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# Natural Killer Cell Protocols

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**Kerry S. Campbell**

**Marco Colonna**



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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.

## 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 mM), 2-mercaptoethanol ( $5 \times 10^{-5}$  M), penicillin (50 g/mL), streptomycin (50 g/mL), neomycin (100 g/mL) (all from Gibco-BRL) and 10% fetal calf serum (FCS).
4. ACK buffer: 0.16 M NH<sub>4</sub>Cl, 0.1 mM Na<sub>2</sub>EDTA, 0.01 M KHCO<sub>3</sub>.
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-Fc $\gamma$ II/III receptors) hybridoma supernatant to prevent nonspecific staining (available as FcBlock™ from Pharmingen, San Diego, CA), phycoerythrin (PE)-labeled anti-CD3 (145.2C11), fluoresoithiocyanate (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<sup>plus</sup> [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<sub>15</sub>, Roche Molecular Biochemicals, cat. no. 814270, Mannheim, Germany).
11. RNase inhibitor (such as RNAGuard, 33 U/ L, Pharmacia, cat. no. 27-0815-01, Uppsala, Sweden).
12. Reverse transcriptase and buffer (such as AMV RT, 20 U/ L, Roche Molecular Biochemicals #109 118).
13. Taq polymerase (such as AmpliTaq, 5 U/ L, 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<sup>1</sup>/<sub>2</sub>-gauge 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<sub>2</sub> 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 500g.
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 500g, 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<sub>2</sub> incubator.
12. Elute nylon wool nonadherent cells with 7–10 mL of prewarmed medium (*see Note 1*). Centrifuge for 5 min at 500g.
13. Resuspend the cell pellet in 10 mL of medium containing rIL-2 at 250 ng/mL. Transfer to a small (25-cm<sup>2</sup>) tissue culture flask and culture in a CO<sub>2</sub> incubator for 3 d.
14. Harvest LAKs. Adherent cells are detached by incubating for a few minutes with cold PBS containing 1.5 mM EDTA. Pool nonadherent and adherent cells.
15. Count viable cells, centrifuge for 5 min at 500g, and resuspend at 10<sup>6</sup> 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<sup>6</sup> cells/25 µL.
18. Wash as above and resuspend at 2 × 10<sup>6</sup> cells/mL for single cell sorting.
19. Sort single CD3<sup>-</sup> NK1.1<sup>+</sup> 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<sub>2</sub> 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

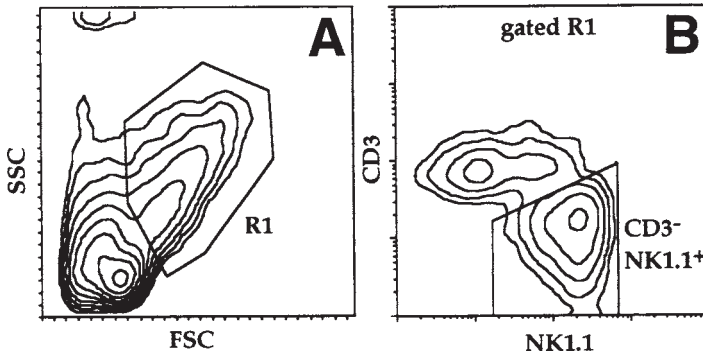


Fig. 1. Lymphokine-activated Killer cells used for NK cell cloning. Forward (FSC) and side scatter gate (SSC) of d 3 lymphokine activated cells are shown in (A). Cell surface expression of CD3 and NK1.1 is assessed in blast cells (R1) (identified based on an elevated FSC /SSC). To derive short term NK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

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

4. Add 40  $\mu$ L of chloroform, shake by hand for 15 s, and incubate for 2–3 min at room temperature.
5. Centrifuge in a cooled (4  $^{\circ}$ C) microfuge for 15 min at 12,000g.
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  $\mu$ L of isopropanol, mix, and incubate at room temperature for 10 min.
8. Centrifuge in a cooled (4  $^{\circ}$ C) microfuge for 10 min at 12,000g.
9. Wash the RNA pellet by adding 1 mL of 70% EtOH, mix and centrifuge in a cooled (4  $^{\circ}$ C) microfuge for 5 min at 7500g.
10. Air-dry RNA pellet for 5–10 min.

