Mouse Ly-49D Recognizes H-2D<sup>d</sup> and Activates Natural Killer Cell Cytotoxicity

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Summary

Although activation of natural killer (NK) cytotoxicity is generally inhibited by target major histocompatibility complex (MHC) class I expression, subtle features of NK allorecognition suggest that NK cells possess receptors that are activated by target MHC I. The mouse Ly-49D receptor has been shown to activate NK cytotoxicity, although recognition of MHC class I has not been demonstrated previously. To define Ly-49D–ligand interactions, we transfected the mouse Ly-49D receptor into the rat NK line, RNK-16 (RNK.mLy-49D). As expected, anti–Ly-49D monoclonal antibody 12A8 specifically stimulated redirected lysis of the Fc receptor–bearing rat target YB2/0 by RNK.mLy-49D transfectants. RNK.mLy-49D effectors were tested against YB2/0 targets transfected with the mouse MHC I alleles H-2D<sup>d</sup>, D<sup>b</sup>, K<sup>k</sup>, or K<sup>b</sup>. RNK.mLy-49D cells lysed YB2/0.D<sup>d</sup> targets more efficiently than untransfected YB2/0 or YB2/0 transfected with D<sup>b</sup>, K<sup>k</sup>, or K<sup>b</sup>. This augmented lysis of H-2D<sup>d</sup> targets was specifically inhibited by F(ab′)<sub>2</sub> anti–Ly-49D (12A8) and F(ab′)<sub>2</sub> anti–H-2D<sup>d</sup> (34-5-8S). RNK.mLy-49D effectors were also able to specifically lyse Concanavalin A blasts isolated from H-2<sup>d</sup> mice (BALB/c, B10.D2, and DBA/2) but not from H-2<sup>b</sup> or H-2<sup>k</sup> mice. These experiments show that the activating receptor Ly-49D specifically interacts with the MHC I antigen, H-2D<sup>d</sup>, demonstrating the existence of alloactivating receptors on murine NK cells.

Key words: natural killer cells • major histocompatibility complex • receptors • cytotoxicity • rodent

Natural killer (NK) lymphocytes have the ability to recognize and to lyse various targets, including neoplastic and hematopoietic cells, although the specific activating receptors involved remain poorly characterized (1-4). In contrast to allogeneic killing by CD8<sup>+</sup> T lymphocytes, NK cytotoxicity is generally inhibited, rather than activated, by the expression of class I MHC antigens on targets (5). Inhibition of natural killing is mediated by polymorphic inhibitory NK cell receptors, which are specific for distinct MHC class I alleles (6-8). Each of the known MHC-binding inhibitory NK cell receptors is a member of a family of molecules (killer cell inhibitory receptor [KIR], Ly-49, or CD94/NKG2) with related sequences (8-12). Within each family, receptors that mediate inhibitory function all contain a cytoplasmic immunoreceptor tyrosine based inhibitory motif (ITIM). However, each family also includes related receptors that lack ITIMs (4, 8, 13), and recent studies have shown that some of these receptors can activate NK cells (14, 15).

Although the target ligands for these stimulatory receptors have not yet been defined, their extracellular domains share homology with the corresponding domains of MHC-binding inhibitory receptors, suggesting that activating receptors may also bind MHC class I antigens (4, 8, 16-18). Consistent with this hypothesis, functional studies in mice and rats suggest that NK cells, like T cells, may express receptors that activate cytotoxicity in response to MHC class I ligands (19-22).

The first two authors contributed equally to this work.

Abbreviation used in this paper: ITIM, immunoreceptor tyrosine based inhibitory motif.
We have studied the mouse Ly-49D receptor, which was shown by Mason et al. to activate NK cells (14). Initial attempts to define the ligand specificity of Ly-49D in vitro were unsuccessful, although in vivo studies have implicated Ly-49D + NK cells in the clearance of allogeneic targets (14, 23). It is likely that attempts to define MHC ligands for Ly-49D using Ly-49D-enriched primary mouse NK cells have been complicated by the coexpression of inhibitory MHC-binding NK receptors (14). To define the specificity of mouse Ly-49D, we transfected the Ly-49D receptor into the rat NK cell tumor line, RNK-16, which expresses none of the known mouse inhibitory alloreceptors. We examined the cytotoxicity of RNK.mLy-49D transfectants against a panel of targets expressing polymorphic alleles of murine MHC class I. We demonstrate here that Ly-49D specifically stimulates cytotoxicity of targets expressing H-2Dd, but not of targets expressing H-2a or H-2k MHC antigens. This H-2Dd-specific killing can be blocked by F(ab')2 anti-Ly-49D or F(ab')2 anti-H-2Dd. These experiments identify Ly-49D as an activating receptor for mouse H-2Dd, and therefore demonstrate the existence of alloactivating receptors on murine NK cells.

