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Recognition of Class I MHC by a Rat Ly49 NK Cell Receptor Is Dependent on the Identity of the P2 Anchor Amino Acid of Bound Peptide

Brian J. Ma and Kevin P. Kane

Members of the rodent Ly49 receptor family control NK cell responsiveness and demonstrate allele specificity for MHC class I (MHC-I) ligands. For example, the rat Ly49i2 inhibitory NK cell receptor binds RT1-A1β but not other rat MHC class Iα or Ib molecules. RT1-A1β preferentially binds peptides with proline at the second, or P2, position, which defines it as an HLA-B7 supertype MHC-I molecule. Previously, our laboratory showed that mutations within the MHC-I supertype-defining B-pocket of RT1-A1β could lead to alterations in P2 anchor residues of the peptide repertoire bound by RT1-A1β and loss of recognition by Ly49i2. Although suggestive of peptide involvement, it was unclear whether the peptide P2 anchor residue or alteration of the RT1-A1β primary sequence influenced Ly49i2 recognition. Therefore, we directly investigated the role of the P2 anchor residue of RT1-A1β–bound peptides in Ly49i2 recognition. First, fluorescent multimers generated by refolding soluble recombinant RT1-A1β with synthetic peptides differing only at the P2 anchor residue were examined for binding to Ly49i2 NK cell transfectants. Second, cytotoxicity by Ly49i2-expressing NK cells toward RT1-A1β target cells expressing RT1-A1β bound with peptides that only differ at the P2 anchor residue was evaluated. Our results demonstrate that Ly49i2 recognizes RT1-A1β bound with peptides that have Pro or Val at P2, whereas little or no recognition is observed when RT1-A1β is complexed with peptide bearing Glu at P2. Thus, the identity of the P2 peptide anchor residue is an integral component of MHC-I recognition by Ly49i2.

Dr. W. Jefferys (University of British Columbia, Vancouver, BC, Canada). Three 9-mer peptides, NPRKVTAYL (P22), NQRKVTAYL (P24), and NVRKVTAYL (P25), were commercially synthesized (GenScript) for peptide loading of RT1-A1. Purified STOK2 (rat IgG2a; anti-Ly49i2) (11) was purchased from BD Biosciences. Tissue culture supernatant of the YRS/5212 hybridoma (rat IgG2b; anti–RT1-A1) (16) was purchased from Serotec. Purified 4D11 (rat IgG2a; anti–Ly49G) and F4/80 (rat IgG2b; anti–F4/80) were produced from hybridomas in this laboratory and used as isotype controls. FITC-coupled and R-PE–coupled mouse anti-rat IgG were purchased from Jackson Immunoresearch Laboratories.

Expression, refolding, and purification of RT1-A1

The cDNA encoding the extracelluar region of RT1-A1 mature α-chain (residues 1–277) was ligated to a sequence that encoded a C-terminal BirA recognition tag (17) and was inserted into the bacterial expression vector pET21a (Novagen). The cDNA encoding the mature rat β-2m cloned from a FVO rat cDNA library was also subcloned into pET21a. Sequences were confirmed by DNA sequencing using an ABI 37300 DNA Analyzer (Applied Biosystems). These plasmid DNAs were then transformed into Escherichia coli strain BL21(DE3) for protein expression. The bacteria cultured in lysogeny broth supplemented with 50 μg/ml ampicillin were induced with 0.5 mM isopropyl-1-thio-galactopyranoside and further supplemented with 20 μg/ml biotin for 4 h. Bacteria were pelleted and then treated with 1 ml of media containing 10 μg/ml lysozyme for 1 h at room temperature in TE (0.1 M Tris, 0.1 mM EDTA) supplemented with 1% Triton X-100 and then lysed by sonication. Pellets containing the inclusion bodies were washed once with TE with 1 M Tris, pH 8, and then dissolved overnight at 4˚C in 8 M urea. Protein concentrations were determined by using absorbance at 280 nm and molar extinction coefficients of 67,760 and 16,740 for RT1-A1c and RT1-A2m, respectively.

The refolding of soluble RT1-A1 was achieved by rapid dilution of a mixture of 0.5 μmol RT1-A1c H chain and 1 μmol rat β-2m into 400 ml arginine buffer (0.4 M l-arginine-HCl, 0.1 M Tris, pH 8, 2 mM EDTA, 5 mM reduced glutathione, and 0.5 mM oxidized glutathione) in the presence or absence of 10 mg protein at 4˚C. With peptide present, this resulted in a molar ratio of 1:2.20 of H chain to β-2m to peptide. The mixture was allowed to stir for 2 h and then concentrated by ultrafiltration. Concentrated proteins were dialyzed against 100 mM NaCl, 100 mM Tris, pH 8.5, and then purified by size exclusion chromatography on a HiLoad Superdex-75 26/60 prep grade column (GE Healthcare).

