Diversity of NK Cell Receptor Repertoire in Adult and Neonatal Mice

Akira Kubota, Satoko Kubota, Stefan Lohwasser, Dixie L. Mager and Fumio Takei

*J Immunol* 1999; 163:212-216; ;
http://www.jimmunol.org/content/163/1/212

---

**References**  This article cites 31 articles, 15 of which you can access for free at: http://www.jimmunol.org/content/163/1/212.full#ref-list-1

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Diversity of NK Cell Receptor Repertoire in Adult and Neonatal Mice

Akira Kubota,2* Satoko Kubota,2* Stefan Lohwasser,* Dixie L. Mager,*† and Fumio Takei3*‡

Murine NK cytotoxicity is regulated by two families of MHC class I-specific receptors, namely Ly49 and CD94/NKG2. We developed a single-cell RT-PCR method to analyze expression of all known Ly49 and NKG2A genes in individual NK cells and determined the receptor repertoires of NK cells from adult and neonatal (1-wk-old) C57BL/6 mice. In adult mouse NK cells, up to six different receptors were coexpressed in random combinations. Of 62 NK cells examined, 42 different patterns of receptor expression were observed. Most of them expressed at least one Ly49, whereas NKG2A was detected in 32% of the cells. Over 75% of them expressed Ly49C, I, or NKG2A, which are thought to recognize self-class I MHC (H-2k). Coexpression of multiple Ly49 receptors and NKG2A was stochastic. In contrast, very few neonatal NK cells expressed any Ly49, but almost 60% of them expressed NKG2A. These results demonstrate that adult NK cells are quite heterogeneous and have diverse receptor repertoires. They also suggest that the expression of NKG2A precedes Ly49 expression in NK cell ontogeny, and NKG2A is a major inhibitory receptor in neonatal NK cells. The Journal of Immunology, 1999, 163: 212–216.

Multiple receptors specific for MHC class I molecules have been identified on NK cells. Human NK cells express killer cell-inhibitory receptors (KIRs)3 that belong to the Ig superfamily (1, 2) and the CD94/NKG2 heterodimers that belong to the C-type lectin superfamily. KIRs recognize specific MHC class I molecules, whereas CD94/NKG2A interacts with the nonclassical MHC class I HLA-E that presents peptides derived from the leader sequences of the classical MHC class I proteins (3–6). In the mouse, the Ly49 family of C-type lectins has been identified to be NK cell receptors for MHC class I (7). Ten Ly49 molecules have been identified thus far (7, 8). Among them, Ly49A has been shown to recognize H-2Dk and Dd and inhibit NK cytotoxicity against target cells expressing those MHC class I molecules (9), whereas Ly49C is an inhibitory receptor with much broader specificity for MHC class I, including those of H-2d,b,k and H-2s haplotypes (10, 11). Ly49G is also an inhibitory receptor specific for Dd and Dk (12). Ly49D also recognizes Dd, but it lacks the immune receptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic domain, associated with DAP12, and forms an activating receptor (13, 14). The specificities of other Ly49 molecules for MHC class I have not been established. Recently, murine homologues of NKG2 have also been cloned (15, 16). Mouse NKG2A/CD94 recognizes the nonclassical MHC class I molecule Qa-1l,5 the murine homologue of HLA-E (15). Murine NKG2A contains an ITIM in the cytoplasmic tail and seems to function as an inhibitory receptor (15).

The expression of multiple receptors with different specificities for MHC class I are thought to generate diverse specificities of NK cells to recognize target cells missing various self-MHC class I molecules and at the same time to maintain self-tolerance of NK cells. Recently, the receptor repertoires of human NK clones have been analyzed, and the results supported this concept (17). Unlike data on human NK cells, relatively little is currently known about murine NK cell receptor repertoires because of difficulties in establishing mouse NK cell clones. Murine NK cell receptor expression patterns have previously been examined by flow cytometric analysis of bulk NK cell populations using anti-Ly49 mAbs (18, 19). However, these studies are limited by the lack of mAbs to some receptors, cross-reactivities of some of the mAbs, and difficulty in simultaneously detecting more than three receptors on individual NK cells. In this study, we have developed a single-cell RT-PCR method to analyze the expression of receptor genes in individual NK cells. The results demonstrate a highly diverse pattern of coexpression of multiple receptors in individual murine NK cells from adult mice, suggesting a stochastic mechanism for receptor expression. In contrast, neonatal NK cells mostly express NKG2A but not Ly49.