Materials and Methods

Cells. RNK-16, a spontaneous NK cell leukemia from F344 rats, was a gift from Craig Reynolds (NCI, Frederick, MD) and was adapted for in vitro growth in crPM1 (RPMI 1640 supplemented with 10% heat-inactivated FCS, 25 μM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) (24). Tumor target cell lines cultured in crPM1 included YAC-1 (mouse lymphoma, H-2a) and Y B2/0 (rat myeloma) from the American Type Culture Collection. The mouse Ly-49A transfectant of RNK-16, RNK.mLy-49A (previously described as RNK-mLy-49A; reference 25), was grown in crPM1 supplemented with 1 mg/ml of G418 (Boehringer Mannheim). Transfected RNK-16 effectors and transfected YB2/0 targets were maintained in 1 mg/ml G418, but were grown in crPM1 without G418 for at least 2 d before functional assays.

Antibodies and Flow Cytometry. mAbs to mouse Ly-49D (12A8, rat IgG2a), OVA (2C7, rat IgG2a), or H-2Dd (34-5-8S, mouse IgG2a) were produced from their respective hybridoma lines. Antibodies were partially purified from ascites by ammonium sulfate precipitation. F(ab')2 fragments were generated by pepsin digestion (26), and completion of digestion was verified by SDS-PAGE and silver staining. For fluorescence analysis, mAbs were used at a concentration of 1 μg/106 cells. Fluorescence analysis was performed using a FACScan®.

Cytotoxicity Assays. Specific lysis of NK targets was determined by a standard 4-h 51Cr-release assay as previously described (27). In brief, target cells were harvested and labeled for 1 h at 37°C with 200 μCi of 51Cr (Amersham). Complete RPMI 1640 supplemented with 10% heat-inactivated FCS, 25 μM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin was used at a concentration of 25 μg/106 effectors or with intact antibody at a concentration of 10 μg/106 effectors before the addition of targets.

Vectors and cDNA. The Ly-49D cDNA was obtained by PCR amplification from cDNA prepared from 7-d-IL-2-activated LAK cells from C57BL/6 mice using the primers 5'-TCACAGAAATCTCAAGGACAT-3' and the primer 5'-TTTT-CTTGAACACTCACCT-3'. The Ly-49D cDNA obtained is identical to the Ly-49D cDNA previously published in the extracellular and transmembrane domains (17). There is a 9-nucleotide insertion at the 3' end of the coding sequence for the cytoplasmic domain in the Ly-49D cDNA obtained and used in these studies, which is identical to the alternatively spliced long Ly-49D cDNA sequence published by Silver et al. (28). The protein sequence of the 44-amino acid cytoplasmic domain of the Ly-49D cDNA obtained reads METQEDTFSARVFHHSSGLONEMRLKETKRPEKA (the initial published sequence is identical except it lacks the underlined 3-amino acid insertion). This Ly-49D cDNA PCR product was subcloned into the TA cloning vector, pCR2.1.
(Invitrogen), shuttled through the vector pSP72 (Promega) using the 5' Hind III/Xba I sites, and then ligated into the Xho I site in the expression vector BSR αEN (a gift from A. Shaw and M. Olszowy, Washington University, St. Louis, MO). Constructs were confirmed by sequencing in both directions before transfection. Vectors used to transfet YB2/0 target cells with H-2\(D^d\), H-2\(D^b\), and chimeric H-2\(D^{a,b}\) class I MHC have been described previously (29). The chimeric molecules were made by exon shuffling between genomic clones of H-2\(D^d\) and H-2\(D^b\) using standard methods (30), and details of the construction have been described previously (29). The chimeric molecule that we have termed D\(^{a,b}\) encodes the \(\alpha_1\) and \(\alpha_2\) domains of H-2\(D^d\) with the \(\alpha_3\) domain of H-2\(D^b\). The chimeric molecule that we have termed D\(^{b,a}\) encodes the \(\alpha_1\) and \(\alpha_2\) domains of H-2\(D^b\) with the \(\alpha_3\) domain of H-2\(D^d\).