Epitope and thermal stability analysis

Refolding of RT1-A1α to acquire the 5212 recognized epitope was assessed by flow cytometry. Latex beads (5-μm diameter; Interfacial Dynamics Corp.) were coated with avidin and used to capture equal amounts offolded RT1-A1α by its biotin tag. Beads were incubated with YRS/5212 for 1 h at 4˚C, were washed twice with 1% BSA in PBS, and incubated with FITC-coupled mouse anti-rat IgG for 30 min before flow cytometric analysis. Flow cytometric acquisition was completed using a Becton Dickinson FACSCalibur and CellQuest software. Analyses were performed using FCS Express (De Novo Software).

In thermal stability studies, refolded RT1-A1α was again captured onto avidin-coated latex beads. Half of the beads were incubated at 37˚C for 1 h, and the other half was incubated at 4˚C for the same amount of time. After incubation, both sets of beads were stained in parallel with YRS/5212 followed by FITC-coupled secondary Ab and analyzed by flow cytometry as described earlier.

Protein analysis

Proteins were analyzed by glycine SDS-PAGE (18). About 2.5 μg protein was boiled in 1× SDS-PAGE sample buffer and run on a 15% polyacrylamide minigel. Proteins were visualized by staining the gel with Gelcode Blue (Pierce). To visualize the presence of peptide in the refolded RT1-A1α, tricine SDS-PAGE was used as previously described (19, 20). Fifteen micrograms of protein were boiled in 1× tricine sample buffer and then run on a 16.5% tricine SDS polyacrylamide minigel at 100 V for 3.75 h. Proteins were visualized by first fixing in 5% glutaraldehyde solution for 1 h and then staining with Gelcode Blue. All proteins were quantified using the BCA protein assay kit (Pierce).

Peptide elution and amino acid quantification

Five nanomoles of each purified rat MHC-I–peptide complex (in 100 mM Tris, pH 8.5, 100 mM NaCl) was diluted to 100 μl, and 10 μl of glacial acetic acid (10% final) was added to acidify the solution and elute the peptides. Peptides were then filtered through 5-kDa molecular mass cutoff Biomax Ultrafree 0.5 ml centrifugal ultrafiltration membranes (Millipore).

The retentate was reduced to ~25 μl, then another 100 μl 10% glacial acetic acid was added, and the filtration was repeated twice more. The filtrate was then recovered for amino acid quantification. Peptides were hydrolyzed under vacuum in 6 M HCl, 0.1% phenol for 1 h at 160˚C. Amino acids were then quantified using a Beckman 6300 amino acid analyzer with postcolumn ninhydrin detection. Efficiency was calculated by the addition of 12 nmol norleucine just before the hydrolysis step. Asn, Leu, and Val were considered the most stable amino acids after hydrolysis and were used to calculate the amount of peptide in the original sample.

Production and use of RT1-A1 multimers

RT1-A1α was multimerized by mixing 40.5 μg monomers in five additions with 11.9 μg Extravidin–PE conjugate (Sigma) in 5-min intervals to prepare 100 μg multimers. To assess the interaction of RT1-A1α with Ly49i2, 5 × 105 RNK.49i2 or RNK-16 cells were incubated with 0.0, 0.125, 0.25, 0.5, 1, 5, or 10 μg multimers in 50 μl RNK medium (RMPI 1640 supplemented with 10% FCS, penicillin, streptomycin, l-Glu, and 50 μM 2-mercaptoethanol) for 1 h at 4˚C. As a negative control, RNK.49i2 and RNK-16 were also incubated with 5 μg Extravidin–PE conjugate. The cells were then pelleted and washed twice before flow cytometric analysis as described earlier. Ly49i2 expression was assessed in parallel by Ab staining with STOK2 followed by secondary staining with PE-coupled mouse anti-rat IgG.

Generation of RNK-16 effector cells expressing chimeric Ly49i2 receptors

A chimeric receptor consisting of the intracellular and transmembrane domains of Ly49i2 and the extracellular domain of Ly49i2 (Ly49i2W) was created from the respective encoding cDNA using two rounds of PCR amplification. The first round consisted of amplification of the intracellular- and transmembrane-encoding domains of Ly49i2 using the 5’ primer 5′-GGCCTTCGAGACCATCTGATTGAGCAGAGGTCTAC-3′, which adds an Xhol restriction site, and 3’ primer 5′-CATGATTATAGCTGAACTGGTTCACAAGCACCTGAGACAATTAC-3′, which includes a priming sequence for the 5′ end of the Ly49i2 ectodomain for the second round of PCR. The amplified product was separated by gel electrophoresis, and the band corresponding with the product was purified. This was used as the 5′ primer for the second round of PCR with cDNA encoding Ly49i2W and 3’ primer 5′-GGCTCTATACAGCAAGAGACTGTGACCC-3′, which adds an XbaI restriction site to the 3′ end of the final construct. The final amplified product was separated by gel electrophoresis, and the band corresponding with the correct product was excised and purified. This was then digested by restriction enzymes and inserted into the BsrRE vector (provided by Dr. A. Shaw, Washington University, St. Louis, MO) at the XhoI–XbaI sites to create BSR.Ly49i2W. The identity of the insert was verified by DNA sequencing. RNK-16 cells were stably transfected with the construct as previously described (21). Positive expression of Ly49i2W was confirmed by flow cytometry using the YR5/12 mAb followed by FITC-conjugated mouse anti-rat IgG2b Ab by flow cytometry.

