Acquisition of Ly49 Receptor Expression by Developing Natural Killer Cells

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Summary

The formation of the repertoire of mouse natural killer (NK) cell receptors for major histocompatibility complex (MHC) class I molecules was investigated by determining the developmental pattern of Ly49 receptor expression. During the first days after birth, few or no splenic NK cells express Ly49A, Ly49C, Ly49G2, or Ly49I receptors. The proportion of $Ly49^+$ splenic NK cells gradually rises to adult levels during the first 6–8 wk of life. The appearance of appreciable numbers of splenic $Ly49^+$ NK cells coincides with the appearance of NK activity at 3–4 wk. After in vivo transfer, NK cells not expressing specific Ly49 receptors can give rise to NK cells that do, and cells expressing one of these four Ly49 receptors can give rise to cells expressing others. Once initiated, expression of a Ly49 receptor is stable for at least 10 d after in vivo transfer. Hence, initiation of Ly49 receptor expression occurs successively. Interestingly, expression of one of the receptors tested, Ly49A, did not occur after in vivo transfer of $Ly49A^-$ cells. One possible explanation for these data is that the order of Ly49 receptor expression by NK cells is nonrandom. The results provide a framework for evaluating models of NK cell repertoire formation, and how the repertoire is molded by host class I MHC molecules.

NK cell lytic activity is often inhibited by MHC class I molecules expressed by target cells. It is believed that this mechanism allows the immune system to destroy cells that downregulate class I expression due to infection or transformation (1). Most or all natural killer cells in mice express one or more members of the Ly49 receptor family, a group of closely related and genetically linked MHC class I–specific inhibitory receptors (2). The capacity of NK cells to attack target cells that lack MHC class I expression, while sparing cells that express self–MHC class I molecules, depends in large part on inhibitory recognition of MHC molecules by Ly49 receptors.

mAb reagents to some Ly49 receptors have been used to show that they are expressed on overlapping subsets of natural killer cells (3–5). An NK cell can express multiple Ly49 receptors, including Ly49 receptors that do not recognize self–MHC class I molecules. The overall pattern of expression of different Ly49 receptors suggests that a stochastic mechanism governs the initial choice of which Ly49 receptors a NK cell expresses (6). Nevertheless, the repertoire is not wholly stochastic, since the frequencies of NK cells expressing different Ly49 receptors in a mouse are clearly influenced by host MHC class I expression (4, 7, 8). The MHC-dependent alterations in the Ly49 repertoire are likely to reflect mechanisms that ensure that NK cells are useful and self-tolerant in the context of the limited set of MHC molecules the host happens to inherit. These processes, and how they integrate with NK cell maturation, are currently poorly understood.

Indeed, the NK cell differentiation process is itself poorly understood. Unlike T cells, NK cells require neither a thymus (9) nor V(D)J recombination (10) for their development. Nevertheless, NK cells appear to be most closely related to the T cell lineage. Single clones of human $CD34^{bright}$ $CD3^-CD4^-CD8^-$ thymocytes are capable of giving rise to both NK and T cells (11). A population of similar phenotype isolated from mouse fetal thymocytes also appears able to give rise to both NK and T cells (12). These immature populations generally differentiate into T cells when placed in a thymic environment and NK cells when placed into other environments, suggesting that the environment in which the cells develop influences their ultimate fate. Early stages of NK cell development are generally thought to occur in the bone marrow, where NK cells make up ~2–4% of the cells present. The presence of a proper bone marrow microenvironment is thought to be necessary for proper NK function, since mice treated with agents that affect the bone marrow, such as ^{89}Sr (13) or estradiol (14), are unable to fully support the maturation of NK cells. However, this microenvironment has proved exceedingly difficult to define. Nor is there a detailed picture of the different stages in NK cell development.

A central issue in murine NK cell development concerns how Ly49 receptor expression is coupled to NK cell matu-

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ration and education processes. Several models can be envisaged. One possibility is that all Ly49 receptors to be expressed by an individual NK cell are initially expressed more or less simultaneously at a specific stage of differentiation. Such a pattern would fit well with models in which Ly49 receptor expression precedes selection steps or anergy induction processes that result in a self-tolerant yet sensitive population of mature NK cells (6). A second possibility is that Ly49 receptor expression occurs successively, such that a developing NK cell gradually accumulates receptors. This pattern of expression would be consistent with models in which NK cell education is directly coupled with receptor gene induction. For example, as the NK cell accumulates receptors, it may be concurrently tested for whether the expressed receptors react with self–MHC class I molecules, with a positive test causing termination of new receptor expression (and possibly maturation of the NK cell; reference 6). A third possibility is that all Ly49 receptors are initially expressed in an NK cell progenitor, and expression of some of these receptors is subsequently extinguished.

In vitro models of NK cell maturation have been assessed, but no induction of new Ly49 receptor expression was observed in these studies (reference 15 and Dorfman, J.R., and D.H. Raulet, unpublished data). Hence, after documenting the ontogeny of Ly49 receptor expression, we have investigated NK cell maturation and Ly49 receptor expression in vivo with the use of a cell transfer protocol, similar to that used in establishing the sequence of cell differentiation steps in thymic T cell development (16, 17). NK cell subsets bearing an allotypic Ly5 marker were transferred to sublethally irradiated mice. After time for subsequent maturation, the phenotype of progeny cells was determined. The results provide evidence for intermediates in NK cell differentiation in which expression of Ly49 receptors is ongoing. The pattern of expression suggests that Ly49 receptors may be expressed successively rather than in a single burst, and that receptor expression, once it occurs, is quite stable. Finally, the results indicate that different rules govern induction of different Ly49 receptors, and raise the possibility that there may be a nonrandom order in which different Ly49 genes are induced.

Materials and Methods

Mice. C57BL/6NCr (B6) mice were bred in the animal facility of the University of California (Berkeley, CA). B6 Ly5.1 mice were purchased from the National Cancer Institutes (Frederick, MD) where they are listed as B6 Ly5.2 mice according to the previous nomenclature. BALB.B mice were kindly provided by Dr. I. Weismann (Stanford University, Palo Alto, CA).

