Identification of the Ly49L protein: evidence for activating counterparts to inhibitory Ly49 proteins

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Abstract: Previous studies have indicated that NK cells from different strains of inbred mice may express distinct Ly49 repertoires. Screening of NK cells from the CBA/J mouse for inhibitory and activating Ly49s revealed a novel DAP12-associated receptor that was immunoprecipitated with the Ly49G-specific mAb 4D11. Degenerate primers were designed to amplify and clone Ly49 cDNAs from CBA/J NK cells. A novel activating Ly49 cDNA was identified, which bears strong homology to the partially sequenced *Ly49l* gene found in C57BL/6 mice. Transfection of *Ly49l* into a DAP12$^+$ cell line and subsequent immunoprecipitation experiments showed that Ly49L is likely the activating Ly49 detected by the 4D11 antibody in CBA/J NK cells. Antibody-mediated cross-linking of Ly49L induced DAP12 phosphorylation, providing evidence that Ly49L is a functional activating receptor. Comparison of the extracellular domains of Ly49 family members indicates that all known activating members have an inhibitory counterpart with a highly related extracellular region. *J. Leukoc. Biol.* 68: 765–771; 2000.

Key Words: *NK cells · Ly49 · cell surface receptors · signal transduction*

INTRODUCTION

Natural killer (NK) cells have evolved a variety of receptors that bind to major histocompatibility complex (MHC) class I and regulate cytotoxicity toward potential target cells. The most studied group of NK receptors in mice is the Ly49 family of type II transmembrane, disulfide-linked homodimeric glycoproteins. Currently, the membership of this family extends from Ly49A through P and is divided into activating and inhibitory receptors [1–4]. The inhibitory receptors are characterized by a V/IxYxxL/V motif in their cytoplasmic region that is known as an immunoreceptor tyrosine-based inhibitory motif (ITIM). The ITIM becomes phosphorylated in response to receptor ligation, leading to recruitment of SHP-1 phosphatase and attenuation of intracellular signaling [5–7]. Such inhibitory Ly49s have been shown to suppress NK cytotoxicity toward targets expressing specific MHC class I ligands [8–10]. When NK cells express inhibitory and activating Ly49s of the same MHC specificity, the negative signal is dominant, and lysis of the target cell is inhibited [11].

Activating Ly49s lack an ITIM and instead contain an arginine residue in their transmembrane region that associates with the signal-transducing protein DAP12 [12, 13]. DAP12 contains an immunoreceptor tyrosine-based activation motif (ITAM) and transmits activating signals resulting in phosphorylation of various substrates [14–17]. Furthermore, the activating receptors induce intracellular calcium release and cytokine production in response to antibody-mediated cross-linking or ligation by specific MHC class I ligands. When expressed on NK cells, activating Ly49s enhance the killing of target cells bearing the appropriate MHC class I molecule [11, 18]. Presently, the known activating Ly49 molecules include Ly49D, H, and P. Three additional ITIM-lacking, potential activating Ly49 candidates (k, l, and n) are known from C57BL/6 (B6) genomic sequence fragments [3].

Ly49d has been cloned from B6 mice, and the Ly49D protein delivers stimulatory signals leading to the lysis of target cells bearing H2-D^d, D^r, or D^{sp2} MHC class I [1, 11, 18]. In agreement with the noted MHC specificity, depletion of Ly49D$^+$ NK cells abrogates the ability of H-2^b mice to reject H-2^d bone marrow cells [19]. Ly49D has been shown also to mediate xenogeneic lysis of Chinese hamster ovary cells and rat lymphoblasts in an MHC-dependent fashion [20, 21]. *Ly49h* cDNAs have been cloned from B6 and B6/CBA F1 hybrids, and Ly49H signals via DAP12, leading to the phosphorylation of various substrates such as DAP12 and Syk [2, 15, 16]. As shown for Ly49D, stimulation through Ly49H leads to intracellular calcium mobilization and cytokine secretion [16]. Ly49P was identified in 129/J mice, and it shares with the other activating Ly49s the ability to associate with DAP12 as well as the induction of DAP12 phosphorylation and intracellular calcium flux in response to antibody-mediated cross-linking [4].

A previous study has shown that the currently available Ly49-specific monoclonal antibodies (mAbs) display strain-specific differences by flow cytometric analysis [22]. In a screen of 12 strains of inbred mice with the Ly49D-specific mAb 4E5, only half of the strains tested showed a positively staining NK population. Immunoprecipitation experiments designed to identify activating and inhibitory Ly49 molecules have shown that NK cells from non-B6 mouse strains express

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cross-reactive yet functionally opposed Ly49 molecules. The mAb YE1/48 immunoprecipitates an inhibitory Ly49 in B6 mice (Ly49A), whereas an activating Ly49 is detected by this antibody in 129/J mice (Ly49P). The nature of a novel Ly49G-related activating receptor (Ly49L) detected by mAb 4D11 in CBA/J NK cells is the subject of the current study.

MATERIALS AND METHODS

Antibodies

The following mAbs were used: YE1/48 and YE1/32 (Ly49A) [23]; 4E5 (Ly49D) [13]; 4D11 (Ly49G2) [10]; 5E6 (Ly49C/I) [24]; 12A8 (Ly49A/D) [12]; and 1F8 (Ly49H/C/I), a generous gift of Vinay Kumar (University of Texas, Dallas, TX). Biotinylated 4G10 antibody, which recognizes phosphotyrosine, was purchased from Upstate Biochemical Inc. (UBI; Lake Placid, NY). Rabbit antirat immunoglobulin G (IgG) was used as a cross-linking reagent (Capell Laboratories, Oxford, PA). DX5 (pan-NK cell) and DX37 (antihuman DAP12) mAbs were kind gifts from Lewis Lanier (DNAX, Palo Alto, CA).

Mouse NK cells and tumor cell lines

NK cells from 129/J, B6, C3H/He, and CBA/J mice were enriched from spleens of 12- to 16-week-old animals by collecting nylon wool nonadherent cells and depleting T cells using mAbs to CD8 and CD4 plus complement. Routinely, 75–80% of the resulting cells were $DX5^+$. Mouse NK cells were cultured for 7–10 days in RPMI 1640 medium supplemented with 1000 cetus units/ml interleukin (IL)-2 (Hoffman LaRoche, Nutley, NJ), 10% fetal calf serum (FCS), nonessential amino acids, sodium pyruvate, HEPES, β-mercaptoethanol, 2 mM L-glutamine, and 100 units/ml penicillin plus 100 μg/ml streptomycin. The human fetal liver cell line CP [16], which is $DAP12^+$, $CD56^+$, $CD16^-$, and $CD3^-$, was maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS), glutamine, and antibiotics.