Generation of RMA-S cells expressing RT1-A1α

The cDNA encoding RT1-A1α was previously cloned from a cDNA library of the PVG strain rat (22) and inserted into the expression vector pC neo (Promega). Stable transfectants were generated by resuspending RMA-S cells (23) in 0.4 ml RMPI 1640 and electroporating 20 μg plasmid in 4-mm-gap cuvettes at 180 mV and 960 μF with a Genepulser (Bio-Rad). Transfected cells were transferred into 96-well microtiter plates and grown in the presence of 0.5 mg/ml G418. Cells surviving G418 selection were cloned by limiting dilution and incubated overnight at 26˚C to enhance RT1-A1β cell surface expression. Expression of RT1-A1α was assessed by flow cytometry using the YRS/5212 Ab followed by FITC-conjugated mouse anti-rat IgG secondary Ab.

Peptide loading and cytotoxicity assays

RMA-S cells transfected with RT1-A1α were cultured overnight at 26˚C, and the surface MHC-I molecules were loaded with specific peptide by incubation in the presence of 10 μM peptides, similar to what has been previously described (15). These target cells were subsequently labeled at 37˚C with 100–150 μCi Na111CrO4 (111Cr; Mandel) for 1 h. The cells were then extensively washed, and 1 × 105 target cells were incubated for 4 h at 37˚C with varying E:T ratios with Ly49i2W-expressing RNK-16 cells in 96-well V-bottom microtiter plates. After incubation, plates were centrifuged for 5 min. Supernatants (25 μl) were transferred to 96-well plates, and 100 μl scintillant (OptiPhase “SuperMix”; PerkinElmer) was added. The plates were analyzed in a β-counter (MicroBeta; PerkinElmer). Percent specific lysis was calculated as [(experimental release) – (spontaneous release)] / ([total release] – [spontaneous release]) × 100%

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release)/(maximal release) – (spontaneous release)] × 100. All cytotoxicity assays were performed a minimum of three separate times.

Results

Design of peptides

MHC class Ia molecules of several mammalian species including primates and rodents can be classified into approximately nine supertypes based on their preference for defined peptide anchor residues bound within specific pockets of the MHC-I peptide binding groove (9, 10). The rat MHC class Ia molecule RT1-A1⁵ can be classified into the HLA-B7 supertype based on its preference for binding nonamer peptides with proline as the second residue from the N terminus at the P2 anchor position (Fig. 1A) (12). The P2 residue of bound peptide is typically held in the B-pocket, and RT1-A1⁵ shares the B-pocket amino acid residues Y7, N63, I66, Y67, and Y99 seen with other molecules of the HLA-B7 supertype (9). Previous work from our laboratory showed that site-directed mutations of polymorphic residues D9 and S24 in the B-supertype (9). Previous work from our laboratory showed that site-directed mutations of polymorphic residues D9 and S24 demonstrated that the proportion of bound peptides bearing proline at the P2 anchor position was reduced relative to peptides eluted from wild-type RT1-A1⁵ (13). In the case of mutant D9A, a significant increase in valine was observed at the P2 anchor position in bound peptides. Valine is a previously reported secondary P2 anchor residue for RT1-A1⁵ and other HLA-B7 supertype members (Fig. 1A) (12, 23, 24). As for the S24A mutant, a significant increase in glutamine as the P2 anchor with a yield equivalent to proline was found at this position in bound peptides (13). Glutamine is neither a previously reported secondary P2 anchor residue for RT1-A1⁵ nor an accepted HLA-B7 supertype P2 residue (9, 23). These results raised the interesting possibility that the identity of the P2 anchor residue of bound peptides may be critical for Ly49i2 recognition of RT1-A1⁵, where substitution from a proline to a non-B7 supertype P2 residue (9, 23). As for the S24A mutant, a significant increase in glutamine as the P2 anchor with a yield equivalent to proline was found at this position in bound peptides (13). Glutamine is neither a previously reported secondary P2 anchor residue for RT1-A1⁵ nor an accepted HLA-B7 supertype P2 residue (9, 23). These results raised the interesting possibility that the identity of the P2 anchor residue of bound peptides may be critical for Ly49i2 recognition of RT1-A1⁵, where substitution from a proline to a non-B7 supertype P2 residue or possibly to a secondary B7 supertype P2 residue in bound peptides may be sufficient to disrupt recognition. We first explored this concept by using fluorescent RT1-A1⁵ multimers loaded with peptides that were otherwise identical except for containing different residues at the P2 position. Peptides were designed based on the “ideal RT1-A1⁵ binding peptide” previously identified by Stevens et al. (12, 24). Their studies involved peptide elution and Edman degradation sequencing with the most abundant residue found at each peptide position used to design a nonamer (NPRKVTAYL) that showed high binding affinity (Fig. 1B) and thus received the designation “ideal” (12). Their results also showed that proline was the predominant anchor residue found at P2 in natural peptides eluted from RT1-A1⁵. To test the importance of bound peptide P2 anchor residue identity in Ly49i2 recognition of RT1-A1⁵, we synthesized peptides for binding to RT1-A1⁵ that shared the “ideal RT1-A1⁵ binding peptide” sequence, with the exception that they each differed in the amino acid Pro, Gln, or Val at the P2 anchor position: NPRKVTAYL (P2P), NQRKVTAYL (P2Q), and NVRKVTAYL (P2V) (Fig. 1B). The Gln and Val were chosen as alternative P2 anchor residues, as they were increased in peptides bound to the mutant RT1-A1⁵ molecules S24A and D9A that were not recognized by Ly49i2 (13). Furthermore, Val but not Gln is a previously reported secondary P2 anchor residue for HLA-B7 supertype MHC molecules, including RT1-A1⁵ (Fig. 1B).

