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Clonal Acquisition of Inhibitory Ly49 Receptors on Developing NK Cells Is Successively Restricted and Regulated by Stromal Class I MHC

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Summary

We report an in vitro stroma-dependent system for the clonal growth and differentiation of natural killer (NK) cells from lymphoid-restricted bone marrow progenitors or bone marrow NK1.1$^+$ cells. Strikingly, the potential to initiate expression of specific Ly49 receptors becomes increasingly restricted as NK cells develop. Moreover, when NK cells express a Ly49 receptor specific for stromal cell class I MHC, they are less likely to initiate expression of another Ly49 receptor in the clonal culture system. The results indicate multiple roles for stromal cells in NK cell development, in supporting clonal growth, in initiation of Ly49 receptor expression, and in formation of the NK cell receptor repertoire.

Introduction

Natural killer (NK) cells lyse a variety of target cells without prior immunization (Trinchieri, 1989). Self MHC class I molecules expressed by target cells can inhibit NK cell attack. Consequently, NK cells are prevented from killing normal self cells but can attack self cells that have extinguished expression of class I MHC molecules as a consequence of infection, mutation, or transformation (Ljunggren and Karre, 1990). In the mouse, receptors for class I include the CD94/NKG2 and Ly49 receptor families. At least nine closely related Ly49 receptors have been identified (Ly49A-I), which are encoded within the NK complex on mouse chromosome 6 (Yokoyama, 1995; Brown et al., 1997; McQueen et al., 1998). Ly49 receptors are expressed on overlapping subsets of NK cells such that a single NK cell may express several receptors (reviewed in Raulet et al., 1997; Kubota et al., 1999). Most of the Ly49 receptors have been shown to exhibit specificity for different class I alleles, with each binding one or more of the MHC class Ia molecules tested (Daniels et al., 1994; Takei et al., 1997; Hanke et al., 1999).

Evidence suggests that differentiating NK cells traverse a developmental stage in which expression of Ly49 receptors is initiated in a successive and cumulative fashion (Dorfman and Raulet, 1998). Highly purified splenic NK1.1$^+$CD3$^-$ cells, which did or did not express various Ly49 receptors, were transferred into sublethally irradiated mice. After 10 days, a fraction of the NK1.1$^+$CD3$^-$Ly49A$^-$C$^-$G2$^-$I$^-$ donor cells had initiated expression of Ly49G2 or Ly49C/I. Initiation of Ly49G2 or Ly49C/I expression was also observed with transferred Ly49A$^+$C$^-$G2$^-$I$^-$ NK cells. The transferred Ly49A$^+$ cells maintained expression of Ly49A, suggesting that expression, once it occurs, is quite stable. It was concluded that NK cells expressing one Ly49 receptor could go on to express others in a cumulative fashion.

The finding that Ly49 receptors are expressed in a cumulative and successive fashion by developing NK cells fulfills a prediction of the "sequential expression model" of NK cell development. This model proposed that the successive accumulation of different Ly49 receptors by developing NK cells is terminated once the cell expresses receptors with sufficient avidity for self MHC class I molecules (Held and Raulet, 1997; Raulet et al., 1997). Independent evidence shows that the repertoire of inhibitory receptors expressed by NK cells is influenced by the MHC class I alleles expressed in an animal. Specifically, NK cells expressing multiple Ly49 receptors specific for an MHC ligand are less frequent in mice that contain the ligand than in mice that do not. For example, Ly49A$^+$G2$^+$ double-positive NK cells were 2- to 4-fold less abundant in H-2^d mice, which express strong ligands for these receptors, than in H-2^b or $\beta_2m^{-/-}$ mice, which do not (Held and Raulet, 1997; Hoglund et al., 1997; Raulet et al., 1997). Moreover, in mice expressing a transgenically encoded Ly49A receptor expressed in all NK cells, the frequency of NK cells expressing the endogenous Ly49G2 receptor was reduced by 2- to 3-fold in H-2^d mice compared to H-2^b and $\beta_2m^{-/-}$ mice (Held and Raulet, 1997; Raulet et al., 1997). We proposed that engagement of Ly49A by D^d inhibited developing NK cells from initiating expression of other receptors. By limiting the number of self MHC–specific receptors that NK cells express, such a mechanism would endow NK cells with a greater capacity to discriminate body cells that extinguish expression of some, but not all, self class I MHC proteins (Raulet et al., 1997).

The question arises whether the sequence in which Ly49 receptors are expressed by NK cells is random or, alternatively, is ordered in some way. An ordered pattern of receptor expression could impact the final repertoire considerably. The possibility that the process is at least partly ordered was suggested by the finding that while transferred NK1.1$^+$Ly49G2/C/I$^-$ splenocytes clearly initiated expression of Ly49G2 and/or Ly49C/I, transferred NK1.1$^+$Ly49A$^-$ splenocytes never initiated expression of Ly49A (Dorfman and Raulet, 1998). As one possibility to explain this discrepancy, we proposed that commitment to initiate Ly49A expression is limited to an early stage of NK cell development, before expression of NK1.1 or before NK cells migrate from the bone marrow to the spleen. In this model, initiation of Ly49G2 or Ly49C/I expression occurs at a later stage, after NK1.1 is expressed.

The signals that induce Ly49 expression in developing

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NK cells are poorly characterized. Complete NK cell development in mice requires interactions between committed NK progenitors and the bone marrow microenvironment (Hackett et al., 1986; reviewed in Raulet, 1999). Among the signals produced by bone marrow stromal cells, IL-15 and its receptor were recently shown to play an important role in NK cell maturation both in the human and murine systems (Mrózek et al., 1996; Jaleco et al., 1997; Williams et al., 1997; Lodolce et al., 1998). Indeed, IL-15 was shown to induce human NK cells to express CD94/NKG2 receptors (Mingari et al., 1997). However, while development of murine NK cells from progenitor cells in vitro was stimulated by IL-15 in concert with other cytokines, initiation of Ly49 expression did not occur (Williams et al., 1997). Nor did IL-15 efficiently induce the expression of the human KIR family of inhibitory receptors on human NK cells (Mingari et al., 1997). These data suggested that additional stromal signals or cytokines are necessary to induce Ly49 receptor expression by developing NK cells.