Transfections. YB2/0 or RNK-16 cells were transfected by electroporation as previously described, using cesium-purified genomic plasmids or QIagen-purified (QIagen) cDNA plasmids using standard methods (25, 29).

Mice: Strain C3HBL/6, BALB/c, BALB.B10 (C.B10-H-2\(d\)/LiMcdJ), BALB.K (C.B10-H-2\(b\)/LiMcdJ), B10.D2, B10.BR, B10.S, DBA/2, and C3H mice were obtained from The Jackson Laboratory and were used at 6–8 wk of age.

B10.S, DBA/2, and C3H mice were obtained from The Jackson Laboratory and were used at 6–8 wk of age.

**Results**

Mouse Ly-49D Functions as an Activating Receptor in RNK-16 Cells. To examine Ly-49D–ligand interactions in the absence of mouse inhibitory NK cell receptors, we transfected the mouse Ly-49D receptor into RNK-16, a rat tumor cell line with phenotypic and functional characteristics of rat NK cells (24). Cell-surface staining of a representative RNK.mLy-49D clone with the anti-Ly-49D mAb, 12A8, is shown in Fig. 1 B. In murine NK cells, the Ly-49D receptor has previously been demonstrated to mediate "redirected lysis" of FcR-bearing targets in the presence of the anti-Ly-49D mAb, 12A8 (14). As shown in Fig. 1 D, killing of the FcR-bearing target YB2/0 by the RNK.mLy-49D transfectant was similarly stimulated by anti-Ly-49D mAb, but not by isotype-matched control mAb. Neither F(ab')\(_2\) anti-Ly-49D nor control F(ab')\(_2\) 2C7 had any effect on cytotoxicity (panel F), indicating that the binding of the intact mAb to target FcR is required for augmentation of killing of YB2/0 (redirected lysis). Lysis of YB2/0 by wild-type RNK-16 cells was unchanged by mAb anti-Ly-49D, by control mAb (panel E), or by F(ab')\(_2\) fragments derived from either antibody (panel G). These studies demonstrate that the mouse Ly-49D receptor can function as an activating receptor in RNK-16.

RNK.mLy-49D transfectants were also tested against the standard NK cell tumor target, YAC-1. Interestingly, killing of YAC-1 by RNK.mLy-49D was decreased compared with killing by RNK-16 (Fig. 1, G and H). The anti-Ly-49D mAb, 12A8, had no effect on the lysis of YAC-1 by RNK.mLy-49D transfectants (data not shown).

