Cross-Species Dependence of Ly49 Recognition on the Supertype Defining B-Pocket of a Class I MHC Molecule

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Cross-Species Dependence of Ly49 Recognition on the Supertype Defining B-Pocket of a Class I MHC Molecule

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Ly49 recognition of MHC class I (MHC I) can be allele specific. However, the site of interaction on MHC I consists of highly conserved solvent-exposed amino acids, leaving it unclear how allele specificity occurs. In examining the specificity of mouse and rat Ly49, we noticed that MHC I ligands for mouse Ly49G and W, and the rat Ly49i2, typically share the HLA-B7 supertype, defined by a B-pocket that prefers a proline at position 2 in bound peptides. Through mutagenesis, we show that the supertype-defining B-pocket of RT1-A1ε controls its allele-specific recognition by the syngeneic rat Ly49i2 inhibitory receptor and xenogeneic mouse inhibitory Ly49G and activating Ly49W receptors. Single amino acid substitutions in the B-pocket that did not prevent peptide binding disrupted Ly49 recognition. In contrast, single mutations in other regions of the peptide-binding groove had no effect. We provide a model whereby the B-pocket dictates the conformation of conserved residues at the Ly49 interaction site below, defining Ly49 allele specificity for MHC I. Therefore, at least some Ly49 may recognize supertypes, detectable even across species, and are sensitive to polymorphisms in the supertype-defining B-pocket. This would ensure that expression of specific MHC I supertypes capable of Ag presentation to T cells is sensed by NK cells, and if lacking, targets a cell for elimination, suggesting a supertype-mediated link between innate and adaptive immunity. The Journal of Immunology, 2006, 177: 8578–8586.

N atural killer cells are large granular lymphocytes that play an important role in innate immunity against transformed and virally infected cells through both cytolytic and cytokine-mediated effector responses (1). Murine and human NK cells express a number of receptors on their cell surfaces, including Ly49 and killer cell Ig-like receptors (KIR), respectively. Although structurally distinct, both of these receptor types are polygenic and have activating and inhibitory members capable of recognizing MHC class I (MHC I) proteins (2, 3). Inhibitory receptors function according to the “missing self hypothesis,” surveying potential target cells for expression of self MHC I molecules and transmitting inhibitory signals that prevent NK cell activation and target cytolysis (4). In rodents, upon loss of MHC I expression, due to virus infection or cell transformation, inhibitory Ly49 may not be engaged and lysis and cytokine production can occur through engagement of activating Ly49 or other activating NK cell receptors (5). In addition to MHC I, a virally encoded MHC I homolog was shown to be a ligand for both activating and inhibitory Ly49 (6, 7).

Ly49 are MHC I allele specific, each having specificity for a small repertoire of MHC I alleles that are generally recognized in a peptide-independent manner (8–15). What determines allele specificity of Ly49 is not understood. The original cocrystal of Ly49A and its ligand H-2D^d showed two potential interaction sites on the MHC I molecule (16) (see Fig. 5A). Site 1 is located at one end of the polymorphic peptide-binding groove and was initially favored as the interaction site, conferring allele specificity for Ly49 recognition (16). Later, site 2 was shown to be the main interaction site through mutagenesis of single solvent-exposed residues under the peptide-binding platform and in the α3 domain of the MHC I H chain, as well as residues within β2-microglobulin (17, 18). Because site 2 is so highly conserved between different MHC I alleles, it is difficult to explain allele specificity by Ly49 at this recognition site.

Murine and primate (including human) MHC I alleles can be classified into about nine different supertypes based on their preference for specific amino acid residues within the bound peptide, which act to anchor it within the peptide-binding groove, in specific anchoring pockets (19). The equal distribution of supertypes across species and even human ethnicities emphasizes the importance of supertypes for the effective function of the immune system against invading pathogens (20–22). Previously, we identified a xenogeneic rat MHC I ligand, RT1-A1ε, for two mouse Ly49, the activating Ly49W and the BALB/c allele of the inhibitory Ly49G (23). The RT1-A1ε molecule is also the natural ligand for the syngeneic inhibitory rat Ly49i2 (24). We noticed that there was a common supertype between MHC I alleles that was recognized by these two mouse Ly49 and the rat Ly49i2. Possibly then, the distinctive molecular characteristics associated with this supertype could be critical for recognition by these rodent Ly49.

Through single amino acid mutations of RT1-A1ε, we show here that both xenogeneic mouse and syngeneic rat Ly49 recognition are exquisitely sensitive to even conservative changes in polymorphic residues within the supertype defining B-pocket. In contrast, nonconservative single mutations within the peptide-binding groove, but outside of the B-pocket, have no effect. We also show that both rat syngeneic and mouse xenogeneic Ly49 recognition occurs at site 2, in a manner analogous to mouse syngeneic Ly49 recognition. Crystal structure conformations of rodent MHC I alleles indicate that alterations in the B-pocket may result in changes in the conformation of solvent-exposed residues at...
site 2, that articulate with the B-pocket, thereby affecting Ly49 recognition. That mouse Ly49 recognize a MHC I supertype across species indicates that this may be a fundamental property of Ly49 interaction with MHC I that transcends species-specific recognition, in that the supertype defining pocket(s) of MHC I offers identity to NK cells of innate immunity, while preserving conformations essential to MHC restriction for TCR interaction and adaptive immunity.