Antibodies and Reagents. Anti-CD4 (RL-172; reference 18), anti-CD8 (AD4-15; reference 19), anti–class II (I-$A^{b,d}$) (BP107; reference 20), anti-NK1.1 (PK136; reference 21), anti-CD3 (22), anti-FcRγII/III (2.4G2; reference 23), and the Ly49A-specific mAb A1 (24) have all been described. The anti-Ly49A mAb JR9-318 (25) was kindly provided by Dr. J. Roland (Institut Pasteur, Paris, France). The mAb SW5E6 (26, 27) that recognizes both Ly49C and Ly49I was generously provided by Drs. Vinay Kumar and Michael Bennett (University of Texas, Dallas, TX) and the hybridoma producing the anti-Ly49G2 mAb 4D11 (28) was purchased from American Type Culture Collection (Rockville, MD). Antibodies were purified from hybridoma supernatants by affinity chromatography over protein A–sepharose or protein G–agarose except JR9-318, which was purified from ascites by ammonium sulfate fractionation followed by ion exchange chromatography. Antibodies were conjugated to fluorescein, biotin, R-PE, or allophycocyanin. The following reagents were purchased: streptavidin tricolor (Caltag Laboratories, South San Francisco, CA), streptavidin RED613 (GIBCO BRL, Gaithersburg, MD), and, for some experiments, anti-NK1.1 PE (PharMingen, San Diego, CA), anti-CD3-FITC, and anti-CD3 biotin (GIBCO BRL).

Isolation of NK Cell Subsets for Transfers. Splenocytes from 40–150 B6 mice aged 18–45 d were pooled and then enriched for NK cells under aseptic conditions as described below. For contaminant control experiments, splenocytes were also isolated from 15–30 B6 Ly5.1 mice and enriched for NK cells. In brief, cells were passed over nylon wool treated with guinea pig complement, rabbit complement, and anti-CD4, anti-CD8, anti–I-A^b antibodies. Viable cells were recovered and stained for NK1.1, CD3ε, Ly49A, Ly49C/I and Ly49G2. The stained populations were sorted on an ELITE flow cytometer (Coulter Immunology, Hialeah, FL). Three- or four-color sorting was used, with different antibodies conjugated to the same fluorescent dye as needed. All isolated populations were sorted $NK1.1^+CD3^-$ except in some replicates of the $Ly49A^-C^-G2^+I^-$ (Ly49G2 single positive cell) experiments and one replicate of the $Ly49A^+C^-G2^-I^-$ (Ly49A single positive cell) experiments. In these cases, the $NK1.1^+$ population was sorted without any selection against residual $NK1.1^+$ $CD3^+$ cells. NK cell subsets isolated from 4–10-d-old mice were isolated in the same manner without use of the complement kill.

In Vivo Transfer and Subsequent Analysis of Isolated NK Subsets. Cells were recovered from the flow cytometer, counted on a hemacytometer and then injected intraveneously (subocularly) into Ly5 congenic mice under metofane anesthesia 2–4 h after they received a single sublethal irradiation dose (600 RAD). After 10 d, spleens and often thymuses were excised and analyzed. No purification of splenocytes was necessary. All samples were preincubated with anti-FcRγII/III antibody to block FcRγII/III-mediated binding. Donor cells were never found in thymuses (≤1/10,000 events, $n = 4$ for $Ly49A^-C^-G2^-I^-$ NK cell–injected mice). When spleens were analyzed 20 d after transfer, splenocytes were enriched for donor cells by passage over nylon wool before analysis. Flow cytometry was performed on a flow cytometer (model XL-MCL; Coulter Immunology). HBSS-injected controls were analyzed in parallel in each experiment and few or no donor-type events were detected in these mice.

Isolation and Transfer of Fetal Liver and NK-depleted Bone Marrow Cells. Fetal liver cells were isolated from day 14 B6 fetuses. Bone marrow cells were isolated from 4-wk-old B6 mice by irrigation of the femurs and tibias and were incubated with PK136-biotin followed by streptavidin conjugated magnetic microbeads followed by passage over a steel wool column using a magnetic activated cell sorting (MACS) apparatus (all MACS beads and apparatuses were purchased from Miltenyi Biotech GmbH, Sunnyvale, CA). Conditions were chosen that resulted in the loss of $Ly49C/I^+$ cells from bone marrow cells and NK-enriched splenocytes in pilot experiments. Fetal liver cells and NK-depleted bone marrow cells were injected into lethally irradiated (980 RAD) B6 Ly5.1 mice as described above for NK subsets. 10–12 wk after reconstitution, donor origin NK cells in spleens from host mice were analyzed for Ly49 receptor expression.

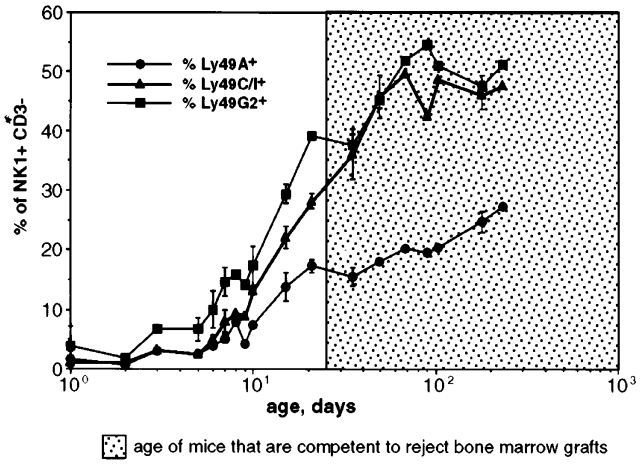

Figure 1. Ontogeny of expression of Ly49 receptors by splenic NK cells. Early splenic NK1.1$^+$CD3$^-$ cells do not express Ly49 receptors and gradually acquire them during postnatal life. The proportion of NK1.1$^+$CD3$^-$ cells (± SD where applicable) expressing the indicated receptor(s) is shown. The same trends were not observed when splenic NK1.1$^+$ T (NK1.1$^+$CD3$^+$) cells were examined (data not shown).

Results

The Proportion of NK Cells Expressing Each Ly49 Receptor Increases during Ontogeny. Cells with the NK phenotype (NK1.1$^+$CD3$^-$) were enriched from splenocytes of B6 mice of various ages, and the percentage of NK cells that stained with each of three Ly49 receptor-specific antibodies was assessed. The percentage of NK cells expressing any particular Ly49 receptor increased over ontogeny, reaching appreciable levels by 2–3 wk of age, and reaching adult levels at 6–8 wk of age (Fig. 1). A similar pattern was observed for NK1.1$^+$CD3$^-$ cells from the bone marrow, except that smaller percentages stained with the antibodies at each age point studied (data not shown).