Materials and Methods

Mice

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Abs and flow cytometry

The hybridomas PK136 (anti-NK1.1) and 2.4G2 (anti-Fc receptor) were obtained from American Type Culture Collection (Manassas, VA). The mAbs YE1/48 (anti-Ly49A) and 5E6 (anti-Ly49C and I) have been described (11). The anti-Ly49G mAb 4D11 (12) was kindly provided by Dr. Stephen Anderson (National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD). Anti-CD3-ITC4 was purchased from BLO/CAv Scientific (Mississauga, Canada). For sorting of NK cells, nylon wool-nonadherent spleen cells were first incubated with unlabeled 2.4G2 to block the Fc receptor and then stained for NK1.1 and CD3. NK1.1+ CD3+ single cells were sorted into microtiter wells on a FACStar Plus cell sorter (Becton Dickinson, San Jose, CA).

* Address correspondence and reprint requests to Dr. Fumio Takei, Terry Fox Laboratory, British Columbia Cancer Research Center, 601 West 10th Avenue, Vancouver, British Columbia, V5Z 1L3 Canada. E-mail address: fumio@terryfox.ubc.ca

** A.K. and S.K. contributed equally to this work.

† A recipient of the Leukemia Research Fund of Canada. S.L. is a recipient of a Deutsche Forschungsgemeinschaft fellowship.

‡ A.K. and S.K. contributed equally to this work.

3 Abbreviations used in this paper: KIR, killer cell-inhibitory receptor; ITIM, immune receptor tyrosine-based inhibitory motif.

Copyright © 1999 by The American Association of Immunologists
RT-PCR. Total cDNA was generated from each well and amplified by a first-round PCR as described in Materials and Methods. The amplified cDNA was then subjected to a second round of PCR to amplify NK cell receptor cDNAs. For all Ly49 except Ly49B, consensus primers were used, whereas specific primers were used to amplify Ly49B and NKG2A. The individual receptor cDNAs thus amplified were identified by specific oligonucleotide probes by Southern hybridization. To verify the efficiency and specificity of this method, the second-round PCR was first applied to individual cDNA clones. As shown in Fig. 1 A, all known Ly49 as well as NKG2A cDNAs were efficiently amplified by the PCR, and the oligonucleotide probes specifically detected the corresponding cDNAs. A mixture of all Ly49 cDNAs (Ly49A–Ly49J) was also subjected to the second-round PCR, and the amplified Ly49 cDNAs were detected by the oligonucleotide probes. All the Ly49 cDNAs were amplified at similar efficiencies, indicating that the second-round PCR is not biased toward preferential amplification of some of the receptor genes because of competition among different Ly49 genes for PCR amplification (Fig. 1 B). Also, all the receptor genes were efficiently amplified when 1000 NK cells were subjected to this RT-PCR method.

Individual NK cells were analyzed by the single-cell RT-PCR, using β-actin transcripts as a positive control. Only those in which β-actin cDNA was detected were used for the analyses of the NK receptor expression. The receptor expression was considered positive when the bands of the predicted sizes for full length cDNAs were visible after overnight exposure of the Southern blot filters. A longer exposure resulted in cross-hybridization of the probes. This method revealed complex patterns of the NK cell receptor gene expression in individual NK cells (Table I). The frequencies of NK cells expressing Ly49A, -C/I, or -G mRNA determined by the single-cell RT-PCR were compared with those determined by flow cytometric analysis of polyclonal NK cells (Fig. 2). The values obtained by the two independent methods are comparable, indicating that the single-cell RT-PCR method introduced little bias toward the detection of particular NK cell receptor expression.

