

# Expression of Different Members of the Ly-49 Gene Family Defines Distinct Natural Killer Cell Subsets and Cell Adhesion Properties

By Jack Brennan,\*<sup>§</sup> Dixie Mager,\*<sup>§</sup> Wilfred Jefferies,<sup>†¶§\*\*</sup> and Fumio Takei\*<sup>||</sup>

From the \*Terry Fox Laboratory, British Columbia Cancer Agency, <sup>†</sup>Biotechnology Laboratory, and the Departments of <sup>§</sup>Medical Genetics, <sup>||</sup>Pathology, <sup>¶</sup>Microbiology and Immunology, and <sup>\*\*</sup>Zoology, the University of British Columbia, Vancouver, British Columbia V5Z 1L3, Canada

## Summary

The murine Ly-49 antigen belongs to a family of type II transmembrane molecules containing lectin-like domains. The original member of this family, Ly-49A, has been demonstrated to be expressed by a subpopulation of natural killer (NK) cells, bind certain class I major histocompatibility complexes (MHC), and act as a negative regulator of lytic activity. The expression patterns and functional activities of the other Ly-49s, however, is unknown. We extended the study of this family by isolating cDNAs encoding two new Ly-49 molecules. The reactivity of these and previously identified Ly-49 molecules with NK antibodies was tested in a COS cell expression system. YE1/32 and YE1/48 bound Ly-49A specifically, and 5E6 reacted only with Ly-49C. Three-color flow cytometric analysis demonstrated that Ly-49A and Ly-49C expression defines complex, but distinct subsets within NK1.1<sup>+</sup> cells. Some NK1.1<sup>-</sup>CD3<sup>+</sup> as well as NK1.1<sup>-</sup>CD3<sup>-</sup> cells expressing Ly-49A or C were also detected. Analysis of MHC congenic strains of mice demonstrated that YE1/32<sup>+</sup> and YE1/48<sup>+</sup> NK cells are not deleted, as has been shown with the Ly-49A mAb A1. Furthermore, COS cells transfected with Ly-49A bound H-2<sup>d</sup> and H-2<sup>k</sup> cell lines, whereas Ly-49C transfectants bound H-2<sup>d</sup>, H-2<sup>k</sup>, H-2<sup>b</sup>, and H-2<sup>s</sup>. The antibodies 5E6 and 34-1-2S (anti-class I MHC) inhibited the binding of Ly-49C to an H-2<sup>s</sup> cell line. These results imply that the NK cell antigens Ly-49A and C bind to different repertoires of class I MHC molecules.

Murine NK cells are capable of recognizing and eliminating diverse cell types, including tumor cell lines, virally infected cells, and MHC-disparate bone marrow grafts (1, 2). Although NK cell cytotoxicity is not MHC restricted, the expression of certain class I MHC on target cells may provide protection from NK lysis. It has therefore been hypothesized that NK cells possess receptors that deliver negative signals upon interaction with class I MHC molecules, and that NK cells normally function by recognizing an absence of self (3, 4). Recently, an NK cell surface molecule termed Ly-49 expressed on ~20% of NK1.1<sup>+</sup> cells from C57BL/6 mice, has been proposed to be a receptor on NK cells that recognizes class I MHC molecules on target cells and transduces negative signals to NK cells. Ly-49<sup>+</sup> NK cells were shown to be unable to lyse tumor targets expressing H-2D<sup>d</sup>, and this inhibitory effect was reversed with either the anti-Ly-49 mAb A1 or an Ab against the  $\alpha 1/\alpha 2$  domain of H-2D<sup>d</sup> (5). The adhesion of Ly-49<sup>+</sup> lymphoma cells to purified and immobilized D<sup>d</sup> and D<sup>k</sup>, which is inhibitable with the Ab A1, has also been demonstrated (6). These results further indicate that Ly-49 is an NK cell receptor for class I MHC on target cells.

Ly-49 is actually a family of closely related molecules that share similar amino acid and nucleotide sequences. We have termed the original member of this family Ly-49A and two other related molecules Ly-49B and Ly-49C (7). The Ly-49 family has sequence similarity with another family of NK cell-associated molecules termed NKR-P1. They both encode type II transmembrane proteins with lectin-like domains (8). Furthermore, these genes are closely linked on a distal segment of chromosome 6, indicating the presence of an NK cell-associated gene complex (9, 10). In the case of CMV infection, different strains of mice differ in the ability of their NK cells to eliminate virally infected cells. This genetic difference maps to the Ly-49/NKR-P1 locus, and has been hypothesized to be the result of differences between strains in the repertoire of polymorphic NK cell receptors (11, 12). Although the role of Ly-49A in NK cell functions has been studied, functional roles of other members of the Ly-49 family are yet to be determined.

The 5E6 antigen is expressed by the subpopulation of NK cells that mediates the rejection of bone marrow cells from H-2<sup>d</sup>, but not H-2<sup>b</sup>, mice transplanted into irradiated hosts (13). The genes regulating the rejection have been located

within the MHC and are thought to encode hemopoietic histocompatibility (Hh)<sup>1</sup> antigens or to regulate the expression of Hh antigens (2). However, the identity of Hh antigens and their receptors on NK cells are unknown. The 5E6 antigen has recently been reported to be a member of the Ly-49 family (14), suggesting that it may function as a receptor molecule on NK cells.

In this report we describe the cloning of two additional members of the Ly-49 multigene family as well as the specificities of three of the existing Ly-49 Abs, YE1/32, YE1/48, and SW5E6. Our results suggest that 5E6 is identical to Ly-49C, and that murine NK cells can be divided into subpopulations on the basis of expression of Ly-49A and Ly-49C. Furthermore, Ly-49A and Ly-49C mediate adhesion to distinct class I MHC molecules, implicating important roles for these receptors in NK cell function.

## Materials and Methods

**Mice.** C57BL/6, C57BL/10 (B10), B10.A, B10.BR, and B10.D2 mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

**NK Cell Preparations.** Activated splenic NK cells were isolated by a two step adherence system. Single cell suspensions of spleen cells were suspended in ammonium chloride to lyse RBC, followed by a 24-h incubation to remove adherent cells according to their adherence to plastic. The nonadherent layer was then cultured for 3 d in the presence of IL-2 (1,000 U/ml). Activation of NK cells with IL-2 has been shown to specifically induce their adherence to plastic (15). After 3 d all nonadherent cells were removed and the remaining cells were cultured with IL-2 for 4–6 d. This procedure results in a cell population that is 70–80% NK1.1<sup>+</sup>.

