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Ly-49W, an Activating Receptor of Nonobese Diabetic Mice With Close Homology to the Inhibitory Receptor Ly-49G, Recognizes H-2D<sup>k</sup> and H-2D<sup>d</sup> 1

Elizabeth T. Silver, Dong-Er Gong, Bart Hazes, and Kevin P. Kane 2

The diversity and ligand specificity of activating Ly-49 receptors expressed by murine NK cells are largely unknown. We cloned a new Ly-49-activating receptor, expressed by NK cells of the nonobese diabetic mouse strain, which we have designated Ly-49W. Ly-49W is highly related to the known inhibitory receptor Ly-49G in its carbohydrate recognition domain, exhibiting 97.6% amino acid identity in this region. We demonstrate that the 4D11 and Cwy-3 Abs, thought to be Ly-49G specific, also recognize Ly-49W. Rat RNK-16 cells transfected with Ly-49W mediated reverse Ab-dependent cellular cytotoxicity of FcR-positive target cells, indicating that Ly-49W can activate NK-mediated lysis. We further show that Ly-49W is allo-MHC specific: Ly-49W transfectants of RNK-16 only lysed Con A blasts expressing H-2<sup>k</sup> or H-2<sup>d</sup> haplotypes, and Ab-blocking experiments indicated that H-2D<sup>k</sup> and D<sup>d</sup> are ligands for Ly-49W. Ly-49W is the first activating Ly-49 receptor demonstrated to recognize an H-2<sup>k</sup> class I product. Ly-49G and Ly-49W represent a new pair of NK receptors with very similar ligand-binding domains, but opposite signaling functions. The Journal of Immunology, 2001, 166: 2333–2341.

Natural killer cells function as a first line of defense to eliminate tumor cells or virally infected cells without prior sensitization (1, 2). NK cells also mediate alloge- neic bone marrow rejection as well as hybrid resistance, wherein an F<sub>1</sub> mouse rejects a bone marrow transplant from a parent (3, 4). The cytotoxicity of NK cells appears to be regulated by opposing signals generated upon interaction with potential target cells. Positive signals are induced upon the interaction of activating receptor(s) with largely undefined target cell surface ligands (5–7), while NK activation is negatively regulated when inhibitory receptors bind class I MHC molecules on the target cell (8). NK cells can lyse cells with deficient self class I expression, but spare cells that express normal levels of self class I MHC molecules. Such observations have resulted in the “missing self” hypothesis (9), which holds as one of its tenets an in vivo selection process in which each mature NK cell will express at least one inhibitory receptor recognizing self class I MHC to ensure NK cell self-tolerance (9, 10).

NK cells express inhibitory receptors from the Ig- and the C-type lectin superfamilies, with the human killer Ig-related receptor (KIR) 3 and the murine Ly-49 families being the most highly characterized of the Ig- and lectin-like NK-inhibitory receptors, respectively (11). While KIR and Ly-49 are structurally dissimilar, they are functionally equivalent, serving as inhibitory receptors that can distinguish class I alleles in their MHC recognition. A common feature of inhibitory receptors is the presence of an immunoreceptor tyrosine-based inhibitory motif (ITIM) (12). Engagement of the inhibitory receptor with its class I ligand on the target cell results in tyrosine phosphorylation of the ITIM (13, 14). The phosphorylated ITIM recruits the tyrosine phosphatase SHP-1, which then dephosphorylates plasma membrane-proximal targets in the NK activation cascade, thus blocking NK activation (13, 14).

All NK cell-inhibitory receptor families contain members that lack ITIM sequences. The ITIM-lacking molecules have a charged residue in their transmembrane domains to allow interaction with signaling adaptor molecules (12). For the KIR and Ly-49 families, this adaptor is the DAP12 signaling molecule that contains an immunoreceptor tyrosine-based activation motif (11, 15–17). DAP12 interacts with the ITIM-lacking receptors via a noncovalent interac-
tion in the transmembrane domain (11, 17). Ligation of the re-
ceptor recruits and activates Syk kinase, leading to subsequent downstream activation events, target cell cytolysis, and cytokine production (18–20). Some Ly-49 proteins can be grouped into inhibitory/activating pairs based on high sequence identities in their external domains (12). The functional significance of these pairs is unknown, but the considerable resemblance suggests that they recognize the same ligands. Ly-49D and Ly-49P are the only activating Ly-49 receptors for which a ligand has been determined. Ly-49D and Ly-49P recognize the H-2D<sup>d</sup> class I MHC molecule, similar to the ITIM-containing inhibitory receptors, Ly-49A and Ly-49G (21–25).

Studies of Ly-49 receptor function have largely been confined to the C57BL/6 (B6) mouse strain. In this study, we examined Ly-49 gene expression and function in the nonobese diabetic (NOD) mouse, to explore strain-to-strain variation in Ly-49 expression, and because of its well-established immune dysregulation and spontaneous onset of autoimmune diabetes (26). We describe Ly-49W, a novel activating Ly-49 receptor expressed in NOD mice with high homology in its external domain to the inhibitory Ly-49G receptor. We also demonstrate that Ly-49W recognizes class

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3 Abbreviations used in this paper: KIR, killer Ig-related receptor; ADCC, Ab-dependent cellular cytotoxicity; CRD, carbohydrate recognition domain; ITIM, immunoreceptor tyrosine-based inhibitory motif; NOD, nonobese diabetic; NOR, nonobese diabetes-resistant; PA, protein A; PG, protein G; rADCC, reverse ADCC.
I MHC molecules with strong and moderate reactivity toward H-2D\(b\) and H-2D\(d\), respectively. This is the first report of an activating Ly-49 molecule recognizing an H-2K\(d\) product and provides further support for the possibility that activating Ly-49 receptors recognize class I MHC ligands.