To investigate the process of receptor expression during development of murine NK cells, we have developed a system in which murine NK cells or earlier precursor cells can be efficiently cloned. This was accomplished with a modification of the NK long-term bone marrow culture system, in which precursors are cultured in the presence of cytokines and a bone marrow stromal layer (van den Brink et al., 1990; Delfino et al., 1996). Using this system, we demonstrate that the induction of Ly49 receptors on developing NK cells in vitro requires signals from bone marrow stromal cells and that Ly49 receptor expression by developing NK cells occurs successively and cumulatively. The data suggest that NK cell development is characterized by successive restrictions in the capacity to initiate expression of different Ly49 receptors. Importantly, we also show that stromal class I MHC expression influences the cumulative acquisition of Ly49 receptors during NK cell differentiation.

Results

A Short-Term Clonogenic Assay for Analysis of NK Cell Development

To examine the initiation of expression of Ly49 receptors in developing NK cells, we devised an in vitro system in which bone marrow precursor cells, lacking expression of various Ly49 receptors, could be cloned in tissue culture. Two bone marrow populations were compared: a population with an NK phenotype ($NK1.1^+DX5^+CD3^-CD19^-$), which had been depleted of cells expressing Ly49A, Ly49C, Ly49F, Ly49G2, and Ly49I (hereafter called $NK1.1^+DX5^+Ly49^-$ cells for convenience), and a population defined as a less committed, lymphoid-restricted progenitor cell ($c\text{-}kit^+$ $Sca\text{-}2^+$ Lineage marker$^-$ [Lin^-]) (Antica et al., 1994; Williams et al., 1997). The lineage markers included CD3, CD19, Gr-1, TER-119, Mac-1, and NK1.1. Typical sorting parameters of both populations are shown in Figures 1A and 1B. Post-sort analyses indicated that the sorted populations were greater than 89%–93% pure, with most of the contaminants being non-NK cells (Figures 1A and 1B).

The sorted populations were cultured at limiting dilution in wells containing irradiated stromal cells and cytokines (c-kit ligand, IL-7, flt-3 ligand, and IL-15). The

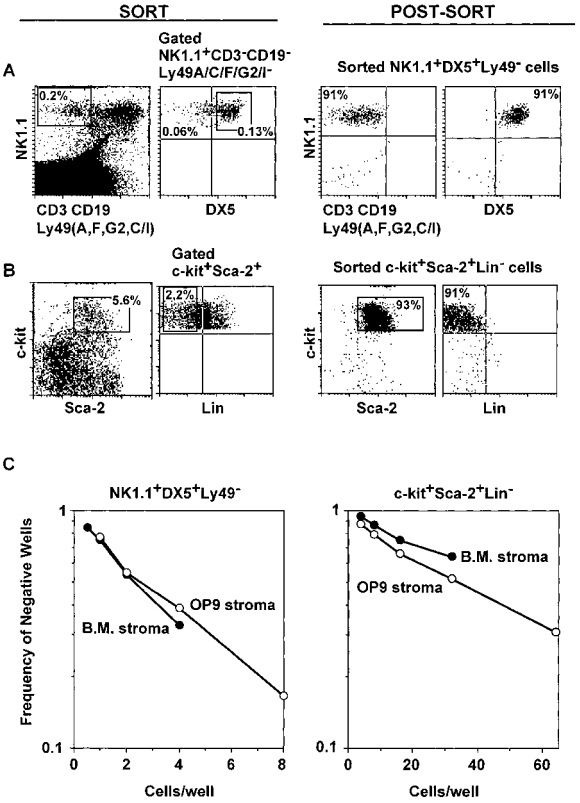

Figure 1. Clonal Growth of Sorted Bone Marrow–Derived Cell Populations Employed for Analysis of NK Cell Development

Percoll gradient purified bone marrow cells were further purified by cell sorting for the indicated markers and cultured at limiting dilution in the presence of stromal cells and cytokines.

(A) Gates for three-color sorting of $NK1.1^+CD3^-CD19^-Ly49A/C/F/G2/I^-$ cells that express DX5 are shown (left panels). Reanalysis of the sorted $NK1.1^+DX5^+CD3^-CD19^-Ly49A/C/F/G2/I^-$ population revealed a 91% pure population (right panels).

(B) Gates for three-color sorting of $c\text{-}kit^+Sca\text{-}2^+$ cells that are also negative for lineage markers, including NK1.1, are shown (left panels). Reanalysis of the sorted $c\text{-}kit^+Sca\text{-}2^+Lin^-$ population revealed a 91%–93% pure population (right panels).

(C) Clonal growth of sorted $NK1.1^+DX5^+Ly49A/C/F/G2/I^-$ and $c\text{-}kit^+Sca\text{-}2^+Lin^-$ precursor cells. Both populations were cultured at limiting dilution with either irradiated B6 bone marrow stromal cells (closed circles) or irradiated OP9 stromal cells (open circles) in the presence of c-kit ligand, IL-7, flt-3 ligand, and IL-15. The number of growing wells containing $NK1.1^+$ cells was determined. The frequency of wells that did not contain growing $NK1.1^+$ cells at day 10 is plotted against the number of input cells. Results are representative of three independent experiments each.

