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*J Immunol* 2001; 166:5034-5043; doi: 10.4049/jimmunol.166.8.5034

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Class I MHC-Binding Characteristics of the 129/J Ly49 Repertoire

Andrew P. Makrigiannis,* Amanda T. Pau,* Ali Saleh,* Robin Winkler-Pickett,* John R. Ortaldo,* and Stephen K. Anderson2*†

The Ly49 family of NK cell receptors and its MHC-binding characteristics have only been well characterized in C57BL/6 (B6) mice. Previous studies have shown that 129/J mice express unique Ly49 genes that are not found in the B6 strain. Screening of a 129/J cDNA library led to the discovery of 10 distinct full-length Ly49-related coding sequences (Ly49e, g, i, o, p, r, s, t, u, and v). Although 129/J mice share identical class I MHC (Kb and Dr) transcripts with B6 mice, only one Ly49 is identical in the two strains (Ly49E). In addition to the previously characterized Ly49P, two new activating Ly49 proteins were discovered, Ly49R and U. The MHC specificity of the total 129/J Ly49 repertoire was evaluated with soluble class I MHC tetramers and found to be distinct compared with the B6 Ly49 repertoire. Ly49V bound to many types of class I MHC, suggesting that Ly49V+ NK cells may monitor host cells for a global down-regulation in MHC levels. An activating receptor, Ly49R, was shown to bind soluble class I molecules to a moderate degree, a result not previously observed for other activating Ly49 proteins. Furthermore, tetramer-binding results were confirmed functionally with cytotoxicity assays using sorted 129/J NK cells. This study shows that the Ly49 repertoire and its MHC-binding characteristics can be very different among inbred mouse strains. Ly49 divergence should be considered when using 129-derived embryonic stem cells for the production of gene-targeted mice, especially when an immune or NK-derived phenotype is under scrutiny. The Journal of Immunology, 2001, 166: 5034–5043.

Natural killer cells are an important component of the innate immune system, providing protection against intracellular infection and neoplasia through direct cytotoxic mechanisms and the secretion of cytokines (1). Efforts to identify receptors on NK cells that regulate cytotoxicity have yielded many interesting proteins. There are three major families of MHC-binding receptors on NK cells: killer cell Ig-like receptors (KIRs), and two lectin-like families, CD94/NKG2 and Ly49. KIR and Ly49 molecules have been found to bind class I MHC, and CD94/NKG2 heterodimers recognize the nonclassical MHC class I ligands HLA-E in humans and Qa-1 in mice (2). Although CD94/NKG2 are expressed in both species, KIRs are only expressed in humans and Ly49s only in rodents. Despite their lack of genetic homology, KIR and Ly49 function appeared to have evolved in a convergent manner. KIR repertoires vary significantly between individuals such that different types and numbers of genes are expressed (3). Recently, the genomic sequence from two different KIR haplotypes was reported, and certain KIR genes are present or absent depending on the individual genotype (4). In contrast, little is known concerning Ly49 genotypic variation in rodents.

The Ly49 family represents a group of receptors expressed on murine NK, NK T cells, and some memory CD8+ T cells (5–7). The most extensively studied repertoire belongs to C57BL/6 (B6) mice. In these mice, Ly49A,B6, E,B6 are known to be expressed, whereas only gene fragments and/or aberrant mRNAs have been detected for Ly49K,B6, P,B6, m,B6, and n,B6 (5, 6, 8–10). We have previously shown that other mouse strains possess Ly49 molecules not found in B6 mice. Ly49O and P were found to be expressed in 129/J mice and Ly49L was found in CBA/J and C3H/He mice, yet none of these were detected in B6 mice using immunoprecipitation or RT-PCR (11, 12). This suggests that the Ly49 repertoire is extremely polymorphic between inbred mouse strains, even those sharing the same MHC haplotype such as 129/J and B6 (H-2b). In fact, Southern blotting with Ly49 single-exon probes has shown striking differences in the Ly49 gene content between mouse strains (9). A survey of inbred mouse strains with Ly49-specific Abs revealed that Ly49 epitope expression varies greatly. For example, a mAb that recognizes Ly49G2B64 (4D11) was found to stain DX5+CD3+ NK cells from nine of nine strains tested, whereas 4E5 (Ly49D16) stained NK cells from only three strains (B6, 129/J, and SJL) (13). Whether this is a result of allelic divergence or absent/silent genes is unknown.

