population is obtained with a high degree of homogeneity. **(F)** Homogeneity is assessed by gel electrophoresis of phage DNA fragments from single clones obtained after PCR and restriction enzyme digestion. The two patterns shown are clones from an anti-NKAT2–Ig–Fc selection and an anti-NKAT4–Ig–Fc selection, respectively. The restriction patterns obtained from the two clones employed in the scFv ELISA (**Fig. 2b**) are indicated.


A
Human IgG
NKAT4-Ig-Fc
Absorbance at 450 nm
0,2
0,15
0,1
0,05
0
PBS
Library
1 st
2 nd
3 rd
4 th
5 th


B
anti-HLA-B35 scFv
anti-NKAT4 scFv clone 19
anti-NKAT4 scFv clone 44
5.133
Absorbance at 450 nm
0,45
0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
NKAT1-IgG1
NKAT2-IgG1
NKAT3-IgG1
NKAT4-IgG1
Human IgG
Human Ig-Fc


C
anti-MBP scFv
scHUB1 scFv
mAb GL183
Cell count
log Fluorescence

clones follows established procedures. Conditions that we have found suitable for PCR of individual clones are described in **Subheading 2.2.**

4. Notes

1. Before starting to work with phage display libraries, it is advisable to become acquainted with basic microbiological and molecular biological techniques. We *found Current Protocols in Molecular Biology* ***(24)*** useful and a practical source of information. These books (3 vols.) contain not only background information in the field of molecular biology, but they are an excellent, permanently updated source of methodological instructions as well.
2. For carrying out successful selections, it is of paramount importance to check the complexity of the library. As shown in **Fig. 1** (*top left*), the DNA of at least 20 phage clones should be analyzed by gel electrophoresis before starting a selection. The patterns should all be different. If this is not the case, do not start the selection procedure. Proceed only with a new sample of the library, of course after checking its complexity.
3. Phage attach at the fragile sex pili of the bacteria. To provide optimal conditions for infection, make sure that all *E. coli* cultures that are used to propagate phage are grown at 37°C and in log phase, i.e., have an OD_{600} of about 0.4–0.5.
4. Make sure that cross-contamination with different phage populations is avoided. Soak all glassware and plastic materials that were used to grow infected bacteria at least for 1 h in 2% (v/v) hypochlorite. Wash extensively afterwards and autoclave.
5. The first round of selection is the most important. Make sure that the phage titer after this round is at least 10^4. If this is not the case, either repeat from **Subheading 3.4., step 9** on, or, possibly better, start from the beginning with a new sample of the library.
6. If a complex antigen is employed as target for the selections, it may be advisable to use a "simple" antigen (e.g., a peptide) as control for the performance of the library. This can be done in parallel with the "real" selection. In all assay procedures, the inclusion of negative control reagents is advisable. These reagents are usually supplied together with the library.

Acknowledgments

We thank Dr. Marco Colonna for providing NKAT-transfected and -producing cells as well as for monoclonal antibodies. We are grateful to Dr. Dietmar Mischke

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Fig. 2 Examples of results from specific selections. **(A)** Phage ELISA reveals enrichment of antigen (here, NKAT4–Ig–Fc)-specific phage already after the third round of selection. Reactivity with a crossreacting antigen (human IgG) does not increase in parallel. **(B)** scFv ELISA with different scFv fragments and the MAb 5.133 (anti-p70 KIR such as KIR3DL1 [NKAT3] or KIR3DL2 [NKAT4]) reveals different degrees of crossreactivity with other KIRs and related proteins. **(C)** Flow cytometric analysis of two scFv fragments (scHUB1 is directed specifically against NKAT2) and the MAb GL183 (anti-NKAT2) on NKAT2-transfected Jurkat cells.

for commenting on the manuscript. This work was financially supported by the European Union (BMH4-CT96-1105; BIO4-97-2196; BMH4-CT98-3732), the Berliner Krebsgesellschaft, the Sonnenfeld-Stiftung, Berlin and by a stipend of the Senate Berlin (to Q.X.).

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20

Binding of Soluble KIR-Fc Fusion Proteins to HLA Class I

Christine C. Winter and Eric O. Long

1. Introduction

Individual natural killer cells express diverse combinations of cell surface receptors, including members of the killer cell immunoglobulin (Ig)-like receptor (KIR) family and of the CD94/NKG2 receptor type. Thus, it is often difficult to assign a precise ligand specificity to any given receptor on an NK cell. This difficulty can be circumvented by using an assay that detects direct binding of soluble forms of individual KIR to transfected cell lines that express single HLA class I allotypes.

The binding assay requires the production of soluble molecules by fusion to the Fc portion of an Ig molecule. This approach provides multiple advantages. Fusion proteins are expressed as homodimers linked via disulfide bridges in the hinge region of the Fc. This provides an enhancement in the ability to detect binding over that obtained with a monomeric soluble receptor. In addition, as immunoglobulins are normally secreted at high levels, fusion of other Ig domains onto an Fc typically results in efficient secretion. The Fc domain provides an easy means for purification using protein A-Sepharose and for detection of the soluble receptor-ligand interaction by flow cytometry using standard anti-IgG1 fluorescein isothiocyanate FITC conjugates. An important parameter in designing the fusion protein is the precise point at which the molecule of interest is joined to the Fc. Differences of a single amino acid have been noted with other proteins to affect folding and expression of the fusion protein. In the case of KIR, our first attempt was successful, possibly owing to luck or to the presence of a stem between the last Ig domain and the transmembrane region of KIR which may have eliminated steric constraints. A few examples

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
Edited by: K. S. Campbell and M. Colonna © Humana Press Inc., Totowa, NJ

of other Fc-fusion proteins that have been used to measure ligand binding by flow cytometry are CD44 ***(1)***, B7 ***(2)***, and CD28 ***(2)***.

In addition to determining ligand specificities, the assay described here can be used to test the effect of amino acid substitutions, or other mutations, on the binding of KIR to HLA class I. It is possible to generate a mutated cDNA for KIR, to express it in a soluble form, and to determine its binding to HLA class I in 2–3 wk, provided all the steps work as expected. Such an approach has been used to determine the critical amino acids involved in the ability of KIR2D to discriminate among the two groups of HLA-C allotypes, and in the different affinities of related KIR2DL and KIR2DS molecules for the same HLA-C molecule ***(3–5)***.

Soluble forms of molecules can also be used to identify unknown ligands by "expression cloning." This approach was used successfully to determine that a viral MHC class I homolog binds to an Ig-like receptor expressed mainly on monocytes and macrophages ***(6)***. This new family of Ig-like receptors have been called immunoglobulin-like transcripts (ILTs), leukocyte immunoglobin-like receptor (LIR), or MIR ***(6–8)***. In turn, soluble forms of ILT, made as fusion proteins with Fc, were shown to bind HLA class I molecules ***(6,9,10)***.

The sensitivity of this binding assay is limited. For instance, it was difficult to detect binding of soluble forms of KIR3DL1 and KIR3DL2 to their HLA ligand ***(11,12)***. Another limitation of this assay is that it does not provide a measurement of affinity or stoichiometry. A different assay has been used to determine the binding of KIR to HLA-C in solution ***(13,14)***. These molecules were expressed in *E.coli*, extracted in urea, refolded, and tested for binding in solution. KIR-HLA-C complexes were visualized by electrophoresis in "native" gels (i.e., polyacrylamide gels without detergent). Careful quantitation and titration of the soluble molecules revealed that KIR-HLA-C bind at a 1:1 ratio ***(13)***.

In brief, the generation of a receptor as an Ig fusion protein involves the following steps: (1) subcloning the cDNA encoding the receptor into an expression vector that provides cloning sites between a signal sequence and the coding region of the Fc portion of IgG, (2) transfection into cells that provide efficient transient expression (e.g., COS cells), (3) purification over a protein A column, and (4) quantitation and testing for integrity. As a source of ligand, HLA class I-negative 721.221 cells ***(15)*** that have been transfected with individual HLA class I genes are generally preferred. However, this HLA-A, -B, and -C negative cell line has retained expression of the nonclassical HLA class I molecules HLA-E and HLA-F. Chapter 6 in this volume describes how 721.221 transfectants were generated. Another, more cumbersome approach, is to use transporter associated with antigen presentation (TAP)-deficient mutant cells (e.g., RMA-S, T2) on which HLA class I molecules can be stabi-

lized by the addition of exogenous peptides *(16)*. Except when specifically pointed out, the protocols allow some latitude. Standard procedures in recombinant DNA technology and in tissue culture of cells are not described in detail here.

2. Materials

2.1. Generation of Ig Fusion Proteins

2.1.1. DNA Constructs

1. Several versions of an eukaryotic expression vector encoding the hinge region, CH2, and CH3 Ig domains exist. The Cd51neg1 vector *(1)* used here encodes the signal sequence of the CD5 antigen, followed by *NheI* and *BamHI* cloning sites, and a genomic fragment with the exons and introns for the hinge, CH2, and CH3 of human IgG1. Cloning into the *NheI* site positions the insert exactly at the point of signal sequence cleavage, such that the mature protein retains its natural amino terminus (*see* **Note 1**).
2. MC1061 competent bacteria containing the P3 episome (Invitrogen, Carlsbad, CA) to propagate Cd51neg1.
3. Oligonucleotides designed to amplify the desired receptor, including a 5' *NheI* and a 3' *BamHI* restriction site for in-frame cloning.
4. Template DNA (cDNA clone of the desired KIR).
5. Polymerase chain reaction (PCR) machine (Perkin Elmer, Branchburg, NJ).
6. GeneAmp kit, PCR reagents (Perkin Elmer).
7. Restriction enzymes and DNA ligase.
8. DNA purification Kit (Qiagen, Chatsworth, CA).

2.1.2. Transfection

1. COS-7 cells (American Type Culture Collection [ATCC], Rockville, MD). These cells should be subcloned to obtain a highly transfectable clone. Freeze multiple vials of such a clone and thaw a new vial once the cells have been in continuous culture for a few months, or whenever transfection efficiency drops.
2. Cell culture medium: The basic cell culture medium used throughout the transfection procedure is serum-free Dulbecco's modified Eagle's medium (DMEM; Life Technologies, formerly Gibco-BRL, Grand Island, NY) supplemented with 2 m*M* L-glutamine (Life Technologies) and 0.05 mg/mL of gentamicin (Life Technologies) (SF-DMEM). A 100× stock solution of L-glutamine is stored frozen at –20°C and thawed just prior to use. A 100× gentamicin solution can be stored at room temperature.
3. Growth medium: SF-DMEM supplemented with 10% (v/v) fetal calf serum (FCS; Life Technologies) that has been inactivated by heating at 56°C for 1 h.
4. Transfection medium (TFM) prepared as follows:
   a. Supplement SF-DMEM with 0.01 *M* HEPES, pH 7.4 (Life Technologies). A 100× stock solution of HEPES can be stored indefinitely at 4°C.
   b. Add DEAE-Dextran (Sigma, St. Louis, MO) at a final concentration of 400 µg/mL. A 100× stock solution of DEAE-Dextran can be prepared by dissolving 0.8 g

of DEAE-Dextran in 20 mL of distilled water. This solution can be aliquoted and stored at –20°C for at least 6 mo.

c. Add chloroquine (Sigma) at a final concentration of 100 μ*M*. A 1000× stock solution of chloroquine can be prepared by dissolving 0.52 g of chloroquine in 10 mL of distilled water. This solution can be aliquoted and stored at –20°C for up to 1 yr.

d. Sterilize the TFM by filtration through a 0.2-μm filter (Nalge Nunc International, Rochester, NY). Add the DNA at a final concentration of 0.4 mg/mL (*see* **Note 2**). Although it is usually not necessary, the DNA can be precipitated in ethanol prior to transfection as a safeguard for sterility.

For quick reference, a 250-mL batch (*see* **Note 3**) of TFM contains the following:

250 mL of SF-DMEM
2.5 mL of 100× (1 *M*) HEPES
2.5 mL of 100× (40 mg/mL) DEAE-Dextran
0.25 mL of 1000× (100 m*M*) chloroquine
100 μg of the DNA of interest (10 μg/flask)

5. Phosphate-buffered saline (PBS; Life Technologies).
6. DNA uptake solution: PBS supplemented with 10% (v/v) dimethyl sulfoxide (DMSO; Sigma) (10% DMSO/PBS). The solution should be sterilized by filtration through a 0.2-μm filter and can be stored at 4°C.
7. 162 cm^2 flasks (Costar, Beverly, MA).
8. Tabletop centrifuge (Sorvall RT 6000D, Dupont, Wilmington, DE)

2.1.3. Purification/Quantification

1. Sterile PBS, pH 8.0 (*see* **Note 4**).
2. Ethanol: 200 Proof ethyl alcohol (Warner-Graham, Cockeysville, MD).
3. Timer (PGC Scientifics, Gaithersburg, MD).
4. Ring stand (PGC Scientifics).
5. Protein A-Sepharose beads (Sigma) prepared as follows: Hydrate 1.5 g of Sepharose beads with 15 mL PBS for at least 4 h. Centrifuge the resulting slurry at 210*g* for 5 min in a 15-mL conical tube displaying written gradations (Sarsted, Newton, NC). The beads will have swollen to a volume of approx 6 mL. Remove liquid and wash beads 3× in PBS. Resuspend the beads in approx 6 mL of PBS to produce a 1:1 slurry. This can be stored up to 6 mo at 4°C.
6. Flex-columns measuring 0.7 × 10 cm (Kontes Scientific Glassware, Vineland, NJ).
7. Polyethylene tubing with an inner diameter of 1.4 mm (Thomas Scientific, Swedesboro, NJ) assembled as follows: Cut one end of the tubing on the diagonal and insert it into a 6 in. Pasteur pipet (Kimble, Toledo, OH). Secure the assembly with masking tape. Fit the other end with a low pressure adapter (Bio-Rad, Hercules, CA), compatible with the tip at the top of the column.
8. 0.05 *M* Sodium citrate (Sigma), pH 3.0.
9. 2 *M* Tris-HCl (ICN Biomedicals, Aurora, OH), pH 8.0.
10. Clear 12 × 75-mm tubes (Falcon 2052, Becton Dickinson Labware, Lincoln Park, NJ).

11. Centriprep-30 concentrators (Amicon, B0everly, MA).
12. Micro BCA Protein Assay reagent kit (Pierce, Rockford, IL).
13. Microplate reader (Molecular Devices, Menlo Park, CA).

2.1.4. Confirm Integrity of Fusion Proteins by Measuring Antibody Reactivity in Enzyme-Linked Immunosorbent Assay (ELISA)

1. PBS, pH 7.4.
2. ELISA wash solution: PBS supplemented with 0.05% (v/v) Tween-20 (Sigma) (PBS/T).
3. AffiniPure goat anti-human IgG, Fcγ fragment specific, supplied at 1.7 mg/mL (Jackson ImmunoResearch Laboratories, West Grove, PA).
4. KIR-Fc transfection supernatants or purified protein preps to be tested.
5. KIR specific mouse monoclonal antibodies, e.g., purified GL183 and EB6 anti-KIR2D antibodies diluted to 0.2 mg/mL (Immunotech, Westbrook ME).
6. Alkaline phosphatase-conjugated AffiniPure $F(ab')_2$ fragment Goat antimouse IgG (H&L), supplied at 0.6 mg/mL (Jackson ImmunoResearch Laboratories,).
7. Diethanolamine substrate buffer (5× concentrate) (Pierce).
8. ImmunoPure PNPP substrate tablets (*p*-nitrophenyl phosphate disodium salt tablets) (Pierce).
9. Immulon two flat-bottom, 96-well plates (DYNATECH Laboratories,Chantilly, VA).
10. ELISA or Microplate reader (Molecular Devices, Menlo Park, CA).

2.2. Growing HLA Class I Transfected Target Cell Lines

1. A large panel of 721.221 cells transfected with different HLA class I genes has been generated and described by Gumperz (Chapter 6). Other cell lines that express a well-defined HLA class I allotype are also suitable. The TAP-deficient mouse cell line RMA-S transfected with HLA class I can be used to evaluate the role of specific peptides in KIR binding ***(16)***.
2. B-cell culture medium: Iscove's medium with 25 m*M* HEPES without L-glutamine (BIOFLUIDS, Rockville, MD), supplemented with 10% (v/v) heat-inactivated FCS and 2 m*M* L-glutamine.
3. G418 (geneticin, Life Technologies): This is the selecting antibiotic for the pSRα-neo expression vector. G418 is supplied at 5 g/bottle but only a fraction of the total solid is active. Each lot indicates the active portion. For example, 682 μg/g indicates that only 68.2% (or 3.41 g) of the total prep is active G418. To prepare a working stock solution, dissolve 250 mg of active G418/mL of H_2O. Anticipate that the 5 g of powder will account for up to 2.5 mL of the final volume. This solution should be sterilized by filtration through a 0.2 μm filter. It can be aliquoted and stored at –20°C for up to 1 yr. Depending on the cell line, G418 is generally used at a final concentration of 1–1.5 mg/mL.
4. Hygromycin B (Calbiochem, La Jolla, CA): This is the selecting antibiotic for the pHEBo expression vector. A working stock solution of 20,000 U/mL in H_2O can be stored at 4°C for at least 1 yr. Depending on the cell line, Hygromycin B is generally used at a final concentration of 200–300 U/mL.

2.3. Binding Assay

1. Flat-bottom tissue culture treated 96-well plates (Costar).
2. Multichannel pipettor (Costar).
3. PBS.
4. Flow cytometry medium: PBS supplemented with 2% (v/v) heat-inactivated FCS.
5. Flow cytometry tubes: Clear 12 × 75 mm tubes (Falcon 2052).
6. Fixative: PBS supplemented with 2% *N*,*N*-dimethyl formamide (Sigma).
7. Anti-HLA class I antibody: Monoclonal antibody (MAb) W6/32 (American Type Culture Collection).
8. FITC-conjugated AffiniPure F(ab')$_2$ fragment goat antihuman IgG, Fcγ fragment specific, supplied at 1.5 mg/mL (Jackson ImmunoResearch Laboratories).
9. Goat antimouse IgG FITC: FITC-conjugated AffiniPure F(ab')$_2$ fragment goat antimouse IgG (H&L), supplied at 1.5 mg/mL (Jackson ImmunoResearch Laboratories).

3. Methods

3.1. Generation of Ig Fusion Proteins

3.1.1. DNA Constructs

1. Sequences encoding the extracellular portion of KIR are amplified by PCR using a forward primer corresponding to sequences immediately downstream of the signal sequence cleavage site and containing an *Nhe*1 site and a backward primer immediately upstream of the transmembrane region and containing a *BamH*1 site. The amplification products are digested with *Nhe*1 and *BamH*1 and ligated into the *Nhe*1-*BamH*1-digested Cd51neg1 expression vector in frame with the leader peptide of CD5 and the artificial splice site allowing fusion of the KIR cDNA to the genomic DNA encoding the hinge, CH2, and CH3 region of human IgG1.
2. The ligation products are transformed into the bacterial strain MC1061/P3 (*see* **Note 5**) and transfection quality DNA is prepared using Qiagen columns.

3.1.2. Transfection

1. Plate 1 × 10^7 COS-7 cells per 162 cm^2 flask in growth medium and incubate in a humidified incubator at 37°C with 5% CO_2 overnight (*see* **Note 6**).
2. The next day (d 0), remove growth medium from flasks.
3. Wash cells 1× with SF-DMEM to remove residual serum as this lowers the transfection efficiency.
4. Add 25 mL of TFM and place in 37°C incubator for 2 h.
5. Remove TFM and add 12 mL of 10% DMSO/PBS and incubate at room temperature for exactly 2 min. DMSO is very toxic to the cells.
6. Remove 10% DMSO/PBS and add 25 mL of growth medium. Return flasks to the 37° C incubator overnight. At this point, the cells will have a granular appearance.
7. The next day (d 1) remove growth medium and add 25 mL of SF-DMEM to each flask (*see* **Note 7**).

8. On d 4, the medium has started to turn orange. Harvest the culture supernatants to sterile, 50-mL conical tubes (Falcon 2098, Beckton Dickinson Labware) and refeed flasks with 25 mL of SF-DMEM. Clarify supernatants by centrifugation (1300*g*, 15 min) to remove residual debris that can cause problems with column flow during purification. Discard the pelleted material and store supernatants at 4°C in tightly covered sterile container to avoid exposure to air and shifts in pH (*see* **Note 8**).
9. Harvest and clarify supernatants again on d 8 and pool with the d 4 supernatants. These supernatants are ready to be purified and can be stored at 4°C for months if necessary (*see* **Note 9**).

3.1.3. Protein Purification/Quantification

Everything must be sterile during this procedure (*see* **Note 10**).

1. Assemble Flex-column onto a ring stand.
2. Pipet 2–2.5 mL of the 1:1 slurry of Protein A-Sepharose into the column. Gently add 4 mL of PBS. As the column flows, the 1–1.5 mL of Sepharose beads settle at the bottom. Allow another 10 mL of PBS to flow through the column to pack the beads well.
3. Sterilize the tubing by dropping the end attached to the Pasteur pipet into a container of ethanol. Attach a sterile syringe to the end fitted with the adapter and pull on the plunger to draw ethanol through the line.
4. Move the Pasteur pipet into a container of sterile PBS and use the syringe to flush the line with PBS. This removes the ethanol from the line and also primes the line with PBS.
5. After priming with PBS, attach the end of the tubing fitted with the adapter to the tip at the top of the column. Raise the container holding the PBS above the column so that the column flows by gravity.
6. The flow rate is controlled by changing the height of the container relative to the column. Establish the flow rate by increasing the height of the container of PBS above the column until PBS flows at a rate of 1 mL/min.
7. To begin loading the column, move the Pasteur pipet from the container of PBS into the container holding the supernatants to be purified very quickly so as to not break the siphon. Secure the tubing to the container with tape so that it does not accidentally slip out during the run. Keep the supernatants covered with foil during the run to keep them clean (*see* **Note 11**).
8. After all of the supernatants to be purified have entered the column, quickly move the Pasteur pipet into sterile PBS. To restore the siphon, use a sterile syringe to remove the air in the line and proceed as in **steps 4** and **5**. Wash the column with at least 15 mL of PBS.
9. To elute the protein, run the PBS wash through the column until it is just above the top of the Sepharose beads. Add 3 mL of 0.05 *M* Na citrate, pH 3.0, and collect fractions immediately.
10. Collect 1 mL at a time into clear 12 × 75 mm tubes containing 100 µL of 2 *M* Tris-HCl, pH 8.0, to neutralize the acid. The protein bound to the column is typi-

cally eluted within the first five fractions. Protein determinations can be performed on each fraction in a spectrophotometer at an OD of 280 nm.

11. Remove a small aliquot to verify that the pH is close to 7.4. At this point, the protein can be safely stored at 4°C for at least a few days.
12. Wash column with 20 mL of PBS and store in 0.02% azide at 4°C for future use.
13. Transfer the diluted protein to a Centriprep-30 concentrator. Centrifuge at 1500*g* for 15 min. Open the concentrator and remove the first 5 mL of effluent. Repeat this cycle 3 or 4× until the final volume remaining is approx 1 mL. Add 15 mL of sterile PBS and repeat the concentration step. This step is necessary to replace the Tris-containing buffer with sterile PBS. Tris interferes with the reagents used in the protein determination assay.
14. Transfer concentrated protein to a sterile tube and store at 4°C.
15. Use the Micro BCA Protein Assay reagent kit to measure the protein concentration (*see* **Note 12**). The main advantage to using this kit is that the assay is done in microtiter plates. Thus, only small amounts of protein are required and the results for multiple samples are obtained quickly on a microplate reader.

3.1.4. Determination of Antibody Reactivity in ELISA

1. All additions are made in 100-µL aliquots and all dilutions are made in PBS.
2. Coat the wells of a flat-bottom 96 well-plate with 2.5 µg of goat anti-human IgG Fcγ specific antibody (at 25 µg/mL) and incubate overnight at 4°C (*see* **Note 13**).
3. Remove the liquid from the wells by flicking the plates over a large receptacle. Blot the plates dry on a small stack of paper towels.
4. Add KIR-Fc transfection supernatants or dilutions of the purified protein preps to be tested and incubate for 1 h at room temperature. Start with 1 µg/well followed by serial fivefold dilutions. All subsequent steps can be done at room temperature.
5. Flick plates and wash 3× with PBS/T.
6. Add appropriate dilutions of KIR specific mouse MAb, e.g., purified GL183 and EB6 can be used at 1 ng/mL and 40 ng/mL, respectively, and incubate for 1 h.
7. Flick plates and wash 3× in PBS/T.
8. Add alkaline phosphatase-conjugated goat anti-mouse IgG diluted 1:10,000 and incubate for 20 min.
9. Flick plates and wash 3× with PBS/T.
10. Add substrate for alkaline phosphatase prepared according to suppliers instructions. (In brief, for one plate: Dissolve two PNPP tablets in 8 mL of water and add 2 mL of 5× substrate buffer). Color will begin to develop in less than 5 min and a final reading at 405 nm of light can be made within 20–30 min.

3.2. Growing Transfectants

1. Late in the day, rapidly thaw a 1-mL vial of 721.221 cells (frozen at 3×10^6 cells/mL) and immediately transfer the cells to a 15-mL conical tube (Sarsted) containing 10 mL of B-cell medium. Pellet the cells by centrifugation at 210*g* for 5 min. Remove the supernatant and resuspend the cells in 10 mL of B-cell medium containing appropriate concentrations of Hygromycin or G418 depending on the

transfectant. Transfer the cells to a 25 cm^2 flask (Costar) and place the flask in the upright position in a humidified incubator at 37°C with 5–10% CO_2 overnight. The next morning, transfer 7 mL of the overnight culture to a new flask containing 7 mL of fresh B-cell medium.

2. Preparing cells for the binding assay: For best results, the cells used in the binding assay should be in log phase and close to 100% viable. This can be accomplished by diluting the cells 1:1 into fresh medium every 24 h. Each point in the binding assay uses 2×10^5 cells. If the culture volume reaches 20 mL or greater, the cells should be carried in an upright 75 cm^2 flask (Costar) (*see* **Note 14**).

3.3. Binding Assay

All the reagents should be at 4°C and the assay takes place on ice.

1. Count cells and prepare at 2×10^6 cells/mL in flow cytometry medium.
2. Dilute the receptor: The binding of most KIR-Ig fusion proteins can be measured at 50 μg/mL final concentration. The fusion proteins are diluted to 10 μg/100 μL in flow cytometry medium.
3. Place a flat-bottom 96-well plate on ice and dispense the cell suspension at 100 μL/well for a final of 2×10^5 cells/well.
4. Add 100 μL of the diluted receptor to the cells by holding the pipettor perpendicular to the center of the well and dispensing the volume with a single push of the plunger. There should be no additional mixing. Incubate for 1 h (*see* **Note 15**).
5. Dilute goat anti-human IgG FITC and goat anti-mouse IgG FITC at 1:100 in flow cytometry medium.
6. Centrifuge the plate at 160–210*g* for 3–4 min.
7. Flick supernatants into a large receptacle, blot once on a small stack of paper towels, and immediately add 100 μL of the goat anti-human IgG FITC dilution dispensed into the center of well and incubate for 20 min.
8. Add 100 μL of the goat anti-mouse IgG FITC dilution to cells that have been incubated with the W6/32 MAb alone to assess HLA-class I expression.
9. Centrifuge, flick, blot dry, and dispense 200 μL of flow cytometry medium into the center of the well to wash and centrifuge immediately.
10. Flick, blot dry, and add 200 μL of fixative. Gently resuspend the cells.
11. Transfer the cells to flow cytometry tubes to which 150 μL of PBS has been added.
12. Analyze by flow cytometry.

4. Notes

1. The inclusion of a signal sequence in Cd51neg1 is convenient for the expression of truncated receptors that lack the amino-terminal domain ***(12).*** Cd51neg1, derived from the CDM7 vector, carries a CMV/T7 hybrid promoter, cloning sites flanking a stuffer, a splice site and polyadenylation signal, the SV40 origin of replication (for amplification in COS cells), two fragments (πVX ori and M13 ori) not relevant to this chapter, and a suppressor (sup) F region. This plasmid

does not carry antibiotic resistance genes but confers resistance to ampicillin and tetracycline in bacteria that carry the P3 episome, by suppressing mutations in both resistance genes with sup F.

2. Do not filter the DNA. When the DNA is first added to the TFM, it sometimes appears to precipitate. This occurrence has not affected the outcome of the transfection.
3. Typically, 10 flasks (at 25 mL/flask) are transfected for production of a given protein. Thus a standard size batch of TFM is 250 mL.
4. Although it is not absolutely necessary (pH 7.4 also works sufficiently well), pH 8.0 is optimal for binding of IgG1 to Protein A-Sepharose and should be used for best results.
5. For convenience, the transformation can be plated on LB plates containing 50–100 mg/mL ampicillin without tetracycline.When colonies are picked and grown in liquid culture, LB broth containing 12.5 mg/mL ampicillin and 7.5 mg/mL of tetracycline should be used.
6. COS-7 cells may be maintained as follows: Plate 4×10^6 cells in 50 mL of growth medium in a 162 cm^2 flask and incubate in a humidified incubator at 37°C with 5% CO_2. After 5–7 d the medium will be quite orange and the cells should be trypsinized and replated at a 1:10 dilution. When growing well, $4–6 \times 10^7$ cells/flask is a typical yield.
7. The cells will look progressively worse and some will detach. However, a sufficient number of cells remain and continue to produce protein.
8. The supernatants can be tested in ELISA now (or any time after d 2) to ensure that the protein of interest has been produced. Alternatively, if no antibody is available for use in ELISA, the protein can be visualized by expression on a small scale with metabolic labeling, and direct immunoprecipitation with Protein A beads.
9. The protein may precipitate during storage. This does not occur often, and it is usually obvious upon visual inspection. Inspect visually before use. If in doubt about the integrity of the protein, reevaluate by ELISA.
10. The run can be performed at room temperature or at 4°C but all the reagents should be equilibrated to the chosen temperature before starting. Otherwise, bubbles tend to form within the Protein A beads in the column and can interfere with column flow and protein binding.
11. At a flow rate of 1 mL/min, 500 mL of supernatants will take 8–9 h. Because washing after the run and harvesting can take 1–2 h, it is convenient to run the column overnight. To prevent the column from drying, allow the tubing to extend below the level of the column.
12. The binding capacity of protein A is very high. For human IgG, 1 mL of hydrated Protein A-Sepharose will bind approx 8 mg of antibody. The yield of each KIR-Fc protein is generally consistent between transfections but can vary among different KIR-Fc proteins. Yields ranged from 1–10 μg/mL of transfection sup or 0.5–5 mg total in 500 mL.
13. Be sure to use PBS and not PBS/T because Tween-20 interferes with the binding of the antibody to the plastic. The plates can be used for at least 2 mo if they have been wrapped to prevent evaporation, and stored at 4°C.

14. Typically, cells thawed on a Monday, and passed 1:1 every day, are almost 100% viable and ready to use on Friday of that week. Generally, the cells should be kept at or below 0.3×10^6/mL. The cells to be used in the binding assay are usually not kept in culture for extended periods of time because overgrowth of some of the transfected cells (e.g., the .221-Cw4 transfectant) correlates with unusual bimodal profiles on flow cytometry analysis of KIR-Fc binding.
15. For best results, the assay should be conducted with a minimum of mixing. Pipetting the mixture up and down correlates with high background fluorescence.

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21

Genetic Analysis of a Highly Homologous Gene Family

The Killer Cell Immunoglobulin-Like Receptors

Michael J. Wilson, Michaela Torkar, and John Trowsdale

1. Introduction

Human natural killer (NK) cells express an array of inhibitory and activating receptors some of which interact with major histocompatibility complex (MHC) class I molecules. Expression of these receptors varies at a clonal level generating subsets of NK cells with respect to their receptor repertoire. These receptors can be divided into two broad groups: members of the C-type lectin family and those from the immunoglobulin superfamily (IgSF). The C-type lectins comprise the NKG2 family encoded by five genes (*NKG2A*, *-C*, *-D*, *-E*, and *-F*, *NKG2B* is an alternatively spliced transcript derived from *NKG2A*) and CD94. CD94 can form a heterodimer with the *NKG2A*, B, C and E proteins and either *NKG2A*, *-B*, or *-C* with CD94 interact with the nonclassical class I molecule HLA-E ***(1)***. The NK cell receptors that are members of the IgSF are called the killer cell immunoglobulin-like receptors (KIRs), and a related set of loci is called the immunoglobulin-like transcripts (ILTs). Both the KIRs and the ILTs are encoded by tightly clustered groups of genes on human chromosome 19q13.4 in the leukocyte receptor complex (**ref. *2*** and ***3***). The KIRs are expressed on NK cells and a subset of T cells, whereas to date only one ILT, ILT-2, has been shown to be expressed on NK cells ***(4,5)***.

Since the first KIR cDNA was described, more than 100 highly homologous cDNAs have been deposited in databases. Comparison of these sequences using alignment programs such as Clustal ***(6)*** allows grouping of individual transcripts into 11 subgroups ***(7)***. These have been designated a specific nomenclature based on the number of Ig domains (2D or 3D) and the presence or absence of immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in the

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
Edited by: K. S. Campbell and M. Colonna © Humana Press Inc., Totowa, NJ

long or short cytoplasmic tail (designated L or S), respectively *(8)*. The last number determines the group to which these receptors belong; i.e., 2DL1-4, 2DS1-5, 3DL1-2, and 3DS1. Although alignment of cDNAs is a useful method of grouping transcripts, it is not always obvious as to whether cDNAs are truly encoded by individual genes or arise from polymorphism in the same locus. To date only three KIR genes have been characterized by sequence analysis, including one that has no corresponding transcript (**refs. *3,9,10*).** The presence of loci that cannot be accounted for by cDNAs and therefore may be pseudogenes, and the high homology of transcripts presumably from different subgroups demonstrates that the number of loci encoding such a plethora of cDNAs can be resolved only by genomic analysis.

Recent studies have shown that KIR loci, unlike CD94 and the NKG2 families show variation in the number of genes in different individuals. Furthermore, these articles also describe clonal variation of expression of KIR and NKG2 cDNAs using allele-specific probes *(11,12)*. Therefore, it will be of interest to study disease associations with NK receptor variations, in view of their pivotal role in interfacing between the innate and the adaptive immune systems.

The procedures described in this chapter are used to chracterize the KIR in detail, including the 5'-untranslated regions and promoters, as well as some of the coding sequence. Once an initial KIR map has been established using genomic clones derived from one individual, the methods may be modified for the determination of the profile of KIR genes in any individual. Ideally, this will be done on a haplotype basis because of the heterogeneity in heterozygotes. This may be achieved in one of three ways: (1) analysis of single haplotypes by introducing chromosomes into rodent cells *(13)*, (2) by identifying true homozygotes for the KIR complex, or (3) analysis of phase by linkage of families. The methodology described can also be adapted for the examination of other gene families such as the closely linked ILT gene cluster.

The strategy described below can be summarized as: (1) isolation of genomic clones, (2) analysis of of these clones by long-range polymerase chain reaction (PCR) using redundant and specific primers to determine which genes are present, and (3) design of locus specific primers to link adjacent genes.

2. Materials

1. All chemicals are obtained from Merck except where indicated. Gloves should always be worn with molecular biology techniques to prevent contamination. Extreme care should be taken with all chemicals. The most toxic are indicated by an asterisk.
2. Human genomic DNA libraries are available from a variety of sources–from companies such as Research Genetics (North America) (http://www.resgen.com) or

from academically based central resources such as Roswell Park Cancer Institute (http://bacpac.med.buffalo.edu/) in the United States and the Human Genome Mapping Resource Centre (HGMP) (http://www.hgmp.mrc.ac.uk) and the Reference Library Database (RLDB) (http://www.rzpd.de) in Europe. Genomic DNA libraries are made in either YAC, PAC, BAC, or cosmid vectors. Libraries can be screened either by hybridization of high density gridded filters or using PCR primers for screening pools of clones. The protocols described in **Section 3** are used for PAC, BAC, or cosmid clones but can be used equally for YAC clones following isolation of the DNA. The advantage of PACs and BACs is the low number of rearranged clones in these libraries compared with cosmids and YACs.

