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Interactions of Ly49 Family Receptors with MHC Class I Ligands in trans and cis

Léonardo Scarpellino,* Franziska Oeschger,* Philippe Guillaume,* Jérôme D. Coudert, Frédéric Lévy,* Georges Leclercq,* and Werner Held2*

The Ly49A NK cell receptor interacts with MHC class I (MHC-I) molecules on target cells and negatively regulates NK cell-mediated target cell lysis. We have recently shown that the MHC-I ligand-binding capacity of the Ly49A NK cell receptor is controlled by the NK cells’ own MHC-I. To see whether this property was unique to Ly49A, we have investigated the binding of soluble MHC-I multimers to the Ly49 family receptors expressed in MHC-I-deficient and -sufficient C57BL/6 mice. In this study, we confirm the binding of classical MHC-I to the inhibitory Ly49A, C and I receptors, and demonstrate that detectable MHC-I binding to MHC-I-deficient NK cells is exclusively mediated by these three receptors. We did not detect significant multimer binding to stably transfected or NK cell-expressed Ly49D, E, F, G, and H receptors. Yet, we identified the more distantly related Ly49B and Ly49Q, which are not expressed by NK cells, as two novel MHC-I receptors in mice. Furthermore, we show using MHC-I-deficient mice that the NK cells’ own MHC-I significantly masks the Ly49A and Ly49C, but not the Ly49I receptor. Nevertheless, Ly49I was partly masked on transfected tumor cells, suggesting that the structure of Ly49I is compatible in principal with cis binding of MHC-I. Finally, masking of Ly49Q by cis MHC-I was minor, whereas masking of Ly49B was not detected. These data significantly extend the MHC-I specificity of Ly49 family receptors and show that the accessibility of most, but not all, MHC-I-binding Ly49 receptors is modulated by the expression of MHC-I in cis. The Journal of Immunology, 2007, 178: 1277–1284.

Natural killer cells detect alterations in the expression of “self” ligands on host cells. NK cells are activated (via ill-defined receptors) upon the encounter of most normal host cells, yet inhibitory receptors specific for MHC class I (MHC-I) molecules prevent their lysis. MHC-I loss relieves NK cell inhibition, consequently allowing target cell lysis (“missing-self recognition”). In addition, “stressed” host cells may express ligands for the NKG2D NK cell activation receptor allowing target cells lysis even if MHC-I is expressed normally. Thus, the integration of activating and inhibitory signals determines whether or not NK cell-mediated target cell lysis occurs.

Inhibitory MHC-I receptors in the mouse include Ly49 family members, which are specific for classical MHC-I molecules. In addition, heterodimeric CD94/NKG2 receptors are specific for the nonclassical Qa-1B (1). Ly49 receptors are homodimeric type II, C-type lectin-like molecules encoded in the NK gene complex on chromosome 6. This locus contains variable numbers of polymorphic Ly49 genes in different mouse strains. In C57BL/6 (B6) mice, 14 Ly49 genes have been identified, four of which are pseudogenes (2). Whereas the majority of the Ly49 receptors are expressed by NK cells, Ly49B and Ly49Q, which are located at either end of the Ly49 gene complex, are not expressed by NK cells (3, 4). Rather, Ly49Q is found on myeloid lineage cells (4). Based on structural criteria and/or functional data, eight of the Ly49 family receptors in B6 mice are inhibitory, whereas two family members, Ly49D and Ly49H, activate NK cells.

Several but not all Ly49 receptors have been shown to interact with classical MHC-I molecules (Refs. 5, 6 and references therein). Evidence based on the crystal structure of Ly49A/Dd and Ly49C/Kb complexes together with site-directed mutagenesis analyses have shown that Ly49 receptors bind MHC-I laterally, making contacts with residues of the α2 and α3 domains and with β2-microglobulin (β2m) (termed site 2) (7–10). The specificity of Ly49 receptors for the polymorphic MHC-I molecules has been determined using various approaches including bone marrow graft rejection experiments, functional in vitro tests, adhesion assays, and MHC multimer binding (5, 6, 11–13). The available data are based to a significant extent on the transient overexpression of Ly49 receptors and/or staining with soluble MHC-I multimer complexed with human β2m. However, recent data indicate that the binding of MHC-I multimers to Ly49 receptors is influenced by species-specific residues in β2m (8, 10, 14). In addition, the cell surface levels of transiently transfected Ly49 receptors often exceed those of NK cells. Indeed, very little information is available regarding the binding of MHC-I multimer to NK cells. Such analyses are also complicated by the fact that individual NK cells can coexpress multiple distinct Ly49 receptors.

