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Specificity and function of activating Ly-49 receptors

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Summary: Inhibitory Ly-49 receptors allow murine natural killer (NK) cells to kill cells with aberrant class I MHC expression while sparing normal cells. This is accomplished by their recognition of specific class I MHC products and prevention of NK-cell lysis of cells that present a normal repertoire of class I MHC ligands – "the missing self hypothesis". However, Ly-49 receptors that lack the cytoplasmic immunoreceptor tyrosine-based inhibitory motif, which is required for inhibition of killing, have also been described. These receptors were found to stimulate NK killing and are therefore referred to as activating Ly-49 receptors. Interestingly, the activating receptors have class I MHC-binding domains that are nearly indistinguishable from those of the inhibiting receptors, and binding to class I MHC has now been demonstrated for three activating receptors. Presently, there is no defined physiological role for activating Ly-49 receptors. Here we present an overview of current knowledge regarding the diversity, structure and function of activating Ly-49 receptors with a focus on class I MHC specificity, and we discuss their potential role(s) in natural resistance.

Introduction

Natural killer (NK) cells express receptors capable of binding classical class I MHC proteins and regulating NK-cell functions (1). In humans, the killer immunoglobulin-related (KIR) receptors mediate these functions, while in mice, Ly-49 proteins, which are type II transmembrane proteins of the C-type lectin superfamily, carry out these activities (2, 3). Only a single Ly-49 gene, *Ly-49L*, has been identified in the human genome. *Ly-49L* is unlikely to encode a functional protein since its transcripts are not appropriately spliced, resulting in a truncated protein product missing a large portion of the C-terminal class I MHC recognition domain (4). The *gp49* genes encode immunoglobulin-related receptors which show some structural homology with human KIR and are expressed by human and mouse NK cells (5–7); however, no KIR-related genes have been identified in the mouse genome. The KIR and Ly-49 receptors are capable of distinguishing individual classical class I MHC alleles (8, 9), while members of both receptor families can either positively or negatively regu-

late NK function; thus, despite major structural differences, Ly-49 and KIR receptor families are functionally equivalent.

The most characterized members of the Ly-49 family are the inhibitory receptors that include Ly-49A, C, and G (8, 10–15). Ly-49 inhibitory receptors engage class I MHC molecules on normal cells and negatively regulate NK function (8, 15–17). This type of inhibition is lacking when tumor cells or virally infected cells with downregulated class I MHC expression are encountered, and NK-mediated target cell lysis generally ensues. Such a function of inhibitory Ly-49 receptors is consistent with a role in NK-cell destruction of pathologically altered cells attempting to evade T-cell recognition by downregulating class I MHC expression and, in essence, fits the basic tenets of the "missing self" hypothesis first proposed by Ljunggren & Kärre for NK-cell surveillance (18).

Ly-49 receptors are expressed at the cell surface as transmembrane disulfide-bonded homodimers, with each (~40–45 kDa) subunit displaying a membrane proximal stalk region and a carboxy-terminal carbohydrate recognition domain (CRD) (12, 19, 20). Ly-49 receptors do not conserve the residues involved in Ca^{++} co-ordination and the classical carbohydrate binding site of typical C-type lectins; however, they may still bind carbohydrates (21). In the case of inhibitory Ly-49 receptors, each subunit, as with other inhibitory NK-cell receptors, contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) (2). Upon receptor engagement, the ITIM becomes phosphorylated on tyrosine, which in turn recruits src homology 2 domain-containing tyrosine phosphatase (SHP)-1 (2). The phosphatase activity of the SHP-1 is activated upon Ly-49 binding, and SHP-1 dephosphorylates tyrosine-phorphorylated proteins involved in the NK activation cascade, thus blocking the response (*Fig. 1*) (15). In contrast, a number of ITIM-lacking members of the Ly-49 family have been identified that, when engaged, activate NK cells to lyse target cells or produce cytokines (22–27). Activating Ly-49 receptors associate with a small (12 kDa) disulfide-bonded transmembrane homodimeric signaling adaptor protein, DAP12, through an intramembrane non-covalent charge interaction (28). Each DAP12 subunit contains an immunoreceptor tyrosine-based activating motif (ITAM). Upon engagement of an activating Ly-49 receptor, the ITAMs of the associated DAP12 become phosphorylated on tyrosine and recruit the Syk tyrosine kinase, which in turn initiates a cascade of signaling events leading to NK activation (*Fig. 1*) (29). It appears that during NK contact with healthy cells, inhibitory receptor signals are dominant over activating receptor signals, preventing autoaggression and thus maintaining NK self tolerance (2, 30).