3. Hybridization reagents:
   a. Hybridization buffer: Mix 0.5 *M* $NaHPO_4$ pH 7.2; 1 m*M* EDTA; and 7% sodium dodecyl sulfate (SDS). 1 *M* $NaHPO_4$, pH 7.2, is made up by adding 134 g of Na_2HPO_4 with 4 mL of 85% H_3PO_4*/L of double-distilled H_2O (ddH_2O).
   b. 20× Saline sodium citrate (SSC): 1× SSC = 0.15 *M* NaCl and 0.015 *M* sodium citrate in water.
   c. 20% SDS in ddH_2O made up in a fume hood.
   d. Random primer labeling kit (Promega).
   e. [α-^{32}P]dCTP, 10 mCi/mL (Amersham).
   f. X-Ray film (Kodak).
4. Charged nylon membrane (Hybond N+, Amersham).
5. Media for growing bacteria:
   a. Luria-Bertani broth (LB): Mix 10 g of bacto-tryptone, 5 g of bacto yeast extract, and 10 g of NaCl/L of ddH_2O. Autoclave before use.
   b. LB agar: Prior to autoclaving LB broth, add 15g/L of bacto-agar.
   c. Kanamycin (kan) (Sigma): Mix 30 mg/mL in ddH_2O; used at 1/1000 in LB or LB agar.
   d. X-Gal* (5-bromo-4-chloro-3-indolyl-β-D-galactosidase): Mix 20 mg/mL in dimethylformamide*; use 80 µL/20mL molten LB agar (i.e., per agar plate).
6. Alkaline lysis solutions:
   a. P1: Mix 15 m*M* Tris, pH 8.0; 10 m*M* EDTA (pH 8.0); and 100 µg/mL RNase A (Sigma) in ddH_2O. Filter sterilize and store at 4°C.
   b. P2: Mix 0.2 *N* NaOH and 1% SDS in ddH_2O. Make fresh on the day of use.
   c. P3: Mix 600 mL of 5 *M* potassium acetate (autoclaved); 115 mL of glacial acetic acid*; and 285 mL of ddH_2O. Store at 4°C.
7. Oligonucleotides (Genosys) to be used as PCR primers are made up as stocks at 50 pmol/µL for PCR and 3.2 pmole/µL for sequencing. *See* **Table 1** for sequences used.
8. PCR:
   a. Expand™ Long Template PCR System (Boehringer Mannheim).
   b. Dideoxy nucleic acids stock—10 m*M* dATP, 10 m*M* dCTP, 10 m*M* dGTP, 10 m*M* dTTP (Gibco-BRL).
   c. Thin-walled PCR tubes (Robbins Scientific).
   d. Thermocycling PCR machine (Applied Biosystems).
   e. Human genomic DNA (Promega).

Table 1
Oligonucleotides Used for PCR and Sequence Analysis

Primer	Sequence 5'–3'
K1	GAGATTGGATCTGAGACGTGTT
K2	ATCTTTCTTTCCAGGGTTC
K3	TGGCCAGGCCCCCTGCAGCA
K4	CAGGACAAGCCCTTCCTGTCTG
K5	GTGACCATGATCACCATGGGG
K6	CCTTCCCTCCTGGCCCACCCA
K7	TCTCCATCAGTCGCATGY
K8	TGCACAGAGAGGGGAAGTA
K9	TCACTCCCCCTATCAGTTT
K10	CCTCAAAGATTTCCACNGAG
K11	CAGTGGGTGAAGGCCAACTAT
K12	TGATTGGGACCTCAGTGGTCA
K13	TGGCAGCACCAGCGATGAAGG
K14	ACCAAGAGCCTGCAGGGAACA
K15	TGTTCCCTGCAGTCTCTTGGT
K16	CCTCAGTGTGATYGCAGCCTC
M13R	CAGGAAACAGCTATGAC
T7	TAATACGACTTCATATAGGG

These sequences are common to all KIRs and the primers should amplify all loci. **Figure 1** demonstrates the positions of oligonucleotides within exons on a KIR locus.

9. Electrophoresis of DNA fragments:
   a. Agarose (Gibco-BRL).
   b. 10× TBE buffer: Mix 108 g of Tris Base, 55 g of boric acid, 40 mL of 0.5 *M* EDTA, pH 8.0, per liter of ddH_20
   c. Ethidium bromide* (Sigma): Mix 10 mg/mL in ddH_2O.
   d. DNA molecular size standards 1-Kb ladder and λ*Hin*dIII (Gibco-BRL).
   e. 6× DNA loading dye: Mix 0.25% bromophenol blue, 0.25% Xylene cyanol FF, and 15% Ficoll (Pharmacia) in ddH_2O.
10. Gel extraction kit (Qiagen).
11. Nucleotide removal kit (Qiagen).
12. Dye terminator sequence kit (ABI/Perkin Elmer).
14. Automated DNA sequencer (ABI/Perkin Elmer). If access is not available then DNA can be sequenced through many molecular biology companies.

3. Methods

Many methods described here are adapted from protocols in Sambrook et al. ***(14)***.

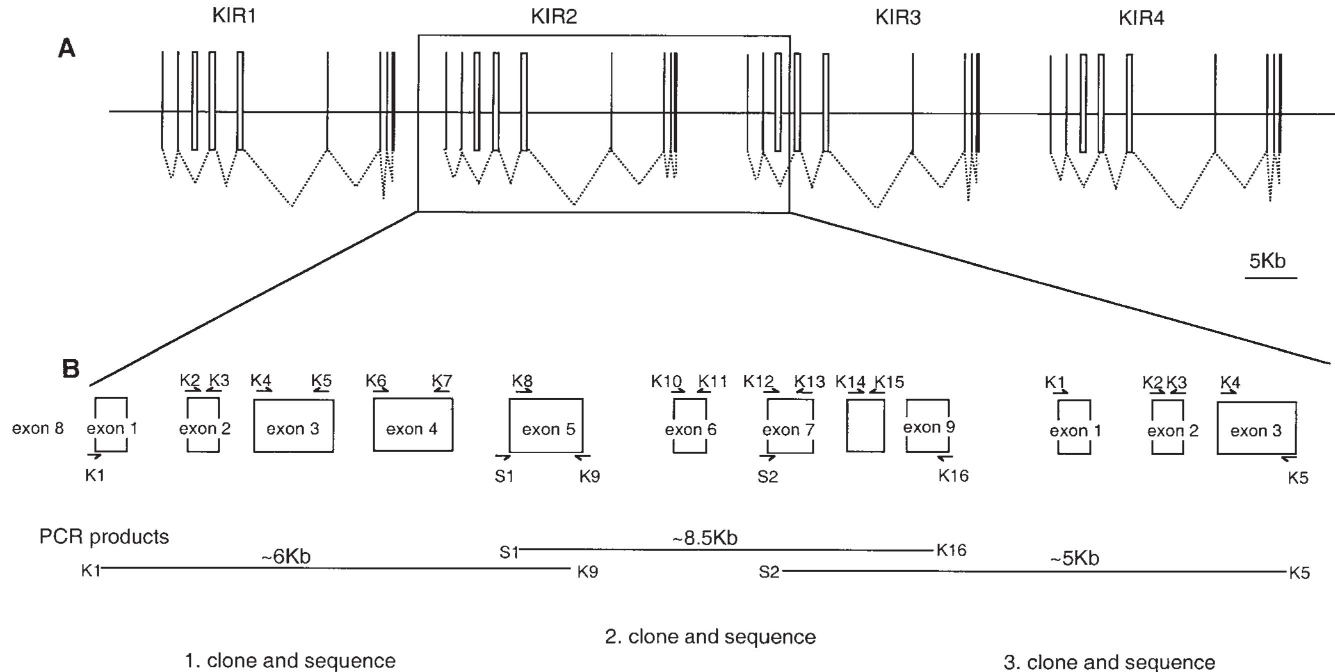

Fig. 1. Schematic diagram of cloning strategy for KIR loci from a genomic DNA clone. Individuals within the population exhibit variable number of KIR loci ranging from six to ten *(10)*. In this example, **(A)** shows genomic organization of four KIR loci with each box representing an exon. In the case of the 2D KIRs, exon 3 is a pseudo exon with an in frame stop codon, while 2DL4 has no exon comparable to exon 4 of other KIR loci. **(B)** A schematic representation of the PCR strategy for determining the nucleotide sequence of unknown KIR loci. Step 1: amplification of DNA fragments from exon 1 to 5 of all KIRs using redundant primers K1 and K9. These DNA fragments are cloned into a TA vector and sequenced using redundant primers K1–K9. Step 2: From sequence analysis flanking exon 5, specific primers S1a, S1b, S1c, S1d are synthesized (**Fig. 2**) and used in combination with redundant primer K16 in exon 9. The cloning and sequencing is repeated and further specific primers S2a, S2b, S2c, S2d for each KIR are used in combination with redundant primer K5, and reverse to exon 3 to link up adjacent KIR loci.

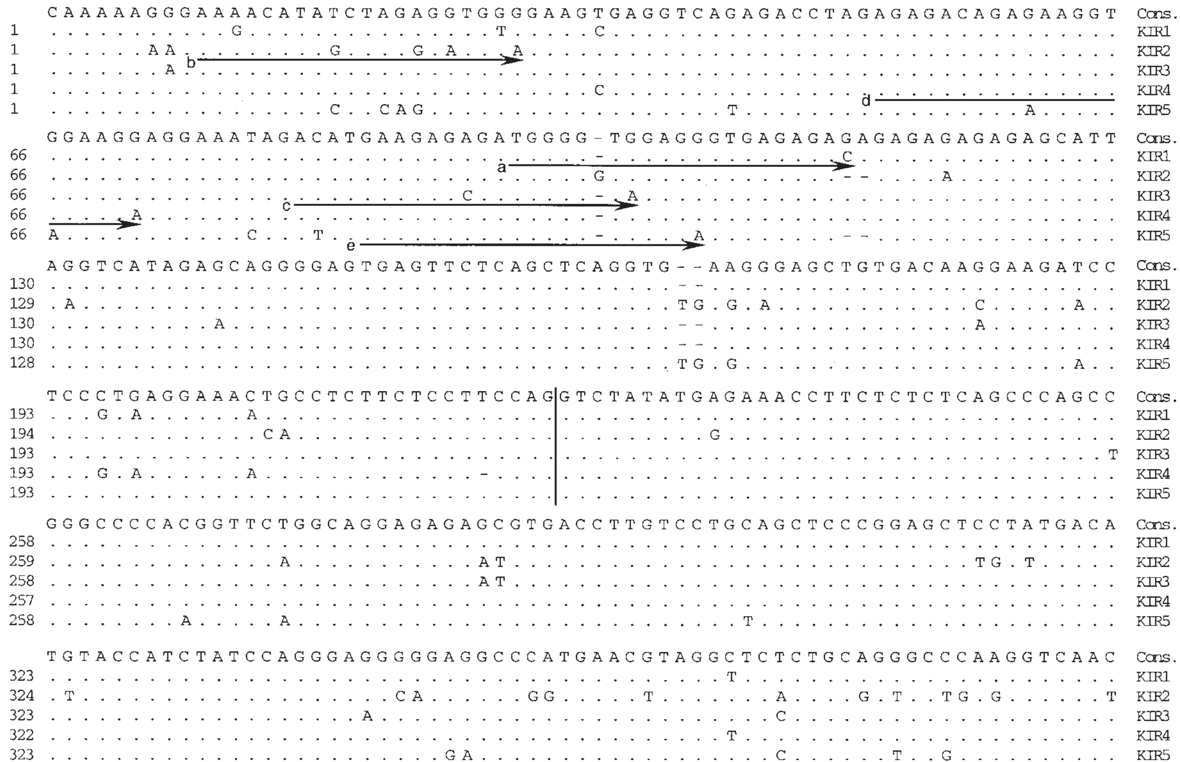

Fig. 2. Design of locus-specific forward S1 primers from the 5' region, flanking exon 5. The intron exon boundary is marked by a line between the sequences and residues matching the consensus (cons.) are indicated by dots. Underlined sequences demonstrate primers designed such that unique nucleotide residues are at the 3' end of the oligonucleotide and the annealing temperature is at least 60°C. Individual primers for KIR loci 1–5 are labeled with lowercase letters. Sequences are derived from synthesis of products with primers K1 and K9 and can be compared to genomic KIR sequence deposited in databases (such as GenBank accesssion nos. U97177, AF001881, and AF001885).

3.1. Identification and Preliminary Analysis of KIR-Positive Clones

3.1.1. Isolation of KIR-Positive Genomic Clones

Genomic DNA libraries can be screened using PCR on pools of clones or by using radiolabeled probes on high-density gridded filters. In the case of the KIRs, redundant KIR primers can be used for either approach. The primers K4 and K5 (**Table 1** and **Fig. 1**), specific for exon 3 (and pseudo exon 3 in the case of 2 Ig domain KIR's) are used to amplify a redundant KIR probe by PCR using the protocol outlined in **Table 2**. The annealing temperature is 60°C, the extension time is 30 s, and 300 ng of human genomic DNA is used as a template.

To screen filters by hybridization, the 270-bp PCR product is purified using a Qiagen nucleotide removal kit. DNA is quantitated on a 1.5% agarose gel and 50 ng of purified DNA is radiolabeled with [α-^{32}P]dCTP using a random hexamer labeling kit. Library filters are prehybridized for at least 30 min at 65°C in hybridization buffer, the probe is denatured by adding NaOH to a final concentration of 0.2 *M*, and the solution is incubated at room temperature for 5 min. The denatured probe is then added to the hybridization solution and incubated overnight at 65°C. The following day filters are washed twice in 2× SSC, 0.1% SDS at room temperature and then exposed to film at –70°C overnight. The low-stringency washing conditions are used to ensure a high background, allowing for easier identification of clones on high-density gridded filters. Clones are identified by using the code supplied with the filters and positive clones are ordered from the original source. Three to six positive clones would be expected from a 3× coverage genomic PAC DNA library.

3.1.2. Isolation of PAC DNA

PAC clones are generally transported streaked out on an agar plate. To ensure that PAC DNA is from a single clone, bacteria are restreaked for single colonies onto a LB agar/kan plate. Several clones are picked into 5 mL of LB broth/kan in a 15-mL snap-cap polypropylene tube, and grown for 16 h in a shaking incubator at 37°C at 250 rpm. The following day, glycerol stocks should be made by taking 850 µL of culture and adding 150 µL of sterile glycerol, mixing well by several inversions, and stored at –70°C. The remaining culture is used for isolation of DNA. This is based on a modified alkaline lysis method as follows.

1. Pellet bacteria by centrifugation at 3500*g*. Discard supernatant and resuspend pellet in 300 µL of P1 solution. Transfer resuspended bacteria to a 1.5-mL Eppendorf tube.
2. To lyse bacteria add 300 µL of P2 solution and gently shake tube to mix the contents. Leave at room temperature for no longer than 5 min. The appearance of the suspension should change from very turbid to almost translucent following lysis.

Table 2
General PCR Protocol

Reagent	1×	Final conc.
10 m*M* dNTP	1 µL	200 m*M*
10× buffer (including $MgCl_2$)	5 µL	1×
TAQ (3.5 U/µL)	0.75 µL	0.0525 U/µL
fwd Primer (50 pmol/µL)	1 µL	1 mM
rev Primer (50 pmol/µL)	1 µL	1 mM
Template: PAC (20 ng/µL) or	1 µL	0.4 ng/µL
Genomic DNA (300 ng/µL)	1 µL	6 ng/µL
ddH_2O	40.25 µL	
Total	50 µL	

A premix containing all the common reagents is made for each tube. It is very important to mix well by vortexing this thoroughly. Premix is based upon the amount of reagent for one reaction. When calculating the volumes of each component, an extra reaction should be factored in to account for pipetting errors. For all PCRs, the following protocol is followed: 92°C for 2 min, followed by 30 cycles of: 92°C for 10 s, 60°C for 30 s, and 68°C for 1 min/Kb of product to be amplified. For long-range PCR, an additional extension time of 7 min at 68°C is added at the end of the 30 cycles.

3. Slowly add 300 µL of P3 solution to each tube and gently shake during addition. A thick white precipitate of protein and *E. coli* DNA will form. After adding P3 solution to every tube, place the tubes on ice for at least 10 min. Place tubes in a microfuge and centrifuge at 16,000*g* for 10 min at 4°C.
4. Remove tubes from microfuge and place on ice. Transfer supernatant, avoiding white precipitated material, to a 1.5-mL Eppendorf tube. Add 800 µL of isopropanol and mix by inverting the tube several times. Centrifuge in a microfuge at 4°C at 16,000*g* for 5 min.
5. Remove supernatant and add 1 mL of 70% ethanol to each tube. Invert tubes several times to wash the DNA pellets. Centrifuge at 16,000*g* in a cold microfuge for 5 min.
6. Remove as much of the supernatant by aspiration rather than pouring the supernatant off.
7. Air-dry pellets at room temperature. When the DNA pellets turn from white to translucent in appearance, i.e., when most of the ethanol has evaporated, add 100 µL of TE buffer. Do not use a narrow-bore pipet tip to resuspend DNA sample; rather, allow the solution to sit in the tube with occasional tapping of the bottom of the tube.

3.1.3. Analysis of Isolated DNA

Use 10 µL of each DNA solution to analyze on a 0.7% agarose gel with λ*Hin*dIII DNA markers. The PAC DNA should migrate just above the largest marker fragment (23 Kb). To determine the minimum number of loci present, 10 ng of PAC DNA is digested with either *Hin*dIII, *Eco*RI, or *Bam*HI and frag-

ments separated on a 0.7% agarose gel at 30 V in TBE for 18 h. The gel is photographed with a marking ruler, and DNA is capillary transferred to a Nylon membrane (Hybond N+) by Southern blotting with 0.4 *M* NaOH as a buffer. The blot is hybridized with the DNA fragment generated with K4 and K5, then washed at high stringency (0.1× SSC, 0.1% SDS, 65°C). Expected results can be seen in **Fig. 3**.

*3.2. PCR Analysis of Genomic Clones (*see *Note 1)*

1. To isolate a single KIR locus from a genomic clone long-range PCR is employed, dividing the gene into two overlapping sections (**Fig. 1**) (*see* **Note 2**). Initially, exon 1 to exon 5 is amplified using primers K1 and K9 (extension time of 7 min) producing a fragment of approximately 6 kb for most KIRs and 4 Kb for KIR2DL4 (*see* **Note 3**). PCR products are separated on a 0.7% agarose gel and DNA fragments of the expected size are excised and purified using a Qiagen gel purification kit. Following purification, PCR products are cloned into the TA cloning vector, transformed into *E. coli* and plated on LB agar kan/X-Gal. Forty white colonies (recombinants) are picked into 5 mL of LB broth/kan and grown overnight in a shaking incubator at 37°C. Glycerol stocks are prepared and plasmid DNA are isolated from bacterial cultures as described in **Subheading 3.1.2.** Plasmid DNA is quantitated on a 0.7% agarose, 1× TBE gel.
2. Sequence analysis is performed on an ABI 377 or 373 automated DNA sequencer using 400 ng plasmid DNA with a dye terminator kit. To minimize the number of sequencing reactions, initially a limited number of clones are analyzed using the end primers T7 and M13R (*see* **Note 4**). Analyses of these clones will allow different loci to be determined. If a number of different loci are not identified then further clones should be analyzed. Once different loci have been determined, then full exon sequence may be performed using the primers K1 to K9 (**Fig. 1**). Following sequence analysis of each clone, edited sequences are compared (*see* **Notes 4** and **5**). The quality of the sequence must be high as often the difference between KIR loci is limited to five or six bases per 300. The expected result with respect to the number of KIRs should be comparable to the results of the Southern blot (*see* **Note 6**).
3. Cloning the second part of the KIR genes. Once all loci have had the nucleotide sequence of exons 1 to 5 deduced, specific primers (S1a, b, c, etc.) for the flanking region 5' of exon 5 can be designed for use in combination with a redundant reverse primer designed to exon 9 (K16). As seen in **Fig. 2**, locus specific primer design is based upon having unique nucleotide sequence at the 3'-end of the oligonucleotide. The other criterion for primer design for long range PCR is an annealing temperature above 60°C to enhance specificity and unique sequence following the primer to allow for assured linking of the two parts of the gene. PCR is performed using the long-range PCR and the locus-specific primers (S1a, b, c, d, e) with redundant primer K16 (**Fig. 1**). Once again products are cloned into the TA vector and sequenced. As specific products are expected, the number of clones analyzed can be limited to three per primer set (glycerol stocks should

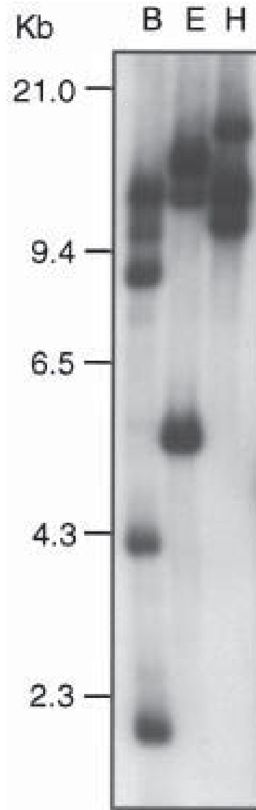

Fig. 3. Southern blot of PAC DNA using a KIR single exon probe. A KIR positive PAC clone was digested with *Bam*HI (B), *Eco*RI (E), and *Hin*dIII (H), then fragments separated on a 0.7% agarose gel. DNA was blotted onto a charged nylon membrane and hybridized with a single exon probe homologous to all known KIR loci. The data are interpreted to indicate that there are at least five KIR loci on this particular PAC clone.

be made from additional clones in case they are required). Sequencing is performed with primers M13R, T7, S1, and K8–K16). The expected results are a 100% overlap with the exon 5 sequence from the corresponding clone and contiguous sequence from exons 7–9.

4. Linking the KIR loci. This protocol takes advantage of the head to tail orientation and the close proximity (5Kb) of KIR loci (**Fig. 1**). Primers specific for each locus are designed in a forward orientation in exons 7–9 using the same criteria as in **step 7**. The specific primers (S2a, b, c, etc.) are used in combination with the redundant primer K5, in exon 3 (**Fig. 2**). One set of primers should give no product being that of the last KIR locus on the genomic clone. Once again products are cloned and sequenced and the resulting edited data are compared with

what has been defined from the previous sequence analysis. The gene order can then be deduced by the overlapping contiguous sequence (*see* **Note 6**).

4. Notes

1. An alternative strategy for the KIRs would be to use locus-specific primers as described in Uhrberg et al. ***(10)***. This allows for detection of loci corresponding to published cDNAs to be determined on a genomic clone. However, although this is a useful addition to the techniques described previously it does not account for KIR loci that do not yet have corresponding cDNAs, such as KIRCI ***(3)*** or for unknown polymorphisms.
2. The whole KIR gene could be sythesized by long-range PCR as described by Selvakumar et al. ***(8)***. However, in our hands this approach is quite problematic owing to the somewhat unpredictable nature of amplification of DNA fragments greater than 12 kb using PCR. In addition, the cloning of 15-kb DNA fragments into standard cloning vectors can also be very inconsistent. In the case of the ILTs this is more likely to succeed owing to the smaller size of the ILT genes, being no greater than 7 kb ***(3)***.
3. This protocol is based on the assumption that all KIR genes have all exons present except for KIR2DL4, KIRCI, and another unpublished sequence which are missing exons 4, 6, and 2, respectively. Long-range PCR often gives rise to smaller spurious products in addition to DNA fragments of the expected size. In our experience it is best to ignore these as nonspecific products unless they are the only detectable DNA fragment, as it is extremely time consuming to sequence every PCR product synthesized.
4. PCR amplification can introduce base substitution errors, although the use of a proofreading enzyme as is present in the Long Ranger Kit usually circumvents this problem. If this is suspected, then comparison of sequence from several clones should make PCR errors obvious. Alternatively, combining the products of several PCR reactions with the same primers will lower the risk of errors from base substitutions occurring in the initial PCR cycles.
5. Sequence analysis must be performed with strict consultation to the trace data as the nucleotide differences between KIR loci can be very small. It is most time efficient to edit the sequence prior to comparison with other clones. A useful cross reference for alignment of KIR cDNAs is the article by Steffens et al. ***(15)***.
6. The number of bands on a KIR Southern blot should be the minimum number of loci present on the PAC clone. Often, additional loci are detected through sequence analysis.

5. Troubleshooting

In our experience some regions of KIR genes are easier to amplify than others and often one locus will not amplify despite having the correct sequence with redundant primers. Therefore, alternative strategies can involve using a

combination of intermediate redundant primers from other exons (e.g., K2–8); however, it is best to avoid exons 2 and 6 as these are very short and are not present in all KIR loci ***(3)***.

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22

Use of Vaccinia Virus for Functional Gene Transfer in Natural Killer Cells

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1. Introduction

Vaccinia virus-mediated gene transfer is a powerful technique to test the function of individual receptors in natural killer (NK) cells. By transient expression of genes encoding NK receptors, one can obtain high levels of protein synthesis, while maintaining NK cell functions. The vaccinia virus expression system is useful for the analysis of wild-type and mutant forms of NK receptors and for the study of structure–function relationships as they relate to receptor–ligand interactions and signaling events.

There is a multiplicity of killer cell Ig-like receptors (KIRs), including isoforms that activate or inhibit NK cells, and the pattern of expression of these receptors is often complex. Studies of the specificities and functional effects of individual receptors are possible using the vaccinia virus expression system because one can achieve homogeneous infection of NK cells and the lytic ability of these NK cells on infection is not compromized. Furthermore, infection does not interfere with the negative signaling machinery, such that analysis of KIR function in cytotoxicity assays is possible. Although this expression system is best suited for functional studies, biochemical analysis of signaling events and protein–protein interactions mediated by the intracytoplasmic tail is also possible. However, such biochemical experiments may be limited by the number of cells that can be infected with a vaccinia virus preparation. This expression system requires fine-tuning. The dose of virus and the time of infection are important parameters. This chapter describes how to harness the power of vaccinia virus mediated gene transfer in experiments using human NK cell lines and clones as well as mouse bulk NK cell cultures.

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
Edited by: K. S. Campbell and M. Colonna © Humana Press Inc., Totowa, NJ

The vaccinia virus expression system is unusual in that transcription occurs in the cytoplasm of the cell rather than in the nucleus. The virion contains all the proteins needed for the transcription of early genes. DNA replication begins after the early phase of infection and leads to the intermediate and late phases of gene expression. Vaccinia virus infects most mammalian cell lines. While there are some cytopathic effects associated with the late phase of the infection, they can be avoided by the use of short infection times. The recombinant vaccinia virus vector (e.g., psc65) contains a compound early/late promoter such that the recombinant protein is expressed very early after infection.

The construction and propagation of recombinant vaccinia viruses have been described in detail ***(1)*** and are not covered in this chapter. In brief, cells (e.g., CV-1) are infected with wild-type vaccinia virus and subsequently transfected with a plasmid containing the gene of interest under the control of a vaccinia virus promoter. The plasmid DNA undergoes homologous recombination with the thymidine kinase gene of vaccinia virus. Selection of recombinant vaccinia viruses on the basis of a thymidine kinase negative phenotype and the coexpression of the *E. coli lacZ* gene (carried by the plasmid in addition to the gene of interest) permits color screening of recombinant plaques in TK^- cells using X-Gal. Blue plaques are isolated and recombinant virus stock is amplified. Purified virus is then used for the infection protocol detailed in this chapter. After a short infection of NK cells with the recombinant vaccinia viruses, cells are used in cytotoxicity assays to assess receptor function. In parallel, a small aliquot of the infected cells is monitored for homogeneous infection by antibody staining and flow cytometry.

2. Materials

2.1. Vaccinia Virus Infection of NK Cells

1. Infection medium: The basal medium used for infection is Iscove's medium (Gibco-BRL, Life Technologies, Baltimore, MD). Bovine serum albumin (Albumin Fraction V, no. 70195, United States Biochemical, Cleveland, OH), is added at a final concentration of 0.5%. Nonessential amino acids (NEAA, 100×, Gibco-BRL) are added at a final concentration of 1× and glutamine (Gibco-BRL; 200 m*M*) is added to give a final concentration of 2 m*M*. The infection medium is supplemented with 50 U/mL of recombinant interleukin-2 (rIL-2) immediately prior to use.
2. Wash medium: Iscove's, supplemented with 10% fetal bovine serum (FBS; Gibco-BRL; inactivated by heating at 56°C for 1 h), 2 m*M* glutamine, and 50 U/mL of rIL-2.
3. 15-mL Polystyrene conical screw-cap tubes (Falcon 2095, Becton Dickinson Labware, San Jose, CA).
4. Sonicator (Ultrasonic Processor, Heat Systems, Farmington, NY).

2.2. Flow Cytometry

1. Staining buffer: Dulbecco's phosphate-buffered saline (DPBS; Gibco-BRL) supplemented with FBS to a final concentration of 1%.
2. Falcon polystyrene, round-bottom tubes (Falcon 2052).
3. Fixative buffer: DPBS supplemented with 1% paraformaldehyde (Mallinckrodt, Phillipsburg, NJ); stable at 4°C for up to 1 mo.
4. Propidium iodide (Sigma-Aldrich, St. Louis, MO): make up a 20 mg/mL of stock (store at 4°C) and use 10 µL/tube of stained cells.

2.3. Cytotoxicity Assays

1. Cytotoxicity assay medium: RPMI 1640 supplemented with 5% FBS, 2 m*M* glutamine, and 40 µg/mL of gentamicin (Gibco-BRL).
2. $Na_2{}^{51}Cr0_4$ (5 mCi, no. CJS4V, Amersham, Arlington Heights, IL).
3. 2% (v/v) Triton X-100 (Sigma) in water.
4. Nonsterile, 96-well V-bottom microtiter plates with lids compatible with harvesting system (Costar 4797, Corning-Costar, Cambridge, MA).
5. Multiwick supernatant harvesting system (Skatron, Sterling, VA).

3. Methods

3.1. Vaccinia Virus Infection of NK Cells

1. Use NK cell lines or clones that are growing vigorously in logarithmic phase at a concentration of $3–5 \times 10^5$/mL. The cells should be very healthy with very high viability (*see* **Note 1**).
2. Count NK cells using a hemacytometer. Use $0.5–1 \times 10^6$ cells per infection point. One can scale up the number of cells and virus added proportionately for larger experiments. Include an uninfected control and a positive control infection to provide an indication of the sensitivity of the particular NK line or clone to infection with vaccinia (*see* **Note 2**). Remove enough cells for the experiment.
3. Wash cells 3× with prewarmed (in a 37°C waterbath) infection medium by centrifugation (~250*g* at room temperature for 10 min). The extensive washing is to ensure complete removal of residual fetal calf serum that would reduce the efficiency of infection.
4. During the washes in **step 3**, thaw an aliquot of each of the purified recombinant vaccinia viruses on ice (*see* **Note 3**). Virus to be sonicated should be in a sealed tube such as a Nunc freezing vial. Sonicate the viruses (1 min at full power in 0.5-s bursts) in an ice-water bath to disaggregate the virus (*see* **Note 4**).
5. Resuspend NK cells at 1×10^6/mL in infection medium containing 50 U of rIL-2 (*see* **Note 5**). Aliquot 1 mL of cells per experimental point in a 15-mL conical tube. Add the lowest dose of virus required for complete infection and vortex gently to mix.
6. Place samples on a rotator placed inside a 37°C, 5% CO_2 incubator. Loosen the lids of the conical tubes and incubate the tubes for 1.5 h (*see* **Note 6**). Periodically (2 or 3× during the infection) flick the tubes to keep the cells well suspended and ensure that the lids are loose.

7. At the end of the incubation, add 10 mL of wash media (at room temperature) to the cells and centrifuge (~250*g* for 10 min) twice to wash away free virus.
8. Count the cells in each infection tube and check cell viability. Remove 2×10^5 cells for antibody staining and flow cytometry (**Subheading 2.2.**) and use the remaining cells in the cytotoxicity assay (**Subheading 2.3.**).

3.2. Flow Cytometry to Assess Complete Infection

Homogeneous expression of recombinant inhibitory NK receptors on the NK cell surface is critical as the lytic activity of even a few uninfected cells would obscure the inhibition mediated by productively infected cells. Antibody staining followed by flow cytometry is performed to ensure complete infection.

1. Transfer 2×10^5 cells to polystyrene round bottom tubes, add 3 mL of staining buffer and centrifuge cells (250*g* for 10 min at 4°C).
2. Aspirate medium and resuspend cells in 100 µL of staining buffer. Add the appropriate concentration of primary antibody directed against the expressed protein (typically 10–50 µg/mL of most MAbs) (*see* **Note 7**). Mix and incubate on ice for 30 min.
3. Remove the primary antibody by adding 3 mL of staining buffer to the tubes and centrifuging the cells (~250*g* for 10 min at 4°C).
4. Aspirate media and resuspend cells in 100 µL of staining buffer. Add an equal volume of the secondary antibody (e.g., isotype matched goat antimouse immunoglobulin-fluorescein isothiocyanate [FITC]) at the appropriate concentration. Incubate on ice for 30 min.
5. Wash cells as described in **step 3** and resuspend in 500 µL of staining buffer for data acquisition on the flow cytometer. If not acquired on the FACS machine right away, cells may be fixed using 1% paraformaldehyde. In this case, cells are resuspended in 500 µL of fixative buffer (*see* **Note 8**).
6. Use propidium iodide exclusion on unfixed cells to discriminate between live and dead cells. Add 10 µL of a 20 mg/mL of stock of propidium iodide to 0.5 mL of cell suspension. After a 5-min incubation, cells may be passed through the flow cytometer (*see* **Note 9**).

3.3. Use of NK Cells Expressing Recombinant Vaccinia in Cytotoxicity Assays

In cytotoxicity assays, NK-sensitive target cells are labeled with ^{51}Cr, washed, and mixed with vaccinia virus-infected NK cells at several effector-to-target ratios. Quantitation of the amount of ^{51}Cr released from killed targets provides an indication of the lytic activity of NK cells or the inhibitory potential of NK receptors expressed on effector cells.

1. During the infection of NK cells with vaccinia virus, set up target cells for labeling with ^{51}Cr (*see* **Note 10**). Count target cells and pellet the required number in a 15-mL tube by centrifugation once at ~250*g* for 10 min at room temperature.

Aspirate the medium completely. Add ^{51}Cr to the pellet (e.g., 10 µL/0.1 mCi/ 5×10^5 cells) and vortex gently to resuspend. Place the loosely capped tubes on a rotator in a 37°C/5% CO_2 incubator for 1 h. Resuspend cells once or twice during the labeling to enhance labeling (*see* **Note 11**). Wash the target cells twice in cytotoxicity assay medium and count the cells. Resuspend the target cells at the appropriate concentration (e.g., 5×10^4/mL). Plate 100 µL of target cells per well of a 96-well plate.