Materials and Methods

Animals

Five- to 8-wk-old female AKR/J (H-2\(k\)), CBA/J (H-2\(b\)), BALB/c (H-2\(d\)), DBA/2J (H-2\(b\)), C57BL/6 (H-2\(b\)), NOD (H-2K\(d\), D\(b\)), nonobese diabetes-resistant (NOR) (H-2K\(d\), D\(b\)), B10.BR (H-2\(k\)), B10.D2 (B10.D2-H2\(d\)/nSn; DBA/2J (H-2\(d\)), C57BL/6 (H-2\(b\)), NOD (H-2K\(d\), D\(b\)), and used to synthesize a cDNA library with the Marathon cDNA Amplitag (Clontech) with an oligo(dT) primer. The cDNA encoding Ly-49W was inserted into the 5' Sac\(I\)/Xba\(I\) sites of the expression vector pBCRzeN (generously provided by Dr. An- drey Shaw, Washington University, St. Louis, MO) and transfected into RK-16 cells using the protocol described by Nakamura et al. (14).

Hybridomas and Abs

Hybridomas producing the following Abs: 4D11 (rat IgG2a, anti-Ly-49G (27); Cwy-3 (IgG1), anti-Ly-49G (28); M1/42 (rat IgG2a), anti-mouse class I MHC (29); 34-5-8S (IgG2a), anti-H-2\(D^{d}\)/α2 domain epitope (30); 34-2-12S (IgG2a) anti-H-2\(D^{d}\)/α3 domain epitope (30); B8-24-3 (IgG1), anti-H-2\(K^{d}\)/31; B27 M1 (IgG2a), H-2\(A\)-B27; B7 (32); and BB7.1 (IgG1), anti-HLA-B7 (33) were obtained from American Type Culture Collection (Manassas, VA), except Cwy-3, which was generated in this laboratory. Abs were prepared by ammonium sulfate precipitation, and PBS dialysis of tissue culture supernatants obtained from hybridomas grown in protein-free hybridoma medium. Purified IgG was purchased from Sigma-Aldrich (Oakville, Canada). FITC-coupled rat anti-mouse IgG, goat anti-rat IgG, and mouse anti-rat IgG were purchased from Jackson ImmunoResearch (West Grove, PA).

Cell lines

RNK-16, a spontaneous F344 rat strain NK cell leukemia cell line (31), was kindly provided by Dr. Mary Nakamura at the University of California at San Francisco. The RNK-16 cells were maintained in RPMI 1640 sup- plemented with 10% FCS, L-glutamine, penicillin, streptomycin, and 5 × 10\(^{-5}\) M 2-ME (RNK medium). COS-7 SV40-transformed African green monkey kidney cells were provided by Dr. John Elliott (University of Alberta). COS-7 cells were grown in Opti-MEM I medium (Life Technol- ogies, Burlington, Ontario, Canada), containing 4% heat-inactivated FCS (Alberta). COS-7 cells were grown in Opti-MEM I medium (Life Technol- ogies, Burlington, Ontario, Canada), containing 4% heat-inactivated FCS (Alberta). COS-7 cells were grown in Opti-MEM I medium (Life Technol- ogies, Burlington, Ontario, Canada), containing 4% heat-inactivated FCS (Alberta).

Cloning of NOD Ly-49 transcripts

Ly-49 transcripts were amplified with Advantage cDNA Polymerase (Clon- tech, Palo Alto, CA) using the sense cloning primer 5'-CTTCAAAGAGATGGTGGCAAGAAGG and the antisense cloning primer 5'-GAGGACTAAGGCGCCAGACCAGAGTCC (in which R = A/G and Y = C/T). To verify the nucleotide sequences of the ends of the open reading frames and to obtain sequence of the noncoding gene-flanking regions for cloning, we performed 5' rapid amplification of cDNA ends of Ly-49W. Poly(A)\(^+\) RNA was isolated from total RNA, obtained from 5' to 8-wk-old female mice, as described by Smith and coworkers (38). Total cellular RNA was isolated with TRIzol reagent (Life Technologies) and reverse transcribed using Superscript II (Life Technologies) with an oligo(dT) primer.

