MHC Class I Mosaic Mice Reveal Insights into Control of Ly49C Inhibitory Receptor Expression in NK Cells


*J Immunol* 1998; 161:6475-6479; ;
http://www.jimmunol.org/content/161/12/6475

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*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
MHC Class I Mosaic Mice Reveal Insights into Control of Ly49C Inhibitory Receptor Expression in NK Cells


We have analyzed lymphocyte development in natural MHC class I chimeric mice, generated through a transgenic approach in β2-microglobulin (β2m)-/- mice. In these mice, MHC class I+ cells coexist with an equal proportion of MHC class I-deficient cells. These MHC class I mosaic mice had normal numbers of CD8+ T cells, which had a target cell specificity similar to that of wild-type mice. Consequently, the mice did not develop any signs of autoimmunity. They also had normal numbers of NK cells. This allowed an examination of the MHC class I influence on the expression of the Ly49C inhibitory receptor on NK cells. This receptor binds to H-2Kb.

It is expressed at low levels on NK cells in wild-type mice of the H-2b haplotype, but at markedly higher levels on NK cells in β2m-/- mice and other strains of mice lacking expression of H-2Kb. Relatively little is known about how MHC class I molecules affect expression of the Ly49 receptors. Through the analysis of the present MHC class I mosaic mice, we demonstrate that the expression levels of Ly49C on NK cells is a consequence not only of MHC class I expression in the environment, but also of the expression of MHC class I molecules by the NK cells themselves. These findings are discussed in relation to the biological role of the calibration of the Ly49 inhibitory receptor expression in relation to self-MHC class I. The Journal of Immunology, 1998, 161: 6475–6479.

The MHC class I molecules affect the development of CD8+ T lymphocytes and NK cells (1–3). This has most clearly been revealed by studies of MHC class I-deficient mice, in particular mice with a disruption in the gene coding for β2-microglobulin (β2m) (4, 5). MHC class I-deficient mice have low numbers of CD8+ T lymphocytes due to impaired positive selection (4, 5). Residual CD8+ T cells in these mice have an altered specificity, most likely as a consequence of selection on low levels of MHC class I ligands and/or an altered MHC class I-presented peptide repertoire (6, reviewed in 2). In contrast to CD8+ T cells, MHC class I-deficient mice have normal numbers of NK cells (7–9, reviewed in 1 and 3). As for CD8+ T cells, the specificity of the NK cells in MHC class I-deficient mice is altered. NK cells from MHC class I-deficient mice are tolerant toward MHC class I-deficient targets, while similar targets are readily killed by NK cells from wild-type mice (7–9, reviewed in 1 and 3).

NK cells recognize MHC class I molecules with specific receptors. Interaction between these receptors and MHC class I molecules on target cells results in an inhibitory signal that prevents killing of the target cells (10–13). In the mouse, MHC class I-specific inhibitory receptors are dimeric C-type lectins that belong to the Ly49 family (14). Recent studies have shown that host MHC class I molecules influence the expression levels of Ly49 receptors on NK cells (15–24). For example, it was recently demonstrated that the Ly49C inhibitory receptor, with a specificity for H-2Kb (25, 26), is expressed at high levels in β2m-/- mice of the H-2b haplotype, but is expressed at significantly lower levels in corresponding wild-type mice and other strains of mice expressing H-2Kb (22, 23). Similar observations have previously been made with the Ly49A receptor (15–17), which has H-2Dd as a ligand (10). Interestingly, different levels of Ly49 inhibitory receptor expression on NK cells correlate with their specificity (27). However, relatively little is known about how MHC class I molecules affect expression of Ly49 receptors.

In an attempt to re-express a functional β2m gene in β2m-/- mice, we generated a panel of founder mice with an unexpected mosaic expression of MHC class I molecules. This allowed us to study lymphocyte development in an environment where MHC class I-positive (MHC class I+) and -deficient cells had coevolved. The analysis of the influence of MHC class I on the expression of the Ly49C inhibitory receptor on NK cells turned out to be of particular interest. In the present study, we demonstrate that expression of the Ly49C inhibitory receptor on NK cells is a consequence not only of MHC class I expression in the environment but also by the expression of MHC class I molecules by the NK cells themselves.