With respect to function, Ly49 molecules can be grouped into activating and inhibitory subgroups. Inhibitory Ly49 sequences can be identified by the presence of an immunoreceptor tyrosine-based inhibitory motif (ITIM; I/VxYxxL/V) in the intracellular domain that recruits Src homology 2 domain-containing phosphatase 1 upon phosphorylation (14, 15). Activating Ly49 molecules (D,B6, H,B6, I,cb/J, and P,129/J) lack an intact ITIM sequence but...
possess an arginine in the transmembrane domain for association with the signal-transducing protein DAP12 (16, 17). The binding of inhibitory Ly49 receptors to MHC ligands results in an inhibition of cytokotoxicity (18, 19). In contrast, NK killing can be triggered via Ly49D<sup>B6</sup>-by H-2<sup>D</sup>-expressing target cells (20, 21). Furthermore, cross-linking of activating Ly49 molecules by specific Ab or MHC ligand results in cytokine production and intracellular calcium ion mobilization (11, 20, 22). In addition to D<sup>D</sup>, Ly49D<sup>B6</sup>-B6 NK cells have been shown to interact with D<sup>D</sup> and D<sup>D</sup>-expressing target cells (23). A recent report has shown that, like Ly49B<sup>B6</sup>, the presence of the nonobese diabetic allele of Ly49P on target cells (23). A recent report has shown that, like Ly49G2<sup>B6</sup>, receptors, such as Ly49G2<sup>B6</sup>, show a very fine specificity toward the known ligand specificities of many B6 Ly49 proteins. Some lines were provided by Drs. J. Ryan and M. Nakamura (University of California at San Francisco). D<sup>D</sup> and D<sup>D</sup>-positive target cells (25–27). One report has also shown that Ly49A<sup>B6</sup> is bound by D<sup>B</sup> tetramers (26). Although the role of inhibitory Ly49 such as Ly49B<sup>B6</sup> and Ly49C<sup>B6</sup> can be explained as the monitoring of “self” in B6 mice, the purpose of inhibitory Ly49 that bind to MHC not present in B6 mice, as well as of activating Ly49 in general, still remains controversial.

Because 129 embryonic stem (ES) cells are used for the production of knockout mice for immunologic research, knowledge of the 129/J Ly49 repertoire is important, especially for studies dealing with NK biology. The 129/J and other closely related 129 strains show very different immunological phenotypes compared with B6 mice, with which they share the same MHC background. For example, 129/J mice are more susceptible to intracellular pathogens, such as the WAl piroplasm and Sendai virus, than B6 mice (28, 29). Also, 129/J mice are more prone to the induction of autoimmune diseases such as experimental autoimmune encephalomyelitis and show greater disease severity in diabetes models than B6 mice (30, 31). In contrast, 129/J mice are relatively resistant to tumor induction by various carcinogenic agents and display a lower spontaneous overall tumor incidence (32–34). It has long been known that 129/J mice are not as capable as B6 mice in tumor rejection of allogeneic and xenogeneic bone marrow transplants (35). Interestingly, the regulation of murine transplant rejection is at least partly dependent on Ly49 expression by NK cells (36). Our initial observations of the 129/J strain show that the Ly49 repertoire is different from B6 mice (11); therefore, elucidation of the total 129/J Ly49 repertoire was conducted to facilitate comparison of B6 and 129/J NK cell function. In addition, we have tested the ability of the newly described receptors to bind seven different types of class I MHC.

Materials and Methods

Mouse NK cells and tumor cell lines

B6 and 129/J mice were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were kept under pathogen-free conditions until use at 20–25 wk of age. Liver NK cells were isolated as previously described (37). CD3<sup>−</sup> DX5<sup>−</sup>-sorted liver NK cells were expanded for 3–5 days in complete RPMI 1640 supplemented with 1000 Cetus U/ml IL-2 (Hoffman-LaRoche, Nutley, NJ). Routinely, 75–80% of the resulting cells were DX5<sup>+</sup>. Cell line 293T is a human kidney epithelial cell line. YB20 and YB/D<sup>D</sup> rat cell lines were provided by Drs. J. Ryan and M. Nakamura (University of California Veterans Affairs Medical Center, San Francisco, CA).

Cloning of Ly49 cDNA from 129/J NK cells

A cDNA library from IL-2-activated 129/J liver NK cells was constructed in the pBK-CMV vector (Stratagene, La Jolla, CA) by Cell and Molecular Technologies (Phillipsburg, NJ). Library screening was conducted with Ly49<sup>29, 30, 31, 32, 33, 34, 35</sup> probes. One hundred positive clones were analyzed, and the following Ly49 sequences were found: Ly49<sup>40, 41, 42</sup> (AF247653), Ly49<sup>26, 25, 24</sup> (AF283875), Ly49<sup>21, 20</sup> (AF283876), Ly49<sup>19, 20</sup> (AF237866), Ly49<sup>19, 20</sup> (AF283877), Ly49<sup>19, 20</sup> (AF283878), Ly49<sup>19, 20</sup> (AF283880), and Ly49<sup>19, 20</sup> (AF283881). Previously assigned GenBank files for Ly49<sup>19, 20</sup> and Ly49<sup>19, 20</sup> (AF146570 and AF146571, respectively) were updated to include S<sup>+</sup> and S<sup>+</sup> untranslated sequence identified in this study.

Plasmids and transfections

Ly49 cDNAs were PCR-subcloned into pEF6/V5-His (pEF6) using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). All subclones were sequenced. The day before transfection, 2.5 × 10<sup>6</sup> 293T cells were seeded in six-well plates. The following day, 293T cells were transfected with FuGENE6 (Roche, Indianapolis, IN) using 1.25 μg each of the indicated combinations of Ly49/pEf6 and mDAP12/pSport (a gift from D. McVicar, National Cancer Institute, Frederick, MD) for activating receptors plus empty pEF6 vector to a total of 5 μg of DNA.