Polymerase chain reaction (PCR) cloning of CBA/J Ly49 cDNA

CBA/J NK cell poly-A mRNA was isolated using the QuickPrep Micro mRNA purification kit (Amersham Pharmacia Biotech, Piscataway, NJ). A total of 1 μg mRNA was used to generate cDNA with the TimeSaver cDNA synthesis kit (Amersham Pharmacia). Degenerate Ly49 primers were used to amplify 50 ng cDNA. The 5′ primer used was: 5′-YACTCCCAMGATGAGTGAGC-3′, and the 3′ primer used was: 5′-RTTGGARARTYAATSAGGR-3′. Thirty cycles of amplification were performed with High Fidelity PCR Supermix (Gibco BRL, Grand Island, NY) at temperatures of 94°C (denaturation) for 30 s, 57°C (annealing) for 30 s, and 73°C (extension) for 1 min. PCR products were cloned into pcDNA3.1/V5/His-TOPO expression vector using the Eukaryotic TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). Clones were sequenced with vector-specific primers using the Sequenase quick-denature kit (Amersham, Cleveland, OH) or by automated DNA sequencing using the Dye Terminator Cycle Sequencing Ready Reaction Kit (ABI, Columbia, MD). Identical *Ly49l* clones were isolated from two separate PCR reactions and sequenced. The *Ly49l1-4* sequences are available from GenBank under the following accession numbers: (*Ly49l1*) AF204265, (*Ly49l2*) AF204266, (*Ly49l3*) AF204267, and (*Ly49l4*) AF204268.

Phylogenetic analysis

Sequence similarities were ascertained using the GrowTree software available on the Web-based University of Wisconsin (Madison, WI) SeqWeb sequence analysis service. Specifically, tree construction was performed using the unweighted pairgroup method with arithmetic mean method, according to the Jukes-Cantor algorithm. The bootstrap values in 1000 replications were estimated using the Phylogeny Inference Package (PHYLIP) version 3.5c. The extracellular (beginning of exon 4 to the stop codon in exon 7) nucleotide-coding regions of the following sequences were used in tree construction: $Ly49c^{B6}$ (U56404), $Ly49c^{BALB/c}$ (U49868), $Ly49i^{B6}$ (U49865; referred to as *Ly49c* in the description), $Ly49j^{B6}$ (AF110492), $Ly49h^{B6}$ (L78253), $Ly49e^{B6}$ (U10091), $Ly49f^{B6}$ (U10092), $Ly49a^{B6}$ (M25812), $Ly49a^{BALB/c}$ (U34890), $Ly49p^{129/J}$ (AF146570), $Ly49o^{129/J}$ (AF146571), $Ly49d^{B6}$ (L78247), $Ly49g^{B6}$ (U10094), and $Ly49b^{B6}$ (U10304).

Ly49-specific reverse transcription (RT)-PCR analysis

Synthesis of cDNA from NK cells of 129/J, B6, and C3H/He mice was done essentially as described above for CBA/J mice. *Ly49l*-specific PCR analysis was conducted with forward primer 5′-GGTGAGGCTTGAGGAGACAG-3′ and (B6-specific) reverse primer 5′-CTTCCCACAAATACAGATGAATGATTTATCACATTTATC-3′ plus (CBA/J-specific) reverse primer 5′-CTTCCCACAAATACAGATGAATGATTTATCACAGTTATC-3′ corresponding to peptides (forward) 22-28 and (reverse) 264-274 of the Ly49L1 aa sequence (see Fig. 2A). PCR was performed with 35 cycles of 94°C for 30 s, 65°C for 15 s, and 72°C for 30 s. *Ly49g*-specific primers used were forward primer 5′-GTTGCAGAAACTAGTGAGGAC-3′ and reverse primer 5′-GATGTATGATTTACCACAGTCC-3′, corresponding to nucleotides (forward) 18-25 and (reverse) 261-268 of the Ly49G1 aa sequence. PCR was performed with 35 cycles of 94°C for 30 s, 55°C for 15 s, and 72°C for 30 s. PCR controls to show the specificity of the *Ly49l* and *g* primers were performed with 1 ng of cloned Ly49 cDNA.

Transient transfections

Ly49a cDNA was cloned into the pSE1 expression vector, *Ly49d* cDNA was cloned into the pEF expression vector, and *Ly49g2* was cloned into the pmKit expression vector. Four million CP cells from a log-phase culture were put in 0.4 ml of RPMI + 10% FBS (no antibiotics) and electroporated with 20 μg of the *Ly49* cDNA expression vector. Electroporation was carried out at 310 mV/960 μF using a GenePulser (Bio-Rad, Hercules, CA). CP cells were analyzed one day after transfection.

Detection of phosphorylated proteins and DAP12

Transiently transfected CP cells or cultured mouse NK cells were stimulated with 0.1 mM pervanadate as previously described [25] for 15 min at 37°C or with specific antibody followed by an isotype-specific cross-linker for 2 min at 37°C. Cells were disrupted in lysis buffer (1.0% Triton X-100, 300 mM NaCl, 50 mM Tris, 2 mM EDTA, 0.4 mM sodium orthovanadate, plus protease inhibitors) and then centrifuged at 15,000 rpm for 30 min at 4°C. Lysates were immunoprecipitated overnight with specific mAb cross-linked to rProtein G Agarose (Gibco BRL). Beads were washed in wash buffer containing 0.2% Triton X-100, and proteins eluted in nonreducing Laemmli buffer and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on precast 10% or 16% Tris-glycine gels (Novex, San Diego, CA). Proteins were transferred to Immobilon-P (Millipore, Bedford, MA), and the blots were blocked with 5% bovine serum albumin (BSA), 0.1% Tween/20 in phosphate-buffered saline (PBS). Biotinylated 4G10 was used to detect phosphoproteins followed by streptavidin-horseradish peroxidase (HRP; UBI). Alternatively, DX37 (mouse antihuman DAP12) was used to detect DAP12 followed by sheep antimouse IgG-HRP (Amersham, Piscataway, NJ). Blots were treated with a chemiluminescent reagent (Amersham, Arlington Heights, IL) and then exposed to BioMax ML film (Kodak, Rochester, NY).