Materials and Methods

Generation of transgenic mice

For the generation of transgenic mice, a 14-kb XhoI fragment containing the β2m gene (28) was used to produce transgenic mice as previously described (29), using β2m-/- mice as egg donors and fertile males. The plasmid containing the mouse β2m gene cloned into pSV2gpt was a kind gift from Dr. P. Robinson (Harrow, U.K.) C57BL/6 (B6) and β2m-/- mice.
(both H-2<sup>b</sup>) were bred and maintained at the Microbiology and Tumor Biology Center, Karolinska Institute, in line with institutional guidelines. β<sub>2m</sub><sup>−/−</sup> mice (5) were originally obtained from The Jackson Laboratory (Bar Harbor, ME). All β<sub>2m</sub><sup>−/−</sup> mice used were backcrossed to a B6 background.

mAbs and flow cytometry
For FACS analysis of splenocytes, erythrocyte-depleted spleen cells from individual mice were resuspended in RPMI 1640 medium supplemented with 10% FCS and loaded onto equilibrated nylon wool (Polyscience, War- rington, PA) columns. The loaded columns were incubated at 37°C for 1 h and the nonadherent cells were subsequently eluted from the column with RPMI 1640/10% FCS. Before staining, cells were washed and resuspended in 200 µl PBS with 1% FCS. For FACS analysis of peripheral blood leukocytes (PBL), approximately 60 µl heparinized blood was collected. Erythrocytes were lysed by three consecutive 5-min incubations in 200 µl of FACS lysis solution (Becton Dickinson, Mountain View, CA; diluted 1:10). Before staining, cells were washed and resuspended as above. Anti-H-2K<sub>b</sub> (AF6-88.5), anti-H-2D<sub>b</sub> (KH95), anti-CD8 (53-6.7), and anti-NK1.1 (PK136) mAbs were obtained from PharMingen (San Diego, CA). Anti-CD4 (KH-CD4) were from Immunokontakt (Frankfurt, Germany). The anti-Ly49C specific mab (4LO3311) has been described (30). All mAbs were used at a concentration of 10 – 20 µg/ml. Incubations with mAbs as well as streptavidin-conjugates (Life Technologies, Paisley, U.K.) for biotinylated molecules were done for 30 min on ice. Cells were analyzed by flow cytometry on a FACScan (Becton Dickinson) flow cytometer.

Immunohistochemistry
Organs or tissue sections were embedded in Tissue-Tek (Sakura Finetek USA, Torrance, CA) and frozen in liquid nitrogen. Frozen sections (6-µm) were cut, air-dried, fixed in ice-cold acetone, dried, and stored at –20°C. Before staining, the sections were rehydrated in Tris-buffered saline and incubated with 0.1% BSA-C (Avrion, Wageningen, the Netherlands) for 15 min to block unspecific protein interactions. The sections were incubated over night with a FITC-conjugated anti-H-2K<sub>b</sub> mAb (AF6-88.5), obtained from PharMingen, at a dilution of 10 µg/ml. For immunohistochemistry, the sections were washed and then incubated for 40 min with a swine anti-rabbit anti-serum conjugated with horseradish peroxidase (P0217), obtained from Dako (Glostrup, Denmark), at a 1:300 dilution. After washing, samples were finally incubated for 40 min with a swine anti-rabbit anti-serum conjugated with horseradish peroxidase (P0217), obtained from Dako, at a 1:40 dilution. Horseradish peroxidase localization was revealed by incubating with a diaminobenzidine substrate for 10 min. The sections were lightly counter-stained with hematoyline before mounting. The images were captured in pseudo color using a digital fluorescence microscope (Leica DM-RXA; Leica, Nussloch, Germany) and processed in Adobe Photoshop (Adobe, Mountain View, CA) to modify colors and to enhance contrast and intensity.

Generation of allospecific CTL
Anti-H-2<sup>b</sup> specific CTL were generated in bulk mixed lymphocyte cultures by using splenocytes from B6, β<sub>2m</sub><sup>−/−</sup> and MHC class I mosaic mice as responders and irradiated splenocytes from DBA/2 mice as stimulators. Cytotoxicity was assessed in a standard 51Cr-release assay.

Generation of LAK cells
For generation of NK cells activated with rIL-2 (referred to as LAK cells), 25 × 10<sup>6</sup> splenocytes were put in 10 ml α-MEM tissue culture medium supplemented with 10% FCS, 10 mM HEPES buffer, 2 × 10<sup>−7</sup> M 2-ME, antibiotics (complete medium), and 1500 U/ml human rIL2 in 25-cm<sup>2</sup> tissue culture bottles at 37°C in 10% CO<sub>2</sub>. After 4 days, nonadherent and adherent cells were removed, washed once, and resuspended in complete medium without rIL-2 and stained for flow cytometry analysis.