Transfection and flow cytometric analysis

COS-7 cells. DNA containing the coding regions of Ly-49 cDNAs was inserted into the Xhol/EcoRI sites of the mammalian expression vector pCI-neo (Promega, Madison, WI). Sequence encoding the mature mouse DAP12 protein was inserted into the pFLAG-CMV-1 expression vector (Sigma-Aldrich) to create an epitope-tagged chimeric protein. Vectors were then transfected into COS cells using LipofectAMINE (Life Tech- nologies). Approximately 48 h after transfection, the cells were incubated with the mAbs 4D11 or Cwy-3, or isotype control Abs M1/42 or B8-24-3, respectively. Secondary Abs, either FITC-labeled goat anti-rat IgG or rat anti-mouse IgG, respectively, were subsequently added for an additional incubation, whereupon the samples were analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

RNK cells. The cDNA encoding Ly-49W was inserted into the Xhol/XbaI sites of the bicistronic vector BSrEZN (generously provided by Dr. An- drey Shaw, Washington University, St. Louis, MO) and transfected into RNK-16 cells using the protocol described by Nakamura et al. (14). In brief, four million cells were transfected with 20 μg of plasmid linearized with SacI by electroporation at 200 μA and 960 μF. Transfected cells were cloned in 96-well microtiter plates in complete RNK medium sup- plemented with 1 mg/ml G418 for drug selection. Expression of Ly-49W on transfectant clones was measured by FACScan analysis with the 4D11 Ab after blocking FcR with normal mouse serum. Reactivity of transfectant clones with Cwy-3 and OX-8 Abs was also determined after blocking purified rat IgG. The BB7.1 Ab was used as an isotype control. Primary Ab binding was detected with mouse anti-rat and rat anti-mouse fluorescein- coupled Abs, respectively, using a FACScan flow cytometer.

Generation of Con A T cell blast target cells

Con A-activated T cell blasts were prepared from spleen cells of various mouse strains. Fifteen million spleen cells were cultured at 5 × 10\(^6\) cells/ml in RPMI 1640 with 10% heat-inactivated FCS, 2-ME, and 3 μg/ml Con A (Sigma-Aldrich) for 48 h. Blast cells were recovered after three washes in RPMI 1640 medium.

Cytotoxicity assays

Target cells were labeled at 37°C with 100–150 μCi of Na\(^{25}\)CrO\(_4\) (37Cr) (Mandel/INEN Life Science Products, Guelph, Canada), for 1 h if tumor cells, or 1.5 h if Con A blast cells. Following extensive washes, 1 × 10\(^3\) 3\(^\text{Cr}\)-labeled target cells were incubated for 4–5 h at 37°C in V-bottom microtiter plates with 1 × 10\(^5\) or 1 × 10\(^4\) confluent cells transduced with Ly-49W at various E:T ratios in triplicate. After the incubation, plates were centrifuged for 5 min, and 100 μl of supernatant was removed and counted in a gamma counter. The percent specific lysis was determined as (experimental release − spontaneous release)/maximal release − spontaneous release) × 100. To perform the reverse Ab-dependent cellular cytotoxicity (rADCC) experiments, untransfected RNK-16 cells and the Ly-49W trans- fectants of RNK-16 were preincubated for 15 min with 20 μg/ml of the Cwy-3 or OX-8 Abs or medium alone before addition of FcR-expressing YB2/0 target cells and subsequent 4-h cytotoxicity assay, as described. For Ab inhibition experiments, Abs were incubated with soluble protein A (PA) (2 μg/10 μg of mAb; Sigma-Aldrich) or a mix of PA and protein G (PG) (4 μg/10 μg of mAb; ICN Pharmaceuticals, Costa Mesa, CA) for 30 min under addition to effecting cells or target cells. Effector cells or target cells were preincubated with the mAb and PA for 15 min before the cytotoxicity assay. In the case of two Abs being employed simultaneously in the same wells in receptor-blocking studies, each Ab was used at the indi- cated concentrations in the figure. The mAbs and PA or PA/PG were present throughout the cytotoxicity assays. All cytotoxicity assays were repeated a minimum of three times.

Results

Cloning of noninhibitory Ly-49 members with homology to Ly-49G

Recent studies suggest that Ly-49 gene expression varies between mouse strains (25, 39, 40). Furthermore, the complex hybridization patterns in Southern blots of various mouse strains suggest that not
all Ly-49 family members have been identified (41). Indeed, investigation of Ly-49 expression in mouse strains such as 129J and CBA/J have resulted in the identification of novel activating Ly-49 molecules (40, 42). In this study, we examine Ly-49 expression in the NOD mouse strain, an animal model of insulin-dependent diabetes mellitus. NOR strain mice are identical with NOD at most, but not all, genetic loci and do not develop diabetes. We designed an RT-PCR strategy to clone cDNAs encoding both activating and inhibitory Ly-49 family members from IL-2-activated NK cells of NOD and NOR strain mice. From this, we obtained cDNAs encoding both ITIM-containing and ITIM-lacking receptors.

The ITIM-containing cDNAs prepared from NOD IL-2-activated NK cells include a novel allele of the inhibitory Ly-49G gene with a nucleotide identity of 98.8% and 98.9%, and an amino acid identity of 97% and 98.1% compared with the C57BL/6 and BALB/c alleles, respectively (Fig. 1A). The NOD Ly-49G allele more closely resembles both the C57BL/6 and BALB/c alleles than they resemble each other (97.9% nucleotide and 95.9% amino acid identities). The NOD transcripts described in this comparison correspond to Ly-49G2, first defined in the C57BL/6 (B6) strain (Fig. 1A). NOR NK cells also express Ly-49G2 (GenBank accession number AF074457) with 100% sequence identity to NOD Ly-49G2. Similar to the B6 strain, NOD NK cells also express alternatively spliced RNA transcripts encoding a larger Ly-49G form, Ly-49G1, in which there is an extension of 13 aa residues in the extracellular membrane-proximal stalk domain of the receptor (GenBank accession number AF283248). In addition, NOD NK cells express Ly-49G transcripts that, through alternative splicing, lack exon 3 encoding the transmembrane segment and a portion of the cytoplasmic domain (GenBank accession number AF283253).