Flow cytometric analysis

Cells were stained using Ly49-specific primary mAb followed by an fluorescein isothiocyanate (FITC)-conjugated goat antirat IgG (H+L) secondary (FGART; Kirkegaard & Perry Lab., Gaithersburg, MD) and analyzed on a FACSort flow cytometer (Becton Dickinson, Mountain View, CA).

RESULTS

Immunoprecipitation analysis of Ly49 proteins in CBA/J NK cells

Previous studies of the 129/J strain of mice led to the discovery of Ly49O and Ly49P and showed that Ly49 expression patterns are strain-dependent [4]. This indicated that, depending on the genetic background of the strain involved, certain Ly49 genes

may be inactivated or deleted. Initial studies of the Ly49 gene family were restricted to the B6 and BALB/c mouse strains; therefore, it is likely that several unknown Ly49 molecules exist. The current study focuses on the CBA/J strain of inbred mice. For the initial screening of Ly49 molecules in this strain, immunoprecipitation with Ly49-specific mAbs and antiphosphotyrosine blotting were performed. IL-2-expanded splenic NK cells from B6 and CBA/J mice were treated with sodium pervanadate to enhance tyrosine phosphorylation of proteins, and then cell lysates were immunoprecipitated with a panel of Ly49-specific mAbs. The immunoprecipitate was then subjected to Western blotting with an antiphosphotyrosine mAb. This analysis detects inhibitory Ly49 molecules at ~100 kDa, but activating Ly49 molecules lack an ITIM, and instead the DAP12 homodimer is detected at ~24 kDa [4]. Ly49D (4E5)- and Ly49H (1F8)-reactive mAb showed the expected receptor-associated DAP12 in B6 mice, but neither detected any phosphoproteins in CBA/J mice (**Fig. 1**). This indicates that Ly49D and Ly49H are not present in the NK cells of this strain or that these mAbs are strain-specific. Surprisingly, DAP12-association in CBA/J NK cells was seen in immunoprecipitations with 4D11 and to a lesser extent, YE1/48. Both of these antibodies only recognize inhibitory Ly49s (G and A, respectively) in B6 NK cells. An activating Ly49 has been detected previously with YE1/48 in 129/J mice (Ly49P) [4], but a 4D11 cross-reacting activating Ly49 has not yet been described.

PCR cloning of Ly49 cDNAs from CBA/J NK cells

To gain a more definitive assessment of the Ly49 molecules expressed in CBA/J mice, a PCR-cloning approach was adopted. Poly-A mRNA from CBA/J NK cells was reverse-transcribed and subjected to PCR with forward and reverse primers compatible with all known Ly49 sequences (with the exception of *Ly49b*) and then cloned into the pcDNA3.1/V5/His-TOPO expression vector. From two separate sets of PCR reactions, ligations, and transformations, a total of 100 colonies was screened for full-length Ly49-sized inserts of which 27 were found. These 27 cDNAs were sequenced, and 14 were found to be the CBA/J allele of *Ly49a*, 3 of *Ly49g*, and 1 of *Ly49c*. The CBA/J version of *Ly49g* was closest to the CB.17-scid sequence (accession S78689) with only two differences (position 248, A–C; position 828, T–C). The CBA/J alleles of *Ly49a* and *c* were identical to the BALB/c counterparts (accession numbers U34890 and U49868, respectively).

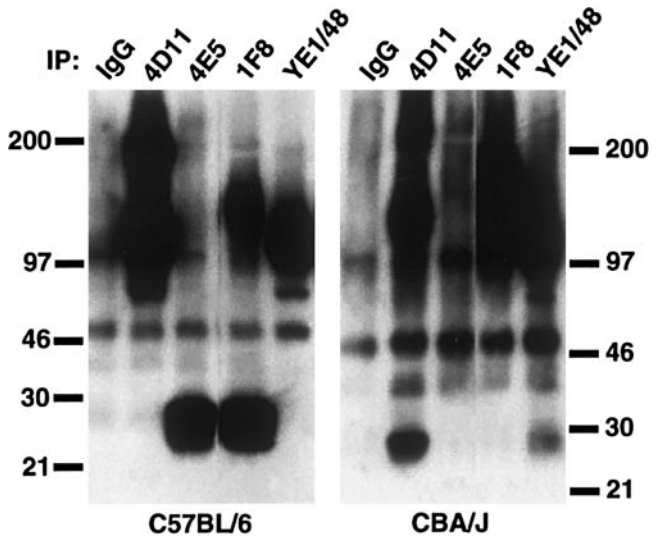

Fig. 1. Detection of mDAP12 coimmunoprecipitated with Ly49-specific mAbs in B6 vs. CBA/J NK cells. NK cells from C57BL/6 (left) and CBA/J (right) mice expanded with IL-2 for 7–10 days were washed and stimulated with pervanadate. Cells were lysed and immunoprecipitated with the indicated anti-Ly49 antibodies. YE1/48 binds to Ly49A, and 4E5 binds to Ly49D in C57BL/6 mice. The immune complexes were run on a nonreducing 10% gel, transferred, and blotted with 4G10 (antiphosphotyrosine antibody). A representative blot of two experiments is shown.

The remaining nine cDNA clones were of a novel activating Ly49 that most closely matched the partially characterized *Ly49l* gene from B6 mice. The B6-*Ly49l* gene is known from 77 bp and 113 bp fragments of the putative exons 2 and 7, respectively [3]. The CBA/J clone was identical to the known *Ly49l* sequence except for a single, nonconservative nucleotide substitution in exon 7. Therefore, we believe we have isolated the CBA/J allele of *Ly49l*. Ly49L lacks the tyrosine residue in the ITIM motif but has an arginine residue in the transmembrane domain that could potentially bind to DAP12 (**Fig. 2A**). Ly49L is extremely similar to Ly49G in the extracellular region. Four different isoforms of Ly49L were discovered. Shown in Figure 2A is the full length Ly49L1 protein sequence which contains a large insertion in the stalk-coding domain (Box-2) that so far has been described only for Ly49G1 [1]. Ly49L2 lacks this large insertion, Ly49L3 lacks three amino acids in the intracellular domain (Box-1), which has also been described for two other activating Ly49s, D and H [26], and Ly49L*4* lacks both of these sequences. Finally, Ly49L has an extra amino acid (position 90; threonine) not shared by any other Ly49. To eliminate possible PCR-related sequence errors, each Ly49 described was isolated from two separate PCR reactions and sequenced.

