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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.

N atural 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/D4 and Ly49C/K6 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 complexes 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 \( \text{D}^4 \) 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 receptor expressed in B6 mice using MHC-I multimers complexed with mouse \( \beta_m \). 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 \( \beta_m \)-deficient B6 mice were purchased from The Jackson Laboratory. Dd (H-2b Dd) transgenic (Tg) mice have been described before (16). Tg mice were backcrossed at least five times to B6 and contained two Ly49 receptors. Therefore, fewer inhibitory receptors are available for functional trans binding and consequently the en- suring inhibitory signaling is relatively weak. This renders NK cells more sensitive to stress-induced activation ligands on \( \text{D}^4 \) 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.

#### Constructs

Ly49 cDNAs in eukaryotic 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, respec- tively, before cloning into a modified pEF-BOS internal ribosome entry site plasmid. Ly49 cDNAs in eukaryotic 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 plasmid. Ly49 cDNAs in eukaryotic 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 plasmid. Ly49 cDNAs in eukaryotic 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 plasmid. Ly49 cDNAs in eukaryotic 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 plasmid. Ly49 cDNAs in eukaryotic 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 plasmid. Ly49 cDNAs in eukaryotic 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 plasmid. Ly49 cDNAs in eukaryotic 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 plasmid.

#### Immunoprecipitation

A total of \( 10 \times 10^6 \) C1498 cells were washed twice in PBS. The cells were lysed on ice for 4 h in Tris buffer (0.133 M citric acid/0.066 M Na2HPO4; pH 3.3) at \( 5 \times 10^6 \) 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.

### 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 \( \beta_m \), our MHC-I complexes were refolded with mouse \( \beta_m \). 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 D\( ^{4} \), Ly49I reacted with K\( ^{b} \)-OVA and K\( ^{d} \)-HA. Somewhat unexpectedly, Ly49A reacted with all MHC-I multimers, even though the binding of D\( ^{4} \), K\( ^{b} \), and K\( ^{d} \) multimers was reduced compared with that of D\( ^{4} \) or D\( ^{4} \) multimers. Moreover, we found that Ly49B also reacted with all MHC-I multimers. In addition, Ly49Q reacted with K\( ^{b} \)-OVA and weakly with D\( ^{4} \)-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-2\( ^{b} \)), 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 Ly-49I multimer binding to 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 \( \beta_m \), our MHC-I complexes were refolded with mouse \( \beta_m \). 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 D\( ^{4} \), Ly49I reacted with K\( ^{b} \)-OVA and K\( ^{d} \)-HA. Somewhat unexpectedly, Ly49A reacted with all MHC-I multimers, even though the binding of D\( ^{4} \), K\( ^{b} \), and K\( ^{d} \) multimers was reduced compared with that of D\( ^{4} \) or D\( ^{4} \) multimers. Moreover, we found that Ly49B also reacted with all MHC-I multimers. In addition, Ly49Q reacted with K\( ^{b} \)-OVA and weakly with D\( ^{4} \)-HIV (Fig. 1). These data identify Ly49B and Ly49Q as two additional murine MHC-I receptors.

### Flow cytometry

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

For surface stainings, the following mAbs were obtained from BD Pharmingen unless otherwise indicated: JR9-318 (anti-Ly49A); provided by J. Roland, Institut Pasteur, Paris, France), Dd11 (anti-Ly49G), 4E5 (anti-Ly49D), 14B11 (anti-Ly49CF/H1), HBF (anti-Ly49I), YLI (anti-Ly49I), 4D12 (anti-Ly49CE) (17), 2E6 (anti-Ly49Q; BML), and polyclonal anti- Ly49B anti-serum was obtained by immunizing rats with a soluble version of the extracellular portion of Ly49B, 145-2C11 (anti-CD3e), PK136 (anti- NK1.1), 34-2-12 (anti-D\( ^{4} \)), SF1.1.1.1 (anti-K\( ^{b} \)), B8.24 (anti-K\( ^{d} \)), and S19.8 (anti-mouse \( \beta_m \)). 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 \( 5 \times 10^6 \) 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.
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 β2m-deficient mice (Fig. 2 and data not shown). We thus determined whether MHC-I multimer binding to β2m-deficient NK cells was accounted for by known NK cell receptors. Dd-HIV binding to β2m-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 Db-LCMV was weak and reduced in part by blocking Ly49A. Db-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. Kd-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 Kk-OVA (data not shown). Thus, MHC-I multimer binding to β2m-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 β2m-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 I. Binding of MHC-I multimers to stable Ly49 transfectants