2. At the end of the1.5-h infection, wash the NK cells twice in wash media. Count cells and resuspend cells at the appropriate effector to target ratio in wash medium containing 100 U/mL of rIL-2. Typically, NK cell effectors expressing recombinant vaccinia are tested at three different effector:target ratios, e.g., 10:1, 5:1, and 2.5:1.
3. Effector cells are plated in a 100 µL volume and the 96-well plates are centrifuged briefly for 1 min at 200*g* to enable contact between the effectors and targets at the bottom of the 96-well plate. Incubate in a humidified 37°C, 5% CO_2 incubator for 3–4 h.
4. Set up in parallel (a) control wells containing targets plus 100 µL medium (to measure spontaneous release) and (b) target cells plus 100 µL of the lysing agent 2% Triton -X100 (to measure maximum releasable ^{51}Cr).
5. Centrifuge plates (200*g* for 5 min). Harvest the supernatants using the Skatron system and count the ^{51}Cr on a gamma scintillation counter.
6. The % specific lysis is calculated as follows:

$$\% \text{ Specific lysis} = \frac{\text{test } ^{51}\text{Cr release} - \text{control } ^{51}\text{Cr release}}{\text{max } ^{51}\text{Cr release} - \text{control } ^{51}\text{Cr release}} \times 100$$

4. Notes

1. The NK cell lines that we have used successfully for the expression of recombinant vaccinia include NK92 ***(2)*** and NK3.3 ***(3)***. Human NK clones used in this protocol were generated from the peripheral blood lymphocytes (PBLs) of normal donors. Unlike T cell clones, most NK clones are only short term (up to 1 mo) cultures that show diminished proliferative and cytolytic capability with time. Therefore, it is critical to use them early after characterization for clonality and endogenous receptor expression. Short-term NK1.1+ mouse NK cell cultures have also been used successfully.
2. It is necessary to do a separate titration of a given virus stock for each NK line or clone tested because vaccinia-associated toxicity and cell surface expression of the protein of interest varies with each clone. A typical initial titration would span 1 plaque forming unit (PFU)/cell to up to 100 PFU/cell depending on the virus preparation. Even after an optimal dose has been identified, it is useful to include doses twofold higher and lower during functional experiments. For example, if the optimal dose is 10 PFU/cell, test at 5, 10, and 20 PFU/cell.
3. Store the purified virus in small aliquots of 100–200 µL to prevent repeated freeze thawing. Although vaccinia virus is very stable, on occasion, we have

noted a drop in infectivity after repeated freeze thawing of larger volumes of virus preparations. Virus should be kept on ice and stocks should be stored at –70°C.

4a. Standard safety level 2 (BL-2) practices should be employed while working with vaccinia virus. Vaccinia virus is very stable and the primary hazard to laboratory personnel is by aerosolization or ingestion of virus. As recommended by the Centers for Disease Control (CDC), individuals working with vaccinia virus should be vaccinated with vaccines provided by the CDC.

4b. Use only purified vaccinia virus for infection of NK cells. Sturdier cells such as some fibroblast lines (e.g., NIH 3T3 cells) fare well upon infection with crude virus preparations. Such preparations are very toxic to NK cells.

5. Do not place the NK cells on ice at any point during the infection procedure. The presence of rIL-2 during the entire infection is critical for obtaining homogeneous infection of NK cells.

6. Infection times may be increased up to 2 h to obtain matched protein expression levels when working with a panel of expressed receptors where some receptors are expressed less efficiently than others. Longer infection times are not recommended when infected cells are to be used for functional cytotoxicity assays. For biochemical analysis of infected NK cells where cells will be lysed at the end of the infection period, infections may proceed for a longer duration (e.g., 4–5 h).

7a. The amount of primary antibody used needs to be titrated along with the secondary antibody to optimize the staining.

7b. Always use an isotype-matched control antibody to detect background fluorescence caused by nonspecific binding.

7c. In the event of high backgrounds caused by MAb binding to Fc receptors on the cell surface, include a short incubation with purified IgG (Sigma) prior to adding the primary MAb.

8. There is variability in the toxicity of different recombinant vaccinia virus preparations. Toxicity can be monitored during flow cytometry by evaluating the forward scatter vs side scatter plots (FSC vs SSC). Because dead cells exhibit decreased forward scatter, a comparison of infected cells with uninfected control cells that undergo the infection procedure will be informative.

9. Titration of different pfu/cell of a given virus stock should yield a dose at which there is uniform cell surface expression with minimal toxicity. Use FACS analysis to identify the PFU/cell at which comparable expression is achieved (i.e., mean fluorescence intensity of the FACS profiles when staining with a given antibody) of different recombinant vaccinia such that the evaluation of the functional data obtained from the cytotoxicity assays is meaningful.

10. Epstein-Barr virus (EBV)-transformed B cell lines or tumor cells are typically used as target cells because they are sturdy enough to incorporate ^{51}Cr without spontaneous lysis and leakage of ^{51}Cr that would lead to high backgrounds.

11a. Standard radiation safety procedures should be followed when working with ^{51}Cr.

Table 1
Use of Vaccinia Virus-Mediated Gene Transfer to Study NK Cell Function

Protein expressed	Cells infected	References
Wildtype KIR	Human NK clones	***(4–6)***
	B cell line	
Mutant KIR	Mouse bulk NK culture	***(7–9)***
	NK cell line (NK92)	
KIR/gp49 chimeric	Mouse bulk NK culture	***(7)***
KIR/FcγRIIb chimeric	NK cell line (NK92)	***(8)***
SHP-1 (dominant neg.)	NK cell line (NK92)	***(8,10,11)***
	Mouse bulk NK culture	
SHIP (dominant neg.)	NK cell line (NK92)	***(8)***
	Mouse bulk NK culture	
Wild-type lck	CD16+ NK cell lines	***(12)***
Lck (dominant neg.)	CD16+ NK cell lines	***(12)***
Wild-type syk	CD16+ NK cell lines	***(12)***
Syk (dominant neg.)	CD16+ NK cell lines	***(13)***
Fyn	CD16+ NK cell lines	***(12)***
c-src	CD16+ NK cell lines	***(12)***

11b. ^{51}Cr labeling can also be done overnight using half the amount of label in a 2-mL volume in a 24-well plate.

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Use of cDNA Library Expression Cloning to Identify Components of Heterodimeric Receptor Complexes

Chiwen Chang and Lewis L. Lanier

1. Introduction

Expression cloning in mammalian host cells has revolutionized immunology. The current protocol was originally developed by Dr. Brian Seed in 1987 ***(1)***, and it has gone through a number of improvements over the years. The most important feature of this technique has been to exploit the exquisite specificity of antibodies for antigens by developing a method permitting gene cloning in a mammalian cell system.

Generally speaking, there are two types of gene cloning strategies based on expression of the encoded protein, depending on the type of vector used for construction of the cDNA library. cDNA libraries can be generated in either phage (e.g., λ gt11) or bacterial plasmid vectors. There are advantages and disadvantages to each method ***(2)***. This chapter focuses on expression cloning using bacterial plasmid vectors in eukaryotic cells, based on the following considerations: (1) The genes we have studied encode membrane receptors on leukocytes. (2) For most of these genes, the cDNAs are fortunately under 5 kb, which is roughly the size limitation for DNA inserts in a plasmid-based cDNA library. (3) Expression cloning of genes encoding cell surface antigens is greatly facilitated by the use of flow cytometry, which permits rapid and precise isolation of antigen-positive cells at low frequency in a population.

An expression cloning experiment in a mammalian cell transfection system consists of four major procedures, starting with construction of a cDNA library using mRNA from a particular cell type that expresses the gene of interest. The library is transfected into an appropriate recipient cell line so that each cell

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
Edited by: K. S. Campbell and M. Colonna © Humana Press Inc., Totowa, NJ

carries one to several recombinant plasmids. After 2–3 d of culture, antigens encoded by the plasmids are expressed on the plasma membrane. Cells that express an antigen of interest are captured by adherence to plastic dishes coated with an antibody or ligand (i.e., panning) ***(3)*** or are stained by the antibody or other reagent (fusion protein, ligand, etc.) and isolated by cell sorting using flow cytometry ***(4)***. Although not outlined in this chapter, the selection procedure can be modified to isolate cDNA that encode secreted proteins (often identified by transfecting host cells with small pools of cDNA and screening the supernatants for the secreted products) or based on strategies to detect cytoplasmic antigens in the transfected cells. Plasmids are recovered from the selected cells and transformed into bacteria for amplification. These amplified plasmids should contain cDNA of interest that are enriched several fold compared to the original library. Usually three or at most four rounds of selection are performed until a single clone that carries the gene of interest is identified. Therefore, the entire procedure is usually completed in less than one month. The following sections will describe details of each procedure.

Although this technique has been used most frequently to identify cDNA encoding membrane receptors that are expressed on the cell surface as a single protein, it can also be modified to clone receptors that contain multiple subunits. If biochemical analysis indicates that the receptor of interest is a complex containing more than a single protein, of which one or more are encoded by genes already cloned, it is possible to cotransfect the known cDNA together with a cDNA library suspected to contain the cDNA for the unknown protein subunit. Alternatively, this can also be accomplished by stably transfecting the host cells (e.g., COS-7 or 293T) with the known cDNA and then using this stable cell line to clone the unknown partner subunit. For example, we recently cloned the cDNA encoding CD94, a membrane protein expressed on all natural killer (NK) cells and a subset of T cells ***(5)***. Although CD94 can be expressed on the cell surface as a single protein, careful biochemical analysis of NK cells indicated that CD94 is expressed on NK cells not as a monomer, but as a heterodimer together with another protein subunit ***(6)***. Therefore, the CD94 cDNA was stably transfected into the 293T cell line and the $CD94^+$ 293T cell line was transfected with a NK cell cDNA library and used to identify the partner subunit, a protein known as NKG2A ***(7)***. It is noteworthy that the NKG2 proteins are not efficiently expressed on the cell surface without CD94, which serves the function of transporting NKG2 to the plasma membrane ***(7)***. Other examples of leukocyte receptors requiring partner subunits for membrane expression include CD8β, which requires CD8α, and CD16 which requires FcεRI-γ or ζ.

1.1. Construction of a cDNA Library

Preparation of high-quality mRNA is critical for the construction of a good cDNA library (*see* **Note 1**). Several companies provide high-quality and easy-to-use kits for both mRNA purification and cDNA library construction. cDNA prepared from the cells of interest are ligated into an appropriate expression vector. Although many different vectors are available, we have successfully used variants of the CDM8 vector, originally developed by Seed ***(8)***, or the SRα-based vectors ***(9)***. Both vectors use strong viral promoters (from CMV or HLTV-I, respectively) and a polyadenylation sequence for high-level expression of the inserted cDNA. Both vectors also contain an SV40 origin of replication (ori) for amplification of the plasmids in mammalian host cells expressing SV40 T antigen. Once cDNA libraries are generated, we typically check the quality of the library by randomly selecting 50 individual plasmids to ensure that all contain cDNA inserts of reasonable length (e.g., >1 kb–~4 kb). In addition, we often perform a "library Southern blot" to confirm that known cDNA expected to be expressed at moderate to low frequency are in fact represented in the library (*see* **Note 2**).

1.2. Transfection of Library DNA into Recipient Cell Lines

The most commonly used recipient cell lines are COS-7 and 293T. Both are adherent epithelial cell lines (COS-7 is simian and 293T human origin) carrying the SV40 T antigen which permits efficient replication of the transfected plasmids having an SV40 ori. Two to three T-175 flasks of recipient cells are plated at ~60–70% confluence the day before transfection to ensure healthy cells for the procedure. Library DNA can be transfected into the cells by various methods, including electroporation, DEAE-Dextran, or commercially available liposomes (our preferred method because of the ease and efficiency of this method when using adherent cell lines). Either 24 or 48 h after transfection, the adherent cells are detached from the plastic dishes using EDTA/trypsin, replated, and then harvested after an additional 24 h of culture. This is done to ensure that the cells can be removed for immunoselection by a more gentle method (e.g., EDTA alone) on the day of panning or cell sorting, avoiding loss of cell viability or potentially destroying the cell surface antigens by using proteases.

1.3. Selection of Recipient Cells Expressing Target Antigen

After 2–3 d of transfection, cells are ready for selection. Harvested cells are stained by antibody or ligand of choice (methods of immunofluorescent staining and flow cytometry are reviewed in **ref. *4***). Stained cells can be selected by panning or cell sorting using flow cytometry. Flow cytometry allows more

specific selection and less background, but requires access to a flow cytometer. When flow cytometry is used for selection, specific controls are essential to ensure that only antigen-positive transfectants are isolated ***(4)***. A small aliquot of the transfected cells is stained with a fluorochrome-conjugated, isotype-matched control antibody or control fusion protein to define the "sort gate" used for selection of antigen-positive transfectants (*see* **Note 3**).

1.4. Amplification of Enriched cDNA

Selected cells are lysed in Hirt solution and plasmids in the supernatant are recovered by ethanol precipitation ***(8)***. These plasmids represent a subpool of the original library and the cDNA of interest should be enriched several fold. Because plasmids precipitated from a few thousand sorted cells are not enough for the next round of transfection, they need to be transformed into bacteria for amplification. Transformed bacteria should be plated on LB agar plates with appropriate antibiotics (*see* **Note 4**). The bacterial colonies are harvested from the plates, pooled, and the recovered amplified plasmids are used for the second round of transfection. At the end of the third or fourth round of selection, the amplified population of plasmids are digested with appropriate restriction enzymes to release the cDNA inserts and analyzed on ethidium bromide-stained agarose gels. Often a dominant cDNA is visualized, providing a candidate for the desired species. If so, single colonies containing this predominant cDNA are isolated and individually tested by transfection into COS-7 or 293T cells to determine if they encode the desired antigen. Generally this is done by isolating plasmids from 5–10 overnight cultures of single bacterial colonies and transfecting each plasmid into recipient cells. Alternatively, if after the third or fourth round of selection no predominant cDNA insert is visualized in the ethidium bromide stained agar gels (but the COS-7 or 293T cells are clearly antigen positive when transfected with this pool of plasmids), we usually prepare 10 subpools of plasmids (with each pool containing plasmids recovered from 10 individual bacterial colonies). These 10 plasmid pools (i.e., representing a total of 100 different bacterial colonies) are transfected into COS-7 or 293T cells (in T25 tissue culture flasks) to identify the pool containing the plasmid encoding the desired antigen. Subsequently, transfection of individual plasmids within the pool of 10 identifies the single cDNA of interest.

2. Materials

2.1. Construction of a cDNA Library

1. Fastrack mRNA isolation kit (InVitroGen, K1593-02).
2. SuperScript cDNA library kit (Gibco-BRL, 18248-013).
3. Plasmid vector (e.g., pJFE14, pCDM8, pcDNA, etc. obtained from InVitroGen, American Type Culture Colledtion [ATCC], or elsewhere).

4. LB agar plates with appropriate antibiotic (compatible with the plasmid expression vector).
5. DNA purification kit (e.g., Qiagen plasmid Maxi kit, 12163).

2.2. Transfection of Library DNA into Recipient Cells

1. Lipofectamine (Gibco-BRL, 18324-012).
2. COS-7 (ATCC no. CRL1651) or 293T recipient cells.
3. Dulbecco's modified Eagle's medium (DMEM) 1× (Mediatech, cat. no. 10-013-CV) supplemented with 10% fetal calf serum (FCS), 1 m*M* L-glutamine, 100 U/mL of penicillin and 100 µg/mL of streptomycin.
4. DMEM (Mediatech, cat. no. 10-013-CV) supplemented with 20% FSC, 1 m*M* L-glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin.
5. 1 m*M* L-Glutamine (Meditech, 25-005-LI).
6. Trypsin/EDTA solution (Gibco-BRL, 45300-027).
7. Penicillin/streptomycin solution (Gibco-BRL; 59-60277P).
8. FCS (Hyclone, SH30071-03).
9. Opti-MEM (Gibco-BRL, 31985-070).
10. T-25 (70 mL), T-75 (250 mL), and T-175 (750 mL) sterile tissue culture flasks (Falcon, cat. no. 3109, 3110 and 3112, respectively)
11. 14-mL 7 × 100 mL sterile round-bottom polystyrene tubes (Falcon, cat. no. 2057).
12. 50-mL Sterile polypropylene conical centrifuge tubes (Falcon, cat. no. 2070).

2.3. Selection of Cells Expressing Target Antigen

1. Antibody, fusion protein, or ligand.
2. Hirt solution: 0.6% SDS, 10 m*M* EDTA.
3. Flow cytometer or antibody-coated panning plates ***(3)***.
4. Dulbecco's phosphate-buffered saline (DPBS; JRH Biosciences, 5921-79P).
5. 0.5 m*M* EDTA in sterile PBS solution (1 mL of 0.5 *M* EDTA stock into 999 mL of sterile PBS solution).
6. 10 m*M* EDTA in sterile PBS solution supplemented with 2% FCS (20 mL of 0.5 *M* EDTA stock and 20 mL of FCS into 960 mL of sterile PBS solution).
7. 0.5 *M* EDTA stock solution.
8. 5 *M* NaCl stock solution.

2.4. Amplification of Enriched cDNA Libraries

1. Phenol/chloroform/isoamyl alcohol solution (Sigma P-3803).
2. Competent bacteria (e.g., DH10B, Gibco-BRL, 18290-015).
3. Electroporation cuvet (Bio-Rad, 165-2089).
4. Gene Pulser (Bio-Rad, 165-2102).
5. SOC medium (Gibco-BRL, 15544-018).
6. 15-cm LB agar plates with appropriate antibiotic (compatible with the plasmid expression vector).
7. tRNA (Gibco-BRL, cat. no. 16051-039), prepare 1 mg/mL stock solution.
8. 3 *M* NaCl solution.
9. Absolute ethanol.

3. Methods

3.1. Construction of a cDNA Library

Follow instructions in the manufacturer's manual for mRNA isolation and cDNA library construction. Quality control the library as described above.

3.2. Transfection of cDNA Library into Recipient Cells

1. Twenty-four hours before transfection, prepare two T175 plastic tissue culture flasks each containing 1×10^7 COS-7 or 293T cells suspended in DMEM supplemented with 10% FCS, 1 m*M* L-glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin.
2. On the day of transfection, in a sterile 50-mL plastic tube mix 42 µg library DNA with 2.8 mL of Opti-MEM. In a second sterile tube, mix 252 µL of Lipofectamine with 2.8 mL of Opti-MEM. Mix contents of the two tubes together and incubate at room temperature for 20 min. Dilute the 5.6-mL mixture with 22.4 mL of Opti-MEM medium.
3. Rinse the COS-7 or 293T adherent cell monolayer once with Opti-MEM medium. Add 14 mL of the DNA mixture to each flask. Place cells in a tissue culture incubator (5% CO_2) at 37°C for 5–6 h.
4. Add 14 mL of DMEM supplemented with 20% FCS, 1 m*M* L-glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin to each flask, then continue incubation at 37°C (5% CO_2) for 18 h (i.e., overnight).
5. Aspirate transfection mixture and gently rinse adherent cell monolayer once with sterile PBS solution.
6. Add 10 mL of trypsin/EDTA solution to each flask and incubate at 37°C (5% CO_2) for 5 min to detach the cells. Shake the flask vigorously to detach the cells and transfer the 10-mL cell suspension to a sterile 50-mL centrifuge tube containing 20 mL of DMEM supplemented with 10% FCS, 1 m*M* L-glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin. Centrifuge in a table-top centrifuge to pellet the cells (e.g., typically 800*g* for 5 min).
7. Aspirate and discard the supernatant and resuspend the transfected cells in 30 mL of DMEM supplemented with 10% FCS, 1 m*M* L-glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin. Place the cells in a T175 tissue culture flask and culture for an additional 24 or 48 h in a tissue culture incubator (5% CO_2) at 37°C.

3.3. Selection of Cells Expressing Target Antigen

1. After 24 or 48 h of culture, aspirate and discard medium and wash cells once with sterile PBS solution.
2. Add 15 mL of 0.5 m*M* EDTA in sterile PBS solution to each flask and incubate at room temperature for 10 min. Vigorously shake the flask to detach the cells and transfer cell suspension to a sterile 15-mL tube and centrifuge in a table-top centrifuge to pellet the cells (e.g., typically 800*g* for 5 min).
3. Aspirate and discard the supernatant. Resuspend the transfected cells in 5 mL of sterile PBS solution.

4. Count the transfected COS-7 or 293T cells and stain with an appropriate amount of antibody or ligand. Typically, incubate the cells with the antibody or ligand on ice for 15 min, wash twice with sterile PBS solution and resuspend in 2–3 mL of 10 m*M* EDTA–PBS solution containing 2% FCS (*see* **Note 5**). As a negative control for flow cytometry, a small aliquot of the transfected cells should also be stained with a fluorochrome-conjugated, isotype-matched control antibody. An additional negative control consists of untransfected or mock-transfected 293T or COS-7 cells that are stained with the specific fluorochrome-conjugated antibody to establish the degree of nonspecific binding of the specific antibody to the untransfected host cells. These negative control stained cells are needed for calibration of the flow cytometer and for establishing the "sort gates" to identify antigen-positive cells.
5. On the flow cytometer, set a "sorting gate" to collect the 1% brightest positive staining cells. Cells can be sorted directly into microfuge tubes containing 400 μL of Hirt solution. After sorting, add 100 μL of 5 *M* NaCl to the HIRT solution and leave on ice for 5 h to overnight. Note that in the first or second round of sorting, positive cells may not be visualized. However, in this case, we typically isolate 10,000 cells that fall within the "positive sort gate," which permits recovery of sufficient plasmid for subsequent amplification.
6. Centrifuge the plasmid containing solution at 12,000*g* for 5 min at 4°C in a refrigerated table-top microfuge.
7. Recover the plasmid containing supernatant carefully and phenol/chloroform/isoamyl alcohol extract once. Recover the upper layer and measure the volume. If desired, add 20 μL of a 1 μg/mL solution of tRNA as a carrier. Add 1/10 vol of 3 *M* NaCl and 2.5 vol of 100% ethanol to precipitate plasmid DNA. Centrifuge the plasmid containing solution at 12,000*g* for 20 min at 4°C in a refrigerated table-top microfuge.
8. Aspirate and discard the supernatant and wash the DNA pellet once with 70% ethanol. Centrifuge the plasmid containing solution at 12,000*g* for 5 min at 4°C in a refrigerated table-top microfuge. Resuspend the DNA pellet in 10 μL of sterile water.

3.4. Amplification of Enriched Plasmid DNA

1. In a chilled 0.2-cm electroporation cuvette (Bio-Rad), add 1 μL of plasmid DNA and 25 μL of competent bacteria (selecting an *E. coli* strain compatible with the plasmid vector). Mix by tapping the cuvet.
2. Gene-Pulser should be set at 2.5 kV, 200 Ω, 25 μF (time constant 4.0–5.0). Slide cuvet into the chamber and apply voltage.
3. Immediately add 1 mm of SOC medium to the mixture and transfer it to a sterile 7 × 100 mm bacterial culture tube. Incubate with shaking at 37°C for 1 h.
4. Plate bacterial transformants on LB plates containing the appropriate antibiotic (compatible with the plasmid vector) at 37°C overnight (*see* **Note 6**).
5. Harvest colonies from LB plates by adding 10–15 mL of LB medium onto each plate. Mix colonies with the medium using a colony spreader. Recover the

medium from the plates and centrifuge bacteria at 3000*g* for 5 min in a Beckman RC5B centrifuge with an SS-34 rotor (or equivalent)

6. Perform plasmid isolation according to manufacturer's manual (e.g., Qiagen plasmid Maxi kit). Determine concentration of plasmid DNA and repeat a second and third round of the selection procedure. Usually the procedures are performed for three or four rounds. When the percentage of antigen-positive transfected cells reaches 15–50%, it is appropriate to begin analyzing individual plasmids or pools of plasmids (*see above*) (*see* **Note 7**).
7. Retest candidate cDNA by transfection of the single, cloned plasmid into COS-7 or 293T cells to verify that the cDNA encodes the antigen recognized by the antibody or ligand.
8. Sequence the cDNA on both strands.
9. Use the cDNA insert as a probe on a Northern blot of normal cells or tissues know to express the antigen to compare the size of the cloned cDNA with the size of transcripts detected by Northern blot analysis.

4. Notes

1. A cDNA library contains an array of cDNA representing genes that are transcribed in a particular cell type. It is important to choose the cell type that expresses large amounts of the antigen of interest as the source of the mRNA. This can be accomplished by identifying cells expressing the highest level of antigen or can be achieved by flow cytometry, repeatedly selecting natural variants within a cell population that express the highest level of antigen.
2. When we have failed to expression clone a cDNA of interest, the most common failure in this procedure is the use of a poor library.
3. Note that in the first or even second round of selection, it is often impossible to visualize the "positive" cells by flow cytometry owing to their low frequency in the population (often <0.05%). However, if the procedure is successful, a discrete population of strongly antigen-positive transfectants is always observed in the third round of selection.
4. It is important to culture the transformed bacteria on agar plates, rather than in liquid broth, because in liquid culture the fastest growing bacteria (often with plasmids lacking cDNA inserts!) will dominate and may result in loss of the plasmids of interest.
5. The EDTA helps prevent cell clumping and the FCS improves cell viability during the manipulations.
6. If you know roughly the titer of the bacterial culture, you can plate out about 50,000 colonies on 10 large (15 cm diameter) LB plates directly. If not, titer the culture first by serial dilution and then plate out enough volume to give you 5000 colonies/plate.
7. If no discrete antigen-positive cells are observed after three or at most four rounds of selection, the experiment has failed. Start again with a first round using the total library or use another cDNA library. Going beyond four rounds of immunoselection is a waste of time. Moreover, when antigen-positive cells are

detectable, there is no advantage to performing additional rounds of selection with the polyclonal pool of enriched plasmids (rather than testing small pools or single plasmids) because continued amplification in COS-7 or 293T cells can generate recombinant cDNA inserts, resulting in artifacts.

Acknowledgments

DNAX Research Institute is supported by Schering Plough Corporation. We thank Brian Seed for generously providing the vectors and libraries that permitted development of the techniques described in this chapter. We also thank Jim Cupp and the DNAX flow cytometry laboratory for expert assistance in developing this method, JoAnn Katheiser for secretarial assistance, and Stuart Tangye and Sasha Lazetic for careful reading of the manuscript.

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Functional Analysis of Natural Killer Cell Receptors in the RNK-16 Rat Leukemic Cell Line

James C. Ryan, Eréne C. Niemi, and Mary C. Nakamura

1. Introduction

An overriding theme of natural killer (NK) cell receptor systems is functional redundancy. Many receptors with similar or divergent functional features may be expressed by an individual NK cell. As such, it may be difficult to define the exact contribution of a particular receptor toward NK cell function. A dearth of stimulating or blocking antibodies against many NK receptors has hampered functional studies, and the available anti-NK cell antibodies are frequently public for several receptors, leading to ambiguities in functional characterizations of their antigens. To study members of the various NK cell receptor families, we have developed an NK cell transfection system using the NK leukemic cell line RNK-16.

RNK-16, first described by Craig Reynolds, is a spontaneous leukemic cell line from F344 rats with structural and functional features of rat NK cells ***(1)***. Like fresh NK cells, RNK-16 expresses NKR-P1 family members and CD45, and is negative for CD3. Like fresh rat NK cells, RNK-16 mediates antibody-dependent cell-mediated cytotoxicity (ADCC) through CD16 on its surface (unpublished personal observations). The rat NK cell activation marker, gp42, which is induced upon interleukin-2 (IL-2) activation on F344 NK cells, is constitutively expressed on RNK-16 ***(2)***. Despite expression of the gp42 activation marker, RNK-16 exhibits a cytolytic profile similar to that of unstimulated F344 NK cells. Standard tumor targets such as YAC-1, P388D1, YB2/0, and B-16S are readily killed, but P815, a classical target for IL-2-activated (but not resting) NK cells, is resistant to RNK-16 lysis. By Northern blot analysis, RNK-16 cells express mRNA species for the putative rat NK alloreceptors Ly 49, CD94, NKG2-A/C, and for the functionally uncharacterized

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
Edited by: K. S. Campbell and M. Colonna © Humana Press Inc., Totowa, NJ

NKR-P2 gene ***(3–5)***. Despite expression of these putative alloreceptors, RNK-16 fails to kill allogeneic concanavalin-A stimulated blast targets from various rat strains ***(6)***.

The RNK-16 cell line can be manipulated experimentally to examine receptor–ligand interactions, signal transduction, site-directed mutagenesis of receptors, and for the derivation of large numbers of clonal rodent NK-like cells. We have exploited RNK-16 predominantly for the functional study of type II NK lectins. We have examined members of the NKR-P1, Ly 49, and CD94/NKG2 families following transfection into RNK-16 ***(7,8)***. Because RNK-16 is a rat RNK cell line, it is naturally deficient in human and mouse NK cell receptors. Nascent or mutated receptors from nonrat species can be transfected into RNK-16 and selectively ligated with species-specific antibodies during functional studies. The expression of some rat NK receptors is strain or subset dependent. Rat receptors that are not expressed on wild type RNK-16 can be transfected and specifically studied using this system. Transfected rat NK cell receptors can also be selectively blocked or triggered using epitope tags or chimeric extracellular domains. In our hands, transfected RNK-16 cells are fully capable of mediating signals that activate, as well as inhibit, NK cell effector functions.

2. Materials

1. RNK-16 cells: These can be obtained from our laboratory via a written request, accompanied by an express mailing billing number. Send requests to:
E. Niemi
Immunology 111-R
Veterans Administration Medical Center
4150 Clement St
San Francisco, CA 94121
FAX (415) 750-6920
e-mail: jryan@itsa.ucsf.edu
2. Complete RPMI for culture of RNK-16: The medium for culture of RNK-16 is RPMI-1640 (Gibco-BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin–streptomycin (from 100× stock; Gibco), L-glutamine (from 100× stock; Gibco), and 5×10^{-5} M 2-mercaptoethanol (2-ME is essential!). This cell line is not IL-2 dependent. The addition of 25 m*M* HEPES buffer is optional, but has no detrimental effect on the cells. Stock 2-ME is prepared by adding 0.035 mL of 2-ME (Sigma, St. Louis, MO) to 10 mL of RPMI and should be stored at 4°C in the dark. Stock 2-ME is added to the media at a 1:1000 concentration.
3. Medium for selective growth in G418: Complete RPMI supplemented with 10 m*M* HEPES and 1 mg/mL of (active) neomycin (G418) (Boehringer Mannheim). (**Note:** Most preps of G418 powder contain approx 700–750 mg of active drug

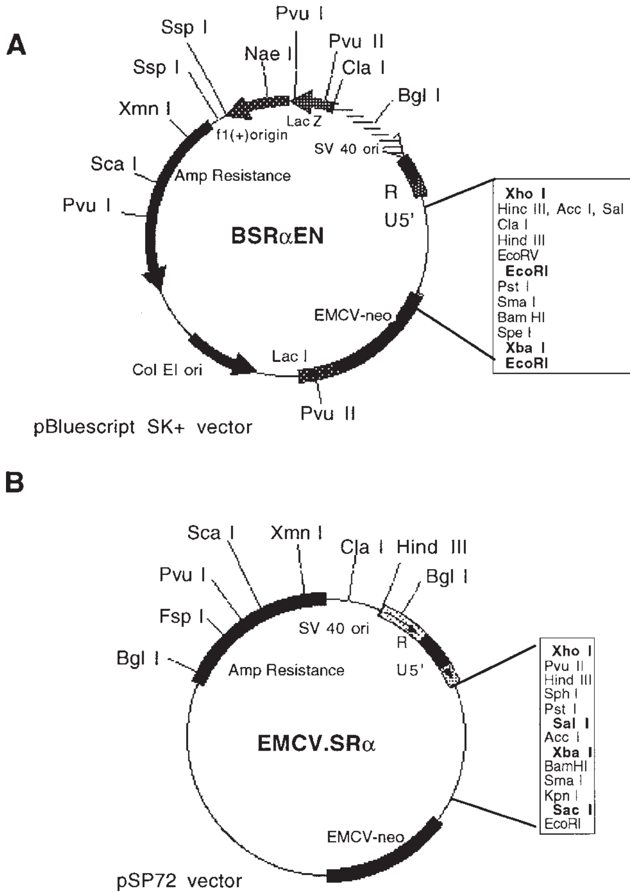

Fig. 1. Vectors for transfection of RNK-16. The BSRαEN and EMCV.SRα vectors were developed by M. Olszowy and A. Shaw at Washington University in St. Louis. Both vectors contain an Srα promotor and the EMCV internal ribosome entry site. The highlighted restriction sites in the polylinker show unique sites useful for cloning. The BSRαEN vector (pBluescript based) can be linearized with *Sac*I, *Cla*I, and *Sca*I digestions. The EMCV.SRα (pSP72 based) can be linearized with *Cla*I and *Sca*I digestions.

per gram. G418 stock solutions of 100 mg/mL in 100 m*M* sterile HEPES, pH 7.0, are added to complete RPMI to produce selection medium).

4. Vectors for transfection: Vectors for RNK-16 transfection appear to require a ribosomal-reentry (IRES) site for reattachment of the polyribosome complex immediately upstream of the neomycin selection gene on the expressed RNA strand. We use the vectors BSRαEN and ECMV (*see* **Fig. 1**), which are from Andrey Shaw (Washington University, St. Louis, MO). They can be obtained from our laboratory, once permission is obtained from Dr. Shaw.

5. Electroporator and cuvets: BTX-600 Elector Cell Manipulator or Bio-Rad Electroporator (with capacitance extender) and the corresponding 2-mm electroporation cuvets (We use BTX cuvets, part no. 01-000295-01).
6. Pristane (Sigma, St. Louis, MO).
7. Qiagen Tip-500 columns and buffers (Qiagen, Chadworth, CA).
8. Plastic culture plates: 96-well flat-bottom plates, 24-well flat-bottom plates, 96-well V-bottom plates (Costar).
9. ^{51}Chromium (Amersham, cat. no. CJS 4V).
10. Classical mouse NK targets: YAC-1, P388D1, B-16S, IC-21, SP2/0, and X-16.C8. All of these lines are available from the American Type Culture Collection ([ATCC], Rockville, MD).
11. Murine tumor targets: P815, C1498, RMA, RMAS, and BW5147.3 (available from ATCC).
12. Rat myeloma target: YB2/0 myeloma (ATCC).
13. Antibodies: rat IgG2b anti-Thy1.2 MAb 30-H12 (Hybridoma available from ATCC, cat. no. TIB 107), mouse IgG2a anti-MHC II monoclonal antibody (MAb) (MAb TU39, Pharmingen, San Diego, CA, cat. no. 32381A).

3. Methods

3.1. Care of Parental RNK-16 Cells In Vitro

RNK-16 cells are grown in a humidified 5% CO_2 atmosphere at 37°C in Complete RPMI. RNK-16 grows as a semiadherent cell line with at least 50% of the cells adherent in a healthy population. Maximum density should be such that a minimum distance of three to four cell diameters separates each cell, and a majority of nonadherent or "floating" cells should be avoided. A confluent culture usually has about $0.3–0.5 \times 10^6$ cells/mL. Cells should be split about every 2–3 d and should not be split more than 1:10. Cells are released gently from flasks using a tissue culture cell scraper. Neither extreme agitation nor trypsin/EDTA solution is used to release cells.

Cells to be used in transfection, a cytotoxicity assay, or any other functional assay should be given fresh media 24 h before the assay.

Cells should be tested initially for cytotoxicity against YAC-1 targets, which are always killed by healthy RNK-16. We have observed that some RNK-16 cell lines can lose cytotoxic function when carried in culture for extended periods (3–6 mo) and new stocks of RNK-16 should be thawed when the cytotoxic function of RNK-16 diminishes. Freezing of RNK-16 can be done in either complete RPMI with 10% dimethyl sulfoxide (DMSO) or 90% FBS and 10% DMSO. We advise freezing multiple aliquots of cells when they are cytolytically active.

3.2. In Vivo Passage of RNK-16 Cells

In the event of loss of cytolytic function by parent cells or transfected cell lines, the cytolytic function of particular RNK-16 cell lines can be enhanced if

needed by an in vivo passage of cells as malignant ascites in pristane-primed F344 rats. If a transfected molecule is immunogenic in F344 rats, expression of that molecule may be downregulated after an in vivo passage. This problem can be avoided by passage in an athymic, nude F344 rat.

