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MHC Class I-Ly49 Interactions Shape the Ly49 Repertoire on Murine NK Cells

Linda Fahlén,* Urban Lendahl,† and Charles L. Sentman‡*

This study aims to determine how the interaction of Ly49 receptors with MHC class I molecules shapes the development of the Ly49 repertoire. We have examined the percentage of NK cells that expressed Ly49A, Ly49G2, and Ly49D in single and double Ly49A/C-transgenic mice on four different MHC backgrounds, H-2b, H-2d, H-2b/d, and β2-microglobulin−/−. The results show that the total numbers of NK cells were not different among the strains. The prior expression of a Ly49 receptor capable of binding to self MHC class I altered the percentage of NK cells expressing endogenous Ly49A, Ly49G2, and Ly49D even in mice in which no MHC ligand was present for the latter receptors. The NK cells in the Ly49-transgenic mice expressed the same level of endogenous Ly49 receptors as wild-type mice of a similar MHC background. In contrast, the number of NK T cells was reduced in mice in which the Ly49 transgene could bind to a MHC class I molecule. The onset of Ly49 receptor expression on NK cells during ontogeny was not altered in the presence of transgenic Ly49 receptors. These data support a sequential model and argue against a selection model for Ly49 repertoire development on NK cells. The Journal of Immunology, 2001, 166: 6585–6592.

It is important to form a NK cell pool that ensures both effetor function and self-tolerance. One mechanism to prevent NK cells from attacking normal cells is for NK cells to express inhibitory receptors that bind to self MHC class I molecules (1, 2). Expression of self MHC class I ligands on a target cell prevents NK cell lysis and cytokine release. Thus, target cells that lack corresponding MHC class I are rendered susceptible for NK cell lysis. Several classes of class I-specific inhibitory receptors have been identified. In the mouse, these receptors belong to two different C-type lectin families, the Ly49 family, which interacts with the classical class I molecules (3), and the CD94/NKG2 family, which interacts with nonclassical class I molecules such as Qa-1 (4, 5). The Ly49 genes are polymorphic and clustered in the NK gene complex on mouse chromosome 6 (6, 7). At least nine members have been identified in the C57BL/6 (B6) genome, Ly49A–I (plus five putative genes Ly49J–N) (8). Fetal NK cells express very few Ly49 receptors at the cell surface, and receptor expression increases gradually to reach adult frequencies by 4 wk of age (9, 10).

The Ly49 family of receptors consists of both inhibitory and activating receptors. The inhibitory receptors contain an immunoreceptor tyrosine-based inhibitory motif in the cytoplasmic tail that, upon receptor cross-linking, recruits Src homology 2 domain-containing tyrosine phosphatase-1 and -2 (11–14). Ly49A is the only Ly49 receptor that, upon receptor cross-linking, recruits DAP12, that contains an immunoreceptor tyrosine-based activation motif (28–30). Upon interaction with H-2Dd, Ly49D delivers a stimulatory signal (28, 31). Inhibitory Ly49 receptors, but not activating ones, are also expressed on subsets of CD1-restricted NK T cells and accumulate on memory CD8 T cells with age (32–35). Both the function and regulation of Ly49 receptors on T cells are not well understood (36).

Recent studies suggest that Ly49A may also interact weakly with H-2b molecules, but the affinity may be too low to observe inhibition of target cell lysis in vitro but sufficient to induce effects on Ly49A receptor expression in vivo (18–21). Ly49C and Ly49G2 are also inhibitory members of the Ly49 family. Ly49C, which has a broad specificity, binds to class I molecules of H-2d, H-2k, H-2b, and H-2r (22–26) but binds most strongly to H-2Kb. Ly49G2 binds to H-2Dd and H-2Ld (27). The activating receptor, Ly49D, has a short cytoplasmic tail that lacks immunoreceptor tyrosine-based inhibitory motifs and forms a complex with an adaptor molecule, DAP12, that contains an immunoreceptor tyrosine-based activation motif (28–30). Upon interaction with H-2Dd, Ly49D delivers a stimulatory signal (28, 31). Inhibitory Ly49 receptors, but not activating ones, are also expressed on subsets of CD1-restricted NK T cells and accumulate on memory CD8 T cells with age (32–35). Both the function and regulation of Ly49 receptors on T cells are not well understood (36).