The ITIM-lacking NOD IL-2-activated NK cDNAs include sequences of two novel Ly-49 transcripts. Both sequences show strong similarity with two genomic fragments from B6 mice that were originally designated Ly-49M (43). To determine the relationship of our new sequences with Ly-49M from the B6 strain, we cloned and sequenced the full Ly-49M transcript from B6 mouse cDNA (Fig. 1B). This showed that Ly-49M is not functionally expressed in B6 mice as there is a premature stop codon near the predicted beginning of exon 4 (Fig. 1B). The B6 Ly-49M sequence differs by only 10 bases and 8 aa from one of our new sequences, corresponding to 98.7% and 97% sequence identity on the DNA and protein levels, respectively. This extent of sequence identity is comparable with that found between alleles of Ly-49G. Thus, we are tentatively designating this NOD Ly-49 product as the NOD allele of Ly-49 m. Interestingly, the NOD gene does not contain the premature stop codon, suggesting that it represents a functional receptor. This is an additional example of variation in Ly-49 expression between different mouse strains.

Our other new NOD Ly-49 transcript also resembles Ly-49M with 18 bases and 15 aa substituted, corresponding to 97.7% and 94.3% sequence identity on the DNA and protein level, respectively. However, this product must represent a distinct gene because we have already shown above that NOD encodes an even more closely related Ly-49M homologue. Hence, we have designated this NOD Ly-49 transcript Ly-49W (Fig. 1A). There are two distinct Ly-49W mRNA transcripts that result from alternative splicing at the beginning of exon 3. We have previously demonstrated that this form of alternative splicing also occurs in Ly-49D and Ly-49H (44). Splice variants containing the coding sequence for cytoplasmic residues Val-Cys-Ser are named Ly-49D1 and Ly-49H1, while those lacking this coding sequence are termed Ly-49D2 and Ly-49H2. Hence, we are using the designations Ly-49W1 and Ly-49W2 for transcripts that encode or lack the Val-Cys-Ser sequence, respectively (Fig. 1B). We have also found both splice variants with identical sequences in the NOD mouse strain (GenBank accession numbers AF074459 and AF074463).

Recently, the complete sequence of a novel receptor, Ly-49L, was determined. Ly-49L is found in CBA/J, C3H (42), and BALB/c mice (Fig. 1B), and its nucleotide and amino acid sequence does not differ in these strains. Ly-49L has 94.3% amino acid sequence identity with Ly-49W (Fig. 1B), the same level of
Ly-49W, an Ly-49G-like activating receptor, recognizes H-2D\textsuperscript{k} and D\textsuperscript{d}

divergence as observed between Ly-49W and Ly-49M. However, Ly-49W and Ly-49L amino acid sequences are identical in exons 2 to 5, with all substitutions taking place in the C-terminal region encoded by exons 6 and 7 (Fig. 1B, and data not shown). The genetic relationship of Ly-49W and Ly-49L is unclear. The overall level of amino acid identity strongly suggests that Ly-49L and Ly-49W are separate genes. It remains a possibility, however, that Ly-49W and Ly-49L are alleles of the same gene, but a substitution of exons 6 and 7 from another gene has occurred in Ly-49L or Ly-49W. Detailed genomic analysis of Ly-49 genes in multiple mouse strains will be necessary to address these possibilities. In any event, the amino acid sequence of the external domain of Ly-49W is closer to that of Ly-49G than those of Ly-49M or Ly-49L.

The carbohydrate recognition domain (CRD) sequence of Ly-49W (exons 5 to 7) is strikingly similar to the same region of the inhibitory receptor Ly-49G, with an amino acid sequence identity of 97.6% compared with the Ly-49G alleles of both the B6 and NOD strains (Fig. 1A). In the case of the stalk region, these numbers are 83.8% and 82.4%, respectively. Interestingly, the cytoplasmic/transmembrane domain is rather different as it lacks the ITIM motif and contains an arginine in the middle of its transmembrane domain (Fig. 1). This is the hallmark of the known activating receptors Ly-49D, Ly-49H, and Ly-49P (17, 25, 40). Below, we will present evidence that Ly-49W is indeed an activating receptor. Accordingly, Ly-49G/Ly-49W form an inhibitory/activating pair with opposite regulatory effects, but closely related CRDs. This is analogous to other Ly-49 inhibitory/activating pairs that have previously been reported (25, 40, 41). The studies described below will examine the function and specificity of Ly-49W.