**Cell Lines.** The cell lines COS-1, A20, R1.1, CTLL-2, GM979, IC-21, P388D1, MBL-2, C1498, YAC-1, 34-1-2S, 2.4G2, and PK136 were purchased from American Type Culture Collection (Rockville, MD). B10/A2/2.2 is an Abelson virus-induced leukemia cell line derived from a B10.A mouse (16). COS-1, A20, R1.1, IC-21, P388D1, MBL-2, C1498, YAC-1, GM979, and B10/A2/2.2 were maintained in DMEM + 5% FCS and 34-1-2S, 2.4G2, and PK136 were cultured in DMEM + 10% FCS. CTLL-2 was maintained in RPMI supplemented with 10% FCS and 20 U/ml IL-2.

**Antibodies.** The mAbs YE1/32 (16), YE1/48 (16), and PK136 (17) were purified from tissue culture supernatants using Protein G Sepharose 4 Fast Flow (Pharmacia LKB, Piscataway, NJ). YE1/32 and YE1/48 were labeled with biotin (Sulfosuccinimidobiotin; Pierce Chemical Co., Rockford, IL) and used for cell staining at 2 µg/ml. PK136 was labeled with FITC and cyanine 5 (Cy5), and used at 8 µg/ml and 25 µg/ml respectively. R-PE-conjugated 5E6 and FITC conjugated CD3 were purchased from PharMingen (San Diego, CA) and were both used at a concentration of 10 µg/ml.

In the adhesion blocking studies, purified 5E6 (PharMingen) and A-20 (anti-Ly-5.2) (18) were used at a concentration of 5 µg/ml, and 34-1-2S (19) was used as hybridoma supernatant. Cells were incubated with the appropriate Ab for 30 min at 37°C before the adhesion assay.

**Flow Cytometry.** All staining procedures were carried out at cell concentrations of 10<sup>7</sup>/ml. Cells were first incubated at 4°C for 30 min with the Ab 2.4G2 (20) to block Fc receptor binding, then

30 minutes with the desired Ab, followed by two washes with PBS containing 2% FCS and 0.1% sodium azide. In the case of biotinylated Abs, streptavidin-PE and streptavidin-FITC were then added and cells incubated for an additional 30 min at 4°C. Indirect secondary Ab staining techniques were used for single-color staining with YE1/32 and YE1/48 only. After the first set of washes, cells were incubated with a goat anti-rat FITC Ab. In all cases the final wash was done with propidium iodide added at a concentration of 1 µg/ml. Dead cells stained with propidium iodide were gated out. The cells were analyzed on a FACStar Plus<sup>®</sup> (Beckton Dickinson & Co., Mountain View, CA) equipped with a 5-W Argon and a 30-mW helium neon laser.

**cDNA Cloning.** PCR primers were designed corresponding to sequences in the 5' and 3' untranslated regions of the Ly-49A cDNA, which amplify the entire open reading frame. The sequences of the oligonucleotides were 5'-AGTACCGCGGCATTGAACTG-AGAACA-3' and 5'-TACTCCGCGGCAGACTAAGTCCAATGG-3'. The first strand of cDNA was generated in a 25-µl mixture containing 10 µg total RNA, 0.5 mM dNTPs, 4 µg/ml random hexamers, 10 mM dithiothreitol, and 200 U of reverse transcriptase RT. The reaction was incubated at 37°C for 1 h followed by 5 min at 95°C to inactivate the enzyme. 1 µl of this reaction was then subjected to PCR in a 50-µl volume of 25 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 1 µM each primer, and 1 U of Taq polymerase. 35 cycles were carried out as follows: 1 min denaturation at 94°C; 1 min annealing at 45°C; and 3 min extension at 72°C. This was followed by a final extension at 72°C for 5 min. The PCR products of expected size (950 bp) were purified from a 1% agarose gel, cut with SstII to remove the primer tails, and subcloned into the SstII site of Bluescript-KS.

Ly-49D was isolated from an amplified (C57BL/6 × CBA)<sub>F1</sub> lung cDNA library purchased from Stratagene (La Jolla, CA). 6 × 10<sup>5</sup> plaques were screened, using a 639-bp PstI Ly-49A fragment (nucleotides 442–1082) as a probe. Overnight hybridizations were done at 55°C in 6× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.5), 0.5% SDS, 0.02% polyvinylpyrrolidone, 0.02% ficoll, 0.02% BSA, 10 µg/ml denatured salmon sperm DNA, and 10<sup>5</sup> cpm/ml denatured probe.

**DNA Sequence Analysis.** cDNA clones were sequenced by the dideoxy method modified for use with T7 polymerase (21). Sequence analysis was performed with the GCG software package (22).

**COS Cell Expression.** cDNAs were cloned into pAX142, a variant of the pAX114 expression vector (23). Plasmid DNA was transfected into COS-1 cells by DEAE-dextran transfection as has been described elsewhere (24). 3 d later cells were analyzed by FACS<sup>®</sup> or assayed for acquired adhesive properties.

**Cell Adhesion Assay.** COS cells were transfected with Ly-49 cDNAs as described above. After 24 h, cells of each transfectant were trypsinized and 2 × 10<sup>5</sup> cells were transferred to 6-cm dishes (3002; Falcon Labware, Oxnard, CA). 48 h later, the adherent layer of cells was washed twice, followed by an overlay of 1.5 ml of test cells at 10<sup>6</sup>–10<sup>7</sup> cells/ml. Cells were incubated for 2 h at 37°C, followed by five washes with prewarmed media.

To quantitate cell adhesion, 5 × 10<sup>6</sup> labeled GM979 cells (1 µCi Na<sup>51</sup>CrO<sub>4</sub>/10<sup>5</sup> cells) were incubated with adherent, transfected COS cells for 2 h. After several washes, cells that remained attached to the adherent layer were lysed with 10% Triton X and radioactivity was determined. Each condition was done in triplicate and error bars show the standard deviation.

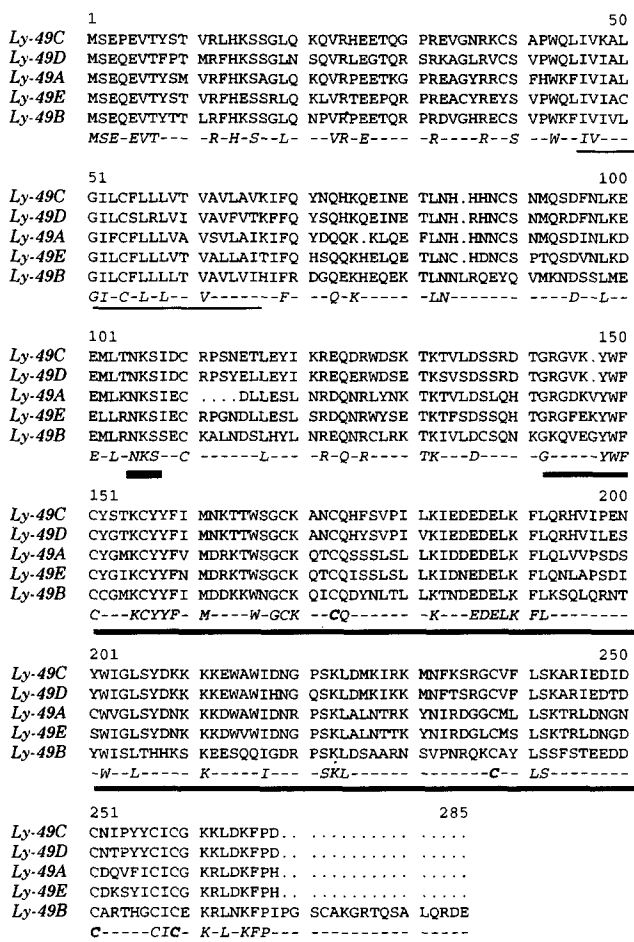
## Results

**Cloning of Additional Members of the Ly-49 Multigene Family.** In a previous study in which we reported the isola-

<sup>1</sup> Abbreviations used in this paper: CRD, carbohydrate recognition domain; Hh, hemopoietic histocompatibility; RT, reverse transcriptase.