In addition to an interaction of NK cell receptors with ligand on potential target cells (trans interaction), we have recently shown that Ly49A interacts with its H-2Dd ligand expressed on the same cell (cis-interaction) (15). Like Ly49A/Dd trans interaction, cis binding occurs via the lateral binding site (site 2),
excluding simultaneous cis and trans binding by individual Ly49A receptors. Therefore, fewer inhibitory receptors are available for functional trans binding and consequently the ensuing inhibitory signaling is relatively weak. This renders NK cells more sensitive to stress-induced activation ligands on Dd target cells and consequently to detect diseased host cells. It is currently not known whether this phenomenon is limited to Ly49A or whether other NK cell receptors are influenced by cis MHC-I.

In this study, we have (re)evaluated the MHC-I-binding capacity of all the Ly49 family receptors expressed in B6 mice using MHC-I multimers complexed with mouse β2m. In addition, we have analyzed MHC-I multimer binding to NK cells, and finally we have tested whether the modulation of ligand binding by the NK cells’ own MHC-I is a common feature of MHC-I-binding Ly49 receptors.

Materials and Methods

Mice

B6 (H-2b) and β2m-deficient B6 mice were purchased from The Jackson Laboratory. Dd (H-2d) transgenic (Tg) mice have been described before (16). Tg mice were backcrossed at least five times to B6 and contained two alleles of the B6 natural killer gene complex on chromosome 6. All mice were older than 6 wk when used for experiments.

Constructs

Ly49 cDNAs in euartropic expression vectors have been described before (15, 17). Ly49B, C, G2, I, and Q were either subcloned directly or PCR amplified from plasmid DNA and B6 day 14 fetal liver cdna, respectively, before cloning into a modified pEF-BOS internal ribosome entry site (7). Expression vectors for Ly49A, Ly49D, E, F, and H were made in our laboratory using primers (restriction sites are underlined): Ly49A antisense, accggccgcc TAATTTATGGATGC; Ly49D sense, actg gcggccgc TTAACTTACATCAC; Ly49E antisense, accggccgc AGCACAATCGCTACTCC; Ly49F sense, actg gcggccgc TTAATTCATCAC; Ly49H antisense, accggccgc TAAATTATGCTTATG; Ly49C antisense, actg gcggccgc TTAATTTATGGATGC; Ly49D sense, actg gcggccgc TTAACTTACATCAC; Ly49E antisense, accggccgc AGCACAATCGCTACTCC; Ly49F sense, actg gcggccgc TTAATTCATCAC; Ly49H antisense, accggccgc TAAATTATGCTTATG; Ly49I antisense, accggccgc TAAATTATGCTTATG; Ly49J antisense, accggccgc TAAATTATGCTTATG; Ly49K antisense, accggccgc TAAATTATGCTTATG; Ly49L antisense, accggccgc TAAATTATGCTTATG; Ly49M antisense, accggccgc TAAATTATGCTTATG; Ly49N antisense, accggccgc TAAATTATGCTTATG; Ly49O antisense, accggccgc TAAATTATGCTTATG; Ly49P antisense, accggccgc TAAATTATGCTTATG; Ly49Q antisense, accggccgc TAAATTATGCTTATG; Ly49R antisense, accggccgc TAAATTATGCTTATG; Ly49S antisense, accggccgc TAAATTATGCTTATG; Ly49T antisense, accggccgc TAAATTATGCTTATG; Ly49U antisense, accggccgc TAAATTATGCTTATG; Ly49V antisense, accggccgc TAAATTATGCTTATG; Ly49W antisense, accggccgc TAAATTATGCTTATG; Ly49X antisense, accggccgc TAAATTATGCTTATG; Ly49Y antisense, accggccgc TAAATTATGCTTATG; Ly49Z antisense, accggccgc TAAATTATGCTTATG.

Ly49 family constructs were transiently expressed in HEK293T cells using lipofectamine transfection reagent (GIBCO, Carlsbad, CA). All constructs were sequenced to ensure that no errors had been introduced.

Cells and transfectants

The following cell lines were used: C1498 (immature NK T cell line (H-2b)) (18) (provided by W. Seaman, University of California, San Francisco, CA), C1498 transfected with Ly49A, Dd, and Ly49A – Dd (15); C4.25 – (a β2m-deficient variant of EL-4 (H-2b)) (19) (provided by H. G. Ljunggren, Karolinska, Sweden) and M145 (TAP-1-deficient cell). These cell lines were transfected by electroporation. Stable transfectants were obtained by three rounds of MACS selection (Miltenyi Biotec) using appropriate mAbs or by selection based on puromycin resistance. Clones were obtained by micro- and by flow cytometry. Ly49 constructs were transiently expressed in HEK293T cells using calcium phosphate-mediated transfection. Ly49D and Ly49H were cotransfected with DAP-12. After 2 days transfected cells were harvested, stained, and analyzed by flow cytometry.