Southern blot analysis of PCR product

PCR products were separated by agarose gel electrophoresis and transferred to nylon membranes. Oligonucleotide probes specific for each known Ly49 cDNA were synthesized so that each had a melting temperature (Tm) of 68°C. Probes for Ly49A–H were previously described (21). The following probes were used: Ly49I, 5′-GAAGACTAGTGAACAAGCAG-3′; Ly49J, 5′-GGAATCTGAACAGATTACATC-3′; NKG2A, 5′-TGGCACAAGTCCCTCGG-3′; and NKG2B, 5′-TGGCACAAGTCCCTCGG-3′. The probes were 32P-end labeled using TdT and hybridized at 5–10°C below Tm to Southern blots, and the blots were washed at room temperature in 3 × SSC, 1% SDS. The filters were exposed to x-ray films at −70°C.

Results

Single-cell RT-PCR analysis of murine NK cell receptors

NK1.1+ CD3+ spleen cells from 8-wk-old C57BL/6 mice were individually sorted into microwell plates by FACS, and expression of NK cell receptor genes in each cell was analyzed by single-cell RT-PCR. Total cDNA was generated from each well and amplified by a first-round PCR as described in Materials and Methods. The amplified cDNA was then subjected to a second round of PCR to amplify NK cell receptor cDNAs. For all Ly49 except Ly49B, consensus primers were used, whereas specific primers were used to amplify Ly49B and NKG2A. The individual receptor cDNAs thus amplified were identified by specific oligonucleotide probes by Southern hybridization. To verify the efficiency and specificity of this method, the second-round PCR was first applied to individual cDNA clones. As shown in Fig. 1 A, all known Ly49 as well as NKG2A cDNAs were efficiently amplified by the PCR, and the oligonucleotide probes specifically detected the corresponding cDNAs. A mixture of all Ly49 cDNAs (Ly49A–Ly49J) was also subjected to the second-round PCR, and the amplified Ly49 cDNAs were detected by the oligonucleotide probes. All the Ly49 cDNAs were amplified at similar efficiencies, indicating that the second-round PCR is not biased toward preferential amplification of some of the receptor genes because of competition among different Ly49 genes for PCR amplification (Fig. 1 B). Also, all the receptor genes were efficiently amplified when 1000 NK cells were subjected to this RT-PCR method.

Individual NK cells were analyzed by the single-cell RT-PCR, using β-actin transcripts as a positive control. Only those in which β-actin cDNA was detected were used for the analyses of the NK receptor expression. The receptor expression was considered positive when the bands of the predicted sizes for full length cDNAs were visible after overnight exposure of the Southern blot filters. A longer exposure resulted in cross-hybridization of the probes. This method revealed complex patterns of the NK cell receptor gene expression in individual NK cells (Table I). The frequencies of NK cells expressing Ly49A, -C/I, or -G mRNA determined by the single-cell RT-PCR were compared with those determined by flow cytometric analysis of polyclonal NK cells (Fig. 2). The values obtained by the two independent methods are comparable, indicating that the single-cell RT-PCR method introduced little bias toward the detection of particular

FIGURE 1. Detection of specific Ly49 mRNA. A, A panel of 10 cloned Ly49 cDNAs (horizontally labeled A–J) were used as templates in PCR reactions, and the PCR products were run on 10 separate agarose gels, blotted, and hybridized to specific oligonucleotide probes (vertically labeled A–J). B, A mixture of different Ly49 cDNA (Ly49A–J, 0.1 ng each), and cDNA generated from 1000 NK cells were used as templates in PCR reactions, and hybridized to specific oligonucleotide probes as mentioned above. C, cDNA generated from each NK cell (labeled 1–10) was used as a template for a second round of PCR to amplify the Ly49 cDNAs. Aliquots of the PCR products were separated by agarose gel electrophoresis, blotted, and hybridized to specific oligonucleotide probes. Individual cells express different patterns of Ly49 expression.
Ly49 expression, and the results likely reflect the expression pattern of the receptors in NK cell populations in vivo.

A total of 62 NK cells from adult B6 mice were analyzed in four experiments for the expression of 10 different Ly49 and NKG2A mRNA by the single-cell RT-PCR method, and the results were compiled to assess the overall expression pattern of NK cell receptors (Fig. 3). The receptor repertoires of individual NK cells are highly diverse. All the NK cells analyzed expressed either Ly49 or NKG2A. Of 62 NK cells analyzed, 30 different patterns of Ly49 expression, 42 patterns when NKG2A was included, were identified. Some (5%) NK cells expressed as many as 6 different NK cell receptors. NKG2A was detected in 32% of NK cells, most of which coexpressed Ly49. This frequency of NKG2A+ adult NK cells is consistent with the finding that 40% of adult NK cells are stained with tetrameric Qa-1b that binds to the CD94/NKG2A heterodimer (15). Two cells expressed only Ly49D, an activating receptor, whereas all other NK cells expressed at least one inhibitory NK cell receptor.