tion of the novel cDNAs Ly-49B and C, analysis of partial genomic clones suggested the existence of other Ly-49 related genes (7). In an effort to further characterize this multigene family, we have isolated additional cDNAs by PCR and library screening. RT-PCR was performed on RNA isolated from IL-2-activated NK cells of B10.A mice. Several clones were isolated, one of which differed from all other Ly-49s in restriction fragment pattern and was therefore analyzed in more detail. In addition, a lung cDNA library, from which Ly-49B and C were isolated, was rescreened using a 639-bp PstI fragment from the 3' end of the Ly-49A cDNA as a probe. This library yielded one clone differing from all other Ly-49 sequences. The cDNA from the lung library has been designated Ly-49D and the PCR generated cDNA from B10.A NK cells is Ly-49E.

An alignment of the predicted proteins of the five Ly-49 molecules shows several well conserved features (Fig. 1). With the exception of Ly-49B, which contains 17 additional COOH-



**Figure 1.** Protein sequence comparisons of the five Ly-49 cDNAs. Only those residues found in all members are indicated in the consensus sequence. A bold C identifies the cysteine residues found in all C-type CRD. (—) Transmembrane region; (---) potential N-linked glycosylation site; and (■) region homologous to C-type CRD. The Ly-49D and E nucleotide sequences are available from EMBL/GenBank/DBJ under accession numbers U12889-U12890.

terminal amino acids, the molecules are almost identical in size. Additionally, there is one N-linked glycosylation site as well as 11 extracellular cysteine residues common to all Ly-49s. These cysteines are likely important in disulfide bond formation which would function to give these molecules similar three dimensional structures. As has been reported previously, Ly-49A contains an extracellular domain homologous to the carbohydrate recognition domain (CRD) of C-type animal lectins (7, 25). Although members of the Ly-49 family lack several of the amino acids common to the CRD of other C-type lectins (26, 27), the four invariant cysteines of this domain are conserved in all Ly-49s.

Table 1 provides pairwise comparisons of the five Ly-49 molecules at the amino acid and nucleotide levels. In all cases homology between members of this family is significantly higher at the nucleotide than at the amino acid level. Ly-49C shares 83% amino acid identity with Ly-49D, a homology higher than that seen between any of the other members of this family. It is interesting to note that the region of striking similarity between Ly-49C and Ly-49D is in the extracellular region (90%), whereas the cytoplasmic and transmembrane regions are equally homologous with all other Ly-49 molecules (65%). Ly-49E shares 76% of its amino acid residues with Ly-49A, with the majority of these falling in the lectin-like domain (83%). All other sequence comparisons between members of this family show similar ranges of identity in the cytoplasmic and transmembrane domains and significantly greater divergence in the extracellular lectin-like domain.

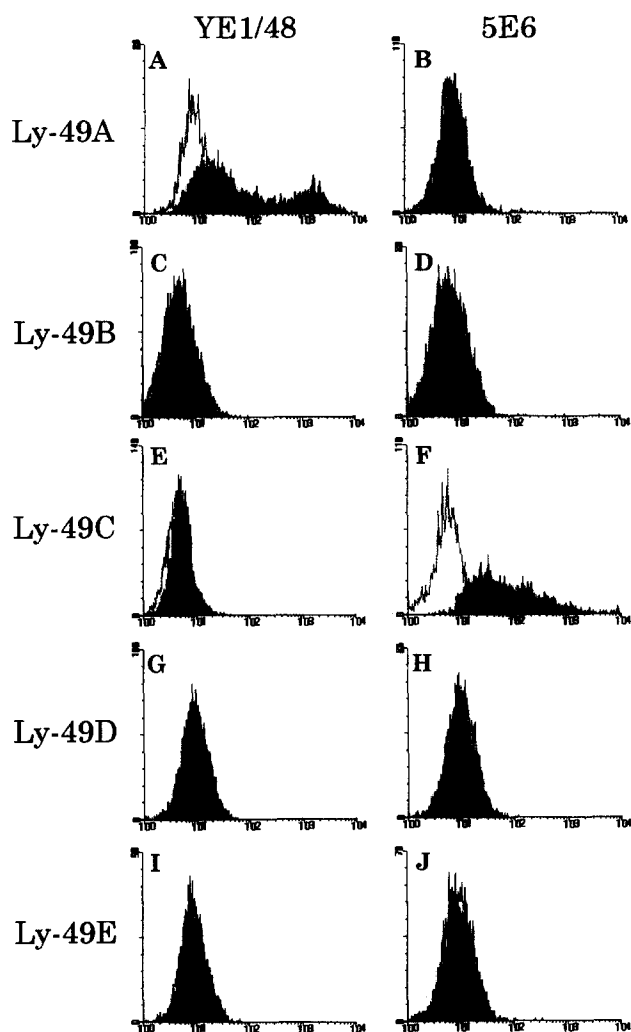
**COS Cell Expression of Ly-49 cDNAs and Reactivity with Ly-49 Antibodies.** To determine whether different anti-Ly-49 Abs can specifically recognize unique members of this multigene family, we expressed all five of our Ly-49 cDNAs in COS cells and tested for reactivity with YE1/32, YE1/48, and 5E6 (Fig. 2). Both YE1/48 (Fig. 2 A) and YE1/32 (not shown) react specifically with Ly-49A and no other members of the family. 5E6, which has recently been shown to bind an Ly-49 molecule (14), reacts specifically with Ly-49C (Fig. 2 D). This lack of crossreactivity between related but distinct proteins indicates that these Abs should prove useful in dissections of the Ly-49 family.