The timing of the appearance of Ly49$^+$ NK cells in the spleen closely matches the appearance of NK activity in the mouse as assayed by their competence to reject bone marrow grafts (17–22 d; reference 29) or as assayed by the appearance of in vitro NK activity in the spleen (18–26 d; references 14, 30). These findings raised the possibility that the NK cells found in younger (<3 wk) mice, few of which express Ly49 receptors, are largely inactive. However, it is not known whether the Ly49$^-$ NK cells in young mice are deficient in functional activity, as suggested by Hackett et al. (14), or are simply too infrequent to mediate substantial NK cell activity, as suggested by their lower absolute cell numbers in the spleen during early life (data not shown).

Acquisition of Ly49 Receptor Expression by NK1.1$^+$ Ly49A$^-$C$^-$G2$^-$I$^-$ Cells. NK cells that express the tested Ly49 receptors could arise directly from NK1.1$^-$ progenitor cells. Alternatively, they could arise from NK1.1$^+$CD3$^-$ cells that do not express these Ly49 receptors. We employed a cell transfer system to assess whether NK1.1$^+$CD3$^-$ cells that do not express the tested Ly49 receptors can subsequently acquire them.

Splenocytes from 40–150 B6 mice of 18–45 d of age were pooled, enriched for NK cells, and then stained and sorted for NK1$^+$CD3$^-$ Ly49A$^-$C$^-$G2$^-$I$^-$ cells. Sorted populations were reanalyzed at the completion of the sort; the reanalysis of a representative experiment is shown in Fig. 2*a*. Sorted cells were injected i.v. into B6 Ly5.1 mice

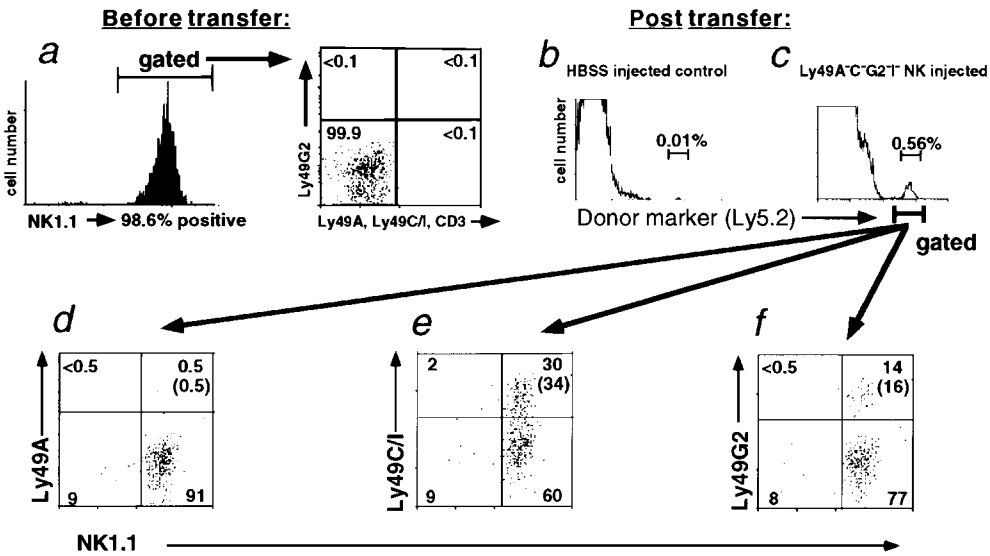

Figure 2. Ly49A$^-$C$^-$G2$^-$I$^-$ NK cells express some Ly49 receptors after in vivo transfer. Ly49A$^-$C$^-$G2$^-$I$^-$ NK cells were purified by cell sorting from B6-derived, NK-enriched splenocytes and then transferred into Ly5 congenic mice as described in the Materials and Methods. (*a*) Post-sort analysis (before transfer) from a representative experiment. (*b* and *c*) Splenocytes from an HBSS injected control (*b*) or a recipient (*c*) were stained for Ly5.2, the donor marker. (*d–f*) Donor-derived cells were stained for NK1.1 vs. Ly49A (*d*), Ly49C/I (*e*), or Ly49G2 (*f*). Numbers indicate the percentage of events in the corresponding quadrant except the number in parentheses, which indicates the percentage of donor NK1.1$^+$ cells in the corresponding quadrant. Cells in the donor gate also failed to stain with antibody specific for the host-type Ly5.1 allotype (not shown).

2–3 h after the mice were sublethally irradiated. After 10 d, the spleens of the host mice were excised and splenocytes were stained without enrichment. Donor cells were recognized by an anti-Ly5.2 antibody, and generally constituted ~0.5% of the splenocytes (Fig. 2, *b* and *c*). The donor cells were generally >90% NK1.1$^+$. The number of donor-type cells recovered ranged from 2–20% of the number injected, with an average of ~10%.

10 d after transfer, ~90% of the donor-derived cells were NK1.1$^+$CD3$^-$ cells, suggesting that the donor NK phenotype is quite stable. No CD3$^+$ donor cells were detected in the spleen and no donor cells of any type were detected in the thymuses of the recipient mice. Significantly, although essentially none of the donor-derived cells expressed Ly49A, Ly49C/I, or Ly49G2 at the time of transfer, an appreciable fraction expressed Ly49C/I (Fig. 2 *e*) or Ly49G2 (Fig. 2 *f*) after transfer. Surprisingly, none of the donor cells expressed Ly49A after transfer (Fig. 2 *d*, and see below).

The results of 11 replicates of this experiment are summarized in the first line of Table 1, including two experiments in which donor cells of B6 origin were injected into BALB.B mice, and one experiment in which donor cells of B6 Ly5.1 origin were injected into B6 mice. Ly49C/I and Ly49G2 receptors were consistently expressed by a significant fraction of cells after transfer, whereas no significant expression of Ly49A was observed in any of the experiments. Control mice were routinely injected with HBSS in parallel and then analyzed at the same time that host mice were analyzed. Few or no cells that stained with the donor-specific antibodies were detected in these mice. Furthermore, control antibodies specific for the host cell marker did not stain the donor-derived cells, indicating that the staining procedure was specific for donor-type cells. These results suggest that Ly49C/I and Ly49G2 expression was induced on a fraction of transferred splenic NK1.1$^+$CD3$^-$ cells, whereas Ly49A was not. It is notable that Ly49C and Ly49I receptors are H-2^b–reactive receptors (31, 32; Hanke, T., and D.H. Raulet, unpublished data), and hence self-reactive in these experiments, whereas Ly49G2 is not H-2^b reactive (5). Therefore, the capacity of a receptor to be expressed after transfer is not dependent on it being self-specific.