Abs and soluble class I MHC tetramers

The following mAbs were used for two-color flow cytometry: FITC-labeled 12A8, which binds to Ly49A<sup>B6</sup>/D<sup>B6</sup> (39) and PE-labeled 4E5 (Ly49B<sup>B6</sup>/D<sup>B6</sup> (16). YE1/48, YE1/32 (Ly49A<sup>B6</sup>/B6, 4D11 (Ly49G2<sup>B6</sup>/B6) (19), 5E6 (Ly49h<sup>B6</sup>/D<sup>3</sup>) (41), and A1 (Ly49B<sup>B6</sup>/D<sup>B6</sup> (42) were all used as primary staining reagents followed by FITC-conjugated goat anti-rat IgG secondary (Kirkegaard & Perry Laboratories, Gaithersburg, MD) or, in the case of 5E6 and A1, FITC-conjugated goat anti-mouse IgG was used as a secondary reagent (Kirkegaard & Perry Laboratories). A generous gift from V. Kumar, University of Chicago (Chicago, IL), 1F8 (Ly49B<sup>B6</sup>/D<sup>3</sup>PE) was FITC-labeled, and PE-12A8 was also used for single-color analyses. Biotinylated 4G10 Ab, which recognizes phosphotyrosine, was purchased from Upstate Biotechnology (Lake Placid, NY). Detection of phosphorylated proteins was conducted as previously described (11). DX5 (pan-NK cell) mAb was a kind gift from L.Lanier (University of California at San Francisco). D<sup>D</sup> expression was analyzed using FITC-conjugated 34-5-85 (BD PharMingen, San Diego, CA). Strepavidin-PE conjugated murine class I MHC tetramers D<sup>D</sup>, D<sup>D</sup>, D<sup>3</sup>, L<sup>3</sup>, K<sup>3</sup>, K<sup>3</sup>, and K<sup>3</sup> were provided by the National Institute of Allergy and Infectious Diseases Tetramer Facility/National Institutes of Health AIDS Research and Reference Reagent Program (Yerkes Regional Primate Research Center, Atlanta, GA). Tetramers were refolded in the presence of peptide previously shown to form a stable tetrameric structure and are as follows: D<sup>D</sup>, GP33–41 (KAVYNFATC) of lymphocytic choriomeningitis virus (43); D<sup>D</sup>, GP160–169 (RGPGRAFVTI) of HIV-1 (44); D<sup>D</sup>, MT389–397 (RLRLGRTLLL) of polyoma virus (45); K<sup>B</sup>, N324–332 (FAGPNYPAL) of sendai virus (46); K<sup>B</sup>, NPP147–155 (TYYQTRALV) of influenza virus (47); K<sup>B</sup>, Ha255–262 (FESTGNLI) of murine gammaretrovirus (48); and K<sup>B</sup>, NP118–126 (RPQASGVM) of lymphocytic choriomeningitis virus (49). Class 1 MHC H chain and human β<sub>2</sub>-microglobulin were both produced in bacteria using the pET expression system.

Flow cytometry and cytotoxicity assays

NK cells and transfected 293T were stained with the Abs described above (for 30 min on ice) and MHC tetramers (for 30 min on ice at 37°C) and analyzed on a FACSort (BD Biosciences, Mountain View, CA). Incubations longer than 30 min did not increase tetramer-binding levels. NK cells used for cytotoxic analyses were sorted on a MoFlo (Cytomation, Ft. Collins, CO). Cytotoxicity assays were conducted as previously described (19).

Results

mAb 4E5 detects activating and inhibitory Ly49 molecules in 129/J mice

We have previously observed that anti-Ly49 immunoprecipitates of 129/J NK cells yield different phosphotyrosine-binding patterns when compared with NK cells from B6 mice (11). Specifically, immunoprecipitation with mAb 4E5, which only binds the activating Ly49D receptor in the B6 strain, results in the coimmunoprecipitation of both activating and inhibitory Ly49 molecules in 129/J mice (11). To better characterize the Ly49D<sup>B6</sup>-like receptors in 129/J mice, two-color flow cytometric analysis was conducted with 4E5 and 12A8. A mAb that can bind to Ly49D<sup>B6</sup> as well as Ly49A<sup>B6</sup>. 12A8 has been shown to react with a subpopulation of 129/J NK cells (13, 39). Two-color staining with 12A8 and 4E5 of
B6 NK cells results in four populations, a double-negative, two double-positive, and a 4E5+12A8+ single-positive population (Fig. 1A). The single-positive population in the lower right quadrant represents Ly49A+D+ NK cells, whereas the upper right quadrant contains Ly49A+D+ (12A8high) and Ly49A-D+ (12A8low) populations. In contrast, when the same Abs are used to stain 129/J NK cells, a very different pattern is observed (Fig. 1C). The 4E5-12A8+ population is not present, and instead a 4E5+12A8+ population appears (upper left quadrant). Although there are still two double-positive populations in 129/J mice, the 12A8high population is gone, and instead a 4E5high population is now present. NK cells from a first generation cross of B6 and 129/J mice show that all populations of the parent mice are present, yet the single-positive populations are diminished (Fig. 1B).