The heterogeneous pattern of Ly49 receptors seen on NK cells suggests that they are expressed in a stochastic way. Several inhibitory receptors have been reported to be coexpressed at the surface of a given NK cell. Single-cell PCR demonstrated that murine NK cells could express up to six different inhibitory receptors and showed a highly diverse pattern of coexpression (37). The percentage of NK cells expressing two distinct Ly49 receptors can be predicted from the product of their frequencies in the total NK cell population (38). Earlier reports have shown that the repertoire of inhibitory receptor expression by NK cells is influenced by the MHC class I expression in the host. First, there is a higher proportion of Ly49+ NK cells in mice deficient in β2-microglobulin (β2m)3 compared with β2m+ mice (39, 40). Second, introducing an H-2Dd transgene in B6 (H-2b) mice allowed specific subsets of NK cells to develop that were inhibited by H-2Dd but not by H-2b and, thus, rejected bone marrow transplants from B6 mice (41). Third, NK cells that express multiple H-2d-specific Ly49 receptors are significantly less frequent in H-2d mice compared with H-2b mice (20). These results suggest that there is a mechanism...
that disfavors coexpression of multiple self class I specific receptors on NK cells and that NK cells need to adapt to the MHC environment in which they develop. To allow NK cells to be more sensitive to alterations in only one of the class I alleles, NK cells may avoid expressing too many different inhibitory receptors for self MHC. Both selection and sequential models have been proposed to account for the development of a useful Ly49 repertoire in mice (38). The selection model proposes that NK cells express Ly49 receptors, and then those cells with at least one, but not too many, receptors that bind to self MHC are selected for survival. The sequential model proposes that NK cells sequentially express Ly49 receptors and are continuously selected for expression of receptors that bind to self MHC. We have studied how one or two inhibitory receptors expressed on all NK and T cells influence the expression and regulation of the endogenous Ly49 molecules, both inhibitory and activating types, using Ly49A-, Ly49C-, and Ly49A/C-transgenic mice. To investigate how host MHC class I molecules effect the NK repertoire, we introduced the transgenes onto four different MHC class I haplotypes, H-2b, H-2d, H-2ab, and β2m−/− (H-2b). We examined the numbers of NK cells and NK T cells, the percentage of different Ly49 receptors, and the level of receptor expression. We discuss our results in relation to the selection and sequential models for Ly49 repertoire development.

## Materials and Methods

### Animals

B6 H-2b mice were purchased from Bomholtgård Breeding and Research Center (Ry, Denmark). B10.D2 H-2b mice and β2m−/− mice (on B6 background) were purchased from The Jackson Laboratory (Bar Harbor, ME). D2 répétition K17, and KTb−/− mice were kindly provided by F. Lemmouner (Institut Pasteur, Paris, France) and have been previously described (42). Mice were bred and maintained at the animal facility at Umeå University (Umeå, Sweden), the animal house at the Swedish Defence Research Agency (Umeå, Sweden), or at the animal house at Microbiology and Tumor Biology Center (Karolinska Institute, Stockholm, Sweden). All animal work was approved by the local Animal Ethical Committee (Umeå, Sweden).

### Production of transgenic mice

A 950-bp Ly49C cDNA clone was cloned into VACD2 (43) and used to produce V49C-transgenic mice using the same promoter construct (19). Several different founder lines were generated, and two of them were then backcrossed to B10.D2 and selected for expression of H-2a and no expression of H-2b. B6V49A-transgenic mice were produced onto a B6 background directly and have been previously described (19). Staining peripheral blood lymphocytes (on B6 background) were purchased from The Jackson Laboratory (Bar Harbor, ME) and have been previously described (42).

### Antibodies

Antibodies used were 2G2 (anti-FcRy), biotin (B)-conjugated 5E6 (anti-Ly49C), and anti-CD4 were also purchased from BD PharMingen. RED670-conjugated streptavidin was purchased from Life Technologies (Täby, Sweden). Ly49C rabbit antiserum (CTO64) was the kind gift of Dr. M. Bennett, University of Texas South-Western Medical Center (Dallas, TX).

### Flow cytometry

To inhibit nonspecific binding of Abs to the FcRy, spleen cells depleted of erythrocytes and liver cells were incubated with anti-FcRy Abs for 20 min at 4°C before staining with specific Abs. Briefly, cells (10^6) were incubated with primary Abs for 30 min at 4°C. After washing with staining buffer (PBS containing 1% FCS), cells were incubated with streptavidin conjugates for 30 min at 4°C. When using the Ly49C rabbit antiserum for detecting Ly49C expression, an additional incubation with B-donkey anti-rabbit Ab was included. After a final wash, cells were resuspended in PBS and analyzed using a FACS Calibur (BD Biosciences, San Jose, CA). Viable lymphocytes are shown after gating on forward and side scatter.

