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Brief Definitive Report

Activating Ly-49D and Inhibitory Ly-49A Natural Killer Cell Receptors Demonstrate Distinct Requirements for Interaction with H2-D^d

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Abstract

The activating Ly-49D receptor and the inhibitory Ly-49A receptor mediate opposing effects on natural killer (NK) cell cytotoxicity after interaction with the same major histocompatibility complex ligand, H2-D^d. To compare Ly-49D and Ly-49A interactions with H2-D^d, we created mutations in H2-D^d and examined the functional ability of these mutants to activate lysis through Ly-49D or to inhibit lysis through Ly-49A. Specific single amino acid changes in either the H2-D^d α_1 helix or the α_2 helix abrogated Ly-49D–mediated cytotoxicity, but these changes had no significant effect on Ly-49A–dependent inhibition. Each of three α_2 domain mutations in the floor of the peptide binding groove reduced functional recognition by either Ly-49D or Ly-49A, but all three were required to fully abrogate inhibition by Ly-49A. Our studies indicate that Ly-49D/H2-D^d interactions require distinct determinants compared with Ly-49A/H2-D^d interactions. These differences have important implications for the integration of activating and inhibitory signals in NK cells.

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

Introduction

NK cell function is regulated by a balance of signals delivered through activating and inhibitory receptors (1). NK cytotoxicity is generally inhibited by class I MHC (MHC I) on targets, but recent studies have identified both inhibitory and activating receptors for MHC I encoded within the same gene families (1).

In mice, the Ly-49 gene family includes both inhibitory and activating receptors for MHC I, expressed on overlapping subsets of NK cells (2, 3). Two members of this receptor family, Ly-49A and Ly-49D, recognize the same MHC I allele, H2-D^d, but mediate opposing effects; Ly-49A inhibits NK function, whereas Ly-49D activates it (4–6). Ly-49A and Ly-49D are 86% homologous in the extracellular domain, and mAb specific for the α_1/α_2-H2-D^d domains blocks functional interaction of H2-D^d with either Ly-49A or Ly-49D (4–7).

To compare structural requirements for functional recognition of H2-D^d by Ly-49D and Ly-49A, we prepared a series of mutated H2-D^d molecules in which specific H2-D^d residues were altered to the corresponding residue in H2-D^b (not a ligand for either Ly-49A or Ly-49D; references 4, 6). We expressed these mutants in the rat target YB2/0 and examined their functional interaction with mouse Ly-49A or Ly-49D expressed on rat RNK-16 cells. We found that single amino acid mutations throughout the α_1 and α_2 helices interrupted recognition of H2-D^d by Ly-49D but not Ly-49A. Combinatorial mutagenesis of α_2 residues in the β-pleats was required to abrogate functional recognition of H2-D^d by Ly-49A. Thus, this opposing receptor pair demonstrates distinctions in their requirements for ligand recognition.

Materials and Methods

Cells. RNK-16, YAC-1, and YB2/0 were cultured in cRPMI-1640. RNK-16 transfectants RNK.Ly-49A and RNK.Ly-49D have been described previously (6, 8).

Antibodies and Flow Cytometry. mAbs A1 (anti–mouse Ly-49A), PK136 (anti-NK1.1), 12A8 (anti–mouse Ly-49D and anti–mouse Ly-49A–specific), 2C7 (antiovalbumin), and 34-5-8S (anti–H2-D^d-α_1/α_2) were partially purified from ascites by am-

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monium sulfate precipitation. Anti–H2-D^d-α_3 (34-2-12S) was from PharMingen. F(ab′)$_2$ fragments were generated by pepsin digestion as previously described (6). Flow cytometry analysis used a Becton Dickinson FACScan™.

Cytotoxicity Assays. NK cytolysis of targets was assessed in triplicate using standard 4-h ^{51}Cr-release assays (8). For mAb inhibition, effectors were preincubated 15 min at room temperature with F(ab′)$_2$ (25 µg/10^6 effectors) or intact antibody (10 µg/10^6 effectors) before target addition. Results shown are representative of at least three separate experiments.