Materials and Methods

Hybridomas and mAbs

The hybridoma producing the Ab 4D11 (rat IgG2a), anti-Ly49GLBALB/c (9), was obtained from American Type Culture Collection. The Cw3-1 (IgG1), anti-Ly49W hybridoma was generated in this laboratory (25). Abs were prepared from ammonium sulfate precipitates as described (11). Purified STOK2 (rat IgG2a), anti-Ly49I2 (24, 26) Ab was purchased from BD Biosciences/BD Pharmingen. The RT1-A1R reactive YR5/12 (rat IgG2b) hybridoma supernatant (27) was purchased from Serotec.

Cell lines

YB2/0, a nonscreening rat myeloma, was obtained from American Type Culture Collection. The YB2/0 cell line was maintained in DMEM supplemented with 10% FCS, L-glutamine, penicillin, streptomycin, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids. RNK-16 was a spontaneous F344 rat strain NK cell leukemia cell line (28). RNK-16 cells were maintained in RPMI 1640 supplemented with 10% FCS, L-glutamine, penicillin, streptomycin, and 5 × 10^-3 M 2-ME. RNK-16 effector cells expressing murine Ly49 were generated by this laboratory as described (12, 29). All transfected RNK-16 and YB2/0 cells were maintained under G418 selection until 48 h before cytotoxicity assays. Transfected cells were grown in the absence of G418 for at least 48 h before cytotoxicity assays.

Cloning and transfection of Ly49I2

PVG rats were obtained from Harlan Sprague Dawley. Experiments were approved by the animal Welfare and Policy Committee of the University of Alberta (Edmonton, Canada). Total RNA was isolated from 1 × 10^7 PVG rat lymphokine-activated killer cells using a RNAeasy Protect minikit (Qiagen). cDNA was produced using PowerScript reverse transcriptase (BD Clontech) with an oligo(dT) primer. Ly49I2 was amplified with Ad-(12, 29) and maintained under G418 selection until 48 h before cytotoxicity assays. 4D11 (rat IgG2a), anti-Ly49GBALB/c (9), STOK2 (anti-Ly49i2) followed with untransfected RNK-16 cells using primary Abs 4D11 (anti-Ly49IG2a), and anti-Ly49GBALB/c) plus R-PE-conjugated AffinPure F(ab')2 donkey anti-rat IgG secondary Ab (Jackson ImmunoResearch Laboratories). Expression of Ly49 by RNK-16 cells was monitored compared with untransfected RNK-16 cells using primary Abs 4D11 (anti-Ly49GLBALB/c), Cw3-1 (IgG1), and STOK2 (anti-Ly49I2), followed by FITC-conjugated anti-rat or anti-mouse secondary Ab as required. All cells were incubated with normal mouse serum (for rat primary Abs) or rat IgG (for mouse primary Abs), to block FcRs before staining.

Cytotoxicity assays

Target cells were labeled with 100–150 μCi Na^25CrO_4 (Mandel) at 37°C for 1 h. Targets were washed three times with RPMI 1640 and 1 × 10^6 of Na^25CrO_4-labeled cells were incubated with RNK-16 or transfected RNK-16 cells for 4 h at 37°C in V-bottom microtiter plates at various E:T ratios in triplicate. After incubation, plates were centrifuged for 5 min at 1500 rpm and 25 μl of supernatant collected and counted in a MicroBeta Trilux liquid scintillation counter (PerkinElmer). Percent-specific lysis was determined as (experimental release − spontaneous release)/(maximal release − spontaneous release) × 100%.

Peptide elutions

Transfectants of YB2/0-expressing His-tagged wild-type or B-pocket mutants of RT1-A1R were each grown in 20 L in spinner culture flasks to a total of ~ 1 × 10^10 cells. Cells were centrifuged and washed in wash buffer (10 mM Tris (pH 7.7) and 150 mM NaCl) before being lysed at room temperature for 30 min in lysis buffer (wash buffer plus 1.5% Nonidet P-40 and 0.5 mM PMSF). Ly49 was centrifuged for 30 min at 20,000 × g at 4°C before adding 5 mM imidazole plus 1.5 ml Ni-NTA agarose beads (Qiagen) to the supernatant, with mixing, for 90 min at 4°C. Beads were then washed five times with lysis buffer plus 20 mM imidazole before one final wash with wash buffer. Peptides were then eluted using 10% glacial acetic acid for 5 min. Beads were removed using a 0.22-μm low protein binding filter and MHC I H chain and β2-microglobulin were removed using an Amicon Ultra filter device MW5000 (Millipore). Samples were diluted to 0.1 M acetic acid and sent for Edman degradation at the Nucleic Acids and Protein Synthesis (NAPS) facility at the University of British Columbia (Vancouver, Canada) and the Advanced Protein Technology Centre Peptide Sequencing Facility at the Hospital for Sick Children (Toronto, Canada).