Expression and purification of soluble RT1-A1⁵ monomers

A fusion protein of the extracellular domain of RT1-A1⁵ with a C-terminal BirA recognition sequence (17) was overexpressed in E. coli. Using identical conditions, RT1-A1⁵ and rat β₂m were refolded in the presence of either P2P, P2Q, P2V, or no peptide (see Materials and Methods). Refolded proteins were purified by size exclusion chromatography (Fig. 2A). Refolding of RT1-A1⁵ in the presence of each of the three peptides produced nearly the same elution profile with a prominent peak at ∼158 ml retention volume (~47 kDa) corresponding with the peptide–RT1-A1⁵–β₂m complex. By contrast, folding of RT1-A1⁵ in the absence of peptide produced a much smaller peak at ∼154 ml corresponding with the RT1-A1⁵–β₂m complex and a much higher peak at the void volume corresponding with protein aggregation. This is consistent with what has been reported previously for folding of recombinant MHC-I molecules (24, 25) and demonstrates that RT1-A1⁵ requires peptide to refold efficiently. The 47-kDa fractions were pooled, concentrated, and analyzed by SDS-PAGE (Fig. 2B). The results show two bands, one consistent with H chain RT1-A1⁵ at ~34 kDa and a second consistent with β₂m at 12 kDa after refolding with each of the peptides, P2P, P2Q, and P2V. This confirms that the peak that eluted at ∼158 ml was indeed refolded MHC-I complexes. In addition, the pooled fractions of RT1-A1⁵ refolded in the absence of peptide also separated into H chain and β₂m indicating that empty RT1-A1⁵ complexes were produced.

Purified soluble RT1-A1⁵ monomers show conformational integrity, thermal stability, and contain peptides

As a prerequisite to using the soluble RT1-A1⁵–peptide complexes in receptor binding studies, we assessed them for conformational integrity, thermal stability, and for peptide content after acid elution. Equivalent quantities of RT1-A1⁵ refolded with P2P, P2Q, P2V, or no peptide were captured on avidin-coated beads and incubated at 4 or 37°C for 1 h before staining with an RT1-A1⁵-specific mAb, YR5/12. Similar staining levels for RT1-A1⁵ refolded with P2P, P2Q, and P2V were observed, indicating that all three RT1-A1⁵ monomers folded to equal levels and displayed a serological epitope recognized by YR5/12 (Fig. 3A). Staining by the YR5/12 Ab was only slightly diminished for RT1-A1⁵ bound with P2P, P2Q, or P2V after incubation at 37°C compared with incubation at 4°C, and Ab staining was equivalent to RT1-A1⁵

FIGURE 1. RT1-A1⁵ has a preference for binding B7-supertype peptides. A. The HLA-B7 supertype (top) and RT1-A1⁵ (bottom) peptide anchor residue binding preferences are shown (boxed). Both HLA-B7 and RT1-A1⁵ have preference for proline at the P2 position and have leucine as a preferred amino acid residue at the C terminus. Secondary anchor residues appear below primary anchor residues. The B7 supertype has a more variable amino acid binding at the C terminus (A, L, I, V, M, F, W, Y), but only the binding preference for the prototypical member, HLA-B7, is shown for simplicity. B. Three nonamer peptides were used in this study. P2P is the “ideal” peptide sequence previously reported (12). P2Q and P2V have the same sequence, except glutamine or valine has been substituted at the P2 position.

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bound with each of the three peptides at the same temperature (Fig. 3A). Thus, RT1-A1c refolded in the presence of any of the three peptides showed equal thermal stability. In contrast, the RT1-A1c refolded in the absence of peptide showed substantially reduced thermal stability, as shown by the significantly diminished YR5/12 staining after incubation at 37˚C (Fig. 3A). Therefore, all three peptides had an equal ability to stabilize against thermal disruption of the RT1-A1c complex.