H2-D^d Mutations. Mutations S73W, T80N, A81L, R83G, and N86Q were generated using standard recombinant PCR with overlapping primers encoding the mutation, with external 5′ primer-CTGCTCCTGCTGCTGGCGGCCGCCCTG and external 3′ primer-AGCCCAGGGCCCAGCACC, using *Pwo* polymerase (Roche) and H2-D^d cDNA as template (pD^{d}SEL.FIX 34; a gift from F. Karlhofer, University of Vienna, Vienna, Austria). The 646-bp PCR fragment replaced the corresponding fragment in wild-type H2-D^d cDNA in pD^{d}SEL.FIX 34 by restriction sites 5′-NotI/3′-ApaI. The mutated H2-D^d cDNA was excised 5′-SalI/3′-EcoRI, and ligated into the expression vector pHβAPr-1-neo, 5′-SalI/3′-HindIII, after 3′ Klenow treatment. Other mutations were created using the QuikChange Site-Directed Mutagenesis kit (Stratagene) using overlapping primers encoding the mutation in H2-D^d-PUC19. Mutated H2-D^d cDNAs were subcloned into the expression vector BSRαEN (a gift from A. Shaw, Washington University, St. Louis, MO). All mutations were verified by sequencing.

Transfections. YB2/0 were transfected as previously described (8, 9) and selected for similar expression levels by staining with mAb 34-2-12S. At least two different transfectants expressing each H2-D^d mutant were functionally tested, although results from one of each are presented. YB2/0 targets expressing H2-D^d, H2-D^b, or chimeric H2-D$^{d/b}$ MHC I have been described previously (9).
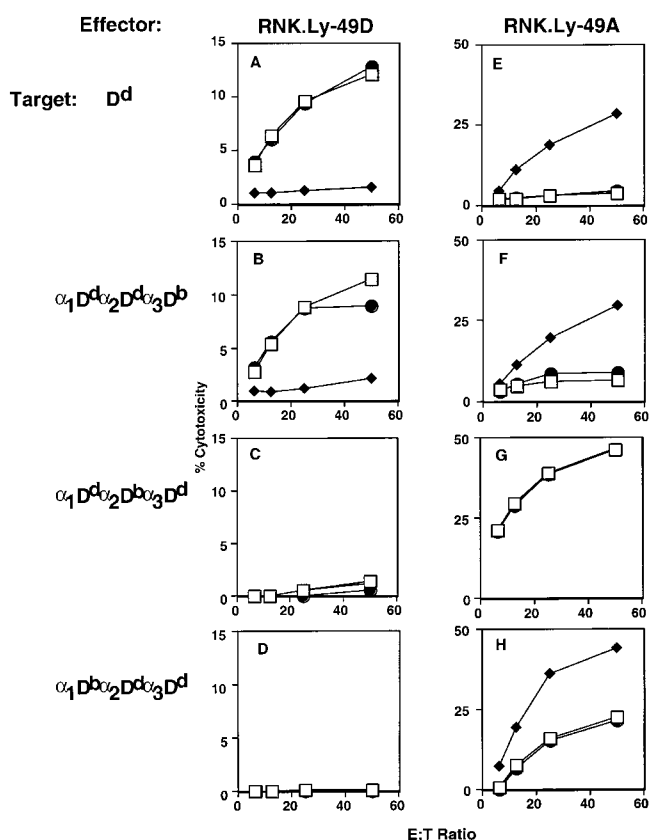

Figure 1. Functional recognition by Ly-49D requires both the α_1 and α_2 domains of H2-D^d. Cytotoxicity by RNK.Ly-49D (left) and RNK.Ly-49A (right) effectors was tested in the presence of blocking 12A8 F(ab′)$_2$ fragments (◆, anti-Ly-49A/Ly-49D), isotype-matched control F(ab′)$_2$ fragments (●, 2C7), or media (□). Targets were YB2/0 cells transfected with H2-D^d or H2-D$^{d/b}$ chimeric molecules as indicated.