Results

RT1-A1R recognition by a syngeneic rat inhibitory receptor and xenogeneic mouse inhibitory and activating receptors expressed on RNK-16 cells

Recognition of syngeneic MHC I molecules by mouse and rat Ly49 can be MHC I allele specific (30, 31). However, recognition of polymorphic residues of MHC I molecules in determining allele specific recognition by Ly49 is poorly understood. We previously identified allele specific xenoreactivity of the mouse inhibitory Ly49GLBALB/c and the activating Ly49W for the PVG rat classical MHC I molecule RT1-A1R (23). From these findings, we set out to characterize the MHC I motifs that confer xenorecognition and to compare them to those required for syngeneic recognition of RT1-A1R by its natural syngeneic PVG rat strain receptor, Ly49I2 (24), which is also allele specific. In doing so, we aimed to obtain fundamental insights into Ly49 recognition of MHC I ligands. RNK-16 cells, which exhibit equal background cytotoxicity toward YB2/0 cells or YB2/0 cells expressing the rat MHC I molecule RT1-A1R (data not shown), were transfected with the mouse Ly49GLBALB/c inhibitory receptor, the mouse Ly49W-activating receptor, or the rat Ly49I2 inhibitory receptor (Fig. 1A). Recognition of RT1-A1R-expressing YB2/0 cells by the inhibitory receptors Ly49GLBALB/c and Ly49I2 is demonstrated by the nearly complete reduction in cytotoxicity by RNK-16 transfectants expressing these receptors, in comparison to normal YB2/0 cells (Fig. 1B). Recognition of RT1-A1R by the activating receptor Ly49W is observable through a substantial increase in cytotoxicity of YB2/0 cells expressing RT1-A1R, in comparison to the background of unaltered YB2/0 cells (Fig. 1B). Thus, the rat MHC I molecule, RT1-A1R, is recognized by inhibitory and activating xenogeneic mouse Ly49 and by syngeneic Ly49I2 as reported (23, 24).
Xenogeneic mouse and syngeneic rat Ly49 recognition of RT1-A1c follows a pattern similar to mouse syngeneic recognition

To characterize the MHC I motifs that confer xenogeneic Ly49 recognition, and to apply these findings to the identification of a motif that determines the allele specificity of Ly49, we needed to determine whether xenogeneic recognition occurs similarly to syngeneic recognition. The original cocrystal structure of Ly49A and H-2Dd identified two different potential interaction sites on the MHC I molecule for recognition by Ly49A (16) (see Fig. 5A). Site 1 is located at the end of the peptide-binding groove, is polymorphic among MHC I molecules, and was initially a favorable candidate for mediating MHC I allelic specificity of Ly49. Site 2 is nonpolymorphic and located under the peptide-binding platform, involving contact sites in all three domains of the H chain as well as residues within β2-microglobulin. Mutagenesis of mouse MHC I residues has shown that site 2, and not site 1, is important for recognition by syngeneic mouse Ly49 (17, 18). To determine whether rat syngeneic or xenogeneic Ly49 recognition of RT1-A1c involved site 1, we mutated RT1-A1c at the previously identified site 1 residue, R169 (see Fig. 5B) (16), and expressed this mutant to similar levels as wild-type RT1-A1c on YB2/0 cells (Fig. 2B). This residue was previously shown to affect recognition of H-2Dd by the activating receptor Ly49A (17). A wild-type RT1-A1c R169A double mutant and expressed it to similar levels as wild-type RT1-A1c (shaded histogram) compared with untransfected YB2/0 cells (open histogram). B, RNK-16 cells transfected with Ly49GBALB/c, Ly49W, or Ly49i2 were compared for cytotoxicity against YB2/0 cells and YB2/0 cells transfected with RT1-A1c. Data represent the mean of triplicate wells ± SD and are representative of three independent experiments.

molecule by all three receptors (Fig. 2B). This finding is in common with the recognition of H-2Dd by Ly49A, which also is not affected by this site 1 mutation (17, 33). To assess whether rat syngeneic and xenogeneic recognition occurs at site 2 we mutated RT1-A1c to alanine at two different residues, R6 and R111 (see Fig. 5B), which are conserved in rat and mouse MHC I molecules. These residues were previously shown to be important for Ly49A recognition of H-2Dd (16, 17). Both mutants were expressed on YB2/0 cells at similar levels compared with the wild-type RT1-A1c (Fig. 2A). We found that xenogeneic recognition by Ly49GBALB/c and Ly49W followed a similar pattern to the previously demonstrated mouse syngeneic recognition of H-2Dd by Ly49A (17) with R6A completely disrupting recognition and R111A having a partial effect (Fig. 2B). However, syngeneic recognition of RT1-A1c by Ly49i2 was not affected by the site 2, R6A, or R111A mutations (Fig. 2B). Additionally, a double R6A R111A mutant of RT1-A1c did not result in any loss of recognition by Ly49i2 (data not shown). Residue K243, located in the α3 domain of the MHC I molecule, has previously been shown to be important for Ly49A recognition of H-2Dd (17) but did not disrupt recognition of RT1-A1c by Ly49i2 as a single mutant (data not shown). We then mutated RT1-A1c to create a R6A K243A double mutant and expressed it to similar levels as wild-type RT1-A1c on YB2/0 cells (Fig. 2C). The double site 2
Xenogenic and syngeneic Ly49 recognition is sensitive to changes in B-pocket residues of RT1-A1

