Ly-49W, an Activating Receptor of Nonobese Diabetic Mice With Close Homology to the Inhibitory Receptor Ly-49G, Recognizes H-2D$^{k}$ and H-2D$^{d}$

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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$^{k}$ or H-2$^{d}$ haplotypes, and Ab-blocking experiments indicated that H-2D$^{k}$ and D$^{d}$ are ligands for Ly-49W. Ly-49W is the first activating Ly-49 receptor demonstrated to recognize an H-2$^{k}$ 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.

N atural 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 allogeneic bone marrow rejection as well as hybrid resistance, wherein an F$_{1}$ 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 (ITAM) (11, 15–17). DAP12 interacts with the ITIM-lacking receptors via a covalent inter- action in the transmembrane domain (11, 17). Ligation of the receptor 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$^{b}$ 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...
I MHC molecules with strong and moderate reactive toward H-2D\(^b\) and H-2D\(^e\), respectively. This is the first report of an activating Ly-49 molecule recognizing an H-2\(^k\) 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\(^e\)), C57BL/6 (H-2\(^e\)), NOD (H-2K\(^d\),D\(^e\)), nonobese diabetes-resistant (NOR) (H-2K\(^D\),D\(^e\)), B10.R (H-2\(^e\)), B10.D2 (B10.D2-H\(^2\)Sn; H-2\(^b\)), B10.S (H-2\(^e\)), B10.C57BL/10 (H-2); B10.d/b (B10.HTG-H2g/2Cy; H-2K\(^D\),D\(^e\)), and B10.b/d (B10.A-H\(^2\)H\(^2\)-T18-1 (5R)/SgSnJ; H-2K\(^b\),D\(^d\)).

**COS-7 cells** were grown in Opti-MEM I medium (Life Technologies). Monkey kidney cells were provided by Dr. John Elliott (University of California, San Francisco). The RNK-16 cells were maintained in RPMI 1640 supplemented with 10% FCS, l-glutamine, penicillin, streptomycin, and 5 \(\times\) 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 Technologies, Burlington, Ontario, Canada), containing 4% heat-inactivated FCS (Medicorp, Montreal, Canada) and 5 \(\times\) 10\(^{-5}\) M 2-ME.