To determine the nature of the Ly49 molecules expressed by the sorted populations in Fig. 1C, proteins were isolated from pervanadate-treated NK cells, immunoprecipitated with Ly49-specific mAb, separated by SDS-PAGE under nonreducing conditions, and detected by anti-phosphotyrosine blotting. We have previously shown that this method will detect inhibitory Ly49 proteins as a ~110-kDa band due to a phosphorylated ITIM. In addition, a ~28-kDa band (DAP12) is communoprecipitated from activating Ly49 molecules (22). Activating Ly49 receptors have no ITIM; therefore, they are not directly phosphorylated, and their presence is detected indirectly through DAP12 association. The results of two such experiments are shown in Fig. 2, A and B. Immunoprecipitation of unsorted B6 splenic NK cells with 4E5 only detected the DAP12 molecule associated with the activating receptor, Ly49D. In contrast to B6 mice, unsorted 129/J NK cells showed both activating and inhibitory receptors when immunoprecipitated with 4E5 as previously reported (11). Due to concerns that different organs might contain different NK populations, bulk spleen and liver 129/J NK cells were compared and shown to contain both types of 4E5-reactive receptors (Fig. 2A). The type of receptor represented in the sorted populations of Fig. 1C was then analyzed. The upper left quadrant containing 4E5+12A8- cells was found to contain only inhibitory-type receptors (Fig. 2A). This suggests that 129/J mice possess an inhibitory-type Ly49 that is recognized by 4E5 but not 12A8.

Immunoprecipitation with 4E5 of the 4E5+12A8+ populations (both 4E5-high and -low) resulted in the detection of both inhibitory and activating forms (Fig. 2A). The 4E5-low 12A8+ population was found to contain an activating receptor that was immunoprecipitated by both 4E5 and 12A8 (Fig. 2B). The appearance of a ~60-kDa band is not always reproducible, as noted by its absence in Fig. 2A, and is most likely an Ig H chain breakdown product. The 4E5-high 12A8+ population contained the 4E5/12A8-reactive activating receptor as well as a 4E5+12A8- inhibitory molecule (Fig. 2B), most probably the same one observed in the 4E5+12A8- population (Fig. 2A). These experiments suggest the existence of at least two different, independently expressed Ly49D129/J-related molecules in 129/J mice. It appears that 12A8 only reacts with the activating receptor, whereas 4E5 reacts with both. The existence of a 4E5-reactive inhibitory receptor corroborates two-color flow cytometric analyses showing that 129/J, but not B6, mice contain a small but measurable population of 4E5+CD3+ T cells (data not shown). In B6 mice, expression of Ly49D is not detectable on T cells (50). The discovery that 4E5 recognizes an inhibitory Ly49 protein in 129/J mice supports observations that activating Ly49 receptors are not expressed on T cells.

The 129/J mice express at least 10 different Ly49-related genes

To further characterize the 129/J Ly49 repertoire, a cDNA library was constructed using mRNA from IL-2-activated 129/J liver NK cells. This library was then probed with Ly49e, Ly49f, Ly49g, and Ly49i full-length coding regions, and 100 positive clones were sequenced. A total of 10 distinct Ly49-related sequences were found. Table 1 shows the percentage of amino acid identity between the new 129/J Ly49 cDNAs and all known B6 Ly49 full-length coding regions. Only one sequence (Ly49e129/J) was found to be highly similar to a B6 Ly49 gene. Ly49e129/J contained only one silent nucleotide difference when compared with the Ly49e129/J nucleotide-coding region. Both Ly49g1 and g2 isoforms were found in 129/J
NK cells, with the Ly49g1 insert sharing 37/39 nucleotides between the 129/J and B6 alleles (data not shown).

Five new Ly49 cDNA sequences were discovered and have been named Ly49r129/J. Based on a previous survey of Ly49 alleles, a boundary was set of at least 95% identity at the protein level for sequences to be considered as potential alleles (51). Until allelic variants are verified by genomic mapping, these new designations should be considered tentative. The predicted amino acid sequences of the novel Ly49 receptors is provided in Fig. 3. Ly49R129/J and U 129/J, are putative activating receptors that lack an ITIM domain and contain a transmembrane arginine residue (Fig. 4).

Tetramer binding analyses indicated that some Ly49 bound to many types of class I MHC, whereas others bound to a few or none. As previously reported for Ly49G2 B6, this was particularly strong, especially Ly49V 129/J to D k (Fig. 5). Surprisingly, Ly49O 129/J and Ly49V 129/J also showed strong reactivity to L d (Fig. 5). Observed differences in expression levels of individual receptors may be due to differences in detection (FITC- vs PE-labeling).

To identify the molecules responsible for the staining and biochemical patterns observed in Figs. 1 and 2, expression constructs were transiently transfected into 293T cells and stained with a panel of Ly49-specific mAb. Three receptors, Ly49G 129/J, R 129/J, and V 129/J, were found to react strongly with 4E5 and/or 12A8 (Fig. 4 and Table II). As predicted by the biochemical data (Fig. 2B), the putative activating Ly49R 129/J was bound by both 4E5 and 12A8, whereas the two putative inhibitory receptors, Ly49G 129/J and V 129/J, were recognized by only 4E5 and not 12A8 (Fig. 4).

Reactivity of 129-Ly49 proteins to Ly49-specific mAb

Table I. Comparison of amino acid identity of 129/J and B6 Ly49 repertoires

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Sequence comparisons were made with SeqWeb Sequence Analysis BestFit (Genetics Computer Group, Madison, WI) using the local homology algorithm of Smith and Waterman.

The highest B6 Ly49 match for each 129/J Ly49 is in boldface. The MHC genotype of 129/J mice was assessed by RT-PCR cloning using B6-derived primers for K b and D b transcripts followed by sequencing. We found that the 129/J alleles for K b and D b are identical with their B6 counterparts over the full coding region at the nucleotide level, suggesting that Ly49 divergence is not a consequence of MHC coevolution (data not shown).