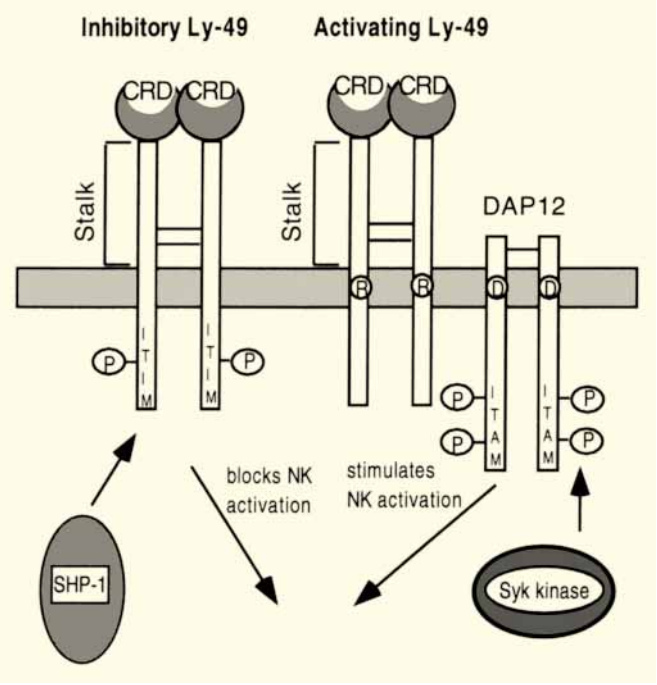

***Fig. 1.* Contrasting signaling activities are associated with inhibitory and activating Ly-49 receptors.** Upon engagement, inhibitory Ly-49 receptors recruit and activate the SHP-1 tyrosine phosphatase, which in turn inhibits NK activation. In contrast, engagement of activating Ly-49 receptors results in recruitment and activation of Syk kinase, leading to NK activation.

A striking property of most NK receptor families, and the Ly-49 family in particular, is the close homology in external domains between many inhibitory and activating receptors within the same gene family, implying that they may have the same or similar ligands, although opposing functions (31). The existence of activating receptors related to MHC-recognizing inhibitory receptors would not be readily predicted by the missing self hypothesis for NK surveillance (18). In this review we explore the diversity, expression, ligand specificity and activities of mouse activating Ly-49 receptors and attempt to understand their role(s) in NK-cell regulation.

Genetic diversity in the Ly-49 family

The *Ly-49* genes compose a large multigene family. From genomic analyses and sequence determination of unique Ly-49 transcripts, it is possible that 20 or more *Ly-49* genes exist within the mouse genome (13, 26, 32–38). Final determination of the number of *Ly-49* genes, as opposed to highly divergent alleles, will require extensive genomic analysis in multiple inbred mouse strains. Of the existing transcripts with substantial sequence differences, 13 are inhibitory or

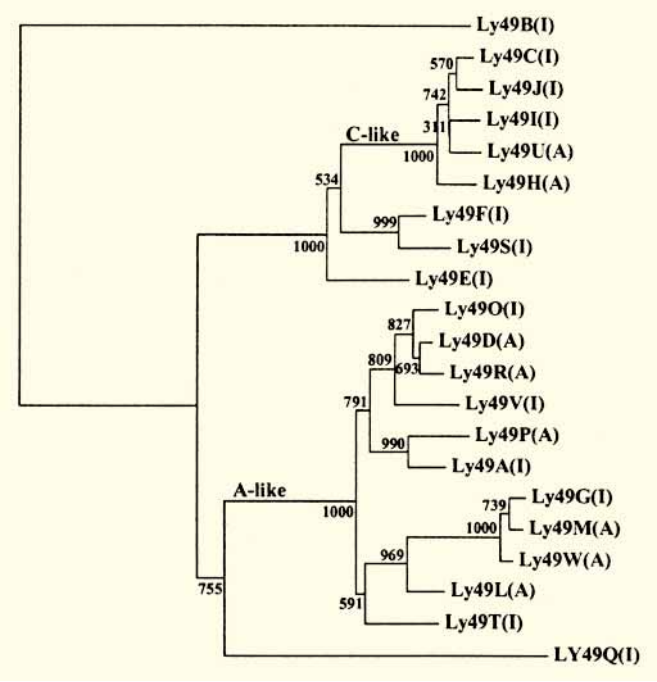

***Fig. 2.* Phylogenetic relationship of the mouse Ly-49 receptor CRDs.** DNA sequences encoding the CRD (exons 5–7) of each Ly-49 receptor type were aligned with CLUSTALW (70), and a bootstrapped phylogeny was calculated. The dendrogram was created by the PHYLO_WIN program (71). It shows the tree with bootstrap values at each node. The bootstrap values indicate how many out of 1,000 bootstrap trials support the given tree. High numbers indicate well-defined subfamilies. (A) or (I) appended to the Ly-49 receptor name indicates whether it is activating or inhibitory in function, respectively. Ly-49K and Ly-49N receptors are not included since only partial CRD sequences are available and no expression of a functional transcript has been demonstrated. Both of these receptors are closely related to Ly-49C and they have N-terminal sequences indicative of activating receptors. Ly-49 members composing this phylogenetic tree are derived from transcripts identified from a number of different mouse strains, and not all Ly-49 members are expressed in a specific inbred mouse strain. The GenBank accession codes of the sequences are as follows: Ly-49A (AF074456), Ly-49B (U10304), Ly-49C (U56404), Ly-49D (AF218078), Ly-49E (U10091), Ly-49F (U10092), Ly-49G (AF074457), Ly-49H (U12889), Ly-49I (AF237686), Ly-49J (AF110492), Ly-49L (AF204266), Ly-49M (AF283252), Ly-49O (AF146571), Ly-49P (AF074458), Ly-49Q (AB033769), Ly-49R (AF288377), Ly-49S (AF288378), Ly-49T (AF288379), Ly-49U (AF288380), Ly-49V (AF288381) and Ly-49W (AF074459).