1. An F344 rat can be primed with a 0.5-mL intraperitoneal injection of Pristane.
2. After 7 d, the rat will be primed for tumor cell injection. Inject IP 10–20 × 10^6 RNK-16 cells i.p. in 0.3 mL of sterile PBS.
3. After 14–21 d, look for the development of ascites in the rat. Tap ascites under sterile conditions and place ascites in a sterile 50-mL conical tube.
4. Fill tube with complete RPMI and centrifuge at 600*g* for 5 min. Wash cells once with complete RPMI by centrifugation.
5. Resuspend cells at about 2 × 10^5 cells/mL (usually about 25 mL) and place in appropriate sized flask (usually a T-75 flask). Incubate cells as usual and split within 1–2 depending on cell growth.

3.3. Transfection of RNK-16

3.3.1. General Considerations

RNK-16 transfects with extremely low efficiency, and as such, can try one's patience. A decision to work with this line should be thought out carefully before one commits time and resources. We have tested a variety of transfection methods, including retrovirus, vaccinia virus, lipofectamine, and electroporation systems. Although both vaccinia and retroviral expression systems have successfully generated transfectants, RNK-16 cells are adversely affected by viral infection. Retroviral infection makes RNK-16 unhealthy, and can inhibit their ability to kill tumor targets. Vaccinia infection activates RNK-16 nonspecifically, perhaps through cytokine induction. Lipofectamine and other liposome-mediated methods have been uniformly unsuccessful in our hands. Electroporation using a particular expression vector is currently our preferred method of transfection.

Although not technically difficult, transfection of RNK-16 using electroporation can be tricky and unpredictable. For unclear reasons, some constructs are more difficult to express than others, and we have been wholly unsuccessful in transfecting some genes into RNK-16. Fortunately, once a molecule has been successfully expressed, RNK-16-transfectants maintain their phenotypes and can be reliably used for functional studies.

The secrets of successful RNK-16-transfection lie in several key points, which are described in the **Notes** (*see* **Note 1**).

3.3.1. Basic Transfection Protocol

1. Prepare expression plasmid by purification over Qiagen Tip-500 according to the standard manufacturer's protocol. After Tip-purification and isopropanol precipi-

tation of purified plasmid, resuspend unwashed pellet in 12 mL of Qiagen Buffer QBT and repeat purification over a second, equilibrated Tip-500. This "double-tip" plasmid purification may be supplanted by purification over two sequential cesium chloride gradients, but we find double-tip Qiagen to be easier, and possibly better. Single Tip-500 purification rarely works as well for RNK-16 transfection, although it is not necessary for transfection of other cells.

2. Linearize 20 μg (per transfection) of sterile plasmid using a restriction enzyme not found in the cDNA insert or in the polylinker (for BSRαEN, we use *Sca*I or *Sac*I). Inactivate the enzyme by heat if possible. If the enzyme cannot be inactivated, it should be removed by GeneClean or by phenol–chloroform extraction, then chloroform extraction, followed by ethanol precipitation.
3. Remove log-phase (split the previous day) RNK-16 cells by gentle scraping, count, wash and resuspend at 7.5–10 × 10^6 cells/mL in complete RPMI. Add 3–4 × 10^6 cells to electroporation cuvets with 2 mm path length (maximum volume is 400 μL/cuvet). Add 20 μg of linearized, sterile plasmid to each cuvet, place briefly on ice and proceed immediately to electroporation.
4. Electroporate in complete RPMI using either a Bio-Rad or BTX eukaryotic electroporator at predetermined settings (*see* **Note 2**). For the Bio-Rad electroporator, we use 120 mV, 960 μF (requires the capacitance extender); and for the BTX electroporator, we use 120 mV, 850 μF, R5 (129 ohms). Place cuvets on ice 15 min, then add the contents of each cuvet to 5 mL of complete RPMI in a 25 cm^2 flask.
5. At 24 h after electroporation, harvest recovered RNK-16 cells, spin to remove spent medium, and resuspend at 10^5 cells/mL in complete RPMI supplemented with 10 m*M* HEPES and 1 mg/mL of (active) neomycin (G418) (*see* **Subheading 2.**).
6. Plate out transfected cells in selection medium into 96-well flat-bottom plates at 100 mL (10^4 cells)/well. Incubate for 4 d at 37°C in a humidified 5% CO_2 incubator, then add 100–125 μL of G418-selection medium to each well. (*Note:* There is usually some evaporation over 4 d, so it is not necessary to remove old medium prior to this supplementation step. There is room for the extra 100–125 μL.)
7. Incubate 6–12 more days in selection medium. Drug-resistant cells emerge in approx 2–20% of wells, 10–16 d after electroporation. (**Note:** As very few wells grow out from each 96-well plate, drug-resistant cells are usually clonal).
8. Transfer actively growing, drug-resistant cells into either two wells of a new 96-well plate or a shallow (0.5 mL) well of a 24-well plate in selection medium. (RNK-16 are extremely sensitive to overdilution at this point, and a relatively high density should be maintained).
9. Assay for the success of transfection by flow cytometry from actively growing 24-well plates. Note that all drug-resistant clones are unlikely to express the desired receptor, so all clones that grow out should be tested for expression (*see* **Notes 3** and **4**).
10. RNK-16 transfectants should be grown in complete G418 selection medium until several days before functional work, when they are grown in complete RPMI without G418. Transfectants should be grown out of G418 for at least 48 h prior to testing in functional assays as G418 may inhibit cytolytic activity.

11. Assay RNK-16 transfectants for lytic capacity against YAC-1. If cells have any lytic potential, they should lyse YAC-1 at 50:1 effector-to-target ratios. RNK-16 cells that fail to kill YAC-1 may have generalized defects in their lytic machinery, rather than receptor-specific effects. Clones demonstrating good YAC-1 lysis are generally best for further functional work.

3.4. Functional Studies Using RNK-16 Transfectants (see Note 4)

3.4.1. Cytotoxicity Assays Using RNK-16

RNK-16 cells are used in standard cytotoxicity assays as described below. A starting effector:target (E:T) ratio of 50:1 is generally optimal for susceptible tumor cell targets. Standard YAC-1 lysis is 30–50% cytotoxicity at an E:T ratio of 50:1. Assays are performed in 96-well V-bottom plates.

1. Target cell preparation: Wash cells in complete RPMI, pellet cells, pour off supernatant except for the last drop (about 200 µL), agitate pellet, and add ^{51}Cr using 200 µCi/1–10 × 10^6 cells. Incubate 37°C for 1 h. Wash 3× in complete RPMI. Dilute cells to 10^5/mL in complete RPMI. Use 100 µL/well (10^4) in the assay.
2. Effector cell preparation: Wash cells in complete RPMI. Dilute to appropriate concentration. For RNK-16, generally start at effector-to-target ratio 50:1 or a starting dilution of 5 × 10^6 cells/mL (100 µL per well). We usually perform the assay with four effector to target cell ratios, or serial twofold dilutions (i.e., 50:1, 25:1, 12.5:1, and 6.25:1).
3. Cytotoxicity assay: We use V-bottom 96-well plates. Plate out effector cells in triplicate (100 µL/well). Add target cells (100 µL/well). Include for controls (also in triplicate), minimum release (100 µL target cells + 100 µL complete RPMI), and maximum release (100 µL target cells + 100 µL 10% saponin). Incubate plates at 37°C for 4 h in a humidified incubator with 5% $C0_2$. Centrifuge microtiter plates to pellet cells at 600*g* for 3 min. Harvest by aspirating 100 µL/well of supernatant without disturbing pellet. Count supernatant in gamma counter, average each set of triplicates, and calculate cytotoxicity according to the following formula:

$$\frac{\text{mean test release} - \text{mean minimum release}}{\text{mean maximum release} - \text{mean minimum release}} \times 100 = \%\ \text{Cytotoxicity}$$

3.4.2. Cellular Targets of RNK-16 Cytotoxicity

We have examined the lytic capacity of the RNK-16 cell line against a variety of rodent cellular targets, which we have published previously ***(9)***. These targets, which are available at the ATCC (Rockville, MD), include the classical mouse NK targets YAC-1, P388D1, B-16S, IC-21, SP2/0, and X-16.C8. The murine tumor targets P815, C1498, RMAS, and BW5147.3 are not reliably susceptible to killing by RNK-16. Concanavalin A (Con A) blasts from most strains of mice are resistant to killing by RNK-16, although RNK-16 will

sometimes kill Con A blasts derived from BALB/c (but not major histocompatibility complex [MHC]-matched B10.D2) mice. Con A blasts may be useful for the examination of murine activating receptors in RNK-16, since the transfection-dependent acquisition of cytotoxicity would be clearly apparent against these normally resistant targets.

The list of susceptible rat targets is less extensive, and we have worked primarily with the moderately susceptible YB2/0 rat myeloma target cell. No allogeneic rat Con A blasts are susceptible to killing by RNK-16. Similarly, RNK-16 does not spontaneously lyse the human targets C1R, 721.221, RPMI 8866, Daudi, or K562.

When examining the effect of a transfected molecule in RNK-16, one must take great care in the selection of the appropriate target. Transfection of RNK-16 with a target-specific activating receptor will enhance cytotoxicity against that target. To clearly see this effect, one should choose a target that is minimally susceptible to killing, so that a clearcut activating effect can be appreciated. If inhibitory effects are to be studied, a target of intermediate or high susceptibility should be examined (you cannot detect inhibition if there is not good activation to start with). Some targets, such as the mouse targets P388D1 and X-16.C8 as well as the rat target YB2/0, are intermediately susceptible to lysis and may be used to study both activating and inhibitory effects.

3.4.3. Transfection of Tumor Targets

A known ligand for a particular receptor can be transfected into an appropriate tumor target. For the examination of mouse alloreceptors on RNK-16, we have transfected the rat YB2/0 target with multiple alleles of murine MHC I. These targets appropriately restrict NK cytotoxicity by mouse Ly-49 transfectants of RNK-16, as well as by sorted Ly-49$^+$ populations of primary mouse NK cells. Because YB2/0 is a rat cell, it naturally expresses no mouse MHC I molecules, and murine receptor-ligand interactions can be dissected at the molecular level.

Ligands for rat NK receptors can be transfected into the intermediately susceptible mouse target X-16.C8, and ligands for human NK receptors can be transfected into the intermediately susceptible rat target YB2/0 or into the the human target 721.221. Although 721.221 is resistant to natural killing by RNK-16, it is susceptible to ADCC (**Subheading 3.4.6.**).

3.4.4. Natural Killing by RNK-16 Transfectants

Once the effector/target combinations have been defined, ^{51}Cr-release killing assays are performed according to standard methods (*see* **Subheading 3.4.1.**). Both effectors and targets should be grown without G418 for at least 2 d prior to killing assays. For the killing of tumors, starting effector-to-target

ratios of 50:1 are used. In each assay, we include the target YAC-1 to ensure that the lytic machinery of all effectors is intact. Because levels of killing can be greatly variable, the receptor specificity of an effect is always examined in the presence and absence of blocking anti-receptor antibodies. For maximal blockade, we use 10–20 μg/10^6 effectors of intact anti-receptor MAb and 20–50 μg/10^6 effectors of anti-receptor $F(ab')_2$.

3.4.5. Redirected Lysis and Inhibition of Fc-Receptor Positive Targets

It is possible to stimulate transfected receptors directly using anti-receptor antibodies bound to the Fc-receptors of particular targets. Agonistic antibodies bound to target Fc-receptors on targets can mimic ligand and modulate cytotoxicity of RNK-16 ***(8)***. If the receptor activates NK cells, receptor-specific antibody augments lysis of Fc-receptor bearing targets (redirected lysis). Conversely, if a receptor inhibits killing, receptor specific antibody may diminish lysis of a susceptible Fc-receptor positive target (redirected inhibition) (*see* **Note 5**). Fc-receptor bearing targets that are resistant to RNK-16 killing such as P815 and Daudi are useful for the examination of redirected lysis, and Fc-receptor bearing targets that are susceptible to lysis (such as P388D1 and X-16.C8) are useful for the detection of either redirected inhibition or redirected lysis. For the stimulation of either redirected lysis or of redirected inhibition, we add MAb at a density of 1 μg/10^6 effectors to the killing assays. Isotype-matched control antibodies are added to control wells in equal concentrations.

3.4.6. ADCC by RNK-16

RNK-16 cells are capable of killing of targets via the low avidity Fc-receptor CD16. We have found that targets coated with antibodies of the mouse IgG2a isotype are nearly uniformly susceptible to ADCC by RNK-16. This effector function can be manipulated in the examination of receptor-specific effects against available panels of RNK-16-resistant targets. We have found that the resistant targets RMA and RMA-S can be lysed via ADCC using a rat IgG2b anti-Thy1.2 MAb (MAb 30-H12). Likewise, we have found that RNK-16 efficiently lyses the resistant target 721.221 when a mouse IgG2a anti-MHC II MAb is added (MAb TU39). For the induction of ADCC, antibodies are added to the killing assays at a concentration of 1 μg/10^6 effector cells.

3.4.7. Signaling Studies

On proper stimulation, a variety of NK cell effector functions can be examined in RNK-16. RNK-16 cells can be stimulated with either target cells or specific antibodies. We have found that RNK-16 cells are faithful to primary rat NK cells in their ability to transduce intracellular signals for receptor-induced tyrosine phosphorylation, phosphoinositide turnover, and calcium

mobilization. RNK-16 can also secrete interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) upon activaton which can be measured by enzyme-linked immunosorbent assay (ELISA) assay (BioSource International, Camarillo, CA). In contrast to primary NK cells, RNK-16 contain very little esterase, and measurements of receptor-stimulated BLT-esterase release have uniformly been unsuccessful. By and large, we have used RNK-16 predominantly to examine cytotoxicity against cellular targets. As stated previously, we have found G418 to be a nonspecific inhibitor of RNK-16 activation, and cells are grown in complete RPMI without G418 for at least 2 d prior to any functional characterizations.

4. Notes

1. Rules for successful RNK transfection. Successful transfection requires adherence to the following guidelines.
   a. Rule I. Take no "short cuts." The protocol described in **Subheading 3.** is the product of 6 yr of painstaking experience and frequent modifications. We have found that deviations from the protocol nearly always result in failure.
   b. Rule II. Your tissue culture facility must be free of mycoplasma. Mycoplasma infection in tissue culture facilities rapidly spreads to RNK-16 stocks. Mycoplasma-infected RNK-16 will never transfect. Note that RNK-16 appears quite healthy in the presence of mycoplasma infections and it is impossible to distinguish infected cells by observation. Mycoplasma-infected RNK-16 will kill tumors such as YAC-1, so do not assume that cells are free of mycoplasma if they kill tumors. To prevent mycoplasma infection in our laboratory, we test all growing cell lines on a monthly or bimonthly basis using one of several commercially available mycoplasma ELISA test kits. Newly acquired cell lines are always grown in a quarantined incubator until their mycoplasma status can be determined. If a mycoplasma infection is detected, all infected cell lines are discarded (if they can be replaced). If unique cell lines cannot be replaced, they are treated under quarantine with Boehringer-Mannheim BM-cyclin (cat. no. 799 050). Mycoplasma can be removed from infected RNK-16 cells by an in vitro passage in the peritoneum of an F344 rat (*see* **Subheading 3.2.**). RNK-16 cells may not survive treatment with the mycoplasma removal agent so it is best to try several methods of disinfection at the same time. The entire tissue culture facility should also be disinfected prior to thawing of cell stocks.
   c. Rule III. The expression vector is critical. We have tested a variety of expression vectors in RNK-16. Vectors for RNK-16 transfection appear to require a ribosomal-reentry site (IRES). This site allows for the reattachment of the polyribosome complex upstream of the neomycin selection gene on the transcribed RNA strand. We use the vectors BSRαEN and EMCV (**Fig. 1**), which were a gift from Andrey Shaw (Washington University, St. Louis, MO).
2. Electroporation settings. The electroporation settings described in the text were designed and tested to kill 50% of electroporated RNK-16 cells after 24 h. Most

electroporators will give similar results, but each machine is different, and a sample electrokilling curve at these settings should be performed before each electroporation. Set up five to six identical cuvets with 3–4 × 10^6 RNK-16 cells but without plasmid. Keep μF, resistance at indicated values, and vary voltage bracketing around 120 mV. (Usually a range of 100–150 mV is adequate). Compare cell numbers to basal (sham-electroporated) cells after 24 h of growth in complete RPMI in 25 cm^2 flasks. Optimal voltage for electroporation reduces live cells to 50% of the number present in the sham-electroporated sample.

3. Cotransfection of RNK-16 with multiple plasmids. It is possible to cotransfect RNK-16 with more than one cDNA. Although we have had limited experience, we have been able to cotransfect RNK-16 with genes encoding human CD94 with its heterodimeric partners NKG2-A or NKG2-C (unpublished data, J. Ryan and L. Lanier). Linearized IRES expression constructs encoding each cDNA are combined in varying ratios prior to electroporation. We have found that each (not just one) cDNA must be in an IRES vector.
4. Testing of RNK-16 transfectants for expression and function. We have found that not all drug-resistant clones will express the desired receptor, not all transfected cells with successful expression maintain cytolytic activity. It is extremely important to test multiple transfectants, from multiple transfections to both obtain the desired transfectants and to be sure of the functional phenotype. Cytolytic activity may be diminished by prolonged culture or a random integration site of the transfected plasmid and cannot be attributed to the transfected gene without multiple clones demonstrating the same phenotype.

   Once successful, cytolytically active transfectants are identified, multiple vials of cells should be frozen, as like the wild-type RNK-16 cell line, transfectants will lose cytolytic activity in long-term culture.
5. Use of antibodies to NK cell receptors in cytotoxicity assays. “Redirected lysis” or “redirected inhibition” may require as little as 1 μg/10^6 effector cells or as much as 20 μg/10^6 effector cells. It is important to remember that the isotype of the antibody may be critical to its ability to bind either to the Fc-receptor bearing target cell or to the Fc-receptors on the RNK-16 cells. There are no antibodies available to block CD16 on rat NK cells. Therefore it is also important to use isotype-matched control antibodies in cytotoxicity assays. Many antibodies against NK cell receptors are stimulatory, but other antibodies do not stimulate the receptor; rather their addition can block receptor function. Interpretation of these experiments can be confusing if it is not known whether an antibody is blocking or stimulatory. For unclear reasons, some inhibitory receptors (such as mouse Ly-49A) fail to mediate redirected inhibition, where other inhibitory receptors (such as human NKG2-A) mediate brisk redirected inhibition.

4.1. Troubleshooting

1. RNK-16 cells are dying in culture.
   a. RNK-16 cells should not be cultured either too densely or too sparsely. Maintain a confluent culture as described in **Subheading 3.** and feed at least every 2 d. Decrease flask size if insufficient cells are present.

   b. 2-ME is essential in the media. RNK-16 cells will die in the absence of 2-ME.
   c. If cells are poorly adherent to plastic flasks, we would recommend changing the brand of plastic tissue culture flasks.
2. RNK-16 cells are not cytolytically active against YAC-1 targets.
   a. Cells may have been maintained in culture for too long a period. Thaw a new aliquot of cells or passage cells in vivo.
   b. Cells may be mycoplasma contaminated; they should be tested.
   c. Cells may be too dense on the day of the assay; split cells into actively growing culture and retest on another day.
   d. Cells were not fed and actively growing on the day of the assay. Replate cells with fresh media and retest on another day.
3. No drug resistant clones are obtained by transfection.
   a. Cells may be mycoplasma contaminated.
   b. Cells may have been transfected when they were not actively growing. Poorer than usual transfection efficiency occurs if cells are too densely growing when transfected.
   c. An alternate vector was used. Try vectors suggested in methods.
   d. Inadequate amount of vector was used in transfection.
4. No transfectants express receptor of interest.
   a. Cells may be mycoplasma contaminated.
   b. An alternate vector was used.
   c. Not enough clones have been screened; try transfection again.
   d. There may be no clear explanation.
5. No transfectants function in cytolytic assays.
   a. Cells may be mycoplasma contaminated.
   b. Wild-type RNK-16 cells that were transfected were losing cytolytic activity; try transfection with freshly thawed cells.
   c. Not enough clones have been screened; try transfection again.
   d. There may be no clear explanation.
   e. Make sure cells grown out of G418 for at least 2 d prior to assay.
   f. See causes in **no. 2**.

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Use of Mutant Jurkat T Cell Lines to Define Human Natural Killer Cell Surface Receptor Signaling Pathways

Bryce A. Binstadt, Brandi L. Williams, Robert T. Abraham, and Paul J. Leibson

1. Introduction

Natural killer (NK) cells and T lymphocytes are derived from a common progenitor cell ***(1)***. This common origin is reflected in mature cells of both lineages: T and NK cells express cell surface receptors utilizing shared signaling chains that couple to similar signal transduction pathways. Genetic approaches to define NK cell receptor signaling pathways are hampered by the limited lifespan of cultured NK cells. We have therefore employed the immortalized human T cell line Jurkat and somatic mutants derived from this cell line as tools for genetic studies of NK cell-receptor signaling pathways.

NK cells kill via either antibody-dependent cell-mediated cytotoxicity (ADCC) or natural killing. For a comprehensive review of signal transduction during ADCC and natural killing, *see* **ref. *2*.** For the purposes of this chapter, only signaling initiated during ADCC shall be discussed. ADCC is initiated by the engagement of FcγRIIIA (CD16) by the Fc portion of antibodies coated on target cells. FcγRIIIA comprises a ligand-binding chain coupled to homo- or heterodimers of zeta and gamma signaling chains. The zeta chain is identical to the T cell antigen receptor complex CD3 a zeta chain, and the gamma chain is identical to the FcεRI gamma chain expressed by mast cells and basophils. Both zeta and gamma contain immunoreceptor tyrosine-based activation motifs (ITAMs) that are critical for signal transduction. Engagement of multisubunit immune recognition receptors such as the T cell antigen receptor, FcεRI, as well as FcγRIIIA results in phosphorylation of the zeta and gamma ITAMs by a Src-family protein tyrosine kinase (PTK) and subsequent recruitment of a

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
Edited by: K. S. Campbell and M. Colonna © Humana Press Inc., Totowa, NJ

src-homology 2 (SH2) domain-containing Syk-family PTK (reviewed in **ref. *2***). Therefore, analysis of early signaling events in one activation receptor system may be extrapolated, on a provisional basis, to other receptor systems.

NK cell activation is inhibited by co-engagement of major histocompatibility complex (MHC) class I-recognizing killer cell immunoglobulin-like receptors (KIR) ***(3)***. KIR are also expressed by a subset of T lymphocytes. The cytoplasmic region of KIR contains an immunoreceptor tyrosine-based inhibitory motif (ITIM), that, when phosphorylated, recruits the SH2 domain-containing protein tyrosine phosphatase SHP-1 ***(4,5)***. SHP-1, in turn, inactivates the PTK-dependent activation signaling pathways of both ADCC and natural killing ***(5)***. Given the identical structure and function of KIR in NK and T cells, we reasoned that the mechanism of KIR signal transduction in both cell types would be shared.

We have taken advantage of the human leukemic T cell line Jurkat to dissect PTK-dependent NK cell receptor signal transduction pathways. NK cells and the Jurkat E6 subclone both express the Src-family PTKs, Lck and Fyn, as well as the Syk-family PTK, ZAP-70. An important difference between the two cell types is that NK cells express Syk, whereas Jurkat E6 does not ***(6)***. Somatic mutants sublines of Jurkat lacking expression of specific PTKs have proven invaluable for the study of NK cell signaling pathways. The PTK expression pattern of these cell lines is summarized in **Table 1**. We have used the Lck-deficient Jurkat derivative, JCaM1 ***(7)***, to show that Lck is required for the tyrosine phosphorylation of the KIR ITIM ***(5)***. The Jurkat derivative, P116, lacks expression of both Syk and ZAP-70 ***(8)***. We have used P116 to demonstrate that tyrosine phosphorylation of KIR requires neither Syk nor ZAP-70 ***(5)***. In addition, significant differences between how Syk and ZAP-70 interact with the zeta signaling chain as well as downstream signal-transducing elements common to T and NK cells have been characterized using the P116 cell line ***(8)***. The reader should refer to these reports ***(5,8)*** for more details regarding how these cell lines have been utilized to analyze specific signaling pathways.

With the definition of more NK cell surface receptors and subunits, a model system in which mechanisms of signal transduction can be dissected genetically will prove particularly useful. We therefore present in this chapter our methods for analysis of signal transduction by NK cell receptors using somatic mutants of the Jurkat T cell leukemia line (*see* **Note 1**).

2. Materials

1. Cells: Cloning and culturing of human NK cells are described elsewhere in this volume (Chapter 1) and by us ***(9)***. The human leukemic T cell Jurkat E6 subclone and the Lck-deficient Jurkat E6 derivative, JCaM1, are available from American Type Culture Collection ([ATCC], Rockville, MD). The isolation and characterization of the ZAP-70-deficient Jurkat E6 derivative, P116, have been described

Table 1
Expression of Selected Protein Tyrosine Kinases by NK Cells and Jurkat Cell Lines

	Protein tyrosine kinase			
Cell type	Lck	Fyn	Syk	ZAP-70
NK cell	+	+	+	+
Jurkat E6	+	+	–	+
JCaM1	–	+	–	+
P116	+	+	–	–

(8). Jurkat lines are grown in RPMI supplemented with 10% bovine calf serum and 0.1% gentamicin. Jurkat cell lines should be maintained at densities less than 5×10^5/mL.

2. Vaccinia viruses: Recombinant vaccinia viruses used to express KIR and other signaling molecules in lymphocytes are described elsewhere in this volume (Chapter 22) and by us ***(5)***. Control WR strain vaccinia virus is available from ATCC.
3. Medium for cell washing and stimulation: Cells may be washed and stimulated either in RPMI supplemented with 0.5 % bovine serum albumin (BSA) or in Hanks balanced salt solution (HBSS) supplemented with 10 m*M* HEPES, pH 7.4.
4. Antibodies for cell stimulation: The anti-CD3-producing hybridoma is available from ATCC. The anti-KIR MAb EB6 and GL183 are available from Immunotech (Westbrook, ME). The anti-KIR monoclonal anitbody (MAb) DX9 and the anti-CD16 MAb 3G8 are available from Pharmingen (San Diego, CA). The crosslinking reagent is goat anti-mouse IgG F(ab')$_2$ fragments (Organon Teknika, West Chester, PA).
5. Lysis buffers and sodium dodecyl sulfate (SDS) sample buffer: Incomplete lysis buffer for whole cell extracts contains 10 m*M* Tris-HCl, pH 7.4, 50 m*M* NaCl, 5 m*M* EDTA, 50 m*M* NaF, 30 m*M* $Na_4P_2O_7$, and 1% Triton X-100. Incomplete lysis buffer for immunoprecipitation experiments contains 20 m*M* Tris-HCl, 40 m*M* NaCl, 5 m*M* EDTA, 50 m*M* NaF, 30 m*M* $Na_4P_2O_7$, and 1% Triton X-100. Both buffers are supplemented on the day of the experiment with phosphatase and protease inhibitors: 1 m*M* Na_3VO_4, 1 m*M* phenyl methylsulfonyl fluoride (PMSF), 5 µg/mL aprotinin, and 10 µg/mL leupeptin. 4× SDS sample buffer contains 0.5 *M* Tris-HCl (pH 6.8), 20% glycerol, 10% SDS, 10% β-mercaptoethanol, and 0.5% bromphenol blue.
6. Reagents for immunoprecipitation and immunoblot: Rabbit antisera to zeta, the KIR cytoplasmic region, Lck, and ZAP-70, as well as other signaling elements have been described ***(5)***. The anti-phosphotyrosine MAb 4G10 is from Upstate Biotechnology (Lake Placid, NY) and is available in both unbound and agarose-bound forms. Rabbit antisera are bound to Protein A–Sepharose beads (Sigma Chemical, St. Louis, MO) for immunoprecipitation analysis. Immunoblots are performed with either sheep anti-mouse IgG or Protein A coupled to horseradish

peroxidase, followed by the ECL detection system (all available from Amersham, Buckinghamshire, England).

7. Electroporation is performed with a BTX model T 820 square-wave electroporator (San Diego, CA).

3. Methods

3.1. Transient Infection of Jurkat and NK Cells with Vaccinia Virus

The use of recombinant vaccinia viruses to express signaling proteins has several advantages. First, the wide tropism of vaccinia allows infection of cell types that are refractory to standard genetic manipulation. Second, several different gene products may be efficiently coexpressed in a short amount of time. Third, nearly the entire population of cells to be studied is infected. The use of vaccinia virus to infect NK cells is described in detail elsewhere in this volume (Chapter 22). The methods that we use for infection of Jurkat cells are identical to those we use for NK cells, except that Jurkat cells generally may be infected for shorter amounts of time.

1. Remove cultured cells from their culture medium by centrifugation and resuspend in serum-free RPMI at a density of 2×10^6 cells/mL.
2. Add vaccinia virus at a multiplicity of infection of 20. Titration of this number for appropriate expression is often necessary (*see* **Notes 2** and **3**).
3. After 1 h of incubation at 37°C, add an equal volume of RPMI supplemented with 20% BCS to bring the final serum concentration to 10%.
4. Return the cells to the incubator for the remainder of the infection time (generally 2–4 h total infection time).

Always take appropriate safety precautions when working with vaccinia virus. Confirm expression of the recombinant vaccinia virus-encoded protein by flow cytometry for surface proteins or by immunoblot analysis of whole cell extracts for cytoplasmic proteins (*see* **Notes 2** and **3**).

3.2. Transient and Stable Transfection of Jurkat Cells with Plasmid DNA

1. Centrifuge cells out of their growth medium and resuspend at 4×10^7 cells/mL in fresh growth medium.
2. Place aliquots containing 1×10^7 cells in 0.4-cm cuvets and add 30 μg of plasmid DNA in a final volume of 300 μL.
3. Electroporate the cells at 300 V (pulse duration, 10 ms).

For transient transfections, co-transfect an appropriate reporter plasmid to determine transfection efficiency. For stable expression, allow cells to rest 24 h following electroporation and then expose to 2 mg/mL G418. Clone the drug-resistant bulk population by standard limiting dilution techniques. Screen subclones for expression of the transfected gene product by immunoblot analysis of whole cell extracts (*see* **Note 2**).

3.3. Cell Stimulation by Surface Receptor Crosslinking

The following protocol is used to stimulate both uninfected cells, vaccinia infected cells, and transfected cells (*see* **Note 4**).

1. Centrifuge cells from their growth medium and wash once in resuspension buffer.
2. Resuspend cells in resuspension buffer (100 µL/sample). For whole cell extracts, use ~1×10^6 cells/sample; for immunoprecipitation analysis, use ~10×10^6 cells/sample. Aliquot cells into 1.5-mL microfuge tubes. Place tubes on ice.
3. Add primary antibody in a small volume (< 10 µL) to a final concentration of 10 µg/mL and vortex-mix gently.
4. Incubate on ice for 3 min.
5. Pellet the cells by centrifugation 20 s at 2900*g* in a microcentrifuge.
6. Aspirate the medium, retaining cell pellet.
7. Gently resuspend cells in 100 µL of crosslinking goat anti-mouse IgG $F(ab')_2$ fragments (1:5 dilution of stock) by pipetting up and down, and place tube immediately in 37°C water bath for desired time of stimulation.
8. Stop the stimulation with either 1 mL of ice-cold detergent-free lysis buffer (for whole-cell extracts) or 1 mL of ice-cold immunoprecipitation lysis buffer.

3.4. Preparation of Whole Cell Extracts

1. Centrifuge cells out of the detergent-free stop solution for 30 s at 15,800*g*.
2. Aspirate stop solution and resuspend the cell pellet in 50 µL of whole-cell extract lysis buffer.
3. Incubate on ice for 10 min.
4. Centrifuge at 4°C for 5 min at 15,800*g* to remove detergent-insoluble fraction.
5. Add supernatant to 25 µL of SDS sample buffer.
6. Samples may be stored at –70°C or boiled 5 min and run on SDS-polyacrylamide gel electrophoresis (PAGE).

3.5. Immunoprecipitation

1. Add 1–10 µL of rabbit antiserum to 1 mL of immunoprecipitation lysis buffer (*see* **Note 5**) containing 20–30 µL of Protein A–Sepharose beads per sample in a 1.5-mL microcentrifuge tube.
2. Rotate beads and antiserum for at least 1 h at 4°C.
3. Pellet beads for 30 s, 15,800*g*, aspirate supernatant, wash in 1 mL of ice-cold lysis buffer (to remove unbound immunoglobulin), repellet the beads, aspirate supernatant, and keep beads on ice.
4. Stimulated cells to which lysis buffer has been added (**Subheading 3.3.**) should be lysed for 10 min on ice and then centrifuged at 4°C for 5 min at 15,800*g* to remove the detergent-insoluble fraction.
5. Transfer supernatant to washed, antiserum-coated beads and rotate for at least 1 h at 4°C.
6. Wash beads 3× with 1 mL of lysis buffer/wash.
7. Elute bound proteins by adding 40 µL of SDS sample buffer and boiling 5 min.
8. Supernatant may be stored at –70°C or boiled 5 min and run on SDS-PAGE.

3.6. SDS-PAGE and Western Immunoblotting

Separation of proteins by SDS-PAGE and analysis by Western immunoblotting are performed according to standard protocols ***(10)***.

4. Notes

1. The interpretation of results using Jurkat T cell lines to study NK cell signaling pathways must be tempered by the knowledge that subtle differences in signaling may exist between the two cell types. Although genetic manipulation of Jurkat provides an easy system to define NK cell signaling pathways initially, attempts should be made to verify that the pathway indeed exists in NK cells. For instance, we used the Lck-deficient Jurkat subclone JCaM1 to show that Lck is necessary for KIR tyrosine phorphorylation in T cells ***(5)***. The lack of Lck-deficient human NK cells prevents us from drawing the same conclusion in NK cells. However, by overexpressing Lck in NK cells using the vaccinia system, we were able to demonstrate that Lck dramatically augments the degree of KIR tyrosine phosphorylation in NK cells ***(5)***, suggesting that Lck is the PTK responsible for KIR tyrosine phosphorylation in both T and NK cells.
2. The level of protein expression achieved by any form of transfection impacts significantly the degree to which that protein either augments or interferes with signal transduction cascades. Therefore, levels of protein expression achieved by either vaccinia virus infection or transfection of plasmid should be analyzed relative to endogenous proteins. For example, reexpression of Lck in JCaM1 should approximate the level of Lck expression in the parental Jurkat cell line. Alternatively, if one desires to compare the efficiency to which either ZAP-70 or Syk restores a given signal in the P116 cell line, the use of epitope-tagged versions of ZAP-70 and Syk provides a convenient means of monitoring the level of protein expression. For the vaccinia virus system, both the time and multiplicity of infection are variables that may be altered to achieve the desired level of expression. Of course, appropriate controls should always be employed, including nonrecombinant vaccinia virus or empty plasmid.
3. If vaccinia virus-driven protein expression is low:
   a. Verify that the viral titer is correct.
   b. Increase the time of infection or the multiplicity of infection, realizing, however, that this may increase the degree of virus-induced cell death.
   c. Ensure that the first hour of infection is in serum-free medium.
   d. Verify that the "recombinant" virus stock does not contain nonrecombinant virus. Recombinant vaccinia viruses are generated using a standard β-galactosidase screen, and this procedure may be repeated on the viral stock. If significant numbers of clear (i.e., nonrecombinant) viral plaques are present, the viral stock should be discarded, and a new recombinant virus clone should be isolated.
4. Experiments involving stimulation of cells by cell surface receptor crosslinking should contain appropriate controls. While some researchers use non-specific IgG_1 myeloma protein as a control for a receptor-specific IgG_1 MAb, we instead

use isotype-matched control antibodies that bind *other* cell surface receptors that are *not* likely to be involved in the signaling pathway under investigation. For instance, as a control for KIR ligation in NK cells, we crosslink the NK cell surface receptor CD56. Because most cell types express MHC class I, a pan-specific anti-class I MAb often can serve as this control. Such controls ensure that any signal observed is not simply due to binding of antibody to the surface of the cell, but rather is due to specific engagement of the receptor in question. These controls are particularly important when studying cells that express FcγR (e.g., CD16 on NK cells) that may be engaged by the Fc portion of antibody. Similarly, to decrease the degree of FcγR engagement, $F(ab')_2$ fragments should be used for as the secondary crosslinking reagent rather than whole antibody.