### Cytotoxicity assays

Erythrocyte depleted splenocytes were cultured in a MEM (Life Technologies) supplemented with 10% FCS, 10 mM HEPES, 2×10^{-5}M 2-ME, 100 U/ml streptomycin, 100 U/ml penicillin, and 1000 U of human rIL-2 (PeproTech, Rockyhill, NJ) for 4 days in 37°C 10% CO₂ and used as effector cells in a conventional 4-h 51 Cr release assay (44). In blocking experiments, effector cells were incubated with 10 μg/ml of F(ab’)₂ anti-Ly49C/I, anti-Ly49D, or anti-Ly49A/I (5E6) for 45 min at 37°C before targets cells were added to the wells as described (19).

### Statistics

Data were analyzed using nonparametric ANOVA methods.

### Results

#### Ly49C-transgenic mice express a functional transgene on all NK and T cells

Ly49C is normally expressed on ~30–35% of NK cells and on a few percent of NK T cells in B6 mice (34, 45). Several diferent MHC class I molecules, in particular H-2Kb, have been shown to be a ligand for Ly49C. To test how expression of Ly49C on all NK cells influences the endogenous Ly49 repertoire, we generated Ly49C-transgenic mice (VA49C) by introducing a Ly49C B6 cDNA under the control of the CD2 promoter. VA49C-transgenic mice expressed Ly49C on all NK and T cells (Fig. 1). The expression level on NK cells was comparable to the endogenous levels seen on the nontransgenic NK cells. This staining pattern resembles what was previously observed for Ly49A in the B6V49A-transgenic mice, generated using the same promoter construct (19). Host expression of an MHC class I ligand leads to reduced surface expression of the corresponding Ly49 receptor on NK cells (46, 47). The expression level of the Ly49C transgene was down-regulated on both T cells and NK cells in H-2ab and H-2db mice, compared with β2m−/− mice, which have very low expression levels of MHC class I. The expression level of Ly49C was lower in the H-2db background than in the H-2ab, suggesting that Ly49C interacts with both H-2ab and H-2db molecules, which is consistent with previous reports (23, 25, 26).

- B-conjugated donkey anti-rabbit was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden).
- FITC–conjugated HB102 (anti-H-2Db), FITC–conjugated anti-CD4, and FITC–conjugated Ly5 (B220) were purchased from Caltag.
- FITC–conjugated PK136 (anti-NK1.1), FITC–conjugated SE6 (anti-Ly49C/I), and FITC–conjugated 4E5 (anti-Ly49D) were purchased from BD PharMingen (Stockholm, Sweden).
- PE–conjugated anti-CD3e and PE–conjugated anti-CD4 were also purchased from BD PharMingen.
- RED670-conjugated streptavidin was purchased from Life Technologies (Täby, Sweden).
- Ly49C rabbit antiserum (CTO64) was the kind gift of Dr. M. Bennett, University of Texas South-Western Medical Center (Dallas, TX).
To test whether the Ly49C-transgenic receptor was able to inhibit killing, cytotoxicity assays were performed using lymphokine-activated (LAK) cells from Ly49C-transgenic mice as effector cells and Con A blasts with different MHC class I expression (H-2d, H-2 b, H-2D b2/2, H-2K b2/2, and H-2D bK b2/2) (Fig. 2). Ly49C-transgenic LAK cells killed both K b2/2 and K bD b2/2 cells equally well and at a similar level as the nontransgenic LAK cells. Although Con A blasts from B6 (H-2 b) and D b2/2 (H-2K b) were killed much better by the nontransgenic cells than by the Ly49C effectors, B10.D2 (H-2 d) Con A blasts (self) were not killed by transgenic or wild-type effector cells. These results suggest that LAK cells from Ly49C-transgenic mice were inhibited by H-2Kb expression on the target cells but not by H-2D b expression. Addition of anti-Ly49C F(ab)2 to the assay restored the cytotoxicity against B6 Con A blasts (data not shown).

Altered NK cell Ly49 repertoire in Ly49A-, Ly49C-, and Ly49A/C-transgenic mice

The sequential and selection models make different predictions for how the Ly49 repertoire would be altered in situations in which all NK cells express one Ly49 receptor that binds to self MHC or two Ly49 receptors that bind to self MHC. To study the formation and regulation of the Ly49 receptor repertoire and to test the two models, we used Ly49C- and Ly49A-transgenic mice. Transgenic mice were bred onto MHC backgrounds so that they expressed a strong ligand for Ly49C (H-2b, B6), a strong ligand for Ly49A (H-2 d, B10.D2), ligands for both Ly49A and Ly49C (H-2 d/b, F1), or very little MHC class I (β2 m2/2) to generate sixteen different Ly49-MHC combinations. These offspring allowed us to study how one or two inhibitory receptors on all NK cells altered the overall Ly49 repertoire in the context of the MHC haplotype of the host. Many of the Ly49A-transgenic mice that were bred onto a H-2d background developed a severe inflammatory disease and died at 3–4 wk of age (19). In this study, the only Ly49A- or Ly49A/C-transgenic mice that expressed H-2αβ included in the analysis were those with no overt signs of this inflammatory condition. No signs of inflammatory disease have been observed in the Ly49C-transgenic mice on any of the MHC backgrounds analyzed thus far. Our data (Fig. 3) describe the expression of endogenous Ly49G2, Ly49A, and Ly49D receptors in the presence of different Ly49 transgenes and MHC class I environments.