Ly-49G-reactive Abs 4D11 and Cwy-3 recognize NOD Ly-49W

Serological determinants may be shared between NOD Ly-49G and Ly-49W due to the high degree of amino acid identity in their external domains. To address this possibility, we transiently expressed cDNAs encoding these molecules in COS cells and performed FACS analysis with Abs reactive with Ly-49G. The 4D11 Ab was initially demonstrated to detect an epitope expressed on several mouse strains (27) and subsequently was determined to recognize Ly-49G (23). The Cwy-3 mAb has been shown to have a much more restricted specificity by recognizing Ly-49G of C57BL/6, but not several other strains (28). Cwy-3 and 4D11 recognize Ly-49G of NOD origin, as indicated by substantial staining of COS cell transfectants with these Abs (Fig. 2, upper panels). Thus, Cwy-3 recognizes the NOD and B6 alleles of Ly-49G. Ly-49W expression on COS cells is not readily detected with Cwy-3 or 4D11 Abs, unless Ly-49W is cotransfected with DAP12 (Fig. 2, lower panels). Thus, Ly-49W is recognized by the Ly-49G-specific mAbs 4D11 and Cwy-3, but, similar to other noninhibitory Ly-49 family members (17, 40), appears to require the accessory molecule DAP12 for efficient cell surface expression.

Ly-49W mediates rADCC

The noninhibitory Ly-49D, Ly-49H, and Ly-49P receptors have been shown to be activating receptors by their ability to mediate rADCC, or redirected lysis, against FeR-expressing targets (25, 45, 46). Because the Abs that bind Ly-49W also recognize the inhibitory receptor Ly-49G, the experimental system we chose (14) employed the RNK-16 NK cell line transfected with mouse Ly-49W as effector cells. In this way, we could be certain there was no Ab cross-reactivity and the effector cells would be Ly-49W\textsuperscript{+}, yet Ly-49G\textsuperscript{−}. Transfected RNK-16 cells were screened by FACS analysis to select for clones stably expressing Ly-49W. A number of transfectant clones expressing Ly-49W were obtained as determined by acquisition of reactivity with the 4D11 and Cwy-3 Abs, and three clones were selected for study (Fig. 3). Cotransfection of mouse DAP12 was not required for expression of Ly-49W on RNK-16 cells as is observed with Ly-49D, another noninhibitory Ly-49 (24), presumably because rat DAP-12 substitutes for the mouse accessory protein. Expression of rat CD8\textsubscript{i}, a constitutively expressed endogenous rat receptor, was retained following transfection with Ly-49W (Fig. 3).

FIGURE 2. Ly-49W is recognized by the 4D11 and Cwy-3 Abs. COS-7 cells transfected with Ly-49W from C57BL/6 (B6) or NOD mice, or Ly-49W alone or Ly-49W cotransfected with murine DAP12 were analyzed by flow cytometry using the Abs 4D11 or Cwy-3 (shaded), or the isotype control Abs M1/42 or B8-24-3 (unshaded), respectively.

FIGURE 3. Expression of Ly-49W on RNK-16 transfectants. FACS analysis of Ly-49W-transfected RNK-16 clones 2C4, 7E8, and 10G5 with the Abs 4D11 or Cwy-3 (shaded), or no first Ab, or BB7.1 isotype control (unshaded), respectively. Expression of rat CD8\textsubscript{i} on RNK-16 cells was analyzed with the OX-8 Ab (shaded) or the isotype control BB7.1 (unshaded). Ab binding was detected with fluorescein-coupled mouse anti-rat or rat anti-mouse Abs.
RNK-16 cells can efficiently lyse the FcR-expressing target cell line YB2/0 (14) (Fig. 4A), but there is often a marked decrease in target cell killing when the RNK-16 cells are transfected with an activating receptor, such as Ly-49D (24). This effect may be due to a competition for, and sequestration of, a limiting activation component, possibly DAP12, by the transfected activating receptor, relative to an activating receptor that is normally involved in YB2/0 recognition. Such a substantial reduction of direct YB2/0 cytosis is seen with two Ly-49W transfectant clones, 2C4 and 10G5 (Fig. 4, B and C). Lysis of FcR-expressing YB2/0 target cells by the Ly-49W-transfected RNK-16 clones 2C4 and 10G5 could be substantially increased when Ly-49W on the effector cells was cross-linked by the Cwy-3 Ab (Fig. 4, B and C). In contrast, cross-linking of effector cell CD8α by the OX-8 Ab did not increase target cell lysis, although there is comparable cell surface expression of CD8α and Ly-49W on each of the transfected RNK clones tested (Fig. 3). These results indicate that Ly-49W can mediate rADCC and can be considered an activating receptor. Because high baseline levels of YB2/0 lysis appear to be retained for the Ly-49W transfectant clone 7E8, perhaps due to a greater abundance of signaling molecules, little augmentation of cytosis by Cwy-3 cross-linking of Ly-49W expressed on 7E8 can be detected with this clone (Fig. 4D). Untransfected RNK-16-mediated lysis of YB2/0 cells was unaffected by the Cwy-3 and OX-8 Abs (Fig. 4A).

The cytolytic activity of Ly-49W-transfected RNK-16 cells is MHC dependent

The H-2D^d class I MHC molecule is a ligand for some inhibitory Ly-49 receptors, including Ly-49G (23) and the activating receptor Ly-49W expressed on RNK-16 cells mediates rADCC with low level except in the case of the highly lytic clone 7E8, which kills H-2^d Con A blasts at a moderate level (Fig. 5A). No lysis of any of the Con A T cell blast targets was observed with the untransfected RNK-16 cells. Together, these results suggest that Ly-49W may recognize H-2^d, and possibly H-2^e, product(s).