5. When attempting to demonstrate coimmunoprecipitation of proteins, the choice of detergent used for cell lysis is an important consideration. Weak protein–protein interactions may be preserved in less stringent detergents than Triton X-100, such as Brij-96 ***(10)***. Regardless of the detergent used, control immunoprecipitations (e.g., using normal rabbit serum) should be performed to ensure that any interaction observed is not due simply to nonspecific binding of the protein in question to immunoglobulin or to the Protein A-Sepharose matrix but rather reflects specific protein–protein interaction.

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Measurement of Calcium Mobilization Responses in Killer Cell/Target Conjugates by FACS Analysis

Salvatore Valitutti and Mark Dessing

1. Introduction

The interaction between killer cells and target cells can be dissected into three steps. First, the two cells form random conjugates mediated by nonspecific interactions between adhesion molecules ***(1)***. Second, specific recognition results in the activation of signal transduction pathways coupled to activating receptors and accessory molecules ***(2)***. This leads to the third step consisting of perforin and/or fas-ligand mediated delivery of the lethal hit ***(3)***. Lethal hit delivery requires a short time; as a consequence killer cells can recycle and kill multiple targets ***(4)***.

Intracellular calcium concentration ($[Ca^{2+}]_i$) increase is an early activation event detectable within seconds after formation of specific conjugates ***(5)***. Owing to the rapidity of Ca^{2+} mobilization, measurement of $[Ca^{2+}]_i$ is a suitable tool to investigate the level of TCR-mediated signal transduction in killer/target cell conjugates.

Measurement of $[Ca^{2+}]_i$ by flow cytometry has several advantages over other methods: (1) It is a fast method. (2) It reveals possible heterogeneity in the responding cell population. (3) It allows the evaluation of several different parameters in parallel with $[Ca^{2+}]_i$ (i.e., the number and stability of specific conjugates, the expression of surface markers on responding and/or on target cells). (4) It allows an easy estimation of the time course of $[Ca^{2+}]_i$ increase.

The Ca^{2+} chelating dye Indo-1 is most frequently employed for $[Ca^{2+}]_i$ measurement by flow cytometry ***(6)***. Cells loaded with Indo-1 are excited in a flow cytometer by the ultraviolet (UV) line (365 nm) of an argon laser. Emission is measured at two wavelengths: 405 nm and 525 nm. Upon $[Ca^{2+}]_i$ increase, the

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
Edited by: K. S. Campbell and M. Colonna © Humana Press Inc., Totowa, NJ

Indo-1 chelates the free Ca^{2+} and changes its emission spectrum: the intensity of the emission at 405 nm increases and at the same time the intensity of emission at 525 nm decreases. The ratioing between the two emission wavelengths (405/525 ratio) provides a measure of the $[Ca^{2+}]_i$ increase in stimulated cells. Because most analyzers on the market employ a low-power argon laser that does not have the UV lines, techniques have been described using the dye Fluo-3 in combination with Fura red (both from Molecular Probes, Eugene OR) that do not require UV excitation ***(7)***. Assuming that these dyes load the cells in equal amounts, ratiometric measurements are possible using the 488 nm line of an argon laser. However, the dye of choice is still Indo-1 because it usually gives a tighter baseline, resulting in more sensitive measurements.

This chapter describes a method to measure $[Ca^{2+}]_i$ in cytotoxic T cells (CTLs)/target cell conjugates that has been applied to investigate Ca^{2+} mobilization in natural killer (NK) cells interacting with stimulatory targets ***(8)***. This method is designed to be performed on a FacsVantage flow cytometer (Becton Dickinson, Mountain View, CA). A technical setup to using a Coulter Elite flow cytometer (Coulter Electronics, Hialeah, FL) for these kinds of experiments has been described previously ***(9)***.

2. Materials

1. Flow cytometer: We use a FacsVantage flow cytometer (Becton Dickinson, Mountain View, CA) fitted with a multiline argon laser (Coherent Innova Enterprise Ion Laser 621, Coherent, Palo Alto, CA). For details of setup, refer to equipment manuals.
2. Software packages as desired (Lysis II, Cellquest, Becton Dickinson)
3. Standard beads: fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), Hoechst, or Indo-1 labeled (Flow Cytometry Standard Corporation, Leiden, The Netherlands), Calibrite beads (Becton Dickinson)
4. Indo-1 AM (Sigma Chemical, St Louis, MO)
5. CTL clones that have been restimulated with feeder cells at least 10–15 d before. Target cells of choice.
6. The tissue culture medium that are commonly used for chromium release assays can be used for $[Ca^{2+}]_i$ measurement. To correctly compare the $[Ca^{2+}]_i$ levels in different samples, it is important to keep a stable pH throughout the assay. Therefore it is convenient to use HEPES buffered media; RPMI 5% FCS containing 10 m*M* HEPES is a suitable medium for $[Ca^{2+}]_i$ measurement in human T cell clones ***(10)***.
7. 5-mL Tubes for FACS analysis (Falcon, Becton Dickinson Labware, Lincoln Park, NJ).

3. Methods

3.1. Optical Setup

The most frequently employed method for optical setup of a flow cytometer, which allows the simultaneous measurement of surface markers and of $[Ca^{2+}]_i$, is described here.

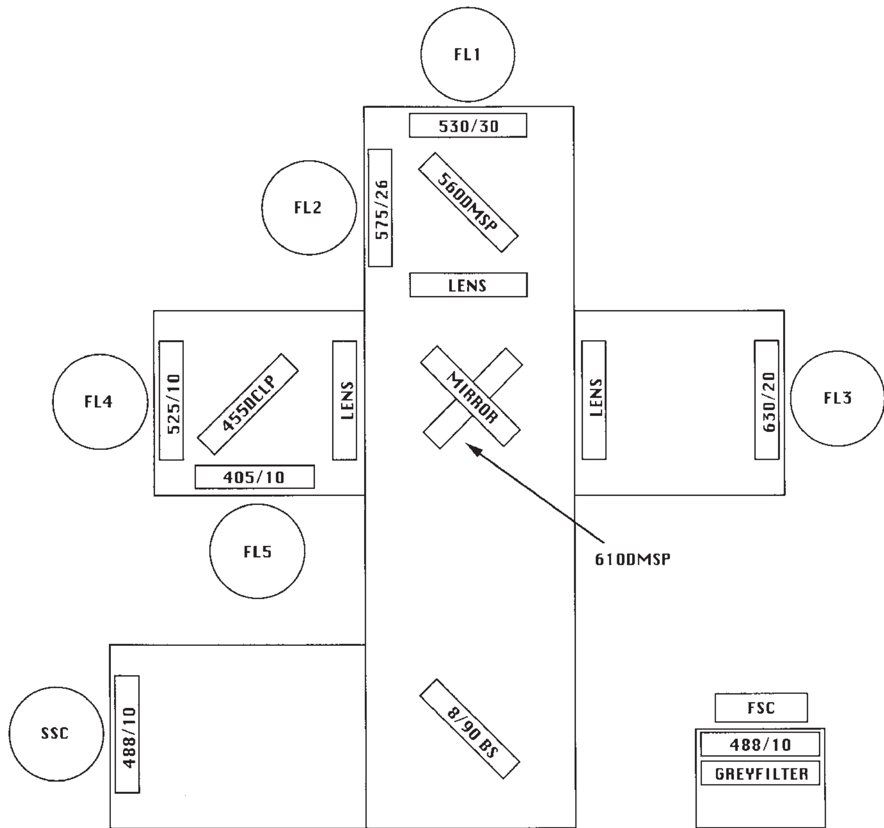

Fig. 1. The optical layout of a flow cytometer for the measurement of $[Ca^{2+}]_i$ in cells loaded with Indo-1. *See text* for explanation.

1. The 488 and the UV lines of a multiline argon laser are split by means of a beam splitter. The 488 nm line is aligned according to the manual. The UV line is lead into the path of the second laser beam via the beam splitter and is also aligned according to standard procedures. The 488 nm beam can now be used to measure forward scatter (FSC), side scatter (SSC), and fluorescence 1–3 (FL1, FL2, and FL3) in the standard way. The UV line is spatially separated from the 488 nm beam and the signals therefore can be measured on fluorescence 4 and 5 (FL4 = 525 nm and FL5 = 405 nm) via the half-mirror (*see* **Fig. 1**).
2. Alignment of the system is done using Calibrite beads in combination with Indo-1- or Hoechst-labeled beads. In this way alignment of both beams can be controlled and spectral overlap can be eliminated.
3. Finally it is important to check if the delay time between the lasers is properly set. The second intensified region on the oscilloscope should be adjusted in such a way that it is centered around the peak of the UV-excited beads.
4. *Note:* Becton Dickinson recommends a filter set consisting of a SP505 as a dichroic mirror, a DF405/20 for the Indo-1 free emission, and a DF485/22 for the

Indo-1 bound emission. The SP505 works, when placed at a 45° angle, as a 450DMSP. Using this filter set one has to use FL4 ratio because the 405 emission is measured on FL4 and 485 emission on FL5.

3.2. Electronic Setup

1. To be able to do ratiometric measurements on a FacsVantage one needs the optional Pulse Processor. There is one for FL1/FL2 and one for FL4/FL5. Essentially, only one is needed because they are identical. In this setup the Pulse Processor board has to be in slot no. 14 in the card cage, to enable the ratioing of FL4 and FL5.
2. Via the software (Lysys II or Cellquest) pulse processing is activated and the appropriate parameters are chosen. With the above described optical setup one has to choose FL5 ratio to get the ratio 405/525 (FL5 divided by FL4).
3. The baseline is set by plotting FL4 against FL5. The calcium response results in a decrease in the FL4 signal (525 nm) and an increase in the FL5 signal (405 nm); therefore one has to start with a high FL4 and a low FL5 setting to eliminate the chance that one of the signals goes out of scale and therefore becomes unusable in a ratio calculation. For that same reason a region is used to gate only on signals between channel 50 and 1020. Signals outside this range are forced full scale by the pulse processor (*see* manual).
4. The angle of the baseline in the FL4 vs FL5 plot determines the FL5 ratio value. If FL5 basal ratio falls in channel 100 and the highest experimental Ca^{2+} rise falls in channel 400, the sensitivity of the measurement is low. To improve the measurement an adjustment of the FL5 ratio gain is necessary. An increase of the gain from 1.00 to 2.00 will move the baseline to channel 200 and the highest response to channel 800, thus improving the resolution.

3.3. Loading of Killer Cells with Indo-1

1. Killer cells are loaded with 5 μ*M* Indo-1 AM for 45 min at 37°C in 5% FCS HEPES-buffered RPMI medium and washed twice. After loading, the cells should be kept in the dark (wrap tube in aluminum foil) at room temperature until use (*see* **Notes 1** and **2**).
2. Shortly before use, T (or NK) cells are aliquoted in 5-mL tubes for FACS analysis and kept in a cell culture incubator at 37°C (5% CO_2). Aliquots containing 5×10^4 T cells/sample are suitable for short acquisition times (2–5 min).
3. Before analysis on the cytofluorimeter CTLs are conjugated with autologous target cells that have been pulsed with the specific peptide ***(10)*** or with target cells expressing allo-antigens on their surface.
4. Alternatively the cells can be stained for surface markers before conjugation with target cells. For this purpose the use of directly fluorochrome-conjugated antibodies is recommended.

3.4. $[Ca^{2+}]_i$ Measurement

1. Before starting sample acquisition, a baseline is set on the 405/525 emission ratio of unstimulated Indo-1-loaded T cells. Because upon $[Ca^{2+}]_i$ increase the emission at 525 nm will decrease and in parallel the emission at 405 will increase

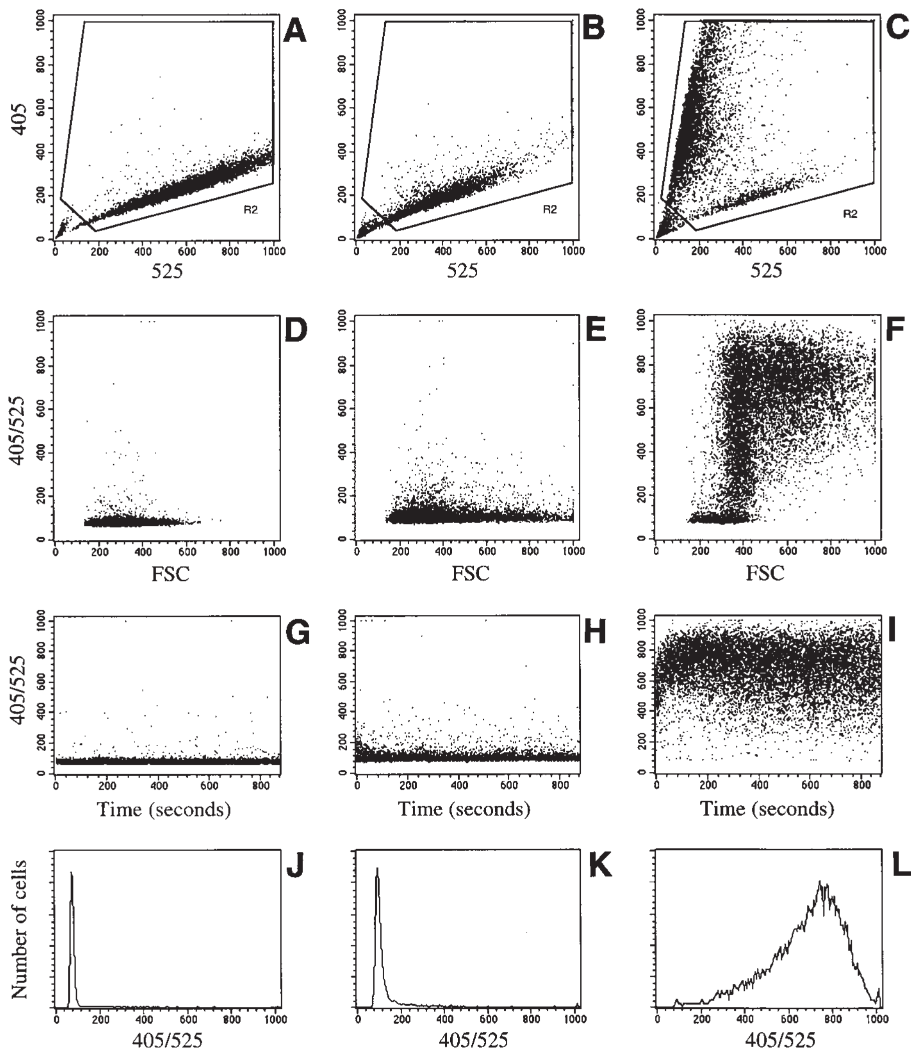

Fig. 2. Measurement of $[Ca^{2+}]_i$ on the flow cytometer. T cells alone: **A**, **D**, **G**, **J**. T cells conjugated with irrelevant targets: **B**, **E**, **H**, **K**. T cells conjugated with specific targets: **C**, **F**, **I**, **L**. (A) Standard baseline setup; (B) the 405/525 ratio does not change upon conjugation between T cells and irrelevant targets; (C) strong increase of the 405/525 ratio upon conjugation with specific target cells. T cells conjugated with irrelevant targets form nonspecific conjugates as detected by their increase in FSC (compare 2D and 2E); upon conjugation with specific targets, T cells not only increase FSC but also undergo $[Ca^{2+}]_i$ increase as detected by a rise of the 405/525 ratio (F). Nonconjugated responding T cells are separated from target cells by the resuspension, which is necessary to avoid clogging of the flow cytometer nozzle. Only Indo-1-loaded cells (R2) and conjugated cells are included in the analysis of Ca^{2+} mobilization in irrelevant and specific conjugates (H, I, K, L). The data are presented either as time course of 404/525 ratio increase (G–I) or as histogram plots of the 405/525 ratio (J–L).

it is necessary to amplify the 525 signal to a high level and set the 405 nm signal to a low level. This will avoid that upon $[Ca^{2+}]_i$ increase the 405/525 ratio will go out of scale (*see* **Subheading 3.2.**). The baseline is measured for a few minutes to verify its stability. The temperature is kept at 37°C throughout the measurement via a water jacket around the sample tube.

2. For sample measurement killer cells are mixed with target cells at 1:2 ratio (5×10^4 CTLs + 10^5 target cells/tube in 500 µL). The cells are centrifuged for 1 min at 1500 rpm to allow conjugate formation, incubated for 1 min at 37°C, resuspended, and swiftly analyzed on the flow cytometer (**Fig. 2A–L**).
3. In case responding cells or target cells have been stained with directly labeled antibodies for detection of surface markers, the flow cytometer must be compensated with the appropriate single positive controls at the beginning of the experiment before setting the 405/525 ratio baseline. Electronic compensation for the detection of one fluorochrome on two PMTs is described in the cytometer equipment manuals (*see* **Note 3**).

4. Notes

1. An important aspect of $[Ca^{2+}]_i$ measurement is temperature control. Detection of $[Ca^{2+}]_i$ increase, in principle, could be performed at room temperature (in particular when soluble stimuli are used). Nevertheless, in this condition the level of Ca^{2+} responses is lower and consequently low $[Ca^{2+}]_i$ increases could be missed. In addition, one should note that conjugate formation is dramatically dependent on temperature; therefore a careful control of temperature is highly recommended for experiments in which killer cell–target cell interactions are studied.
2. Another important parameter that has to be controlled before starting $[Ca^{2+}]_i$ measurement is pH. HEPES-buffered media must be used and killer cells must be kept in the cell culture incubator at 37°C and in the presence of CO_2 until conjugate formation.
3. Note that soluble stimuli (i.e., Ca^{2+} ionophores, stimulatory antibodies, soluble ligands for Ca^{2+} coupled receptors) can remain in the tubing and therefore interfere with the Ca^{2+} responses of the following sample leading to a false $[Ca^{2+}]_i$ increase. To avoid such artifacts it is convenient to run for a few minutes a solution that could clean the tubing and inactivate the previous stimulus (e.g., 0.1 *M* NaOH for 2–3 min followed by phosphate-buffered saline).

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BIAcore Analysis to Test Phosphopeptide-SH2 Domain Interactions

Frédéric Vély, Alain Trautmann, and Eric Vivier

1. Introduction

1.1. SH2 Domains: A Major Role in Intracellular Signaling

The elucidation of intracytoplasmic signaling pathways is a critical step in the precise understanding of cell biology. In this regard, the identification of intracytoplasmic domains, which are specialized in the transmission of biological messages, has been an enormous breakthrough ***(1)***. Among these transducing domains, the src homology 2 (SH2) domains are specialized in the interaction with the phosphorylated forms of tyrosine residues ***(2)***. SH2 domains are classically 100 amino acids long and the three-dimensional structure of several SH2-containing molecules has been resolved. As it is precisely controlled by a subtle balance between protein tyrosine kinases (which phosphorylate) and protein tyrosine phosphatases (which dephosphorylate), the phosphorylation on tyrosine residues is a highly versatile way of dictating the interaction between a given protein and an SH2-containing partner ***(3)***. The inhibitory receptors (IRs) for major histocompatibility complex (MHC) class Ia and Ib molecules expressed on NK cells, such as the killer cell inhibitory receptors (KIRs) and the CD94-NKG2A heterodimers, respectively, mediate their inhibitory function via intracytoplasmic motifs (IVLS)xYxx(LV), the immunoreceptor tyrosine-based inhibitory motifs (ITIMs) ***(4–6)***. Upon engagement of these IRs, the tyrosine residue present in ITIMs is phosphorylated, and is therefore capable of interacting with two distinct SH2-containing proteins: the protein tyrosine phosphatases SHP-1 and SHP-2 . In this chapter, we provide protocols (1) to investigate the direct binding of recombinant soluble SH2 domains to phosphorylated peptides using surface plasmon reso-

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
Edited by: K. S. Campbell and M. Colonna © Humana Press Inc., Totowa, NJ

nance (SPR) technology, as well as (2) to measure the affinity constant that characterizes this interaction ***(7–13)***.

1.2. Surface Plasmon Resonance Technology: A Real-Time Analysis of Biomolecular Interactions

Surface plasmon resonance is used as a biosensor technology for monitoring real time interactions, without prior labelling of the tested biomolecules. BIAcore is an integrated and automatic system including a detection module, a biospecific interface, and a microfluidic cartridge. The working principle starts with the immobilization of one of the interacting molecules, named the ligand, to the interface called sensor chip. The other partner of the interaction, named the analyte, is injected over the surface in a constant flow using the microfluidic cartridge. The most commonly used sensor chips (sensor chip CM5) consist of a glass slide coated on one side by a thin gold film bonded to a carboxymethylated dextran surface via an inert linker layer. These surfaces are suitable for studying interactions between hydrosoluble molecules in a hydrophilic solution. The ligand can be covalently immobilized to the dextran by chemical reactions involving mainly amino groups of the ligand (if necessary other reactional groups can be used, such as thiol and aldehyde). For instance, surfaces covalently coated with streptavidin (sensor chip SA) are appropriate for monitoring the interactions between biotinylated peptides, oligonucleotides, and proteins. Sensor chips for specialized interactions are also available, such as NTA-SAM sensor chips (nitrilotriacetic acid self-assembled monolayer) for capturing histidine-tagged ligand protein, and a flat hydrophobic surface, the sensor chip HPA, for studying membrane-associated ligands. The detection principle relies on the optical phenomenon of SPR. In brief, light directed at an appropriate angle of incidence below the sensor surface is reflected internally at the sensor surface. At the same time, an electromagnetic component of the light, the evanescent wave, is generated. In the BIAcore technology, the evanescent wave causes a dip in the reflected light intensity ***(14)***. When the refractive index and/or thickness of material in contact with the sensor surface changes, the evanescent field is altered. Because the evanescent field decays exponentially away from the surface with a space constant of a few hundred nanometers, it probes only interactions occurring near the sensor–solution interface, and it is insensitive to interactions taking place in the bulk solution, even one micrometer above the sensor surface. Surface plasmon resonance thus allows detection of real time changes in the molecular mass close to the dextran surface induced by the association and dissociation of analyte–ligand molecular complexes. These changes are expressed in resonance units (RUs). A variation of 1000 RU is equivalent to a change in the molecular surface concentration of about 1 ng/mm^2.

2. Materials

1. BIAcore apparatus (BIAcore®).
2. BIAevaluation software (BIAcore®).
3. BIAcore SA sensor chip (or BIAcore CM-5 sensor chip with streptavidin and the amine-coupling kit) (BIAcore®).
4. *N*-Biotinylated phosphopeptides (at least 80% purity upon HPLC separation).
5. Purified SH2 domain protein.
6. P20 surfactant (BIAcore®).
7. HBS running buffer, pH 7.4: 10 m*M* Hepes, 150 m*M* NaCl, 3.4 m*M* EDTA, 0.05% P20 surfactant, and 1 m*M* dithiothreitol (DTT).

3. Methods

3.1. Sensor Chip Surface Preparation

1. Insert a sensor chip SA in the BIAcore apparatus.
2. Equilibrate in HBS.
3. Normalize the chip according to the manufacturer's instructions.

Sensor chips CM-5 can be used after coupling of streptavidin to the dextran (3000 RU) using the Amine-coupling kit.

3.2. Peptide Immobilization

With regard to the SPR technology (*see below*), it is highly recommended to immobilize on sensor chips the partner of a given interaction with the smaller molecular mass: in the case of an interaction between phosphorylated ITIM peptides and soluble SH2 domains, the peptides are immobilized on sensor chips.

1. According to peptide solubility, dilute peptide at a final concentration of 0.01 ng/µL in running buffer.
2. Inject the dilution.

Kinetic constant determinations needs a low level of ligand immobilization to prevent the rebinding of the analyte during the dissociation phase. Do not immobilize more than 0.2 ng/mm^2 of peptide. Serial injections of small volumes allows reaching the appropriate level of immobilization. As an example, an accurate amount of a 15-mer phosphopeptide immobilized on a sensor chip will give a BIAcore signal comprised between 80 and 100 RU.

3.3. Injection of the SH2-Containing Protein

1. Inject the analyte (i.e., SH2 domain-containing protein) diluted at the first concentration in running buffer.
2. Inject different concentrations of the analyte to check the homogeneity of the values of kinetic constants calculated from distinct curves (*see* **Note**).
3. If an estimation of the dissociation constant K_d is known, inject analyte concentrations varying from 0.1 K_d to 100 K_d. To avoid bulk contribution to the refrac-

tive index changes both at the beginning and at the end of the injection, analytes can be dialyzed in running buffer before their use.

4. For kinetic constant determination, a high flow rate is recommended (20–40 µL/min). Inject the analyte during a period of time sufficient to reach the steady state of the SH2-phosphopeptide interaction, which will allow K_d calculation without rate constant determinations (*see* **Subheading 3.5.**). The purity of the injected proteins is important for the quality of the results.
5. At the end of the injection, the dissociation of bound analyte is monitored under the same flow rate for around 10 min. Although not detailed in this chapter, off-rates (k_d) can be experimentally measured; they must be calculated from the early part of the dissociation phase (the first minutes), when the risk of rebinding is less important.
6. Regenerate the surface (as described in **Subheading 3.4.**).
7. Inject the analyte diluted at a second concentration in running buffer. A minimum of four analyte concentrations have to be used.

3.4. Regeneration

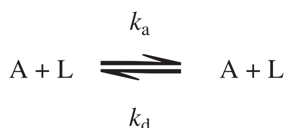
1. Regeneration solutions commonly used are: 10 m*M* NaOH, pH 10.0, 10 m*M* HCl, pH 2.0, or HBS running buffer supplemented with 0.01–0.05% sodium dodecyl sulfate (SDS).
2. Inject the regeneration solution until the level of RU before the injection is reached again.
3. Regeneration is a crucial step in the analysis of interactions using BIAcore. It must lead to a complete dissociation of bound analyte from the immobilized ligand without disrupting the association between biotinylated peptide and streptavidin. Such as regeneration can be checked by twin injections which are separated by a regeneration step; the response (RU) has to be identical for the two injections. Users are advised to start regeneration using the less harsh solution.

3.5. Interpreting Experimental Data

For determination of K_d and IC_{50}, the experimental binding curves have to be overlayed as described in the BIAevaluation handbook.

3.5.1. Principles for Determination of K_d and Kinetic Constants

The specific binding of an analyte A to an immobilized ligand L can be written:



$$A + L \underset{k_d}{\overset{k_a}{\rightleftharpoons}} A + L$$

where k_a (in M^{-1} s^{-1}) and k_d (in s^{-1}) represent the microscopic rates of association and dissociation of A from L, respectively.

The dissociation constant of the ligand is $K_d = k_d/k_a = [A] \cdot [L]/[AL]$.

In such a bimolecular reaction, the rate of formation of AL is given by:

$$d[AL]/dt = k_a.[A] \cdot [L] - k_d \cdot [AL] \quad (1)$$

At any time after addition of a given concentration of analyte, the BIAcore signal R is proportional to the concentration of AL complexes. Under saturating concentrations of A, R will reach a value, R_{max}. Therefore, from **eq. 1**, one can calculate the rate of change of signal R (e.g., reflecting the binding of a SH2 domain to an immobilized phosphopeptide) as being:

$$dR/dt = k_a \cdot [A] \cdot (R_{max} - R) - k_d \cdot R \quad (2)$$

The solution of this equation is:

$$R = R_{max} \cdot (1 - e^{-t \cdot [k_d + k_a \{A\}]}) \quad (3)$$

One then defines t_{on} (or τ_{on}) as:

$$1/t_{on} = k_{on} = k_d + k_a \cdot [A] \quad (4)$$

The experimental time constant t_{on} of the exponential association of A to L is thus related to the microscopic rate constants of the reaction, and **eq. 3** can be written as:

$$R = R_{max} \cdot (1 - e^{-t \cdot k_{on}}) \quad (5)$$

$$R = R_{max} \cdot (1 - e^{-t/t_{on}}) \quad (6)$$

Note that the observed rate of binding, k_{on} (named k_s in BIAevaluation software) differs from the microscopic rate of association k_a because (1) k_{on} takes into account the concentration of the analyte A, whereas k_a is an intrinsic parameter of the reaction, and (2) because the progressive binding of A reflects an equilibrium between its association and dissociation, hence the presence of the term k_d in **Eq. 3**. The observed rate of dissociation, k_{off}, is equal to k_d. It should be constant, whatever the value of [A] before washing.

Based on these principles, the determination of K_d can be derived using two distinct methods: (1) one from binding kinetics (k_{on}) and (2) the other from signals measured at equilibrium (R_{eq}).

3.5.2. Determination of K_d *Using the* k_{on} *Values*

For each value of [A], the BIAevaluation software provides a graphic determination of t_{on} (**Fig. 1a**). From these data, k_{on} can be calculated for each value of [A]. When k_{on} has been measured at different values of [A], from **Eq. 4**, it appears that the slope of the line gives k_a, and the y intercept gives k_d (**Fig. 2**). Thus, simply by measuring a series of associations of A, one can calculate k_a, k_d, and their ratio, K_d.

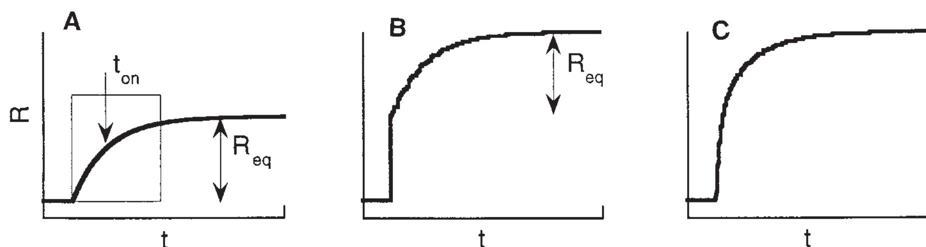


Fig. 1. Sensorgrams corresponding to a bimolecular reaction without change of the refractive index of the bulk solution **(A)**, and with such a change **(B)**. **(C)** Reaction with an order >2, fitted by the sum of two exponentials.

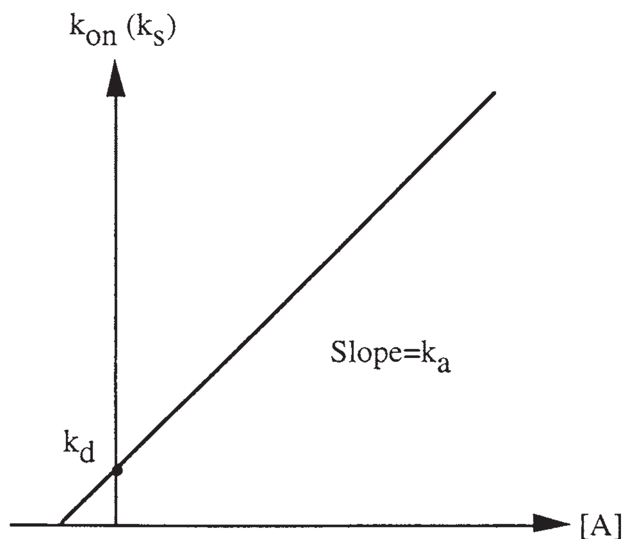


Fig. 2. Determination of rate constants using a linear fit.

3.5.3. Determination of K_d *Using the* R_{eq} *Values*

For a given value of [A], R will increase exponentially until it reaches a value, R_{eq} (**Fig. 1a**). An evaluation of K_d can be obtained from these R_{eq} plateau values.

Indeed, when $R = R_{eq}$, $dR/dt = 0$.

Thus from **Eq. 2**:

$$k_a \cdot [A] \cdot (R_{max} - R_{eq}) = k_d \cdot R_{eq} \quad (8)$$

And therefore:

$$R_{eq} = R_{max} / (1 + K_d/[A]) \quad (9)$$

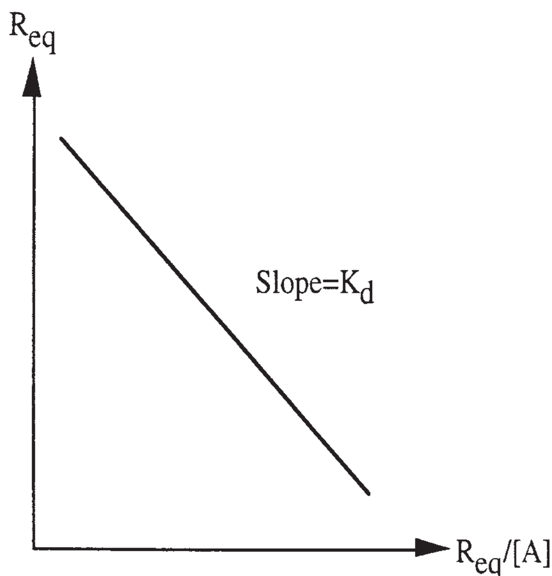


Fig. 3. Direct determination of the dissociation constant K_d using a Scatchard-like representation.

From R_{eq} values obtained at different values of [A], the estimate of K_d can be derived from a nonlinear fit of the data with **Eq. 9**. **Equation 9** can be rearranged to give a Scatchard-like equation (**Fig. 3**), useful to derive K_d from a linear fit of the data (R_{eq} as a function of R_{eq}/[A]):

$$R_{eq} = R_{max} - K_d \cdot R_{eq}/[A] \qquad (10)$$

Two important notes of caution should be considered. First, all the previous calculations are valid only if the recorded signal can be fitted by a simple exponential (**Fig. 1a**). If the signal is, like in **Fig. 1b**, the sum of a constant term and an exponential, it reveals an inappropriate subtraction of a nonspecific signal (owing to differences in the refractive indexes of the solutions and/or to protein adsorption in addition to specific binding). In evaluating K_d from equilibrium data (**Eq. 9**), R_{eq} should not include this constant term, as indicated in **Fig. 1b**. Another important possibility is that the binding of the analyte cannot in reality be described by a bimolecular reaction. If an appropriate fit of the kinetic data requires two exponentials (**Fig. 1c**), all the above analysis becomes invalid. Such a case may occur if there is more than one functional form of the ligand, or of the analyte, or if upon binding there is a conformational change that stabilizes the interaction. If the first step is very rapid, it may not always be easy to distinguish it from the case shown in **Fig. 1b**. Therefore, as a first

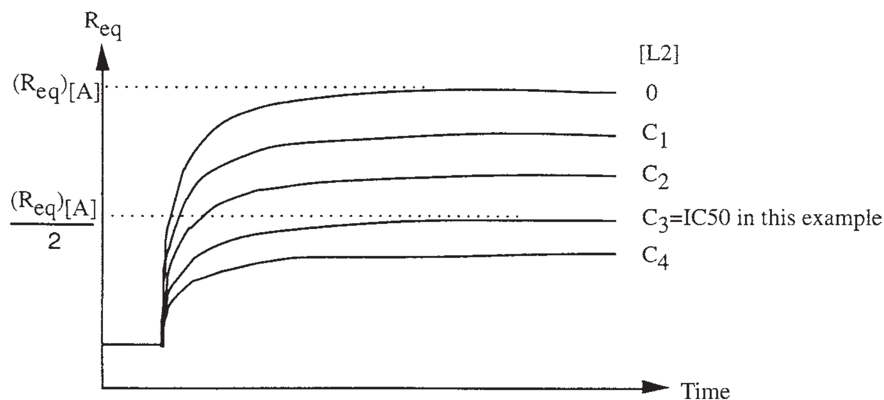


Fig. 4. Relative affinities by IC_{50} determination.

step to check the validity of the K_d determination, the linearity of the function $R_{eq} = R_{max} - K_d \cdot R_{eq}/[A]$ as defined in **Eq. 10** should be verified.