Expression of a Ly49 Receptor Is Stable upon In Vivo Transfer. As shown above, Ly49A$^-$C$^-$G2$^-$I$^-$ NK cells can give rise to Ly49C/G2/I$^+$ NK cells. One possible interpretation of this observation is that cells are constantly turning Ly49C, Ly49G2, and/or Ly49I on and off. To address this possibility, we purified populations of NK cells expressing Ly49A, Ly49C/I, or Ly49G2. These populations were transferred individually into Ly5-congenic mice. In representative experiments, NK1.1$^+$ cells expressing Ly49A (Fig. 3 *a*), Ly49C/I (Fig. 3 *b*), or Ly49G2 (Fig. 3 *c*) were purified. Cells from each sort were injected into sublethally irradiated B6 Ly5.1 mice, and splenocytes from the recipient mice were analyzed after 10 d. As can be readily seen, the large majority of cells of donor origin continued to express

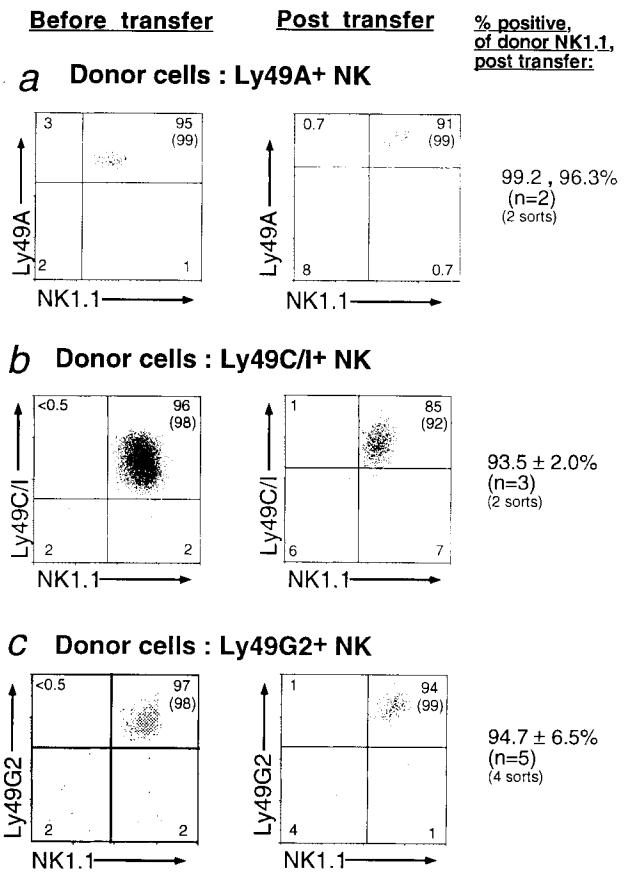

Figure 3. Cells expressing particular Ly49 receptors before transfer continue to express them after in vivo transfer. B6 NK cells sorted for expression of the Ly49A (*a*), Ly49C/I (*b*), or Ly49G2 (*c*) receptors were transferred into B6-Ly5 congenic mice. Analyses of the cells before transfer (*left panels*) and 10 d after transfer (*right panels*) are shown. Note that these analyses were performed on different days on different flow cytometers; therefore, staining intensities are not directly comparable. Numbers in each quadrant indicate percentages of total events in each quadrant, and numbers in parentheses are percentages of donor-derived NK1.1$^+$ cells. On the right side of the panels, a summary is shown of all experiments in which NK cells expressing the indicated receptor were transferred. Ly49G2 summary values include values from experiments in which Ly49G2 single positive cells were injected, while Ly49A values are derived exclusively from experiments in which Ly49A single positive cells were transferred (see Table 2). *a* and Fig. 4 contain data derived from a single experiment. Values are indicated as percentages of donor derived NK1.1$^+$ cells ± SD.

the selected receptor even 10 d after in vivo transfer (Fig. 3). Whether or not this stability extends beyond 10 d remains untested.

Failure of Ly49A$^-$C$^-$G2$^-$I$^-$ NK Cells to Acquire Ly49A after Transfer. It was surprising that donor NK1.1$^+$ Ly49A$^-$C$^-$G2$^-$I$^-$ cells specifically failed to express Ly49A after transfer. The Ly49A stain was effective, because host-derived Ly49A$^+$ NK cells were readily detectable in the same samples (not shown). Furthermore, Ly49A$^+$ NK cells were easily detected among donor-type cells after injection of unsorted, enriched NK cells regardless of whether or not these cells had been stained in the same manner as was used for the sorting experiments (not shown). Also, Ly49A$^+$ NK

Table 1. *Summary of Transfer Experiments with Ly49A$^-$C$^-$G2$^-$I$^-$ NK Cells or Bone Marrow Cells*

Injected cells	Resulting cells, 10 d after injection		
	Percent Ly49A$^+$	Percent Ly49C/I$^+$	Percent Ly49G2$^+$
Ly49A$^-$C$^-$G2$^-$I$^-$ (n = 11) seven sorts; from 18-45-d-old mice	1.0 ± 0.7	27.3 ± 5.3	18.9 ± 7.8
Ly49A$^-$C$^-$G2$^-$I$^-$ (n = 1) from 4–10-d-old mice	0.0	25.5	25.2
Ly49A$^-$C$^-$G2$^-$I$^-$ (n = 1) 20 d after injection	1.2	48.1	28.4
NK-depleted bone marrow (n = 2) 12 wk after injection	16.8 ± 0.4	40.4 ± 3.5	47.0 ± 0.5

Values represent average percentages of donor-derived NK1.1$^+$ cells that express the relevant receptor after *in vivo* transfer. Values are ±SD where applicable.