likely to have that function based on the presence of an ITIM sequence in their cytoplasmic domain, including Ly-49A, B, C, E, F, G, I, J, O, Q, S, T and V, while eight are activating or may have that function based on the presence of a charged residue in the transmembrane segment allowing DAP12 association, including Ly-49D, H, L, M, P, R, U and W. Inhibitory Ly-49 receptors share substantial homology in cytoplasmic and transmembrane domains, and the same relationship is true for activating Ly-49 receptors as a separate group. Activating Ly-49 receptors have external domains, particularly CRDs, which are highly homologous to those of one or more inhibitory Ly-49 receptors. These relationships are readily observed in a phylogenetic tree constructed using nucleotide sequences encoding exons 5, 6 and 7, which constitute the CRD of known Ly-49 receptors (*Fig.* 2). For example, the CRDs of the activating receptors Ly-49M and Ly-49W are highly homologous to that of the inhibitory Ly-49G receptor. Other similar pairings include Ly-49A/P, Ly-49O/D and Ly49C,I,J/H,U (*Fig.* 2). These comparisons suggest that non-homologous recombination has occurred in the *Ly-49* gene family to generate inhibitory and activating *Ly-49* genes with very similar CRDs, and therefore, possibly ligand specificities. In addition, evidence for exon replacement/exchange in activating *Ly-49* genes or alleles has been found recently (38). In the case of Ly-49L and W, their amino acid sequences are identical in exons 2 to 5, but amino acids encoded by exons 6 and 7 are highly divergent (38). These differences may have substantial functional significance, since they occur in an area where receptor specificity is likely to reside. An examination of the dendrogram indicates that activating receptors are only found in the A-like and C-like clusters (*Fig.* 2): no activating receptors resembling Ly-49B, Q or the E/F/S cluster of inhibitory receptors, have been described. The significance of these apparent differences within the Ly-49 family is unclear. It is still possible that activating counterparts of the latter Ly-49 receptors do exist, but are yet to be identified.

The nucleotide sequence relationships of *Ly-49* genes suggest that they are rapidly evolving, but the molecular basis of *Ly-49* gene diversification is not fully understood. It appears that significant diversification has occurred in inhibitory and activating *Ly-49* genes through gene duplication and by undetermined forms of non-homologous gene recombination events and/or gene conversion (26, 36, 38). Similar mechanisms also appear to shape the genes encoding the functionally equivalent human KIR receptors (39–41). Determination and analysis of multiple complete *Ly-49* gene sequences will be needed to gain further insight into possible mechanisms of *Ly-49* gene diversification. Further studies will also be required to understand what may be responsible for *Ly-49* diversification. Properties of *Ly-49* genes themselves and/or adaptive selection processes may promote diversification. Should selection play a role in Ly-49 diversification it may relate to keeping pace with MHC polymorphism and/or responding to viral adaptation.

***Table 1.* Ly-49 expression by strain**

Strain	C57BL/c	BALB/c	NOD	129/J	129/SvJ	C57L/J	C57BL/10J	NZB	CBA/J
MHC haplotype	H-2^b	H-2^d	H-2K^d/D^b	H-2^b	H-2^b	H-2^b	H-2^b	H-2^d	H-2^k
Receptor[1,2]									
Ly-49A	✓	✓	✓						
Ly-49B	✓	✓	✓						
Ly-49C	✓	✓			✓				✓
Ly-49D	✓		✓						
Ly-49E	✓		✓	✓					
Ly-49F	✓								
Ly-49G	✓	✓	✓	✓			✓		
Ly-49H	✓								
Ly-49I	✓				✓			✓	✓
Ly-49J	✓								
Ly-49K	*								
Ly-49L	*	✓							
Ly-49M	*		✓						
Ly-49N	*								
Ly-49O				✓		✓			
Ly-49P			✓	✓					
Ly-49Q	✓								
Ly-49R				✓					
Ly-49S				✓					
Ly-49T				✓					
Ly-49U				✓					
Ly-49V				✓					
Ly-49W			✓						

[1] Activating Ly-49 receptors and their expression are indicated in bold.
[2] A blank indicates untested or unreported except in the case of Ly-49P and W, which are not expressed in the C57BL/6 mouse. An asterisk indicates no viable full-length transcripts detected. Some nucleotide sequences originally reported to be from Ly-49C, are likely to be from Ly-49I due to higher sequence homologies and are indicated as Ly-49I in the table.

Expression of Ly-49 activating receptors

Preliminary RT-PCR analyses indicate that *Ly-49* gene expression by NK cells differs between inbred mouse strains (*Table 1*) (26, 35–38). These results are consistent with strain-specific differences in Ly-49 expression suggested by flow cytometric analysis with Ly-49-specific monoclonal antibodies (42). All strains express a combination of inhibitory and activating Ly-49 receptors, but surprisingly, the specific set of expressed receptors appears to be strain dependent. For example, the inhibitory Ly-49A receptor is expressed by C57BL/6 and various other strains; however, its expression is apparently lacking in the 129 strain (35). Similarly, expression of the Ly-49W activating receptor is readily detected in the non-obese diabetic (NOD) strain but not in the C57BL/6 strain (38). Thus, it seems that inbred mouse strains express partially overlapping repertoires of Ly-49 receptors. Interpretation of RT-PCR results is subject to several limitations, including the potential inability to detect gene expression due to gene polymorphism. However, differential or strain-specific expression of viable transcripts is particularly evident for activating Ly-49 receptors (*Table 1*). An understanding of the molecular basis of strain-specific activating Ly-49 expression is now clear for at least one receptor, Ly-49M. *Ly-49M* was originally identified in the C57BL/6 mouse from genomic fragments (33). A cDNA encoding an apparent allele of *Ly-49M* has recently been cloned from the NOD mouse strain (38). In NOD NK cells, *Ly-49M* is expressed as a viable transcript encoding a full length protein, whereas full-length Ly-49M protein cannot be expressed in the C57BL/6 strain due to the presence of a premature stop codon near the predicted beginning of exon 4 (38). Gene/gene segment deletion or differential regulation of *Ly-49* gene expression at the transcriptional level may also contribute to differences in expression of individual Ly-49 receptors observed between inbred mouse strains, although these possibilities remain to be explored.