Results
Generation and characterization of MHC class I mosaic mice
β<sub>2m</sub><sup>−/−</sup> mice of the H-2<sup>b</sup> haplotype express severely reduced levels of H-2K<sub>b</sub> and D<sub>b</sub> at the cell surface (4, 5). In an attempt to re-express a functional β<sub>2m</sub> gene in β<sub>2m</sub><sup>−/−</sup> mice, we generated a panel of founder mice with an unexpected mosaic expression of MHC class I molecules. Briefly, 13 of 59 founder mice were typed positive for the β<sub>2m</sub> transgene (data not shown). Flow cytometry analysis of PBL showed that 3 of 13 transgene-positive mice (founder mice 33, 34, and 53, respectively) expressed MHC class I molecules on all PBL, resembling the pattern observed in wild-type mice (Fig. 1). Ten of 13 transgene-positive mice had an unexpected mosaic pattern of MHC class I expression, with an overall equal distribution of MHC class I<sup>+</sup> and -deficient cells among PBL (founder mice 7, 11, 29, 36, 37, 44, 47, 49, 51, and 57, respectively). Genetic and molecular analysis of these founder mice, and offspring from these mice, suggested a late integration of the transgene as the most likely explanation for the observed phenotype, leading to expression of the transgene in one of two cells at the two-cell stage of embryonic development. Offspring from the MHC class I mosaic mice, i.e., (MHC class I mosaic × β<sub>2m</sub><sup>−/−</sup>)<sub>F<sub>1</sub></sub> mice, were either entirely MHC class I<sup>+</sup> or MHC class I-deficient, and no β<sub>2m</sub> transgene product could be amplified by PCR from the MHC class I-deficient offspring (data not shown). Taken together, these mice fulfill all criteria of being natural β<sub>2m</sub><sup>−/−</sup> and β<sub>2m</sub><sup>+</sup> chimeras, and will from here be referred to as MHC class I “mosaic mice” when not else noted.

MHC class I expression
In all mice with an MHC class I mosaic expression pattern, the levels of MHC class I expression on MHC class I<sup>+</sup> cells were...
ing areas of MHC class I-deficient cells.

Previously published results have shown normal allospecific and MHC class I-restricted T cell responses (Fig. 2, data not shown). Mosaic expression of MHC class I molecules was sufficient to restore positive selection of CD8\(^+\) T cells in the periphery (Table I). Thus, the mosaic expression of MHC class I molecules was readily detectable among purified subpopulations of T cells, B cells, and NK cells, as revealed by flow cytometry analysis of PBL and single-cell suspensions of lymphoid organs (data not shown). Several mosaic mice were observed for >1 yr, and the MHC class I mosaic phenotype was preserved in all mice over time (data not shown).

Restored development of CD8\(^+\) T lymphocytes and no signs of autoimmunity

All mosaic mice analyzed had normal numbers of CD8\(^+\) T cells in the periphery (Table I). The mosaic MHC class I expression pattern was readily detectable among purified subpopulations of T cells, B cells, and NK cells, as revealed by flow cytometry analysis of PBL and single-cell suspensions of lymphoid organs (data not shown) and by immunohistochemical (Fig. 2, A and B) analysis of different organs and tissues. Several mosaic mice were observed for >1 yr, and the MHC class I mosaic phenotype was preserved in all mice over time (data not shown).

that the specificity of the residual CD8\(^+\) T cells selected in an MHC class I-deficient environment, such as in the \(\beta_2\)m\(^{-/-}\) mouse, differs from that of corresponding CD8\(^+\) T cells selected in wild-type mice in that the former CD8\(^+\) T cells readily kill target cells expressing normal levels of self-MHC class I molecules (2, 6). In light of this observation, it was of interest to note that the CD8\(^+\) T cells from the MHC class I mosaic mice failed to kill target cells merely as a consequence of expression of normal levels of self-MHC class I molecules and that the mice did not have any visible pathological changes as revealed by visual inspection of mice during autopsy or by microscopical examination of tissue sections (data not shown).