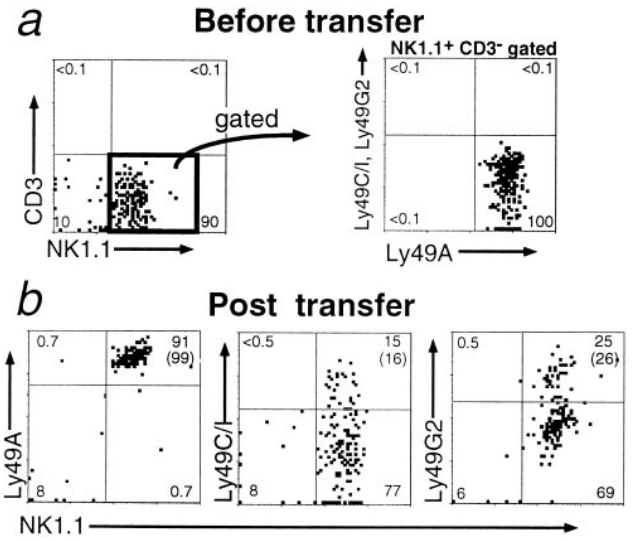

Figure 4. Ly49A single positive (Ly49A$^+$C$^-$G2$^-$I$^-$) NK cells express Ly49C/I and Ly49G2 after in vivo transfer. Ly49A$^+$C$^-$G2$^-$I$^-$ NK cells were purified by cell sorting from B6-derived, NK-enriched splenocytes and then transferred into Ly5 congenic mice as described in Materials and Methods. (*a*) Post-sort analysis (before transfer) from a representative experiment. (*b*) Donor-derived cells from the same experiment 10 d after transfer were stained for NK1.1 vs. Ly49A, Ly49C/I or Ly49G2. Numbers in each quadrant indicate percentages of events in each quadrant except numbers in parentheses, which are percentages of donor-derived NK1.1$^+$ cells. Data in this figure and Fig. 3 *a* are derived from the same experiment.

cells were readily detectable when sorted Ly49A$^+$ NK cells were injected (Fig. 3 *a*). Thus, the failure to detect Ly49A$^+$ NK cells after transfer of Ly49A$^-$C$^-$G2$^-$I$^-$ NK cells does not reflect an inability to detect Ly49A expression.

The possibility was considered that the failure to detect Ly49A$^+$ NK cells after transfer of NK1.1$^+$Ly49A$^-$ cells reflected the relatively advanced age (18–45 d old in different experiments) of the donor mice. However, transferred Ly49A$^-$C$^-$G2$^-$I$^-$ NK cells from 4–10-d-old donors also failed to produce significant numbers of Ly49A$^+$ NK cells (Table 1). Since most endogenous Ly49A$^+$ cells appear in normal mice after 10 d of age (Fig. 1), it appears unlikely that precursors of Ly49A$^+$ NK cells have already disappeared by this age.

Another possibility was that acquisition of Ly49A is delayed compared with the other receptors and had not yet occurred by 10 d after transfer. However, even 20 d after transfer of Ly49A$^-$C$^-$G2$^-$I$^-$ NK cells no Ly49A$^+$ NK cells could be detected (Table 1). In normal mice, Ly49A expression reaches nearly adult levels within 20 d of birth (Fig. 1). There was also the possibility that Ly49A expression occurs immediately after transfer and is subsequently rapidly extinguished. However, as shown above, transferred Ly49A$^+$ NK cells persist and faithfully continue to express Ly49A 10 d after transfer (Fig. 3 *a*). Therefore, it is unlikely that the expression of Ly49A after transfer is transient. Finally, it seemed possible that irradiated mice represent a nonpermissive environment for differentiation of Ly49A$^+$ NK cells from Ly49A$^-$ progenitor cells. However, irradiated mice reconstituted with NK cell–depleted bone marrow cells generated normal numbers of donor-type Ly49A$^+$ NK cells 12 wk after reconstitution (Table 1). Similar results were obtained when fetal liver cells were transferred (data not shown).

The above experiments and considerations suggest that irradiated mice are permissive for acquisition of Ly49A by differentiating NK cells, and that the failure to detect these cells after transfer of Ly49$^-$NK1.1$^+$ cells is unlikely to reflect inadequate detection procedures. It thus appears likely that splenic NK1.1$^+$CD3$^-$ cells are competent to initiate expression of some Ly49 receptors (Ly49C/I and Ly49G2), whereas initiation of Ly49A expression by these cells either does not occur or is grossly delayed (see Discussion).

Successive Expression of Ly49 Receptors. Although the donor cells in the above transfer experiments were Ly49A$^-$C$^-$G2$^-$I$^-$ cells, it is highly likely that at least some of them expressed one of the at least five other members of the Ly49 receptor family, for which specific antibodies were not available. With this in mind, two nonmutually exclusive explanations can be considered for the initiation of Ly49C/I and Ly49G2 receptor expression after transfer of Ly49A$^-$C$^-$G2$^-$I$^-$ NK cells. The first is that the Ly49-expressing cells after transfer were all derived from transferred NK1.1$^+$ progenitor cells that did not express any Ly49 receptors. The competing possibility is that some or all of the Ly49C/I$^+$ and Ly49G2$^+$ cells after transfer were derived from NK cells that already expressed other Ly49 family members at the time of transfer. If this were the case, it would imply that expression of new Ly49 receptors on NK lineage cells occurs rather gradually and successively.

Table 2. *NK Cells Already Expressing an Ly49 Receptor Remain Capable of Expressing New Receptors After In Vivo Transfer*

Injected cells	Resulting cells, 10 d after injection: Percent Ly49A$^+$	Percent Ly49C/I$^+$	Percent Ly49G2$^+$
Ly49A$^+$C$^-$G2$^-$I$^-$ (n = 2) two sorts	99.2, 96.3*	8.6, 27.8	26.7, 87.0
Ly49A$^-$C$^-$I$^-$G2$^+$ (n = 3) three sorts	1.7 ± 2.5	22.6 ± 3.6	92.2 ± 7.7*

Values indicate the precentages of donor NK1.1$^+$ cells that express the relevant receptor(s) after in vivo transfer, ±SD where applicable.
*These values are included in the summary data shown in Fig. 3.