Clustering of inhibitory and activating Ly49 extracellular domains

The striking homology of *Ly49l* to *Ly49g* in the region coding for the extracellular domain was reminiscent of another activating/inhibitory pair, *Ly49a* and *p* [4]. To see if this "domain swapping" is used by other members of the Ly49 family, a phylogenetic tree was constructed for the intracellular and extracellular nucleotide-coding regions. When possible, CBA/J sequences were used; otherwise, an alternative strain was used to provide a complete tree. An interesting clustering effect was seen with this analysis. Comparison of the intracellular domains showed that the activating and inhibitory Ly49 genes formed separate clusters as expected (unpublished results). However, when the extracellular domains were analyzed in this fashion, each of the known activating Ly49 molecules clustered with an inhibitory Ly49 (Fig. 2B). *Ly49l* was grouped with *Ly49g* (this clustering took place even when using the Ly49L2 isoform that lacks the Ly49G1-like insertion), *Ly49p* clustered with *Ly49a*, *Ly49d* clustered with *Ly49o*, and *Ly49h* clustered with the *Ly49i/j/c* subfamily. This suggests that diversity within the Ly49 family is not only because of gene duplication but is also a result of gene recombination. Although *Ly49e* and *f* are not grouped with activating counterparts, there are two potential activating *Ly49c*-related genes in B6 mice [3], suggesting

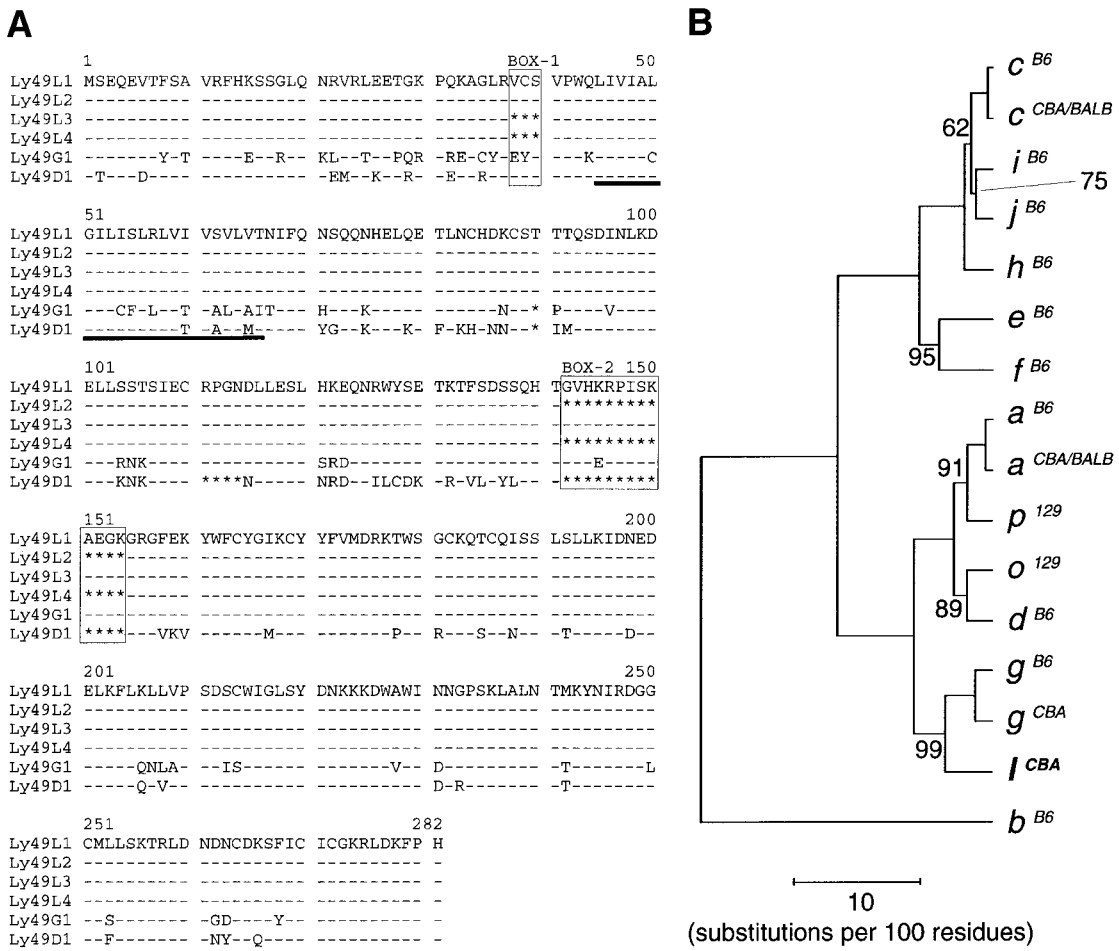

Fig. 2. (A) Proposed amino acid sequence of Ly49L1-4. The amino acid sequences of Ly49L1-4 is compared with Ly49G1 and D1. Dashes indicate regions of identity with Ly49L1. Asterisks represent sequence gaps. The transmembrane region is underlined. L1 has both sets of boxed amino acids, L2 is missing Box-2, L3 is missing Box-1, and L4 is missing Box-1 and -2. cDNAs were isolated from at least two separate cloning experiments to eliminate PCR error. These sequences can be found under GenBank accession numbers (*Ly49l1*) AF204265, (*Ly49l2*) AF204266, (*Ly49l3*) AF204267, and (*Ly49l4*) AF204268. (B) Phylogenetic tree of the nucleotide sequence coding for the extracellular region (exon 4 to termination codon in exon 7) of the various known Ly49 molecules. The *Ly49a* and *c* alleles of CBA/J and BALB/c mice are identical. The isoforms of *Ly49g* and *l* without the large insert in the stalk region (*Ly49g2* and *Ly49l2*) were used to decrease bias in pairing. *Ly49b* was included as an outgroup to artificially root the tree. Numbers at branch nodes represent bootstrap confidence-level percentages of 1000 bootstrap replications. Unlabeled branch nodes had a bootstrap value of 100%. Tree structure was produced using the UPGMA neighbor-joining method of the Web-based University of Wisconsin SeqWeb sequence analysis service.

that the majority of Ly49 family members will have activating/inhibitory counterparts.