Soluble murine MHC class I tetramers reveal ligand specificities of the 129/J Ly49 repertoire

Fluorochrome-labeled soluble murine class I MHC “tetramers” have previously been used to identify the specificity of various members of the B6 Ly49 repertoire (25, 26). Therefore, to investigate the MHC-binding characteristics of the 129/J Ly49 repertoire, the tetramer approach was used. To determine the level of expression, 293T cells transfected with individual Ly49 expression constructs were stained with specific mAb or PE-conjugated murine class I tetramers D k, D d, K b, and L d (Fig. 4). No control staining for Ly49G 129/J expression is shown, due to a lack of Ly49S-reactive Abs (Table II). Otherwise, all transfected cells showed relatively high levels of appropriate Ly49 expression (Fig. 5). Observed differences in expression levels of individual receptors may be due to differences in detection (FITC- vs PE-labeling).

Also, it cannot be assumed that the cross-reactive mAbs used all share similar affinities for the 129/J Ly49 proteins. As a control, tetramers were used to stain 293T cells transiently expressing Ly49B6, C B6, and D B6. Tetramer binding to these three receptors was found to be almost identical with that reported previously (data not shown).

The results of the tetramer binding analyses indicated that some Ly49 bound to many types of class I MHC, whereas others bound to a few or none. As previously reported for Ly49G2 B6, Ly49G2 129/J bound soluble D k (Fig. 5) (25). Ly49G2 129/J also bound to D d and K b, but to a lesser extent. Ly49O 129/J and Ly49V 129/J bound D d and D k. To our knowledge, this is the first report of a MHC tetramer binding to an activating receptor, Ly49R 129/J. The tetramer binding by Ly49O 129/J and V 129/J was particularly strong, especially Ly49V 129/J to D k (Fig. 5). Surprisingly, Ly49R 129/J and Ly49V 129/J also showed strong reactivity to L d (Fig. 5). In binding to D d, L d, and Ly49V 129/J also bound strongly to D d. The ability of Ly49V 129/J and Ly49O 129/J to bind the autologous D d haplotype suggests that they can detect the presence of self-MHC as shown for the related Ly49A B6 (26). In this respect, Ly49V 129/J showed weaker reactivity toward K b, K d, and K b (Fig.
Ly49V 129/J is reminiscent of Ly49C B6 in its ability to bind many types of murine class I MHC (25). Ly49S 129/J, Ly49T 129/J, and Ly49U 129/J showed no significant binding to any MHC tetramer, whereas the third activator, Ly49P 129/J, only showed a low level of binding to Dd (Fig. 5). Finally, Ly49I 129/J-transfected 293T showed the second highest promiscuity with reactivity toward Dk, Kb, Kd, and Kk (Fig. 5). The MHC tetramer-binding data obtained for Ly49O 129/J and V 129/J from transiently transfected human cells was confirmed in stably transfected rat RNK-16 cells (data not shown).

Temperature-dependent binding increases were seen by Ly49R 129/J for Dd, Dk, and Ld (Table III). Because Ly49R 129/J and Ly49D B6 are closely related, this prompted us to perform a similar experiment with Ly49D B6-transfected 293T. No binding of Ly49D B6-expressing 293T was found for any tetramers when incubated on ice as previously reported (24), but, at 37°C, the Dd, Dk, and Ld tetramers were bound (data not shown). This brings into question the ability of other activating Ly49 proteins to bind MHC tetramers at 37°C, but Ly49P 129/J and U 129/J did not show enhanced tetramer binding at the higher incubation temperature (Table III). Interestingly, there was no increase in tetramer binding to Ly49I 129/J at 37°C over that already seen on ice. Also of interest was the significantly increased binding of the H-2b,d,k alleles of H-2K for Ly49V 129/J (Table III). Incubation at physiological temperature shows significant binding of Ly49V 129/J to all soluble class I MHC tetramers used in this study. This suggests that Ly49V 129/J NK cells monitor host cells for a global down-regulation of class I MHC.

Ly49O and/or V 129/J NK cell cytotoxicity is inhibited by class I MHC Dd

Identification of mAb that can bind to the novel 129/J Ly49 proteins allowed the possibility of isolating different NK subpopulations for functional analysis to confirm the observations of the tetramer-binding experiments. When transiently expressed in 293T or stably expressed in RNK-16, Ly49O 129/J and V 129/J expression constructs using the FuGENE6 lipid transfection reagent. Ly49R 129/J was also cotransfected with mDAP12 expression vector. After 1 day, transfected cells were stained with PE-12A8 or PE-4E5 and analyzed on a FACSort. This figure is representative of four similar experiments.
subset should be suppressed by $D^d$-expressing target cells. To test this hypothesis, 129/J NK cells were sorted into $4E5^{12A8^1}$ (Ly49O/V-positive and Ly49R-negative), $4E5^{12A8^2}$ (Ly49O/V-positive and Ly49R-positive), and $4E5^{12A8^2}$ (Ly49O and V-negative and Ly49R-negative) populations and used as effector cells against the BA/2-derived (H-2$d$) P815 target cell. The single- and double-positive populations, which contain Ly49O$^{129/J}$ and/or V$^{129/J}$, lysed P815 poorly (Fig. 6B). In contrast, double-negative NK cells displayed a 4-fold increase in killing against this H-2$d$ target cell (Fig. 6B). The classical NK target cell YAC-1 was used as a control for the cytotoxic potential of the differentially sorted populations. All three populations lysed YAC-1 cells efficiently.