**Hybrids and Abs**

Hybrids producing the following Abs: 4D11 (rat IgG2a), anti-Ly-49G (27); W3-24 (IgG2a, anti-Ly-49G (28); M1/42 (rat IgG2a), anti-mouse class I MHC (29); 34-5-8S (IgG2a), anti-H-2\(^D\)\(^e\)/al2o2 domain epitope (30); 34-2-12S (IgG2a) anti-H-2\(^D\)\(^e\)\(\alpha\) domain epitope (30); B8-24-3 (IgG1), anti-H-2\(^K\)\(^b\) (31); B27 M1 (IgG2a), anti-HLA-B27, B7 (32); and BB7.1 (IgG1), anti-HLA-B7 (33) were obtained from American Type Culture Collection (Manassas, VA), except Cw3, which was generated in this laboratory. Abs were prepared by ammonium sulfate precipitation, and PBS dialysis of tissue culture supernatants obtained from hybridsoma producing in protein-free hybridoma medium. Purified ox-1 (IgG1) anti-rat CD8a (34), 11-4.1 (IgG2a) anti-Kk (35), and 15-5-3S (IgG2a) anti-Dk (36) Abs were purchased from BD Pharmingen (San Diego, CA). Purified rat IgG was purchased from Sigma-Aldrich (Oakville, Canada). FITC-coupled rat anti-mouse IgG, goat anti-rat IgG, and mouse anti-rat fluorescence-coupled Abs, 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 BsrZEn (generously provided by Dr. Andrew 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 \(\mu\)g of plasmid linearized with SacI by electroporation at 200 mV and 960 \(\mu\)F. Transfected cells were cloned in 96-well microtiter plates with complete FCS medium supplemented with 1 mg/ml G418 for drug selection. Expression of Ly-49W in transfected clones was measured by FACS analysis with the 4D11 Ab at various E:T ratios in triplicate. After the incubation, plates were centrifuged for 5 min, and 100 \(\mu\)l of supernatant was removed and counted in a gamma counter. The percent specific lysis was determined as (experimental release -- spontaneous release)/maximum release -- spontaneous release) \times 100. To perform the reverse Ab-dependent cellular cytotoxicity (rADCC) experiments, untransfected RNK-16 cells and the Ly-49W transfected clones of RNK-16 were preincubated for 4--5 h at 37 \(^\circ\)C in V-bottom microtiter plates with RNK-16 cells or RNK-16 cells transfected with Ly-49W at various E:T ratios in triplicate. After the incubation, plates were centrifuged for 5 min, and 100 \(\mu\)l of supernatant was removed and counted in a gamma counter. The percent specific lysis was determined as (experimental release -- spontaneous release)/maximum release -- spontaneous release) \times 100. To perform the reverse Ab-dependent cellular cytotoxicity (rADCC) experiments, untransfected RNK-16 cells and the Ly-49W transfected clones of RNK-16 were preincubated for 15 min with 20 \(\mu\)g/ml of the Cw3-3 and OX-8 Abs was also determined after blocking with purified rat IgG. BB7.1 Abs was used as an isotype control. Primary Ab binding was detected with mouse anti-rat and 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 \(\times\) 10\(^{5}\) cells/ml in RPMI 1640 with 10% heat-inactivated FCS, 2-ME, and 3 \(\mu\)g/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 \(^\circ\)C with 100--150 \(\mu\)Ci of Na\(^{25}\)CrO\(_4\) (Amersham Pharmacia Biotech, Piscataway, NJ) for 30 min. Target cells incubated for 9--10 h at 37 \(^\circ\)C in V-bottom microtiter plates with RNK-16 cells or RNK-16 cells transfected with Ly-49W at various E:T ratios in triplicate. After the incubation, plates were centrifuged for 5 min, and 100 \(\mu\)l of supernatant was removed and counted in a gamma counter. The percent specific lysis was determined as (experimental release -- spontaneous release)/maximum release -- spontaneous release) \times 100. To perform the reverse Ab-dependent cellular cytotoxicity (rADCC) experiments, untransfected RNK-16 cells and the Ly-49W transfected clones of RNK-16 were preincubated for 15 min with 20 \(\mu\)g/ml of the Cw3-3 and OX-8 Abs or medium alone before addition of FcR-expressing YB2/0 target cells and subsequent 4-h cytotoxic assay, as described. For Ab inhibition experiments, Abs were incubated with soluble protein A (PA) (2 \(\mu\)g/10 \(\mu\)l of mAb; Sigma-Aldrich) or a mix of PA and protein G (PG) (4 \(\mu\)g/10 \(\mu\)l of mAb; ICN Pharmaceuticals, Costa Mesa, CA) for 30 min min before addition to effecting cells or target cells. Effecting 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 is used at the indicated concentrations in the figure. The mAbs and PA or PA/Pg are 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-49

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 allelic difference 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-49 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). NOD 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-49G1sp3, 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-49m. 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 NOR 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
to select for clones stably expressing Ly-49W. A number of trans-
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as effector cells. In this way, we could be certain there was no Ab
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Ly-49W mediates rADCC

The noninhibitory Ly-49D, Ly-49H, and Ly-49P receptors have
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49G⁻. Transfected RNK-16 cells were screened by FACS analysis
select for clones stably expressing Ly-49W. A number of trans-
fectant 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, a constitutively ex-
pressed endogenous rat receptor, was retained following transfec-
tion with Ly-49W (Fig. 3).
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 cytolysis 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 cytolysis 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-2Dβ class I MHC molecule is a ligand for some inhibitory Ly-49 receptors, including Ly-49G (23) and the activating receptor Ly-49D (24). To test whether class I MHC molecules, especially Ly-49 receptors, including Ly-49G (23) and the activating receptor Ly-49W, we examined Ly-49D (24). To test whether class I MHC molecules, especially the H-2d haplotype, are recognized by Ly-49W, we examined Ly-49W-mediated killing of Con A-activated T cell blast target cells prepared from splenocytes of mouse strains expressing different MHC haplotypes. None of the Ly-49W transfectant clones lysed B6 Con A targets (H-2b), whereas all of the transfectants lysed Con A blasts generated from the intra-MHC recombinant mouse strains B10.b/d (H-2Kb, H-2Dd) and B10.d/b (H-2Kd, H-2Dd) was compared with the B10 (H-2b)-negative control. Con A-activated T cell blasts from the intra-MHC recombinant that expresses H-2Dβ were lysed, whereas those from the recombinant expressing H-2Kα 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 transfectant clone with two Abs that recognize H-2Dβ: 34-5-8S, which recognizes the Dd a1/a2 domains, and 34-2-12S, which recognizes the Dd α2 domain. Both Dd-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-2Dβ (22). This suggests that Ly-49W interacts with H-2Dβ in the a1/a2 domains, as does Ly-49A. In addition, because the 34-2-12S Ab also blocks Ly-49W interaction with Dd, albeit less efficiently, the α3 domain may also contribute to the interaction, or this Ab may sterically hinder Ly-49W interaction with the Dd a1/a2 domains. In any case, these results indicate that Ly-49W recognizes H-2Dβ.