Results and Discussion

Functional Recognition by Ly-49D Requires Both the α_1 and α_2 Domains of H2-D^d. In previous studies, we examined Ly-49A/H2-D^d interactions with chimeric MHC I that combined exons for H2-D^d (a ligand for Ly-49A) and H2-D^b (not a ligand for Ly-49A) using untransfected RNK-16 and RNK.Ly-49A transfectants (9). In this study, we tested the functional capacity of Ly-49D to recognize targets expressing these chimeric H2-D^b/D^d MHC. As shown in Fig. 1, RNK.Ly-49D transfectants specifically mediated lysis of YB2/0 cells expressing intact H2-D^d or the chimeric MHC, α_1D$^d\alpha_2$D$^d\alpha_3$D^b (Fig. 1, A and B), and lysis was blocked by 12A8 F(ab′)$_2$ (anti–Ly-49D). There was no lysis of targets expressing α_1D$^b\alpha_2$D$^b\alpha_3$D^d (data not shown). Furthermore, RNK.Ly-49D cells failed to lyse targets expressing α_1D$^d\alpha_2$D$^b\alpha_3$D^d (Fig. 1 C), indicating that Ly-49D, like Ly-49A (Fig. 1 G), fails to recognize α_1D^d paired with α_2D^b. RNK.Ly-49D cells also failed to lyse targets expressing α_1D$^b\alpha_2$D$^d\alpha_3$D^d (Fig. 1 D), indicating that Ly-49D, unlike Ly-49A (Fig. 1 H), could not recognize α_2D^d in the context of α_1D^b. Thus, Ly-49D–mediated activation requires the combined α_1 and α_2 domains of H2-D^d, whereas Ly-49A can recognize α_2 from D^d paired with α_1 from D^b.

Recognition of H2-D^d by Ly-49D but Not by Ly-49A Is Highly Sensitive to Specific Single Amino Acid Changes in Either the α_1 or α_2 Helices of H2-D^d. We next substituted individual residues in the α_1 or α_2 helices of H2-D^d with the corresponding H2-D^b residues. We targeted three sites: (i) the COOH-terminal α_1 helix, corresponding to the human MHC I region critical for recognition by killer inhibitory receptors (KIRs; reference 10); (ii) single and paired mutations at positions 73 (α_1) and 156 (α_2), which create a salt bridge in H2-D^b but not in H2-D^d (11, 12); and (iii) residues in the exposed α_2 helix that differ between H2-D^d and H2-D^b. Sites targeted for mutation are shown in Fig. 2. YB2/0 targets transfected with mutated H2-D^d were selected for equivalent expression by binding to 34-2-12S mAb (anti-D^d, α_3-specific; data not shown). Using RNK.Ly-49D or RNK.Ly-49A transfectants as effectors, we assessed the ability of mutant H2-D^d molecules to activate cytotoxicity through Ly-49D or to inhibit cytotoxicity through Ly-49A.

As noted in our previous study, baseline lysis of untransfected YB2/0 target cells by RNK.Ly49D transfectants is much lower than lysis by RNK.Ly-49A transfectants (6). Therefore, we do not base conclusions on differences in ly-
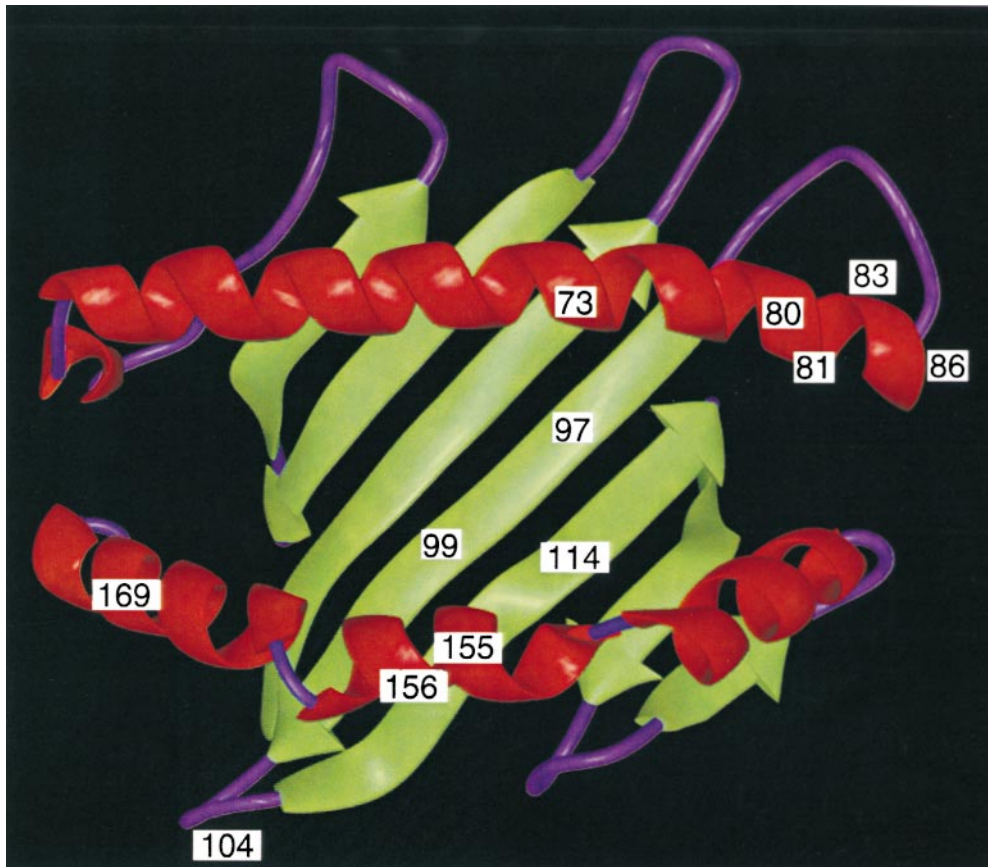
Figure 2. Location of mutated amino acid residues in H2-D^d. The molecular graphics image was created by H. Houtkooper, University of California, San Francisco, Computer Graphics Laboratory, with the MidasPlus modeling system. The image is based on Protein Data Bank coordinates 1bii (26).