### 3.3. Complementary DNA Preparation

1. Resuspend RNA pellet in a total of 7  $\mu$ L of H<sub>2</sub>O containing 0.3  $\mu$ L of oligo-dT (150  $\mu$ M) as a primer.
2. Incubate for 5 min at 72  $^{\circ}$ C.
3. Transfer directly on ice.
4. Add 13  $\mu$ L of reverse transcriptase mix:
 

4.0 $\mu$ L	5 $\times$ concentrated reverse transcriptase buffer
5.0 $\mu$ L	2 mM of each dATP, dCTP, dGTP, and dTTP
2.0 $\mu$ L	0.1 mM DTT
1.1 $\mu$ L	H <sub>2</sub> O

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

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

### 3.4. Polymerase Chain Reaction

1. Take 1 L of the cDNA preparation for PCR.
2. Add 29 L of PCR mix (*see Note 5*):
  - 0.6 L of sense primer (10 mM stock)
  - 0.6 L of antisense primer (10 mM stock)
  - 3 L of 10 x PCR buffer containing 15 mM MgCl<sub>2</sub>
  - 3 L of 2 mM of each dATP, dCTP, dGTP, and dTTP
  - 0.15 L of *Taq* polymerase
  - total volume of 30 L 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 L) 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 L) of the second PCR product. Add 2 U of restriction enzyme plus the appropriate digestion buffer and bring volume to a total of 20 L. 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<sup>6</sup> 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:

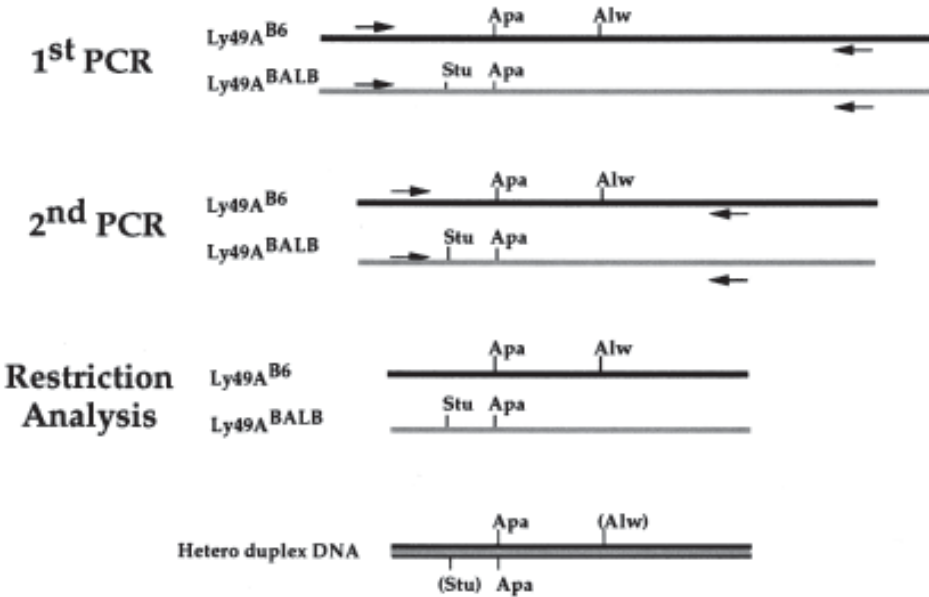


Fig. 2. Analysis of *Ly49A* gene expression in short-term NK cell clones. Complementary DNA templates derived from short-term NK cell clones are subjected to PCR amplification using *Ly49A*-specific primers (1<sup>st</sup> PCR). An aliquot of the first reaction is reamplified using an internal pair of *Ly49A*-specific primers (2<sup>nd</sup> 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:

*Ly49A*-specific

1<sup>st</sup> round 52 sense: 5'-ACCAGAACCCTTCTTG<sup>C/A</sup>TAGC-3'  
 976 antisense: 5'-CAAAACACTTGTTTTGCAAGG-3'  
 2<sup>nd</sup> round 145 sense: 5'-GGAGGTCACTTATTCAATGG-3'  
 535 antisense: 5'-CCATAGCAGAACCAGTATAC-3'

*Ly49C*-specific

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

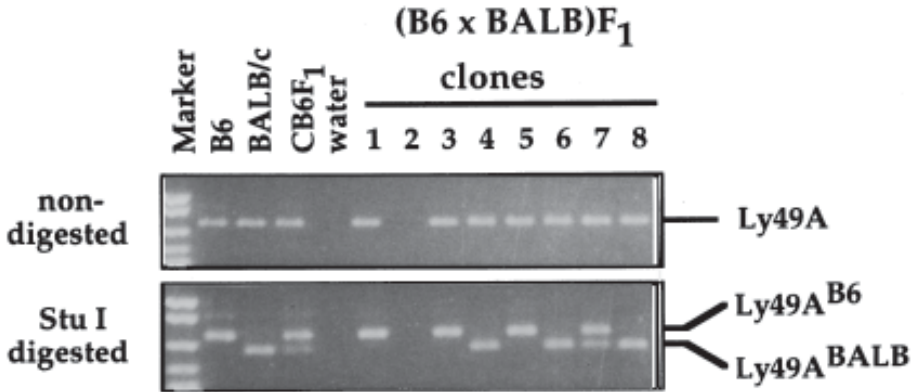


Fig. 3. *Ly49A* gene expression in *Ly49A*<sup>+</sup> short-term NK cell clones. Control amplifications include cDNA derived from B6, BALB/c, and (B6 × BALB/c)<sub>F1</sub> bulk NK cells RNA and no cDNA (water). No PCR product was obtained for clone no. 2. Whereas most clones express either the B6 or 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