To further define the importance of MHC molecules in Ly-49W recognition, we examined the lysis of Con A blasts generated from congenic B10 mouse strains that differ only in their MHC genes, by the Ly-49W transfectants. Striking differences were observed in the lysis of the Con A blasts generated from the B10 congenic mice. The B10.Br (H-2^b) Con A blasts were readily lysed by all three Ly-49W transfectant clones (Fig. 5B). The B10.D2 (H-2^d) Con A blasts were lysed at low to moderate levels, whereas no detectable lysis of B10.5 (H-2^c) Con A blasts was observed (Fig. 5B). These results suggest that Ly-49W is an activating receptor that recognizes one or more H-2^e and H-2^d product(s).

Ly-49W recognizes H-2^d and H-2^d

The previous results suggest that there is an H-2^e product or products recognized by Ly-49W. To determine which H-2^e class I product(s) is recognized by Ly-49W, we attempted to block Ly-49W-mediated lysis of B10.Br (H-2^c) Con A blasts with Abs that recognize either H-2K^d or H-2D^d. Soluble PA was used to bind the AbFc domain to prevent ADCC by the FcR-expressing RNK-16 cells. Neither the 11-4.1 Ab, which recognizes H-2K^b (35), nor the isotype control Ab B27 M1 blocks lysis of B10.Br Con A blasts (Fig. 6). In contrast, the 15-5-5S Ab, which recognizes H-2D^d (36), blocks lysis of B10.Br Con A blasts by both Ly-49W-transfected clones 7E8 and 10G5 in a dose-dependent manner (Fig. 6). These results indicate that Ly-49W recognizes H-2D^d.

Con A blast lysis results using congenic mice suggest that Ly-49W has a low to moderate level of recognition of an H-2^d molecule. The Ly-49W transfectant 7E8 lysed B10.D2 Con A blasts at a moderate level and was therefore used in additional experiments to determine which H-2^d product can be recognized by Ly-49W. We took two complementary approaches to address this issue. First, 7E8 lysis of Con A blasts generated from intra-MHC recombinant mouse strains B10.b/d (H-2K^b, H-2D^d) and B10.d/b (H-2K^b, H-2D^d) was compared with the B10 (H-2^b)-negative control. Con A-activated T cell blasts from the intra-MHC recombinant that expresses H-2^d were lysed, whereas those from the recombinant expressing H-2K^d and the B10 strain were not (Fig. 7A). Second, we attempted to block lysis of B10.D2 Con A blasts by the 7E8 Ly-49W-transfected clone with two Abs that recognize H-2D^d: 34-5-8S, which recognizes the D^d a1/a2 domains, and 34-2-12S, which recognizes the D^d a3 domain. Both D^d-specific Abs blocked Ly-49W-mediated RNK lysis of the B10.D2 blasts (Fig. 7B), whereas the isotype control Ab or protein A had no effect. The 34-5-8S Ab blocking was more effective than 34-2-12S through the titration of these Abs, consistent with previous studies of Ly-49A interactions with H-2D^d (22). This suggests that Ly-49W interacts with H-2D^d in the a1/a2 domains, as does Ly-49A. In addition, because the 34-2-12S Ab also blocks Ly-49W interaction with D^d, albeit less efficiently, the a3 domain may also contribute to the interaction, or this Ab may sterically hinder Ly-49W interaction with the D^d a1/a2 domains. In any case, these results indicate that Ly-49W recognizes H-2D^d.

Lysis of H-2^d-expressing targets by Ly-49W-transfected RNK cells is inhibited by Abs that recognize Ly-49W

The foregoing experiments demonstrated that Ly-49W-transfected RNK cells readily recognize H-2D^d-expressing targets, while untransfected RNK cells do not recognize them at all. To confirm that
the observed lytic activity is mediated by Ly-49W, we examined whether Abs recognizing Ly-49W could block the cytolytic activity. Both of the Ly-49W-recognizing Abs 4D11 and Cwy-3 reduced the killing of B10.BR Con A blasts by clone 10G5 (Fig. 8). The Cwy-3 Ab inhibited the majority of the lysis observed with the Ly-49W transfectant clone 10G5, and it was more effective than 4D11 (Fig. 8). Combining 4D11 and Cwy-3 Abs did not increase inhibition of lysis. Similar results were obtained with other Ly-49W transfectants (data not shown). These results support the role of Ly-49W in mediating RNK lysis of H-2Dk target cells.

Ly-49W NOD transfectants of RNK-16 cells do not lyse NOD or NOR Con A blasts

The NOD and NOR mouse strains express H-2Kd and H-2Dd MHC molecules. Our previous results indicated that neither of these MHC proteins is recognized by Ly-49W (Fig. 7A). To confirm that Ly-49W from NOD and NOR mice does not recognize self MHC, we compared lysis of Con A blasts generated from NOD, NOR, AKR, and B6 mice by Ly-49W-expressing RNK-16 effector cells (Fig. 9). In comparison, we found that NOD or NOR Con A blasts were not lysed by clones 2C4 and 10G5, and lysed to only a very limited extent, if at all, by the more highly cytolytic clone 7E8 (Fig. 9). These results demonstrate that Ly-49W from NOD and NOR mice does not recognize self Ags. We conclude from our studies that Ly-49W of NOD/NOR mice is an activating receptor specific for allogeneic class I MHC molecules.