We next confirmed the presence and equivalent loading of peptide in refolded RT1-A1c. The RT1-A1c refolded with P2P, P2Q, P2V, or no peptide was denatured with sample buffer and separated into its components by tricine SDS-PAGE. A band corresponding with peptide is present in all the refolded RT1-A1c complexes, except for the one refolded without peptide (Fig. 3B).

Peptides eluted with mild acid from equal amounts (5 nmol) of the P2P, P2Q, and P2V–RT1-A1c complexes, respectively, were hydrolyzed and quantified for amino acid content (Fig. 3C). The results show a roughly 1:1 ratio of peptide to MHC in the three different peptide–RT1-A1c complexes. The internal relationships of the amino acid quantification show a 1:1:1 molar ratio of Asn, Leu, and Val for P2P and P2Q and a 1:1:2 molar relationship for P2V, which accurately reflect the amino acid representation in the peptide sequences. The results show slightly higher values for the yield of amino acids compared with theoretical input; however, this fractional difference can be accounted for by small deviations in both protein and amino acid residue quantifications. Nonetheless, the results demonstrate that the refolded peptide–RT1-A1c complexes were similarly loaded and stabilized with the three different peptides, P2P, P2Q, and P2V.

Binding of RT1-A1c multimers to Ly49i2 is determined by the identity of the P2 residue bound in the supertype-defining B-pocket

The Ly49i2 inhibitory NK cell receptor of the PVG rat strain is allele specific in its recognition of rat MHC-I ligands. Using a rat NK leukemia cell line, RNK-16, stably transfected to express Ly49i2, our laboratory previously showed that recognition of RT1-A1c is dependent on the composition of MHC-I residues of the B-pocket, which determines the P2 anchor residue accepted and in turn defines the MHC-I supertype (13). With the refolded RT1-A1c bound with P2P, P2Q, or P2V and RNK.49i2 transfectants, we could now directly determine whether the identity of the P2 peptide residue influences Ly49i2 recognition of RT1-A1c. First, the RNK.49i2 transfectant (RNK.49i2) expresses significant levels of Ly49i2, whereas the parental RNK-16 cells are negative, as measured by staining with the Ly49i2-specific Ab, STOK2 (Fig. 4A). Next, we captured the individual P2P, P2Q, or P2V peptide–RT1-A1c complexes onto avidin–PE conjugates through their biotin tags to form multimeric staining reagents (26). We then incubated the multimers with RNK-16 or RNK.49i2 cells. PE-labeled RT1-A1c multimers bearing the P2P or P2V peptide showed little or no binding to RNK.49i2 cells, demonstrating a need for the presence of peptide for Ly49i2 recognition, and is consistent with what has been reported previously for Ly49A recognition of H-2Dd (27, 28). Notably, RT1-A1c multimers refolded with P2Q peptide also showed little or no binding to RNK.49i2 cells, indicating that the
presence of Gln at P2 could not support RT1-A1c recognition by Ly49i2 (Fig. 4B). An increasing titration of RT1-A1c multimers presenting P2Q peptide showed a slight increase in staining of RNK.49i2 cells, but not much more than that by RT1-A1c bearing no peptide and significantly less than that by RT1-A1c presenting P2P or P2V peptides, which showed dose-dependent increases in RNK.49i2 binding (Fig. 4C). Our findings demonstrate that Ly49i2 can discriminate between RT1-A1c peptide complexes due to differences in bound peptides. Specifically, our results indicate that RT1-A1c bound with peptides that contain the HLA-B7 supertype P2 anchor residue, Pro, or Val, an acceptable secondary supertype residue, are supportive for Ly49i2 recognition of RT1-A1c. In contrast, Gln, which is not an HLA-B7 supertype P2 peptide residue, is insufficient in supporting Ly49i2 recognition of RT1-A1c. It is possible that when bound with Pro or Val anchor residues, RT1-A1c complexes assume specific conformation(s) conducive to Ly49i2 recognition that are not assumed when Gln is the P2 anchor. A strong preference or requirement for Pro or Val bound in the supertype-defining B-pocket of RT1-A1c may be a component of NK cell surveillance for expression of an MHC-I supertype.

RT1-A1c. It is possible that when bound with Pro or Val anchor residues, RT1-A1c complexes assume specific conformation(s) conducive to Ly49i2 recognition that are not assumed when Gln is the P2 anchor. A strong preference or requirement for Pro or Val bound in the supertype-defining B-pocket of RT1-A1c may be a component of NK cell surveillance for expression of an MHC-I supertype.