Ly-49W recognizes H-2Kβ and H-2Dβ

The previous results suggest that there is an H-2β and perhaps an H-2d product or products recognized by Ly-49W. To determine which H-2k class I product(s) is recognized by Ly-49W, we attempted to block Ly-49W-mediated lysis of B10.BR (H-2k) Con A blasts with Abs that recognize either H-2Kk or H-2Dk. Soluble PA was used to bind the Ab Fc domain to prevent ADCC by the FcR-expressing RNK-16 cells. Neither the 11-4.1 Ab, which recognizes H-2Kk (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-2Dk (36), blocks lysis of B10.BR Con A blasts by both Ly-49W transfectant clones 7E8 and 10G5 in a dose-dependent manner (Fig. 6). These results indicate that Ly-49W recognizes H-2Dk.

Con A blast lysis results using congenic mouse suggest that Ly-49W has a low to moderate level of recognition of an H-2d molecule. The Ly-49W transfectant 7E8 lyses B10.D2 Con A blasts at a moderate level and was therefore used in additional experiments to determine which H-2d 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-2Kb, H-2Dd) and B10.d/b (H-2Kd, H-2Dd) was compared with the B10 (H-2b)-negative control. Con A-activated T cell blasts from the intra-MHC recombinant that expresses H-2Dd were lysed, whereas those from the recombinant expressing H-2Kα 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 transfectant clone with two Abs that recognize H-2Dβ: 34-5-8S, which recognizes the Dd a1/a2 domains, and 34-2-12S, which recognizes the Dd α2 domain. Both Dd-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-2Dβ (22). This suggests that Ly-49W interacts with H-2Dβ in the a1/a2 domains, as does Ly-49A. In addition, because the 34-2-12S Ab also blocks Ly-49W interaction with Dd, albeit less efficiently, the α3 domain may also contribute to the interaction, or this Ab may sterically hinder Ly-49W interaction with the Dd a1/a2 domains. In any case, these results indicate that Ly-49W recognizes H-2Dβ.

Lysis of H-2k-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-2Dβ-expressing targets, while untransfected RNK cells do not recognize them at all. To confirm that

**FIGURE 4.** Ly-49W expressed on RNK-16 cells mediates rADCC with the Cwy-3 Ab. RNK-16 cells (A) and the Ly-49W transfectant clones 2C4 (B), 10G5 (C), and 7E8 (D) were incubated with the indicated Abs at 20 μg/ml or with medium for 15 min before addition of FcR-bearing YB2/0 target cells and a 4-h cytotoxicity assay. The data represent the mean of triplicate wells ± SD.
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-2K\(d\) and H-2D\(b\) 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 mice express two new mRNA transcripts encoding activating Ly-49 receptors. For one of these, Ly-49M, this difference in 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 RNK 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.

![FIGURE 6. Ly-49W recognizes H-2D\(b\). Lysis of B10.BR (H-2\(b\)) Con A blasts by the 7E8 and 10G5 Ly-49W transfectants was determined in the presence of PA and various concentrations of 11-4.1 (anti-K\(k\)), 15-5.5S (anti-D\(b\)), or B27 M1 (isotype control) Abs. Abs were preincubated with PA (2 \(\mu\)g/10 \(\mu\)g of mAb) to prevent ADCC. Effector cells were added to provide a 12.5:1 E:T cell ratio. Cytotoxicity was measured after 4 h. Data are the means of triplicate wells ± SD.](image-url)
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...
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-49G and Ly-49D (40); Ly-49C/Ly-49I/Ly-49J 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-49D 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-2d product. Ligands for Ly-49W were determined to be H-2d and, with weaker interaction, H-2d. 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 inhibitory Ly-49G receptor of B6 mice recognizes Dd, Dk, and an undefined H-2D product (55). This work is contradicted by the finding that Ly-49G does not bind soluble H-2Dk tetramers and does not mediate binding to H-2Dk-bearing cells in cell-cell adhesion assays (56). However, the high sequence identity between the CRD of Ly-49G and Ly-49W and our finding that Ly-49W binds H-2Dk suggest that Ly-49G should be reexamined for H-2Dk reactivity.