**Receptor repertoire of neonatal NK cells.**

We also analyzed NK cell receptor expression in neonatal NK cells. NK1.1+CD3− spleen cells from 1-wk-old C57BL/6 mice were sorted as single cells, and 64 NK cells were analyzed for the expression of NK cell receptors by single-cell RT-PCR. The frequency of neonatal NK cells expressing Ly49 was significantly lower than that of adult mice. Only low percentages of cells expressed Ly49A, -C, -H, or -I (Fig. 5). In contrast, almost 60% of neonatal NK cells expressed NKG2A. The difference in the frequency of NKG2A+ cells between adult and neonatal NK cells was statistically significantly ($\chi^2$ test, $p < 0.01$). Therefore, it is likely that NKG2A expression precedes Ly49 expression in NK cell ontogeny and that it is the major inhibitory receptor on neonatal NK cells.

**Discussion**

In this study, we analyzed the expression pattern of Ly49 and NKG2A receptors in individual freshly isolated NK cells by single-cell RT-PCR. The results showed very diverse receptor repertoires among adult NK cells. In contrast, neonatal NK cells showed much simpler receptor repertoires in that almost 60% of them expressed NKG2A but most of them were Ly49−. The single-cell RT-PCR method used in this study was reproducible and provided reliable data on the pattern of multiple receptor expression in individual NK cells. The frequency of NK cells expressing individual Ly49 determined by the single-cell RT-PCR method was slightly higher than that determined by flow cytometric analysis (see Fig. 2). This probably is the result of high sensitivity of the RT-PCR analysis detecting very low levels of Ly49 expression that may not be readily detected by flow cytometric analysis. On
the other hand, the percentages of NKG2A$^+$ cells among adult and neonatal mice were slightly lower than those expected from the binding of Qa-1b tetramer. Qa-1b tetramer, the ligand for CD94/NKG2A (15), bound to $\sim$70% of NK cells from 1-wk-old mice and 40% of adult NK cells (data not shown). However, considering that CD94/NKG2C also binds Qa-1b tetramer (S. Lohwaser, A. Kubota, M. Salcedo, and F. Takei, unpublished results), the frequencies of NKG2A$^+$ NK cells determined by the single-cell RT-PCR are in good agreement with those estimated by Qa-1b binding. Although CD94 expression was not determined in this study because of unknown technical difficulties, these results also suggest that NKG2A+ NK cells likely coexpress CD94. Among the adult and neonatal NK cells analyzed in this study, none expressed Ly49B, -E, or -F. These genes were readily detected by the current method when the number of NK cells to be analyzed was increased to 1000. Therefore, the lack of detection of NK cells expressing these receptors in this study was most probably due to the low frequency of NK cells expressing these genes.

This study revealed, for the first time, the full complexity of the receptor repertoires of murine NK cells. Previous flow cytometric analyses have shown that more than two Ly49 molecules can be coexpressed on some NK cells. However, these studies were limited to the receptors for which specific mAbs are available. The results showed that the frequency of Ly49 coexpression is close to that expected from the individual frequency (product rule) (18, 19), suggesting that different Ly49 genes are randomly activated at a low frequency. Our study extended this product rule to all possible combinations of known murine NK cell receptors, including NKG2A. Although NKG2 and Ly49 belong to separate gene families, they follow the similar product rule. Up to six different receptors have been detected in individual NK cells. There seems to be no limitation to the number of receptors a single NK cell can express. However, the frequency of NK cells expressing several receptors is low simply because of low probability of simultaneously expressing multiple genes, each of which has $<50\%$ probability of expression in NK cells. The stochastic coexpression of multiple NK receptors generates very diverse receptor repertoires as demonstrated by the single-cell RT-PCR analysis. Of 62 adult NK cells examined in this study, 42 different combinations of receptor coexpression were observed, implying a high degree of heterogeneity among NK cells.