Stable transfectants were obtained by electroporation of C1498 cells and puromycin selection.

MHC-I multimers

MHC-I multimers were produced in bacteria, refolded with the peptides indicated below and mouse β2m (unless indicated otherwise), biotinylated, and multimerized using streptavidin-PE using standard techniques (20). The following multimers were used: Kb-OVA (OVA: SIINFEKL), Kd-HA (influenza virus HA 204 –212: LYQNVGTYV), Kd-ERK (mouse ERK: QYIHSANVL), Kd-Pb (Plasmodium berghei CS 253 –260: SYIPSAE (ABA)K I), Dd-HIV (RPGGRAFTVTI), Dd-MT (polyoma virus middle T Ag (MT389 –397: RRLGRTRL)), Dd-LCMV (LCMV gp33: KAVYNFATCGI).

Flow cytometry

Spleens were passed through nylon wool columns, and nonadherent cells were incubated with 24G2 (anti-CD16/32) hybridoma culture supernant to reduce background. Transfectants (2 × 10^6/sample) or splenocytes (10^6 cells/sample) were incubated with MHC-I multimer (at 30 – 40 μg/ml) for 30 min at 4°C.

For surface stainings, the following mAbs were obtained from BD Pharmingen unless otherwise indicated: J9R3-318 (anti-Ly49A; provided by J. Roland, Institut Pasteur, Paris, France), 4D11 (anti-Ly49G), 4E5 (anti-Ly49D), 14B11 (anti-Ly49CF,H1), HBF (anti-Ly49F), YLI (anti-Ly49I), Dd12 (anti-Ly49C,E) (17), 2E6 (anti-Ly49G, MBL), and polyclonal anti-Ly49B anti- serum was obtained by immunizing rats with a soluble version of the extracellular portion of Ly49B, 145-2C11 (anti-CD3ε), PK136 (anti-NK1.1), 34-2-12 (anti-Dd), SF1.1.1.1 (anti-Kb), B8.24 (anti-Kk), and S19.8 (anti-mouse β2m). These Abs were conjugated to different fluorochromes or biotinylated. Biotinylated mAbs were revealed through staining with streptavidin-PE (BD Pharmingen). Anti-Ly49B was revealed using anti-rat IgG-PE (Caltag Laboratories). Three- or four-color flow cytometry was performed using FACScan or FACSCalibur flow cytometers and CellQuest software for data evaluation (BD Biosciences).

Acid treatment

Transfectants or nylon-wool nonadherent spleen cells were acid stripped as described previously (15). Briefly, the cells were washed twice in PBS and resuspended in citrate buffer (0.133 M citric acid/0.066 M Na2HPO4; pH 3.3) at 5 × 10^6 cells/ml for 4 min at room temperature. The treatment was stopped by the addition of an excess of PBS + 5% FCS. After washing, the cells were stained for flow cytometry as detailed above. Acid treatment did not affect cell viability as judged by trypan blue exclusion or forward and side scatter analysis.

Immunoprecipitation

A total of 10 × 10^6 C1498 cells were washed twice in PBS. The cells were lysed on ice for 4 h in Tris buffer (20 mM Tris; pH 8.0) containing 0.3% Triton X-100 and immunoprecipitated overnight using anti-ASV agarose beads (Sigma-Aldrich). After two washes with lysis buffer, immunoprecipitates were resolved on SDS-PAGE (8% nonreducing), transferred onto nitrocellulose membrane, and immuno blotted using Abs to ASV and rabbit anti-pan-class I Abs (R218). For detection, the ECL kit (Amersham) was used according to the manufacturer’s instructions.

Results

Binding of MHC-I multimers to Ly49 family receptors

The specificity of individual Ly49 receptors was tested by transiently transfecting HEK293T cells and by staining them with H-2K and H-2D multimers (Fig. 1). Because Ly49 binding may be dependent on species-specific residues in β2m, our MHC-I complexes were refolded with mouse β2m. Ly49C transfectants stained with all the different MHC-I multimers, in agreement with Ref. 6. This also ensured the quality of the different MHC-I multimers used. In contrast to Ly49C, Ly49D, E, F, and H transfectants did not significantly react with any of the MHC-I multimers. Ly49G2 reacted weakly with Dd, Ly49I reacted with Kk-OVA and Kk-HA. Somewhat unexpectedly, Ly49A reacted with all MHC-I multimers, even though the binding of Dd, Kk, and Kk multimers was reduced compared with that of Dd or Dd multimers. Moreover, we found that Ly49B also reacted with all MHC-I multimers. In addition, Ly49Q reacted with Kk-OVA and weakly with Dd-HIV (Fig. 1). These data identify Ly49B and Ly49Q as two additional murine MHC-I receptors.