Expression of Ly49A and Ly49C transgenes does not alter the percentage of NK cells

Table I shows the percentage of NK cells in the spleens of mice with the different MHC and Ly49 transgene combinations. The total numbers and the percentages of NK1.1+CD3− cells were not altered in the transgenic mice compared with nontransgenic mice.
These data indicate that expression of one or two inhibitory receptors on all NK cells did not change the size of the NK cell pool.

**Ly49G2 expression in Ly49C-transgenic mice**

Ly49C has been shown to interact with ligands of both the H-2^d and H-2^b haplotypes. A decrease in the percentage of Ly49G2^+ NK cells was seen in the Ly49C-transgenic mice of H-2^d and H-2^d/b haplotypes (Fig. 3A). These mice express MHC ligands for both Ly49C and Ly49G2, and the decrease in Ly49G2^+ NK cells may be explained by a need to limit the types of inhibitory receptors that bind to self MHC on a given NK cell. However, in Ly49C transgenic mice on an H-2^b background, there was also a decrease in the percentage of NK cells expressing Ly49G2 (27%) compared with nontransgenic littermates (49%). In Ly49C-transgenic mice on an H-2^b background and on a H-2^d/b haplotype, there was also a decrease in the percentage of NK cells that expressed Ly49G2 compared with the respective nontransgenic littermates (47%) (Fig. 3C). However, the decrease in the percentage of NK cells that expressed Ly49D was less in mice that expressed H-2^d/b. There was no decrease in the percentage of Ly49D^+ NK cells in Ly49C-transgenic mice in the absence of MHC class I expression (β2m^{−/−}). Thus, in mice in which Ly49C can bind to an MHC class I ligand, there was a decrease in Ly49D^+ NK cells that could be somewhat reversed in mice also carrying a ligand for Ly49D. These data suggest that interaction of activating Ly49 receptors with their MHC ligands during NK cell development may lead to survival or expansion of that subset.

**Ly49A expression in Ly49C-transgenic mice**

Ly49A has a strong ligand in H-2^d (H-2^d/b) mice and a weak ligand in H-2^b mice. The frequency of NK cells expressing endogenous Ly49A was decreased in the Ly49C-transgenic mice on all three class I-positive backgrounds, H-2^d, H-2^b, H-2^d/b, compared with the respective nontransgenic littermates (Fig. 3B). The drop in the percentage of Ly49A^+ NK cells in the transgenic mice was very similar between the three different types of class I mice. This was similar as seen for the Ly49G2^+ NK cells in the same mice. No significant difference in the percentage of Ly49A^+ NK cells between Ly49C-transgenic mice expressing strong ligand for Ly49A (H-2^d) or mice expressing a weak ligand (H-2^b) was observed. Surprisingly, expression of Ly49C on all NK cells in β2m^{−/−} mice reduced the percentage of NK cells expressing endogenous Ly49A. This observation would not have been predicted from either a sequential or a selection model for Ly49 repertoire development. Similar to the expression levels of Ly49G2, the expression levels of Ly49A on NK cells were altered by the host MHC haplotype but not by the expression of the Ly49C transgene. That is, the level of Ly49A receptors expressed was similar to the wild-type littermates for each MHC haplotype (data not shown).

**Ly49D expression in Ly49C transgenic mice**

Ly49D is an activating receptor that is normally expressed on 50% of NK cells but is not found on NK T cells. Ly49D interacts with H-2^b molecules. Ly49C-transgenic H-2^b mice showed a significant drop in the percentage of NK cells expressing Ly49D (30%) compared with nontransgenic littermates (47%) (Fig. 3C). However, the decrease in the percentage of NK cells that expressed Ly49D was less in mice that expressed H-2^d/b. There was no decrease in the percentage of Ly49D^+ NK cells in Ly49C-transgenic mice in the absence of MHC class I expression (β2m^{−/−}). Thus, in mice in which Ly49C can bind to an MHC class I ligand, there was a decrease in Ly49D^+ NK cells that could be somewhat reversed in mice also carrying a ligand for Ly49D. These data suggest that interaction of activating Ly49 receptors with their MHC ligands during NK cell development may lead to survival or expansion of that subset.