stroma were established by culturing either syngeneic (B6) or MHC-different (B10.D2; B6-$\beta_2m^{-/-}$) bone marrow cells or the OP9 stromal cell line (Kodama et al., 1994) in the wells. These culture conditions are similar to those used previously to support the proliferation of NK precursor cells in "NK-long-term bone marrow cultures" (NK-LTBMC) (Delfino et al., 1996). Strikingly, the $NK1.1^+DX5^+Ly49^-$ cells yielded growing clones at a surprisingly high efficiency, approximately one in three (Figure 1C). The $c\text{-}kit^+Sca\text{-}2^+Lin^-$ population yielded clones with a lower efficiency, approximately 1 in 55. In both cases, the clones grew for up to 4 weeks, yielding colonies of up to 1×10^5 cells, but eventually ceased growing, even if transplanted onto wells containing new

Table 1. Expression of Ly49 Receptors by Colonies Derived from NK1.1$^+$DX5$^+$Ly49-Depleted Bone Marrow Cells under Limiting Dilution Conditions

Input Cells	Cells per Well	Growing Wells[a]	Number of Wells Tested[b]	Ly5.1/5.2 Mixed Wells[c]	Fraction of Ly49$^+$ Wells[d]			
					A	F	G2	C/I
NK1.1$^+$DX5$^+$Ly49$^-$	4	32/48	26	5/26	0/26	0/26	7/26	11/26
(Ly5.1/5.2 mix);	2	22/48	13	2/13	0/13	0/13	3/13	4/13
Bone marrow stroma	1	48/192	32	2/32	0/32	1/32	6/32	19/32
	0.5	29/192	21	0/21	0/21	0/21	2/21	10/21
Total			92		0/92 (0%)	1/92 (1%)	18/92 (20%)	44/92 (48%)
NK1.1$^+$DX5$^+$Ly49$^-$;	2	46/96	23	NT[e]	0/23	0/23	2/23	12/23
OP9 stroma	1	28/96	15	NT	0/15	0/15	1/15	7/15
Total			38		0/38 (0%)	0/38 (0%)	3/38 (8%)	19/38 (50%)

The indicated input cells were cultured for at least 10 days with cytokines and the indicated stroma and resulting colonies examined by flow cytometry for expression of NK1.1, Ly5.1/5.2 and Ly49A, Ly49F, Ly49G2, and Ly49C/I.

[a] Fraction of wells with visible colonies.

[b] The number of wells tested by flow cytometry.

[c] The fraction of wells containing both Ly5.1$^+$ and Ly5.2$^+$ cells.

[d] The fraction of wells containing $\geq$5% of cells expressing the indicated Ly49 receptor.

[e] Not tested.

stroma and cytokines (data not shown). Similar results were obtained in two other experiments. The cloning efficiency was similar whether bone marrow cells or OP9 cells were employed to generate the stroma (Figure 1C). In comparable cultures lacking stroma, no clones were generated from either progenitor cell population using relatively high numbers of input cells (4 NK1.1$^+$DX5$^+$Ly49$^-$ or 32 c-kit$^+$Sca-2$^+$Lin$^-$ cells/well; data not shown). Preliminary functional tests showed that at least some of the clones exhibit cytotoxicity specific for the NK-sensitive YAC-1 target cells (data not shown). The small burst size of NK clones differentiating under these conditions ($\leq 1 \times 10^5$ cells) precluded a systematic analysis of class I inhibition of the clones.

A variety of evidence indicated that the colonies obtained with both types of precursor cells were clonal in origin. First, both precursor cell populations yielded a linear relationship between the input cell number and the log of the fraction of nongrowing wells, consistent with clonal growth. As a further test of clonality, we initiated cultures with limiting numbers of cells from a 1:1 mixture of precursor cells obtained from B6-Ly5.1 and B6-Ly5.2 mice. The experiment was carried out with either NK1.1$^+$DX5$^+$Ly49$^-$ (Table 1) or c-kit$^+$Sca-2$^+$Lin$^-$ (Table 2) cells as precursors. In both cases, essentially all of the growing wells obtained at low cell inputs contained cells expressing either Ly5.1 or Ly5.2 but not both, as expected for clonal conditions (Tables 1 and 2).

Individual clones were tested for expression of various markers. All of the clones derived from the NK1.1$^+$DX5$^+$Ly49$^-$ population homogeneously expressed NK1.1 (Figure 2A) and were CD3$^-$ (data not shown), identifying them as NK cells. In contrast, the c-kit$^+$Sca-2$^+$Lin$^-$ population yielded both NK lineage and CD19$^+$ (B lineage) cells (Table 2, experiment 2). An average of 76% of the growing wells contained NK1.1$^+$CD3$^-$ cells. About half of these colonies also contained CD19$^+$ cells, which were shown to represent a separate population within the colony by two-color staining (data not shown). The remaining 24% of colonies contained only CD19$^+$ cells. Data indicated that most, if not all, of the mixed colonies were derived from single progenitor cells. First, the mixed NK1.1$^+$/CD19$^+$ colonies were as frequent at the lowest input cell number (4/well) as at the highest (Table 2). Second, when the input cells consisted of a 1:1 mix of Ly5.1$^+$ and Ly5.2$^+$ cells, none of the mixed NK1.1$^+$/CD19$^+$ colonies obtained at low input cell numbers were mixed with respect to Ly5 expression (Table 2, experiment 2). The results demonstrate that under these culture conditions, a single uncommitted c-kit$^+$Sca-2$^+$Lin$^-$ progenitor cell can divide, yielding daughter cells that subsequently differentiate into either NK lineage or B lineage cells.

Initiation of Ly49 Receptor Expression by Developing NK Cells in the Clonogenic Culture System

Expression of Ly49A, F, G2, and C/I by the clones was determined. Significantly, an appreciable fraction of clones derived from NK1.1$^+$DX5$^+$Ly49$^-$ precursors contained cells expressing Ly49G2 or Ly49C/I (Table 1). Staining profiles for representative clones obtained with a low cell input number (0.5–1 cell/well) are shown in Figure 2A. Expression of Ly49G2 and Ly49C/I was almost always heterogeneous, with some of the cells in the well positive and some negative for a given receptor. Some of the clones contained both Ly49C/I$^+$ and Ly49G2$^+$ cells, mostly on separate subpopulations, though coexpression may occur on a small subset in some of the clones (e.g., B12 and B1). Therefore, while the colonies were clonal in origin, they were not homogeneous in Ly49 expression.