To determine whether some of the observed interactions of Ly49 receptors with MHC-I were due to transient over expression in a xenogeneic cell line, we used murine C1498 cells (H-2b), stably transfected with individual Ly49 receptors (Table I). Consistent with the analysis of the transient transfectants, Ly49C and B reacted with all MHC I multimers and Ly49I bound H-2K. Stably transfected Ly49A efficiently bound to Dd and Dd, weakly to Dd, but not at all to Kk or Kk, which is in agreement with published data. In addition, stably transfected Ly49B bound Kk-OVA but not Dd-HIV. Similarly, Ly49G2 failed to bind Dd-HIV. Thus, stable
transfection shows that the Ly49A, B, C, I and Q receptors engage classical MHC-I molecules.

**Binding of MHC-I multimers to NK cells**

Each of the PE-labeled MHC-I multimers identified a subpopulation of 15–70% of NK cells in /H9252 2m-deficient mice (Fig. 2 and data not shown). We thus determined whether MHC-I multimer binding to /H9252 2m-deficient NK cells was accounted for by known NK cell receptors. D d-HIV binding to /H9252 2m-deficient NK cells was blocked partially using mAb JR9 (anti-Ly49A) or mAb 14B11 (anti-Ly49C, F, H, I). The binding was completely blocked upon combining these two mAbs (Fig. 2). Identical data were obtained with Dk-MT (data not shown). The binding of D b-LCMV was weak and reduced in part by blocking Ly49A. D b-LCMV binding was prevented using mAb 14B11 (anti-Ly49C, F, H, I), but unaffected by mAb YLI (anti-Ly49I). Thus, together with the data shown above, Db-LCMV binds NK cells preferentially but weakly via Ly49C, whereby the coexpression of Ly49C with Ly49A seems to enhance multimer binding. K d-HA binding to NK cells was prevented partly using mAb YLI (anti-Ly49I) and completely using mAb 14B11 (anti-Ly49C, F, H, I) (Fig. 2). Identical results were obtained with K b-OVA (data not shown). Thus, MHC-I multimer binding to /H9252 2m-deficient NK cells is completely prevented using a combination of mAb JR9 (anti-Ly49A) and/or 14B11 (anti-Ly49C, F, H, I). Together with the data from the transfectants, this analysis suggests that detectable binding of classical MHC-I to NK cells is mediated by the Ly49A, C and I receptors.

In agreement with these findings, an antiserum raised against a soluble version of the extracellular portion of Ly49B, did not stain splenic NK cells from /H9252 2m-deficient or B6 mice (data not shown), indicating that NK cells do not express Ly49B. This is in agreement with an earlier RT-PCR analysis (3). Similar to Ly49B, Ly49Q is not expressed on NK cells, but is rather found on various myeloid lineage cells, including macrophages and dendritic cells (4).

<table>
<thead>
<tr>
<th>Transfectant</th>
<th>Ab</th>
<th>K b OVA</th>
<th>K d HA</th>
<th>D b LCMV</th>
<th>D d MT</th>
<th>D d HIV</th>
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<td>125</td>
<td>2</td>
<td>2</td>
<td>4</td>
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</tbody>
</table>

* a Data are from one of many independent experiments. Numbers indicate the MFI of staining. Numbers in bold face depict significant staining (>2 fold over controls). All MHC-I multimers were used at 40 µg/ml.
**Modulation of H-2D multimer binding by MHC-I expression**

Next, we compared MHC-I multimer binding to NK cells from MHC-I-deficient and -sufficient mice. As shown above, the D\textsuperscript{d}-HIV multimer efficiently (mean fluorescence intensity (MFI) = 1334) stained the Ly49A receptor on β\textsubscript{2}m-ko NK cells (Fig. 3). The intensity of multimer binding to Ly49A was slightly reduced in H-2\textsuperscript{b} mice (MFI = 1241). In contrast, in H-2\textsuperscript{D\textsuperscript{d}} mice, D\textsuperscript{d}-HIV staining of Ly49A was strongly reduced (MFI = 78), i.e., a reduction of 15-fold as compared with B6 (Fig. 3). Corresponding results were obtained with the D\textsuperscript{d}-MT multimer (data not shown).