Because recognition of RT1-A1w by Ly49i2, Ly49G<sup>BALB/c</sup>, and Ly49W was determined to be occurring at site 2 where there are no obvious polymorphic solvent-exposed residues on the MHC I molecule that would confer allele specificity, we looked beyond amino acid sequence identity for a MHC I motif that could determine recognition of RT1-A1w by both syngeneic and xenogeneic Ly49. MHC I molecules bind peptides of about nine amino acids in length within the peptide-binding groove. Not all of the amino acids within the peptide play an equal role in positioning and stabilizing it within the folded peptide-MHC I complex, with two or three amino acids of the bound peptide acting as “anchor residues” within specific MHC I-binding pockets. MHC I molecules can be grouped into supertypes based on their preference for defined peptide anchor residues bound within specific pockets of the MHC I peptide-binding cleft (19). We noted previously that Ly49G<sup>BALB/c</sup> and Ly49W appear to recognize MHC I ligands of a HLA-B7 supertype (23) that prefer to bind peptides with proline at the second or P2 anchor position (H-2L<sup>d</sup> and RT1-A1<sup>c</sup>) or prefer a P2 residue with a small side chain, such as H-2D<sup>d</sup>, which uses P2 and P3 anchors residues of glycine and proline, respectively (35). Although only one MHC I ligand, RT1-A1w has been identified for Ly49i2 (24), it too may prefer MHC I molecules such as RT1-A1<sup>c</sup>, which bind small P2 residues. With this in mind, we examined the B-pocket of the MHC I molecule as its amino acid composition and three-dimensional structure determines the peptide residues that are capable of acting as P2 anchors. We first noted the presence of a large tyrosine residue at position 67 of RT1-A1<sup>c</sup> (see Fig. 5B), which is not present in other nonrecognized rat MHC I molecules, and could be responsible for the requirement of a small P2 residue to bind within the B-pocket. We mutated this residue to alanine and expressed the RT1-A1<sup>c</sup> Y67A mutant on YB2/0 target cells, matching its expression to that of YB2/0 cells expressing the wild-type RT1-A1<sup>c</sup> molecule (Fig. 3A). When we assayed for recognition of the Y67A mutant by the mouse Ly49G<sup>BALB/c</sup> and Ly49W receptors, we saw that alteration of this residue resulted in the loss of recognition by both xenogeneic receptors. In contrast, Ly49i2 recognition of the Y67A mutant was slightly reduced compared with wild-type RT1-A1<sup>c</sup> molecule (Fig. 3B). We mutated two other B-pocket residues, D9 and S24 (see Fig. 5B), to alanine residues and expressed these mutants on YB2/0 cells at similar levels to that of wild-type RT1-A1<sup>c</sup> (Fig. 3A). We used these additional mutants to determine whether loss of recognition may be due to opening the B-pocket to larger P2 residues, as might occur with Y67A and D9A, or whether recognition could be altered by a more conservative modification of the B-pocket, S24A, that likely would not change the preferred P2 residue. For all three receptors, recognition of the mutant RT1-A1<sup>c</sup> D9A was disrupted, with loss of recognition by the xenogeneic mouse receptors being complete and loss of recognition by the rat syngeneic receptor, Ly49i2, being only slightly less than complete (Fig. 3B). In a similar manner, YB2/0 cells expressing the RT1-A1<sup>c</sup> S24A mutant at comparable levels to wild-type RT1-A1<sup>c</sup> (Fig. 3A) also were not recognized by the inhibitory mouse receptor, Ly49G<sup>BALB/c</sup> nor the mouse activating receptor, Ly49W. Recognition of the S24A mutant by the rat inhibitory receptor, Ly49i2, was also lost, although again to a slightly lesser degree than the mouse receptors (Fig. 3B). Therefore, recognition of RT1-A1<sup>c</sup> by mouse xenogeneic and rat syngeneic receptors is similar, in that recognition can be altered by mutations affecting single polymorphic amino acids within the B-pocket of RT1-A1<sup>c</sup>, including even a fairly conservative modification such as serine to alanine within the supertype-defining B-pocket.

Xenogenic and syngeneic Ly49 recognition of RT1-A1<sup>c</sup> is not sensitive to alterations of non B-pocket residues along the length of the peptide-binding groove

Because xeno- and syngeneic Ly49 recognition of RT1-A1<sup>c</sup> was sensitive to alterations of residues in the B-pocket, we wanted to determine whether the receptors relied on the conformation of the B-pocket specifically or whether their recognition could be altered by changes in other residues within the peptide-binding groove. We selected four different residues for mutagenesis to alanine: F74, F116, D150, and F152 (see Fig. 5B). These residues were chosen for a number of reasons. First, due to their positions on both the sides and bottom of the peptide-binding groove and, second, their location within additional pockets of the peptide-binding groove. These residues were also chosen for their polymorphic nature, large size, or the presence of charge, which would result in significant changes in groove structure upon mutagenesis to alanine. Each of these mutants was expressed on YB2/0 cells at similar expression levels as wild-type RT1-A1<sup>c</sup> (Fig. 4A) and assayed for recognition by the two mouse receptors, Ly49G<sup>BALB/c</sup> and Ly49W, and rat Ly49i2 (Fig. 4B). Mutagenesis of F74, which faces into the groove on the same side as Y67 and lies just outside of the B-pocket, resulted in no loss of recognition by any of the three receptors (Fig. 4B), indicating no reliance on this portion of
the peptide-binding groove for recognition by these receptors. Altering F116, a residue that lies in the center of the floor of the groove underneath residues P6 to P8 of the bound peptide, also did not result in any loss of recognition by either of the xenogeneic Ly49 or rat Ly49i2 (Fig. 4B). Similarly, mutagenesis of D150, which lies on the opposite side of the groove from Y67 but more toward the C-terminal end of the bound peptide, resulted in no loss of recognition by any of the three Ly49 tested (Fig. 4B). Lastly, mutagenesis of F152, which lies nearly opposite to F74 but more toward the center of the groove, also resulted in no loss of recognition by mouse inhibitory Ly49G^BALB/c, mouse activating Ly49W, and rat Ly49i2 (Fig. 4B). Hence, both syngeneic and xenogeneic recognition of RT1-A1c are not affected by the alteration of single residues within additional pockets of the peptide-binding groove, outside of the B-pocket.