**Ly49G2 expression in Ly49A-transgenic mice**

Ly49G2 and Ly49A both bind to H-2^d. In Ly49A H-2^d/b mice in which Ly49A has a strong ligand, the frequency of NK cells expressing Ly49G2, which also has a ligand in these mice, was reduced from 48 to 18% (Fig. 3A). A similar observation has been reported in other Ly49A-transgenic mice (20). There was a decrease in the percentage of Ly49G2^+ NK cells in Ly49A-transgenic mice on the H-2^b background, although it was significantly less than in the presence of H-2^d. This may reflect the different affinities of Ly49A for H-2^d and H-2^b MHC molecules. There was no decrease in the percentage of Ly49G2^+ NK cells in β2m^{−/−} Ly49A-transgenic mice. These data suggest that it is the presence of an MHC ligand for the Ly49A transgene that leads to a reduction in Ly49G2^+ cells.

**Ly49D expression in Ly49A-transgenic mice**

Ly49D and Ly49A both bind to H-2^d. A reduction in the percentage of Ly49D^+ NK cells was seen in Ly49A-transgenic mice on an H-2^b background and on a β2m^{−/−} background (Fig. 3C). The decrease in β2m^{−/−} mice was surprising because the MHC class I ligands are expressed at a very low level. Similar to the observation in Ly49C-transgenic mice, there was not a significant drop in the percentage of Ly49D^+ cells in mice that expressed a ligand for Ly49D (H-2^d/b).

**Ly49G2 expression in Ly49A/C double-transgenic mice**

There were lower percentages of NK cells that expressed Ly49G2 in the Ly49A/C double-transgenic mice on H-2^d and H-2^d/b backgrounds compared with either Ly49A- or Ly49C-transgenic mice (Fig. 3A). These data suggest an additive effect of the two transgenes. The percentage of Ly49G2^+ NK cells was lowest in H-2^d/b Ly49A/C-transgenic mice (11%) in which both Ly49 transgenes have strong MHC ligands. There was no difference in the percentage of NK cells that expressed Ly49G2 in mice with low MHC class I expression (β2m^{−/−}).

**Ly49D expression in Ly49A/C double-transgenic mice**

The percentage of Ly49D^+ NK cells in Ly49A/C double-transgenic mice was similar to that observed in either Ly49C-transgenic mice.
Liver NK cells from H-2b Ly49C-transgenic mice have a desplenic NK cells. A similar observation was also seen in the liver. Ly49 receptors in the transgenic mice was not exclusive for the Ly49 expression on liver NK cells of Ly49C-transgenic mice. Here, the NK T cell numbers were effected by transgene expression. The frequency of liver NK T cells in Ly49-transgenic mice is been shown to be significantly reduced in another H-2d Ly49A-transgenic mice. The reduction of NK T cells in the Ly49C-transgenic mice was largest on the H-2b background in which Ly49C has a strong ligand (Table II). Although there was a decrease in the total number of NK T cells in the liver of Ly49-transgenic mice, the percentage of NK T cells expressing Ly49A or Ly49G2 was not altered in the transgenic mice (Fig. 4). Unlike the observations with NK cells, the number of NK T cells was reduced, but the percentage of different Ly49 subsets was unchanged.

**DeCREASED NUMBER OF LIVER NK T CELLS IN Ly49C-TRANSGENIC MICE**

Table II shows the percentage of NK T cells in the liver of the Ly49-transgenic mice. Here, the NK T cell numbers were effected by transgene expression. The frequency of liver NK T cells has been shown to be significantly reduced in another H-2b Ly49A-transgenic mice as compared with nontransgenic littersmates, whereas the H-2b Ly49A-transgenic mice had normal numbers of NK T cells in the liver (48). Preliminary data with our Ly49A-transgenic mice confirm this observation. A similar decrease in the percentage of NK T cells was also observed for the Ly49C-transgenic mice. The reduction of NK T cells in the Ly49C-transgenic mice was largest on the H-2b background in which Ly49C has a strong ligand (Table II). Although there was a decrease in the total number of NK T cells in the liver of Ly49-transgenic mice, the percentage of NK T cells expressing Ly49A or Ly49G2 was not altered in the transgenic mice (Fig. 4).