To assess whether multimer binding to Ly49A was modulated by the NK cell's own MHC-I, we disrupted MHC-I complexes on the surface of NK cells using a brief exposure to an acidic buffer (15, 21). Disruption of MHC-I complexes is demonstrated by the complete loss of mAb staining for β\textsubscript{2}m (Fig. 3, whereas other markers like NK1.1 were not affected (data not shown). The treatment of H-2\textsuperscript{D\textsuperscript{d}} NK cells significantly improved D\textsuperscript{d} (data not shown) and D\textsuperscript{d} multimer binding (MFI = 866) to Ly49A (Fig. 3 and data not shown).

These data confirm our previous findings using Ly49A transfectants and Ly49A Tg mice (15) and suggest that D\textsuperscript{d} expression on normal NK cells significantly masks the Ly49A receptor. The D\textsuperscript{d} dependence of this effect is demonstrated by the acid treatment of β\textsubscript{2}m-ko or H-2\textsuperscript{D\textsuperscript{d}} NK cells, where D\textsuperscript{d}-HIV or D\textsuperscript{d}-MT multimer binding to Ly49A is not improved (Fig. 3 and data not shown).

An additional Ly49 receptor expressed by NK cells binds to D\textsuperscript{k} and D\textsuperscript{d} multimers. From mAb blocking experiments and the analysis of transfectants, we conclude that this receptor is Ly49C (Table I, Fig. 2, and data not shown). Compared with Ly49A, D\textsuperscript{d}-HIV and D\textsuperscript{d}-MT multimer stain Ly49C expressed on β\textsubscript{2}m-ko NK cells with intermediate efficiency (MFI of 80–200). Thus, D\textsuperscript{k} or D\textsuperscript{d} multimer binding to Ly49C is on average 5- to 7-fold lower than to Ly49A. In H-2\textsuperscript{b} mice, D\textsuperscript{d}-HIV and D\textsuperscript{d}-MT multimer binding to Ly49C is below detection, suggesting that MHC-I expression by NK cells reduces multimer binding to Ly49C at least 10-fold (Fig. 3 and data not shown). Indeed, acid treatment of H-2\textsuperscript{b} NK cells significantly restored multimer binding to Ly49C. Similarly, the weak binding of D\textsuperscript{d}-LCMV to β\textsubscript{2}m-ko NK cells that is preferentially mediated by Ly49C (Fig. 2), was no longer observed in H-2\textsuperscript{b} NK cells (Fig. 3). Again, the D\textsuperscript{d}-LCMV staining improved upon acid stripping of H-2\textsuperscript{b} NK cells (Fig. 3). We conclude that, similar to Ly49A, the self-MHC class Ia molecules expressed by H-2b NK cells (K\textsuperscript{k} and/or D\textsuperscript{b}) mask Ly49C. The fact that Ly49C binds K\textsuperscript{k} much more efficiently than D\textsuperscript{b} (6, 22) (see below) suggests that K\textsuperscript{k} is the MHC-I molecule that exerts this cis effect. Thus, besides Ly49A, the MHC-I ligand binding capacity of Ly49C is strongly modulated by cis MHC-I. D\textsuperscript{d}-HIV or D\textsuperscript{d}-MT binding to Ly49A/C-negative NK cells was not observed (data not shown), suggesting that there is no additional NK cell receptor, which significantly binds these multimers.

**Distinct effect of cis MHC-I on H-2K multimer binding to Ly49C and I**

As shown in Table I, K\textsuperscript{d}-HA and K\textsuperscript{b}-OVA multimer bind to both Ly49I and Ly49C receptors. Because the Ly49C-specific mAb 4LO does not efficiently block multimer binding to Ly49C (data not shown), we used a different approach to compare the ligand binding capacity of Ly49I and Ly49C. Because the two receptors are coexpressed on a significant fraction of NK cells, we evaluated multimer binding to NK cell populations where Ly49I and/or Ly49C-expressing cells were gated out (Fig. 4). Importantly, multimer staining was always done before anti-Ly49 mAb staining.