sis observed between different transfected cell lines. Instead, our conclusions are based on comparisons between lysis by RNK transfectants with and without receptor-specific blocking antibody. The specificity of the effect due to the Ly-49 receptor was demonstrated by reversal of the effect after blockade with receptor-specific mAb (intact or F(ab′)$_2$ fragments). Blocking mAbs (A1, 12A8) did not stain untransfected RNK-16 or IL-2–activated NK cells from F344 rats (the strain from which RNK-16 is derived), indicating that these antibodies do not recognize rat Ly-49 receptors (data not shown). Moreover, receptor-specific mAb had no effect on lysis of YB2/0.H2-D^d transfectants by wild-type RNK-16 cells (9, 13). Finally, blocking mAbs had no effect on lysis of YB2/0 or of YB2/0 transfected with D^b, K^b, or K^k by RNK-16, RNK.Ly-49A, or RNK.Ly-49D (6, 9, and data not shown).

As shown in Fig. 3, single amino acid substitutions in the α$_1$ helix at positions 73, 80, or 83 all abrogated activation of cytolysis through Ly-49D. Mutation at position 81 or removal of the conserved N-linked glycosylation site at position 86 did not abrogate lysis through Ly-49D. In the α$_2$ helix, mutations at positions 155, 156, or 169 also interrupted recognition by Ly-49D. These results demonstrate that Ly-49D–mediated activation is highly sensitive to single amino acid changes in either of the α helices of H2-D^d.

In contrast, inhibitory recognition by Ly-49A was unimpaired by the same single amino acid mutations (Fig. 3). In addition, we found that mutagenesis of residues 73 and 156 had no effect on Ly-49A–mediated inhibition. This conflicts with results of Waldenstrom et al., who found impaired functional recognition by Ly-49A after substitution at these sites with H2-D^b residues, predicted to form a salt bridge across the peptide binding groove (11, 12). This contradiction is unexplained, although their studies used a different target cell and nonclonal effector cells, perhaps detecting an effect not specific to the Ly-49A receptor.

Mutations in the Floor of the Peptide Binding Groove of H2-D^d Abrogate Functional Recognition of H2-D^d by both Ly-49D and Ly-49A. Previous studies have demonstrated that mAb 34-5-8S (H2-D^d α$_1$/α$_2$-domain specific; reference 14), blocks recognition of H2-D^d by both Ly-49A (4, 7) and Ly-49D (5, 6) and recognizes an epitope including residues 92–116 in the α$_2$ domain (9, 15). Therefore, we mutated H2-D^d at sites within this region that differed between H2-D^d and H2-D^b.