Strikingly, while many NK1.1$^+$DX5$^+$Ly49$^-$ precursor cells initiated expression of Ly49G2 and/or Ly49C/I expression in culture, none initiated expression of Ly49A and only one initiated expression of Ly49F. Thus, NK1.1$^+$DX5$^+$Ly49$^-$ precursor cells differentiating in vitro

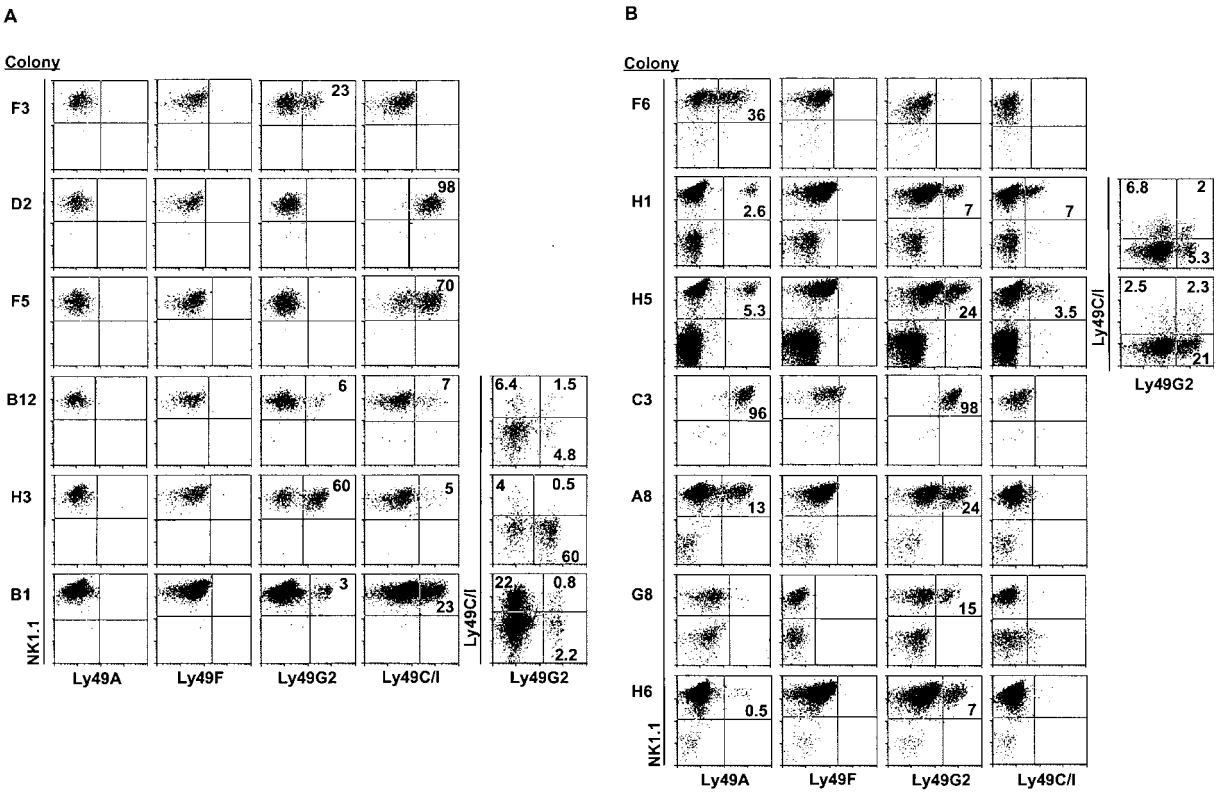

Figure 2. Ly49 Expression by Clones Derived In Vitro from NK1.1$^+$DX5$^+$Ly49A/C/F/G2/I$^-$ or c-kit$^+$Sca-2$^+$Lin$^-$ Precursor Cells

(A) Representative clones derived from NK1.1$^+$DX5$^+$Ly49A/C/F/G2/I$^-$ precursor cells, derived at 1 input cell per well (clones F3, D2, and B1) or 0.5 cell per well (clones F5, B12, H3) are shown. Percentages are indicated in the quadrants.

(B) Representative clones derived from c-kit$^+$Sca-2$^+$Lin$^-$ precursor cells, derived at 32 input cells/well (clones F6, H1, H5, H6), 16 cells/well (clones C3, G8), or 8 cells/well (clone A8) are shown. For H1 and H5 clones, double staining with anti-Ly49G2 and anti-Ly49C/I is shown on gated NK1.1$^+$ cells. Some clones contained a CD19$^+$ population. Percentages correspond to percent of NK1.1$^+$ cells in the colonies.

exhibit potential to initiate expression of Ly49G2 and Ly49C/I, but little or no potential to express Ly49A or Ly49F.