In β\textsubscript{2}m-ko NK cells, K\textsuperscript{d}-HA binding to Ly49C (MFI = 264) was ~3-fold more efficient than to Ly49I (MFI = 81) (Fig. 4). K\textsuperscript{d}-HA binding to Ly49C/I-negative NK cells was not observed (Fig. 4), demonstrating that there is no additional NK cell receptor...
responding results (data not shown). Ly49I binding by Kb-OVA subset coexpressing Ly49I and C. Staining with Kd-HA (gray histogram) is negatively gated for Ly49I and/or Ly49C. This excludes the NK cell bound to Ly49C (Fig. 5). Thus, Ly49I binding to Kd is peptide-selective, which is in agreement with a previous report using transient Ly49I transfectants (6).

which significantly binds this multimer. In H-2b NK cells, Kd-HA multimer binding to Ly49C was very low (MFI <18), indicating a >10-fold reduction of the ligand binding capacity of Ly49C, consistent with the data shown above (Fig. 3). Corresponding data were obtained with the Kd-OVA multimer (data not shown) and also recently reported by (23). Removal of MHC-I molecules by acid stripping significantly restored Kd-HA binding to Ly49C (MFI = 63) (Fig. 4).

In contrast to Ly49C, the capacity of Ly49I to bind Kd-HA was only marginally reduced in H-2b (MFI = 67) as compared with β2-m-k0 NK cells (MFI = 81). Acid stripping of H-2b NK cells did not significantly improve Kd-HA binding to Ly49I (MFI = 63) (Fig. 4). Thus, the NK cell’s MHC-I does not significantly impact ligand binding by Ly49I, whereas ligand binding to Ly49C is strongly (>10-fold) reduced. The Kd-OVA multimer yielded corresponding results (data not shown). Ly49I binding by Kd-OVA multimer was not observed by (23), which is likely due to an inferior staining efficacy of the multimer used in this study.

Basis for deficient cis MHC-I binding by Ly49I

To address the basis for the finding that cis MHC-I had no effect on the ligand binding capacity of Ly49I, we tested the binding of a series of soluble H-2K complexes to Ly49I. Although binding of Kd-OVA and Kd-HA was readily detected, Kd-Cw3 (data not shown), Kd-ERK, and Kd-Pb failed to bind Ly49I expressed on β2-m-deficient NK cells (Fig. 5). Yet, all these multimers efficiently bound to Ly49C (Fig. 5). Thus, Ly49I binding to Kd is peptide-selective, which is in agreement with a previous report using transient Ly49I transfectants (6).

As shown above, Kd-HA refolded with murine β2m efficiently binds Ly49I expressed on β2-m-deficient NK cells. The same multimer refolded with human β2m failed to bind Ly49I, yet readily bound to Ly49C (Fig. 5). Thus, similar to Ly49A, MHC-I binding by Ly49I is influenced by β2m and thus seems to occur via the lateral binding site 2. The peptide selectivity of Ly49I binding may be based on peptide-induced conformational changes in Kd. From a total of two Kd and eight Kd multimers tested, only three bind Ly49I (Fig. 5 and Ref. 6), suggesting that MHC-I complexes compatible with Ly49I binding are relatively rare.

Based on the above findings, deficient masking of Ly49I may be accounted for by a low abundance of appropriate peptides in NK cells, which are compatible with Ly49I binding in cis. Alternatively, structural constraints may prevent Ly49I masking. To address this issue, we stably transfected Ly49I into C1498 (H-2b). Consistent with the possibility that Ly49I is partially masked by the cis MHC-I of C1498 tumor cells, Kd-OVA and Kd-HA binding to Ly49I significantly improved upon acid stripping (176 ± 44 and 141 ± 12% of nonstripped, respectively) (Table II). Next, C4.4.25 (H-2b, β2-m-deficient EL-4 variant) cells were stably transfected with Ly49I together with mouse or human β2m. Acid stripping of Ly49I β2-m-deficient C4.4.25 cells did not improve multimer staining. Rather, ligand binding was actually somewhat reduced (68–79% of nonstripped) (Table II), suggesting that acid treatment has a minor negative effect on ligand binding by Ly49I. Nevertheless, after the introduction of mouse β2m into the above cells, a significant improvement of multimer staining was observed following acid stripping (180–247% of nonstripped). In contrast, after the introduction of human β2m, there was no significant improvement of multimer staining following acid stripping (85–112% of nonstripped). These data show that Ly49I is partly masked by cis MHC-I of C1498 and mouse β2m-C4.4.25 (EL-4) tumor cells, suggesting that the structure of Ly49I is compatible with cis association. The data raise the possibility that in contrast to primary NK cells, the two tumor cell lines express peptide pools, which are skewed toward peptides that are compatible with...
cis binding of Ly49 to MHC-I. Indeed, the dependence of cis and trans binding on mouse β₂m argues that the two types of interactions occur via a similar (if not identical) binding site.