**Expression of YE1/48 and 5E6 Defines Distinct NK Subsets.** We next examined the expression of Ly-49A and Ly-49C on NK cells from C57BL/6 mice by three-color

**Table 1.** Homologies Between Members of the Ly-49 Family

	Ly-49A	Ly-49B	Ly-49C	Ly-49D	Ly-49E
Ly-49A	--	70	81	78	88
Ly-49B	52	--	71	70	71
Ly-49C	65	52	--	91	80
Ly-49D	59	49	83	--	78
Ly-49E	76	50	63	59	--

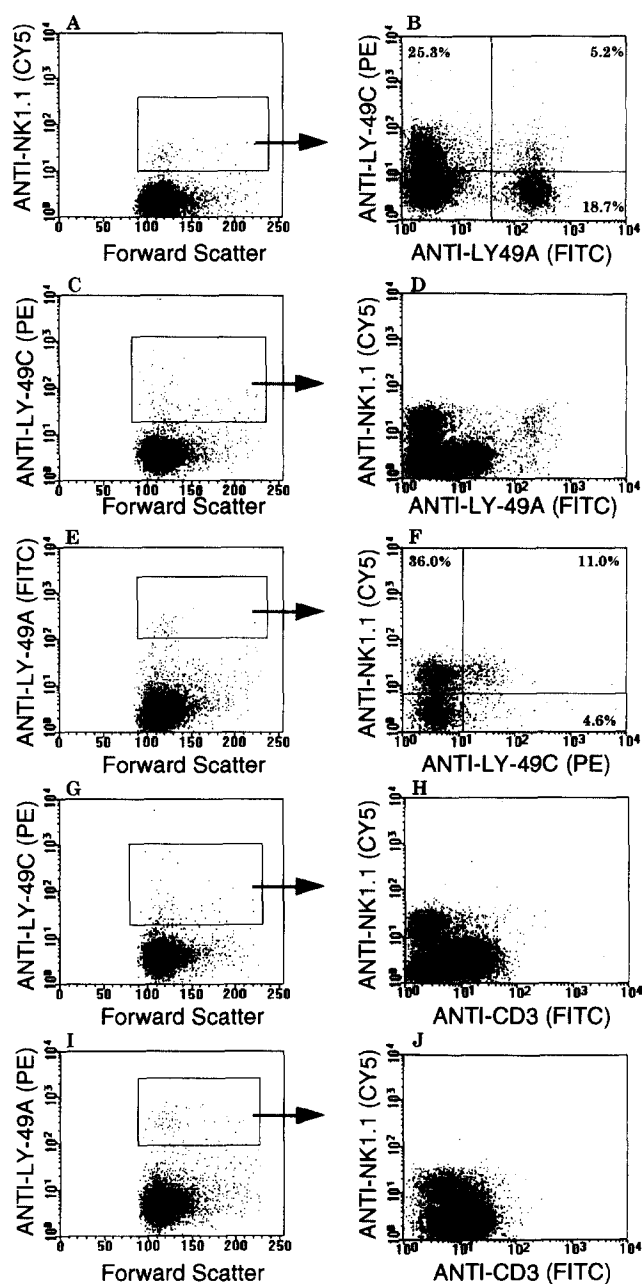
Percent nucleotide identity within the coding region is above the dashes, and percent amino acid identity is below.



**Figure 2.** COS cell expression of the Ly-49 cDNAs. Each of the cDNAs were transiently expressed in COS cells, and tested for reactivity with the Abs YE1/48 and 5E6 after 3 d of growth. *Solid* histograms indicate COS cells transfected with Ly-49 cDNAs, and *empty* histograms are cells transfected with the vector PAX142.

FACS<sup>®</sup> analysis using YE1/48, 5E6, and NK1.1. Fig. 3 B demonstrates that NK1.1<sup>+</sup> cells are either Ly-49A<sup>-</sup>Ly-49C<sup>-</sup> (50.8%), Ly-49A<sup>+</sup>Ly-49C<sup>-</sup> (18.7%), Ly-49C<sup>+</sup>Ly-49A<sup>-</sup> (25.3%), or Ly-49A<sup>+</sup>Ly-49C<sup>+</sup> (5.2%). This is the first demonstration that expression of members of the Ly-49 family of molecules defines separate NK cell subsets. Analysis of NK1.1 and Ly-49A within Ly-49C<sup>+</sup> cells showed a complex pattern of expression (Fig. 3 D). Two Ly-49A<sup>+</sup> populations were evident within the Ly-49C<sup>+</sup> population, one Ly-49A “high” (most of which are NK1.1<sup>+</sup>) and the other Ly-49A “low” (mostly NK1.1<sup>-</sup>). Ly-49C<sup>+</sup>Ly-49A<sup>-</sup> cells were also divided into NK1.1<sup>-</sup> and NK1.1<sup>+</sup> populations. Similarly, Ly-49A high cells could be subdivided into NK1.1<sup>-</sup>Ly-49C<sup>-</sup>, NK1.1<sup>+</sup>Ly-49C<sup>-</sup>, NK1.1<sup>+</sup>Ly-49C<sup>+</sup>, and NK1.1<sup>-</sup>Ly-49C<sup>+</sup> populations (Fig. 3 F).

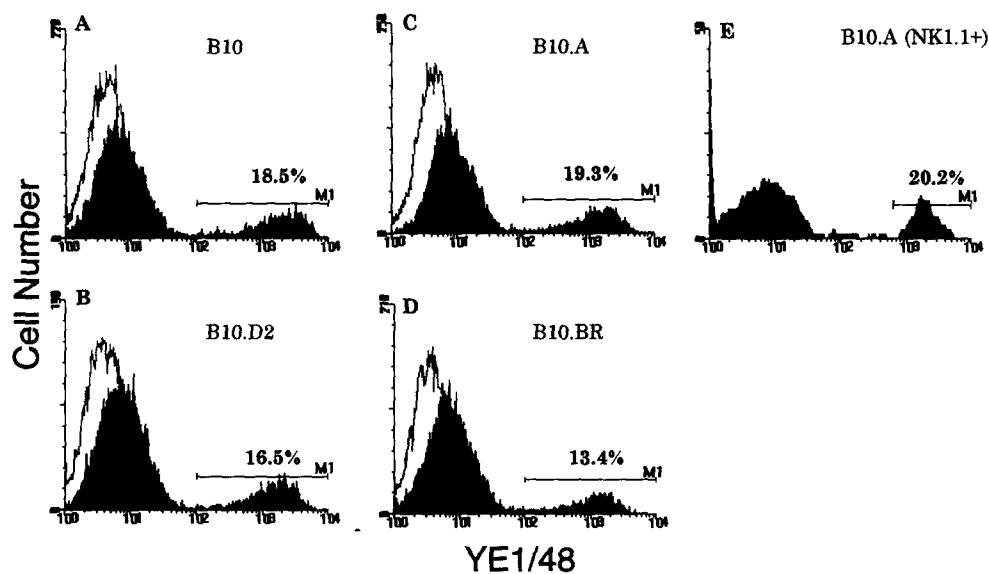
Gating on Ly-49A<sup>+</sup> or Ly-49C<sup>+</sup> spleen cells has shown that significant numbers of these cells are NK1.1 negative



**Figure 3.** Phenotypic analysis of Ly-49A and Ly-49C populations in C57BL/6 spleen cells. Staining profiles of total splenocytes are shown in parts A, C, E, G, and I. *Boxes* indicate gated populations from which 5,000 events were collected for analysis in parts B, D, F, H, and J.