### Table II. Relative binding of Ly49-specific mAb to 129/J Ly49 receptors

<table>
<thead>
<tr>
<th></th>
<th>YE1/48 (A)</th>
<th>YE1/32 (A)</th>
<th>A1 (A)</th>
<th>12A8 (A/D)</th>
<th>4E5 (D)</th>
<th>4D11 (G)</th>
<th>5E6 (C/I)</th>
<th>1F8 (C/I/H)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ly49G2$^{129/J}$</td>
<td>+/−</td>
<td>+/−</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Ly49I$^{129/J}$</td>
<td>−/−</td>
<td>−/−</td>
<td>−</td>
<td>−</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Ly49O$^{129/J}$</td>
<td>−/−</td>
<td>−/−</td>
<td>−</td>
<td>−</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Ly49P$^{129/J}$</td>
<td>−/−</td>
<td>−/−</td>
<td>−</td>
<td>−</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Ly49R$^{129/J}$</td>
<td>+/−</td>
<td>+/−</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Ly49S$^{129/J}$</td>
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<td>−/−</td>
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<tr>
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<td>+/−</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Ly49U$^{129/J}$</td>
<td>−/−</td>
<td>−/−</td>
<td>−</td>
<td>−</td>
<td>++</td>
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<tr>
<td>Ly49V$^{129/J}$</td>
<td>+/−</td>
<td>+/−</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

* All Ly49 cDNAs were subcloned into the pEF6 expression vector, transiently expressed in 293T, and stained with the indicated mAb.
* B6 Ly49 specificity of mAb are indicated in parentheses.
* Ab binding was rated from “+++” as being high to “−” for no reactivity.

FIGURE 5. Binding of soluble murine MHC class I tetramers to 129J Ly49-transfected 293T cells. The 129J Ly49 cDNA/pEF6 expression vector constructs were transiently transfected into 293T cells and, the next day, were incubated on ice for 30 min with a specific mAb or PE-conjugated MHC tetramers. Cells were then washed and analyzed on a FACSsort. Gray-filled curves represent staining of the mAb or tetramer of 293T cells transfected with “empty” pEF6 vector. Black-filled curves show the binding of Ly49/pEF6-transfected 293T cells.
at low E:T ratios in a fashion comparable to unsorted splenic B6 NK cells (Fig. 6A). This indicates that the 4E5-reactive subpopulation contains a receptor that can inhibit NK cytotoxicity in the presence of a H-2d-derived ligand.

To prove that class I was responsible for the observed inhibition, similar populations of 129/J NK cells were used as effectors against the rat myeloma YB20 and a stably transfected derivative, YB/Dd, expressing physiological levels of murine Dd (Fig. 6C). Both 4E5^12A8^ (Ly49O/V-positive and R-negative) and 4E5^12A8^- (Ly49O/V-negative and R-negative) NK cells displayed similar levels of cytotoxicity toward the YB20 parental line (Fig. 6D). In contrast, lysis by the 4E5^12A8^- subpopulation was severely inhibited against the Dd-expressing YB20 transfectant in comparison to the lysis by the 4E5^12A8^- subpopulation (Fig. 6E). This suggests that Dd is a ligand for Ly49O 129/J and/or V 129/J.

Unsorted 129/J NK cells also displayed decreased cytotoxicity toward YB/Dd compared with the sorted 4E5^12A8^- population (Fig. 6E). This is consistent with the expression of Ly49G, O, and V on unsorted 129/J NK cells, especially Ly49G^{129/J}, which is an abundant 129/J receptor that can also bind the Dd tetramer (Fig. 5). Cytotoxicity and cytokine secretion of Ly49D^B6 NK cells in response to Dd-expressing target cells can be blocked by preincubation with 4E5-F(ab')_2 (20, 23). Because 4E5 also recognizes Ly49O^{129/J}/V^{129/J}, the inhibitory signal should also be affected by such treatment. Indeed, blockade with 4E5-F(ab')_2 resulted in the restoration of cytotoxicity by the 4E5^12A8^- population to levels obtained by the 4E5^12A8^- population toward YB/Dd (Fig. 6F). We found that 4E5 F(ab')_2 did not affect the lysis of YB/Dd induced by 4E5^12A8^- cells. Collectively, these results indicate that Ly49G^{129/J} and/or V^{129/J} bind to murine class I Dd and signal in a manner which inhibits NK cell cytotoxicity.

**Discussion**

The order of all known B6 Ly49 genes was recently confirmed, along with the identification of a new Ly49d-like gene fragment (54). This fragment may represent the B6 allele of Ly49o, v, or r.