3.5.4. Relative Affinity Determination (IC_{50})

It may sometimes be useful to know the relative affinities of a series of phosphopeptides (L1, L2, ...L*n*) for a given SH2-containing protein "A." This can be done by competition assays with one immobilized phosphopeptide (e.g., L1). The binding of A to L1 will be reduced in the presence of L2, due to the formation of AL2 complexes. IC_{50} is the concentration of L2 which causes a twofold decrease of R_{eq}, as compared to its value in the absence of L2 (**Fig. 4**). Note that in such an assay, the constant of dissociation of A from L2 can be very different from IC_{50}. However, for two peptides, their K_d values are in the same ratio as their IC_{50}, under the condition that the experiment has been done far from the saturation of the immobilized ligand by A. Therefore, one can simply rank the affinities of several phosphopeptides (L2,......, L*n*) for a given SH2-containing molecule A, via the evaluation of their IC_{50} toward the L1-A association.

In these experiments, a constant concentration of the analyte ([A]) is incubated with a variable concentration of competitor peptide L2. The various mixtures are then sequentially injected on the sensor chip coated with the reference peptide L1. IC_{50} is the concentration of L2 that leads to a twofold decrease of the binding at the equilibrium observed $(R_{eq})_{[A]}$ for the injection of the same concentration of the analyte without preincubation with L2. Best results are obtained using nonbiotinylated competitor peptides to avoid direct association of these peptides with immobilized streptavidin. If only biotinylated peptides are available, free biotin binding sites of the streptavidin can be saturated by biotin injection.

4. Note

Several controls have to be performed to verify the specificity of the interaction between SH2-containing proteins and phosphopeptides. First, injections of the same concentrations of the analyte on a chip coated with the corresponding nonphosphorylated homologue peptide are important to validate the phosphopeptide specificity of these interactions. If this peptide is not available, it is possible to use an irrelevant peptide. For each injection, the irrelevant signal must be subtracted from the relevant one. Second, it is important to inject an irrelevant protein to check the SH2 domain specificity of the interaction. For example, if the SH2-containing protein is expressed as a glutathione-S-transferase (GST) fusion protein, an irrelevant GST fusion protein should be used.

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Analysis and Use of Mutant Mice Exhibiting Natural Killer Cell Defects

Toru Miyazaki and Kerry S. Campbell

1. Introduction

Natural killer (NK) cells mediate host defense through recognition and lysis of tumors, virally infected cells and "missing self" hematopoietic blast cells ***(1)***. Various in vitro experimental systems to assay NK cell function have been established, which indeed contribute to determining the functional capacities of NK cells under either normal or abnormal biological conditions. However, as our biological knowledge and technologies develop, the evaluation of NK function directly in vivo has become more essential. Because the function of each cell type within the immune network, including NK cells, is tightly interrelated, isolated cells might not exhibit normal physiological function when cultured in plastic dishes.

Therefore, mutant mice that harbor a functional and/or developmental defect in the NK cell lineage are quite useful to assess roles for NK cells in vivo. So far, a number of mutant mice with NK cell defects have been generated either via gene knockout or transgenic techniques. Here, we briefly review the characteristics of several mutant mice that exhibit altered NK cell function/development and provide some related techniques for their use and functional analysis. Anyone utilizing these mice should thoroughly investigate the current literature to assess the extent of defects in NK cells and other leukocytes.

1.1. Beige Mice

Beige mice are C57Bl/6 mice that have developed a spontaneous autosomal recessive mutation that results in increased susceptibility to infection. In 1979, the mice were shown to exhibit profound deficiencies in NK cell function, while cytotoxic and helper T-cell responses and B-cell function appeared nor-

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
Edited by: K. S. Campbell and M. Colonna © Humana Press Inc., Totowa, NJ

mal ***(2)***. This was the earliest example of a selective NK cell deficient mouse, and the strain has been historically valuable in demonstrating the important role of NK cells in host defense against tumors and viral infections. Early work with the mouse noted phenotypic similarities to Chediak–Higashi syndrome (CHS) in humans ***(3)***. CHS is a rare disorder characterized by severe immunodeficiency with neutropenia and lack of NK cell function ***(3)***. Patients with CHS exhibit giant inclusion bodies and organelles in many cell types, which are the result of defects in protein sorting. Recent work has identified the *beige* gene and pathologic mutations in that gene are also evident in CHS patients ***(4)***. The *beige* gene product exhibits homology to the yeast vacuolar sorting protein, VPS15, pointing to a defect in protein transport that appears to be most disruptive to NK cells and neutrophils. NK cells from *beige* mice demonstrate normal numbers and normal target cell conjugate formation, but lack cytolytic function in both natural cytotoxic and antibody-dependent cytotoxic responses owing to a defect in degranulation capacity ***(2,5)***.

1.2. β2-microglobulin deficient (β2m$^{-/-}$) mice

β2m$^{-/-}$ mice were generated by inactivating the β2m gene via homologous recombination in ES cells ***(6)***. Because β2m is a critical element of the class I major histocompatibility complex (MHC) molecule, cells from β2m$^{-/-}$ mice harbor extremely low levels of class I, because of instability of the class I complexes either on the cell surface or during the transport inside cells. Owing to the low expression of class I molecules, blasts derived from β2m$^{-/-}$ T cells or fetal liver cells are susceptible to destruction by NK cells both in vivo and in vitro (*see* **Subheading 3.**) ***(7)***. At least one reason explaining this susceptibility is activation of NK cells due to the lack of inhibitory signals which are mediated through Ly49–class I MHC interactions ***(8)***.

Paradoxically, however, NK cells from β2m$^{-/-}$ mice were shown to be present in normal numbers, but functionally deficient ***(7)***. β2m$^{-/-}$ NK cells fail to lyse concanavalin A (Con A) T-cell blasts from either β2m$^{-/-}$ or H2-K$^{-/-}$D$^{-/-}$ mice in vitro ***(7, 9)***. Furthermore, bone marrow cells from allogeneic mice are not rejected when grafted into β2m$^{-/-}$ mice in vivo ***(7)***. Recently, H2-K$^{-/-}$D$^{-/-}$ double-knockout mice, in which class I MHC expression is entirely diminished, were shown to harbor a similar NK cell dysfunction toward Con A T-cell blasts, suggesting that the reduced NK cell activity seen in β2m$^{-/-}$ mice is indeed owing to the reduced or lack of class I expression, and not to any secondary effects resulting from the lack of β2m ***(9)***. These experimental results are, however, easily predicted by the fact that β2m$^{-/-}$ mice show no autoimmune disease caused by autologous attack by NK cells. Thus, β2m$^{-/-}$ mice exhibit a striking deficiency in NK activity of recognizing "missing self" class I MHC, and these data suggest that NK development in the absence of MHC

class I disrupts the recognition of the MHC class I negative cells, and renders the cells tolerant to self. Importantly, β2m$^{-/-}$ NK cells are not entirely tolerant, as they retain the ability to lyse several commonly used MHC class I-deficient NK tumor target cells, although the capacity of lysis is somewhat reduced as compared to normal NK cells ***(7,9)***. Interestingly, the inhibitory receptors in β2m$^{-/-}$ mice appear to exhibit enhanced MHC class-I-mediated inhibition toward class I bearing target cells, presuMAbly owing to higher expression levels of these inhibitory receptors in the NK cells that develop in the absence of ligand ***(9)***. One possibility to explain the NK cell dysfunction in MHC class I deficient mice is that some activation receptors, in contrast to the inhibitory receptors, are dysfunctional toward self. Further studies are required to clarify whether the repertoire of or an anergic state of activation receptors is responsible.

1.3. Lymphocyte Activation Gene (Lag)-3 *Deficient Mice*

The *Lag-3* gene was initially discovered in activated NK cells by differential screening ***(10)***. Later, it was shown to be expressed just as prominently in stimulated T cells. The gene encodes a transmembrane protein of the immunoglobilin (Ig) superfamily, consisting of four extracellular Ig-like domains, the most external with an unusual looplike insertion. Both *Lag-3* and its product (LAG-3) exhibit similarities to the CD4 gene and protein; the genes map close together in mouse and human genomes and the proteins share stretches of sequence homology ***(10)***. Some experiments showed that LAG-3, like CD4, interacts with MHC class II molecules. These similarities provoked speculation that LAG-3 plays a role in controlling T cell responses. Hence, *Lag-3*$^{-/-}$ mice were generated, originally to investigate the potential function of LAG-3 in T cell development and/or function ***(11)***.

Although T cell function in *Lag-3*$^{-/-}$ mice was entirely normal, *Lag-3*$^{-/-}$ mice harbored an interesting and complex defect in NK cell function ***(11)***. NK cells from *Lag-3*$^{-/-}$ mice exhibited reduced or virtually lacked the ability to lyse some tumor cell targets, such as the T cell lymphoma line YAC-1, the macrophage tumor line IC-21, and J774 cells. Interestingly, in contrast to their reduced activity against these tumor targets, *Lag-3*$^{-/-}$ NK cells harbored entirely normal capacity to recognize and kill other target cells expressing lower levels of or lacking class I MHC molecules either in vivo or in vitro. *Lag-3*$^{-/-}$ NK cells lyse β2m$^{-/-}$ ConA T cell blasts comparable to wild-type NK cells, and both irradiated wild-type and *Lag-3*$^{-/-}$ mice rejected grafted bone marrow cells comparably. These experimental results suggested that the natural-killing system operates in two modes. LAG-3 was important for the lysis of tumor targets, whereas the targets whose lysis was independent of LAG-3 were nontransformed cells taunting NK cells by an absence of class I MHC expression. This supports previous observations that NK cell lysis of some tumor

lines is only partially or not at all affected by the abundance of class I expression on the target cell surface.

So far, the ligand(s) of LAG-3 on tumor cells has not been identified. Although LAG-3 was reported to interact with class II MHC ***(12)***, class II is not likely the only ligand that activates the LAG-3 signaling pathway for tumor lysis in NK cells, considering the generally low levels of or lack of expression of class II on diverse tumor cells. Alternatively, because the cytoplasmic domain of LAG-3 is very short and contains no typical motifs for signal transduction, it may function as a coreceptor, like CD4, for the NK–tumor interaction that initiates tumor specific lysis by NK cells.

1.4. Interferon-Regulatory Factor-1 (IRF-1) Deficient Mice

IRF-1 is a transcription factor, the expression of which is induced by interferons and other cytokines. Mice deficient in IRF-1 generate normal numbers of NK cells, but these cells exhibit greatly reduced cytolytic activity both in vitro and in vivo ***(13)***. The defect in these mice appears to be in the microenvironment in which NK cells develop in vivo, as functional NK cells can be generated from IRF-$1^{-/-}$ bone marrow cells when cultured in the presence of IL-15. Because induction of IL-15 expression is dependent on IRF-1, this cytokine is a good candidate for a missing factor that is required for normal development of functional NK cells in these mice ***(14)***.

1.5. Interleukin (IL)-2 and IL-2 Receptor Mutant Mice

It is well established that IL-2 is a critical cytokine for activation of NK cells. The IL-2 receptor is composed of a complex of α, β, and γ chains. NK cells from mice deficient of the IL-2 receptor β-chain (which is also a component of the IL-15 receptor) exhibit greatly reduced or virtually lack various functions, such as tumor lysis or recognition of reduced class I expression ***(15)***. Development of NK cells in IL-2 receptor β-chain-deficient mice is impaired, as the numbers of NK cells is also reduced in the mutant mice.

The IL-2 receptor γ chain is also a component of IL-4, IL-7, IL-9, and IL-15 receptors. Thus, signals mediated by those various cytokines are lost or impaired in $\gamma c^{-/-}$ mice ***(16–18)***. While numbers of mature T cells and B cells are reduced, $\gamma c^{-/-}$ mice completely lack mature NK cells in peripheral organs, as assessed by staining for NK-specific surface markers such as DX-5 and NK-1.1. This indicates critical involvement of signal transduction through γc-chain in the development of NK cells, although the differentiation stage at which the NK development stops in these mice is still controversial. Mutant mice expressing a truncated form of the common γ-chain (lacking the cytoplasmic domain) are also devoid of detectable NK1.1$^+$ NK cells, while T and B cell numbers are greatly reduced ***(19)***.

Interestingly, NK cell function in IL-2-deficient mice is normal ***(20)***. Transgenic mice overexpressing both IL-2 and the IL-2 receptor (Tac) under the control of the class I MHC-promoter exhibit increased numbers of NK cells and enhanced function in tumor cell lysis ***(21)***.

1.6. IL-12 and IL-18 Deficient Mice

Despite a lack of amino acid sequence homology, IL-12 and IL-18 are macrophage-derived cytokines that share similar properties of inducing interferon-γ (IFNγ) production and enhancing NK cell activity and proliferation of activated T cells. In accordance with their shared functional capacities, however, mice deficient in either interleukin exhibit significant impairment in both inducible IFNγ production and NK cell activity ***(22)***. Double-knockout mice, lacking both interleukins, have even further impairment of NK cell activity and inducible IFNγ production capacity ***(22)***. Type 1 T helper (Th1) cell responses are also reduced in the same manner in these interleukin-deficient mice.

1.7. Perforin and Granzyme Knockout Mice

Perforin is a membrane pore-forming polypeptide that is secreted from effector cells, such as cytotoxic T lymphocytes (CTLs) and NK cells, when these effector cells interact with target cells and initiates target cell lysis. Mice lacking perforin show greatly reduced, but not lack of, lysis by NK cells of either tumor cells or class I MHC-deficient target cells ***(23–25)***. Target cell-stimulated granzyme release is normal in perforin-deficient NK cells ***(24)***.

Granzymes-A and -B are serine proteases, which exist with perforin in the vesicular granules of CTLs and NK cells and enter target cells through perforin-generated pores to initiate DNA fragmentation. Granzyme-A and -B single-knockout mice exhibit normal NK cell development and virtually normal lytic activity against target cells ***(26–28)***. NK cells from granzyme-A × Granzyme-B double-knockout mice also exhibit virtually normal lytic capacity (when measured in chromium release assays), but capacity to elicit target cell DNA fragmentation is entirely eliminated ***(28)***. Target cell DNA fragmentation capacity is normal in granzyme-A-deficient NK cells, and reduced only in short-term but not long-term assays of granzyme-B-deficient mice.

These results indicate that target cell lysis is mediated by pore formation by perforin and that the granzymes-A and -B collectively induce DNA damage. The residual lytic capacity of NK cells from perforin-deficient mice indicates that alternative cytolytic pathways exist. The most probable alternative killing mechanism is CD95 (Fas)-dependent killing. Lymphokine-activated killer (LAK) cells from mice lacking both perforin and Fas ligand (PKO/*gld* mice) lack lytic activity in short-term cytotoxicity assays, whereas long-term assays exhibit residual lysis that was shown to be tumor necrosis factor (TNF) depen-

dent ***(29)***. Thus, NK-mediated target cell lysis can be mediated through perforin, Fas, and/or TNF.

1.8. Ikaros-Deficient and Recombination-Deficient Mice

The *Ikaros* gene is a zinc-finger transcription factor, the expression of which is restricted to hematopoietic cells both in fetus and adult. Two kinds of *Ikaros*-mutant mice were generated: one is a null mutant generated by deleting the DNA-binding domain encoded by exons 3 and 4 and another was designed to lack the C-terminal zinc-finger dimerization domain by deleting exon 7 ***(30,31)***. Both types of mutants lack detectable NK cells, and NK cell activity against tumor cell targets is absent from the spleens of *Ikaros*$^{-/-}$ mice. Mature B cells are also absent in *Ikaros*$^{-/-}$ mice, while mature T cells are greatly reduced, indicating that all three of these cell lineages are derived from a common precursor that is dependent on Ikaros for normal development. In contrast, myeloid and erythroid lineages are not disrupted in these mice. *Ikaros*$^{-/-}$ mice lacking exons 3 and 4 generally die within 4 wk after birth ***(30)***, whereas disrupting exon 7 results in mice that can live 4 mo and breed ***(31)***.

Lymphocytes from *RAG*-deficient and severe combined immunodeficiency (SCID) mice exhibit complete lack of or dramatically reduced capacities to rearrange the variable domain genes of their antigen receptors, respectively ***(32–34)***. The *RAG-1* and *RAG-2* genes are specifically expressed in lymphocytes and encode critical elements for generating site-specific DNA strand breaks at recombination elements of variable region genes ***(33,34)***. Therefore, *RAG*-deficient mice are totally devoid of mature T and B cells, which require antigen receptor rearrangements during development. The causative defect in the SCID mouse has been identified in the DNA-dependent protein kinase gene, which plays an important role in the recombination process ***(35,36)***. The mutation in SCID mice results in extremely inefficient DNA recombination of immunoglobulin or T cell receptor variable genes ***(32)***, although some "leaky" development of mature cells is observed, particularly in older animals. Although T and B cell development is dramatically disrupted in these mouse strains, NK cell numbers and functions are normal. These observations indicate that DNA recombination is not required for NK cell development and function, and the mice provide unique models of mice lacking B and T cell functions, while exhibiting normal NK cell functions.

1.9. CD3ε Transgenic Mice

Transgenic mice have been generated that express the human CD3ε gene with endogenous promoters in high copy number ***(37)***. Interestingly, these mice (tgε26) completely lack both T cells and NK cells ***(37)***. The T cell and NK cell deficiencies appear to be due to effects on signal transduction components of

early differentiating precursor cells, as a transgene composed of the transmembrane and cytoplasmic domains of CD3ε also eliminated T cell and NK cell development, while expression of only a portion of the extracellular domain did not affect development of either population ***(37)***.

2. Materials

1. Mice: All mutant mice in this chapter are summarized in Table 1.
2. Antibodies: Anti-mouse NK 1.1 monoclonal antibodies (MAbs) (PK136), anti-DX5 MAb, and anti-CD45RO MAb (B220) are available from Pharmingen. Other monoclonals that can be used as culture supernatant are anti-CD4 MAb (RL-172; **ref. *38***), anti-CD8 MAb (M31; **ref. *39***), and anti-class II MHC MAb (BP-107; **ref. *40***; American Type Culture Collection).
3. Concanavalin A (Con A) (Sigma).
4. Polyinosinic-Polycytidylic acid [Poly (I:C)] (Sigma).
5. Histopaque-1077 (Sigma).
6. Lo-Tox complement and Lympholyte-M are available from CedarLane (Hornby, Ontario, Canada).
7. Complete culture medium: Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) including 10% fetal calf serum (FCS), 1× β-mercaptethanol (diluted from 1000× stock solution purchased from Gibco), 1× penicillin–streptomycin (Gibco), and 1× L-glutamine (diluted from 100× stock solution from Gibco).

3. Methods

3.1. In Vitro Lysis of β2m$^{-/-}$ Con A Blast Cells by NK Cells

3.1.1. Preparation of Con A Blast Cells

1. To purify MHC class I-deficient splenic T cells, spleen cells (1×10^7) from β2m$^{-/-}$ mice are incubated with 1–5 μg/mL of anti-class II MHC MAbs for 30 min on ice in 10 mL of complete DMEM.
2. Cells are washed with phosphate-buffered saline (PBS; Gibco; without FCS) two times (by centrifugation at 300–350*g* for 4 min) and then resuspended in 10 mL of 10% Cedarlane Lo-Tox complement in PBS. Cell suspension is incubated at 37°C for 1 h.
3. After the incubation, cells are washed once with complete DMEM medium and resuspended in 5 mL of the same medium. The suspension is loaded gently on 5 mL of Lympholyte-M in a 15-mL tube, and centrifuged for 20 min at 1000–1500*g*.
4. After centrifugation, cells at the interphase are isolated with a Pasteur-pipet and washed twice with complete DMEM.
5. Purified T cells are cultured in complete DMEM including 5 μg/mL of Con A for 36–48 h at 37°C.
6. After culture, dead cells are excluded as described in **step 3**. Resulting ConA blast cells are resuspend in complete culture medium at 10^6 cells/mL in a 15-mL tube.

Table 1
List of the Mutant Mice

Mouse	NK cell status	T cell status	B cell status	Reference	Source
Beige	normal numbers, not cytolytic	normal	normal	***2,3,5***	Taconic, Harlan
$\beta 2m^{-/-}$	normal numbers, fail to kill ConA blasts, but kill some tumors	cytotoxic T cell deficiency	normal	***7,9***	Jackson, Taconic
Lag-$3^{-/-}$	deficient lysis of certain tumors, while others lysed normally	normal	normal	***11***	Dr. C. Benoist (e-mail: cb@igbmc.u-strasbg.fr)
IRF-$1^{-/-}$	normal numbers, greatly reduced cytotoxicity	normal	normal	***14***	Jackson
IL-$2R\beta^{-/-}$	reduced number and functionally impaired	reduced number	normal	***15***	Jackson
IL-$2R\gamma c^{-/-}$	no detectable cells	reduced numbers	reduced numbers	***16–19***	corresponding author
IL-$12^{-/-}$	significantly reduced function	decreased Th1 response	not tested	***22***	corresponding author
IL-$18^{-/-}$	significantly reduced function	decreased Th1 response	not tested	***22***	corresponding author
$perforin^{-/-}$	greatly reduced lytic function	decreased cytotoxic function	normal	***23–25***	Taconic
Granzyme-$A^{-/-}$	normal development and virtually normal lytic function	virtually normal	normal	***26,28***	corresponding author

Granzyme-B$^{-/-}$	normal development and virtually normal lytic function	virtually normal	normal	***27,28***	Jackson
Ikaros$^{-/-}$	absent	greatly reduced	absent	***30,31***	corresponding author
RAG-1$^{-/-}$	normal	absent	absent	***34***	Jackson
RAG-2$^{-/-}$	normal	absent	absent	***33***	Taconic
SCID	normal	almost absent	almost absent	***32***	Jackson, Taconic, Harlan, Charles River
CD3ε transgenic	absent	absent	normal	***37***	Jackson

Commercial sources of certain mice are: Jackson Laboratory (jaxmice.jax.org/index.shtml), Taconic (www.taconic.com), Harlan Sprague Dawley (www.harlan.com), and Charles River (www.criver.com).

7. One milliliter of the Con A blast cell suspension is mixed with 200 μCi of ^{51}Cr, and incubated at 37°C for 1 h with tapping of the tube every 15 min.
8. After the incubation, cells are washed 3× with complete culture medium, and resuspended in the medium at 10^5 cells/mL.

3.1.2. Preparation of Effector NK Cells

1. Effector NK cells should be prepared from spleen cells of mice into which 100 μg of Poly (I:C) was injected intraperitoneally 48 h before to activate NK cells (*see* **Note 1**).
2. A spleen cell suspension prepared from these mice is incubated with 1–5 μg/mL each of anti-CD4, anti-CD8, anti-class II, and anti-CD45RO (B220) MAbs in complete DMEM for 30 min on ice.
3. Cells are washed with PBS (without FCS) two times (by centrifugation at 300–350*g*, for 4 min), and then resuspended in 10 mL of 10% concentration of Cedarlane Lo-Tox complement in PBS. The cell suspension is then incubated at 37°C for 1 h.
4. After the incubation, cells are washed once with complete DMEM and resuspended in 5 mL of the medium. The suspension is loaded gently on 5 mL of Lympholyte-M in a 15-mL tube, and then centrifuged for 20 min at 1000–1500*g*.
5. After centrifugation, cells at the interphase are isolated with a Pasteur pipet and washed twice with complete DMEM, and resuspended in the medium at 10^6 cells/mL.

3.1.3. Cytotoxicity Assay

Lysis is assayed by incubating the mixtures of effector NK cells and ^{51}Cr-labeled target blast cells for 4–6 h at various effector:target cell ratios, and counting the cpm of supernatants of the cultures as described in Chapters 8, 13, 16, 29–31.

3.2. In Vivo Rejection of β2m$^{-/-}$ Bone Marrow Cells

1. Bone marrow cells are prepared from either β2m$^{-/-}$ or wild-type mice.
2. Contaminating mature T cells are depleted by treating cells with anti-CD4 and anti-CD8 MAbs and complement (*see* **Subheading 3.1.2.**).
3. Removal of dead cells and red cells should be done as described in **Subheading 3.1.**
4. Viable bone marrow cells (5 million) are suspended in 200 μL of PBS, and grafted intravenously into 900-rad irradiated recipient mice.
5. On day 5, 3 μCi of 5-^{125}I-iodo-2'-deoxyuridine (^{125}I-UdR) is inoculated by intraperitoneal injection.
6. At 24 h, the animals are killed and radioactivity incorporated into the spleens is measured in a γ-counter. The measured radioactivity denotes the proliferation of grafted bone marrow cells. Control counts (CPM of spleens from mice with no cells grafted) should be subtracted from each experimental count.

4. Notes

1. Poly I:C induces NK activity by increasing IFNγ production in mice. Basal NK activity in mice housed under clean conditions may be extremely low, and therefore poly I:C pretreatment of the mice may be essential to adequately measure NK cell activity.

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29

Isolation and Analysis of Natural Killer Cells in Chickens

Thomas W. F. Göbel

1. Introduction

Chicken natural killer (NK) cells have been characterized mainly based on functional properties in a variety of systems (reviewed in **refs. *1*,*2***). A detailed analysis of their ontogeny, phenotype, function, tissue distribution and the receptors involved in killing of target cells has been difficult owing to the lack of NK cell specific monoclonal antibodies (MAbs). In addition, a chicken interleukin-2 (IL-2) homologue essential to establish in vitro NK cell lines has only recently been identified ***(3)***.

We have characterized $CD8^+$ cells in the chicken embryonic spleen at a developmental stage where T cells have not yet migrated to the periphery ***(4)***. These embryonic spleen cells were expanded in vitro in the presence of conditioned medium containing growth promoting cytokines and were further characterized as avian NK cell homologues by their morphology, phenotype, and function. Embryonic avian NK cells express surface CD8αα homodimers, but no T or B cell lineage specific antigens such as T cell antigen receptor or immunoglobulin.

Based on these criteria, NK cells in adult chickens are mainly located in the intestinal epithelial lymphocyte population (IEL). In contrast to mammals, only small numbers of NK cells are present in other peripheral tissues ***(5)***. The intestinal epithelium is a valuable source to obtain sufficient numbers of NK cells for immunizations, functional analyses, and in vitro culture, as roughly 50% of all IELs are characterized as NK cells by their phenotype and function. A MAb specific for IEL NK cells has recently been developed ***(5)*** and will be an important tool to isolate and analyze chicken NK cells in more detail.

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
Edited by: K. S. Campbell and M. Colonna © Humana Press Inc., Totowa, NJ

This chapter outlines the isolation and culture of splenic NK cells from chick embryos, the preparation and selective enrichment of adult intestinal NK cells, the generation of virally transformed NK cell lines, and a cytotoxicity assay for functional NK cell analysis.

2. Materials

2.1. Isolation and Culture of Embryonic NK Cells

1. Dulbecco's PBS (Gibco BRL, Grand Island, NY).
2. Ficoll-Paque® (Pharmacia, Pitscataway, NJ).
3. Serum-free medium: Iscove's modified Dulbecco's medium (IMDM; Gibco- BRL) supplemented with 0.5% bovine serum albumin (BSA).
4. Concanavalin A (Con A, Pharmacia). Dissolve ConA at 1 mg/mL of sterile water, aliquot and freeze at –20°C. Thaw only once.
5. Ultrafiltration cell equipped with 30 kDa membrane (Amicon, Berverly, MA).
6. Complete medium: IMDM supplemented with 8% fetal calf serum (FCS), 2% chicken serum, 50 IU/mL of penicillin, 50 µg/mL of streptomycin and 2 m*M* of glutamine (all reagents: Gibco BRL).

2.2. Isolation of IEL

1. PBS, 10× stock solution (1 L): 80 g NaCl, 2 g KCl, 11.5 g $Na_2HPO_4 \cdot 7H_2O$, 2 g KH_2PO_4, dilute 1:10 before usage, the pH should be around 7.4.
2. PBS/DTT/EDTA solution: PBS supplemented with 10 m*M* of DTT and 0.1 m*M* of EDTA and prewarmed to 37°C. DTT should be prepared freshly.
3. Nylon wool column: 0.7 g of prewashed nylon wool fiber (Polysciences, Warrington, PA) are packed into a 5-mL syringe and compressed with the plunger to 2 mL. The column is prewetted with 20 mL of PBS immediately before usage.
4. Ficoll-Paque® (Pharmacia).
5. FACS Buffer: PBS supplemented with 1% BSA.

2.3. Enrichment of Intestinal NK Cells

1. 28-4 MAb (murine IgG_3) reacts with a 35 kDa molecule on intestinal NK cells ***(5)***.
2. MACS buffer: PBS supplemented with 5 m*M* EDTA and 0.5% BSA. Degas buffer before use. It is important to keep the buffer on ice during the entire procedure.
3. Goat-anti-mouse-IgG_3-biotin-conjugate, 1:100 final dilution (Southern Biotechnology Associates, Birmingham, AL).
4. Streptavidin-FITC-conjugate 1:100 final dilution (Southern Biotechnology Associates).
5. Vario MACS magnet unit (Miltenyi Biotech, Auburn, CA).
6. Biotin-conjugated microbeads (Miltenyi Biotech).
7. MACS BS columns (Miltenyi Biotech).

2.4. Generation of Virally Transformed NK Cell Lines

1. Complete medium (*see* **Subheading 2.1.**).

2. S_2A_3 viral supernatant: S_2A_3 cells are cultured at 1×10^7 cells/mL complete medium for 4 h and the supernatant is harvested by centrifugation at 500*g* for 10 min followed by filtration through a 0.2-µm sterile filter ***(6)***.

2.5. Cytotoxicity Assay

1. Complete medium (*see* **Subheading 2.1.**).
2. LSCC-RP9 cells are maintained in complete medium and passaged regularly.
3. ^{51}Cr: Sodium chromate in aqueous solution containing 0.9% NaCl, specific activity 10 mCi/mL (Amersham, Arlington Heights, IL).
4. Triton® X-100, 10% aqueous solution (Pierce, Rockford, IL).
5. Scatron supernatant collection system (Scatron Instruments, Norway).

3. Methods

3.1. Isolation and Culture of Embryonic NK Cells

3.1.1. Preparation of Conditioned Medium

1. Remove the spleen from an adult chicken and put it into 50 mL of sterile Dulbecco's PBS in a petri dish.
2. Tease the spleen through a fine steel mesh with a plunger from a 10-mL syringe to obtain a single cell suspension, transfer the suspension in a 50-mL tube and sediment larger fragments for 10 min on ice. Transfer cell suspension to a new tube.
3. Centrifuge at 250*g* for 10 min, remove the supernatant and resuspend the cell pellet in 20 mL of Dulbecco's PBS. Distribute 10-mL aliquots in 50-mL tubes.
4. Carefully underlay the cell suspension with 10 mL Ficoll-Paque® and centrifuge at 650*g* for 12 min.
5. Collect the interphase and wash three times by resuspending the cells in Dulbecco's PBS and centrifugation at 250*g* for 10 min.
6. Following the last wash resuspend the cell pellet at 1×10^7 cells/mL serum free medium supplemented with 10 µg/mL Con A, distribute 100 mL cell suspension in 175 cm^2 tissue culture flasks and incubate at 37°C in 5% CO_2 for 24 h (*see* **Note 1**).
7. Centrifuge the cultured cells at 500*g* for 10 min and filter the supernatant through a sterile 0.2-µm filter.
8. Filter the supernatant through a 30 kDa membrane and collect effluent. This effluent is used as conditioned medium (*see* **Note 2**).
9. Store aliquots at –70°C.

3.1.2. Isolation and Culture of Embryonic NK Cells

1. Incubate the eggs for 14 d (*see* **Note 3**).
2. Remove the embryonic spleen and collect the spleens in a Petri dish in Dulbecco's PBS (*see* **Note 4**).
3. Mince the spleens between the ends of two frosted glass slides.
4. Collect the cell suspension in a 15-mL tube and sediment larger fragments 10 min on ice.

5. Collect the supernatant in 15-mL tube and centrifuge at 250*g* for 10 min.
6. Resuspend the cell pellet in 5 mL Dulbecco's PBS, carefully underlay with 5 mL Ficoll-Paque® and centrifuge at 650*g* for 12 min.
7. Collect the interphase and wash three times by resuspending the cells in Dulbecco's PBS and centrifugation at 250*g* for 10 min.
8. Following the last wash resuspend the cells, count and adjust the cell concentration to 1×10^6 cells/mL in complete medium supplemented with 10% conditioned medium (**Subheading 3.1.1.**) and distribute 3 mL cell suspension per well of a 6-well tissue culture plate. Incubate at 37°C in 5% CO_2.
9. The cells should be split every 2 d to maintain the cell density (*see* **Note 5**).

3.2. Isolation of IEL

1. Take out the duodenal loop of an adult chicken and remove the remaining fat and pancreatic tissue (*see* **Note 6**).
2. Flush the intestine five times with 20 mL of PBS using a syringe.
3. Cut the intestine longitudinally and in 1-cm long pieces.
4. Wash the pieces five times by gently swirling in 100 mL PBS and decanting soluble matter.
5. Transfer the pieces in 50 mL of prewarmed PBS/DTT/EDTA solution and vigorously stir with a magnetic stirring bar for 30 min at 37°C (*see* **Note 7**).
6. Collect the supernatant and sediment larger fragments for 5 min.
7. Centrifuge at 250*g* for 10 min and resuspend the cell pellet in 20 mL.
8. Prewet the nylon-wool column with PBS and pass the cell suspension through the column. Collect the flow through and centrifuge at 250*g* for 10 min (*see* **Note 8**).
9. Wash the cells twice by resuspending in PBS and centrifugation at 250*g* for 10 min.
10. Resuspend the cells in 20 mL PBS.
11. Distribute 10 mL of the cell suspension per 50-mL tube and underlay with 10 mL Ficoll-Paque® (*see* **Note 9**).
12. Centrifuge at 650*g* for 12 min.
13. Collect the interphase and wash three times as above.
14. Resuspend the cells in FACS buffer for phenotypic analysis or directly in 28-4 MAb for selective enrichment (*see* **Note 10**).

3.3. Enrichment of Intestinal NK Cells

1. Carry out **steps 1–13** as outlined in **Subheading 3.2.**
2. Resuspend 10^8 cells/mL 28-4 MAb supernatant (*see* **Note 11**).
3. Incubate the cells on ice for 15 min.
4. Wash once by adding 15 mL of cold MACS buffer and centrifuge at 250*g* for 10 min at 4°C.
5. Resuspend 10^8 cells/mL goat-anti-mouse-IgG_3-biotin-conjugate and incubate for 15 min on ice.
6. Wash the cells as in **step 4**.
7. Resuspend 10^8 cells/mL streptavidin-FITC-conjugate and incubate for 15 min on ice.
8. Wash the cells as in **step 4**.

9. Resuspend 2×10^8 cells/mL MACS buffer, add 10 µl/mL biotin conjugated microbeads.
10. Incubate the cells for 10 min on ice.
11. Wash the cells as in **step 4**.
12. Prepare a MACS BS column during centrifugation by washing the column with 15 mL MACS buffer (*see* **Note 12**).
13. Resuspend the cell pellet in 2 mL MACS buffer and directly apply the cell suspension to a BS column equipped with a 19-G needle.
14. Wash the column with 15 mL MACS buffer.
15. Remove the column from MACS, backflush the cells to the top and replace the column.
16. Wash the column with 15 mL MACS buffer.
17. Repeat **step 15** and change the 19-G needle to an 18-G needle (*see* **Note 13**).
18. Wash the column with 15 mL MACS buffer.
19. Remove the column from the MACS, backflush the cells, remove the 18-G needle and elute the cells in a 15 mL tube using 15 mL MACS buffer.
20. Centrifuge the cells at 250*g* for 10 min.
21. Resuspend the cells in appropriate buffer or medium (*see* **Note 14**).