Low levels of Ly49C receptor expression on MHC class I-positive and -deficient NK cells

The MHC class I mosaic mice had normal numbers of NK cells. Given a mouse model with normal numbers of NK1.1\(^+\) cells and mosaic MHC class I expression, it became of particular interest to study the expression of the Ly49C receptor in these mice. This is the only known Ly49 inhibitory receptor with specificity for an H-2\(b\) class I molecule. Splenic NK1.1\(^+\) cells from \(\beta_2\)m\(^{-/-}\) mice express high levels of Ly49C, while expression is markedly lower on NK1.1\(^+\) cells from B6 mice (Fig. 4 and Table II; Ref. 23). When Ly49C expression was assessed on NK1.1\(^+\) splenocytes and PBL from the mosaic mice, it was observed that levels of Ly49C receptor expression were low on all NK1.1\(^+\) cells, resembling the expression levels of those observed in B6 mice (Fig. 4). However, detailed comparison indicated that levels of Ly49C expression on the MHC class I-deficient/NK1.1\(^+\) splenocytes from the mosaic mice were on average somewhat higher than the levels on the MHC class I\(^+/\)NK1.1\(^+\) cells (Fig. 4 and Table II). Nevertheless, the levels of Ly49C expression were still significantly lower on the MHC class I-deficient/NK1.1\(^+\) population from the mosaic mice than the levels observed on corresponding MHC class I-deficient

![FIGURE 2. Mosaic expression of MHC class I molecules in tissue sections from \(\beta_2\)m\(^{-/-}\) mutant mice rendered transgenic for an intact \(\beta_2\)m gene. Immunohistochemical analysis of liver (A) and intestines (B) from one representative MHC class I mosaic mouse (mouse 57) stained with Abs against H-2K\(b\). The figures show a mosaic pattern of MHC class I expression, made up by areas of MHC class I\(^+\) cells intermixed with corresponding areas of MHC class I-deficient cells.](image)

| Table I. Numbers of CD8\(^+\) and CD4\(^+\) lymphocytes in the spleen of B6, \(\beta_2\)m\(^{-/-}\), and MHC class I mosaic mice |
|-----------------|-----------------|-----------------|
|                 | Mice            |                 |
| CD8\(^+\)CD4\(^+\) | B6             | \(\beta_2\)m\(^{-/-}\) | MHC class I mosaic |
| CD8\(^+\)CD4\(^+\) | 14.1 (4.5)\(^a\) | 1.6 (0.6)\(^a\)  | 15.0 (3.6)\(^a\)  |
| CD8\(^+\)CD4\(^+\) | 15.6 (2.2) | 25.4 (4.0) | 19.0 (3.9) |

\(^a\) Mean percentage of positive cells (± SD); \(n = 3\) for B6 and \(\beta_2\)m\(^{-/-}\) mice are \(n = 6\) for the MHC class I mosaic mice.

![FIGURE 3. Generation of allospecific CTL from B6, \(\beta_2\)m\(^{-/-}\), and MHC class I mosaic mice. Anti-H-2\(d\) allospecific CTL were generated from B6, \(\beta_2\)m\(^{-/-}\), and MHC class I mosaic mice (mice 37 and 47) in primary mixed lymphocyte cultures and were tested against P815 targets (H-2\(d\)).](image)
Discussion

In the present study, we have examined lymphocyte development in MHC class I mosaic mice generated through a transgenic approach using the Ly49C inhibitory receptor. The present results demonstrate that calibration of expression of the Ly49C inhibitory receptor is a direct consequence of the expression of MHC class I molecules on surrounding cells, an observation in line with the initial observations made in studies of the regulation of Ly49A inhibitory receptor expression in bone marrow chimeric mice (15, 16). Furthermore, they demonstrate that expression of Ly49C is affected by MHC class I molecules expressed by the NK cells themselves.

Differential expression of Ly49 receptors in vivo may be important to ensure proper regulation of NK cells, i.e., the ability to detect cells expressing reduced levels of certain or all MHC class I gene products, yet allowing maintenance of self-tolerance (15–17, 27, 31). It may at first glance seem paradoxical that a certain receptor is expressed at lower levels in mice expressing the ligand in vivo are lost in vitro during culture in IL-2.