When compared with the receptor repertoires of human NK cells (17), our study revealed strong similarities between murine and human NK cell receptor repertoires despite their structural difference. Both human and murine NK cells express the nonclassical MHC class I-specific receptors CD94/NKG2 and classical

---

**Table II. Comparison of observed and expected frequencies of NK cell receptor pairs**

<table>
<thead>
<tr>
<th>Receptors</th>
<th>Ly49A (0.34)</th>
<th>Ly49C (0.48)</th>
<th>Ly49D (0.44)</th>
<th>Ly49G (0.45)</th>
<th>Ly49H (0.16)</th>
<th>Ly49I (0.35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ly49A</td>
<td>Obs.$^a$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0.34)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ly49C</td>
<td>Obs. 0.10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0.48)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ly49D</td>
<td>Obs. 0.19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0.44)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ly49G</td>
<td>Obs. 0.18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0.45)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ly49H</td>
<td>Obs. 0.08</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0.16)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ly49I</td>
<td>Obs. 0.13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0.35)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NKG2A</td>
<td>Obs. 0.10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0.32)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Obs., observed; Exp., expected.

$^b$ The underlined values differ significantly ($p < 0.01$) from that expected by random association. Statistical significance was determined using a $2 \times 2$ analysis.

---

**FIGURE 4. Frequency of adult NK cells expressing different numbers of receptors.** The results were calculated from 62 adult NK cells analyzed in Fig. 2. ■, percentages of NK cells expressing any Ly49 or NKG2A; □, those of inhibitory receptors (Ly49A, -C, -E, -F, -G, -I, and -J and NKG2A).

**FIGURE 5. Frequency of neonatal (1-wk-old) and adult (8-wk-old) mouse NK cells expressing Ly49 and NKG2A.** Sixty-four neonatal (■) and 62 adult (□) NK cells were analyzed by the single-cell RT-PCR and the frequencies of cells expressing individual receptors were compared.
MHC class I-specific receptors, the Ly49 family of C-type lectins are expressed on mouse and human NK cells and humans would have evolved independently since the separation of these two species, which may explain why structurally different NK cell receptors are used. The similarity between human and murine NK cell receptor repertoires indicates an evolutionary advantage of the system. It allows NK cells to express not only self-specific receptors but also multiple receptors in various combinations. The stochastic coexpression of multiple receptors in individual NK cells would generate a high degree of diversity in overall NK cell specificities.

It has been proposed that NK cells are potentially self-reactive but maintained to be self-tolerant because of inhibitory receptors specific for self-MHC class I (17, 24, 25). According to this hypothesis, every NK cell must express at least one inhibitory receptor recognizing self-MHC class I molecules (26). In C57BL/6 mice, Ly49C and possibly Ly49I (R. Lian, D. L. Mager, and F. Takei, unpublished results) recognize self-MHC. The SE6 mAb that recognizes both Ly49C and -I stains only ~60% of NK cells. Therefore, the recently characterized murine NKG2A may be an important inhibitory receptor for the recognition of self-MHC. The NKG2A/C9d4 heterodimer has been shown to bind to non-classical MHC class I Qa-1 (15). Our studies have shown that NKG2A mRNA is detected in 32% of adult NK cells. However, 23% of adult NK cells tested in this study did not express Ly49C or Ly49I, or NKG2A mRNA. These results suggest that murine NK cells may express additional self-MHC-specific inhibitory receptors yet to be identified.

The proportion of NK cells expressing each Ly49 receptor is low at birth and increases during ontogeny (27, 28). Consistent with this, the frequency of Ly49-expressing NK cells in neonatal mice was low in our assay. It has been reported that fetal and neonatal NK cells cultured in the presence of cytokines in a stroma-free system are deficient in Ly49 expression but preferentially kill tumor cells and blast cells deficient in the expression of MHC class I molecules (27–30). This suggested that fetal and neonatal NK cells may express inhibitory receptors other than Ly49. Indeed, our current results showed that many neonatal NK cells express NKG2A mRNA. It is likely that C9d4/NKG2A plays a major role in self-recognition of NK cells in early ontogeny, as suggested by the highly conserved features of Qa-1 and HLA-E (31, 32).

Acknowledgments

We thank Karina McQueen for oligonucleotide probes.

References