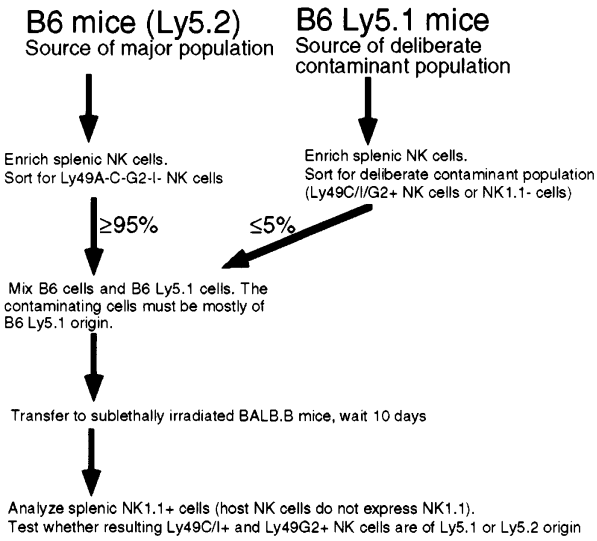

Figure 5. Experimental design to assess contribution of cellular contaminants to Ly49C/I or G2$^+$ populations present after cell transfer. Ly49A$^-$C$^-$G2$^-$I$^-$ NK1.1$^+$ cells were purposefully contaminated with tracer Ly5 congenic cells, either Ly49C/I or G2$^+$ NK cells or NK1.1$^-$ cells, and transferred into irradiated BALB.B recipients. The contribution of the added contaminating cells to the population 10 d after transfer was then determined.

To investigate whether Ly49 receptors can be expressed successively, we purified Ly49A$^+$C$^-$G2$^-$I$^-$ cells (referred to below as Ly49A single positive cells) and transferred them to Ly5 congenic mice. After 10 d, an appreciable fraction of the cells expressed Ly49C/I or Ly49G2 (Fig. 4, Table 2). In other experiments, Ly49A$^-$C$^-$G2$^+$I$^-$ cells (referred to below as Ly49G2 single positive cells) were purified and transferred. 10 d after transfer, an appreciable fraction of these cells expressed Ly49C and/or Ly49 (Table 2). Thus, splenic NK cells that already express at least one Ly49 receptor were capable of expressing new Ly49 receptors after in vivo transfer. These data suggest that Ly49 receptors may be expressed successively during NK cell development. The newly expressed receptors that we observed included receptor(s) that are self-specific (Ly49C and/or Ly49I) and a receptor that is not self-specific (Ly49G2). Thus, there was no correlation between the presence of a MHC class I ligand for the newly expressed receptor and the ability of the NK cell to initiate its expression. However, when Ly49G2$^+$ NK cells were transferred, few or none of them expressed Ly49A 10 d after transfer (Table 2). These data extend the earlier results indicating that transferred NK1.1$^+$Ly49A$^-$ cells do not give rise to Ly49A$^+$ cells.

Ly49-expressing Cells after Transfer Are Not Derived from Contaminating Ly49C/I or G2$^+$ Cells or NK1.1$^-$ Cells in the Transferred Population. The previous data suggest that NK1.1$^+$ CD3$^-$ cells that do not express a Ly49 receptor can acquire it after in vivo transfer. However, it remained possible that the Ly49C/I or G2$^+$ cells after transfer were derived not from the majority Ly49A$^-$C$^-$G2$^-$I$^-$ NK1.1$^+$ CD3$^-$ population, but from contaminating cells in the inoculum, which might selectively survive or expand after transfer. One possibility was that the Ly49C/I or G2$^+$ cells after transfer were derived from small numbers of contaminating Ly49C/I or G2$^+$ cells in the inoculum. To address this possibility, the inoculum population (Ly49A$^-$C$^-$G2$^-$I$^-$ NK cells from B6 mice) was purposefully contaminated with a defined percentage of sorted Ly49C/I or G2$^+$ NK cells from B6-Ly5.2, as diagrammed in Fig. 5. The Ly5 difference allowed us to evaluate the contribution of the contaminants to the resulting Ly49C/I or G2$^+$ NK cells after transfer. To distinguish both types of donor cells from host-type NK cells after transfer, the cells were inoculated into irradiated BALB.B recipients that fail to express NK1.1 on their NK cells. Thus, cells that expressed NK1.1 were sure to be of donor origin, and whether they were derived from the added Ly49C/I or G2$^+$ contaminant population could be ascertained based on Ly5 expression.

Post-sort analysis indicated that the Ly49A$^-$C$^-$G2$^-$I$^-$ NK cells purified from B6 (Ly5.2) splenocytes contained ≤0.1% Ly49C/I or G2$^+$ NK contaminants (not shown). This population was spiked with sorted Ly49C/I or G2$^+$ NK cells from B6-Ly5.1 mice, so as to achieve 2% contamination, a >20-fold excess (2%/≤0.1%) compared with B6-derived contaminants (Table 3). After flow cytometric analysis to confirm the contamination level, the cell mixture was inoculated into BALB.B mice. Although the contaminant population accounted for ≥95% of the Ly49C/I or G2$^+$ NK cells at the time of injection, 10 d after transfer they accounted for only ~15% of the resulting Ly49C/I$^+$ NK cells and 5–20% of the resulting Ly49G2$^+$ cells (Table 3). These data indicate that contaminating Ly49C/I or G2$^+$ NK cells in the inoculum cannot account for the large majority of Ly49C/I or G2$^+$ NK cells in the population after transfer, especially when it is recalled that the Ly5.1$^+$ contaminating cells were added at a >20-fold excess compared with contaminating B6 origin Ly49C/I or G2$^+$ cells. Therefore, the large majority of Ly49C/I or G2$^+$ NK cells after transfer were derived from Ly49A$^-$C$^-$G2$^-$I$^-$ cells in the inoculum.