The P2 residue of RT1-A1c–bound peptide affects cytolytic activation of RNK cells by Ly49i2

We next wanted to examine the consequences of the altered Ly49i2 recognition of RT1-A1c on cell-mediated cytolytic function. We
used the TAP-2–deficient mouse T lymphoma cell line, RMA-S, as target cells, which allowed us to control the peptide exogenously loaded into the MHC-I molecules on the cell surface (15). RMA-S cells were stably transfected with RT1-A1\(^c\), and cell surface expression was confirmed by staining with the YR5/12 mAb after incubating cells with peptide and analyzing by flow cytometry (Fig. 5A). The behavior of RT1-A1\(^c\) on the transfected RMA-S (RMA-S.A1\(^c\)) cells in the presence or absence of peptide was similar to what has been described for endogenous H-2\(K^b\) on RMA-S (15). Specifically, in the absence of exogenously added peptide, RMA-S.A1\(^c\) cells display low cell surface levels of RT1-A1\(^c\) after incubation at 37\(^\circ\)C, indicating thermal instability of the peptide-deficient MHC-I molecules (Fig. 5A). However, in the presence of any of the three peptides, P2P, P2Q, or P2V, RT1-A1\(^c\) is able to express stably to similar high levels on the RMA-S.A1\(^c\) cell surface for at least 4 h (Fig. 5A). For cytotoxicity experiments, we used the rat NK cell line to assess ligand recognition by Ly49i2. Because RNK-16 does not recognize RMA-S cells, we could not assess receptor functionality by transfecting Ly49i2 as an inhibitory receptor (data not shown). Instead, we recombinantly converted Ly49i2 into an activating receptor by fusing its extracellular domain to the transmembrane and intracellular domains of the activating mouse Ly49W receptor. The chimeric activating receptor, Ly49Wi2, would contain the Ly49i2 ectodomain and therefore retain the recognition properties of Ly49i2 but transmit an activating rather than an inhibitory signal upon ligand engagement. Our laboratory successfully used this strategy previously to convert mouse inhibitory receptors into activating receptors that retain the ligand specificity of the original inhibitory receptor (29). RNK-16 cells were stably transfected with Ly49Wi2 (RNK-49Wi2), and expression was confirmed by staining with anti-Ly49i2 mAb, STOK2 (Fig. 5B). We next assessed the role of the P2 anchor residue on cytolytic activity mediated through Ly49Wi2. RMA-S.A1\(^c\) cells were incubated overnight at 26\(^\circ\)C to maximize surface expression of empty RT1-A1\(^c\) molecules. The cells were then incubated in the presence of P2P, P2V, P2Q, or no peptide and then used as targets in cytotoxicity assays with RNK-49Wi2 cells (Fig. 5C). No recognition or cytolysis was observed when RMA-S.A1\(^c\) cells were incubated without peptide. However, when RT1-A1\(^c\) expressed by the RMA-S transfectants presented either P2P or P2V, RNK-49Wi2 cells were able to recognize the MHC-I molecules and mediate substantial lysis of targets. In contrast, when RT1-A1\(^c\) presented P2Q, which contains the non-B7 supertype P2 residue, a significantly depressed level of cytolysis was mediated by the RNK-49Wi2 cells. Thus, the pattern of RT1-A1\(^c\)–peptide complex recognition by Ly49i2 observed with soluble multimers was also found in target cell lysis, where RT1-A1\(^c\) bound with peptides bearing supertype P2 anchor residues, Pro or Val, are efficient Ly49i2 ligands, whereas RT1-A1\(^c\) bound with a non-supertype P2 anchor residue is not. These results indicate that the identity of the P2 anchor residue of bound peptide plays an important role in Ly49i2 recognition of its RT1-A1\(^c\) ligand.

**Discussion**

RT1-A1\(^c\) preferentially binds peptides with a proline residue at P2, which defines it as an HLA-B7 supertype (9, 10). Previously, our laboratory showed that point mutations in the B-pocket of RT1-A1\(^c\) preferentially bind RT1-A1\(^c\)–peptide complexes with a proline residue at P2, which defines it as an HLA-B7 supertype (9, 10). Previously, our laboratory showed that point mutations in the B-pocket of RT1-A1\(^c\) preferentially bind RT1-A1\(^c\)–peptide complexes with a proline residue at P2, which defines it as an HLA-B7 supertype (9, 10).
A1Δ resulted in a loss of protection from NK cell lysis mediated by Ly49i2 (13). These mutations were accompanied by alterations in the P2 anchor residues of the RT1-A1Δ-bound peptides. This raised the possibility that bound peptide could influence MHC recognition by Ly49i2 and the hypothesis that Ly49i2 will only recognize RT1-A1Δ that is bound with peptides bearing the HLA-B7 supertype-defining P2 residue Pro or possibly the secondary HLA-B7 supertype residue Val but not P2 anchor residues that are not normally associated with the HLA-B7 supertype (13). Requirements for Ly49i2 recognition of MHC-I could include MHC-I bound with 1) no peptide; 2) any peptide; 3) a peptide with an acceptable B7 supertype P2 anchor; or 4) specifically only Pro at P2. Using three synthesized nonamer peptides that differed only at the P2 residues, we were able to refold each with RT1-A1Δ and showed that each peptide was loaded into the RT1-A1Δ to an equivalent extent. Furthermore, all three RT1-A1Δ–peptide complexes were recognized by the RT1-A1Δ–specific mAb YR5/12 (27, 31), and it was possible that the contribution of bound peptide occupancy. Previously, we suggested that Ly49i2 recognition of MHC-I could be viewed as unexpected, given that the Ly49A/H-2DΔ and Ly49C/H-2KΔ cocrystal structures revealed that Ly49 receptors could interact with, or specificity for, MHC-I ligands by multiple Ly49 receptors.