NK cells are heterogeneous with respect to expression of individual Ly-49 receptors, with NK subsets expressing par-

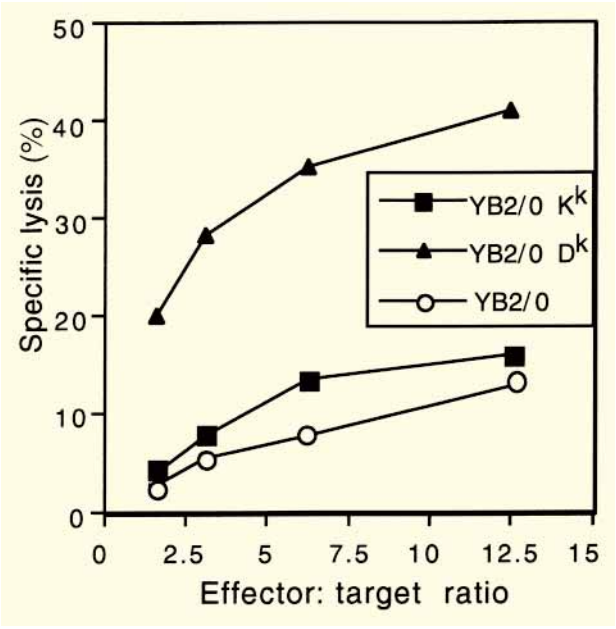

***Fig. 3.* Ly-49W activates NK-cell-mediated cytotoxicity by recognizing H-2D^k.** The Ly-49W-transfected rat NK-cell leukemia, RNK-16, was incubated at the indicated effector to target cell ratios with the rat YB2/0 cell line, YB2/0 transfected with H-2D^k, or YB2/0 transfected with H-2K^k. Cytotoxicity was measured after 4 h. Data are the means of triplicate wells.

tially overlapping repertoires of multiple Ly-49 receptors. It has been demonstrated that the percentage of NK cells expressing any two inhibitory receptors can be predicted from the product of their frequencies within the entire NK-cell population (the product rule) (43). This relationship suggests that the expression of Ly-49 inhibitory receptors is stochastic in nature. In contrast, recent evidence suggests that expression of activating Ly-49 receptors is not stochastic, since it appears that there is preferential co-expression of multiple activating Ly-49 receptors on the same subsets of NK cells (27). These investigations were limited to an examination of Ly-49D and H expression; similar co-expression studies with a number of additional activating Ly-49 receptors will be necessary to confirm such a non-stochastic relationship. Preferential co-expression of Ly-49 activators could have functional significance, since it may allow simultaneous recognition of multiple and perhaps distinct ligands of low density or low affinity by a single NK cell. This could allow for combined interaction above a threshold level that is sufficient for activation, which is not reached with engagement of a single ligand. A separate study has found that expression of the activator Ly-49D is also non-stochastic, but in another sense: it is co-expressed disproportionately on NK-cell subsets that also bear inhibitory receptors Ly-49A and/or G, which share class I MHC ligand specificity (H-2D^d) with Ly-49D (44). The functional significance of this type of co-expression will need to be determined, particularly since this relationship is reported in a mouse strain that does not express H-2D^d (44). It is perhaps possible that co-ordinated expression of specific Ly-49 activators and inhibitor genes is determined by a specific developmental selection process or by co-ordinated gene promoter regulation.

The temporal pattern of activating Ly-49 receptor expression could provide clues to their function. The expression and function of two activating Ly-49 receptors, Ly-49D and H on NK cells, in relation to ontogeny (birth through the first few weeks of age) were recently reported (27, 44). Although there are some discrepancies in these reports, expression of Ly-49D and H appear to initiate soon after birth and gradually increase, generally similar to the temporal expression pattern of inhibitory Ly-49 receptors. The two Ly-49 activating receptors were also shown to be functional as soon as they were expressed (44). These results suggest that the expression of activating and inhibitory Ly-49 receptors is not temporally dissociated. Thus, there is no evidence to suggest that prior expression of Ly-49 activators occurs or is necessary for expression of the inhibitory Ly-49 receptor repertoire by NK cells. This same conclusion was reached based on the normal inhibitory Ly-49 receptor repertoire expressed on NK cells of mice with non-functional DAP12 (45).