Residues in this region proved critical for recognition of H2-D^d by both Ly-49D and Ly-49A. Each of three mutations, W97Q, A99S, and W114L, abrogated activation by Ly-49D (Fig. 4, G–I) and had a partial effect on functional inhibitory recognition by Ly-49A (B–D). However, the combination of all three mutations (97, 99, and 114) was required to completely abrogate inhibitory recognition by Ly-49A (Fig. 4 E). The triple A97Q/A99S/W114L mutation and the single W97Q mutation were expressed at a slightly lower level than the wild-type H2-D^d (Fig. 5, A and C), because higher expressing transfectants could not
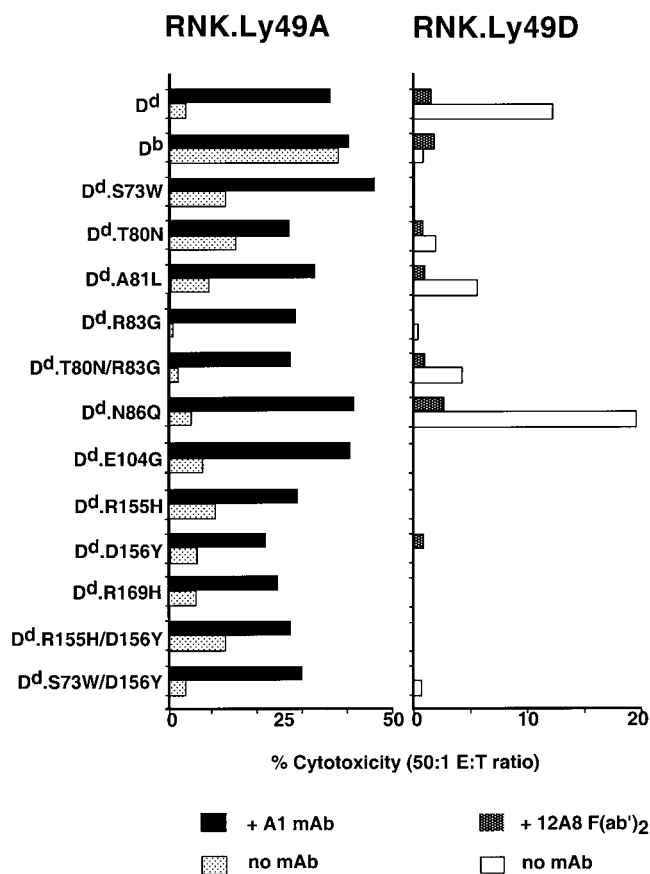
RNK.Ly49A   **RNK.Ly49D**

% Cytotoxicity (50:1 E:T ratio)

■ + A1 mAb   ▨ + 12A8 F(ab')₂
▨ no mAb   ▢ no mAb

Figure 3. Recognition of H2-D^d by Ly-49D but not by Ly-49A is highly sensitive to specific single amino acid changes in either the α_1 domain or the α_2 domain of H2-D^d. RNK.Ly-49D (right) and RNK.Ly-49A (left) effectors were tested for lysis of YB2/0 targets transfected with intact H2-D^d, H2-D^b, or H2-D^d mutated to the corresponding residue of H2-D^b as indicated in the left-hand column. Lysis of target cells is shown at an E/T ratio of 50:1. Lysis by RNK.Ly-49A effectors was assessed in the presence of anti–Ly-49A (A1, black bars) or no blocking mAb (spotted bars). Lysis by RNK.Ly-49D effectors was assessed in the presence of anti–Ly-49D F(ab')₂ fragments (12A8, hatched bars) or no antibody (white bars).
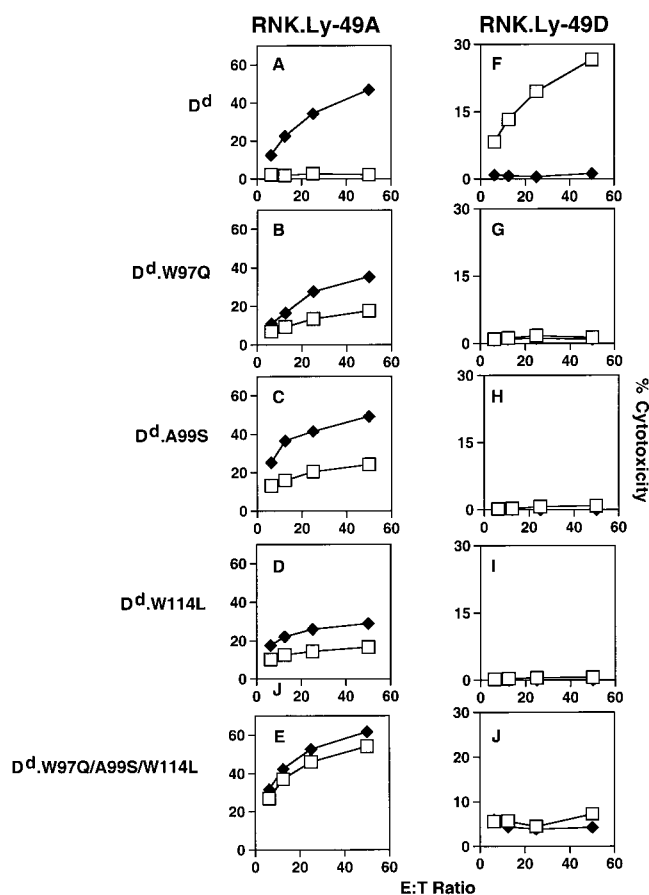