The influence of different peptides on Ly49 recognition of MHC-I could be viewed as unexpected, given that the Ly49A/H-2DΔ and Ly49C/H-2KΔ cocrystal structures revealed that Ly49 receptors could interact with, or specificity for, MHC-I ligands by multiple Ly49 receptors.
only polymorphic residue in the β1 strand differing between RT1-A1h and Aα is residue 9 (Asp and Tyr, respectively). The Asp9 on the β1 strand interfaces the peptide-binding groove B-pocket and the β2m binding site on RT1-A1h. In RT1-Aα, Tyr9 does not contact β2m. Notably, we have previously mutated Asp9 to Ala in A1h, and this disrupted Ly49i2 recognition, despite this mutant maintaining the correct peptide P2 anchor residue, Pro or Val, in bound peptides (13). This suggests that Asp9 has a role in Ly49i2 recognition independent of its influence on RT1-A1h binding of peptides with specific P2 anchor residues. This may involve facilitating an RT1-A1h conformation conducive to direct interaction of Ly49i2 with the RT1-A1h H chain and/or maintaining β2m in an associated orientation that promotes interaction with Ly49i2, perhaps in conjunction with A1h residues Arg6 and Phe8. Further to the possible role of β2m in Ly49i2 recognition, the conformation of the S4 strand of rodent β2m, which includes residues Phe56, Ser57, and Lys58 that contact the H chain β1 strand, is flexible and can be dramatically different between different rodent MHC-I allele products, resulting in different β2m contacts with the H chain (38). By contrast, the human β2m S4 strand has a single conformation (38). Also, the orientation of β2m and the MHC-I H chain α1/α2 peptide binding domains can differ significantly between rodent MHC-I molecules compared with human (39). Such variation in the nature of β2m orientation and association with the H chain in rodents may provide identity and specificity for Ly49 interactions not used in humans. Thus, rodent β2m may be a transmitter of structural changes in the peptide binding groove (e.g., from the B-pocket bound with different peptide residues)—via MHC-I β1 strand residues—that confers specificity to Ly49 interactions. In the case of the peptides we have examined, P2P and P2V bound in the RT1-A1h B-pocket, through conformational effects communicated through the β1 strand, may be conducive to association of β2m to the H chain in such an orientation, conformation, or affinity that it promotes Ly49i2 interaction. In contrast, it is possible that P2Q would not in such an orientation, conformation, or affinity that it promotes Ly49i2, could indirectly discriminate between the shapes of these peptides—via MHC-I complex conformation, and likely how the MHC-I is perceived by multiple receptor types.

A recent report has indicated that residues 211–231 encompassing the L3 loop on mouse Ly49 determines the allele specificity for H-2Kb (41). In mouse Ly49C, part of this L3 loop forms an α-helix, which is not seen in mouse Ly49A or Ly49G. Exchange of the Ly49C L3/α3 loop into Ly49A led to a gain in recognition of H-2Kb (41). Notably, the L3 loop interacts with MHC-I on the platform below the peptide binding groove in an area proximal to the Aα-, B-, and D-pockets. It is therefore possible that the L3 loop of mouse Ly49, and equivalent sequences in rat Ly49 such as Ly49i2, could indirectly discriminate between the shapes of these pockets through their conformational effects on neighboring MHC-I H chain solvent-exposed residues. Crystal structures of RT1-A1h bound with peptides differing in P2 anchor residues, which either do or do not support Ly49i2 binding (e.g., with Pro versus Gln at the P2 position), may be informative in this regard and further enhanced if in conjunction with a cocryystal of Ly49i2 and RT1-A1h bound with a peptide bearing Pro at the P2 position.

With the potential involvement of β2m in rat Ly49i2 recognition of MHC-I molecules and the possibility that RT1-A1h-bound peptides influence this interaction, it is worth pointing out that FCS, which contains bovine β2m, was used in our Ly49i2 tetramer binding and cytotoxicity assays. Exogenous β2m (e.g., bovine) has a potential to exchange with endogenous β2m (e.g., rodent) bound to MHC-I molecules on the cell surface (42), and therefore some percentage of the RT1-A1h in our assays contains bovine β2m rather than rat β2m, which could influence receptor recognition. It has been shown that mouse Ly49 will not recognize mouse MHC-I when associated with human β2m (7). This is because human β2m lacks S4 strand residues that are involved in rodent-specific recognition by Ly49i2 (43). Bovine β2m, like human β2m, lacks the rodent β2m-specific S4 strand residues and thus if associated with RT1-A1h would likely not be recognized by Ly49i2. However, despite the presence of exogenous bovine β2m from FCS in our assays, we still observed significant recognition by rat Ly49i2 of rat MHC-I complexed with peptides in both multimer binding and cytotoxicity assays. In the case of the Ly49i2 multimer assays, the recombinant RT1-A1h complexes were produced with rat β2m, and the binding assays were carried out at 4°C, a temperature that allows very little β2m exchange (44). As for the cytotoxicity experiments, there would be greater β2m exchange; however, in the current study, rat Ly49 recognition is also robustly observed and with the same relative patterns with the different bound peptides found in the Ly49i2 multimer assays. This may be analogous to mouse Ly49A recognition, where despite human β2m replacing ∼35% of the mouse β2m on mouse MHC-I, Ly49A recognition was entirely unaffected and completely blockable by Ab to mouse β2m (7).