To understand fully the expression patterns and functions of activating Ly-49 receptors (as well as inhibitory Ly-49s) will require the development of Ly-49 receptor-specific antibody reagents. Many existing antibodies, such as A1, YE1/48, and 4D11, thought to be specific for inhibitory Ly-49 receptors such as Ly-49A and G, actually cross-react with a number of Ly-49 activating receptors, including Ly-49L, P and W (26, 35, 37, 38). Therefore, antibody staining with these reagents does not allow expression of an inhibitory (or activating) Ly-49 receptor to be assumed. Generation of monoclonal antibodies that are specific for L, P, W and other newly identified Ly-49 activating receptors should greatly enhance our ability to understand their expression and functions.

Class I molecules are ligands for Ly-49 activating receptors

It is well established that class I MHC molecules are ligands for inhibitory Ly-49 receptors. For example, it has been demonstrated that Ly-49A recognizes H-2D^d and D^k, while

Ly-49G binds D^d, D^r and possibly an H-2^k product, and Ly-49C recognizes a fairly broad spectrum of class I MHC molecules (10, 11, 24, 46). Nakamura et al. first demonstrated that a Ly-49 activating receptor, Ly-49D, can recognize H-$2D^d$, leading to NK activation and target cell lysis (23). Ly-49D, like the inhibitory Ly-49 receptors studied, is allele specific in its recognition of class I ligands in that D^d, D^r and D^{sp2} serve as ligands, but other allele products such as D^b do not (24). More recently, we demonstrated that Ly-49P and Ly-49W also recognize class I MHC ligands and activate NK-cell-mediated lysis. Like Ly-49D, Ly-49P recognizes D^d, but also weakly interacts with D^k, similar to its inhibitory counterpart Ly-49A (26). In contrast, Ly-49W readily recognizes D^k (*Fig.* 3) and, to a lesser extent, D^d (38). Ly-49P and W do not recognize a number of other class I allele products (26, 38), indicating that they, like Ly-49D, are allele specific in their class I MHC recognition. It remains to be determined whether other Ly-49 activating receptors such as Ly-49H, L and U interact with class I MHC proteins. However, given the strong sequence conservation in CRD domains, it is likely that class I MHC binding is a feature shared by most if not all Ly-49 receptors.

Molecular basis of activating Ly-49 interactions with class I ligands

The co-crystal structure of the inhibitory Ly-49A receptor bound to D^d (47) may serve as a model for interactions of all Ly-49A-like receptors with their ligands (*Fig.* 2). The co-crystal structure, solved using engineered, bacterially expressed truncated forms of Ly-49A and D^d, indicates that Ly-49A can bind D^d at two distinct sites. Site 1 encompasses areas on the N terminus and C terminus of the D^d α1 and α2 domains, respectively (47). A second site of interaction on D^d was identified, which involves residues from all three class I heavy chain domains as well as β2 microglobulin. These residues line the cavity below the peptide-binding platform and partially overlap with the site for CD8 binding by T cells (47). In one study, site-directed mutagenesis of D^d involving single point mutants in site 1 or site 2 supports the involvement of site 2 residues in Ly-49A interaction, measured in a direct binding assay using Ly-49A tetramers and functional assays using Ly-$49A^+$ NK cells (48). In another study that focused on possible site 1 interactions, a single amino acid change of a non-contact residue, position 52 in D^d, resulted in reduced binding affinity of Ly-49A, possibly by altering the conformation of site 1 residues such as Q54 (49). The residues in site 2 that were identified to be important for Ly-49A binding are non-polymorphic and are thus shared by class I MHC allele products whether they are Ly-49A ligands or not. Therefore, the identified site 2 residues may contribute to Ly-49A-D^d binding affinity but do not directly determine the allele specificity of Ly-49A interaction. The class I MHC residues determining the allele specificity of Ly-49A interaction are not determined, but they may be near to or affecting the conformation of site 2, in or near site 1, or at additional polymorphic sites.

Ly-49P and Ly-49W display subtle differences in their fine specificities for class I MHC ligands. In NK functional assays, Ly-49P, like its inhibitory counterpart Ly-49A, interacts strongly with D^d and weakly with D^k, while Ly-49W interacts weakly with D^d and strongly with D^k (26, 38). An examination of sequence differences between D^d and D^k in the context of the Ly-49A-D^d co-crystal structure indicates that only five polymorphic residues are located in the Ly-49-MHC contact interface (S2, E104, K173, N174 and E227 in D^d and P2, G104, E173, L174 and D227 in D^k. Since Ly-49A and P are predicted to use identical residues to interact with class I MHC, we can use the co-crystal structure directly to interpret the specificity determinants of Ly-49P. For Ly-49W, we can use the co-crystal structure as a template to study the effects of its deviations from the Ly-49A sequence. Positions 2, 104 and 227 are located in the site 2 interface, whereas positions 173 and 174 mediate interactions at site 1. Of these residues, only positions 104, 173 and 174 interact with residues of the Ly-49 receptor that are not conserved between Ly-49P and W. The glutamate at position 104 of D^d forms a salt bridge with arginine 223 on Ly-49P. This interaction cannot be formed with D^k, due to the substitution of glycine. This could explain the preferred binding of Ly-49P to D^d. However, mutagenesis data argue against an important role for Glu104 in the interaction of D^d with Ly-49A and presumably Ly-49P (50). In Ly-49W, Arg223 is replaced by the much smaller threonine residue, which can no longer interact with D^d or D^k. Thus, the polymorphism at position 104 in site 2 cannot explain the preference of Ly-49W for D^k over D^d. Accordingly, although there is evidence for a role of site 2 in Ly-49A binding and cytotoxicity (48), it is not clear that site 2 confers the class I specificity of Ly-49 receptors. In site 1, Lys173 of D^d points into a negatively charged pocket formed by Asp241 and Asn242 of Ly-49P (*Fig.* 4*A*). This favorable interaction is not possible with D^k due to the substitution of Lys173 by glutamate. Similarly, Asn174 of D^d makes a hydrogen bond to the Gln273 of Ly-49P that would not exist in D^k due to the Asn174 to Leu substitution. These differences, especially the Lys173 to Glu substitution, could explain the