Ly49L is expressed in CBA/J but not B6 NK cells

The B6-*Ly49l* sequences were originally identified from genomic fragments [3]. To our knowledge, no expression of this gene has been described for B6 mice. To determine if Ly49L is expressed in B6 and other strains of mice, an RT-PCR-based approach was developed. Primers were designed that could bind and amplify B6 and CBA/J allelic forms of *Ly49l*-derived cDNA, and similar primers were designed for *Ly49g* as a positive control. Previous studies have shown that Ly49G is expressed on many different strains of mice [22]. The specificity of this assay is shown when both sets of primers are used on cloned Ly49 cDNAs (**Fig. 3**, left panels). To test for *Ly49l* and *g* transcripts in primary cells, NK cell cDNA from various mouse strains was produced and then analyzed by this approach. As expected, *Ly49g* was found in all four strains tested. It is interesting that *Ly49l* transcripts were only detected in CBA/J and C3H/He but not 129/J or B6 NK cells (Fig. 3, right panels). The PCR products obtained in this analysis were cloned and sequenced to confirm their identity with *Ly49l*. These results suggest that Ly49L expression is restricted to certain strains of inbred mice.

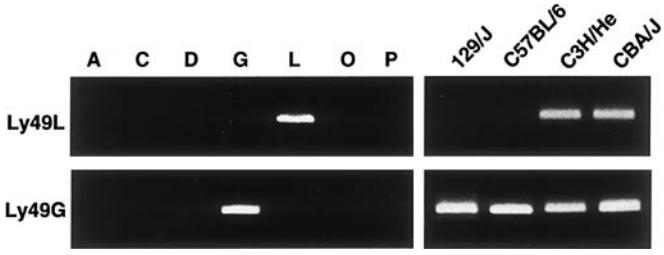

Fig. 3. Detection of *Ly49l* mRNA in various mouse strains. Poly-A RNA was isolated from the listed strains of mice, reverse-transcribed, and then amplified in the presence of *Ly49l*- or *Ly49g*-specific primers (right). These primers were designed to be capable of binding and amplifying all known alleles of these genes. As a control to show primer specificity, 1 ng of various vector-contained Ly49 cDNAs was amplified using the same primers (left).

Ly49L antibody reactivity and DAP12 association

To further characterize Ly49L, the reactivity of currently available Ly49 mAbs was analyzed by flow cytometry of the DAP12$^+$ CP cell line transiently transfected with Ly49L-expression constructs. Flow cytometric analysis demonstrated that Ly49L2 was bound by YE1/48 weakly but not 4E5 or 4D11 (**Fig. 4A**). 12A8 and 5E6 were tested also but showed no reactivity against Ly49L2 (unpublished results). Ly49L1 had an antibody-reactivity profile that was similar to Ly49L2 (unpublished results). The novel activating receptor predicted to exist in CBA/J mice (Fig. 1) was immunoprecipitable by 4D11, so the inability of 4D11 to recognize *Ly49l*-transfected cells was unexpected. Therefore, the ability of the Ly49 mAbs to coimmunoprecipitate Ly49L and DAP12 from Ly49L2-transfected CP cells was examined. Cell lysates were immunoprecipitated with a panel of Ly49 mAb, and a Western blot was performed with an antihuman DAP12 mAb. It was found that DAP12 was detected in cell lysates immunoprecipitated with YE1/48 as well as 4D11 mAbs (Fig. 4B). The 4D11 reactivity seen under these conditions suggests that Ly49L is the novel CBA/J-activating Ly49 observed in NK cells. Ly49L1 was used in this type of experiment also and found to associate with DAP12; however, detection was less efficient (unpublished results).

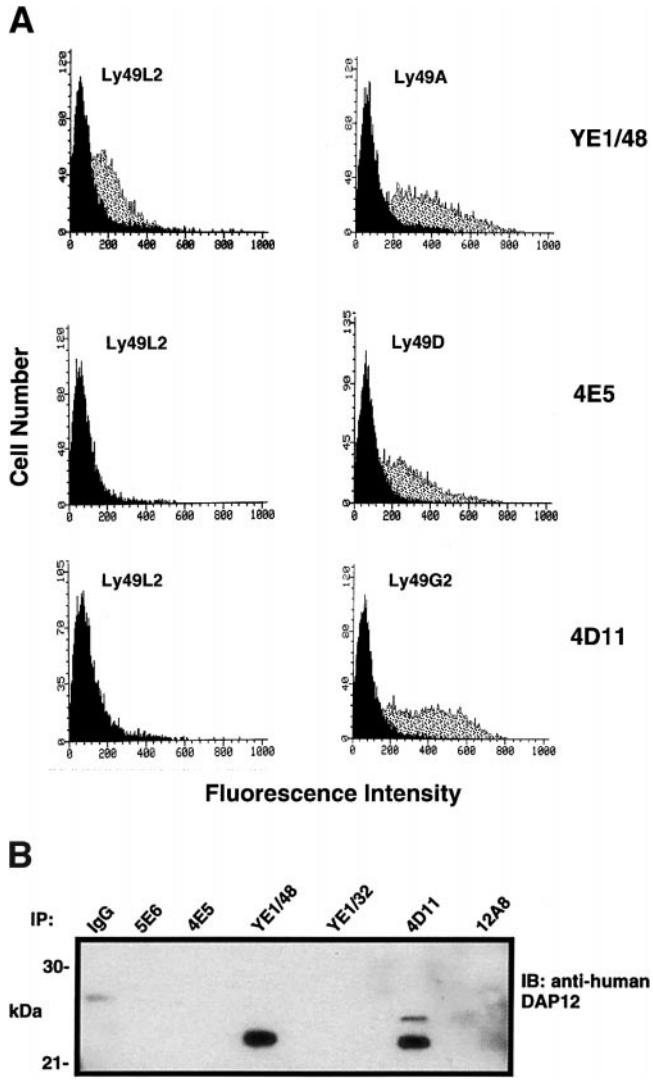

Fig. 4. Reactivity of Ly49L2 to a panel of anti-Ly49 mAb. (A) Fluorescein-activated cell sorter (FACS) analysis. CP cells were transfected with Ly49L2, Ly49A, Ly49G2, or Ly49D. Cells were stained with the indicated mAb followed by an FITC-labeled secondary. YE1/48 is specific for Ly49A, 4E5 is specific for Ly49D, and 4D11 reacts with Ly49G. (B) Immunoprecipitation analysis. CP cells were transfected with Ly49L2 by electroporation. Cell lysates were immunoprecipitated with the indicated mAb. Immune complexes were run under nonreducing conditions, transferred, and blotted with DX37 (antihuman DAP12).