1<sup>st</sup> round 113 sense: 5'-CTCAACTGTGAGATTTTCATG-3'  
 814 antisense: 5'-TGTATGATTTATCACAGTCC-3'  
 2<sup>nd</sup> 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 simultaneously 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, *Stu I* 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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# 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](http://www.atcc.org)) and the Developmental Studies Hybridoma Bank (DSHB, Iowa City, IA; [www.uiowa.edu/~dshbwww](http://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)

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 ( $\beta$ 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- $\beta$ 2	CD122	mouse IgG2a	PharMingen (18,24)
Mik- $\beta$ 3	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  $\gamma\delta$  TCR<sup>+</sup> T cells and about 50% of CD8<sup>+</sup> 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](http://www.atcc.org)) and the Developmental Studies Hybridoma Bank (DSHB, Iowa City, IA; [www.uiowa.edu/~dshbwww](http://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 <sup>d</sup> , D <sup>k</sup> , Inhibitory) (specific for B6 but not BALB allele)	mouse IgG2a	PharMingen (1-4)
JR9-318	Ly49A (D <sup>d</sup> , D <sup>k</sup> , Inhibitory)	mouse IgG1	J. Roland (5)
YE1/32	Ly49A (D <sup>d</sup> , D <sup>k</sup> , Inhibitory)	rat IgG	F. Takei (6)
YE1/48	Ly49A (D <sup>d</sup> , D <sup>k</sup> , Inhibitory)	rat IgG2c	F. Takei (6,7)
12A8	Ly49A (D <sup>d</sup> , D <sup>k</sup> , Inhibitory) Ly49D (D <sup>d</sup> , L <sup>d</sup> , D <sup>Sp2</sup> , Activating)	rat IgG2a	J. Ortaldo (8,13)
4D11	Ly49A, weak (D <sup>d</sup> , D <sup>k</sup> , Inhibitory) Ly49G2 (D <sup>d</sup> , Inhibitory)	rat IgG2a	ATCC (9,14)
4LO3311	Ly49C (K <sup>b</sup> , D <sup>d</sup> , K <sup>d</sup> , Inhibitory)	mouse IgG3	S. Lemieux (10,11)
SW5E6	Ly49C (K <sup>b</sup> , D <sup>d</sup> , K <sup>d</sup> , Inhibitory) Ly49I (K <sup>b</sup> , H-2 <sup>d</sup> )	mouse IgG2a	PharMingen (10,12) (T. George, V. Kumar, and M. Bennett, unpublished)
SW9C10	Ly49G2 (D <sup>d</sup> , Inhibitory)	mouse IgM	V. Kumar (15)
4E5	Ly49D (D <sup>d</sup> , L <sup>d</sup> , D <sup>Sp2</sup> , 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<sup>+</sup> T cells) only in distinct strains of mice. NKR-P1C is expressed

## Mouse NK Cell Antibodies

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 ( <b>18,19</b> )
1C10	NKR-PIA, B, & C from B6 mice	mouse IgG1	Exalpha( <b>31</b> )

### 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 ( <b>20</b> )
10/78	rat NKR-P1A	mouse IgG1	Cedarlane, PharMingen, Beckman Coulter, Accurate, Serotec ( <b>21</b> )

### Antimouse CD16 (Fc $\gamma$ RIII)

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 (Fc $\gamma$ RII) on B cells and myeloid cells.

Clone	Specificity	Species/Isotype	Source/Reference
2.4G2	mouse CD16/CD32	rat IgG2b	PharMingen, ATCC ( <b>22–24</b> )
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 $\beta$ Chain and IL-15 Receptor $\beta$ 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 <sup>+</sup> 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 expression. 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-2R $\beta$  subunit, which lacks IL-2R $\alpha$  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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## Target Cell Lines

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## 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<sup>2+</sup>-dependent) cytotoxicity (**3,4**). Source: ATCC.

C1R—MHC class I-deficient  $\gamma$ -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 Fc $\gamma$ RI and Fc $\gamma$ RII, 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<sup>+</sup>/CD4<sup>+</sup> Fas (CD95)<sup>+</sup> 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 Fc $\gamma$ RII/III negative, while clone P815y is Fc $\gamma$ RII/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<sup>b</sup>, K<sup>b</sup>, and  $\beta$ 2-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<sup>b</sup>. 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<sup>a,b</sup>

Receptor	HLA Specificity	Mass designation	Alternative Names (and closely related (sequences) <sup>c</sup>
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. <sup>d</sup>	NKAT9
KIR3DS1	unknown	N.D.	NKAT10, KIR-123FM, C97.12#5, D97.10

<sup>a</sup>KIR was originally adopted as an acronym for “**K**iller **C**ell **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 “**K**iller **C**ell **A**ctivating **R**eceptors. To avoid renaming the receptors entirely, the acronym KIR was adopted to denote **K**iller **C**ell **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.

<sup>b</sup>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.

<sup>c</sup>Alternative names are those originally designated by different investigators that separately cloned the receptors.

<sup>d</sup>N.D. = not determined.