Figure 4. Mutations in the floor of the peptide binding groove of H2-D^d abrogate functional recognition of H2-D^d by both Ly-49D and Ly-49A. Lysis by RNK.Ly-49D (right) and RNK.Ly-49A (left) effectors were tested in the presence of blocking 12A8 F(ab')₂ fragments (◆, anti-Ly-49A/Ly-49D) or no antibody (□) against YB2/0 targets expressing H2-D^d or mutated H2-D^d as indicated in the left-hand column. In panels E and J, H2-D^d is mutated at all three sites (97, 99, and 114).

This loop contains polymorphic residues at positions 103, 104, and 107. Mutation at position 104 abrogated recognition by Ly-49D, but it did not alter recognition by Ly-49A (Fig. 3). This result again demonstrates the sensitivity of Ly-49D to mutations throughout H2-D^d, and the contrasting insensitivity of Ly-49A.

Mutations in H2-D^d Demonstrate that Functional Recognition by Ly-49A or Ly-49D Does Not Strictly Correlate with Binding by the mAb 34-5-8S. Like Ly-49A, binding by mAb 34-5-8S to H2-D^d requires a conformational epitope dependent on occupancy of the peptide binding groove but not on peptide sequence, and 34-5-8S is the only known anti–H2-D^d mAb that blocks Ly-49A/H2-D^d interactions (7). We examined 34-5-8S binding to the H2-D^d mutants that differed in their functional recognition by Ly-49A and Ly-49D. The triple mutation W97Q/A99S/W114L in H2-D^d, which abrogated functional recognition by Ly-49A and Ly-49D, also eliminated binding by 34-5-8S (Fig. 5 D). However, we did not observe an exact correlation between binding to 34-5-8S and functional inhibition through Ly-
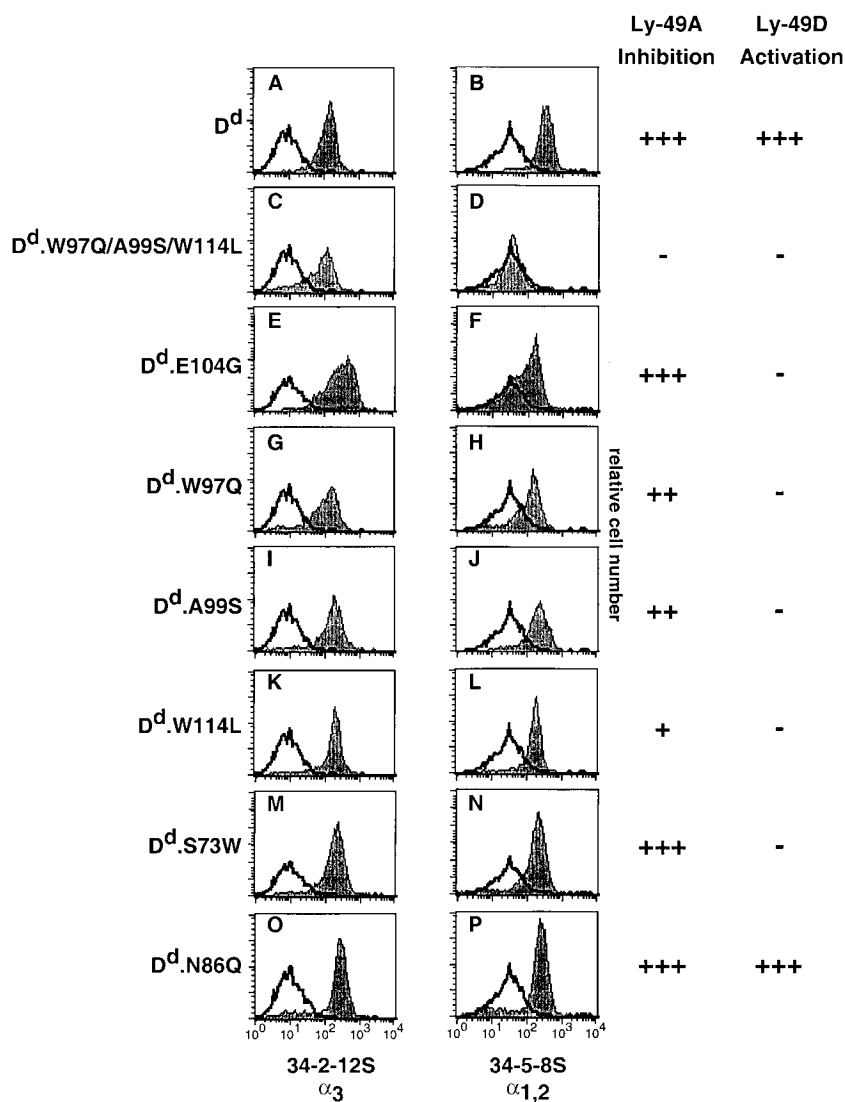
be obtained. Thus, it remains possible that a triple mutant expressed at higher levels might retain some Ly-49A inhibition.