3.4. Generation of Virally Transformed NK Cell Lines

1. Isolate embryonic or intestinal NK cells as described in **Subheadings 3.1.–3.3.**
2. Aliquot 0.5 mL of 1×10^6 cells/mL complete medium in a 24-well tissue culture plate.
3. Add 0.5 mL of S_2A_3 viral supernatant. Incubate the plate at 37°C in 5% CO_2 for 5–8 d until cell colonies are visible (*see* **Note 15**).
4. When the colonies have grown confluent, transfer them to a 25 cm^2 tissue culture flask, add 10 mL fresh complete medium and expand the cell lines.
5. To obtain single clones, harvest the cells from the tissue culture flask by centrifugation at 250*g* for 10 min in a 15-mL tube.
6. Collect the supernatant and sterile filter through a 0.2-µm filter.
7. Resuspend the cell pellet in 10 mL complete medium and count the cells.
8. Dilute the cells to 10 cells/mL complete medium and distribute 100 µL of the cell suspension on a 96-well flat bottom tissue culture plate. Add 100 µL of the supernatant obtained in **step 6** (*see* **Note 16**).
9. Incubate at 37°C in 5% CO_2 for about 2 wk until colonies are visible and expand those wells, where only one colony is present (*see* **Note 17**).

3.5. Cytotoxicity Assay

1. Isolate enriched intestinal NK cells as described in **Subheading 3.3.**
2. Prepare serial dilution's of NK cells in a 96-well round bottom tissue culture plate: Prepare the plate by adding 100 µL complete medium to rows B to G. Add 200 µL of 1×10^7 cells/mL in row A. Twofold dilutions are generated by serially transferring 100 µL from row A to B, and so on up to row G. Add 100 µL complete medium to wells H1 to H6 (they will serve as control of spontaneous lysis), leave wells H7 to H12 empty (they will serve as control of total lysis; *see* **Note 18**).
3. Harvest the LSCC-RP9 cells, count and centrifuge at 250*g* for 10 min (*see* **Note 19**).

4. Resuspend the cells at 1×10^7 cells/mL complete medium.
5. Combine 100 µL FCS, 100µL LSCC-RP9 cells, and 50 µL ^{51}Cr in a 15-mL tube.
6. Incubate the cells at 37°C in 5% CO_2 for 45 min.
7. Add 15 mL complete medium and centrifuge the cells at 250*g* for 10 min.
8. Discard the supernatant and resuspend the cell pellet in 15 mL prewarmed complete medium.
9. Incubate at 37°C in 5% CO_2 for 45 min (*see* **Note 20**).
10. Wash twice as in **step 7**.
11. Resuspend the cells in 5 mL prewarmed complete medium, count the cells and dilute them to 1×10^5 cells/ml complete medium.
12. Add 100 µL of the labeled target cell suspension to all wells of plate prepared in **step 2**.
13. Centrifuge at 50*g* for 2 min and incubate at 37°C in 5% CO_2 for 4 h (*see* **Note 21**).
14. Add 100 µL of 2 % Triton X-100 to wells H7 to H12.
15. Centrifuge the plate at 250*g* for 5 min.
16. Harvest the plate using the Scatron supernatant collection system.
17. Count the radioactivity and calculate the specific cytotoxicity (*see* **Note 22**).

4. Notes

1. The average cell yield of one spleen is about 2×10^9 cells, resulting in 200 mL of conditioned medium. Following the 24 h incubation the quality of cell stimulation is judged microscopically. The majority of cells should have formed clumps of cell blasts.
2. Removal of larger inhibitory components is essential for good stimulation.
3. It is critical to maintain the eggs at 14°C before incubation to obtain a large batch of eggs at an identical developmental stage. Day 14 eggs are used because from d 15 on T cells migrate from the thymus into the spleen and would therefore contaminate the cultures.
4. The embryonic spleen can be easily removed by flipping over the stomach and removing the spleen with the ends of bent forceps.
5. About 5×10^5 cells are obtained per embryonic spleen, the cells vigorously proliferate during the first week.
6. Six to 8 wk old chickens are the best source, because the frequency of NK cells seems to drop with age.
7. The method described is a modification of a previously published protocol *(7)*, however, DTT and EDTA are simultaneously used.
8. The nylon wool separation is a critical step and the column should be prepared with care. Filtration through a column that is too compact results in cell loss, whereas a loosely packed column does not remove enough epithelial cells. The cell suspension should be gray following this step. If it still appears yellow, repeat the nylon wool step.
9. Some investigators use a two step Percoll gradient instead of Ficoll-Paque®, however, when we compared both gradients similar cell preparations were obtained.
10. An example of an average IEL isolation is shown in **Fig. 1**. It is important to note that there is some variability between preparations. IEL prepared according to this method are sufficiently enriched for immunofluorescence analysis, since the lym-

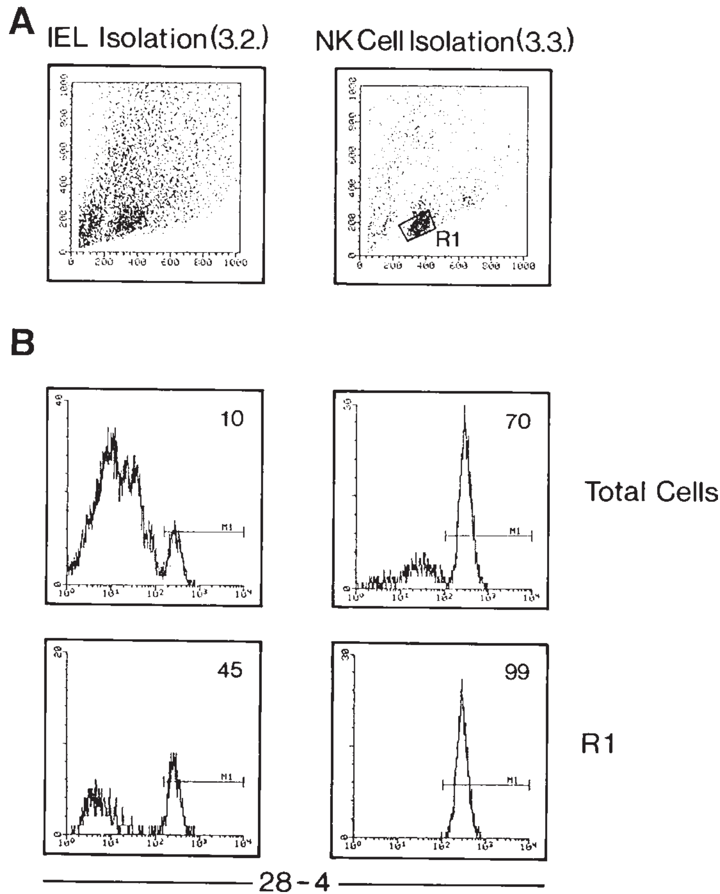

Fig. 1. IEL isolation and enrichment of NK cells. **(A)** Light scatter characteristics of IEL and enriched NK cells as prepared in **Subheadings 3.2.** and **3.3.** Forward scatter is on the x-axis and side scatter is on the y-axis of these dot plots. **(B)** 28-4 immunofluorescence analysis of total cells (upper panels) and cells within the lymphocyte gate R1 as shown in A (lower panels). The relative percentage of 28-4$^+$ cells in the marked region is indicated in the graphs.

phocyte population can be easily gated. As a marker for lymphocytes and an internal standard to compare different preparations, immunofluorescence analysis should always include staining with an anti-panCD45 MAb (Southern Biotechnology Associates) as a marker for total leukocytes. The relative frequencies of different cell populations should be expressed as percentage of CD45$^+$ cells. The contamination by cells from the lamina propria is controlled by immunofluorescence staining with an anti-immunoglobulin-light chain reagent (Southern Biotechnology Associates), since B cells are only found in the lamina propria, but not in IEL.

11. The IEL preparation obtained in **Subheading 2.2.** is still heavily contaminated with gut epithelial cells and the lymphocytes are a mixture of CD3$^+$ T cells and

28-4$^+$ NK cells. The positive selection using the 28-4 MAb highly enriches NK cells. The 28-4 MAb does not modulate NK cell activity.

12. The basic handling of the MACS system is described in the protocols provided by the company. We therefore focus on important modifications.
13. The flow rate as controlled by the different needles is critical for the purity of the sorted cells. Using an 18-G needle generates a fast flow rate, which is important to remove most of the epithelial cells.
14. An example of a typical positive selection is shown in Fig. 1. There is still some contamination with epithelial cells, but within the lymphocyte gate, virtually all cells represent 28-4$^+$ NK cells. This method can be adapted to specific needs of an experiment using different MAbs to positively select the cell population of interest. For instance, using CD45 enriches T cells and NK cells which can be further separated on a cell sorter to test both populations simultaneously in a cytotoxicity assay.
15. The S_2A_3 cells are the source of the replication-defective avian reticuloendotheliosis virus strain T and the chicken syncitial virus as a helper virus.
16. In our experience it is critical to supplement the medium with the supernatant from the polyclonal cell lines, most likely containing growth promoting cytokines.
17. At this point the cell clones are normally very stable and can be maintained in culture for months without phenotypic changes. Although the cell lines seem to be nonfunctional in cytotoxicity assays and some antigens (e.g., CD8) are lost during the initial transformation, the clones are fast growing and ideal for numerous studies like immunoprecipitations, protein purification, RNA isolation and signaling experiments.
18. This dilution series will result in an effector:target ratio of 100:1 to 1.6 :1, a range that is normally sufficient for the analysis. If the effector cell numbers are limited, an effector:target ratio of 25:1 should be used as a start point. The NK cell enrichment and the target cell preparation are described separately here, however, they can be performed simultaneously as follows: After the IEL isolation, the enrichment of NK cells and the labeling of target cells is performed in parallel.
19. The LSCC-RP9 cells are derived from a lymphoid tumor induced by Rous associated virus 2, and they are commonly used as target cells ***(1,8)***. The cell line originates from a B^2B^{15} MHC haplotype. It is critical that the cells are maintained in highly proliferative status and that they are passaged at the day before labeling. The viability of the cells should be at least 90%, otherwise viable cells should be purified on a Ficoll-Paque® gradient before the labeling procedure.
20. This step dramatically decreases the spontaneous cytotoxicity of the LSCC-RP9 target cells.
21. The centrifugation step ensures the close contact of target and effector cells. It is critical that it is performed in a centrifuge at room temperature.
22. The specific cytotoxicity is calculated according to the formula: specific release = [(test release – spontaneous release)/(total release – spontaneous release)] × 100. The spontaneous cytotoxicity usually ranges around 10% of the total release and

should not exceed 20%. The assay is normally performed in triplicates, and difference between individual wells should be less than 5%. An effector : target ratio of 50:1 yields typically 40% specific cytotoxicity in a 4 h assay using enriched NK cells as effectors. The assay can be modified according to specific needs. MAb can be tested for their function by preincubation with either target or effector cells. The effect of cytokines can be tested by preincubation of the effector cells.

Acknowledgments

The Basel Institute for Immunology was founded and is supported by F. Hoffmann-La Roche & Co. Ltd., CH 4005 Basel, Switzerland.

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Analyzing Nonspecific Cytotoxic Cells in Fish

Sandra Ristow, Donald L. Evans, and Liliana Jaso-Friedmann

1. Introduction

The nonspecific cytotoxic cell (NCC) is believed to be the evolutionary precursor of the mammalian natural killer (NK) cell. Evidence for a phylogenetic linkage between NCCs of teleost fish and NK cells from higher vertebrates was first obtained functionally; that is, NCCs lyse many of the same target cells as human (K562), mouse (YAC-1) and rat NK cells ***(1,2)***. The best parallels between NK and NCC are found in the target cell recognition of NCC vs adherent lymphokine (interleukin-2 [IL-2]) activated killer cells (ALAK). Both ALAK and NCC kill promiscuously. Their wide range of targets include many different histological types of allogeneic tumor cells, xenogeneic tumor targets, protozoan parasites, as well as certain virus transformed cells ***(3,4)***. Beyond the observations that both types of cells kill the same targets, two other properties link NK cells with NCC.

First, studies of the morphology of NK cells in mammals have provided insight into a possible phylogenetic relationship between these cells and NCC. Although the predominant NK morphology described for most species is that of large granular lymphocytes (LGLs), porcine, and avian NK cells and fish NCCs have NK-like cell activity exhibited by small agranular lymphocytes. Unlike fish, the porcine and avian (chicken) species also have other cells with NK activity that have LGL morphology. Second, the most common property of cells used to compare similarities is cell phenotype. Almost without exception, most antibodies, including those with cluster of differentiation (CD) activities, are not useful for detection of interspecies crossreactivities. One exception to this rule is a monoclonal antibody (MAb) generated against catfish NCC, the antibody 5C6 ***(5,6)***. This MAb detects a 32-kDa protein on catfish NCC and is

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
Edited by: K. S. Campbell and M. Colonna © Humana Press Inc., Totowa, NJ

also reactive with this receptor homologue on NK cells from many different species including humans, rats, pigs, cattle, and chickens.

NCCs were first described in fish almost 15 yr ago by their ability to kill nonadherent transformed human B cells (Daudi, P3HR-1, IM-9). Subsequently, studies of NCCs in catfish and their effector cell counterparts in the frog, rainbow trout, tilapia, damselfish, and carp have demonstrated that these cells are widespread in cold-blooded vertebrates ***(7,8)***. Of additional interest is that both marine and freshwater, as well as warm water and cold water teleosts, possess these cells. Also, because vertebrates lower on the phylogenetic scale (i.e., anurans) have NCC-like cells, the assumption is that cells with such widespread occurrence may be essential participants in innate responses.

2. Materials

2.1. Fish

1. Catfish: Outbred catfish of mixed sex are purchased from local commercial suppliers. The optimum size for obtaining peak cytotoxicity with catfish cells is 10–15 g.
2. Rainbow trout: Trout of mixed sex are also purchased from hatcheries. (It is preferable to use rainbow trout larger than 15 g in size).

2.2. Anesthetics

1. Anesthetic solutions for catfish: 3-Aminobenzoic acid diluted to 100–200 ppm is used to anesthetize catfish. A dilution of 0.5 g/L of 3-aminobenzoic acid will kill catfish.
2. Anesthetic solution for trout: 2-phenoxyethanol (Sigma no. P1126) (3 mL in 4 gal of water from the culture system) is used for anesthetizing rainbow trout (*see* **Note 1**).

2.3. Isolation of Nonspecific Cytotoxic Cells

1. Dissection instruments: Sterile scalpels and scalpel handles, sterile surgical scissors, and sterile forceps are needed for dissection.
2. Plasticware: Sterile plastic Petri dishes are required for holding dissected tissues. Fifteen- and 50-mL sterile centrifuge tubes and 96-well round- or flat-bottomed microtiter plates are essential for the performance of assays. In addition, sterile plastic pipets (1 and 10 mL) and micropipetor tips are required (100–200 µL, 500 µL).
3. Blood drawing equipment for trout: 3 mL disposable plastic sterile syringes containing 100 U of sodium heparin are utilized. Fourteen-gage needles are needed to perform heart puncture on large rainbow trout weighing 500–1000 g.
4. Screens: Sterile 100 mesh screens are necessary for separating the lymphocyte populations from the tissue matrix from spleens and anterior kidneys. Screens may be flat for placement over a tube or other vessel. Alternatively, they may be bent into a cup like form, which some investigators find to be more useful.

5. Hemacytometer: Hemacytometers are necessary for counting cells, both effectors and targets (*see* **Note 2**).
6. Medium for washes and assay of catfish cells: Two different media are used for experiments with catfish cells. Catfish complete medium contains RPMI 1640 (Cellgro, Media Tech, Washington, DC) supplemented with 10% fetal bovine serum (FBS). Complete medium is used to dilute and deliver target and effector cells. Buffered saline PBS/BSA/Glucose (phosphate-buffered saline [PBS] pH 7.4, 0.5% bovine serum albumin [BSA], 20 m*M* glucose] may be used as alternative to complete catfish medium (*see* **Note 3**).
7. Medium for washes and assay of rainbow trout cells: Unsupplemented RPMI 1640 at 4°C is used for washes. RPMI 1640 supplemented with 10% FBS containing 100 U/mL of penicillin and 100 µg/mL of streptomycin is the complete medium for the assay.
8. Centrifuge: A tabletop centrifuge such as the Beckman GPR is utilized to wash and separate the cell populations.
9. Separation medium for catfish NCCs: Percoll (Sigma Chemical, St. Louis, MO).
10. Separation medium for rainbow trout NCCs: Histopaque 1.077 (Sigma no. 1077-1).

2.4. Target Cells

1. Target cells: All target cells may be obtained from the American Type Tissue Culture Collection (ATCC). Targets most sensitive to NCC lysis are Epstein-Barr virus transformed human B cells (Daudi, P3HR-1). Other targets include U937, HL-60, YAC-1, MOLT-4, K562, and IM-9.
2. Radioactive label for targets: A sterile solution of ^{51}Cr as sodium chromate is obtained from New England Nuclear (NEZ 030S), or Amersham (CJS.11, Arlington Heights, IL). The specific activity of this material is (14.8-44.4TBq)/g (NEZ 030S).
3. Geiger counter: A Geiger counter such as the Ludlum model 3 survey meter with the 44-3 probe is needed for detection of contamination with ^{51}Cr and unshielded sources.
4. Lead safety wraps: The ^{51}Cr solution should be kept in its lead container and tubes containing high concentrations of isotope should be wrapped in malleable lead shielding.
5. Disposable gloves are necessary for handling isotopes.

2.5. Cytototoxicity and Conjugation Assays

1. Incubators for catfish NCC assays: Cytotoxicity assays for catfish are conducted in a 37°C incubator.
2. Incubators for trout NCC assays: A low-temperature incubator (achieving 12–18°C) normally used for assays of biological oxygen demand is necessary.
3. Gas boxes: Gas boxes (C.B.S. Scientific, Delmar, CA) are required for incubating plates if the incubator does not have a CO_2 gassing system.
4. Carbon dioxide: Tanks of 5% CO_2 in air are used for gassing plates containing the assays before placing them in the incubator.
5. 2% Solution of sodium dodecyl sulfate.

6. 0.2% Solution of trypan blue.
7. Disposable counting tubes appropriate for collection of supernatants from assay.
8. Gamma counter: Access to a gamma counting device with a ^{51}Cr channel is required for counting supernatants from each test.
9. Agarose for catfish conjugation assay: Agarose must be Grade A (i.e., devoid of any charges), which can be purchased from Calbiochem. Generally speaking, any high-quality agarose is suitable.

3. Method

3.1. Culture of Fish

Fish whose lymphocytes are to be used in NCC assays must be kept under the best possible conditions to allow the production of fish populations with highly consistent levels of health and behavior. Success will be assured providing proper husbandry practices are maintained.

3.1.1. Culture of Catfish

1. Maintain catfish at ambient water temperatures in the range 5–28°C. Optimum temperature for obtaining high levels of active NCCs is in the 15–20°C range (*see* **Note 4**).
2. Feed catfish on a regular basis with pelleted Salmon Crumbles no. 4 (Zeigler Brothers, Gardners, PA.) (*see* **Note 5**).
3. Keep catfish rearing facilities dark and quiet. Restrict the traffic.
4. Perform water quality checks 2–3× weekly. Test temperatures, pH, oxygen levels, nitrites, and ammonia nitrogen. Oxygen levels must be adequate and compressors and diffusers should be working optimally to ensure oxygen saturation.

3.1.2. Culture of Trout

1. Maintain the water temperature between 4°C and 20°C (*see* **Note 6**).
2. Maintain rainbow trout on an adequate diet such as Oregon Moist Pellet, Rangen, or Biodiet (*see* **Note 7**).
3. Maintain trout in raceways or in recirculating systems.
4. Adjust lighting systems in the area so they are diurnal and mimic the season.

3.1.3. Rules for Maintaining Healthy Fish Populations (see **Note 8**)

1. Do not return experimental animals to the holding tanks after use.
2. Never use fish that are undergoing treatments for disease outbreaks for experiments.
3. Never return unused fish to the wild.

3.2. Anesthetizing or Killing Fish

3.2.1. Anesthetizing Catfish

1. Anesthetize catfish by immersion in 3-aminobenzoic acid ethyl ester. Catfish are sedated at 0.1–0.2 g/L (100–200 ppm).

2. Kill catfish by immersion in 3-aminobenzoic acid ethyl ester at 500–1000 ppm (0.5 g/L).

3.2.2. Anesthetizing Trout

Anesthetize trout with 3 mL of 2-phenoxyethanol in 4 gal of water from the system in which the trout are maintained. Immersion of trout in a bucket of solution usually produces sedation in about 2 min (*see* **Note 9**).

3.3. Retrieval of Lymphocytes from Tissues

If you are testing peripheral blood NCC levels, you may withdraw blood in a heparinized syringe from the trout or catfish by either heart puncture or through the caudal vein. It is best to withdraw blood when the fish is anesthetized rather than after killing it. It is therefore important that the technique for anesthetizing fish described in **Subheading 2.2.** is followed rigorously.

3.3.1. Bleeding Fish from the Heart

1. Anesthetize the fish (*see* **Note 9**).
2. Place the fish on its back, holding the fish in place on a drape.
3. Locate the soft tissue just below the jaw and insert the heparinized syringe 1/4 in. below the "V" in the jaw.
4. Insert the needle and slowly withdraw the plunger until the blood streams from the heart back into the syringe. One and one-half milliliters of blood may be removed from a 1000 g trout (*see* **Note 10**).

3.3.2. Alternative Method for Withdrawing Peripheral Blood by the Caudal Vein

1. Anesthetize the fish (*see* **Note 9**).
2. Insert a needle into the caudal vein located about 1/4 in. below the lateral line directly above the anus.
3. Insert the needle into the anesthetized fish and slowly pull back on the plunger. The blood will stream back into the barrel of the syringe.

3.3.3. Centrifuging and Washing the Peripheral Blood Lymphocytes of the Trout

1. Remove the needle from the syringe and expel the blood into a sterile 15-mL centrifuge tube containing a fivefold volume of RPMI 1640.
2. Place the sample over a bed of ice.
3. Underlay the mixture with 6 mL of Histopaque 1.077 (at 20°C).
4. Centrifuge the layers at 1500*g* at 15°C for 40 min with the brake on the centrifuge in the "off" position.
5. Retrieve the lymphocytes at the interface of the two liquids utilizing a sterile Pasteur pipet.

6. Wash the cells three times in 15 mL of RPMI 1640 and resuspend them in 2 mL of complete medium.
7. Adjust the cell numbers to 1×10^7/mL utilizing a hemacytometer (*see* **Note 2**).

3.3.4. Recovery of Cells from the Anterior Kidney of a Trout

1. Dissect the anterior kidney from the killed fish by first placing the fish on a paper drape.
2. Pour 70% ethanol over the surface of the fish.
3. Using a scalpel, forceps, and surgical scissors cut away the muscle tissue on the side of the fish behind the operculum so that the internal organs are exposed.
4. Cut the heart and liver away from the body cavity which will expose the anterior kidney next to the spine.
5. With another sterile scissors and fine forceps, remove no more than the anterior third of the pulpy organ.
6. Place the tissue on a sterile screen above a Petri dish containing 5–10 mL RPMI 1640.
7. Gently press the tissue through the screen with a rubber tipped plunger from a 3- or 5-mL plastic syringe.
8. Rinse the screen to loosen adherent lymphocytes and collect the screened tissue in RPMI 1640 in a plastic centrifuge tube.
9. Underlay the mixture with 6 mL of Histopaque and centrifuge at 1500*g* for 40 min with the brake off.
10. Collect the cells and rinse them once in RPMI 1640.
11. Resuspend the cells in 2 mL of complete medium.
12. Count and adjust cell numbers to 1×10^7/mL.

3.3.5. Recovery of Cells from the Spleen of the Trout

1. Dissect the spleen from the fat just below the stomach, removing as much of the fatty tissue as possible.
2. Place the spleen on a sterile 100-mesh screen and snip the tissue into small pieces.
3. Proceed as described previously with the anterior kidney tissue (**steps 6–8**), pressing the materials through the screen.
4. Isolate the lymphocytes at the interface between the Histopaque and the RPMI 1640.

3.3.6. Modifications of the Lymphocyte Isolation Procedure for Catfish NCCs

1. Layer a total of 3×10^7 total cells in 2 mL of medium over 4 mL of 45.5% Percoll in a sterile 15-mL centrifuge tube.
2. Centrifuge the layers for 20–30 min at 400*g*.
3. Harvest the cells from the Percoll interphase with a Pasteur pipet.
4. Wash and count the cells. Wash the cells in the same media used in the assay (either PBS/BSA/glucose or RPMI-1640/10% FBS).
5. Dilute the cells appropriately in assay medium such that the effector:target ratios of 160:1, 80:1, 40:1, and 20:1 may be achieved (*see* **Note 11**).
6. Deliver the cells to the microwell plates in 100-µL aliquots.

3.4. Preparation of Target Populations

3.4.1. Tissue Culture

1. Maintain target cells as continuous cell lines in RPMI 1640 with 10% FBS at 5% CO_2 and in a 37°C incubator (*see* **Note 12**).
2. Split the targets at least 18 h prior to labeling (*see* **Note 13**).
3. Count the cells with trypan blue and make sure that viability is greater than 90% (*see* **Note 14**).

3.4.2. Labeling Target Cells with ^{51}Chromium

1. Count out a minimum of 10×10^6 cells for labeling.
2. Centrifuge cells at 300*g* for 10 min into a soft pellet.
3. Pour off excess medium.
4. Add 200 µCi of ^{51}Cr and RPMI 1640 medium to the pellet to a final volume of 1 mL.
5. Cap the suspension and vortex-mix.
6. Place the suspension of cells in the incubator for 1–2 h (*see* **Note 15**). Minimum labeling time is 1–2 h, but may be increased if the age of the chromium is approaching one half-life.
7. Hand vortex-mix or shake the tube every 30 min.
8. Wash the cells free of unincorporated ^{51}Cr by washing in plain RPMI 1640 medium and centrifuging the cells three times at 300*g*.
9. Count the cells in a hemocytometer.
10. Resuspend the cells at 10^5/mL in complete medium (*see* **Note 14**).

3.5. Assembling the Cytotoxicity Assay

1. Dispense 10^4 washed ^{51}Cr-labeled target cells in 100-µL aliquots into the round bottom wells of the 96-well microtiter plates.
2. Dispense the effector lymphocytes in 100-µL aliquots into the wells at appropriate effector target ratios (i.e., 160:1; 80:1; 40:1; 20:1 or 100:1; 50:1; 25:1; 12.5:1).
3. Perform each test in triplicate or quadruplicate to achieve statistical significance.
4. Designate at least four wells on each plate for measurement of complete release of the radionucleide. These wells contain 100 µL of target cells plus 100 µL of 2% sodium dodecyl sulfate.
5. Designate at least four wells on each plate for a spontaneous release test. These wells contain 10^4 target cells plus 100 µL of complete medium.
6. After adding effectors to target cells the plate is centrifuged for 3 min at 200*g* before incubation.

3.6. Incubating and Harvesting the Cytotoxicity Assay

1. Incubate the assay plates for the designated number of hours (4–18 h for trout NCCs; 6 h for catfish NCCs) under a 5% CO_2 atmosphere (*see* **Note 16**). Assays of catfish NCCs are conducted at 25–37°C for 4–6 h.
2. Harvest the assays by pipetting 100 µL of supernatant from each well into glass tubes.

3. Count each sample in the gamma counter.
4. Calculate the percent specific chromium release by the following formula:

$$\%\text{ specific release} = \frac{\text{CPM(experimental)} - \text{CPM(spontaneous)}}{\text{CPM(total)} - \text{CPM(spontaneous)}} \times 100$$

where CPM = counts per min, and spontaneous release equals radioactivity in wells receiving target cells plus 100 µL of complete medium. Total release is the counts in the wells receiving 100 µL of 2% sodium dodecyl sulfate.

3.7. Conjugate Formation and Recycling Capacity Analysis for Catfish NCCs

The details of this procedure have previously been published ***(9)***.

1. Equilibrate the catfish NCCs and target cells (2×10^6 cells/mL of each) at 22°C.
2. Mix 100 µL of NCC and 200 µL of targets at 22°C for 5 min.
3. Centrifuge the cells together at 200*g* for 5 min.
4. Remove the supernatant carefully.
5. Resuspend the pellet in 50 µL RPMI-1640 (10% FBS).
6. Add 50 µL of 1.3% agarose at 39°C and resuspend the effectors and target cells.
7. Spread the cells onto a precoated (2.0% agarose) slide.
8. Air-dry the slides for 1–2 min.
9. Submerge the slides in RPMI-1640 at 26°C for 3 h.
10. Stain the slides in 0.2% trypan blue for 5 min.
11. Wash the slides with PBS.
12. Fix the slides in 0.5% formalin.
13. Determine the percentage of target binding cells (conjugates) by counting 300 NCCs and note the number of NCCs bound to target cells.
14. Determine the cytotoxicity by counting the number of dead targets in approx 100 conjugates.
15. Determine spontaneous death by counting the number of dead targets on slides without added effector cells.
16. Calculate the percentage of killed conjugates. The percentage of killed conjugates = (% dead targets in conjugates) – (% spontaneous dead targets × % dead targets in conjugates).
17. Determine the percentage of active NCCs by multiplying the percentage of target binding cells by the percentage of dead conjugates.

3.8. Calculation of V_{max} and K_m

The cytotoxicity assay to determine V_{max} and K_{m} is performed in round-bottomed microtiter plates using a final volume of 200 µL.

1. Make a series of six 1:2 dilutions of target cells (TC) with the highest concentration at 4×10^6 cells/mL.
2. Add 100 µL of each dilution to each well along with 1×10^6 effector cells in a 100-µL volume. (Make four replicates).

3. Incubate effectors and conjugates for 3 h.
4. Determine the % specific release.
5. Obtain the number of TCs killed by the following formula: Number of TCs killed = % killed TCs × total number TCs present in a given microtiter well.
6. Use the following formula to plot the dose–response curve, which is expressed by Michaelis–Menten kinetics *(7)*:

$$V = \frac{V_{max} \times T}{K_m + T}$$

where V = no. of killed TCs, T = starting no. of TCs, V_{max} = no. of TCs killed when the TC concentration approaches infinity, K_m = no. of TCs that produce $V_{max}/2$.

The V_{max} and K_m for the varying E:T ratios is determined by the Lineweaver–Burk equation *(7)*.

$$\frac{1}{V} = \frac{K_m}{V_{max}} \times \frac{1}{T} + \frac{1}{V_{max}}$$

1/V is plotted against $1/T$ to give a straight line. V_{max} is the reciprocal of the Y-intercept. K_m is the negative reciprocal of the X-intercept.

3.9. Maximum Recycling Capacity (MRC)

The MRC of NK cells or NCC represents the relative absolute numbers of these cells required to kill targets within a given period of time. Low numbers of NK cells would be required to kill targets if a single NK cell could recycle between many different target cells. If, however, following a single "hit" of an NK cell and a target, the effector failed to dissociate and contact a second target, more effectors would be required to achieve the same level of conjugates compared to a recycling NK cell. An MRC value could then be used to compare NCC and NK cells regarding: numbers of cells recruited from lymphoid tissue during protozoan infections; numbers of cells required to kill an individual tumor cell; killing efficiencies; and mechanisms of killing. For example, in a 3-h cytotoxicity assay, mammalian NK cells have been shown to recycle an average of 2.8× in the presence of MOLT-4 cells and 3.8× for K-562 targets. NCCs however, do not recycle in a 3-h assay in the presence of NC-37 targets. The MRC can be calculated using the following equation:

$$\text{MRC} = V_{max}/(\%\ \text{active NCC} \times \text{no. of effectors}).$$

4. Notes

1. Although MS-222, tricaine methanesulfonate, is routinely used in anesthetizing trout, the authors have found that lymphocytes from 2-phenoxyethanol-anesthetized trout display better NCC killing.

2. We have found it useful when enumerating cells to count 10 fields (including the center grid which is usually reserved for counting red blood cells) and then multiplying the number of cells counted by 1000 and then multiplying by the dilution. This gives a value equal to the number of cells per milliliter. Accuracy in determining cell counts is very important and is greatly amplified by counting 10 fields.
3. In the older literature, catfish medium was made up to an osmolality different from the regular formulation supplied for mammalian cell culture. Recently, it has been found that there is no difference in cell lysis when the regular formulations are used in the assay.
4. Maintaining catfish at temperatures above 28°C is stressful and can be immunosuppressive.
5. Catfish weighing less than 5 g are fed daily and those larger than 15 g may be fed on alternate days. Overfeeding should be avoided.
6. Optimum temperatures for raising trout range between 12°C and 17°C. Temperatures above this range stress the fish.
7. Food pellets are supplied in various sizes to accommodate different sizes of fish. It is important not to overfeed trout because extra food and feces in the water contribute to the biological oxygen demand.
8. Fish are among the most fastidious and difficult animals to maintain under laboratory conditions. They are exceptionally sensitive to changes in water quality, overcrowding, temperature changes, noise, light, and the presence of human beings. Stress can lead to marked changes in NCC response; i.e., stressed channel catfish do not have active cytotoxic NCCs in the anterior kidney tissue. In most instances where one is unable to obtain positive cytotoxic results, there is an underlying stressor at work. After removal of the stressor, it may take as long as 3 wk for the fish to regain normal levels of cytotoxic activity.
9. Watch for the trout to gently drift to the surface. When properly anesthetized, trout will not startle when touched. If blood is collected by either heart puncture or from the caudal vein, the fish must be properly anesthetized to obtain a good sample.
10. The trout will recover if this method is used and if no more than 1.5 mL of blood is withdrawn.
11. To achieve the 160:1 target:effector ratio, the initial concentration of effectors to be delivered is 1.6×10^7. Likewise, the initial concentration of effectors is 1.0×10^7 for a 100:1 effector:target ratio.
12. Cells may be incubated with or without antibiotics. Antibiotics consist of penicillin (100 U/mL) and streptomycin (100 μg/mL).
13. Split ratios are low and are a characteristic of each individual target cell line. The split ratio must be sufficiently low so that at the time of harvest the indicator in the media will not have turned yellow (acid), but is a rose color.
14. Proper timing for cell labeling is critical because cells must be in log phase of growth and intact to hold the ^{51}Cr label. If viability is less than 90% the assay should not continue.
15. 1×10^7 YAC-1 targets (used for trout NCC) are labeled with 200 μCi of ^{51}Cr for 1.5 h in 0.5 mL of RPMI 1640 at 37°C in a 5% CO_2 incubator. The tube may be placed in a malleable lead wrap to minimize exposure of workers to the radiation.

16. Assays of trout NCCs may be run at various temperatures from 10°C to 22°C. Incubate the assays with rainbow trout effectors 4–18 h. Eighteen hours is usually required for complete killing.

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Natural Killer Cells and Immunotoxicology

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1. Introduction

Immunotoxicology can be simply defined as the study of adverse effects to the immune system. However, because of the complexity of the normal immune system, adverse effects by a xenobiotic can alter the system in a variety of ways: suppression, upregulation as in the case of xenobiotic-induced autoimmunity or hypersensitivity, or specific alterations of certain types of cells. During the last 20 yr, many observations have been made concerning chemical or drug-induced immunosuppression ***(1–3)***. Examples of these include polyhalogenated aromatic hydrocarbons such as TCDD, polycyclic aromatic hydrocarbons such as benzo(a)pyrene, and drugs (opioids, cannabinoids, and lithium). Typically, alterations to the immune system are manifested in decreased host resistance. The immune system works in a concerted way to prevent invasion by a foreign substance. Therefore, when the system is damaged, either the defenses break down so that the foreign agent can enter the body and cause disease, or the immune system itself may attack the body as in the case of hypersensitivity.

Natural killer (NK) cells, as components of the innate immune system, are the first line of defense against cancerous cells and infectious agents. In other words, they do not require prior exposure to the antigen to elicit a response. In fact, NK function was originally defined by the cell's ability to kill NK-sensitive tumor cells (ex. YAC-1 cells). NK cells are also able to lyse virus- or bacteria-infected fibroblasts, epithelial cells, and monocytes as a means to eliminating the infectious agent. In addition, NK cells can eliminate other foreign organisms through the release of cytokines, which recruit and activate neutrophils ***(4)***. Because NK cells have such a unique, nonspecific role for the early elimination of infectious agents and immune surveillance against certain

From: *Methods in Molecular Biology, vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*
Edited by: K. S. Campbell and M. Colonna © Humana Press Inc., Totowa, NJ

tumor types, alterations of NK function can be an important determinant in the evaluation of xenobiotics for immunotoxicity.