No alteration in the ontogeny of Ly49 receptors of Ly49C-transgenic mice

The expression of Ly49 receptors on the cell surface begins around 4 or 5 days after birth and increases gradually over the next 4 wk until the Ly49 percentage reaches the frequencies found on adult NK cells (9, 10). To investigate whether the expression of a Ly49 receptor on all NK cells altered the onset or time span of acquisition of Ly49 receptors, we studied the proportion of NK cells expressing Ly49A, Ly49G2, and Ly49D in Ly49C-transgenic (H-2b) mice from 10–22 days of age. Ly49C transgene expression was detected at high levels on splenic NK cells from 4-day-old mice and before endogenous Ly49 expression was detected by flow cytometry (data not shown). The onset of endogenous receptor expression on the NK cells did not differ between the Ly49C-transgenic and nontransgenic littersmates. This was true for both activating and inhibitory receptors (Fig. 5, A and B). Both the time of onset as well as the time span necessary until adult frequencies of Ly49+ subsets were reached was not effected by Ly49C transgene expression.

**Discussion**

We have used an in vivo transgenic system expressing Ly49A or Ly49C cDNAs under the control of the human CD2 promoter to investigate how the interaction of Ly49 receptors and MHC class I molecules alters the Ly49 repertoire in vivo. Our data demonstrate that the prior expression of a Ly49 receptor able to bind to self MHC class I reduced the percentage of NK cells that expressed endogenous Ly49 receptors even in mice in which no MHC ligand was present for these endogenous Ly49 receptors. NK cell effector functions can be regulated by MHC, and the “missing self” theory proposes that this interaction via inhibitory receptors is a key mechanism that allows NK cells to distinguish between self and nonself. In order for such a system to be functional, NK cells must express at least one inhibitory receptor for self MHC. Although expression of too many inhibitory receptors for self MHC would not be obviously harmful, the observation that the MHC haplotype of the host alters Ly49 receptor expression suggests that the MHC shapes the Ly49 repertoire to produce a

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

**Percentage of NK or NK T cells from liver expressing endogenous Ly49G2 and Ly49A.** Lymphocytes from liver were isolated from Ly49A (A), Ly49C (C), and Ly49A/C (A/C)-transgenic mice and nontransgenic H-2a mice (NTg), and the percentage of NK1.1+ CD3+ and NK1.1+ CD3+ cells expressing Ly49G2 or Ly49A were analyzed by flow cytometry. Each point represents analysis from one mouse, and the horizontal lines represent the mean value for each group. Statistical analysis, *, p < 0.005

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<th>Transgene</th>
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<td>6.9 ± 3.4 (5)</td>
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<td>Ly49A/Ly49C</td>
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a NTg, Nontransgenic.

b Percentage of NK1.1+ CD3+ cells among isolated liver lymphocytes ± SD.

Numbers in parentheses represent number of mice in each group.

No alteration in the ontogeny of Ly49 receptors of Ly49C-transgenic mice

The expression of Ly49 receptors on the cell surface begins around 4 or 5 days after birth and increases gradually over the next 4 wk until the Ly49 percentage reaches the frequencies found on adult NK cells (9, 10). To investigate whether the expression of a Ly49 receptor on all NK cells altered the onset or time span of acquisition of Ly49 receptors, we studied the proportion of NK cells expressing Ly49A, Ly49G2, and Ly49D in Ly49C-transgenic (H-2b) mice from 10–22 days of age. Ly49C transgene expression was detected at high levels on splenic NK cells from 4-day-old mice and before endogenous Ly49 expression was detected by flow cytometry (data not shown). The onset of endogenous receptor expression on the NK cells did not differ between the Ly49C-transgenic and nontransgenic littersmates. This was true for both activating and inhibitory receptors (Fig. 5, A and B). Both the time of onset as well as the time span necessary until adult frequencies of Ly49+ subsets were reached was not effected by Ly49C transgene expression.

**Discussion**

We have used an in vivo transgenic system expressing Ly49A or Ly49C cDNAs under the control of the human CD2 promoter to investigate how the interaction of Ly49 receptors and MHC class I molecules alters the Ly49 repertoire in vivo. Our data demonstrate that the prior expression of a Ly49 receptor able to bind to self MHC class I reduced the percentage of NK cells that expressed endogenous Ly49 receptors even in mice in which no MHC ligand was present for these endogenous Ly49 receptors. NK cell effector functions can be regulated by MHC, and the “missing self” theory proposes that this interaction via inhibitory receptors is a key mechanism that allows NK cells to distinguish between self and nonself. In order for such a system to be functional, NK cells must express at least one inhibitory receptor for self MHC. Although expression of too many inhibitory receptors for self MHC would not be obviously harmful, the observation that the MHC haplotype of the host alters Ly49 receptor expression suggests that the MHC shapes the Ly49 repertoire to produce a
functional NK cell pool within the host environment. Two nonmutually exclusive models have been proposed to explain how the Ly49 repertoire is shaped by host MHC class I molecules (38). The sequential receptor expression model states that receptors are stochastically expressed and tested for recognition of self MHC class I molecules. Cells continuously express new Ly49 receptors until sufficient interaction with self MHC is achieved. Cells failing to express Ly49 receptors that interact sufficiently with self MHC do not survive. The other model is the two-step selection model stating that Ly49 receptors are stochastically expressed and cells are subsequently tested for necessary self recognition. Similar to the selection mechanism for T cells, useful NK cells that express at least one inhibitory receptor are favored, and NK cells that express multiple receptors for self are disfavored or eliminated. Hence, the final NK cell pool consists of NK cells that express a limited number of self MHC inhibitory receptors.