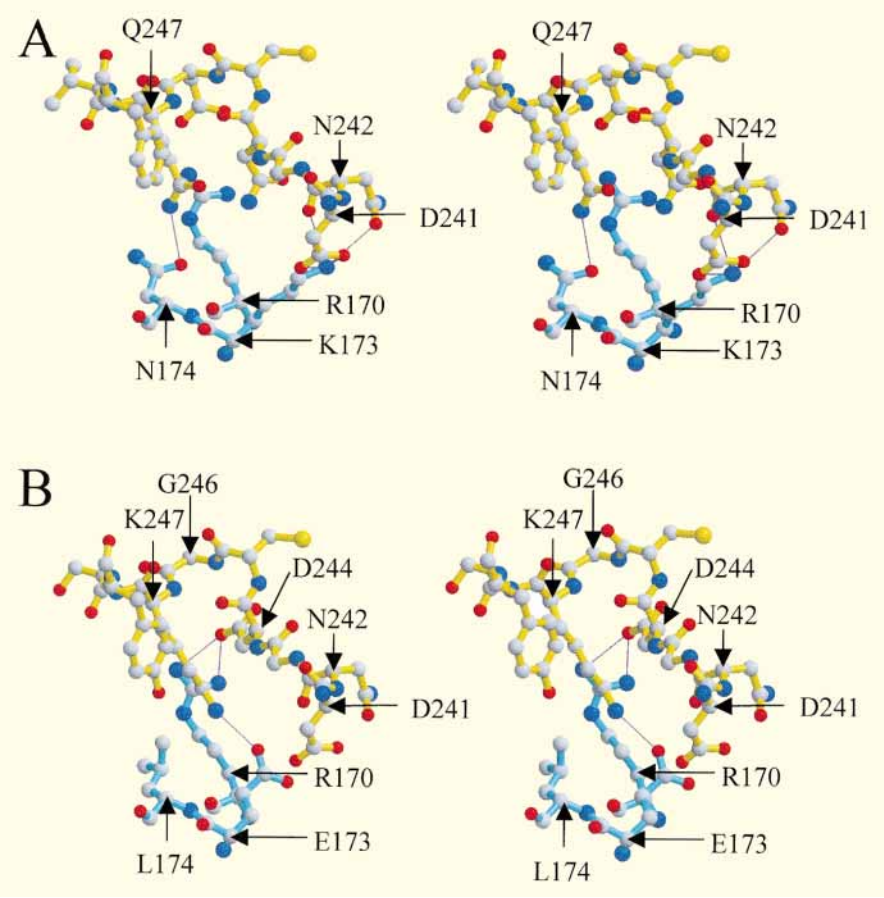

***Fig. 4.* Possible class I MHC specificity-defining determinants in Ly-49P and Ly-49W.** Class I MHC and Ly-49 residues are shown with cyan and yellow bonds, respectively. Important hydrogen bond and/or salt bridge interactions are highlighted with thin purple lines. **A. Stereodiagram of the Ly-49P/D^d interface in the site 1 contact.** Lysine 173 and asparagine 174 of the D^d heavy chain both make hydrogen bonds to Ly-49P (as with Ly-49A). Note the extensive interactions of lysine 173, including a salt bridge with aspartate 241 of Ly-49P. **B. Stereodiagram of a model of the Ly-49W/D^k interface in the site 1 contact.** The substitution of lysine 173 in D^d by glutamate in D^k is compensated by the substitution of glutamine 247 in Ly-49P/A by lysine in Ly-49W, leading to the formation of a new salt bridge. Aspartate 244 of Ly-49W (asparagine in Ly-49P/A) adopts a new sidechain conformation to create space for the lysine 247 sidechain and to reduce charge repulsion with glutamate 173 of D^k. Space for this sidechain rearrangement is created by the substitution of aspartate 246 in Ly-49P/A by glycine in Ly-49W. In its new conformation, aspartate 244 of Ly-49W functionally replaces aspartate 246 of Ly-49P in forming the salt bridge with arginine 170.

reduced affinity of Ly-49P for D^k. In Ly-49W, five positions involved in the site 1 interface are polymorphic with Ly-49P, all in the hexapeptide 244–249 (NcDQVF in Ly-49P and DcGKSY in Ly-49W). The substitution of lysine at position 247 (glutamine in Ly-49P) is of particular interest, as it can specifically interact with Glu173 of D^k. Using the program Xfit (51), we could change Gln247 of Ly-49A/P and Lys173 of D^d in the crystal structure to lysine found in Ly-49W and glutamate found in D^k, respectively. Using preferred side chain rotamers, the two oppositely charged sidechains could be brought to within 3.5 Å of each other (*Fig. 4B*). It is possible that this new salt bridge contributes to the preferred binding of Ly-49W to D^k. The Asp246 to glycine substitution, which is unique to Ly-49W, may be a further adaptation to stabilize this salt bridge (*Fig. 4B*). The Asp246 of Ly-49A/P forms a salt bridge with Arg 170 of D^d in the crystal structure. In Ly-49W, this interaction would be lost, but Asp244 (Asn244 in Ly-49A/P) can alter its sidechain conformation so that it becomes the functional equivalent of Asp246 in Ly-49A/P. This creates space for the side chain of Lys247 and avoids electrostatic repulsion between Asp244 and Glu173 of D^k (*Fig. 4B*). Site-directed mutagenesis studies and a co-crystal structure of Ly-49W bound to D^k can determine the accuracy of these predictions.