<table>
<thead>
<tr>
<th>Transfectant</th>
<th>Ab</th>
<th>Kb-Ova</th>
<th>Kd-HA</th>
<th>Db-LCMV</th>
<th>Dk-MT</th>
<th>Dd-HIV</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td></td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Ly49A</td>
<td>JR9</td>
<td>1616</td>
<td>3</td>
<td>3</td>
<td>8</td>
<td>286</td>
</tr>
<tr>
<td>Ly49B</td>
<td>Ab</td>
<td>77</td>
<td>431</td>
<td>224</td>
<td>191</td>
<td>324</td>
</tr>
<tr>
<td>Ly49C</td>
<td>4LO</td>
<td>1618</td>
<td>734</td>
<td>801</td>
<td>314</td>
<td>395</td>
</tr>
<tr>
<td>Ly49G2</td>
<td>4D11</td>
<td>1292</td>
<td>4</td>
<td>43</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Ly49I</td>
<td>YLI</td>
<td>469</td>
<td>12</td>
<td>41</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Ly49Q</td>
<td>2E6</td>
<td>148</td>
<td>125</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

*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 Dd-HIV multimer efficiently (mean fluorescence intensity (MFI) = 1334) stained the Ly49A receptor on β2m-ko NK cells (Fig. 3). The intensity of multimer binding to Ly49A was slightly reduced in H-2b mice (MFI = 1241). In contrast, in H-2b Dd mice, Dd-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 Dd-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 β2m (Fig. 3), whereas other markers like NK1.1 were not affected (data not shown). The treatment of H-2b Dd NK cells significantly improved Dd (data not shown) and Dd 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 Dd expression on normal NK cells significantly masks the Ly49A receptor. The Dd dependence of this effect is demonstrated by the acid treatment of β2m-ko or H-2b NK cells, where Dd-HIV or Dd-MT multimer binding to Ly49A does not improve (Fig. 3 and data not shown).

An additional Ly49 receptor expressed by NK cells binds to Dk and Dd 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, Dd-HIV and Dd-MT multimer stain Ly49C expressed on β2m-ko NK cells with intermediate efficiency (MFI of 80–200). Thus, Dk or Dd multimer binding to Ly49C is on average 5- to 7-fold lower than to Ly49A. In H-2b mice, Dd-HIV and Dd-MT multimer binding to Ly49C was 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-2b NK cells significantly restored multimer binding to Ly49C. Similarly, the weak binding of Dd-LCMV to β2m-ko NK cells that is preferentially mediated by Ly49C (Fig. 2), was no longer observed in H-2b NK cells (Fig. 3). Again, the Dd-LCMV staining improved upon acid stripping of H-2b NK cells (Fig. 3). We conclude that, similar to Ly49A, the self-MHC class Ia molecules expressed by H-2b NK cells (Kk and/or Dk) mask Ly49C. The fact that Ly49C binds Kk much more efficiently than Dd (6, 22) (see below) suggests that Kk 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. Dd-HIV or Dd-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, Kk-HA and Kk-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 β2m-ko NK cells, Kk-HA binding to Ly49C (MFI = 264) was ∼3-fold more efficient than to Ly49I (MFI = 81) (Fig. 4). Kk-HA binding to Ly49C/I-negative NK cells was not observed (Fig. 4), demonstrating that there is no additional NK cell receptor.
which significantly binds this multimer. In H-2b NK cells, Kd-HA multimer binding to Ly49C was very low (MFI = 63), 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 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 β2m-deficient 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 β2m-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).
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-2d-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 Kb-OVA Ly49B and Ly49Q was modulated by cis to evaluate the possibility that the ligand-binding capacity of Ly49B is not masked by cis MHC-I. The possibility that cis MHC-I partially masks Ly49Q. In contrast to Ly49Q, Kb-OVA or D₄-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 Ly49Q, but not by Ly49B, 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 coimmunoprecipitated. As shown in Fig. 6, MHC-I was readily detected in Ly49A immunoprecipitates when cells coexpressed D₄, but not when they lacked D₄, 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 D₄ 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, D₄ (and D₄) binding to Ly49C was 5– to 7-fold less efficient. As compared with Ly49C, K₄-HA (and K₆-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>Kb-OVA Strip</th>
<th>Kb-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>C4.4.25</td>
<td>Ly49I</td>
<td>No</td>
<td>68 ± 14</td>
<td>79 ± 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4.4.25</td>
<td>Ly49I 2mβ₂m</td>
<td>H-2b</td>
<td>180 ± 39</td>
<td>247 ± 67</td>
<td>1 ± 1</td>
<td></td>
</tr>
<tr>
<td>C4.4.25</td>
<td>Ly49I huβ₂m</td>
<td>H-2b</td>
<td>85 ± 6</td>
<td>112 ± 31</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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*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.
presence of D\textsuperscript{b} in cis reduced D\textsuperscript{b} multimer binding of Ly49A \textasciitilde 15-fold, and acid stripping improved multimer binding 11-fold. The presence of H-2\textsuperscript{b} (most likely K\textsuperscript{b}) reduced K\textsuperscript{b} (or K\textsuperscript{d}) multimer binding of Ly49C at least 10-fold and acid stripping improved it 4- to 5-fold. The cis effect on Ly49C cannot be determined more accurately because K\textsuperscript{b} (or K\textsuperscript{d}) multimer binding to Ly49C in H-2\textsuperscript{b} mice is at the limit of detection.