The possibility that the Ly49$^+$ clones were derived from Ly49$^+$ contaminants in the input cell population is highly unlikely. Since Ly49 expression, once initiated, appears to be quite stable (Dorfman and Raulet, 1998; see below), colonies derived from Ly49$^+$ contaminants would not be expected to be heterogeneous for Ly49 expression, in contrast with the data (Figure 2A). Furthermore, the NK1.1$^+$ input cells were essentially devoid of Ly49A/C/F/G2/I$^+$ cells as assessed by post-sort analysis. In addition, semiquantitative RT-PCR analysis indicated that the extent of contamination of the input cells by cells expressing one or the other Ly49 receptor was always less than 5%, and often less than 0.5% (data not shown). Even if the cloning efficiency of Ly49$^+$ cells was greater than that of Ly49$^-$ cells, the high overall cloning efficiency we observed (approximately 30%) is incompatible with the possibility that the Ly49$^+$ colonies arose from contaminants. Moreover, direct comparisons indicated that the cloning efficiency of purified Ly49$^+$ cells is no higher than that of Ly49$^-$ cells (see below; Table 3). As a further indication of this point, when we employed 1:10 cell mixtures of NK1.1$^+$Ly49$^-$ cells from Ly5.1 mice and NK1.1$^+$Ly49$^+$ (Ly49G2$^+$ or Ly49C/I$^+$) cells from Ly5.2 mice in a cloning experiment, Ly5.2$^+$ clones represented 5%–20% of the total, as would be predicted if the two populations exhibited similar cloning efficiency (data not shown). Taken together, the data demonstrate that clonogenic NK1.1$^+$DX5$^+$Ly49$^-$ precursor cells give rise to colonies containing Ly49C/I$^+$ or Ly49G2$^+$ NK cells in vitro.

Developing c-kit$^+$Sca-2$^+$Lin$^-$ Progenitor Cells Can Initiate Ly49A Expression In Vitro

The finding that NK1.1$^+$DX5$^+$Ly49$^-$ precursor cells give rise to clones expressing Ly49G2 or Ly49C, but not Ly49A, is consistent with the results of our earlier study, which showed that splenic NK1.1$^+$Ly49A/C/G2/I$^-$ cells, after transfer in vivo to irradiated recipient mice, gave rise to Ly49G2$^+$ and Ly49C/I$^+$ NK cells but not to Ly49A$^+$ cells. One possible explanation for these data is that commitment to expression of Ly49A occurs at an early stage in development, perhaps even before expression of NK1.1. This model predicts that less restricted progenitor cells, at a stage prior to NK1.1 expression, should give rise to Ly49A$^+$ clones in vitro. The analysis of NK1.1$^+$ clones derived from c-kit$^+$Sca-2$^+$Lin$^-$ progenitors verified this prediction. Among the colonies obtained with these cells, we detected NK cells expressing each of the four Ly49 receptors tested, including Ly49A

Table 2. Expression of Ly49 Receptors and Markers by Colonies Derived from c-kit$^+$Sca-2$^+$Lin$^-$ Precursor Cells at Limiting Dilution

Input Cells	Cells per Well	Growing Wells[a]	CD19$^+$ Wells[b]	NK1.1$^+$ Wells[c]	NK1.1$^+$/CD19$^+$ Wells[d]	Ly5.1/5.2 Mixed Wells[e]	Fraction of Ly49$^+$ Wells[f] A	F	G2	C/I
c-kit$^+$Sca-2$^+$Lin$^-$	32	42/96	NT[g]	34/42	NT	NT	5/14	0/14	9/14	3/14
(NK1.1$^-$);	16	35/96	NT	24/35	NT	NT	0/7	0/7	3/7	2/7
Bone marrow	8	16/96	NT	12/16	NT	NT	1/2	0/2	1/2	1/2
Stroma	4	7/96	NT	4/7	NT	NT	1/1	0/1	1/1	1/1
Totals				74/100 (74%)			7/24 (29%)	0/24 (0%)	14/24 (58%)	7/24 (29%)
c-kit$^+$Sca-2$^+$Lin$^-$	64	83/96	58/83	66/83	41/83	3/15	2/13	0/13	5/13	5/13
(NK1.1$^-$Ly49$^-$)	32	57/96	30/57	46/57	19/57	3/22	7/20	2/20	13/20	7/20
(Ly5.1/5.2 mix);	16	49/96	31/49	32/49	14/49	1/22	5/20	1/20	11/20	6/20
OP9 stroma	8	27/96	18/27	20/27	11/27	1/7	1/4	0/4	2/4	1/4
	4	15/96	9/15	12/15	6/15	0/7	1/7	0/7	4/7	1/7
Totals			146/231 (63%)	176/231 (76%)	91/231 (39%)		16/64 (25%)	3/64 (4%)	35/64 (54%)	20/64 (31%)

The indicated input cells were cultured for at least 10 days with the indicated stroma and resulting colonies examined by flow cytometry for expression of CD19, NK1.1, Ly5.1/5.2, Ly49A, Ly49F, Ly49G2, and Ly49C/I.
[a] Wells with visible colonies/wells plated.
[b] Wells containing CD19$^+$ cells/growing wells.
[c] Wells containing NK1.1$^+$ cells/growing wells.
[d] Wells containing both NK1.1$^+$ and CD19$^+$ cells/growing wells.
[e] Wells containing both Ly5.1$^+$ and Ly5.2$^+$ cells/wells tested.
[f] Wells containing ≥5% of cells expressing the indicated Ly49 receptor/wells tested.
[g] Not tested.

and Ly49F (Table 2; Figure 2B). Comparable Ly49 expression was observed in colonies containing only NK cells and colonies containing both NK cells and CD19$^+$ cells, indicating that the culture conditions support initiation of Ly49 expression on NK cells as they differentiate from relatively undifferentiated progenitor cells. Although c-kit$^+$Sca-2$^+$Lin$^-$ cells do not express Ly49 receptors (data not shown), antibodies against Ly49A/C/F/G2/I were included in the cocktail of lineage-specific antibodies in some sorting experiments (e.g., Table 2, experiment 2). The frequencies of clones expressing each Ly49 receptor was 25%–29% for Ly49A, 0%–4% for Ly49F, 54%–58% for Ly49G2, and 29%–31% for Ly49C/I. As seen with the NK1.1$^+$ precursor cells, most of the clones were heterogeneous in Ly49 expression, with only a fraction of the NK1.1$^+$ cells in each well expressing any given receptor (Figure 2B). In addition, we also observed clones in which two or three Ly49 receptors were expressed by cells in the population. In most such cases, expression of different Ly49 receptors within a colony was predominantly nonoverlapping, though some overlap was observed (e.g., clones H1 and H5). In rare cases, extensive overlap was observed, as in clone C3 where nearly all the cells coexpressed Ly49A and Ly49G2 (Figure 2B).