(Fig. 3, D and F). We have examined these populations for expression of CD3 (Fig. 3, H and J). Three-color analysis shows that substantial portions of both Ly-49A<sup>+</sup> and Ly-49C<sup>+</sup> cells are CD3<sup>+</sup>, mainly in the NK1.1<sup>-</sup> window. However, in each case there remains a significant number of Ly-49A<sup>+</sup> and Ly-49C<sup>+</sup> cells that are negative for both NK1.1 and CD3.

*YE1/32 and YE1/48 Expression on NK Cells of B10 MHC Congenic Mice.* It has been reported previously that Ly-49<sup>+</sup> NK cells defined by the mouse mAb A1 are deleted in certain



**Figure 4.** Equivalent expression of YE1/48 on NK cells of MHC congenic mice. Day 9 IL-2-activated NK cells (A–D) stained with mAb YE1/48 and a secondary Ab (solid histograms) or stained with secondary Ab alone (empty histograms). NK1.1<sup>+</sup> gated fresh spleen cells stained with biotinylated YE1/48 followed by Streptavidin-PE (E).

MHC congenic strains of mice (B10.A, B10.D2, and B10.BR), indicating an MHC-based selection of the NK repertoire (28). FACS<sup>®</sup> analysis of IL-2-activated NK cells, however, demonstrated that the YE1/48<sup>+</sup> population is not altered in these strains of mice (Fig. 4, A–D). In addition, two color analysis of freshly isolated splenic NK cells indicates that YE1/48<sup>+</sup> NK cells are not deleted in B10.A mice (Fig. 4 E). This expression pattern is in stark contrast to that of the Ab A1, which stains 15–20% of B10 NK1.1<sup>+</sup> cells, but is entirely absent in B10.A mice (28). Analysis of YE1/48 expression within the NK1.1<sup>+</sup> gate of fresh spleen cells clearly demonstrates that the same 15–20% population persists regardless of haplotype. Identical staining was observed with both YE1/48 and YE1/32 (not shown).

**Ly-49A and Ly-49C Mediate Cell Adhesion.** Recent studies have demonstrated a physical interaction between Ly-49A and certain class I MHC molecules (D<sup>d</sup> and D<sup>k</sup>) (5, 6). To determine whether Ly-49C also functions as an adhesion molecule and to further characterize the binding patterns of Ly-49A, we have examined several cell lines for their ability to adhere to COS cells transfected with each of these Ly-49 cDNAs (Table 2). The haplotypes of the cell lines were H-2<sup>d</sup> (A20 and P388D1), H-2<sup>k</sup> (R1.1), H-2<sup>b</sup> (C1498, CTLL-2, IC-21, and MBL-2), H-2<sup>s</sup> (GM979), and H-2<sup>a</sup> (B10/A2/2.2 and YAC-1). As expected, Ly-49A transfected COS cells bound the H-2<sup>d</sup> and H-2<sup>k</sup> cell lines, but failed to bind to H-2<sup>b</sup>, H-2<sup>s</sup>, and H-2<sup>a</sup>. Ly-49C transfectants bound all the cells tested, with the exception of the two H-2<sup>a</sup> cell lines (which also did not bind Ly-49A). Fig. 5 shows typical binding observed by H-2<sup>d</sup> (A20) and H-2<sup>b</sup> (CTLL-2) cell lines. These results demonstrate that Ly-49C, like Ly-49A, functions as a receptor molecule, and that these molecules have nonidentical ligand specificities.

**Antibodies to Class I MHC and Ly-49C Block the Adhesion of GM979 to Ly-49C.** After demonstrating distinct adhesion specificities for Ly-49A and C, we next investigated whether the ligand for Ly-49C is a class I MHC molecule.

In this set of experiments we employed the H-2<sup>s</sup> cell line GM979, which was shown to bind Ly-49C but not Ly-49A. The anti-class I MHC Ab 34-1-2S almost entirely abolished Ly-49C-mediated adhesion (Fig. 6), whereas an anti-Ly-5.2 Ab had minimal effect. The anti-Ly-49C Ab 5E6 also completely blocked the binding of GM979 to transfected COS cells (Fig. 6).