**B-pocket mutants that disrupt syngeneic recognition are loaded with fewer peptides having proline at P2 while xenogeneic recognition can be disrupted without a change in P2 preference**

By altering residues within the B-pocket of RT1-A1c, we were potentially changing the size, charge, and conformation of the pocket such that it no longer preferentially bound proline as its P2 anchor residue (36). Because Ly49C recognition of H-2K^b^ has been shown to be dependent on the binding of specific peptides to H-2K^b^ (37), potentially, our RT1-A1c mutants were altering the B-pocket in such a manner that peptides with a different P2 anchor residue were binding and altering the RT1-A1c conformation and disrupting Ly49 recognition. To investigate this possibility, we eluted peptides from wild-type RT1-A1c molecules and each of the B-pocket mutants expressed in the YB2/0 cell line. The yield and frequency of specific amino acids at defined positions within peptides, eluted in bulk, were determined by N-terminal Edman degradation sequencing and were compared with known elution profiles from PVG rat splenocytes (36, 38), which express this allele product naturally. The sequencing we obtained from wild-type RT1-A1c expressed on YB2/0 cells agreed well with published results, showing a significantly higher molar yield of proline at P2 compared with any other residue (Table I). Despite the change of a bulky tyrosine residue to a small alanine residue, which could hypothetically open the B-pocket to larger P2 residues, we still saw proline as the dominant P2 anchor residue being present in nearly half of peptides eluted from the Y67A mutant, which was able to disrupt xenogeneic recognition only (Table I). The RT1-A1c D9A mutant potentially removed a charge constraint on which residues could act as P2 anchors of peptides that bound this RT1-A1c mutant. In this case, we continued to see proline as the dominant P2 anchor although its yield was reduced in comparison to wild-type and the Y67A mutant (Table I) and the P2 yield of valine, a previously reported secondary RT1-A1c P2 anchor and HLA-B7 supertype P2 residue (35, 36, 38), was increased.

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Xenogeneic and syngeneic Ly49 recognition of RT1-A1c is not sensitive to alterations in the peptide-binding groove outside of the anchor binding B-pocket of the MHC I molecule. A, Wild-type RT1-A1c and its peptide-binding groove mutants F74A, F116A, D150A, and F152A were each expressed to similar levels on YB2/0 cells. Transfected YB2/0 cells (shaded histograms) were assessed for RT1-A1c-specific YR5/12 Ab staining compared with untransfected YB2/0 cells (open histograms). B, RNK-16 cells transfected with Ly49G^BALB/c^, Ly49W, or Ly49i2 were compared for cytotoxicity against YB2/0 cells transfected with the indicated RT1-A1c peptide-binding groove mutants and wild-type RT1-A1c. Data represent the mean of triplicate wells ± SD and are representative of three independent experiments.

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**Table I. P2 residues of peptides eluted from wild-type and mutant RT1-A1c molecules**

<table>
<thead>
<tr>
<th>MHC I Molecule^a^</th>
<th>RT1-A1c</th>
<th>A1c Y67A</th>
<th>A1c D9A</th>
<th>A1c S24A</th>
</tr>
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<tbody>
<tr>
<td><strong>P2</strong></td>
<td>% Yield</td>
<td>% Yield</td>
<td>% Yield</td>
<td>% Yield</td>
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<tr>
<td>P</td>
<td>52</td>
<td>P</td>
<td>31</td>
<td>Q</td>
</tr>
<tr>
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<td>11</td>
<td>Q</td>
<td>17</td>
<td>V</td>
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<td>V</td>
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<td>L</td>
<td>14</td>
<td>R</td>
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<td>8/8</td>
<td>I</td>
<td>12</td>
<td>T/Q</td>
</tr>
<tr>
<td>A/I</td>
<td>5/5</td>
<td>V</td>
<td>7</td>
<td>K</td>
</tr>
<tr>
<td>F</td>
<td>2</td>
<td>E/F</td>
<td>2/2</td>
<td>I/T</td>
</tr>
<tr>
<td>Y</td>
<td>1</td>
<td></td>
<td></td>
<td>7/7</td>
</tr>
</tbody>
</table>

^a His tagged and purified from YB2/0 transfectants using Ni-NTA beads.

^b Residues found at position 2 as determined by Edman degradation of peptides eluted from wild-type RT1-A1c or the designated MHC I mutants. Residues found in >25% of eluted peptides (in bold) were considered as dominant anchor residues.

^c The P2 molar yield is expressed as the percentage of total molar yield from amino acids showing a picomole increase at P2.
to nearly 25%. Similarly, peptides eluted from RT1-A1\(^*\) S24A, a mutant that we did not expect to see any alteration in the P2 residue, as the mutation is quite conservative, also continued to show proline as a dominant anchor residue at the P2 position (Table I). Glutamine also appeared as a codominant anchor residue at P2 in peptides eluted from the S24A mutant (Table I). Glutamine is not a previously reported P2 anchor residue for RT1-A1\(^*\) nor an accepted HLA-B7 supertype P2 residue (19, 35). This may indicate that the S24A mutant, while maintaining a B7-supertype anchor profile in \(\sim 25\%\) of loaded peptides, also bound in similar proportion to peptides with a specific non-B7 anchor residue, possibly contributing to the loss of Ly49 recognition of this mutant through an overall reduction of RT1-A1\(^*\) molecules loaded with peptide of the B7 supertype. Together, the peptide sequencing results suggest that the single B-pocket residue mutant Y67A, introduced in RT1-A1\(^*\) through mutagenesis, could affect xenogeneic Ly49 recognition without a change in the dominant P2 anchor residue of bound peptides. In contrast, syngeneic recognition was disrupted only by B-pocket mutants that showed a reduced yield of proline at P2, suggesting that disruption of syngeneic recognition may require the increased loading of peptides without proline as a P2 anchor residue. Our results indicate that recognition of the RT1-A1\(^*\) molecule by syngeneic and xenogeneic Ly49 is dependent on, and extremely sensitive to, alterations in MHC I amino acids that specifically contribute to the composition of the polymorphic, peptide anchor residue-binding, and supertype-defining, B-pocket. These findings indicate that murine NK cells may play an important role in sensing either alterations in or loss of MHC I of a specific supertype.