Successive and Restricted Potential for Ly49 Expression by Developing NK Cells

We next investigated whether cells that express one of the Ly49 receptors can initiate expression of other

Table 3. Initiation of Additional Ly49 Expression by NK1.1$^+$ Cells Expressing Ly49A and Ly49G2 Cultured at Limiting Dilution

Input Cells		Cells per Well	Growing Wells[a]	Number of Wells tested[b]	Fraction of Ly49$^+$ Wells[c] A	F	G2	C/I
NK1.1$^+$Ly49A$^+$ (Ly49F/G2/C/I)$^-$	Exp. #1	1	33/96	16	15/16 (93.7%)	8/16 (50%)	2/16 (12%)	8/16 (50%)
	Exp. #2	1	70/288	28	24/28 (85%)	15/28 (53%)	5/28 (17%)	15/28 (53%)
NK1.1$^+$Ly49G2$^+$ (Ly49A/F/C/I)$^-$	Exp. #1	1	29/96	14	0/14 (0%)	0/14 (0%)	14/14 (100%)	3/14 (21%)
	Exp. #2	1	90/288	78	0/78 (0%)	0/78 (0%)	78/78 (100%)	22/78 (28%)

The indicated input cells were cultured for at least 10 days with OP9 stromal cells and cytokines and the resulting colonies examined by flow cytometry for expression of NK1.1 and Ly49A, Ly49F, Ly49G2, and Ly49C/I.
[a] Wells with visible colonies.
[b] The number of wells tested by flow cytometry.
[c] The number of wells containing ≥5% of cells expressing the indicated Ly49 receptor.

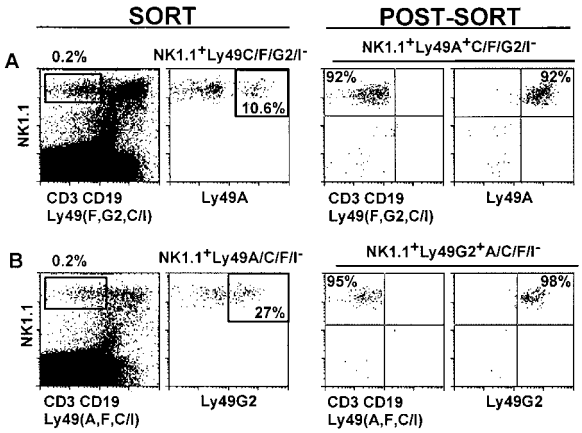

Figure 3. Sorted Ly49$^+$ Bone Marrow Cell Populations Employed for Analysis of NK Cell Development

(A) CD3$^-$CD19$^-$NK1.1$^+$ B6-derived bone marrow cells were sorted for the expression of Ly49A, but not Ly49F, G2, C/I. Post-sort analysis indicated a purity of 92%.

(B) CD3$^-$CD19$^-$NK1.1$^+$ B6-derived bone marrow cells were sorted for the expression of Ly49G2, but not Ly49A, F, C/I. Post-sort analysis indicated a purity of 95%–98%.

receptors in the clonogenic assay. NK1.1$^+$CD3$^-$CD19$^-$ cells that express Ly49A, but not Ly49C/F/G2/I, were sorted from the bone marrow to high purity (Figure 3A) and subsequently cloned at 1 cell/well. The cloning efficiency averaged 29% (Table 3), similar to that of NK1.1$^+$DX5$^+$Ly49$^-$ NK cells. When tested after at least 10 days of culture, an average of 90% of the clones homogeneously expressed Ly49A, consistent with the purity of the input cells (Table 3; Figure 3A; data not shown). Therefore, Ly49A expression, once initiated, is quite stable. Significantly, a fraction of the clones had initiated expression of each of the other Ly49 receptors tested, including Ly49F (52%), Ly49G2 (16%), and Ly49C/I (52%) (Table 3). Thus, Ly49A$^+$C/F/G2/I$^-$ NK cells from the bone marrow exhibit the potential to subsequently initiate expression of Ly49F, Ly49G2, and Ly49C/I in vitro.

To extend the analysis, Ly49G2$^+$A/C/F/I$^-$ NK cells were sorted to high purity (Figure 3B) and cloned. Again, the cloning efficiency was high (31%; Table 3). All the clones homogeneously expressed Ly49G2. Strikingly, 27% of the colonies contained Ly49C/I$^+$ cells, typically on a fraction of the cells. However, none of the clones expressed Ly49A or Ly49F. These data suggest that Ly49 receptors are expressed successively but that the potential to initiate expression of certain Ly49 receptors is lost in an orderly fashion as development proceeds.

Influence of Stromal Class I MHC on Cumulative Ly49 Expression Potential

We next tested the effect of stromal class I MHC expression on the successive expression of Ly49 receptors in the clonogenic assay. We used precursors from Ly49A transgenic mice and analyzed subsequent expression of the Ly49G2 receptor during differentiation in vitro. Both of these receptors are reactive with H-2^d ligands. The Ly49A transgene is driven by a class I promoter and Ig enhancer and is expressed by all NK cells in vivo. Uncommitted c-kit$^+$Sca-2$^+$Lin$^-$ precursors were used because they are immature cells and exhibit the potential to generate a diverse repertoire. Accordingly, Ly49A^{tg}c-kit$^+$Sca-2$^+$Lin$^-$ precursors were sorted and cloned onto irradiated bone marrow stromal cells derived from B10.D2 (H-2^d), B6 (H-2^b), and class I–deficient ($\beta_2m^{-/-}$) mice. In addition, to reveal any effects of class I MHC expression by the growing NK cells themselves, MHC-congenic precursors were used that either did or did not express H-2D^d, a known high-affinity ligand for the transgenic Ly49A receptor (Ly49A^{tg}B10.D2 or Ly49A$^{tg}\beta_2m^{-/-}$ precursors, respectively). Precursor cells were seeded at 30 cells/well. The resulting cloning efficiencies were similar for all conditions.