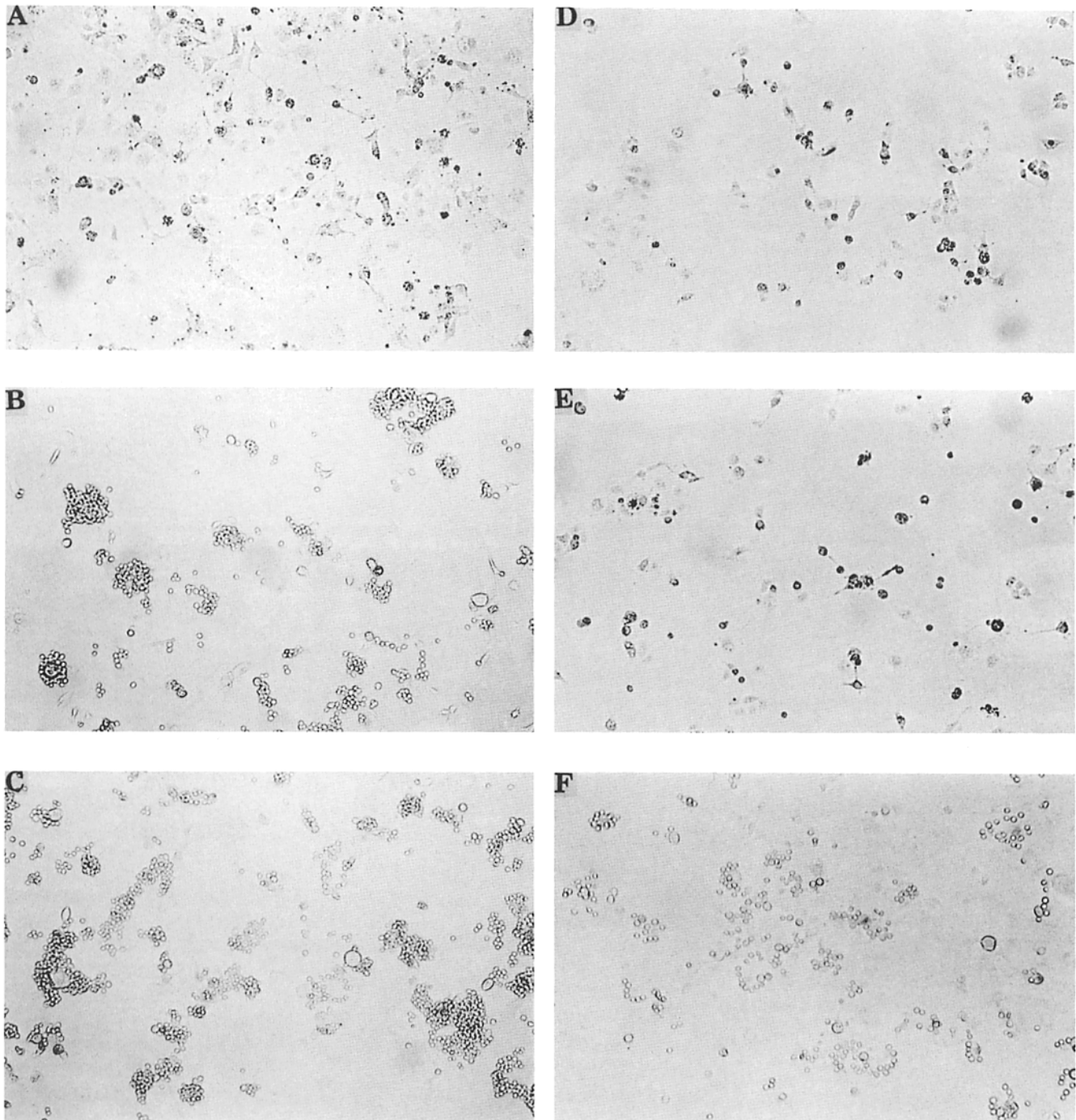
## Discussion

Previous Ly-49 studies have focused on the original member of this multigene family. Ly-49A has been demonstrated to

**Table 2.** Adhesive Properties of Ly-49A and Ly-49C Expressing COS Cells

Cell line	Cell type	H-2	Ly-49A	Ly-49C
A20	B cell	d	+	+
P388D1	Macrophage	d	+	+
R1.1	T cell	k	+	+
CTLL-2	T cell	b	–	+
C1498	Lymphoma	b	–	+
IC-21	Macrophage	b	–	+
MBL-2	T cell	b	–	+
GM979	Erythroleukemia	s	–	+
Yac-1	T cell	a	–	–
		(K <sup>k</sup> D <sup>d</sup> )		
B10A/A2/2.2	Immature myeloid	a	–	–

Transfected COS cells were incubated with the above cell lines for 2 h at 37° C. Adhesion (+ or –) was evaluated after five washes as described in Fig. 5.

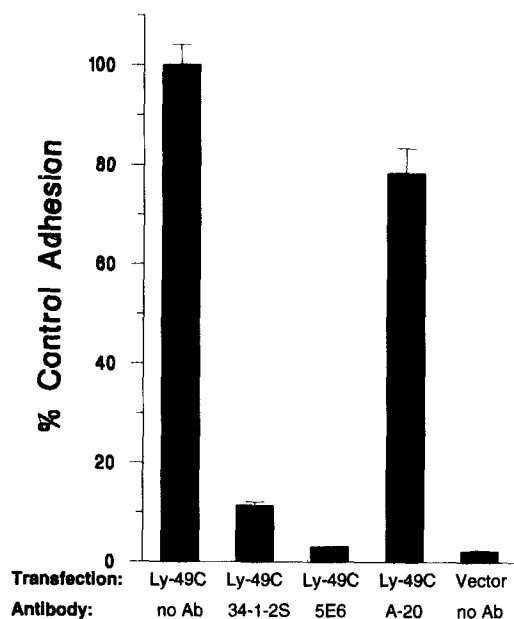


**Figure 5.** Adhesion of cell lines to COS cells expressing Ly-49A and Ly-49C. COS cells transfected with the vector PAX142 (*A* and *D*), Ly-49A (*B* and *E*), and Ly-49C (*C* and *F*), were overlaid with the cell lines A20 (*A-C*) or CTLL-2 (*D-F*). Cells were photographed after five washes after a 2-h coincubation at 37°C.

function as a receptor on NK cells from C57BL/6 mice for certain MHC class I molecules on target cells and to transduce negative signals (5). Since MHC molecules are highly polymorphic, and the Ly-49 family consists of at least several highly related genes, it is tempting to speculate that all members of the Ly-49 multigene family are expressed by murine NK cell populations and function as receptors for MHC

on target cells. Our present study has indeed shown that another Ly-49 molecule, Ly-49C, is also primarily expressed on NK cells and binds to class I MHC on target cells.

The initial molecular characterization of Ly-49 demonstrated that the cDNA hybridizes to multiple bands on a genomic Southern blot. These bands likely represent separate genes rather than a complex intron-exon structure, because even



**Figure 6.** Ly-49C-mediated adhesion to the cell line GM979 (H-2<sup>b</sup>) is blocked by anti-class I MHC and anti-Ly-49C Abs.  $5 \times 10^5$  <sup>51</sup>Cr-labeled GM979 cells were added to COS cells transfected with Ly-49C. GM979 was preincubated with 34-1-2S hybridoma supernatant (anti-class I MHC), A-20 (anti-Ly-5.2), or with no Ab (media alone). In the case of 5E6 blocking, COS cells were preincubated with 5  $\mu$ g/ml of purified Ab. The mean and SD are from triplicate plates and represent the percent binding relative to control (GM979 binding to COS cells transfected with Ly-49C).

small fragments of the Ly-49A cDNA hybridized to multiple bands (25, 29). We have continued the elucidation of this complex family by isolating two novel Ly-49 cDNAs. Ly-49D was cloned from the same (C57BL/6  $\times$  CBA)F<sub>1</sub> lung cDNA library which yielded Ly-49B and Ly-49C (7). Although Southern blot analysis between mouse strains indicates that this family may be polymorphic (30), the sequence differences between the cDNAs we have isolated is likely too great to be allelic. Ly-49E was cloned from a B10.A mouse, whereas Ly-49A was originally cloned from C57BL/6 T lymphoma lines (25, 29). Genomic Southern blot analysis with the Ly-49A cDNA has shown that these two strains share the same RFLP pattern (30). Although the expression patterns of these genes have not been fully determined, all five cloned Ly-49 genes are detected in NK-enriched spleen cells by RT-PCR, suggesting that all of them may be expressed in NK cells (Masterman, S., and F. Takei, unpublished observation).

COS cell expression of these five Ly-49 cDNAs has allowed for a precise definition of three Ly-49 mAbs. YE1/32 and YE1/48 react with Ly-49A, and 5E6 reacts with Ly-49C. This lack of crossreactivity between related molecules has allowed us to determine the identity of the 5E6 antigen and dissect NK subsets based upon expression of these Ly-49 molecules. We have shown that within NK1.1<sup>+</sup> spleen cells, there are both Ly-49A single positive, Ly-49C single positive, as well as double negative and double positive cells. This is the first demonstration that expression of distinct members

of the Ly-49 family of molecules defines separate NK cell subsets.