This experiment shows that Ly49L can be immunoprecipitated by 4D11 and YE1/48 also in the absence of Ly49G, demonstrating that binding is direct and not the result of heterodimer formation despite the shared sequence similarity. The discrepancy between flow cytometric analysis and immunoprecipitation using 4D11 may be attributable to a low affinity for Ly49L; thus, the longer binding times used in the immunoprecipitation experiment may allow detection of Ly49L. The CP cells used in the transient transfection model may have been a factor also in the failure of 4D11 to bind Ly49L in flow analysis. CP cells are of human origin, and differences in glycosylation as well as other posttranslational modifications may account for the lack of 4D11 reactivity. 4D11 may be able to bind Ly49L on the surface of mouse NK cells, because 4D11 can induce a modest $[Ca^{2+}]_i$ mobilization as well as interferon-γ (IFN-γ) secretion in CBA/J NK cells (unpublished results).

Antibody-mediated cross-linking of Ly49L2 leads to DAP12 phosphorylation

Cross-linking of Ly49D by specific antibody leads to DAP12 phosphorylation [16]. To see if Ly49L was a functional activating receptor also, Ly49L2-transfected CP cells were subjected to cross-linking with YE1/48 mAb. Cross-linking of Ly49D with 4E5, followed by immunoprecipitation and detection of phosphorylated proteins, reveals a "ladder" of the various DAP12 phosphorylation states. Similarly, cross-linking Ly49L2 with YE1/48 yielded such a ladder also (**Fig. 5**). A western blot run in parallel shows that equal levels of DAP12 are present in cells cross-linked with specific or irrelevant mAb. These data suggest that Ly49L2 is a functional activating receptor in CBA/J mice. As expected, a band at 100 kDa is not present because of the lack of tyrosine residues in the intracellular domain of Ly49L.

DISCUSSION

The role of activating Ly49 molecules in general is still unknown. The occurrence of NK cells expressing activating Ly49s with allogeneic MHC specificity is perplexing. For example, Ly49D-expressing NK cells can kill target cells bearing H-2^d, but Ly49D is expressed in B6 mice that are of the H-2^b haplotype. The MHC specificities of Ly49H, L, and P are unknown, but it would be interesting to see if these molecules are also allospecific. The functional role of the allospecificity of Ly49D is shown in the inability of Ly49D-depleted H-2^b mice to reject H-2^d bone marrow cell allografts [19]. A situation in which mice would require Ly49-mediated protection against heterologous transfer is not easy to envisage. However, such protection may be needed at the maternal/fetal interface, es-

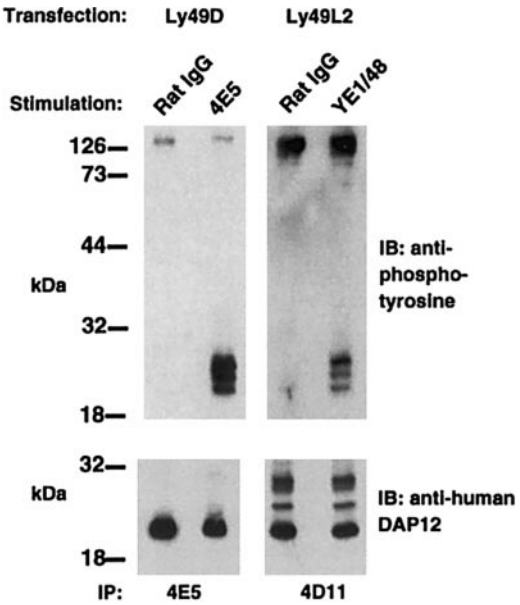

Fig. 5. Antibody-mediated cross-linking of Ly49L2 results in mDAP12 phosphorylation. CP cells were transfected with the indicated cDNAs in expression vectors by electroporation. Cells were treated with specific mAb or irrelevant rat IgG followed by a 2 min incubation with an isotype-specific secondary antibody at 37°C. Cell lysates were immunoprecipitated with 4E5 or 4D11, specific for Ly49D and L2, respectively (by immunoprecipitation only for Ly49L2). Immune complexes were run under nonreducing conditions, transferred, and blotted with 4G10 (antiphosphotyrosine) or DX37 (antihuman DAP12).

pecially in a normal situation (as opposed to inbred mice) where the fetus is likely to differ from the mother in one or more histocompatibility antigens. It has long been known that NK cells are abundant in the mucosal decidual tissues of the uterus. NK allospecificity may play a role in protecting the mother from invasion by fetal cells. However, there is still no explanation for the presence of activating Ly49 molecules in male mice or on circulating NK cells in general.

In a recent study of MHC class I binding by Ly49s, no binding of Ly49D or H to MHC class I was detected using cell-cell or MHC-tetramer binding assays [27]. This shows that the affinity of the activating receptors for MHC class I is considerably lower than the inhibitory Ly49s. This may suggest that class I MHC is not the real *in vivo* ligand for activating Ly49 receptors, or that the affinity is lower for activating receptors to prevent autoimmunity. Another alternative is that activating receptors bind to an MHC class I-related ligand and possess weak cross-reactivity with MHC class I. Perhaps the activating receptors evolved from the inhibitory receptors to provide a mechanism whereby NK cells could eliminate NK-resistant viruses expressing molecules that mimic MHC class I [28]. Although inhibitory receptors bind to constitutively expressed ligands to suppress NK cell activation, activating receptors would respond to a newly synthesized molecule, a product of a native stress-induced gene or originating from a pathogen.