Discussion

The B6 strain has served as a prototype for the study of Ly-49 gene expression and function. However, apparent strain-specific transcripts encoding novel activating receptors have recently been described: Ly-49P from the 129/J and NOD mouse strains (25, 40), and Ly-49L from CBA/J, C3H, and BALB/c mice (42) (Fig. 1B). Viable transcripts for none of these receptors have been found in the B6 mouse. We demonstrate in this study that NOD and NOR mouse strains express H-2Kd and H-2Dd MHC molecules. Our previous results indicated that neither of these MHC proteins is recognized by Ly-49W (Fig. 7A). To confirm that Ly-49W from NOD and NOR mice does not recognize self MHC, we compared lysis of Con A blasts generated from NOD, NOR, AKR, and B6 mice by Ly-49W transfectants. As in previous experiments, AKR Con A blasts served as very good targets, while B6 blasts were not lysed by the Ly-49W-expressing RNK-16 effector cells (Fig. 9). In comparison, we found that NOD or NOR Con A blasts were not lysed by clones 2C4 and 10G5, and lysed to only a very limited extent, if at all, by the more highly cytolytic clone 7E8 (Fig. 9). These results demonstrate that Ly-49W from NOD and NOR mice does not recognize self Ags. We conclude from our studies that Ly-49W of NOD/NOR mice is an activating receptor specific for allogeneic class I MHC molecules.
is due to a gene defect rather than to different levels of gene transcription.

Ly-49L, M, and W are closely related genes as indicated by sequence comparisons, suggesting that they result from relatively recent gene duplication and perhaps exon exchange. The ability of Ly-49 genes to undergo duplication and genetic recombination is similar to the functionally equivalent KIR receptors in primates, perhaps in response to similar evolutionary pressures (47–50). Evidence of human KIR gene recombination involving genes widely separated in the genome (47, 48) supports a contribution of gene conversion or nonhomologous recombination in the generation of receptor diversity. There are specific areas within the KIR gene complex in which these variations occur, suggesting that hot spots of gene duplication in the KIR gene complex may exist (48). The Ly-49 gene complex may have similar variability in different mouse strains, as the results in this study and others suggest (25, 40, 42). The relatively recent formation of Ly-49L, M, and W by gene duplication and exon exchange events suggest that these genes may be located near a similar hot spot in the NK gene complex region (51) encoding Ly-49 genes. Thus, gene duplication and nonhomologous recombination or gene conversion may contribute to the generation of polymorphism in both Ly-49 and KIR gene families.

In addition to substantial homology between Ly-49W, Ly-49M, and Ly-49L, there is a striking similarity of the Ly-49W CRD with that of the Ly-49G inhibitory receptor. For exons 5 to 7, encoding

![Figure 7](image_url)

**Figure 7.** Lysis of H-2d Con A target cells by Ly-49W-transfected RNK cells is dependent on H-2Dd recognition. A, Lysis by Ly-49W-transfected RNK-16 clone 7E8 of Con A blasts generated from intra-MHC recombinants B10.d/b (H-2Kb and Dd), B10.b/d (H-2Kb and Dd), and the B10 (H-2Kb and Dd) control strain. B, Lysis of B10.D2 Con A blasts by 7E8 in the presence of medium or PA in control wells (□), or PA plus 34-5-8S (anti-Dd α1/α2 domain, □), 34-2-12S (anti-Dd α3 domain, □), or isotype control Ab B27 M1 (anti-HLA B27, ■) at the indicated concentrations. Abs were preincubated with PA (2 μg/10 μg of Ab) to prevent ADCC. Abs and PA remained in the wells throughout the assay. The E:T ratio in B was 12.5:1. Cytotoxicity was measured after 4 h. Data are the means of triplicate wells ± SD.

![Figure 8](image_url)

**Figure 8.** Lysis of H-2Dk-expressing targets by Ly-49W-transfected RNK-16 is inhibited by Abs that recognize Ly-49W. Cytotoxicity of B10.BR (H-2k) Con A T cell blasts by the Ly-49W-transfected RNK-16 clone 10G5 was measured in the presence of medium or PA/PG alone (□), or PA/PG with OX-8 (■), 4D11 (□), Cwy-3 (□), or 4D11 plus Cwy-3 (□), at the indicated concentrations. Abs were preincubated with PA/PG (4 μg/10 μg Ab) for 30 min before addition to effector cells. Effector cells were incubated with the mAbs and PA/PG for 15 min before the cytotoxicity assay. The PA/PG and Abs remained in the wells throughout the assay. The E:T ratio was 12.5:1. Cytotoxicity was measured after 4 h. Data are the means of triplicate wells ± SD.