Using mutagenesis and NK functional assays, Nakamura et al. (50) showed that mutations in the α1 and α2 domains of D^d destabilize binding by Ly-49D to a much larger extent than an inhibitory counterpart, Ly-49A. This could be due to a lower affinity of Ly-49D for D^d, leading to less tolerance for small changes in the D^d ligand, or perhaps a significant difference in the structural requirements for Ly-49D and possibly other activators compared to related inhibitory receptors. Also unresolved is whether the nature of bound peptides influences interactions of activating Ly-49 receptors with class I ligands. The Ly-49A and C inhibitory receptors differ in their requirements for class I ligand binding. Ly-49A interacts with D^d bound to any peptide ligand (52), whereas Ly-49C appears to be sensitive to the nature of bound peptide (53), or may prefer binding to peptide-receptive class I ligands (54). Related activating Ly-49 receptors may display similar requirements, but this remains to be determined. Furthermore, the possible function of class I MHC carbohydrate structures in

interactions of inhibiting and activating Ly-49 receptors with class I ligands remains unresolved. Inhibitory Ly-49 receptors can bind mutagenized class I ligands that lack N-linked carbohydrate (11, 21). Ly-49 receptors are also capable of interacting directly with carbohydrate structures, and modification of carbohydrates on class I ligands can reduce the level of Ly-49 receptor interaction (21, 55–57). It is possible that the class I carbohydrate moieties contribute to the affinity but not necessarily the specificity of Ly-49 interaction.

Several questions remain with respect to how activating (and inhibitory) Ly-49 receptors interact with their class I ligands, not the least of which is whether co-crystal site 1 or site 2 is determining allele specificity of Ly-49 interactions *in trans*, i.e. where Ly-49 expressed on an NK cell interacts with a class I ligand on a potential target cell.

Function of Ly-49 activators

The role of class I MHC-recognizing Ly-49 activating receptors in murine NK-cell regulation is unknown. Their existence is not readily predicted by the missing self hypothesis, at least in its current form (18). For example, it is clear that NK cells express a variety of activating receptors specific for non-MHC ligands that could function in concert with class I-specific inhibitory receptors to identify pathologically altered cells with a global or allele-specific downregulation of MHC expression (58, 59). Class I MHC-recognizing activating receptors would not appear to be necessary for this type of surveillance. One possible role for class I MHC-recognizing Ly-49 activating receptors is the enhancement of inhibitory signals delivered through co-expressed inhibitory receptors. This may be achieved by activating Ly-49 receptors increasing the avidity of target cell interaction using their external domains and/or enhancing the recruitment of SHP-1 or associated signaling, as a result of transmembrane signals delivered through the associated ITAM-containing DAP12 adapter protein (*Fig. 5A*). Such an enhancement of inhibitory signals could occur whether the activating Ly-49 receptor has the same or different class I allele product specificity of the co-expressed inhibitory receptor(s), as long as class I ligands for both the activating and inhibitory receptors are expressed on the same target cell (*Fig. 5A*). It remains to be determined whether such a function is applicable to activating Ly-49 receptors.

A second possible function of activating Ly-49 receptors is that they act in conjunction with class I-recognizing inhibitory receptors to detect the loss of expression of a subset of class I alleles or only a single class I MHC allele (*Fig. 5B*).