Ly49B is not masked by cis MHC-I

Finally, we used stable C1498 (H-2b) and/or H-2b/Dd) transfectants to evaluate the possibility that the ligand-binding capacity of Ly49B and Ly49Q was modulated by cis MHC-I. We noted a minor but consistent and significant improvement of K⁺-OVA multimer binding upon acid stripping of Ly49Q transfectants (112 ± 4% of nonstripped) (Table II), consistent with the possibility that cis MHC-I partially masks Ly49Q. In contrast to Ly49Q, K⁺-OVA or Dd-MT binding to Ly49B did not improve at all upon acid stripping. Rather, ligand binding was actually somewhat reduced (78–86% of nonstripped) (Table II), consistent with the above observation that ligand binding by Ly49B receptors is slightly acid sensitive. These data indicate that MHC-I ligand binding by Ly49B, but not by Ly49QB, is modulated by cis MHC-I.

Physical association of Ly49 receptors with MHC-I in cis

Acid stripping combined with MHC-I multimer staining provided evidence that Ly49 receptors interact to a variable extent with MHC-I in cis. To generate more direct evidence for associations in cis, we immunoprecipitated Ly49 receptors from stable transfectants using intracellular VSV tags and tested whether MHC-I co-immunoprecipitated. As shown in Fig. 6, MHC-I was readily detected in Ly49A immunoprecipitates when cells coexpressed Dd, but not when they lacked Dd, consistent with earlier results (15). Moreover, MHC-I was abundant in Ly49C, lower in Ly49Q and detectable in Ly49I precipitates. No MHC-I was detected in association with Ly49B.

Because no MHC-I was detectable in Ly49B immunoprecipitates, this excludes the possibility that Ly49-MHC-I association occurs after cell lysis. In agreement with this notion, no MHC-I was previously detected in Ly49A immunoprecipitates when Ly49A and Dd cells were mixed before lyses (15), demonstrating that coimmunoprecipitation detects only cis associations between Ly49 and MHC-I. Collectively, the data are consistent with the above receptor masking analyses and show that Ly49A, Ly49C, Ly49I, and Ly49Q, but not Ly49B, associate with MHC-I in cis.

Discussion

In this study, we have evaluated the specificity of Ly49 family receptors for MHC-I using a set of soluble, fluorescent MHC-I multimers refolded with mouse β₂m. We show that detectable binding of classical MHC-I to primary NK cells is mediated by the Ly49A, C and I receptors. In addition, we identify Ly49B and Ly49Q, which are not expressed by NK cells, as two novel receptors specific for classical MHC-I molecules in mice.

We observed no significant MHC-I multimer binding to Ly49D, E, F, and H receptors, whereas binding to Ly49G2 was only detectable upon transient overexpression, consistent with Ref. 6. Notwithstanding, there is functional evidence for MHC-I recognition by the Ly49G and D receptors expressed on NK cells (24–26). Moreover, transfected Ly49F was previously shown to mediate weak MHC-I interaction in cellular adhesion assays (6). Thus, the available data suggest that the majority of Ly49 receptors expressed in B6 mice are specific for classical MHC-I molecules. No MHC-I ligand has been identified for the activating Ly49H, which binds to the MCMV m157 protein (27, 28), and no ligand is known for Ly49E, which is predominantly expressed in fetal NK cells and certain TCRγδ T cells (17, 29).

MHC-I binding of Ly49A, C and I on NK cells

Staining of primary NK cells from β₂m-ko mice with soluble multivalent MHC-I indicated that MHC-I binds exclusively to the Ly49A, C and I receptors. As compared with Ly49A, Dd (and Dd) binding to Ly49C was 5- to 7-fold less efficient. As compared with Ly49C, K⁺-HA (and K⁺-OVA) staining of Ly49I was ~4-fold reduced.

In addition to the binding of MHC-I in trans, we have addressed whether the accessibility of Ly49 receptors was modulated by MHC-I expression in cis. Indeed, our previous findings regarding Ly49A masking (15) was confirmed and extended to normal, MHC-deficient and MHC-different NK cells. In addition, we show that Ly49C is also strongly masked by cis MHC-I ligand. The

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Table II. Acid stripping of Ly49 transfectants