With the availability of the Ly-49A/H-2Dd complex co-crystal structure (57), it is now possible to correlate sequence variation among Ly-49 and class I MHC molecules with their known interaction specificities. Ly-49A has a known binding specificity for H-2Dd with only weak binding to H-2Dk (22, 56). Conversely, we have demonstrated in this study that Ly-49W reacts strongly to Dk and only weakly to Dd. 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 Glu173-Leu174 in Dd). 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 (NCDDQVF 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-2Dd 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-49W, a class I MHC-specific activating receptor, contribute to this goal.

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References

1. Ortaldo, J. R., and D. L. Longo. 1988. Human natural lymphocyte effector cells: definition, analysis of activity, and clinical effectiveness. J. Natl. Cancer Inst. 80:999.
2. Trinchieri, G. 1989. Biology of natural killer cells. Adv. Immunol. 47:187.
3. Yu, Y. Y., V. Kumar, and M. Bennett. 1992. Murine natural killer cells and narrow graft rejection. Annu. Rev. Immunol. 10:189.
4. George, T., Y. Y. Yu, J. Liu, C. Davenport, S. Lemieux, E. Stoneman, P. A. Mathew, V. Kumar, and M. Bennett. 1997. Allorrecognition by murine natural killer cells: lysins of T-lymphoblasts and rejection of bone-marrow grafts. Immunol. Rev. 155:29.
5. Ryan, J. C., E. C. Niemi, M. C. Nakamura, and W. E. Seanman. 1995. NKR-P1A is a target-specific receptor that activates natural killer cell cytotoxicity. J. Exp. Med. 181:1911.
6. Moretta, A., C. Bottino, R. Millo, and R. Biaosi. 1999. HLA-specific and non-HLA-specific human NK receptors. Curr. Top. Microbiol. Immunol. 244:69.
7. Bottino, C., R. Biaosi, R. Millo, L. Moretta, and A. Moretta. 2000. The human natural cytotoxicity receptors (NCR) that induce HLA class I-independent NK cell triggering. Hum. Immunol. 61:1.
8. Long, E. O. 1999. Regulation of immune responses through inhibitory receptors. Annu. Rev. Immunol. 17:875.
9. Ljunggren, H. G., and K. Karre. 1990. In search of the ‘missing self’: MHC natural cytotoxicity receptors (NCR) that induce HLA class I-independent NK cell recognition. Immunol. Today 11:237.
10. Raulet, D. H. 1999. Development and tolerance of natural killer cells. Curr. Opin. Immunol. 11:129.
11. Lanier, L. L. 1998. NK cell receptors. Annu. Rev. Immunol. 16:359.
12. Vely, F., and E. Vivier. 1997. Conservation of structural features reveals the existence of a large family of inhibitory cell surface receptors and noninhibitory, activatory counterparts. J. Immunol. 159:2075.
13. Burshtyn, D. N., A. M. Scharenberg, N. Wagtman, S. Rajapojakul, K. Berruda, T. Yi, J. P. Kinet, and E. O. Long. 1996. Recruitment of tyrosine phosphatase SHP-2 to the activating NK receptor Ly49A. J. Immunol. 157:3277.
14. Nakamura, M. C., E. C. Niemi, M. J. Fisher, L. D. Shultz, W. E. Seaman, and J. C. Ryan. 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.
15. Olcese, L., A. Cambiaggi, G. Semenzato, C. Bottino, A. Moretta, and E. Vivier. 1997. Human killer cell activator receptors for MHC class I molecules are included in a multimeric complex expressed by natural killer cells. J. Immunol. 158:5083.
16. Tomaselio, E., L. Olcese, F. Vely, C. Geourgeon, M. Bley, A. Moqrich, D. Gautheret, M. Djalali, M. G. Mattei, and E. Vivier. 1998. Gene structure, expression pattern, and biological activity of mouse killer cell activating receptor-associated protein (KARAP)/DAP-12. J. Biol. Chem. 273:54115.
17. Smith, K. M., J. Wu, A. B. Bakker, J. H. Phillips, and L. L. Lanier. 1998. Ly-49D and Ly-49H associate with mouse DAP12 and form activating receptors. J. Immunol. 161:7.