![Figure 9](image_url)

**Figure 9.** Ly-49W<sup>NOD</sup> RNK-16 transfectants do not lyse NOD or NOR Con A T cell blasts. RNK-16 and individual Ly-49W<sup>NOD</sup> transfectant clones, 2C4, 7E8, and 10G5, were assayed for cytotoxicity against Con A T cell blasts from AKR/J, C57BL/6, NOD, and NOR mouse strains, at the indicated E:T cell ratios. Cytotoxicity was measured after 4 h. Data are the means of triplicate wells ± SD.
the CRD, the amino acid sequence identity between Ly-49W and G is 97.6%. The sequence conservation drops to 83.8% for exon 4, which encodes the less well-conserved stalk region. For exons 2 and 3, the amino acid sequence identity is only 58.2% and, unlike Ly-49W and M, Ly-49G contains the ITIM motif in its cytoplasmic domain. Based on these results, Ly-49G and Ly-49W and M should be considered as inhibitory/activating pairs of Ly-49 receptors, similar to other examples such as: Ly-49A and Ly-49P (25, 40); Ly-49O and Ly-49D (40); Ly-49C/Ly-49/ly-49 and Ly-49H (41); NKG2-A and NKG2-C/NKG2-E (52, 53); NKR-P1B and NKR-P1A/NKR-P1C (54). The generation of inhibiting/activating receptor gene pairs may involve nonhomologous gene recombination events or gene conversion.

Ly-49W joins Ly-49D and Ly-49P as activating Ly-49 receptors that recognize class I MHC ligands (24, 25). Our findings with Ly-49W strengthen the conclusion that activating Ly-49 receptors, just like inhibitory Ly-49 receptors, recognize class I MHC ligands. We have demonstrated that Ly-49W is the first activating Ly-49 receptor to recognize an H-2Dk product. Ligands for Ly-49W were determined to be H-2Dd and, with weaker interaction, H-2Dk. This novel recognition pattern contrasts with Ly-49D and Ly-49P, which only react significantly with H-2Dd (24, 25). From NK lytic assays using Con A blast target cells, it was determined that the finding that Ly-49G does not bind soluble H-2Dk tetramers and our finding that Ly-49W binds to H-2Dd with only weak binding to H-2Dk (22, 56). Conversely, we have demonstrated in this study that Ly-49W reacts strongly to Dd and only weakly to Dk. Among the Dd residues that interact directly with Ly-49A in the crystal structure, only two are not conserved in Dd (Lys173, Asn174 in Dd and Glu172-Leu174 in Dk). Similarly, among the Ly-49A residues that interact directly with Dd in the crystal structure, five positions are not conserved in Ly-49W, all in the hexapeptide 244–249 (NCDQVF in Ly-49A and DCGKSY in Ly-49W). Molecular modeling suggests that compensating changes within these sequences may confer the differential class I MHC specificities/affinities of Ly-49A and Ly-49W (not shown).

Self-tolerance of NK cells is believed to be maintained by expression and function of inhibitory receptors specific for self class I MHC proteins (10). Activating receptors may react with self MHC as well, and tolerance could be maintained by coexpression of inhibitory receptors that dominantly suppress activating receptor signals. For instance, it has been reported that tolerance of Ly-49D+ NK cells of self cells expressing H-2Dd is most likely maintained by the coexpression of Ly-49G and other Ly-49-inhibitory receptors (55). We demonstrate in this study that Ly-49W does not interact with self class I MHC proteins of the NOD/NOR mouse strains, but is instead alloreactive. For this reason, self-tolerance in NOD/NOR strains is not likely to be affected by Ly-49W. It remains to be determined whether, and if so how, Ly-49W+ NK cells are tolerant in F1 animals such as CBA × NOD, in which an identified Ly-49W ligand is expressed.

We found that the Cwy-3 and 4D11 Abs were both reactive with Ly-49W, an activating receptor, and there were differences between the results obtained using Cwy-3 and 4D11. While rADCC using RNK-16 transfectants of Ly-49W could be demonstrated with the Cwy-3 Ab, the 4D11 Ab was unable to mediate rADCC (data not shown). Additionally, the Cwy-3 Ab was somewhat better at blocking Ly-49W-mediated lysis of H-2d Con A blasts than 4D11 (Fig. 8). Differences in Ab affinity and/or site of attachment are most likely the cause of these different results. Our results suggest that it cannot be assumed that the inhibitory Ly-49G receptor is what is recognized by 4D11 in every mouse strain, because in addition, or instead, it may recognize an activating Ly-49 such as M, L, W, or related receptors in certain strains.

The existence of activating receptors that recognize class I MHC molecules is not readily predicted by the missing self hypothesis, which prompts the question: what is the role of MHC-specific activators in NK cell function? Activating Ly-49 members may function in conjunction with inhibitory receptors by recruiting kinases to phosphorylate ITIM sequences following ligand binding, thus augmenting the recruitment of SHP-1 and thereby the function of coexpressed inhibitory receptors. This possibility remains to be explored. The expression of inhibitory Ly-49 receptors occurs through a stochastic process that obeys a simple statistical product rule for expression of multiple Ly-49 receptors (10). In contrast, a recent report provides evidence to suggest that there is nonstochastic expression of two Ly-49 activators, D and H, in that there is a greater tendency for them to be coexpressed on NK cells (46). These observations suggest that Ly-49 activators may coordinate their activities and possibly function independent of inhibitory Ly-49 receptors in some NK cells (46). However, this analysis involved only two Ly-49-activating receptors, and it is now clear that several more exist. Further studies that include additional activating Ly-49 receptors will be necessary before a paradigm can be established for the function of this form of Ly-49 receptor. Identifying the extent and diversity of the Ly-49 family, both activating and inhibitory members, as well as their expression patterns and ligand specificities, should provide an opportunity to fully understand Ly-49 receptor functions. Identification and characterization of Ly-49, a class I MHC-specific activating receptor, contribute to this goal.

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