The study of Ly49 expression in various mouse strains has lead to an accumulation of evidence indicating that Ly49 expression is strain-specific. The current study demonstrates that Ly49L is expressed in CBA/J and C3H/He but not 129/J or B6 NK cells. Similarly, previous studies have shown that Ly49P is expressed in 129/J but not B6 mice [4]. Ly49P is cross-reactive with YE1/48, and so the DAP12-associating Ly49 seen in Figure 1 could be Ly49L and/or Ly49P. However, *Ly49p* was not detected in CBA/J NK cells by PCR cloning using primers capable of isolating *Ly49p* from 129/J mice. Ly49A and D are expressed in B6 mice, but there is no evidence from biochemical studies for the presence of Ly49A in 129 strains of mice [4, 29, 30] or for Ly49D expression in CBA/J NK cells (Fig. 1, 4E5 immunoprecipitation lane). No Ly49H protein was detected in CBA/J immunoprecipitation experiments (Fig. 1), and no *Ly49h* cDNAs were cloned. Therefore, the *Ly49h* cDNA cloned from B6-CBA F1 mice [2] is probably of B6 origin.

The observed differences in Ly49 repertoire between inbred mouse strains raises questions relating to the mechanism of selective Ly49-gene expression. It is possible that the genes of the unexpressed Ly49s in the various strains are completely missing. A Southern blotting study of *Ly49c* and *a*-related genes in a panel of mouse strains has indicated significant differences in gene number between strains [3]. Another possibility is gene silencing, as evidenced by the presence of the *Ly49l* gene in B6 mice, but no detectable *Ly49l* mRNA. The B6 *Ly49l* gene has been only partially characterized, so the mechanism of silencing is unknown [3]. Transcripts of the *Ly49k* and *n* genes in B6 mice were shown to contain incomplete open-reading frames as a result of missing exons or premature stop codons [31]. In addition, an improperly spliced transcript of *Ly49m* containing a stop codon at the beginning of exon 4 was isolated from a B6 NK cell cDNA library (unpublished results). Therefore, it appears that inactivation by mutation has silenced several of the B6 Ly49 genes. The inability to detect *Ly49l* transcripts using PCR primers corresponding to known exons suggests that the *Ly49l* promoter activity may be altered in B6 mice because of mutation of cis-acting elements or transcription factors. The TCF-1 transcription factor has been implicated in *Ly49a* transcription, however it does not appear to be required for the transcription of other Ly49s [30]. Recently, we have found that unlike the *Ly49a* promoter, the *Ly49i* promoter does not contain TCF-1 binding sites (unpublished results). The observation that TCF-1 knockout mice show no difference in the level of Ly49C/I expression supports this finding [30]. Perhaps there are specific transcription factors required for the expression of individual Ly49s, and the B6 mouse is missing an *Ly49l*-specific transcription factor. An understanding of the *Ly49l* gene defect in B6 mice will require the complete characterization of the *Ly49l* locus in B6 and CBA/J mice.

Another possible mechanism governing the expression of various Ly49 molecules is the MHC haplotype environment of the NK cells, if one assumes that NK cell selection occurs somewhat like thymic selection for T cells. There is considerable evidence indicating that an MHC-dependent education process operates during NK cell Ly49 repertoire development [32]. Ly49L was found to be expressed in CBA/J and C3H/He mice, which are of the H-2^k background, but there was no expression in B6 or 129/J mice, both of the H-2^b background. We cannot discount the possibility that the Ly49 expression pattern and the MHC haplotype are the product of the two strains sharing a common ancestor and, therefore, genotypes. The observation that a highly related inhibitory Ly49 exists for each activating Ly49 may bear on this argument (Fig. 2B). If one assumes that the MHC specificities are similar for each

activating and inhibitory partner, then this could serve as a basis for selection through MHC. If an NK cell expresses a self-reactive activating Ly49, then the survival of that NK cell may depend on the coexpression of its inhibitory partner. Alternatively, other inhibitory receptors with similar specificities could provide a negative signal, preventing the development of self-reactive NK cells. The generation of mice lacking activating Ly49 receptors may provide insight into the true functional role of activating Ly49 family members.