Mice Ly-49D Is Activated by H-2\(D^d\) on YB2/0 Targets. To examine the MHC restriction of Ly-49D, we tested the RNK.mLy-49D transfectant in cytotoxicity assays against a panel of rat YB2/0 myeloma target cells transfected with one of the murine H-2 alleles D\(^d\), D\(^b\), K\(b\), or K\(k\). As seen in Fig. 2, RNK.mLy-49D effectors lysed YB2/0.D\(^d\) targets more efficiently than targets expressing any other H-2 allele.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** The Ly-49D receptor activates lysis of H-2\(D^d\)-expressing YB2/0 target cells. Standard 4-h cytotoxicity assays were performed using either RNK.mLy-49D transfectants (A–D), wild-type RNK-16 (E–H), or RNK.mLy-49A transfectants (I–L) as effectors. Each effector was tested against a panel of YB2/0 cells transfected with one of the MHC class I alleles as targets: panels A, E, and I, H-2\(D^d\); panels B, F, and J, D\(^b\); panels C, G, and K, K\(b\); panels D, H, and L, K\(k\). Effector cells were preincubated with media alone (●), anti-Ly-49D (12A8) F(ab')\(_2\) fragments (●), or control (2C7) F(ab')\(_2\) fragments (○).
H-2D\(d\) and H-2Db were recombined to create chimeric class I molecules. The RNK.mLy-49D effectors was specifically prepared from BALB.B10 (H-2b; panel C), C57BL/6 (H-2 or Kk), and B10.D2 (H-2d; panel B) mice, but not Con A blasts from C3H (H-2k) or B10.S (H-2s) mice. Ly-49A cells were unable to lyse YB2/0.Dd cells, as they were inhibited through the Ly-49A receptor. Because the 12A8 mAb binds to Ly-49A as well as to Ly-49D (14), we were able examine the effects of 12A8 on lysis by RNK.mLy-49A cells. As shown in Fig. 2 A, F(ab\(^{'})\)\(2\) 12A8 blocked H-2D\(d\)-specific activation of cytotoxicity by RNK.mLy-49D cells, but reversed H-2D\(d\)-specific inhibition of lysis by RNK.mLy-49A cells (panel I). Thus, the activating Ly-49D receptor and the inhibitory Ly-49A receptor share ligand specificity for H-2D\(d\) on target cells, and the interaction between ligand H-2D\(d\) and either Ly-49A or Ly-49D can be blocked by F(ab\(^{'})\)\(2\) 12A8. Neither F(ab\(^{'})\)\(2\) 12A8 nor control F(ab\(^{'})\)\(2\) 2C7 had any significant effect on the lysis of YB2/0.Dd, K\(b\), or K\(k\) targets by any effector (panels B–D, F–H, and J–L).

RNK.mLy-49D Cells Are Activated by H-2D\(d\) Con A Blasts. We next extended our observations regarding the lysis of transfected cells by examining the lysis of Con A blasts derived from mice of various H-2 haplotypes. For these studies we tested RNK.mLy-49D effectors in cytotoxicity assays against Con A blasts prepared from MHC congenic resistant strains of BALB/c and C57BL/10 mice.

As shown in Fig. 3, RNK.mLy-49D cells effectively lysed Con A blasts prepared from BALB/c (H-2\(d\); panel A) and B10.D2 (H-2\(d\); panel B) mice, but not Con A blasts prepared from BALB.B10 (H-2\(b\); panel C), C57BL/10 (H-2\(b\); panel D), BALB.K (H-2\(k\); panel E), or B10.BR (H-2\(k\); panel F) mice. In experiments not shown, RNK.mLy-49D effectors also lysed Con A blasts from DBA/2 (H-2\(k\)) mice but not blasts from C3H (H-2\(k\)) or B10.S (H-2\(k\)) mice. Lysis of all H-2D\(d\) blasts by RNK.mLy-49D effectors was inhibited by anti-Ly-49D mAb 2C7 (panels A and B). As we have previously shown, RNK.mLy-49A transfectants were tested as effector cells for lysis of Con A blast targets prepared from mice that were congenic resistant at the H-2 locus. Con A blast targets were prepared from splenocytes isolated from mice expressing either H-2\(d\) (BALB/c, B10.D2; panels A and B), H-2\(b\) (BALB.B, C57BL/10; panels C and D), or H-2\(k\) (BALB.K, B10.BR; panels E and F). Assays were performed after preincubation of effector cells with media alone (○), anti-Ly-49D mAb (12A8) (●), or control mAb (2C7) (□) before addition of targets.

**Figure 3.** The Ly-49D receptor activates lysis of H-2\(d\)-expressing Con A blasts. RNK.mLy-49D transfectants were tested as effector cells for lysis of Con A blast targets prepared from mice that were congenic resistant at the H-2 locus. Con A blasts were prepared from splenocytes isolated from mice expressing either H-2\(d\) (BALB/c, B10.D2; panels A and B), H-2\(b\) (BALB.B, C57BL/10; panels C and D), or H-2\(k\) (BALB.K, B10.BR; panels E and F). Assays were performed after preincubation of effector cells with media alone (□), anti-Ly-49D mAb (12A8) (●), or control mAb (2C7) (○) before addition of targets.
We, therefore, determined whether F(ab')₂ 34-5-8S could inhibit the Ly-49D/H-2Dd interaction as well. As shown in Fig. 5, activation of RNK.mLy-49D effectors by YB2/0.Dd was inhibited by F(ab')₂ 34-5-8S (panel C), whereas lysis of YB2/0.Dd by RNK.mLy-49A effectors was restored in the presence of F(ab')₂ 34-5-8S. RNK-16 lysis of YB2/0.Dd was unaffected by F(ab')₂ 34-5-8S.