**Discussion**

Ly49 generally recognize MHC I molecules in an allele-specific manner (30, 31), yet Matsumoto et al. (17) showed that the highly conserved site 2 region, which lies beneath the peptide-binding groove and includes residues in all three domains of the MHC I H chain and \(\beta_2\)-microglobulin (Fig. 5A), was the major site of interaction for Ly49 with MHC I molecules. This raises two questions: how do Ly49 distinguish between polymorphic MHC I molecules and how has there been expansion of the sites for optimal interaction with Ly49? By identifying RT1-A1\(^*\) as a xenogeneic rat MHC I ligand for the mouse receptors Ly49G and Ly49W (23), we thought that a polymorphic epitope that was conserved between species but differed between MHC I alleles might be apparent to explain the discrimination between rodent MHC I alleles by these receptors. In examination of site 2 of RT1-A1\(^*\), with known ligands and nonligands for Ly49G and W, we found that between the rat and mouse, only two polymorphisms occur at site 2: Pro\(^2\) (H-2D\(^d\), D\(^k\), L\(^d\), K\(^d\)) or Ser\(^2\) (H-2D\(^d\), RT1-A1\(^*\), A\(^*\)) and Met\(^{138}\) (H-2D\(^d\), D\(^a\), D\(^b\), L\(^d\), K\(^d\)) or Phe\(^{138}\) (RT1-A1\(^*\), A\(^*\)). The polymorphisms at positions 2 and 138 do not differentiate Ly49G or W ligands from nonligands, because Ly49G recognizes H-2D\(^d\), D\(^b\), L\(^d\), and RT1-A1\(^*\), Ly49W recognizes only H-2D\(^d\), D\(^b\), and RT1-A1\(^*\), and neither receptor recognizes H-2D\(^d\), K\(^d\), or RT1-A1\(^*\). Thus, it was not apparent how these polymorphisms could explain the allele-specific recognition patterns observed.

What is in common between the MHC I alleles recognized by Ly49G, W, and i2 is a similarity of supertype. MHC I molecules of numerous species are classified into about nine different supertypes based on the peptide residues that act to “anchor” the peptide into the MHC I molecule’s peptide-binding groove (19). Most MHC I molecules tightly bind the second (P2) and last residues (P9) of the peptide in the anchor binding B- and F-pockets of the MHC I molecule, respectively, although other anchor/pocket combinations can occur. H-2L\(^d\) fits perfectly into what is known as the HLA-B7 supertype, binding Pro as P2 in the B-pocket and Leu or Phe as P9 in the F-pocket of the MHC I molecule (35). Interestingly, we noted that the xenogeneic ligand, RT1-A1\(^*\), also fits into the HLA-B7 supertype (36) and although H-2D\(^d\) is not a perfect example, it also best fits into the B7 supertype, binding small residues (Gly and Pro) in its anchoring B and C pockets, respectively (35). In contrast, nonrecognized alleles did not fit the HLA-B7 supertype, and varied from it considerably. The H-2D\(^d\) and K\(^d\) molecules use the P5 residue to anchor peptides and prefer Asn and Phe/Tyr peptide anchors, respectively. RT1-A1\(^*\) also does not fit the HLA-B7 supertype and although it uses the B-pocket to anchor peptides it binds larger amino acids such as Leu, Gln, and Met as preferred anchor residues (35). This led us to examine whether similarities in the MHC I B-pocket, which are shared among species but differ between MHC I alleles, could be the means by which Ly49 differentiate between MHC I alleles. Indeed, upon examination of site 2 residues that lie beneath and articulate with the B-pocket of recognized alleles (RT1-A1\(^*\) and H-2D\(^d\)) with those of nonrecognized alleles (RT1-A\(^*\), H-2D\(^d\), H-2K\(^d\)), it is apparent that solvent exposed side chains available for interaction at site 2 have an essentially identical conformational structure and position in RT1-A1\(^*\) and H-2D\(^d\) and a completely different conformational structure and position in RT1-A\(^*\), H-2D\(^d\), and K\(^d\) (Fig. 5C). Also, H-2L\(^d\), which is recognized weakly by Ly49G (29) and not by Ly49W (data not shown), has a similar but not identical conformational structure of the solvent-exposed residues below the B-pocket, compared with RT1-A1\(^*\) and H-2D\(^d\), that may explain its less than optimal interaction with Ly49G and W (Fig. 5C).