Investigation of NK cell activity in immunotoxicological evaluations has become standard in delineating a chemical's effect on natural immunity. Many xenobiotics affect NK activity in addition to other immune parameters, resulting in a cumulative effect on the animal's resistance to infection. However, it has also been shown that certain chemicals selectively affect NK cells. Smialowicz et al. showed that nickel (Ni) suppressed NK activity in both male and female rats, and for both allogeneic W/Fu-G1 target cells as well as xenogeneic YAC-1 target cells. No other measured immune parameters were affected, including proliferative responses to B- and T-cell mitogens and primary immunogloblulin M (IgM) response to sRBC. Yet, the Ni-induced suppression of NK activity was manifested by an increase in mortality of rats injected with MADB106 tumor cells ***(5)***. This study exemplifies that inhibition of NK activity alone is sufficient to cause an increased susceptibility to tumors. This increased tumor burden associated with increased suppression of NK activity was also demonstrated by Wilson ***(6)***. It was demonstrated that at least a 50–60% or greater inhibition of activity is required before changes in host resistance to B16F10 melanoma are apparent. Therefore, it is important to investigate any potential for toxicity to NK cells during an immunotoxicological evaluation of a xenobiotic.

2. Materials

2.1. Phenotyping by Flow Cytometry

1. Spleens from xenobiotic-treated and/or untreated mice.
2. Complete RPMI medium: RPMI 1640, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 m*M* of L-glutamine, 15 m*M* HEPES buffer, and 10 mL/L of 7.5% sodium bicarbonate solution (all from Gibco, Grand Island, NY). RPMI complete is supplemented with 10% fetal bovine serum (FBS), and 50 m*M* of 2-mercaptoethanol (2-ME; Sigma, St. Louis, MO).
3. 96-well U-bottomed microtiter plate.
4. Staining buffer: 100 mL of phosphate-buffered saline (PBS), 1 g of bovine serum albumin (BSA; Sigma), 1 mL of NaN_3 (10% solution; Sigma).
5. Monoclonal antibodies diluted 1:100 in staining buffer: fluorescein isothiocyanate (FITC)-conjugated anti-CD3ε (clone 145-2C11; hamster IgG; PharMingen), phycoerythrin (PE)-conjugated anti-NK1.1 (clone PK136; mouse $IgG_{2a}\kappa$; PharMingen) and purified anti-CD16/CD32 (clone 2.4G2; rat $IgG_{2b}\kappa$; PharMingen) to prevent nonspecific binding to Fc receptors.
6. Propidium iodide (PI; Sigma): Stock solution: 0.1 mg/mL of PI diluted in staining buffer; working solution: 1:20 dilution of stock solution in staining buffer.

2.2. ^{51}Chromium (^{51}Cr) Release Assay

1. NK cells: spleen cells from 6–8-wk old animals (B6C3F1 mice) that have been xenobiotic treated and/or untreated.

2. Freshly passaged (within 2–3 d) YAC-1 Mouse Moloney leukemia cell line (American Type Culture Collection; Rockville, MD).
3. ^{51}Cr (Na_2CrO_4), 1 mCi/mL (New England Nuclear or Amersham).
4. Hanks' balanced salt solution (HBSS; GIBCO).
5. RPMI 1640 medium (GIBCO).
6. FBS (Hyclone Labs).
7. Penicillin/streptomycin (GIBCO).
8. L-Glutamine (GIBCO).
9. 2-ME (Sigma).
10. *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (Sigma).
11. 0.1% Triton X-100 (Sigma T9284).
12. Trypan blue (0.04%; Sigma).
13. U-bottomed polystyrene 96-well microtiter plates with lids.
14. 8.8 × 45 mm plastic microtiter tubes and holder.
15. Gamma counter.

3. Methods

3.1. Phenotyping by Flow Cytometry

3.1.1. Preparation of Cells

1. Vehicle or treated mice are killed and spleens placed into 3 mL of HBSS.
2. The spleens are prepared into a single-cell suspension by mincing the spleens between the frosted ends of microscope slides.
3. After centrifugation at 300*g* for 10 min, cells are resuspended in complete RPMI medium and counted.
4. Into each well of a 96-well microtiter plate, 1×10^6 cells are pipetted. The volume in each well is increased up to 200 µL with staining buffer and the plate is centrifuged at 350*g* for 2 min.
5. Prior to the addition of antibodies, the supernatant is removed and the plate is mixed gently to disengage the cells from the plate surface.

3.1.2. Staining of Cells

1. Fluorescence-conjugated antibodies to NK1.1 (PE) and CD3ε (FITC) are diluted 1:100 and 100 µL are added to the appropriate wells on the microtiter plate. The plate is kept on ice for 30–45 min in the dark.
2. After the incubation period, the plate is centrifuged at 350*g* for 2 min and the supernatant is removed from each well.
3. The plate is gently mixed and the cells are resuspended in a solution of PI.
4. After 5 min of incubation in the dark, the plate is centrifuged and the cells are resuspended in staining buffer. The cells are now ready for analysis on the flow cytometer.

3.1.3. Analysis of NK Cells

Nonviable cells (PI-positive cells) are excluded by either live gating or by gating during analysis. The percentage of positive staining cells can be calcu-

lated from both four quadrant dot plot (log green fluorescence vs log orange fluorescence) and histogram displays. Data are also presented as the absolute numbers of cells per spleen. This is determined by multiplying the percentage of positive cells by the total number of cells in the spleen.

3.2. ^{51}Cr Release Assay

3.2.1. Preparation of Target Cells

1. The target cell, YAC-1 cells, are grown in continuous culture in complete RPMI at 37°C in 5% CO_2, and maintained in log growth phase.
2. On the assay day, approx 1×10^7 YAC-1 cells are sedimented in a 15-mL conical tube at 300*g* for 10 min.
3. The supernatant is removed and the cells are resuspended in 500 μCi of $Na_2{}^{51}CrO_4$ (500 μL) and incubated for 90 min at 37°C in 5% CO_2. Cells are gently mixed every 10–15 min during the incubation period.
4. The cells are then washed once in room temperature HBSS and once in complete RPMI by centrifugation at 300*g* for 10 min. After washing, the cells are resuspended in 15 mL of complete RPMI and allowed to sit at room temperature for 30 min.
5. The cells are washed twice more in complete RPMI prior to counting with a hemacytometer. Viability is determined by trypan blue exclusion and is typically greater than 90%. Cell concentration is adjusted to 1×10^5 cells/mL

3.2.2. Preparation of Effector Cells

1. Vehicle or treated mice are killed and spleens placed into 3 mL of HBSS.
2. The spleens are prepared into a single-cell suspension by mincing the spleens between the frosted ends of microscope slides.
3. After centrifugation at 300*g* for 10 min, cells are resuspended in complete RPMI media and counted. A final concentration of 2×10^7 cells/mL is prepared in complete RPMI.

3.2.3. Preparation of Plate

1. A sample of 200 μL of the spleen cell suspension is added to the first two wells of each row of a 96-well U-bottomed culture plate and 100 μL of complete RPMI is added to the remaining wells.
2. Serial dilutions (100 μL to adjacent wells) are performed to yield final effector:target (E:T) ratios of 200:1, 100:1, 50:1, 25:1, 12.5:1, and 6.25:1.
3. To each well, 100 μL (1×10^4 cells) of the ^{51}Chromium-labeled YAC-1 target cells are added. For determination of maximum release, 100 μL of target cells are added to 100 μL of a 0.1% Tween solution. To determine spontaneous release, 100 μL of target cells are added to 100 μL of complete RPMI. Replicates of 12 wells were run for both maximum and spontaneous release.
4. After a 4-h incubation at 37°C in 5% CO_2, the plates are centrifuged at 300*g* for 10 min and 100 μL of the supernatant is aspirated and transferred to microtiter tubes *(7)*.

3.2.4. Analysis of Data

Counts per minute (CPM) are determined for each tube on a gamma counter. Percent specific release or percent lysis of the target cells is calculated as follows:

$$\% \text{ Specific release} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

Data are also expressed as lytic units which is defined as the number of cells required to lyse a given percentage of the target cells. This allows for the conversion of the % specific release at each E:T ratio into a single value. Lytic units are calculated from values derived from the Allfit Program *(8)*, which generates a best fit curve based on individual curves derived from the different E:T ratios for each animal within a group. The curved is generated from the formula shown below:

$$Y = \frac{a - d}{1 + (X/c)b} + d$$

where Y is the% specific release, X is the number of cells required to yield Y, a is the minimum response (i.e., at $X = 0$), d is the maximum response and c is the value that yields a % specific release midway between a and d. The data are expressed as either lytic units per 10^7 splenocytes or per spleen. The routine use of this assay in immunotoxicology evaluations requires continually reproducible results. We have noted through the years that numerous factors can contribute to variability of the assay (*see* **Notes 1–5**). Our historical control ranges of cytotoxicity responses by rodent NK cells can be found in **Notes 6** and **7**.

4. Notes

4.1. Sources of Variability

1. Variability due to species/source of effector cells: Several different species can be used in the assessment of NK cell activity in an immunotoxicological evaluation. Our laboratory typically uses mouse or rat. The assay can also be done using primates, including humans. For analysis of NK function in the rodent system, spleens are usually used as the source of effector cells. For primates and sometimes rats, blood is used to collect effector cells. Historically, blood yields higher NK activity than spleen.
2. Variability due to animal strain: When using rodents for the chromium release assay, it is important to consider the strain of animal. For example, certain mouse strains have higher spontaneous activity than other strains. C3H, CBA, and nude mice are several strains with high activity.
3. Variability due to age of animals: Age can sometimes be a factor in determination of NK cell activity using the ^{51}Chromium release assay. There is spontane-

ous NK activity in spleens of rodents between 4 and 8 wk of age. After this time, activity decreases. Therefore, it is important to use mice or rats that are 4–8 wk old for determination of an immunotoxic effect to NK cells. However, to observe an NK cell enhancing effect, animals that are 10 wk or older should be used so that the background activity is low ***(4)***.

4. Variability due to target cells used: The YAC-1 leukemia cell line is the only target cell that our laboratory has used. For these cells, several things must be kept in mind concerning when they are used in the assay. The minimum time between passages is 5 d and the maximum time is 15 d. This ensures that the cells are maintained in the log growth phase. In addition, the cells should be used in the assay 2 d after passage.
5. Variability due to circannual rhythms: Interestingly, there have been reports of observed differences in NK cell activity depending on the time of year. Pati et al. reported that the maximum and minimum splenic NK cell activities were observed in January–February and July–August, respectively. The data were collected from a total of 356 mice over a period of 5 yr ***(9)***. Our laboratory has also observed this type of seasonal variability with NK activity.

4.2. Historical Control Ranges in Our Laboratory

6. Historical control range for mouse (B6C3F1 Female): The range of % lysis for spleen cells at an effector: target cell ratio of 100:1 is between 5% and 15%.
7. Historical control range for rat (Fischer 344 Female): The range of % lysis for spleen cells at an effector: target cell ratio of 100:1 is between 10 and 25% and 20% and 30% in the peripheral blood.

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Appendices

I. Antibodies to Human Natural Killer Cell Receptors

The listed sources of antibodies are original authors, companies or hybridoma bank resources. The hybridoma bank resources listed are American Type Culture Collection (ATCC, Manassas, VA; www.atcc.org and the Developmental Studies Hybridoma Bank (DSHB, Iowa City, IA; www.uiowa.edu/~dshbwww).

Anti-Killer Cell Immunoglobulin-Like Receptor (KIR, CD158)

KIR constitutes a family of polymorphic gene products that exhibit variable inter-individual and inter-clonal expression on NK cells and some subsets of T cells. Expression is genetically determined and does not correlate with MHC class I haplotype. Antibodies to some isoforms of KIR have not been reported, and the cross reactivities of most antibodies should be appreciated when typing receptor expression on NK cells. Some additional crossreactivities of these antibodies have been reported on KIR transfectants, which should also be appreciated.

Clone	Specificity	Species/Isotype	Source/Reference
EB6	KIR2DL1, KIR2DS1 (CD158a)	mouse IgG1	Beckman Coulter, Serotec ***(1)***
GL183	KIR2DL3, KIR2DS2 KIR2DL2(CD158b)	mouse IgG1	Beckman Coulter, Serotec ***(1)***
DX9	KIR3DL1	mouse IgG1	Biosource, Becton Dickinson, PharMingen ***(2)***
Z27.3.7	KIR3DL1	mouse IgG1	Beckman Coulter ***(3,4)***
HP-3E4	KIR2DL1, KIR2DS1, KIR2DS4	mouse IgM	Becton Dickinson, Biosource, PharMingen ***(5–7)***

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Clone	Specificity	Species/Isotype	Source/Reference
5.133	KIR3DL1, KIR3DL2, KIR2DS4	mouse IgG1	M. Colonna, Basel ***(7)***
Q66	KIR3DL2	mouse IgM	Beckman Coulter ***(8)***
DX31	KIR3DL2	mouse IgG2a	L. Lanier, San Francisco, CA ***(6)***
PAX250	KIR2DS4	mouse IgG1	L. Moretta, Genova ***(9)***
FES172	KIR2DS4	mouse IgG2a	Beckman Coulter ***(9)***

Anti-CD94/NKG2

CD94 (*Kp43*) is expressed as a heterodimer with various isoforms of NKG2 family of polypeptides. The NKG2 isoforms are expressed variably between clones within an individual on most NK cells and a subset of T cells.

Clone	Specificity	Species/Isotype	Source/Reference
HP-3B1	CD94	mouse IgG2a	Beckman Coulter, Serotec ***(10,11)***
HP-3D9	CD94	mouse IgG1	PharMingen ***(10)***
39B10	CD94	mouse IgG1	M. Colonna, Basel (unpublished)
39C10	CD94	mouse IgG1	M. Colonna, Basel (unpublished)
Z199	NKG2A	mouse IgG	Beckman Coulter ***(11)***

Anti-CD161 (NKR-P1A)

NKR-P1A is found on most human NK cells and a subset of T cells. The *NKR-P1A* gene is the only gene identified in man, but the presence of additional genes in both mouse and rat suggests the possible existence of a family of homologous genes.

Clone	Specificity	Species/Isotype	Source/Reference
DX1	NKR-P1A	mouse IgG1	L. Lanier, San Francisco, CA ***(12)***
191B8	NKR-P1A	mouse IgG2a	Beckman Coulter ***(13)***
B199.2	NKR-P1A	mouse IgGZb	B. Perussia, Philadelphia ***(14)***

Anti-CD16 (FcγRIIIA)

The transmembrane (and signalling competent) form of CD16 is expressed on human NK cells, a subset of T cells, macrophages, and mast cells. A glycosyl-phosphatidylinositol (GPI)-linked form of CD16 (*FcγRIIIB*) has also been identified on neutrophils.

Clone	Specificity	Species/Isotype	Source/Reference
3G8	CD16	mouse IgG1	PharMingen, Beckman Coulter, Accurate, Serotec, Caltag ***(15)***
B-E16	CD16	mouse IgG2a	Biosource
B73.1	CD16	mouse IgG1	Becton Dickinson ***(16)***
Leu 11	CD16	mouse IgG1	Becton Dickinson ***(17)***

*Anti-CD56 (*N-CAM*)*

CD56 is an isoform of the neural cell adhesion molecule (*N-CAM*) that is expressed in the brain. The leukocyte isoform of CD56 is expressed on NK cells and subsets of T cells in humans, but not mouse or rat.

Clone	Specificity	Species/Isotype	Source/Reference
B-A19	CD56	mouse IgG1	Biosource
B159.5	CD56	mouse IgG1	PharMingen, Accurate ***(18,19)***
N901 (NKH-1)	CD56	mouse IgG1	Beckman Coulter ***(20)***
3B8 (NKH-1A)	CD56	mouse IgM	Beckman Coulter
T-199	CD56	mouse IgG1	Beckman Coulter, Accurate ***(21)***
NKI-nbl-1	CD56	mouse IgG1	Caltag, Accurate
Leu 19	CD56	mouse IgG1	Becton Dickinson ***(22)***
C5.9	CD56	mouse IgG2b	Exalpha

Anti-CD122 (β Chain of the IL-2 and IL-15 Receptors)

CD122 is expressed constitutively on NK cells, T cells, monocytes, and some B cell lines. Expression increases on activated T cells.

Clone	Specificity	Species/Isotype	Source/Reference
CF1	CD122	mouse IgG1	Beckman Coulter ***(23)***
2RB	CD122	mouse IgG1	Beckman Coulter
Mik-β2	CD122	mouse IgG2a	PharMingen ***(18,24)***
Mik-β3	CD122	mouse IgG1	PharMingen ***(18,24)***

Anti-Integrins: LFA-1 (CD11a/CD18, $\alpha_L\beta_2$) and Mac-1 (CD11b/CD18, $\alpha_M\beta_2$)

CD18 is expressed as a heterodimer with either *CD11a*, *CD11b*, or *CD11c*. CD11a/CD18 dimers (LFA-1) are expressed on lymphocytes, granulocytes, monocytes, and macrophages. CD11b/CD18 dimers (Mac-1) are found on NK cells and myeloid cells. Some antibodies to these integrins can block NK cell conjugation with target cells, thereby preventing natural cytotoxic responses.

Clone	Specificity	Species/Isotype	Source/Reference
25.3	CD11a	mouse IgG1	Beckman Coulter
TS1/22.1.1.13	CD11a	mouse IgG1	ATCC, DSHB ***(25)***
ICRF44 (44)	CD11b	mouse IgG1	PharMingen, Serotec, Southern Biotechnology
94 (Mo1)	CD11b	mouse IgM	Beckman Coulter ***(26)***
Bear1	CD11b	mouse IgG1	Beckman Coulter, Caltag, Accurate
TS1/18.1.2.11	CD18	mouse IgG1	ATCC, DSHB ***(25)***
7E4	CD18	mouse IgG1	Beckman Coulter ***(27)***
CLB-LFA-1/1	CD18	mouse IgG	Accurate, Caltag ***(28)***

Anti-CD2

CD2 is expressed on almost all human T cells, thymocytes, and NK cells.

Clone	Specificity	Species/Isotype	Source/Reference
OKT11	CD2	mouse IgG1	ATCC
35.1	CD2	mouse IgG2a	ATCC *(29)*
TS2/18.1.1	CD2	mouse IgG1	ATCC, DSHB ***(25)***

Novel Receptors

Several monoclonal antibodies have recently been reported that bind to NK cell surface molecules and trigger NK cell activation or inhibition. These receptors may contribute significantly to NK cell activation and inhibition during natural cytotoxicity. The C1.7 monoclonal antibody recognizes 2B4, which is expressed on almost all NK cells and γδ TCR^+ T cells and about 50% of $CD8^+$ T cells. Antibody crosslinking with C1.7 triggers granule independent killing. NKp46 is reported to be expressed exclusively on NK cells in either resting or activated states ***(31)***. NKp44 is not expressed on fresh PBL, but NK cell-specific expression reportedly increases with in vitro culture in IL-2 ***(32)***. LAIR-1 (p40) is reportedly expressed on 60–80% of peripheral blood leukocytes (PBL), including all NK cells ***(33–35)***.

Clone	Specificity	Species/Isotype	Source/Reference
C1.7	2B4, activation	mouse IgG1	Beckman Coulter ***(30)***
BAB281	NKp46, activation	mouse IgG1	A. Moretta, Genova ***(31)***
Z231	NKp44, activation	mouse IgG1	A. Moretta, Genova ***(32)***
NKTA255	LAIR-1 (p40), inhibition	mouse IgG1	A. Moretta, Genova ***(33,34)***
NKTA72	LAIR-1 (p40), inhibition	mouse IgG1	A. Moretta, Genova ***(33)***
1F1	LAIR-1 (p40), inhibition	mouse IgG1	A. Moretta, Genova ***(33)***
1B1	LAIR-1 (p40), inhibition	mouse IgG1	A. Moretta, Genova ***(33)***
DX26	LAIR-1 (p40), inhibition	mouse IgG1	J. Phillips, Palo Alto ***(35)***

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II. Antibodies to Mouse and Rat Natural Killer Cell Receptors

The listed sources of antibodies are original authors, companies, or hybridoma bank resources. The hybridoma bank resources listed are American Type Culture Collection (ATCC, Manassas, VA; www.atcc.org) and the Developmental Studies Hybridoma Bank (DSHB, Iowa City, IA; www.uiowa.edu/~dshbwww).

Antimouse Ly49

Mouse Ly49 receptors are expressed variably on subsets of NK cells and a small subset of T cells. At least nine separate genes have been identified with distinct MHC class I binding specificities as designated below. It should be noted that the data summarized below has been obtained from direct binding studies, in vitro functional assays, or in vivo depletion studies. The results from these three types of assays do not always correspond. Individual references should be consulted to determine how a given specificity was defined. Antibodies to some isoforms have not been reported, and the cross reactivities of most antibodies should be appreciated when typing *Ly49* expression on NK cell clones (*see* Chapter 2).

Clone	Specificity (H-2 ligand, signal)	Species/Isotype	Source/Refs.
A1	Ly49A (D^d, D^k, Inhibitory) (specific for B6 but not BALB allele)	mouse IgG2a	PharMingen ***(1–4)***
JR9-318	Ly49A (D^d, D^k, Inhibitory)	mouse IgG1	J. Roland ***(5)***
YE1/32	Ly49A (D^d, D^k, Inhibitory)	rat IgG	F. Takei ***(6)***
YEI/48	Ly49A (D^d, D^k, Inhibitory)	rat IgG2c	F. Takei ***(6,7)***
12A8	Ly49A (D^d, D^k, Inhibitory) Ly49D (D^d, L^d, D^{Sp2}, Activating)	rat IgG2a	J. Ortaldo ***(8,13)***
4D11	Ly49A, weak (D^d, D^k, Inhibitory) Ly49G2 (D^d, Inhibitory)	rat IgG2a	ATCC ***(9,14)***
4LO3311	Ly49C (K^b, D^d, K^d, Inhibitory)	mouse IgG3	S. Lemieux ***(10,11)***
SW5E6	Ly49C (K^b, D^d, K^d, Inhibitory)	mouse IgG2a	PharMingen ***(10,12)***
	Ly49I (K^b, H-2^d)		(T. George, V. Kumar, and M. Bennett, unpublished)
SW9C10	Ly49G2 (D^d, Inhibitory)	mouse IgM	V. Kumar ***(15)***
4E5	Ly49D (D^d, L^d, D^{Sp2}, non-inhibitory)	rat IgG2a	J. Ortaldo ***(16,17)***

Antimouse NKR-P1C (NK1.1, CD161)

NKR-P1C is found on most murine NK cells and a subset of T cells (many times designated as NK1.1$^+$ T cells) only in distinct strains of mice. NKR-P1C is expressed

in the following strains of mice: C57BL, FVB/N, NZB, SJL, C57BR, and C57L (NOT in A, AKR, BALB/c, CBA/J, C3H, C58, DBA/1, DBA/2, or 129 strains).

Clone	Specificity	Species/Isotype	Source/Reference
PK136	mouse NKR-P1C	mouse IgG2a	ATCC, PharMingen, Cedarlane, Serotec, Southern Biotechnology Associates, Accurate, Caltag, Leinco Technologies, Beckman Coulter ***(18,19)***
1C10	NKR-PIA, B, & C from B6 mice	mouse 1gG1	Exalpha***(31)***

Antirat NKR-P1A (CD161)

High level expression of NKR-P1A is found on all rat NK cells and low level expression is seen on most rat neutrophils, a subset of T cells, and reportedly on activated monocytes and a subset of dendritic cells. Antibodies specific to other rat isoforms have not yet been reported.

Clone	Specificity	Species/Isotype	Source/Reference
3.2.3	rat NKR-P1A	mouse IgG1	Harlan Bioproducts for Science, Endogen ***(20)***
10/78	rat NKR-P1A	mouse IgG1	Cedarlane, PharMingen, Beckman Coulter, Accurate, Serotec ***(21)***

Antimouse CD16 (FcγRIII)

The transmembrane form of CD16 is expressed on mouse NK cells, macrophages neutrophils, myeloid precursors, and a subset of thymocytes. As opposed to humans, no glycosylphosphatidylinositol (GPI)-linked form has been identified in mouse. It is important to note that the available antibodies also bind CD32 (FcγRII) on B cells and myeloid cells.

Clone	Specificity	Species/Isotype	Source/Reference
2.4G2	mouse CD16/CD32	rat IgG2b	PharMingen, ATCC ***(22–24)***
IOT-17.2	mouse CD16/CD32	mouse IgG2a	Beckman Coulter
IOT-17.1	mouse CD16/CD32	mouse IgM	Beckman Coulter

Anti-CD56 (N-CAM)

Note: As opposed to human, *N-CAM* is not expressed on mouse or rat NK cells.

Antimouse CD122 (IL-2 Receptor β Chain and IL-15 Receptor β Chain)

CD122 is expressed constitutively on NK cells, T-cells, and some B-cell lines. Expression increases on activated T cells.

Clone	Specificity	Species/Isotype	Source/Reference
TM-β1	mouse CD122	rat IgG2b	PharMingen, Serotec ***(25,26)***
5H4	mouse CD122	rat IgG2a	PharMingen ***(26)***

Antimouse Integrins: LFA-1 (CD11a/CD18, $\alpha_L\beta_2$) and Mac-1 (CD11b/CD18, $\alpha_M\beta_2$)

CD18 is expressed as a heterodimer with either *CD11a*, *CD11b*, or *CD11c*. CD11a/CD18 dimers (LFA-1) are expressed on lymphocytes, granulocytes, monocytes, and macrophages. CD11b/CD18 dimers (Mac-1) are found on NK cells and myeloid cells. Some antibodies to these integrins can block NK cell conjugation with target cells and prevent natural cytotoxic responses.

Clone	Specificity	Species/Isotype	Source/Reference
I21/7	mouse CD11a	rat IgG2a	Caltag, Accurate, Serotec, Southern Biotechnology
M17/4.4.11.9	mouse CD11a	rat IgG2a	PharMingen, DSHB ***(27)***
M1/70.15.11.5	mouse CD11b	rat IgG2b	ATCC, Caltag, Accurate, Serotec, DSHB ***(28)***
2E6	mouse CD18	hamster IgG	ATCC ***(29)***
C71/16	mouse CD18	rat IgG2a	Caltag, Accurate, Serotec Southern Biotechnology

Antimouse CD2 (LFA-2)

CD2 is expressed on almost all rodent T cells, thymocytes, and NK cells. It is also expressed on mouse B cells and rat splenic macrophages.

Clone	Specificity	Species/Isotype	Source/Reference
RM2-5	mouse CD2	rat IgG2b	PharMingen ***(30)***
AT37	mouse CD2	rat IgG	Accurate, Serotec

Miscellaneous Antimouse NK Cell Antibodies

Clone	Specificity	Species/Isotype	Source/Refs.
SW2B4	mouse 2B4 on all NK cells and a small T cell subset	mouse IgG2b	PharMingen ***(18)***
DX5	most mouse NK cells and a small T cell subset	rat IgM	PharMingen (J. Phillips and L. Lanier, unpublished)
3A4	mouse NK cell antigen with similar strain distribution as NKR-P1C (*see above*)	mouse IgM	PharMingen ***(18)***

Clone	Specificity	Species/Isotype	Source/Refs.
Asialo GM1	all mouse and rat NK cells, some activated macrophages, and some $CD8^+$ T cells	polyclonal rabbit IgG	Accurate, Cedarlane, Wako

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III. Transformed Natural Killer Cell Lines

Human

NKL—An IL-2-dependent neoplastic NK-like cell line. Expresses ILT2 which is an inhibitory receptor that binds HLA-A, -B, and –G (1).

NK3.3—IL-2-dependent NK-like cell line that exhibits natural killing activity ***(2,3)***.

NK-92—An IL-2-dependent human NK-like cell line that is highly cytolytic and lacks CD16 expresssion. This cell line expresses several KIR receptors ***(4)***.

YT—An IL-2 independent human NK-like cell line. It has come to our attention that many variants of this line exist and some exhibit natural cytotoxicity toward targets that are not normally killed by NK cells, while others do not express typical NK cell markers. One should characterize the line to determine its phenotype before use in functional and biochemical studies ***(5)***.

YT2C2—A subclone of YT, selected for intermediate affinity binding of IL-2 by the IL-2Rb subunit, which lacks IL-2Rα expression ***(6)***.

CP—An IL-2-independent human fetal liver-derived immature lymphoid cell line derived by Dr. Frank Ruscetti (NCI, Frederick, MD). It expresses CD56, but lacks CD 16, CD3, or known KIRs [Winkler-Pickett et al. unpublished results]. CP lacks granzyme-A and -B, expresses metase and perforin, and lacks detectable lytic activity to prototype human NK targets. CP cells can be transfected with mammalian expression vectors ***(7,8)***.

Rat

RNK-16 - An IL-2-independent spontaneous leukemic cell line from F344 rats which exhibits NK cell characteristics. The original line is IL-2 dependent, but many subclones currently available are IL-2 independent ***(9,10)***.

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10. *See* Chapter 24, this volume.

IV. Natural Killer Cell Target Lines

Human

721.221—MHC class I-deficient human EBV-transformed B lymphoblastoid line. These cells do not express endogenous HLA-A, HLA-B or HLA-C class I antigens due to gamma-ray-induced mutations in the HLA complex. This line (and HLA transfectants) is commonly used to measure spontaneous (natural) cytotoxicity by human NK cells ***(1,2)***. Source: R. DeMars.

K562—A human chronic myelogenous leukemia cell line. Classical target cell for spontaneous (granule exocytosis-mediated, Ca^{2+}-dependent) cytotoxicity ***(3,4)***. Source: ATCC.

C1R—MHC class I-deficient γ-irradiated variant of the human EBV-transformed Licr.Lon.Hmy2 B cell line. The cells express HLA-Cw4 and a low levels of a mutant form of HLA-B35, designated B*3503. This line (and HLA transfectants) is commonly used to measure spontaneous (natural) cytotoxicity by human NK cells ***(5,6)***. Source: P. Parham, P. Cresswell.

Daudi—Human Burkitt's B-cell lymphoma line ***(7)***. Source: ATCC.

THP-1—An acute monocytic leukemia cell line that expresses both FcγRI and FcγRII, as well as the complement (C3b) receptor. The cells grow in suspension but are somewhat "sticky." For use in redirected ADCC cytotoxicity assays. Importantly, this cell line is also insensitive to spontaneous (natural) cytotoxicity by IL-2 activated NK cells and clones ***(8)***. Source: ATCC.

RDMC—A human rhabdomyosarcoma, which is likely to be the same as RD. This cell line is adherent and can be used for redirected ADCC cytotoxicity assays using very low E:T ratios (i.e. also sensitive to spontaneous cytotoxicity) ***(9)***. Source: ATCC.

Jurkat—A human $CD3^+/CD4^+$ Fas $(CD95)^+$ T-lymphoid cells line sensitive to spontaneous cytotoxicity by NK cells, both granule exocytosis- and Fas (CD95)-mediated ***(10)***. Source: ATCC.

Mouse

YAC-1—Mouse lymphoma induced by Moloney leukemia virus (MLV) in A/Sn mouse. Classical target cell for spontaneous cytotoxicity. Recent evidence by Petersson et al. suggests that low MHC Class I expression by YAC-1 grown in vitro is due to high constitutive IL-10 production by the cell line ***(11–13)***. Source: ATCC.

P815—DBA/2 murine mastocytoma has been used as a target cell for cytotoxic assays. Clone P815-X2 is FcγRII/III negative, while clone P815y is FcγRII/III positive. Both can be used for ADCC with peripheral blood human NK cells. P815y can be used in reverse cytotoxicity assays, provided low E:T ratios are used. It is also sensitive to spontaneous (natural) killing by IL-2 activated human NK cells ***(14–16)***. Source: ATCC.

RMA-S—A mutant of the murine RMA lymphoma line which has a defect in the TAP-2 transporter, resulting in expression of only 5 to 10% of the wild-type H-2D^b, K^b, and β2-microglobulin molecules ***(17,18)***.

L1210—A murine lymphocytic leukemia of DBA/2 origin that grows in suspension. It can be used, like P815, for ADCC by human NK cells ***(19)***. Source: ATCC.

EL4—Mouse T-lymphoma induced in a C57BL/6N mouse by 9,10-dimethyl-1,2-benzanthracene. This cell line expresses H-2^b. It is important to note that this line also expresses Ly49A ***(20,21)***. Source: ATCC.

Rat

YB2/0—Rat myeloma clone derived from the hybrid myeloma YB2/3HL as selected for absence of Ig secretion. Use of this line is described in Chapter 24 of this volume (22). Source: ATCC.

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V. Killer Cell Immunoglobulin-Like Receptor (KIR) Nomenclature[a,b]

Receptor	HLA Specificity	Mass designation	Alternative Names (and closely related (sequences)[c]
KIR2DL1	Cw2, Cw4, Cw5, Cw6	p58	NKAT1, cl-47-11, cl-42, p58.1,
KIR2DL2	Cw1, Cw3, Cw7, Cw8	p58	NKAT6, cl-43
KIR2DL3	Cw1, Cw3, Cw7, Cw8	p58	NKAT2, cl-6, KIR-023GB p58.2
KIR2DL4	G1	p49	KIR-103AS, KIR-103LP, 15.212, NK3.3#27
KIR3DL1	Bw4, B27	p70	NKAT3, NKB1, AMB11, cl-11, cl-2
KIR3DL2	A3, A11	p70/140	cl-5, AMC5, NKAT4, cl-1.1, cl-17.1c, 8-11c
KIR2DS1	Cw2, Cw4, Cw5, Cw6	p50	EB6act1, EB6act2, p50.1
KIR2DS2	Cw1, Cw3, Cw7, Cw8	p50	NKAT5, cl-49, GL183act1, p50.2
KIR2DS3	unknown	p50	NKAT7, 59C/K3
KIR2DS4	Cw3	p50	NKAT8, cl-39, cl-17, p50.3
KIR2DS5	unknown	N.D.[d]	NKAT9
KIR3DS1	unknown	N.D.	NKAT10, KIR-123FM, C97.12#5, D97.10

[a]KIR was originally adopted as an acronym for "**K**iller Cell **I**nhibitory **R**eceptors," but subsequent studies confirmed that truncated forms of this receptor family were not inhibitory in function. In some reports, these truncated receptors have also been termed "KAR" for **K**iller Cell **A**ctivating **R**eceptors. To avoid renaming the receptors entirely, the acronym KIR was adopted to denote **K**iller Cell **I**mmunoglobulin-like **R**eceptors by several investigators in the field. This nomenclature has been utilized throughout this book. The nomenclature for individual receptors within the KIR family have been designated as "2D" or "3D" according to their number of extracellular immunoglobulin-like domains, which is followed by the letter "L" or "S" for long or short (truncated) cytoplasmic domain, respectively, and a definitive number for that specific receptor within each subgroup.

[b]It should be noted that the diversity of KIR sequences may be much more complex than presented in this table, due to numerous minor sequence polymorphisms that have been identi-

fied in cDNAs from individuals within the population. For an excellent alignment of many of these sequences, *see* Steffens, U., Vyas, Y., Dupont, B., and Selvakumar, A. (1998) Nucleotide and amino acid sequence alignment for human killer cell inhibitory receptors (KIR). *Tissue Antigens* **51,** 398–413 (also currently updated at: http://www.tissue-antigens.dk/kir-text.html). Some of this material was obtained and confirmed using these references.

[c]Alternative names are those originally designated by different investigators that separately cloned the receptors.

[d]N.D. = not determined.