Based on KIR2DL1/HLA-Cw4 and KIR2DL2/HLA-Cw3 co-crystal structures, KIRs make direct contact with the α1 and α2 helices at the top of the peptide binding groove and, particularly for KIR2DL2, also make direct contact with positions 7 and 8 of the bound peptide (45, 46). For KIR2DL2 and KIR2DL3, their binding to HLA-C and inhibition of NK cell function has been shown to be peptide dependent, and the strength of binding/inhibition is strongly influenced by the identity of residues 7 and 8 of HLA-C–bound peptide (47). Thus, peptide influences on KIR recognition are through direct peptide contact with KIR, via peptide residues 7 and 8, which are not anchor residues and instead extend up and out of the peptide binding groove. In the case of rodents, as we have mentioned, β2m can directly contact Ly49 receptors and may confer specificity to MHC-I–Ly49 interactions as a result of MHC-bound peptide-dependent conformational changes imposed on β2m via the floor of the peptide binding groove. The S4 strand of rodent β2m contacting the H chain is flexible and can be dramatically different when bound to different rodent MHC-I allele products (38) and could potentially be further influenced by the nature of the peptide bound in the MHC-I groove. These differences may affect the association and orientation of β2m bound to MHC-I. Such variation may provide identity and specificity for rodent Ly49 interactions. Because the S4 strand of human β2m has only a single conformation, irrespective of the MHC-I allele or nature of bound peptide (38), it would not be conducive to MHC-I ligand discrimination by Ly49 receptors in humans. The purpose of just a single β2m conformation in humans is unclear; however, it could have been a contributor to the evolution of an independent receptor system, the KIRs, to detect and distinguish between MHC-I ligands by means that were not reliant on β2m conformation or orientation. Furthermore, by interacting with MHC-I ligands on top of the peptide binding groove, KIR could directly interact with residue(s) of the MHC-I bound peptide (perhaps providing greater discrimination of peptides) rather than sensing them indirectly as occurs with Ly49. It has also been demonstrated that
HLA-C bound with peptides that induce weak KIR binding antagonize NK cell inhibition by HLA-C–peptide complexes that mediate strong KIR binding (47). It remains to be determined whether RT1-A1–peptide complexes that weakly interact with Ly49I2 also antagonize NK cell inhibition by strongly interacting RT1-A1–peptide complexes.

Like humans, but unlike mice, rat NK cells can express CD8α as homodimers (48). On CD8α+ T cells, CD8 is expressed as an αα homodimer or αβ heterodimer and acts as a coreceptor, simultaneously binding MHC-I with the TCR to enhance responsiveness (49). It is unclear what function CD8 plays on rat NK cells; however, it is presumably capable of binding MHC-I, and at a site below the peptide binding groove involving at least the MHC-I α3 domain (50). Because this is the same area on MHC-I that Ly49 binds, it implies that binding of individual MHC-I molecules by CD8 and CD9 on rat NK cells is mutually exclusive. Our results indicate that Ly49I2 is peptide selective in MHC-I binding, such that a subset of RT1-A1–molecules, perhaps only those with a Pro or Val in the P2 position of bound peptide, are recognized by Ly49I2. In contrast, mouse CD8 is not peptide selective in its interaction with MHC-I ligands (50, 51), as is also likely for rat CD8. Thus, there would likely be some RT1-A1–molecules on the surface of a potential target cell that are ligands for either Ly49I2 or CD8 or others that are ligands only for CD8. This adds complexity to receptor–ligand interactions between NK cells and target cells, perhaps influencing the balance of signals and ultimately whether a target is killed or spared. This would be the case for normal rat NK cells and possibly also with RNK-16 and RNK-16W2 used in our studies, as RNK-16 also naturally expresses CD8α (52, 53).

Finally, because differences in peptides bound to MHC-I can alter Ly49 recognition, as we have shown in this study with rat Ly49I2 and has been shown with mouse Ly49C and Ly49I, this may have implications for immune surveillance. It has been noted that viral infection can alter the repertoire of peptides presented by MHC-I (54, 55). Such changes may alter the type of self-peptide bound by MHC molecules as well as provide substituted or added presentation of virus protein-derived peptides, which may affect the identity of anchor residues of MHC-bound peptides. Should certain viral infections or tumor transformation skew the peptide profile bound to MHC-I in this way, Ly49 recognition could potentially be affected, possibly enhancing or diminishing interactions with inhibitory or activating Ly49 receptors and, correspondingly, NK cell effector functions.

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Disclosures
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References