Interestingly these critical α_2 domain residues lie in the β-pleats forming the floor of the peptide binding groove (16, 17), demonstrating that residues in the H2-D^d peptide binding platform are important in recognition by both Ly-49D and Ly-49A. This was unexpected, as none of these residues is directly solvent accessible (16, 17). The tryptophan residues at positions 97 and 114 form a unique hydrophobic ridge in the floor of the antigen binding groove that severely constrains the bound antigenic peptide at residue P3 (16, 17). Only proline at anchor position 3 fits the steric and hydrophobic constraints of the cleft (16–18). Thus, the mutation of the tryptophans might permit other peptide residues at this anchor site.

The two β-pleats containing these critical sites are connected by a loop that protrudes from beneath the α_2 helix.

Figure 5. Mutations in H2-D^d demonstrate that functional recognition by Ly-49A and Ly-49D does not strictly correlate with binding by the mAb 34-5-8S. YB2/0 tumor target cells expressing H2-D^d or H2-D^d mutated at the indicated residues were stained with an anti–H2-D^d mAb (34-2-12S [α_3] or 34-5-8S [$\alpha_{1,2}$]). Overlay (black line) shows staining of YB2/0 cells transfected with H2-D^b as background which was similar to staining of untransfected YB2/0 cells (not shown). Transfectants were initially selected for equivalent expression as assessed by 34-2-12S binding (A, C, E, G, I, K, M, and O, gray histogram) then assessed for 34-5-8S binding (B, D, F, H, J, L, N, P, gray histogram). For comparison, relative inhibition by Ly-49A and activation by Ly-49D are shown at the right.

49A. Mutant E104G mediated Ly-49A–dependent inhibition but demonstrated less binding to 34-5-8S (Fig. 5 F), whereas W114L retained 34-5-8S binding but mediated decreased inhibition through Ly-49A (Fig. 5 L). Notably, single residue mutations in the α helices of H2-D^d preserved 34-5-8S binding, but failed to activate Ly-49D. Thus, the requirements for binding by 34-5-8S closely overlap with, but are not identical to, the requirements for recognition by Ly-49A. In contrast, the 34-5-8S epitope was not sufficient for recognition by Ly-49D.