By modifying the site 2 residues R6 and R111 (Fig. 5B), we demonstrated that xenogeneic recognition of RT1-A1\(^*\) follows an identical pattern of recognition to the mouse Ly49A/H-2D\(^d\) interaction. Additionally, these site 2 residues lie beneath and articulate with the B-pocket, particularly R6, which when mutated, results in greater disruption of recognition compared with R111, which is less influenced by the B-pocket. This relationship supports the hypothesis that the side chain conformations of these and other solvent-exposed residues that articulate with the B-pocket may become more or less available for optimal interaction with Ly49 depending on B-pocket conformation (Fig. 5C). Ly49i2, like Ly49G and W, did not appear to be reliant on site 1 for recognition of RT1-A1\(^*\), but also did not show a similar sensitivity to single mutations of R6 and R111 at site 2. However, Ly49i2 does recognize RT1-A1\(^*\) at site 2, as a double mutant including the B-pocket associated R6 residue and the previously identified site 2 residue K243 (Fig. 5B) of the class I molecule disrupted recognition, whereas single R6 or K243 (data not shown) mutants had no effect. This slightly altered dependence on site 2 residues by Ly49i2 may be expected, as Ly49i2 differs from Ly49G and W in two important ways that may alter how it interacts with residues below the B-pocket. First, Ly49i2 does not share the glycosylation motif NTT (221–223) that Ly49G and W possess, which is directly adjacent to the interaction site under the B-pocket at site 2 and might lessen the binding strength (39). Second, there is sequence dissimilarity between Ly49i2 and Ly49G/W (12, 24) in a region that could alter precisely how this receptor interacts with residues below the B-pocket. Therefore, Ly49i2 is more likely either interacting with greater affinity than Ly49G and W or has a greater reliance on different B-pocket-associated solvent-exposed residues. Nevertheless, the dependence on B-pocket residues for Ly49i2 recognition is shared with the mouse Ly49G and W receptors.

In support of our hypothesis that both xenogeneic and syngeneic rat recognition was occurring at site 2 through solvent-exposed residues that were influenced by B-pocket conformations, we
found that both xenogeneic recognition by Ly49G and W and syngeneic recognition by Ly49i2 were very sensitive to changes within the B-pocket. By mutating polymorphic residues within the B-pocket of RT1-A1c to alanine, we were able to show that Ly49 could sense changes in floor conformation with the S24A mutant, changes in the charge of the pocket with the D9A mutant, and even showed xenogeneic sensitivity to structural changes higher up in the pocket with the Y67A mutant (Fig. 5

Although it is apparent that mutations in the floor of the B-pocket might directly influence the conformation of solvent-exposed residues used in Ly49 recognition, it is less apparent how a mutation higher in the pocket could disrupt recognition. However, it has been previously suggested that “void” regions in proteins play a role in stability and function by affecting molecular packing in the protein interior (40). The Y67A mutant may affect the molecular packing of the B-pocket in this manner, translating to the solvent-exposed interaction residues and disrupting the xenogeneic interaction at site 2. Mutations in other pockets within the peptide-binding groove of RT1-A1c changing the size (F74A, F152A) and charge (D150A) of pockets within the groove and the conformation of the groove platform (F116A) (Fig. 5B) did not alter recognition, showing a specific reliance on the supertype-defining B-pocket and its potential effects on the conformation of solvent-exposed residues that articulate with it. In examination of earlier work that mutated residues

FIGURE 5. Alterations in the polymorphic and supertype defining B-pocket of RT1-A1c disrupts interaction with Ly49, possibly by altering the conformation of solvent exposed residues at site 2. A, Cocrystal of Ly49A (mauve and teal) and H-2Dd (blue) showing sites 1 and 2 interactions, respectively. B, Depiction of residues mutated in RT1-A1c and comparison of their effects on xenogeneic Ly49G and W recognition (left) vs syngeneic Ly49i2 recognition (right). Total disruption, red; no disruption, green; partial disruption, orange; double mutant disruption, purple. Residues within the peptide-binding groove are shown as spheres, solvent exposed residues as ball and stick. C, Comparison of the conformation of solvent-exposed residues, shown in ball and stick, which articulate with the B-pocket of ligands (RT1-A1c, H-2Dd, Ld) vs nonligands (RT1-Aa, H-2Db, Kb). Side chains are depicted in blue except for R6 and R111, which are depicted in yellow.
within the peptide-binding groove of H-2D\textsuperscript{d}, but not specifically focusing on anchor-binding pockets, we noticed that single mutants outside of the main anchor-binding pockets were also unable to disrupt recognition by Ly49A and it required double and triple mutants close to or within minor anchor-binding pockets to cause partial or complete disruption, respectively (33, 41). In contrast, we demonstrate here that single mutants within the supertype-defining B-pocket of RT1-A\textsuperscript{d}, that do not prevent the MHC I molecule from binding peptide, had significant effects on both xenogeneic mouse and syngeneic rat Ly49 recognition.