Table III. LY49C inhibitory receptor expression on MHC class I+ and MHC class I-deficient NK1.1+ LAK cells

<table>
<thead>
<tr>
<th>MHC Class I Mosaic Mice</th>
<th>B6</th>
<th>β2m−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class I− cells</td>
<td>148</td>
<td>140</td>
</tr>
<tr>
<td>Class I+ cells</td>
<td>997</td>
<td>997</td>
</tr>
<tr>
<td>Expt. 1</td>
<td>99</td>
<td>898</td>
</tr>
<tr>
<td>Expt. 2</td>
<td>286</td>
<td>1621</td>
</tr>
<tr>
<td>Expt. 3</td>
<td>266</td>
<td>857</td>
</tr>
</tbody>
</table>

* Mean fluorescence values of Ly49C (mAb 4LO3311) inhibitory receptor staining on NK1.1+ LAK cells from B6, β2m−/−, and MHC class I+ and MHC class I− cells, respectively, from MHC class I mosaic mice (Expt. 1, mosaic mice 37 and 47; Expt. 2, mosaic mice 29 and 36; Expt. 3, mosaic mouse 57). Background staining was ≤7 in all cases.

MHC class I+/NK1.1+ splenocytes from the same mosaic mouse (Table II) indicated a potentially interesting role for MHC class I molecules in the NK cells themselves in determining expression levels of the Ly49C receptor. This notion was substantiated in experiments where NK cells were cultured for 4 days in rIL-2-generating LAK cells. In LAK cell cultures, marked changes in expression of Ly49C in the MHC class I-deficient population of NK1.1+ cells from the mosaic mice were observed (Table III). These cells now expressed high levels of Ly49C, resembling those observed on NK1.1+ LAK cells from β2m−/− mice. In contrast, the Ly49C expression on corresponding MHC class I+NK1.1+ cells from the same LAK cell culture was similar to what was observed in B6 LAK cell cultures (Table III). This result strengthens the notion that MHC class I molecules on the NK cells themselves may affect the expression of Ly49C molecules. The results further suggest that the environmental factors that may serve to repress Ly49C expression on MHC class I-deficient/NK1.1+ cells in vivo are lost in vitro during culture in IL-2.
to this model, expression of low levels of an inhibitory receptor would make the NK cell more prone toward recognizing the difference between MHC class Ihigh and MHC class Ilow targets by only being able to receive an appropriate inhibitory signal from the MHC class Ihigh targets. In contrast, NK cells with high levels of expression of an inhibitory receptor would receive an inhibitory signal from both MHC class Ihigh and MHC class Ilow targets, making such NK cells relatively less useful. A recent study comparing the reactivity of Ly49Ahigh and Ly49Alow NK cells supports this notion (27). In line with the reasoning above, it remains an open question how the MHC class I-deficient cells in the MHC class I mosaic mice manage to avoid NK cell-mediated rejection. It may be so that NK cell tolerance is also controlled at levels other than the expression of Ly49 receptors (discussed in Refs. 23, 33, 34).

At present, little is known about the molecular mechanisms affecting Ly49C receptor expression. It cannot be excluded that they operate on an post-transcriptional level (20, 21). The present study strengthens the notion that MHC class I molecules expressed in the environment are involved in calibration of expression of (at least some) Ly49 receptor(s) (15–17, 20, 21, 24) and demonstrates this to be the case for the Ly49C receptor. The present study also suggests that MHC class I molecules expressed by the NK cells themselves may affect expression of the Ly49C receptors. This appears particularly effective under circumstances when calibration due to environmental factors function less well, such as during 4-day cultures of NK cells in IL-2. It is important to note that these results are not specific for the Ly49C receptor. Käse and collaborators have obtained similar results in their analysis of the expression of the H-2Dβ-binding receptor Ly49A in a transgenic strain of B6 mice expressing an H-2Dβ gene product in a mosaic pattern (35). Taken together, through the analysis of MHC class I mosaic mice generated through a transgenic approach in β2m−/− mice, the present results suggest that expression of the Ly49C inhibitory receptor on NK cells is calibrated in response to expression of MHC class I molecules in the environment as well as to the expression of MHC class I molecules expressed by the NK cells themselves.

Acknowledgments

We thank Drs. A. Käse, P. Höglund, and K. Kärre as well as Dr. C. Sentman for fruitful discussions and for communicating unpublished results; E. Nilsson for excellent help with microinjections; M. L. Solberg and M. Hagelin for technical assistance; and Drs. A. D. Diehl and L. Van Kae for providing comments to the present manuscript.

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