Although the functional significance of these NK cell subsets is yet to be established, previous work has ascribed different functional properties to Ly-49A<sup>+</sup> and Ly-49C<sup>+</sup> NK cells. IL-2-activated Ly-49A<sup>+</sup> and Ly-49A<sup>-</sup> NK cells are equal in their ability to lyse H-2<sup>b</sup> tumor target cell lines, whereas Ly-49A<sup>+</sup> NK cells are specifically unable to lyse H-2<sup>d</sup> and H-2<sup>k</sup> targets. It has been demonstrated that expression of D<sup>d</sup> on the target cell surface protects the cell from lysis by Ly-49A<sup>+</sup> NK cells (5). Ly-49C<sup>+</sup> (5E6<sup>+</sup>) NK cells, on the other hand, mediate the rejection of H-2<sup>d</sup>, but not H-2<sup>b</sup>, bone marrow grafts in lethally irradiated recipients (13). Our demonstration that these NK antigens are expressed on distinct subsets confirms the prediction that NK cells may be subdivided into separate functional compartments. The fact that the cell surface molecules that allow for this precise subdivision belong to the same family of molecules suggests that they themselves may be receptors that confer a functional property on the subset. This has been shown to be the case for Ly-49A. It is interesting to note that there are a small number of NK1.1<sup>+</sup> cells double positive for Ly-49A and Ly-49C. It remains to be seen if this population has a unique functional characteristic or whether it represents a stage in NK cell development.

Ly-49C<sup>+</sup> spleen cells can be divided into complex subsets, including Ly-49A negative, Ly-49A low, and Ly-49A high populations, each of which can further be divided into NK1.1<sup>+</sup> and NK1.1<sup>-</sup> subsets. Significant numbers of Ly-49A<sup>+</sup> and Ly-49C<sup>+</sup> cells coexpress CD3 and NK1.1, demonstrating a true sharing of markers that have been regarded as NK specific (NK1.1) or T cell specific (CD3). Some of the cells expressing Ly-49A or C are NK1.1<sup>-</sup> and include CD3<sup>+</sup> as well as CD3<sup>-</sup> cells. The importance of these populations is unclear, but they too may represent stages in NK cell development.

Our Ly-49A expression data with YE1/32 and YE1/48 has shown an interesting difference when compared with the mAb A1. A1<sup>+</sup> cells represent 15–20% of NK1.1<sup>+</sup> cells in B10 mice, but are absent in the MHC congenic strain B10.A, demonstrating an MHC based selection of the NK repertoire (28). Our results have shown, however, that NK1.1<sup>+</sup> YE1/32<sup>+</sup> and NK1.1<sup>+</sup> YE1/48<sup>+</sup> cells are not deleted in B10.A or any other MHC congenic strains of mice tested (B10.BR and B10.D2). This discrepancy in the expression patterns of these Ly-49 Abs suggests that YE1/32 and YE1/48 are capable of recognizing an epitope not recognized by A1 and not negatively selected by the H-2<sup>d</sup>, H-2<sup>k</sup>, or H-2<sup>a</sup> haplotypes. It is not known whether this epitope is carried by an additional, and as yet uncharacterized, member of this highly related family of molecules or if it is generated by a posttranslational modification of Ly-49A, perhaps by formation of dimers with other Ly-49 molecules.

We have shown that both Ly-49A and Ly-49C function as cell adhesion molecules when expressed in COS cells. The binding pattern of Ly-49A correlates well with the data on lysis by Ly-49A<sup>+</sup> NK cells. These cells kill H-2<sup>b</sup> targets, but

not H-2<sup>d</sup> or H-2<sup>k</sup> (5). Our data demonstrates that a ligand for Ly-49A is expressed on H-2<sup>d</sup> and H-2<sup>k</sup>, but not H-2<sup>b</sup> cell lines. The lack of binding to H-2<sup>b</sup> implies a lack of protective class I molecules, which explains why these cells are killed by Ly-49A<sup>+</sup> NK cells. Ly-49A also failed to bind the H-2<sup>s</sup> and H-2<sup>a</sup> cell lines. The H-2<sup>a</sup> haplotype is a hybrid one (K<sup>k</sup>, D<sup>d</sup>), and it was therefore unexpected that Ly-49A would not bind a cell expressing D<sup>d</sup>. Interestingly, these H-2<sup>a</sup> cell lines were the only ones tested to which Ly-49C did not bind, although binding was observed to H-2<sup>k</sup>, H-2<sup>d</sup>, H-2<sup>b</sup>, and H-2<sup>s</sup> lines. It should be noted that the two H-2<sup>a</sup> cell lines expressed D<sup>d</sup> as expected (data not shown). It is unknown if the inability of these H-2<sup>a</sup> cell lines to bind Ly-49A and Ly-49C is a peculiar property of these particular cell lines, or the H-2<sup>a</sup> haplotype in general.

The demonstration that anti-class I MHC antibody in-

hibits Ly-49C-mediated adhesion supports the hypothesis that Ly-49 is a family of class I binding molecules expressed on subsets of NK cells. Although Ly-49C binds a wider range of haplotypes than Ly-49A, it is not known which class I molecules serve as ligands. The adhesive properties of Ly-49C are of significance in light of the functional characterization of the 5E6<sup>+</sup> NK subset. These cells comprise the entire population responsible for the rejection of H-2<sup>d</sup> but not H-2<sup>b</sup> bone marrow grafts. Our data shows that Ly-49C is capable of binding a molecule on H-2<sup>d</sup> cell lines. The fact that 5E6<sup>+</sup> cells eliminate H-2<sup>d</sup> grafts indicate that the interaction of Ly-49C with its H-2<sup>d</sup> ligand may not negatively regulate the NK cell as does the Ly-49A receptor-ligand interaction. The identification of the class I MHC ligand for Ly-49C as well as the significance of this interaction in NK cell function are currently under investigation.

---

We thank John Dunlop and Doug Freeman for their work in cloning and sequencing Ly-49D cDNA; Dr. Peter Lansdorp for the purified A-20 Ab; and Dr. Simon Wong for a critical reading of the manuscript.

This work was supported by a joint grant from the Cancer Research Society of Canada to F. Takei and D. Mager, with core support provided by the British Columbia Cancer Agency, and grants to W. Jefferies from the Medical Research Council of Canada and the National Cancer Institute of Canada.

Address correspondence to Dr. Fumio Takei, Terry Fox Laboratory, British Columbia Cancer Agency, 601 West 10th Avenue, Vancouver, B.C. V5Z 1L3 Canada.

*Note Added in Proof:* The cloning of several novel Ly-49 cDNAs has recently been reported (31), and these have been designated Ly-49D, E, F, and G. Our Ly-49D is distinct from all of these sequences, and will be termed Ly-49H in future studies. Our Ly-49E corresponds to the Ly-49G group of cDNA clones, which likely arise from alternate splicing of the same gene.