Our studies can now be interpreted in light of the recent publication of the crystal structure of the Ly49A receptor bound to H2-D^d (19). In these studies, Ly-49A was observed to contact H2-D^d through two distinct interfaces. At the first site, predicted to be important in NK/target cell interactions, Ly-49A binds to one side of the exposed MHC I α-helices that form the peptide binding platform, but does not contact the peptide itself. Regions of Ly-49A involved in this site of H2-D^d interaction include the loop preceding the β3 strand, strand β4, and the connecting

loop to β5. Interestingly, this is a region where the sequences of Ly49A and Ly49D diverge, with differences at residues 234, 243, 244, and 248 (19). Notably, Ly-49D contains two large aromatic residues that are not found in Ly-49A: phenylalanine at 234 and tyrosine at 244. If Ly-49D interacts with H2-D^d at the same interface, the presence of these residues might destabilize binding by Ly-49D, rendering it more sensitive to slight alterations in H2-D^d.

The Ly-49A–binding site on H2-D^d includes regions in both the α_1 helix and α_2 helix adjacent to the N-linked glycosylation site at position 176. Within this site, allelic differences between H2-D^d and H2-D^b are found only at positions 50 and 169 (20). Notably these are both charged arginine residues in H2-D^d. We found that mutation R169H did not abrogate Ly-49A–mediated interactions (Fig. 2). The residue at position 50 also may not be critical individually, since a chimeric MHC with the entire α_1 region of H2-D^b in place of H2-D^d-α_1 still mediates inhibition through Ly-49A (9). Thus, although both residues may be important in recognition of H2-D^d by Ly-49A, the

allelic specificity may indeed depend on conformational interactions independent of the contact residues. In support of the latter, we found critical residues in H2-D^d that were located in the floor of the peptide binding groove.

Ly-49A recognition of H2-D^d depends on occupancy of the peptide binding groove, yet Ly-49A/H2-D^d interactions are sustained by divergent peptides bound to H2-D^d (7, 21). The functionally significant mutations we have identified in the floor of the peptide binding platform may alter the conformation of the platform itself either directly or through changes in bound peptide, possibly through alteration of the P3 binding pocket.

Interestingly, recent studies using soluble H2 tetramers have found that MHC I recognition by other inhibitory Ly-49 receptors is influenced by certain peptides (22), similar to findings regarding MHC I recognition by human KIR (23). Involvement of peptide in MHC I recognition by Ly-49D and other activating receptors has yet to be defined.

The crystal structure of Ly-49A and H2-D^d revealed a second site of interaction involving a cavity beneath the H2-D^d peptide binding platform that partially includes the CD8 binding site (19). There are no polymorphic H2-D^d residues in this contact region to account for the allelic specificity observed in Ly-49A interactions, but it remains possible that conformation of this region is affected significantly by distant polymorphisms. It was proposed that the second site of Ly-49A and MHC I interaction might be important in interactions on the NK cell itself (19). We did not address this hypothesis because our studies tested only interactions between Ly-49A and H2-D^d on different cells.

Our demonstration that Ly-49A and Ly-49D differ markedly in their requirements for binding to H2-D^d is consistent with evidence that activating and inhibitory receptors differ in their binding kinetics and affinities. Studies with soluble human KIR receptors revealed differences in the kinetics of MHC I binding, with activating receptors binding so weakly that rates were difficult to quantify (24–26). Recent studies were also unable to detect binding of activating Ly-49D to soluble tetrameric H2-D^d (22). Our finding that functional recognition of H2-D^d by Ly-49D is extremely sensitive to single amino acid changes in H2-D^d may reflect a relatively low-avidity receptor/ligand interaction that can be easily disrupted by subtle changes in H2-D^d. Alternatively, binding by activating receptors may require involvement of specific peptides or coreceptors.

Both activating and inhibitory receptors have been identified within each of the major MHC I binding NK receptor families (KIR, Ly-49, CD94/NKG2) in humans and rodents, suggesting an important role for these opposing molecules in balancing receptor function. Aside from H2-D^d, Ly-49D responds to other mouse MHC I as well as certain xenogeneic ligands (5, 13, 27). Thus, Ly-49D may recognize a diverse repertoire that only partially overlaps with inhibitory Ly-49 receptor ligands. Our demonstration that Ly-49D and Ly-49A differ in ligand recognition has implications regarding the integration of NK responses, because Ly-49A and Ly-49D receptors may be affected differentially by changes in MHC I antigens or, possibly, by peptides bound to MHC I during immune surveillance.

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