Each clone was examined for the percentage of NK cells that expressed Ly49G2 (Figures 4A and 4B). In the case of Ly49A$^{tg}\beta_2m^{-/-}$ precursors, the percentages varied considerably among the clones from each group, but the average percentage of Ly49G2$^+$ NK cells was significantly higher on stromal cells that lacked MHC expression ($\beta_2m^{-/-}$ stroma; Figure 4A, 34% $\pm$ 5%) than on stroma that expressed a strong MHC ligand for the transgenic receptor (B10.D2 stroma; Figure 4A, 8% $\pm$ 2%). Interestingly, the percentage of Ly49G2$^+$ NK cells on H-2^b stromal cells (17% $\pm$ 4%), a haplotype not known to interact strongly with either Ly49 receptor (Daniels et al., 1994; Takei et al., 1997; Hanke et al., 1999), was significantly lower than that observed with $\beta_2m^{-/-}$ stroma. Notably, the effects of the stromal cells were similar if the precursor cells themselves expressed endogenous H-2D^d ligand for the transgenic Ly49A receptor (Ly49A^{tg}B10.D2 precursors; Figure 4B). In this case, due primarily to the analysis of fewer clones, only the results comparing $\beta_2m^{-/-}$ and H-2^d stromal cells scored as a statistically significant difference. Nevertheless, the similarity of the trend demonstrates that the MHC haplotype of the bone marrow stromal cells plays a predominant role in determining Ly49 receptor expression during NK cell differentiation in vitro.

Similar trends were observed when we plotted the frequency of clones containing few or no ($\leq$5%) Ly49G2$^+$ NK cells (threshold indicated by dotted line in Figures 4A and 4B). None of the clones that developed on $\beta_2m^{-/-}$ stroma met this criterion (Figure 4C), suggesting that the daughters of progenitor cells exhibit a very high potential to express Ly49G2 as they differentiate in the absence of MHC ligands. In contrast, 70% of clones that developed on H-2^d stromal cells contained few or no Ly49G2$^+$ NK cells (Figure 4C). Thus, expression of a strong MHC ligand for the Ly49A transgene (H-2D^d) resulted in a reduced likelihood of Ly49G2 expression. Again, an intermediate result was obtained when the stromal cells expressed H-2^b (Figure 4C; 23%), and little or no effect could be attributed to MHC expression by the cloned NK cells themselves (compare Figure 4C versus Figure 4D). Thus, in the clonogenic assay, the MHC haplotype of the stromal cells critically influences the cumulative potential for Ly49 receptor expression during NK cell differentiation.

We next examined whether the levels of Ly49 surface expression were also modulated by MHC ligand (Figures 4E and 4F). When Ly49A$^{tg}\beta_2m^{-/-}$ precursors were used, Ly49G2 mean fluorescence intensity (MFI) values were significantly higher on $\beta_2m^{-/-}$ stroma (Figure 4E; 76 $\pm$

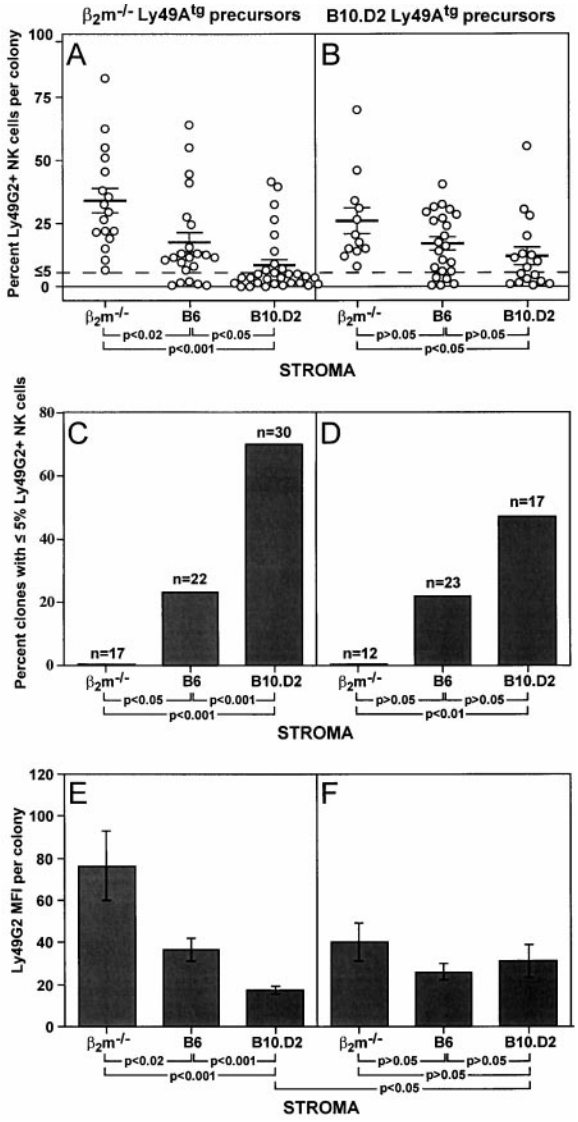

Figure 4. Influence of Stromal Class I MHC on the Cumulative Expression of Ly49G2 on Clonally Differentiating Ly49A^{tg} c-kit$^+$Sca-2$^+$Lin$^-$ Precursors

(A and B) The percentage of NK cells expressing Ly49G2 in each colony from Ly49A^{tg} multipotent precursors that differentiated on class I MHC-different stromal cells are indicated. Bone marrow precursors (Ly49A^{tg}c-kit$^+$Sca-2$^+$Lin$^-$) were sorted and cloned onto irradiated bone marrow stromal cells derived from B6 (H-2^b), B10.D2 (H-2^d), and $\beta_2m^{-/-}$ mice. Colonies were analyzed for Ly49G2 expression (on NK1.1$^+$ cells) by flow cytometry. Each circle represents one colony; the mean percentages (thick bars) ± SEM are shown. Statistical significance values based on the two-tailed Student's t test are indicated. The dotted line represents the ≤5% threshold used for plotting (C) and (D).