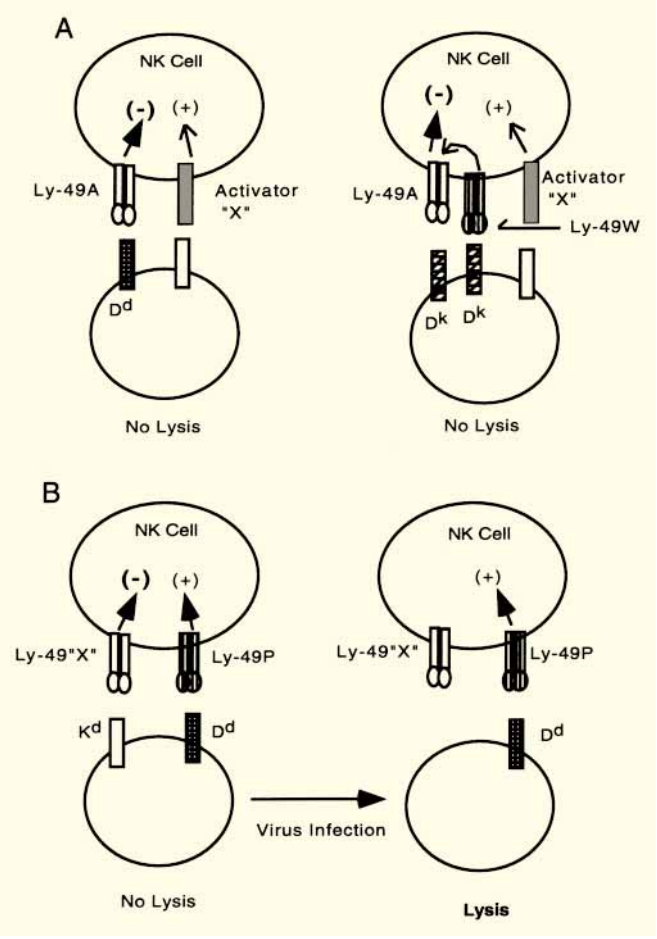

Fig. 5. Models of possible activating Ly-49 receptor functions. **A.** One possible function of activating Ly-49 receptors is to enhance the inhibition signal induced by co-expressed inhibitory receptors. In the case of a high affinity interaction such as Ly-49A and D^d, leading to efficient signaling, this would be unnecessary. However, it is possible that inhibitory receptor interactions with lower affinity ligands, such as might be the case between Ly-49A and D^k, may not always efficiently downregulate NK functions. Activating Ly-49 receptors, such as Ly-49W, may augment the inhibitory response of a co-expressed inhibitory receptor, through enhanced avidity of interaction with target class I ligands and/or impacts on signal regulation by the inhibitory receptor. **B**. A second possibility is that activating Ly-49 receptors, such as Ly-49P, activate NK cells by engaging remaining class I allele products, e.g. D^d, when inhibition through a hypothetical inhibitory Ly-49 receptor "X", for example, is lost due to selective downregulation of its distinct class I ligand K^d.

This function would require that the activating Ly-49 receptor recognize a self class I allele distinct from those recognized by co-expressed inhibitory receptors and would result in class I MHC-dependent activation of the NK cell. In such a role, the activating Ly-49 receptor would be functional in the absence of adequate NK inhibitory receptor engagement and in this sense would function in a similar manner to non-MHC-

specific activating receptors. One notable difference, however, is that activating Ly-49 receptors are capable of recognizing constitutively expressed classical class I MHC molecules, whereas the ligands of a major and broadly expressed NK activating receptor, NKG2D, are induced by stress-related stimuli (60–62). The presence of activating Ly-49 receptors would bypass a strict requirement for *de novo* synthesis of NK-cell activating ligands. The ability to activate NK cells in response to the loss of a single class I allele could be necessary to combat viruses of the type that downregulate expression of only one or a subset of class I alleles or products (63). Some viruses could be selective in targeting the expression of class I molecules in instances where only one or a subset of class I molecules may be involved in presentation of dominant antigen epitopes of that virus to T cells. However, if the virus allows expression of the remaining cohort of class I allele products on the infected cells, protection from attack by many NK cells could still be possible through inhibitory receptor engagement. Some subsets of NK cells may express an activating Ly-49 receptor that recognizes one of the remaining class I allele products and become activated. This would be effective with NK cells that lack inhibitory receptor engagement, as a result of the selective loss of class I allele expression on the virally infected cells (*Fig* 5B). The ability to detect selective alterations in class I allele product expression, potentially offered by activating Ly-49 receptors, is in apparent contrast with CD94/NKG2 receptors. The heterodimeric CD94/NKG2A inhibitory receptor may generally recognize global loss of class I expression (particularly in outbred mice). This may be the case since the Qdm peptide (which when bound to Qa-1^b forms the ligand of mouse CD94/NKG2 receptors), is derived from leader sequences of multiple class I MHC allele products (41, 64–66). Additional experiments examining NK function in the context of viral challenge will be necessary to determine whether Ly-49 activating receptors are involved in activation of NK cells when a cell loses expression of a single class I MHC allele or subset of alleles.

Should Ly-49 activating receptors function as enhancers of inhibitory receptor function, then it is conceivable that this activity would be favored by preferential co-expression of Ly-49 activating and inhibitory receptors with the same class I MHC specificity on the same NK-cell subsets, as suggested by the preliminary results of Ortaldo et al. (44). In contrast, if activating Ly-49 receptors activate NK cells in the context of class I allele loss, then stochastic expression of activating and inhibitory Ly-49 receptors would presumably maximize this function by enhancing the probability of obtaining NK subsets co-expressing an activating receptor and an inhibitory receptor that recognize different self class I alleles. In either event, such functions are dependent on the expression of activating Ly-49 receptors that recognize self class I molecules. In the inbred mouse strains examined thus far, however, only alloreactive activating Ly-49 receptor expression by NK cells has been observed (23, 24, 26, 30, 38). It is unclear whether the lack of self MHC-reactive Ly-49 activating receptor expression is a fortuitous consequence of inbreeding or has functional significance. The latter raises other possibilities. For example, although activating Ly-49 receptors are capable of recognizing native class I molecules, they may bind with greater affinity class I molecules that have been specifically altered in pathological circumstances, or virally encoded MHC homologs (67, 68). The observations that the Ly-49D activating receptor may differ in its requirements for interaction with class I ligands compared to an inhibitory counterpart, and activating NK receptors appear to have substantially lower affinities for class I ligands also recognized by corresponding inhibitory receptors (50, 69), may be consistent with such possibilities.

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