These data show that the activating Ly-49D receptor shares some aspects of ligand recognition with the inhibitory Ly-49A receptor. Both receptors recognize the α₁/α₂ domains of H-2Dd, and F(ab')₂ anti–H-2Dd 34-5-8S can block the interaction of H-2Dd with either Ly-49A or Ly-49D. However, the ligand specificity of Ly-49D does not completely mirror that of Ly-49A, as Ly-49D–mediated cytotoxicity is not activated by H-2k blasts, which express the Ly-49A ligand H-2Dk (33–35). These data suggest that the structural features that restrict the allelic specificities of Ly-49D and Ly-49A are similar, but not identical.

Discussion

Cytotoxicity by NK cells is generally inhibited by the expression of class I MHC antigens on target cells. The “missing self” hypothesis proposes that loss of target cell MHC class I may lead to the unopposed activation of natural killing and eventually to target cell lysis (5). However, several previous lines of evidence suggest that rodent NK cells may possess activating receptors in addition to inhibitory receptors for MHC-encoded target structures. First, irradiated C57BL/6 (H-2b) mice rapidly reject bone marrow from H-2Dd transgenic C57BL/6 mice, unless recipient mice are depleted of NK cells before transplantation (19, 20). Thus, the transgenic expression of H-2Dd on C57BL/6 blasts is associated with the selective acquisition of susceptibility to lysis by mouse NK cells. Second, genetic studies in rats have shown that NK cells are activated by structures encoded within the MHC locus, and NK cells from the PVG (RT1c) rat strain efficiently lyse blast targets from LEW (RT1l) rats, but not from mutant LEW.LM1 (RT1lm1) rats, which have a homozygous 100-kb deletion in the nonclassical RT1.C region (21). These data suggest that the ability of NK cells to lyse allogeneic blast targets may directly involve activating receptors for target MHC.

A gene controlling NK alloactivating responses in rats has been linked to the Ly-49 gene family in NK alloresponder PVG rats backcrossed to DA rats, which are selectively de-
efficient in NK allorecognition. These studies implicate Ly-49-like molecules in the activation of cytotoxicity by target MHC antigens (22).

Mason et al. previously demonstrated that Ly-49D can activate NK cell cytotoxicity, but the specific activation of Ly-49D+ NK cells by target MHC class I antigens was not demonstrated in vitro (14). Subsequent in vivo studies revealed that depletion of Ly-49D+ cells from C57BL/6 mice prevented their ability to reject H-2Dd bone marrow grafts (23), consistent with the hypothesis that these cells are functionally activated by MHC-encoded structures. We examined target-induced Ly-49D activation using RNK.mLy-49D transfectants, which can activate NK cell cytotoxicity through the Ly-49D receptor. Interestingly, when compared with wild-type RNK-16, RNK.mLy-49D effectors demonstrated diminished lysis of YAC-1 and YB2/0, as well as diminished antibody-dependent cellular cytotoxicity and diminished redirected lysis through the rat NKR-P1A receptor (data not shown). This change was not associated with changes in expression of NKR-P1A on the RNK-16 transfectants (data not shown). Because these changes in cytolytic specificity were seen in three different RNK.mLy-49D clones, it seems unlikely that they were unique to Ly-49D integration sites in stable transfectants, although this possibility cannot be ruled out completely. It is also possible that the overexpression of the Ly-49D activating receptor leads to sequestration of signaling intermediates required for activation, which, in turn, leads to a decrease in lysis through other activating receptors. However, we were easily able to observe specific activation through Ly-49D, demonstrating that the cytolytic capacity of RNK.mLy-49D cells was intact.