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**Table III. Temperature-dependent binding of soluble murine class I MHC to 129/J Ly49 receptors**

<table>
<thead>
<tr>
<th></th>
<th>D^d</th>
<th>D^e</th>
<th>D^k</th>
<th>K^b</th>
<th>K^d</th>
<th>K^k</th>
<th>L^d</th>
</tr>
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<tbody>
<tr>
<td>G2</td>
<td>0/17</td>
<td>24/35</td>
<td>12/22</td>
<td>3/0</td>
<td>12/20</td>
<td>2/0</td>
<td>3/8</td>
</tr>
<tr>
<td>I</td>
<td>0/0</td>
<td>0/0</td>
<td>10/13</td>
<td>14/13</td>
<td>46/28</td>
<td>7/11</td>
<td>0/0</td>
</tr>
<tr>
<td>O</td>
<td>7/28</td>
<td>39/44</td>
<td>16/25</td>
<td>0/2</td>
<td>0/0</td>
<td>0/0</td>
<td>46/54</td>
</tr>
<tr>
<td>P</td>
<td>0/5</td>
<td>5/3</td>
<td>4/0</td>
<td>0/0</td>
<td>0/0</td>
<td>3/2</td>
<td>2/0</td>
</tr>
<tr>
<td>R</td>
<td>0/18</td>
<td>21/34</td>
<td>15/31</td>
<td>0/0</td>
<td>1/0</td>
<td>5/4</td>
<td>8/34</td>
</tr>
<tr>
<td>S</td>
<td>0/0</td>
<td>0/0</td>
<td>2/0</td>
<td>0/0</td>
<td>0/0</td>
<td>1/1</td>
<td>0/0</td>
</tr>
<tr>
<td>U</td>
<td>0/1</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/2</td>
</tr>
<tr>
<td>V</td>
<td>74/72</td>
<td>67/76</td>
<td>68/68</td>
<td>28/74</td>
<td>8/36</td>
<td>16/43</td>
<td>81/80</td>
</tr>
</tbody>
</table>

*a* Tetramer binding was calculated by subtracting the percentage of binding to 293T cells transfected with empty pEF6 vector from the percentage of binding to 293T cells transfected with pEF6-Ly49 construct. Background binding to mock-transfected cells was typically <2%.

*b* Binding values are shown as specific binding on ice/specific binding at 37°C.
In addition, we have isolated B6 genomic DNA fragments containing exon 7 of what may be the genes for Ly49p<sup>129/J</sup> and Ly49v<sup>129/J</sup> (S. K. Anderson, unpublished observations). However, this still leaves open the possibility that the majority of Ly49 cDNAs described in this study, with the exception of Ly49<sub>129/J</sub>, are the products of 129/J-specific genes. There are some pairs that may represent alleles, for example, Ly49<sub>B6</sub><sup>129/J</sup> and Ly49<sub>B6</sub><sup>129/J</sup>. The order of 129/J Ly49 genes in the 129/J genome would shed light on whether some of the newly described genes are in fact alleles. Comparison of Ly49 cDNAs between various strains has shown that 99% nucleotide homology exists for several family members (51), suggesting that a very high level of amino acid homology (>98%) is indicative of true Ly49 alleles. Based on a low level of homology to their closest relative, Ly49<sub>B6</sub><sup>129/J</sup>, Ly49<sub>L</sub><sup>129/J</sup>, and Ly49<sub>P</sub><sup>129/J</sup> are almost certainly new genes. We have found selective Ly49 gene expression in several mouse strains. For example, Ly49<sub>i</sub> transcripts were detected in CBA/J and C3H/HeJ but not B6 NK cells, even though at least two exons of Ly49<i>i</i> have been found in this strain's genome (9, 12). Therefore, it is possible that the Ly49<i>p</i>, <i>s</i>, <i>t</i>, and <i>v</i> genes are also present, but silent, in B6 mice.

The ITIM of Ly49<sub>v</sub> (VTYSTM) is different from that of all other known inhibitory Ly49 proteins (VYXXV) and is identical with the binding motif for phosphatidylinositol (PI)3-kinase (YxxM) (Fig. 3). To test for possible PI3-kinase recruitment, we peroxidase-treated I<sup>252</sup>-Ly49<sub>v</sub> stable transfectants, immunoprecipitated with a pan-Ly49 antiserum, separated proteins by SDS-PAGE, and blotted with an anti-PI3 kinase (anti-p85 subunit) mAb. Although p85 was readily detectable in whole-cell lysate, there was no evidence of its existence in immunoprecipitated samples. We subsequently blotted with anti-phosphotyrosine and observed the phosphorylated form of Ly49<sub>v</sub> (data not shown). Therefore, the ITIM of Ly49<sub>v</sub> is phosphorylated but does not seem to associate with PI3-kinase.

Compared with the divergence of the other nine 129/J Ly49 genes, the conservation of Ly49e between B6 and 129/J is intriguing. The conservation may emphasize an important and perhaps separate function for this Ly49 gene. It is interesting to note that Ly49e transcripts, unlike Ly49a–d, g, and i, are readily detectable in B6 fetal thymic and thymus NK preparations (55). The divergence of the other Ly49 genes between 129/J and B6 may reflect the fact that these strains are very distantly related, despite sharing the same MHC haplotype (56).