Table 3. *Contribution of Added Contaminants to NK Cell Populations after In Vivo Transfer*

	Inoculum		Post-transfer			
	Percent Ly49C/G2/I$^+$ cells		Percent Ly49C/I$^+$ cells		Percent Ly49G2$^+$ cells	
Recipient	Ly5.2 (B6 origin)	Ly5.1 (added)	Ly5.2 (B6 origin)	Ly5.1 (added)	Ly5.2 (B6 origin)	Ly5.1 (added)
1	<0.1%	2%	26.2%	4.5%	35.5%	2.2%
2	<0.1%	2%	23.4%	3.9%	23.8%	5.5%

A similar strategy (Fig. 5) was used to address the possibility that the Ly49C/I or G2$^+$ NK cells observed after transfer arose from contaminating NK1.1$^-$ cells in the inoculum. The spleen contains hematopoietic stem cells and possibly other more restricted lymphoid progenitors, so it was possible that these cells differentiated into Ly49C/I or G2$^+$ NK cells after transfer. Post-sort analysis indicated that a purified population of B6-derived Ly49A$^-$C$^-$G2$^-$I$^-$ NK cells contained ~2.1% NK1.1$^-$ cells, a value similar to that seen in other experiments (e.g., 1.4% in Fig. 2). NK1.1$^-$ splenocytes from B6-Ly5.1 mice were added to this population so as to achieve 5% contamination, a 2.5-fold excess compared with the NK1.1$^-$ contaminants already present. The added NK1.1$^-$ splenocytes had been sorted from a population that was initially enriched for NK cells using the same procedure as was used for the NK1.1$^+$ population. After flow cytometric analysis to confirm the cell composition (not shown) the mixture was injected into irradiated BALB.B mice. 10 d after transfer, NK1.1$^+$ (donor) cells were tested for expression of Ly5 markers (Fig. 6). Although 70% of the contaminating NK1.1$^-$ cells in the inoculum were from the Ly5.1$^+$ donor, essentially none (<2%) of the donor-derived NK1.1$^+$ cells after transfer were Ly5.1$^+$. Significant numbers of Ly5.1$^+$ cells were observed in only one of the two recipients, and these were overwhelmingly NK1.1$^-$ (Fig. 6). None of the Ly5.1$^+$ cells after transfer detectably expressed Ly49C/I or Ly49G2. We conclude that neither contaminating NK1.1$^-$ precursors, nor contaminating Ly49C/I or G2$^+$ cells, can account for the vast majority of Ly49C/I or G2$^+$, NK1.1$^+$ cells observed after transfer of Ly49A$^-$C$^-$G2$^-$I$^-$ NK1.1$^+$ cells. It follows that the Ly49C/I or G2$^+$ cells after transfer were derived from NK1.1$^+$ Ly49A$^-$C$^-$G2$^-$I$^-$ cells in the inoculum.

Discussion

Ly49$^+$ Cells Appear Gradually during Ontogeny Among Splenic NK Cells and Their Appearance Is Associated with the Appearance of NK Activity. During ontogeny, the proportion of NK1.1$^+$CD3$^-$ cells expressing each Ly49 receptor starts very low and then gradually rises, reaching adult levels by approximately six weeks of age. At the adult stage, >80% of NK cells express one or more of the detectable Ly49 receptors (data not shown). Functional tests revealed that the 20% that were negative for Ly49A, C, G, and I were nevertheless as lytic against YAC-1 tumor target cells as Ly49A, C, G2 or I$^+$ effector cells (data not shown), and it is likely that many of these cells express one of the five other Ly49 receptors that have been identified that are not detected with the antibodies employed here. In contrast, since so few NK1.1$^+$ CD3$^-$ cells in young mice stained with the anti-Ly49 antibodies, it appears likely that many of these cells fail to express any Ly49 receptors. Whether these cells are nonfunctional and/or immature, or express inhibitory receptors other than Ly49 receptors, such as CD94/NKG2 heterodimers (33, 34), remains to be determined.

Initiation of Ly49 Receptor Expression. The transfer experiments demonstrate that Ly49A$^-$C$^-$G2$^-$I$^-$ NK1.1$^+$ cells can initiate Ly49 receptor expression after transfer in vivo (Fig. 2). The control experiments argue persuasively that the Ly49C/I$^+$ or G2$^+$ cells did not arise from either contaminating Ly49C/I or G2$^+$ cells or NK1.1$^-$ cells in the inoculum (Figs. 5 and 6, Table 3). In the case of transferred NK cells from young (4–10 d old) mice, it is likely that most of

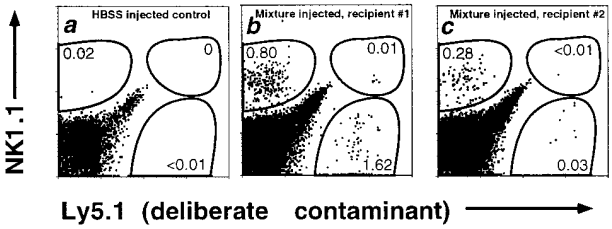

Figure 6. NK cells do not arise from splenic NK1.1$^-$ contaminants in the starting Ly49A$^-$C$^-$G2$^-$I$^-$ NK population. Cells were purified, mixed and inoculated into BALB.B mice as described in Fig. 5. NK cells of B6 and B6 Ly5.1 (marked contaminant) origin were detected by their expression of NK1.1, which is not expressed in BALB.B mice. NK cells derived from the marked contaminant NK1.1$^-$ population were detected by their coordinate expression of Ly5.1 and NK1.1. The marked contaminant cells represented 70% of NK1.1$^-$ cells in the injected population 10 d earlier (not shown). However, few or none of the NK1.1$^+$ cells after transfer were derived from these contaminants. Additionally, none of the Ly49C/G2/I$^+$ NK cells were of B6 Ly5.1 origin (not shown). Numbers denote the percentages of total cells present in each quadrant. Ly5.1$^-$ cells include both host origin and B6 origin cells. The oddly shaped NK1.1$^-$ Ly5.1$^-$ population was an artifact of the flow cytometry analysis, as shown by parallel analysis of BALB.B mice inoculated with HBSS.

the transferred donor cells failed to express any Ly49 receptors, suggesting that Ly49$^-$ NK1.1$^+$ cells can initiate Ly49 receptor expression within 10 d after in vivo transfer. In the case of the older (18–45 d old) donors, the transferred population probably contained substantial numbers of cells expressing undetected Ly49 receptors, so it cannot be directly surmised from this experiment whether the Ly49 expression was initiated on transferred Ly49$^-$ or Ly49$^+$ cells. However, the cumulative expression of Ly49 receptors was demonstrated directly by transferring Ly49 single positive cells (Fig. 4, Table 2). The extent of new receptor expression appeared similar to that of transferred Ly49A$^-$ C$^-$G2$^-$I$^-$ NK cells (Table 1). These results provide support for the notion that Ly49 receptor acquisition is a relatively gradual process, rather than occurring in a burst. Notably, the data as a whole also demonstrate that receptor expression occurs additively, rather than subtractively.