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Transfectant</th>
<th>MHC</th>
<th>K⁺-OVA Strip</th>
<th>K⁺-OVA Nonstrip</th>
<th>β₂m Strip</th>
<th>β₂m Nonstrip</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1498</td>
<td>Ly49I</td>
<td>H-2b</td>
<td>176 ± 44</td>
<td>141 ± 12</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>C1498</td>
<td>Ly49I</td>
<td>H-2d</td>
<td>68 ± 14</td>
<td>79 ± 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1498</td>
<td>Ly49I</td>
<td>H-2d</td>
<td>180 ± 39</td>
<td>247 ± 67</td>
<td>1 ± 1</td>
<td></td>
</tr>
<tr>
<td>C1498</td>
<td>Ly49I</td>
<td>H-2d</td>
<td>85 ± 6</td>
<td>112 ± 31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1498</td>
<td>Ly49Q</td>
<td>H-2bDd</td>
<td>112 ± 4</td>
<td>K⁺-OVA Strip</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1498</td>
<td>Ly49B</td>
<td>H-2b</td>
<td>84 ± 7</td>
<td>78 ± 13</td>
<td>1 ± 1</td>
<td></td>
</tr>
<tr>
<td>C1498</td>
<td>Ly49B</td>
<td>H-2bDd</td>
<td>nd</td>
<td>86 ± 11</td>
<td>1 ± 1</td>
<td></td>
</tr>
</tbody>
</table>

* Stable transfectants were stained with the indicated MHC-I multimer or an anti-β₂m mAb before and after acid stripping. The MFI of staining acid-treated cells was divided by the MFI of untreated cells multiplied by 100. Values represent the mean (±SD) of three or more independent experiments. Values that are significantly >100 indicate a masking effect of cis MHC-I.

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FIGURE 6. Ly49/MHC-I coimmunoprecipitation. Lysates of C1498 or C1498 Dd cells, stably transfected with indicated VSV-tagged Ly49 receptor construct, were immunoprecipitated (ip) with anti-VSV mAb. Precipitates were analyzed by immunoblot using pan class I Abs and using an anti-VSV mAb (to ensure equal Ly49 immunoprecipitation).
been missed in a previous study, in which a hemagglutinin tag was receptors in mice (Fig. 1 and Table I). The former interaction has
Ly49B and Ly49Q as two additional MHC-I complexes compatible with Ly49I binding on tumor cells. In addition, it seems likely that only few MHC-I peptide combinations mediate Ly49I binding in cis. Thus, the relative lack of Ly49I receptor masking suggests that NK cells predominantly express peptides that cannot mediate Ly49I binding and consequently that Ly49I cis binding is weak. The pool of peptides displayed by MHC-I is generated and influenced in part by proteasome-dependent cleavages (30). In preliminary experiments, we have tested whether the immunoproteasome influences Ly49I masking. However, K\(^d\)-HA or K\(^b\)-OVAb multimer binding to Ly49I expressed by LMP-2-deficient NK cells was not altered as cis, but we found no evidence that the NK cells’ MHC masked Ly49I. We considered a number of distinct possibilities to explain this discrepancy including structural constraints in Ly49I, which prevent cis association. Alternatively, it was possible that cis association does not occur when the affinity for Ly49I-MHC-I trans binding is below a certain threshold. Finally, we provide evidence for a third explanation, which is based on the observation that K\(^b\) and K\(^d\)-multimer binding to Ly49I is dependent on the bound peptide (Fig. 5 and Ref. 6). From a total of 2 K\(^b\) and 8 K\(^d\) multimers tested so far, only three bind Ly49I (Fig. 5 and Ref. 6). These data indicate that MHC-I complexes compatible with Ly49I binding are relatively rare. Unfortunately, the available data do not yet allow us to determine whether the discrimination of peptides by Ly49I observed with multimers is confirmed in functional assays. Because only selected MHC-I-peptide combinations bind Ly49I in trans, it seems likely that only few MHC-I-peptide combinations mediate Ly49I binding in cis. This information may provide clues to understand why cis interaction is a feature of some but not all Ly49 receptors and ultimately to understand the precise role of cis interaction for NK cell biology.

In conclusion, in this study we show that binding of classical MHC-I by NK cells is mediated by the inhibitory Ly49A, C, and I receptors. The Ly49A and Ly49C receptors on NK cells are strongly masked by the NK cell’s own MHC-I. In contrast, Ly49I is not detectably masked on NK cells but can be masked on transfecants, likely due to an increased presence of MHC/peptide complexes compatible with Ly49I binding on tumor cells. In addition, we show that the more distantly related Ly49B and Ly49Q receptors, which are not expressed by NK cells, represent novel MHC-I receptors in mice. We also detected effects of cis MHC-I on Ly49Q accessibility, whereas the access to Ly49B was not influenced by cis MHC-I. Thus, the accessibility and consequently the function of several but not all MHC-I binding Ly49 receptors can be modulated by MHC-I molecules, which are expressed in the plane of the same membrane.

Note added in proof. Ly49B expression patterns have just been reported (32).

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Disclosures
The authors have no financial conflict of interest.

References