Interestingly, Ly49<sub>B6</sub><sup>129/J</sup> bound K<sup>b</sup> tetramers, whereas Ly49<sub>v</sub> and Ly49<sub>T</sub> recognized D<sup>d</sup> (Fig. 3 and Table III). Both tetramers represent self-MHC Ags of 129/J mice. Ly49<sub>B6</sub> binds to both K<sup>b</sup> and D<sup>d</sup> in B6 mice (25). In contrast, Ly49<sub>v</sub> did not significantly bind to H<sup>2</sup><sup>b</sup> tetramers (25) but, when overexpressed as a transgene, inhibited the rejection of H<sup>2</sup><sup>b</sup> bone marrow transplants (57). These results show that both B6 and 129/J mice possess multiple inhibitory receptors for self-MHC Ags. However, it is clear that even at physiological temperatures there is wide variation in the binding of different Ly49 molecules for the same class I MHC (Table III). Such variation in binding ability may be a consequence of the peptide being presented, as in the case of Ly49<sub>B6</sub> (25), or even a result of the presence or absence of peptide altogether, as has recently been reported for Ly49<sub>B6</sub> (58).

The three activating Ly49 cDNA species, Ly49P, R, and U, expressed in 129/J mice did not bind the K<sup>b</sup> tetramer, but Ly49<sub>P</sub> recognized D<sup>d</sup> at 37°C (Fig. 3 and Table III). Despite this apparent self-reactivity, Ly49R seems to be expressed at moderate levels and Ly49U, expressed in N<sup>s</sup> (58), K<sup>b</sup>, and L<sup>d</sup> on ice (Fig. 5 and Table III). This suggests that the affinity of different activating receptors for MHC ligands may vary widely. It also appears that the tetramer binding by Ly49<sub>R</sub> is not an anomaly, because we have found that the recently described activating receptor in CBA/J and C3H mice, Ly49L (12), also bound weakly to D<sup>d</sup> tetramers (data not shown). The well-documented ability of Ly49<sup>i</sup> NK cells to confer rapid cytotoxicity and cytokine production in response to D<sup>d</sup>-expressing target cells reveals that tetramer-binding data may actually underestimate the binding potential of Ly49 molecules for in vivo-expressed MHC (20, 21). The increased binding of Ly49<sub>R</sub><sup>129/J</sup> to D<sup>d</sup>, D<sup>d</sup>, and L<sup>d</sup>, as well as the additional binding to D<sup>d</sup> at 37°C (Table III), and our finding that Ly49<sub>B6</sub> binds to D<sup>d</sup> at physiological temperatures supports this position (data not shown).

Recently, the crystal structure of Ly49A<sup>B6</sup> bound to D<sup>d</sup> was deduced by Tormo et al. and the sites of contact between the two proteins predicted (59). The Ly49A<sup>B6</sup> residues required for site 1 interaction with D<sup>d</sup>, predicted to be the trans binding site (between a ligand and receptor on different cells), are fairly well conserved in those 129/J Ly49 proteins that were found to interact with D<sup>d</sup> tetramers. In this study, Ly49G<sup>129/J</sup> O. V. R. and, to a much lesser extent, P, were found to bind D<sup>d</sup> (Fig. 5 and Table III). Of the 12 Ly49<sup>B6</sup> residues thought to be used for D<sup>d</sup> binding in trans, Ly49G<sup>129/J</sup> has 7 of 12, Ly49D<sup>129/J</sup> has 10 of 12, Ly49V<sup>129/J</sup> has 9 of 12, Ly49R<sup>129/J</sup> has 12 of 10, and Ly49P<sup>129/J</sup> has in common 12 of 12. It is interesting to note that the lowest (but still detectable) D<sup>d</sup>-tetramer binding was seen with Ly49P<sup>129/J</sup>, which had all the predicted trans binding sites in common with Ly49A<sup>B6</sup>, but the best binding was seen with Ly49V<sup>129/J</sup>, which had only 9 of 12 binding sites conserved. Also, Ly49G<sup>129/J</sup> had the least number of residues in common with Ly49B<sup>B6</sup> for D<sup>d</sup> binding, 7 of 12, but also bound the D<sup>d</sup> tetramer much better than Ly49P<sup>129/J</sup> (Fig. 5 and Table III). Our study, like that of Tormo et al., used bacterially expressed MHC and the same peptide for presentation by D<sup>d</sup>. This suggests that other as yet unidentified residues may be important for MHC binding by Ly49 proteins. Comparison of the predicted MHC-contacting residues of the two pan-MHC-binding proteins, Ly49C<sup>B6</sup> and Ly49<sup>129/J</sup>, showed that they agreed at only 12 trans residues, despite Ly49C<sup>B6</sup> binding D<sup>d</sup> tetramers very well (25). This suggests that the Ly49C-like family members use an altogether different combination of residues for MHC-binding compared with Ly49A-like family members.

ES cells of 129/SvJ origin are the most widely used in current mouse gene-mutation studies, although most of the functional assays used to characterize the mutant mice were developed in non-129 mouse strains. Because 24% of 121 129/SvJ genomic sequence markers differ from the consensus 129 genotype (60), 129/SvJ is considered to be "contaminated." We have found that 129/SvJ splenocytes express transcripts identical with Ly49P<sup>129/J</sup>, Ly49<sub>S</sub> <sup>129/J</sup>, and Ly49<sub>V</sub> <sup>129/J</sup> (data not shown). This agrees with the prediction that chromosome 6, where the Ly49 gene cluster resides, is of 129 origin in 129/SvJ (60). Our current findings suggest that an ES cell of 129 origin would not be the appropriate choice for deletion of a gene mapping near the Ly49 locus where the phenotype of interest is immunological in nature. Even with many backcrosses to the phenotypically important strain, closely linked genes may be
References

1. Bancroft, G. J. 1993. The role of natural killer cells in innate resistance to in-