Whether the particular NK cells that successively express Ly49 receptors are functionally active before transfer has not been established. The transferred NK cell population includes functional (mature) NK cells, but it is plausible that some of the transferred cells were nonfunctional or immature, and that it was these cells that gave rise to new Ly49 receptor expression after transfer. Alternatively, it remains possible that even functionally active NK cells can express new Ly49 receptors. Whether such a process would be advantageous to the animal is arguable in the context of available information. New Ly49 expression by active mature NK cells may represent a threat to the animal, since the NK cells might eventually adapt to the class I MHC alterations that occur in tumor cells or virus-infected cells (35). For example, many tumor cells have been observed to extinguish expression of some but not all cellular class I molecules (36). It would seem counterproductive if NK cells could adapt by expressing new receptors specific for the remaining class I molecules on these tumor cells. On the other hand, the expression of new receptors by mature NK cells could endow the NK compartment with the capacity to adapt to changing MHC environments, as might occur as NK cells traffic to different tissues where class I MHC levels may vary considerably.

The kinetics of receptor expression observed here fulfill a critical prediction of the sequential receptor expression model of NK cell education, which posits that successive receptor expression on developing NK cells is coupled with ongoing testing of the reactivity of the receptors with self–MHC class I molecules (4, 6, 37). Strong reactions with self–MHC class I molecules would terminate the phase in which receptor expression is initiated, and possibly stimulate maturation of the NK cell. It must be acknowledged, however, that while the results argue for successive expression of receptors on NK cells, they do not address whether interactions with class I MHC molecules impact this process. Therefore, it remains possible that receptors are expressed successively, but that testing of these cells (e.g., selection) does not occur until a later stage. A hybrid between these models (38) proposes that developing NK cells may be permissive for initiating Ly49 receptor expression for only a discrete time period in their development, during which MHC class I engagement can terminate the process. Cells that fail to achieve expression of self–MHC class I–specific receptors in this time window would then be silenced.

Ly49 Receptor Expression, Once Initiated, Appears Stable. As noted above, Ly49 receptor expression, once initiated, is stable for at least 10 d in vivo, consistent with previous in vitro results (39). Although 2–7% of the transferred Ly49$^+$ NK cell population were negative for the applicable Ly49 receptor 10 d after transfer (Fig. 3, Table 2), we suspect that these cells may have arisen from contaminating NK cells that did not express the relevant Ly49 receptor at the time of transfer. Indeed, small numbers of NK1.1$^+$Ly49$^-$ cells could be detected in the donor Ly49$^+$ populations in most experiments (Fig. 3). However, we cannot rule out the possibility that small numbers of Ly49$^+$ cells extinguish Ly49 expression by 10 d after transfer. Minimally, the data argue that extinction of receptor expression is much rarer after transfer than initiation of receptor expression, since 19–27% of donor cells initiated expression of a Ly49 receptor after transfer, whereas only $\leqslant$2–7% of cells, if that, lost receptor expression after transfer. The extent to which Ly49 gene expression is stable in vivo over periods longer than 10 d is untested.

The stability of Ly49 receptor expression, after initiation, seems consistent with the demands of NK cells to maintain self-tolerance. If self–MHC class I–specific receptors were readily extinguished, NK cells would be constantly reverting to an autoaggressive state. However, a previous study with MHC-mosaic mice indicates that NK cell tolerance can be rapidly reversed when NK cells are cultured in the absence of a relevant MHC ligand (40). Our data suggest that this reversal in tolerance is unlikely to result from repression of Ly49 receptor expression, since we found that Ly49A$^+$ and Ly49G2$^+$ NK cells maintained receptor expression in vivo, even in the absence of a ligand (e.g., D^d). An alternative interpretation noted above is that during their development, some NK cells never attain expression of self-specific Ly49 receptors. These cells may be rendered nonresponsive to autologous cells by a mechanism that does not involve expression of self–MHC class I specific receptors, such as downregulation of receptors or signaling pathways required for NK cell activation. Reversal of this form of tolerance might occur when the MHC ligand is absent, and/or in the presence of IL-2.

The Inability of Transferred Ly49A$^-$ Cells to Initiate Expression of Ly49A. The specific failure of transferred Ly49A$^-$ NK cells to give rise to Ly49A$^+$ NK cells was unexpected. The failure of Ly49A to be expressed was not obviously related to its inability to recognize H-2^b molecules; Ly49G2 expression was initiated in the transfer experiments, and H-2^b mice lack a ligand for Ly49G2. Furthermore, expression of both Ly49A and Ly49G2 occurs during normal NK cell differentiation in H-2^b mice (Fig. 1), and also during NK cell differentiation after transfer of bone marrow stem cells to irradiated mice (Table 1). The data suggest that splenic NK1.1$^+$ Ly49A$^-$ NK cells have impaired capacity to initiate expression of the Ly49A receptor, while retaining the

capacity to express at least some of the other Ly49 receptors.

One possibility to explain the impaired capacity of transferred cells to initiate Ly49A expression is that there is a specific sequence in which Ly49 genes are expressed, and that Ly49A expression is normally initiated early in the process. Initiation of Ly49A receptor expression may be limited to the stage before NK progenitors express NK1.1, or may occur on $NK1.1^+$ cells before such cells migrate to the spleen. We found it difficult to obtain sufficient numbers of purified bone marrow $Ly49A^-NK1.1^+CD3^-$ cells to test the latter version of the hypothesis. Either of these scenarios could account for our results, since our protocol involved the transfer of splenic $NK1.1^+CD3^-$ cells that may have already passed the stage where cells are permissive for initiation of Ly49A expression. Alternatively, initiation of Ly49A expression, unlike the other receptors tested, may require an in vivo environment that is poorly replicated in the early phases of bone marrow reconstitution in irradiated mice or may be grossly delayed compared with initiation of Ly49C, G2 or I expression.

In conclusion, the results reported here demonstrate a successive acquisition of stable Ly49 receptor expression by developing NK cells. The results have significance both for the mechanisms that initiate receptor expression and the education process that results in a useful NK repertoire. The experimental system we employed may be useful for examining the impact of Ly49 receptor engagement with class I MHC ligands on subsequent Ly49 receptor gene expression in developing NK cells.

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