Data presented to date are consistent with both of these models. Recent data from two in vitro cell culture systems suggest that NK cells begin to express different Ly49 receptors at different times during their development, although the order of receptor expression differed between the two systems (49, 50). The sequential and selection models make different predictions about how expression of a single Ly49 receptor on all NK cells would alter the repertoire development. Data reported from Ly49A-transgenic mice can be predicted from both models (20). Because all of the Ly49 receptors analyzed in those experiments bound to H-2D\textsuperscript{b}, the effect of Ly49A transgene expression on the expression of other Ly49 receptors in hosts in which both transgene and endogenous Ly49 gene expression, there is an MHC regulation of receptor expression. This study shows that there were decreases in the percentages of NK cells that express endogenous Ly49 receptors that bind to H-2D\textsuperscript{b}. This could be due to several reasons, including the observations that the NK cells expressed a Ly49 receptor that bound to self MHC, so additional receptors were not necessary (sequential model), that NK cells that expressed additional receptors that bound to self MHC were disfavored (selection model), or that the endogenous receptors and transgenic receptor competed for binding on the same MHC molecules, thus somehow preventing receptor expression.

In this paper, we have presented data using Ly49C-transgenic mice. Ly49C will bind to H-2K\textsuperscript{b}, thus allowing us to examine a scenario in which only the transgenic Ly49 bound to MHC but not the endogenous Ly49 receptors (e.g., Ly49G2). In this case, we observed that there was a decrease in the expression of Ly49G2 and Ly49D even though these receptors have no ligand in H-2\textsuperscript{b} mice. There was also a decrease in Ly49A expression. Data using tetramers suggest that Ly49A can bind to H-2D\textsuperscript{b} but not to H-2K\textsuperscript{b} (18). Thus, our data indicate that it is the interaction of the transgene with MHC that results in a decrease in the expression of other Ly49 receptors, whether they have an MHC ligand or not. It should be noted that the percentage of NK cells that expressed a given Ly49 receptor was reduced and not the level of the receptors on those NK cells that did express them. These data are not consistent with a selection model because this model predicts that there would be no reason to select against Ly49G2 or Ly49D expression because these receptors do not interact with MHC in H-2\textsuperscript{b} mice. However, a sequential model would predict that all Ly49 receptors would be reduced because all NK cells expressed one functional Ly49 receptor, so there would be no need for additional ones.

We have combined Ly49A- and Ly49C-transgenic mice and examined the Ly49 repertoire in hosts that express ligands for both receptors. The selection model predicts that the expression of multiple self receptors would lead to deletion of those NK cells. Yet in the Ly49A/C double-transgenic mice, we did not observe a decrease in the size of the NK cell pool. It is conceivable that a limited number of unusual NK cells were allowed to survive and expand to repopulate the entire NK cell pool; however, all of the NK cells expressed both Ly49 transgenes and some other Ly49 receptors. We also did not observe a delay in the development of the adult level of Ly49 subsets in Ly49C-transgenic mice. In contrast, the sequential model predicts that, as the level of self receptors is increased, there would be no effect on the total numbers of NK cells but a decrease in the percentage of endogenous receptors expressed. This is consistent with our observations. We have observed that the decrease in the percentage of Ly49G2\textsuperscript{+} NK cells in the Ly49A/C-transgenic mice is additive in comparison with Ly49A- and Ly49C-transgenic mice.

In vitro data suggest that there may be a time element that needs to be considered (49, 50). During NK cell differentiation, there may be a discrete stage in which Ly49 receptor genes can be activated. Interaction between Ly49 receptors and host MHC appears to be a necessary step that may induce further NK cell differentiation and prevents additional Ly49 receptor expression. Thus, expression of a Ly49 transgene that can interact with host MHC may induce differentiation more quickly. However, there would likely remain a period of time when additional Ly49 genes could be expressed. It has been shown that transfer of Ly49\textsuperscript{+} NK cells into irradiated hosts led to the expression of new Ly49 receptors (9). This may reflect the differentiation process or an expansion to fill a niche in an irradiated host. On top of the regulation of receptor gene expression, there is an MHC regulation of receptor expression on the cell surface (46, 51). Because the ability of an inhibitory receptor to prevent effector responses is dependent upon the receptor expression level, and these levels can change on mature NK cells (46, 52, 53), this adds a further level of complexity to the NK cell repertoire. In the experiments presented in this report, the presence of an Ly49 transgene did not alter the level of receptor expression only the percentage of cells that expressed each receptor.