Peptides eluted from the B-pocket mutants of RT1-A\textsuperscript{d} showed that disruption of xenorecognition and syngeneic rat Ly49 recognition through mutagenesis of the anchor-binding and supertype-defining B-pocket may occur through two different mechanisms. First, the Y67A mutant showed little alteration in proline yield as the P2 anchor, which is surprising, as this mutant would potentially open the B-pocket to larger P2 residues. This may be due to a reliance on the overall composition of the B-pocket, or residues deeper in the pocket, for alteration of P2 anchor-binding preference. Because the Y67A mutant showed little or no alteration in P2-binding preference, likely the conformational change induced in the MHC I H chain through the Y67A mutation is the mechanism capable of disrupting xenogeneic but not syngeneic Ly49 recognition of this mutant. Second, although none of the mutants showed an overt change of supertype by complete displacement of proline as a dominant anchor, it is interesting to note that peptides eluted from the S24A and D9A mutants did show a lesser yield of proline at P2 of bound peptides. The S24A mutant is particularly interesting. The reduction in loaded peptides with proline at P2 was balanced by an increase of peptides containing glutamine at P2, a residue that is not recognized as either a P2 anchor for RT1-A\textsuperscript{d} (35) nor as a B7-supertype anchor residue (19). This may indicate that the loss of recognition of this mutant was due to the loss of a recognizable supertype, at sufficient density at the cell surface, for recognition by both syngeneic and xenogeneic Ly49. With D9A, the reduction in proline at P2 was balanced by an increase of valine, which is accepted as a secondary RT1-A\textsuperscript{d} (36) and HLA-B7 supertype anchor residue (19). This suggests that Ly49 recognition of MHC I may also be sensitive to alterations in the overall proportion of MHC I loaded with specific anchor residues without requiring an overt change in supertype. Therefore, in addition to direct conformational changes in the MHC I H chain, induced through B-pocket mutagenesis, the overall reduction of RT1-A\textsuperscript{d} molecules at the cell surface, loaded with peptides with a proline P2 anchor, could be an additional mechanism contributing to the loss of recognition of these mutants by Ly49.

Orihuela et al. (14) demonstrated that Ly49A is not peptide selective, loading H-2D\textsuperscript{d} with various peptides including a "skeletal" Ala peptide that did not disrupt recognition. This peptide still required the presence of H-2D\textsuperscript{d} anchor residues (AGPAAAAAL) to allow proper folding and presentation at the cell surface, but in the context of our present study, may also have preserved a recognizable MHC I supertype associated conformation for Ly49A recognition. It is possible then that the reduction in proline at P2, and therefore the increased presence of MHC I loaded with peptides having either secondary P2 anchors or P2 anchors of a different supertype, was required for the loss of syngeneic, but not xenogeneic Ly49, recognition we observed. An altered repertoire of bound peptides has been proposed to explain the gain of recognition of H-2D\textsuperscript{d} by Ly49 upon murine CMV infection (42). Should this be the case, it might be due to changing the structure of the MHC I molecule through altered dominance of different amino acids as P2 residues, thereby affecting recognition by Ly49.

KIR also survey for changes in the peptide-binding preferences of the MHC I molecule (43) and this may be supertype related as KIR-3DL2 recognizes more than one MHC I allele of the HLA-A3 supertype when loaded with virus-derived peptides, but not self peptides or MHC I of other supertypes (44). Although early work demonstrated human NKR (likely KIR) sensitivity to B-pocket mutations of HLA-B7 (45), it is now clear that KIR interact with HLA nearer to the other anchor residue-binding and supertype-defining F-pocket (46). This suggests that, similar to what we have demonstrated with Ly49 recognition, some KIR may also survey for MHC I of specific supertypes. It is conceivable that KIR may be sensitive to small differences in the conformation of the F-pocket region of different MHC I supertypes in combination with different peptides, as opposed to the B-pocket sensitivity demonstrated here with rodent Ly49. KIR can also recognize xenogeneic MHC I ligands (47) and perhaps this is mediated through common supertype conformations at anchor-binding pockets.

Why would Ly49 survey for MHC I supertypes? The equal distribution of supertypes across species (20, 21) and even human ethnicities, although allele representation within those supertypes varies (48), implies that a certain complement of supertypes is required within a population to provide immune fitness toward a battery of pathogens (49). Because there are no MHC I orthologs between rat and mouse or between rodents and humans (50), and because examination of the B-pocket structure of murine vs primate supertypes shows a large difference in the pocket structures, it appears that convergent evolution has occurred to produce similar supertypes in both mammalian orders. Supertypes likely are important for T cell recognition. It has been shown that the TCR can be affected by more than the solvent-exposed regions of MHC I and changes in anchor-binding pockets affect the structure and conformation of the MHC I surface that interacts with the TCR, altering recognition (51, 52). Therefore, MHC I allele-specific inhibitory Ly49, such as Ly49A, G, and i2 may be sensing whether specific MHC I supertypes are expressed to present peptides with specific anchor residues that would normally be presented to T cells. Loss of relevant supertype interaction with Ly49 inhibitory receptors, for example, would indicate that a cell may be incapable of presenting a major subset of peptides (with specific P2 anchors) to T cells and should be eliminated. In contrast, receptors such as Ly49C that recognized a broader repertoire of MHC I alleles (10), may be surveying more for the presence of properly folded MHC I capable of interacting with B2-microglobulin and the CD8 T cell costimulatory molecule, which shares portions of the Ly49-binding site on MHC I (53).

We demonstrate that some Ly49 are able to detect a supertype, even across species, and are sensitive to alterations specifically in the supertype-defining B-pocket. Therefore, we conclude that MHC I allele specificity of Ly49 can be dictated by conformational differences between MHC I supertypes. Importantly, this would allow NK cells to survey for the presence or absence of MHC I supertypes capable of presenting peptide Ags to T cells and elimination of cells defective in Ag presentation through loss of or alterations in a specific MHC I supertype. Such defects would result in the inability to present immunodominant peptides of a specific supertype or possibly loss of conformations important for MHC restriction. This shared dependence on MHC I supertypes by Ly49 and TCR indicates yet another fundamental link between innate and adaptive immunity. It also provides a potential explanation for the expansion of the Ly49 locus, through pressure to maintain Ly49 capable of detecting conformational differences in the MHC I supertypes that are essential to adaptive immunity.
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Disclosures

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References