18. Brumbaugh, K. M., B. A. Binstadt, D. D. Billadeau, R. A. Schoon, C. J. Dick, R. M. Ten, and P. J. Leibson. 1997. Functional role for Syk tyrosine kinase in natural killer cell-mediated natural cytotoxicity. J. Exp. Med. 186:1965.
19. McVicar, D. W., L. S. Taylor, P. Gosselin, J. J. Willette-Brown, A. I. Mikhael, K. P. Kane. 1999. Tissue distribution of mouse killer cell activating receptor Ly49D. J. Immunol. 162:293.
20. Mason, L. H., J. Willette-Brown, A. T. Mason, D. McVicar, and J. R. Ortaldo. 2000. Interaction of Ly-49D with H-2Dd-targeted cells leads to Dap-12 phosphorylation and B7-2 secretion. J. Immunol. 164:603.
21. Karfohler, F. M., R. K. Ribaudo, and W. M. Yokoyama. 1992. HICH class I alloantigen specificit of Ly-49 IL-2-activated natural killer cells. Nature 366: 60.
22. Trowsdale, J. K. P. 1994. Ly-49 mediates EL4 lymphoma adhesion to isolated class I major histocompatibility complex molecules. J. Exp. Med. 179:1011.
23. Mason, L. H., J. R. Ortaldo, H. A. Young, V. Kumar, M. Bennett, and S. K. Anderson. 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.
24. Nakamura, M. C., P. A. Linnemeyer, E. C. Niemi, L. H. Mason, J. R. Ortaldo, J. C. Ryan, and W. E. Seaman. 1999. Mouse Ly-49D recognizes H-2Dd and activates natural killer cell cytotoxicity. J. Exp. Med. 189:493.
25. Silver, E. T., J. F. Elliott, and K. P. Kane. 1996. Alternatively spliced Ly-49D and H-2Dd transcripts are found in IL-2-activated NK cells. Immunity 4:478.
26. Mason, L. H., S. K. Anderson, W. M. Yokoyama, H. R. Smith, R. Winkler-Pickett, and J. R. Ortaldo. 1996. The Ly-49D receptor activates murine natural killer cells. J. Exp. Med. 184:2119.
27. Smith, H. R., H. H. Chuang, L. L. Wang, M. Salcedo, J. W. Heusel, and W. M. Yokoyama. 2000. Nonstochastic coexpression of activation receptors on murine natural killer cells. J. Exp. Med. 191:1341.
28. Shilling, H. G., K. Lienert-Weidenbach, N. M. Valiante, M. Ulrich, and P. Parham. 1998. Evidence for recombination as a mechanism for KIR diversification. Immunogenetics 48:413.
29. Wilson, M. J., M. Torkar, A. Hause, S. Milne, T. Jones, D. Sheer, S. Beck, and J. Trowsdale. 2000. Plasticity in the organization and sequences of human KIR/ILT gene families. Proc. Natl. Acad. Sci. USA 97:4778.
30. Martin, A. M., E. M. Freitas, C. S. Witt, and F. T. Christiansen. 2000. The KIR gene cluster. Immunogenetics 51:268.
31. Khakoo, S. I., R. Rajalingam, B. P. Shum, K. Weidenbach, L. Flodin, D. G. Muir, F. Canavaza, S. L. Cooper, N. M. Valiante, L. L. Lanier, and P. Parham. 2000. Rapid evolution of NK cell receptor systems demonstrated by comparison of chimpanzees and humans. Immunity 12:667.
32. Yokoyama, W. M., and J. R. Ortaldo. 1995. A family of murine NK cell receptors specific for target cell MHC class I molecules. Semin. Immunol. 7:89.
33. Lohrwaesser, S., P. Harde, D. L. Mager, and F. Takei. 1999. Cloning of murine KIR2DLB, B and C: second family of C-type lectin receptors on murine NK cells. Eur. J. Immunol. 29:755.
34. Silver, E. T., J. C. Lau, and K. P. Kane. 1999. Molecular cloning of mouse KIR2DLB and C. Immunogenetics 49:727.
35. Yokoyama, W. M., and W. E. Seaman. 1993. The Ly-49 and NKR-P1 gene families encoding lectin-like receptors on natural killer cells: the NK gene complex. Annu. Rev. Immunol. 11:513.
36. George, T. C., J. R. Ortaldo, S. Lemieux, V. Kumar, and M. Bennett. 1999. Tolerance and allosecretory activity of the Ly49D subset of murine NK cells. J. Immunol. 163:1859.
37. Hanke, T., H. Takizawa, C. W. McMahon, D. H. Busch, G. E. Palmer, J. D. Miller, J. D. Altman, Y. Liu, D. Cado, F. A. Lemmerson, et al. 1999. Direct assessment of MHC class I binding by seven Ly-49 inhibitory NK cell receptors. Immunity 11:67.
38. Tormo, J., K. Natarajan, D. H. Margulies, and R. A. Marzuzza. 1999. Crystall structure of a lectin-like natural killer cell receptor bound to its MHC class I ligand. Nature 402:623.