Neither of the models for Ly49 repertoire development specifically discusses the expression of activating receptors. It is not
clear whether Ly49 activating receptors are independent receptors capable of activating NK cells alone or coreceptors that magnify or modify activation signals via other receptors. Because these receptors provide activation signals in contrast to inhibitory signals, and cell responses are determined by the interaction between activating and inhibitory signals (54, 55), the effect of Ly49 transgenes on the development of activating receptors, such as Ly49D, is likely to be different from that for inhibitory receptors. Our observations of Ly49D expression suggest that activating receptors may interact with MHC to promote survival and/or expansion of NK cell subsets that express them. One possibility would be that it may be beneficial to maintain a balance between activating and inhibitory receptors so that those activating receptors that bind to self MHC are favored. Thus, one may expect the percentage of NK cells expressing the activating Ly49D to be increased in the Ly49A- and Ly49C-transgenic mice. However, we did not observe any increase in the percentage of NK cells expressing Ly49D in these transgenic mice. Another idea is that activating receptors are only allowed to be expressed after inhibitory receptors to ensure self-tolerance. Until further Abs become available and the MHC specificity of many activating receptors is better understood, the regulation of Ly49 activating receptors will remain unresolved.

One puzzling observation was a decrease in the percentage of NK cells that expressed Ly49A in β2m−/− Ly49C-transgenic mice. There were also alterations observed in Ly49D expression in β2m−/− Ly49A-transgenic mice but not in β2m−/− Ly49C-transgenic mice. However, Ly49G2 expression was not altered in Ly49C-transgenic β2m−/− mice. If the regulation of Ly49 expression was only due to the interaction of Ly49 and classical MHC class I molecules, there should be no alterations in β2m−/− mice. As we have discussed, there are several elements that together explain Ly49 receptor expression and regulation in addition to the direct interaction between Ly49 receptors and MHC class I molecules. Ly49A and Ly49D are known to be regulated by a specific transcription factor, and in vitro data suggest that they may be expressed after Ly49C and Ly49G2 (50, 56). It is possible that Ly49 receptors can interact with other molecules in the absence of β2m, such as MHC class I free heavy chains or nonclassical MHC molecules, and these interactions may alter expression of some Ly49 receptors. It has been observed that NK cells from β2m−/− mice are tolerant to self MHC, but they are very poor at cytotoxicity toward a variety of tumor target cells. Until we understand more about the molecular events involved in the development of NK cells in the absence of β2m, the nature of the regulation of NK cell activity in these mutant mice will remain difficult to explain.

In contrast to the observation that there were alterations in the percentage of NK cells that expressed different Ly49 receptors in the Ly49A-transgenic mice, there were no such effects on the NK T cell population. In the NK T cell population, there was a decrease in the number of NK T cells in mice in which the Ly49 gene was deleted. This was due to the deletion of Ly49 repertoire on NK T cells rather than expression of inhibitory Ly49 receptors. MacDonald and co-workers have observed a similar decrease in liver NK T cells in Ly49A-transgenic mice that expressed H-2Dk (48). These NK T cells did not express the characteristic Vα14 or Vβ chains found on CD1-restricted NK T cells, suggesting that the NK T cells that developed did not have the same specificity. We and others have reported that Ly49 receptor expression can alter T cell development (19, 57). NK T cells do not express activating Ly49 receptors, and the role of the inhibitory Ly49 receptors is known to alter their function when stimulated via their TCRs. However, expression of Ly49 receptors on all NK T cells as they develop could alter the survival of cells with certain TCR specificities with the result that many of the NK T cell pool may fail to survive development. Furthermore, the finding that the Ly49 repertoire on NK T cells is not altered in the Ly49-transgenic mice may be due to the fact that NK T cell development and survival is primarily dependent upon signals received via their TCR complex.

The formation of a properly functioning NK cell pool has important biological consequences. MHC class I molecules have been demonstrated to be able to inhibit and activate murine NK cells via Ly49 receptors. Hence, it is necessary for NK cells to adapt to the host MHC environment to function in an optimal way. One way to achieve this is for the Ly49 receptor repertoire to be shaped by host MHC. Different models have been proposed for how this occurs. Although our data cannot rule out a selection type of model, several of our observations are inconsistent with a strict interpretation of these models. Our observations on the Ly49 repertoire in Ly49A-, Ly49C-, and Ly49A/C-transgenic mice on different MHC haplotypes are consistent with a sequential model of Ly49 development.

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References