**Basis for deficient receptor masking: Ly49I**

Ly49I binds soluble MHC-I in trans, 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\textsuperscript{b}- and K\textsuperscript{d}-multimer binding to Ly49I is dependent on the bound peptide (Fig. 5 and Ref. 6). From a total of 2 K\textsuperscript{b} and 8 K\textsuperscript{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 what renders K\textsuperscript{b} peptides compatible or incompatible with Ly49I binding. Moreover, it remains to be determined 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. 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\textsuperscript{d}-HA or K\textsuperscript{b}-OVA multimer binding to Ly49I expressed by LMP-2-deficient NK cells was not altered as compared with B6 (our unpublished data). Thus, the set of peptides expressed in primary NK cells, which is compatible with MHC-I/Ly49I cis binding, is not significantly altered due to absence of the immunoproteasome.

However, significant Ly49I masking was observed in two tumor cell lines, demonstrating that the Ly49I structure and affinity for ligand are compatible in principal with cis association. Moreover, these data suggest that the peptide pool of these B6-derived tumor cells is skewed toward peptides compatible with Ly49I/MHC-I cis association. Thus, we conclude that a low abundance of such peptides and consequently a low abundance of appropriate MHC-I complexes in primary NK cells can account for the lack of Ly49I cis association.

We can currently only speculate on the importance of the capacity of Ly49I to discriminate peptides associated with H-2K\textsuperscript{b} or K\textsuperscript{d}. Ly49I may be useful to detect cells infected with intracellular pathogens, if these pathogens produce large amounts of peptides that are incompatible with Ly49I binding. Such peptides may compete with the rare peptides derived from self proteins, which engage the Ly49I receptor and normally keep NK cells in check. An excess of noninhibitory peptides would thus abrogate Ly49I-dependent NK cell inhibition and consequently result in the lysis-infected host cells.

**Ly49B and Ly49Q**

We have identified Ly49B and Ly49Q as two additional MHC-I receptors in mice (Fig. 1 and Table I). The former interaction has been missed in a previous study, in which a hemagglutinin tag was added to the Ly49B C terminus (6), which is extracellular. Most likely, this modification abrogates ligand binding by Ly49B, even though a similarly modified Ly49A still binds MHC-I (6). This raises the possibility that MHC-I recognition by Ly49A and Ly49B is significantly different. Because Ly49B efficiently binds K\textsuperscript{d}-HA complexes refolded with human \( \beta_2m \) (our unpublished data), we cannot currently draw any conclusion as to how Ly49B binds MHC-I.

Unlike Ly49A, C, I, and Q, we noted a complete lack of Ly49B receptor masking by MHC-I cis ligand. Ly49B is the most distantly related Ly49 family member with <50% aa identity to the other Ly49 receptors. It is thus possible that the structure of Ly49B is incompatible with cis binding of MHC-I. Along this line, we have previously hypothesized that flexibility of the stalk region of Ly49A plays a critical role in binding MHC-I in cis (15). The swapping of the respective domains of Ly49A and Ly49B should now allow us to test this issue.

It is of interest that Ly49B and Q are two Ly49 family members that are not expressed by NK cells (3, 4) (data not shown). Ly49Q is expressed on myeloid lineage cells, in particular plasmacytoid dendritic cells (4, 31). Thus, cell types other than NK cells use receptor masking to adapt receptor accessibility and consequently receptor function to self-MHC-I. Finally, it is currently not clear where Ly49B is expressed and whether its expression is regulated. 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 transfectants, 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**

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**References**