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REFERENCES

1. Smith, H. R., Karlhofer, F. M., Yokoyama, W. M. (1994) Ly-49 multigene family expressed by IL-2-activated NK cells. J. Immunol. 153, 1068–1079.
2. Brennan, J., Mager, D., Jefferies, W., Takei, F. (1994) Expression of different members of the Ly-49 gene family defines distinct natural killer cell subsets and cell adhesion properties. J. Exp. Med. 180, 2287–2295.
3. McQueen, K. L., Freeman, J. D., Takei, F., Mager, D. L. (1998) Localization of five new Ly49 genes, including three closely related to Ly49c. Immunogenetics 48, 174–183.
4. Makrigiannis, A. P., Gosselin, P., Mason, L. H., Taylor, L. S., McVicar, D. W., Ortaldo, J. R., Anderson, S. K. (1999) Cloning and characterization of a novel activating Ly49 closely related to Ly49A. J. Immunol. 163, 4931–4938.
5. Olcese, L., Lang, P., Vely, F., Cambiaggi, A., Marguet, D., Blery, M., Hippen, K. L., Biassoni, R., Moretta, A., Moretta, L., Cambier, J. C., Vivier, E. (1996) Human and mouse killer-cell inhibitory receptors recruit PTP1C and PTP1D protein tyrosine phosphatases. J. Immunol. 156, 4531–4538.
6. Mason, L. H., Gosselin, P., Anderson, S. K., Fogler, W. E., Ortaldo, J. R., McVicar, D. W. (1997) Differential tyrosine phosphorylation of inhibitory vs. activating Ly-49 receptor proteins and their recruitment of SHP-1 phosphatase. J. Immunol. 159, 4187–4196.
7. Nakamura, M. C., Niemi, E. C., Fisher, M. J., Shultz, L. D., Seaman, W. E., Ryan, J. C. (1997) Mouse Ly-49A interrupts early signaling events in natural killer cell cytotoxicity and functionally associates with the SHP-1 tyrosine phosphatase. J. Exp. Med. 185, 673–684.
8. Karlhofer, F. M., Ribaudo, R. K., Yokoyama, W. M. (1992) MHC class I alloantigen specificity of Ly-49$^+$ IL-2-activated natural killer cells. Nature 358, 66–70.
9. Yu, Y. Y. L., George, T., Dorfman, J. R., Roland, J., Kumar, V., Bennett, M. (1996) The role of Ly49A and 5E6(Ly49C) molecules in hybrid resistance mediated by murine natural killer cells against normal T cell blast. Immunity 4, 67–76.
10. Mason, L. H., Ortaldo, J. R., Young, H. A., Kumar, V., Bennett, M., Anderson, S. K. (1995) Cloning and functional characteristics of murine large granular lymphocyte-1: a member of the Ly-49 gene family (Ly-49G2). J. Exp. Med. 182, 293–303.
11. George, T. C., Mason, L. H., Ortaldo, J. R., Kumar, V., Bennett, M. (1999) Positive recognition of MHC class I molecules by the Ly49D receptor of murine NK cells. J. Immunol. 162, 2035–2043.
12. Mason, L. H., Anderson, S. K., Yokoyama, W. M., Smith, H. R., Winkler-Pickett, R., Ortaldo, J. R. (1996) The Ly-49D receptor activates murine natural killer cells. J. Exp. Med. 184, 2119–2128.
13. Mason, L. H., Willette-Brown, J., Anderson, S. K., Gosselin, P., Shores, E. W., Love, P. E., Ortaldo, J. R., McVicar, D. W. (1998) Characterization of an associated 16-kDa tyrosine phosphoprotein required for Ly-49D signal transduction. J. Immunol. 160, 4148–4152.
14. Lanier, L. L., Corliss, B. C., Wu, J., Leong, C., Phillips, J. H. (1998) Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. Nature 391, 703–707.
15. Smith, K. M., Wu, J., Bakker, A. B., Phillips, J. H., Lanier, L. L. (1998) Ly-49D and Ly-49H associate with mouse DAP12 and form activating receptors. J. Immunol. 161, 7–10.
16. Gosselin, P., Mason, L. H., Willette-Brown, J., Ortaldo, J. R., McVicar, D. W., Anderson, S. K. (1999) Induction of DAP12 phosphorylation, calcium mobilization, and cytokine secretion by the Ly49H receptor. J. Leukoc. Biol. 66, 165–171.
17. McVicar, D. W., Taylor, L. S., Gosselin, P., Willette-Brown, J., Mikhael, A. I., Geahlen, R. L., Nakamura, M. C., Linnemeyer, P., Seaman, W. E., Anderson, S. K., Ortaldo, J. R., Mason, L. H. (1998) DAP12-mediated signal transduction in natural killer cells. A dominant role for the Syk protein-tyrosine kinase. J. Biol. Chem. 273, 32934–32942.
18. Nakamura, M. C., Linnemeyer, P. A., Niemi, E. C., Mason, L. H., Ortaldo, J. R., Ryan, J. C., Seaman, W. E. (1999) Mouse Ly-49D recognizes H-2D^d and activates natural killer cell cytotoxicity. J. Exp. Med. 189, 493–500.
19. Raziuddin, A., Longo, D. L., Mason, L. H., Ortaldo, J. R., Bennett, M., Murphy, W. J. (1998) Differential effects of the rejection of bone marrow allografts by the depletion of activating versus inhibiting Ly-49 natural killer cells subsets. J. Immunol. 160, 87–94.
20. Idris, A. H., Smith, H. R., Mason, L. H., Ortaldo, J. R., Scalzo, A. A., Yokoyama, W. M. (1999) The natural killer gene complex genetic locus Chok encodes Ly-49D, a target recognition receptor that activates natural killing. Proc. Natl. Acad. Sci. USA 96, 6330–6335.
21. Nakamura, M. C., Naper, C., Niemi, E. C., Spusta, S. C., Rolstad, B., Butcher, G. W., Seaman, W. E., Ryan, J. C. (1999) Natural killing of xenogeneic cells mediated by the mouse Ly-49D receptor. J. Immunol. 163, 4694–4700.
22. Ortaldo, J. R., Mason, A. T., Winkler-Pickett, R., Raziuddin, A., Murphy, W. J., Mason, L. H. (1999) Ly-49 receptor expression and functional analysis in multiple mouse strains. J. Leukoc. Biol. 66, 512–520.
23. Takei, F. (1983) Two surface antigens expressed on proliferating mouse T lymphocytes defined by rat monoclonal antibodies. J. Immunol. 130, 2794–2800.
24. Stoneman, E. R., Bennett, M., An, J., Chesnut, K. A., Wakeland, E. K., Scheerer, J. B., Siciliano, M. J., Kumar, V., Mathew, P. A. (1995) Cloning and characterization of 5E6(Ly-49C), a receptor molecule expressed on a subset of murine natural killer cells. J. Exp. Med. 182, 305–313.
25. O'Shea, J. J., McVicar, D. W., Bailey, T. L., Burns, C., Smyth, M. J. (1992) Activation of human peripheral blood T lymphocytes by pharmacological induction of proteintyrosine phosphorylation. Proc. Natl. Acad. Sci. USA 89, 10306–10311.
26. Silver, E. T., Elliott, J. F., Kane, K. P. (1996) Alternatively spliced Ly-49D and H transcripts are found in IL-2-activated NK cells. Immunogenetics 44, 478–482.
27. Hanke, T., Takizawa, H., McMahon, C. W., Busch, D. H., Pamer, E. G., Miller, J. D., Altman, J. D., Liu, Y., Cado, D., Lemonnier, F. A., Bjorkman, P. J., Raulet, D. H. (1999) Direct assessment of MHC class I binding by seven inhibitory NK cell receptors. Immunity 11, 67–77.
28. Cretney, E., Degli-Esposti, M. A., Densley, E. H., Farrell, H. E., Davis-Poynter, N. J., Smyth, M. J. (1999) m144, a murine cytomegalovirus (MCMV)-encoded major histocompatibility complex class I homologue, confers tumor resistance to natural killer cell-mediated rejection. J. Exp. Med. 190, 435–443.
29. Held, W., Roland, J., Raulet, D. H. (1995) Allelic exclusion of Ly49-family genes encoding class I MHC-specific receptors on NK cells. Nature 376, 355–358.
30. Held, W., Kunz, B., Lowin-Kropf, B., van de Wetering, M., Clevers, H. (1999) Clonal acquisition of the Ly49A NK cell receptor is dependent on the trans-acting factor TCF-1. Immunity 11, 433–442.
31. McQueen, K. L., Lohwasser, S., Takei, F., Mager, D. L. (1999) Expression analysis of new Ly49 genes: most transcripts of Ly49j lack the transmembrane domain. Immunogenetics 49, 685–691.
32. Held, W., Raulet, D. H. (1997) Ly49A transgenic mice provide evidence for a major histocompatibility complex-dependent education process in natural killer cell development. J. Exp. Med. 185, 2079–2088.