*Received for publication 28 April 1994 and in revised form 8 August 1994.*

## References

- Trinchieri, G. 1989. Biology of natural killer cells. *Adv. Immunol.* 47:187.
- Yu, Y.Y.L., V. Kumar, and M. Bennett. 1992. Murine natural killer cells and marrow graft rejection. *Annu. Rev. Immunol.* 10:189.
- Ljunggren, H.G., and K. Kärre. 1990. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol. Today.* 11:237.
- Moretta, L., E. Ciccone, A. Moretta, P. Hoglund, C. Ohlen, and K. Kärre. 1992. Allorecognition by NK cells: nonself or no self? *Immunol. Today.* 13:300.
- Karlhofer, F.M., R.K. Ribaud, and W.M. Yokoyama. 1992. MHC class I alloantigen specificity of Ly-49+ IL-2 activated natural killer cells. *Nature (Lond.)* 358:66.
- Kane, K.P. 1994. Ly-49 mediates EL4 lymphoma adhesion to isolated class I major histocompatibility molecules. *J. Exp. Med.* 179:1011.
- Wong, S., J.D. Freeman, C. Kelleher, D. Mager, and F. Takei. 1991. Ly-49 multigene family: New members of a superfamily of type II membrane proteins with lectin-like domains. *J. Immunol.* 147:1417.
- Giorda, R., W.A. Rudert, C. Vavassori, W.H. Chambers, J.C. Hiserodt, and M. Trucco. 1990. NKR-P1, a signal transduction molecule on natural killer cells. *Science (Wash. DC)* 249:1298.
- Giorda, R., and M. Trucco. 1991. Mouse NKR-P1: a family of genes selectively coexpressed in adherent lymphokine-activated killer cells. *J. Immunol.* 147:1701.
- Yokoyama, W.M., J.C. Ryan, J.J. Hunter, H.R.C. Smith, M. Stark, and W.E. Seaman. 1991. cDNA cloning of mouse NKR-P1 and genetic linkage with Ly-49: identification of a natural killer cell gene complex on mouse chromosome 6. *J. Immunol.* 147:3229.
- Scalzo, A.A., N.A. Fitzgerald, A. Simmons, A.B. LaVista, and G.R. Shellam. 1990. *Cmv-1*, a genetic locus that controls murine cytomegalovirus replication in the spleen. *J. Exp. Med.* 171:1469.
- Scalzo, A.A., N.A. Fitzgerald, C.R. Wallace, A.E. Gibbons, Y.C. Smart, R.C. Burton, and G.R. Shellam. 1992. The effect of the *Cmv-1* resistance gene, which is linked to the natural killer cell gene complex, is mediated by natural killer cells. *J. Immunol.* 149:581.
- Sentman, C.L., J. Hackett, Jr., V. Kumar, and M. Bennett. 1989. Identification of a subset of murine natural killer cells that mediates rejection of *Hh-1<sup>d</sup>* but not *Hh-1<sup>b</sup>* bone marrow grafts. *J. Exp. Med.* 170:191.



14. Stoneman, E., V. Kumar, M. Bennett, and P.A. Matthew. 1993. Molecular cloning of an NK cell surface molecule expressed on a subset of cells that mediate specific rejection of Hh-1d positive marrow cells. *J. Immunol.* 150:1465a. (Abstr.)
15. Gunji, Y., N.L. Vujanovic, J.C. Hiserodt, R.B. Herberman, and E. Gorelik. 1989. Generation and characterization of purified adherent lymphokine-activated killer cells in mice. *J. Immunol.* 142:1748.
16. Takei, F. 1983. Two surface antigens expressed on proliferating mouse T lymphocytes defined by rat monoclonal antibodies. *J. Immunol.* 130:2794.
17. Koo, G., and J.R. Peppard. 1984. Establishment of monoclonal anti-Nk-1.1 antibody. *Hybridoma.* 3:301.
18. Shen, F.W. 1981. Monoclonal antibodies to mouse lymphocyte differentiation antigens. In *Monoclonal Antibodies and T Cell Hybridomas: Perspectives and Technical Advances*. G.J. Hämmerling, U. Hämmerling, and J.F. Kearney, editors. Elsevier/North Holland and Biomedical Press, Amsterdam/New York/Oxford. 25-31.
19. Ozato, K., N.M. Mayer, and D.H. Sachs. 1982. Monoclonal antibodies to mouse major histocompatibility antigens IV. A series of hybridoma clones producing anti-H-2<sup>d</sup> antibodies and an examination of expression of H-2<sup>d</sup> antigens on the surface of these cells. *Transplantation (Baltimore).* 34:113.
20. Unkeless, J.C. 1979. Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors. *J. Exp. Med.* 150:580.
21. Tabor, S., and C.C. Richardson. 1987. DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. *Proc. Natl. Acad. Sci. USA.* 84:4767.
22. Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* 12:387.
23. Kay, R., and R.K. Humphries. 1991. New vectors and procedures for isolating cDNAs encoding cell surface proteins by expression cloning in COS cells. *Methods Mol. Cell. Biol.* 2:254.
24. Hammarskjöld, M.L., S.C. Wang, and G. Klein. 1986. High-level expression of the Epstein-Barr virus EBNA1 protein in CV1 cells and human lymphoid cells using a SV40 late replacement vector. *Gene.* 43:41.
25. Yokoyama, W., L.B. Jacobs, O. Kanagawa, E.M. Shevach, and D.I. Cohen. 1989. A murine T lymphocyte antigen belongs to a supergene family of type II integral membrane proteins. *J. Immunol.* 143:1379.
26. Drickamer, K., and M.E. Taylor. 1993. Biology of animal lectins. *Annu. Rev. Cell Biol.* 9:237.
27. Chambers, W.H., T. Adamkiewicz, and J.P. Houchins. 1993. Type II integral membrane proteins with characteristics of C-type animal lectins expressed by natural killer (NK) cells. *Glycobiology.* 3:9.
28. Sykes, M., M.W. Harty, F.M. Karhofer, D.A. Pearson, G. Szot, and W. Yokoyama. 1993. Hematopoietic cells and radioresistant host elements influence natural killer cell differentiation. *J. Exp. Med.* 178:223.
29. Chan, P.Y., and F. Takei. 1989. Molecular cloning and characterization of a novel murine T cell surface antigen, YE1/48. *J. Immunol.* 142:1727.
30. Yokoyama, W.M., P.J. Kehn, D.I. Cohen, and E.M. Shevach. 1990. Chromosomal location of the Ly-49 (A1, YE1/48) multigene family: genetic association with the NK 1.1 antigen. *J. Immunol.* 145:2353.
31. Smith, H.R.C., F.M. Karhofer, and W. Yokoyama. 1994. Ly-49 multigene family expressed by IL-2-activated NK cells. *J. Immunol.* 153:1068.