Our in vitro studies demonstrate that Ly-49D is an activating NK cell receptor specific for H-2Dd. The acquisition of enhanced cytotoxicity against H-2Dd-transfected YB2/0 targets was specifically blocked by F(ab')2 anti-Ly-49D or by F(ab')2 anti-H-2Dd. Activation of NK cells by Ly-49D was not unique to transfected targets, as Ly-49D also stimulated lysis against blasts from H-2b mice, but not against blasts from H-2k or H-2d mice. We considered the possibility that these results might be obtained if Ly-49D were not the activation receptor itself but instead, through its interaction with H-2Dd, facilitated activation through a separate activating receptor. However, it is unlikely that adhesion alone between Ly-49D and H-2Dd accounts for the observed activation of cytotoxicity. First, stimulation of the Ly-49D receptor with antibody is known to induce activation of cytotoxicity by NK cells, as shown here and by others (14). Second, we have previously demonstrated that adhesion between another Ly-49 receptor and H-2Dd is not sufficient to activate NK cell lysis. Specifically, we have previously demonstrated that interaction of an inactive Ly-49A receptor with H-2Dd does not activate NK cell lysis through other receptors on RNK-16 (25). The Ly-49A receptor normally binds to H-2Dd and thereby leads to inhibition of NK cell lysis. Our previous studies showed inhibition of lysis of H-2Dd expressing targets by RNK-16 cells transfected with Ly-49A. We also studied a mutated Ly-49A receptor, containing a point mutation in the cytoplasmic domain of the receptor which disrupts the ITIM motif required for inhibitory function. This mutated Ly-49A receptor expressed on RNK-16 cells failed to inhibit or to augment lysis of H-2Dd-expressing targets. This mutated Ly-49A receptor is unaltered in the extracellular or transmembrane domains so its binding to H-2Dd is presumably unaltered. Thus, engagement of H-2Dd by the mutant Ly-49A receptor does not facilitate the activation of lytic pathways through other receptors on RNK-16.

It is of considerable interest whether the inhibitory Ly-49A receptor and the activating Ly-49D receptor recognize the same or distinct domains of the H-2Dd antigen. As with Ly-49A, Ly-49D-dependent effects required the αβ/αγ2 domains of H-2Dd and could be blocked by an αβ/αγ2-specific F(ab')2 anti-H-2Dd mAb (34-5-8S) (references 32–34, 36). The extracellular domains of Ly-49A and Ly-49D may recognize similar regions of H-2Dd, but only Ly-49A recognizes H-2Dk (33–35). Thus, Ly-49A and Ly-49D appear to exhibit overlapping but distinct allelic specificities for murine class I MHC antigens. Studies by several groups have indicated that Ly-49A does not discriminate between different peptides presented by H-2Dd (32, 37). The role of antigenic peptides in the specificity of Ly-49D–H-2Dd interaction remains to be examined.

It has been speculated that activating Ly-49-like receptors may participate in the recognition of abnormal or virally-encoded MHC-like molecules by NK cells, although this has not been proven (6, 38). However, the physiologic significance of alloactivating NK receptors is unknown. Although their effects may be partially masked by inhibitory NK cell receptors, the identification of in vivo NK alloactivating functions indicates that alloactivation is not completely abrogated by inhibitory MHC receptors in vivo (19–22). A delicate balance between activating and inhibitory alloalloreactors may be influenced by different affinities or structural requirements for ligand binding. Alternatively, expression of opposing alloalloreactors on different NK cell subsets may permit NK alloresponses in vivo. Alloactivating NK cell receptors, in addition to allospecific T cell receptors, likely play a role in the physiologic rejection of foreign cells (6, 7, 23, 39).

In conclusion, our experiments using gene transfer provide the first direct evidence that an activating receptor on murine NK cells specifically directs cytotoxicity against a classical class I MHC ligand. They also provide a model for dissecting the molecular pathways involved in this interaction. Other candidate alloactivating receptors on human NK cells include the Ig-like short domain KIR (killer cell inhibitory receptor) receptors, which may recognize alleles of HLA-C (8, 15, 16), and the activating CD94/NKG2C receptor, which can bind to ligand HLA-E (11). Future studies to define the distribution and activation requirements for these receptors will help us to understand the physiologic functions of alloactivating receptors in NK cell biology.
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