(C and D) The frequency of Ly49A^{tg} clones that generated ≤5% Ly49G2$^+$ NK cells per colony (see [A] and [B], dotted line) are indicated for clones derived on each type of stroma. Statistical significance values based on the two-tailed chi-square (χ^2) test are indicated.

(E and F) The levels of Ly49G2 surface expression are indicated for clones derived on each type of stroma. Colonies were analyzed for the mean fluorescence intensities (MFI ± SEM) of Ly49G2 expression (on NK1.1$^+$Ly49G2$^+$ cells). Statistical significance values based on the two-tailed Student's t test are indicated.

17) than on B6 stroma (37 ± 5) or B10.D2 stroma (17 ± 2). In contrast, no significant differences in MFI were observed when Ly49A^{tg}B10.D2 precursors were used (compare Figure 4E versus Figure 4F). Whether this reflects the lower number of clones analyzed or an influence of the endogenous H-2D^d ligand is unclear. Nonetheless, the trend in Figure 4E demonstrates that the MHC haplotype of the bone marrow stromal cells also plays an important role in determining the levels of Ly49 receptor expression. Interestingly, the same trend was seen with respect to surface expression levels of both the endogenous Ly49G2 receptor (Figures 4E and 4F) and the transgenic Ly49A receptor (data not shown).

Discussion

Proliferation and Ly49 Expression under Defined Clonal Conditions

The results demonstrate that a mixture of cytokines and irradiated stromal cells supports clonal growth and differentiation of NK cells, starting with either NK1.1$^+$ DX5$^+$Ly49$^-$ bone marrow cells or c-kit$^+$Sca-2$^+$Lin$^-$ bone marrow cells. The NK1.1$^+$ precursor cells yielded only NK cell clones, while the c-kit$^+$Sca-2$^+$Lin$^-$ population yielded clonal populations containing either NK cells, B lineage cells, or both. The c-kit$^+$Sca-2$^+$Lin$^-$ population has been previously shown to exhibit potential for NK and B lineage development in vitro (Williams et al., 1997) but is devoid of pluripotent stem cells (Antica et al., 1994). The absence of T lineage differentiation by these cells in the in vitro system is likely to reflect the absence of thymic tissue to support T cell development.

Significantly, our results indicate that stromal cells participate in supporting the proliferation or survival of developing NK cells. Efficient clonal growth in our system required the presence of stromal cells. In a previous study, the same cytokines we employed, in the absence of stromal cells, were able to support the proliferation of developing NK cells cultured at high cell densities (Williams et al., 1997). It is possible that the high cell density cultures contain contaminating stromal cells or that, at high cell density, NK cells can provide themselves with signals necessary for sustained cell growth.

Importantly, the clonogenic culture system supported the initiation of expression of various Ly49 receptors on developing NK cell populations. Ly49 receptor expression occurred using either NK1.1$^+$DX5$^+$Ly49$^-$ bone marrow cells or c-kit$^+$Sca-2$^+$Lin$^-$ cells as precursor cells. It is striking that the same cytokines we used failed to support the initiation of Ly49 expression in high-density NK cell cultures lacking stromal cells (Williams et al., 1997). However, more recent studies in the same system demonstrated Ly49 receptor expression in bulk cultures in the presence of OP9 stromal cells (Williams et al., 1999). In our studies, both primary bone marrow–derived stromal cells and the OP9 stromal cell line supported the initiation of Ly49 receptor expression, and the spectrum of Ly49 receptor expression observed was similar with both types of stromal cells. Together, the data imply that stromal cells provide the signal(s) necessary for both initiation of Ly49 expression and efficient clonal growth.

Interestingly, if a given Ly49 receptor was not detected in a colony on the first day tested, typically day

10 of culture, it was also not observed later, even at day 30 of the culture (data not shown). These findings suggest that initiation of Ly49 expression probably does not occur continuously throughout the culture period, but may be restricted to an early phase. Whether this is a limitation of the in vitro system or reflects a true limitation in the progenitor cells is uncertain.

Developmental Restrictions in the Initiation of Ly49 Expression

Two aspects of our current data fit with the previously proposed "sequential expression model" of NK cell development, which proposed that NK cells sequentially accumulate Ly49 receptors until receptors specific for self MHC class I molecules are expressed, at which time the accumulation of new receptors is inhibited (albeit incompletely) (Held and Raulet, 1997; Raulet et al., 1997). First, essentially all clones derived from Ly49A$^+$ or Ly49G2$^+$ NK cells stably maintained expression of the corresponding receptor, consistent with previous in vivo data (Dorfman and Raulet, 1998). Second, we directly demonstrated that cells expressing Ly49A or Ly49G2 can subsequently initiate expression of a second receptor under clonal conditions.

An exciting aspect of our results is the finding that input progenitor populations vary considerably in their potential to initiate expression of different Ly49 receptors. For example, the NK1.1$^+$DX5$^+$Ly49$^-$ precursor population exhibited potential to initiate expression of Ly49G2 and Ly49C/I receptors, but little or no potential to initiate expression of Ly49A or Ly49F. Two observations indicate that the limited potential of these cells to express different Ly49 receptors is not an artifactual limitation of the culture system. First, the in vitro culture system supported the initiation of expression of all Ly49 receptors tested when c-kit$^+$Sca-2$^+$Lin$^-$ progenitor cells were employed. Second, the in vitro results with bone marrow NK1.1$^+$CD3$^-$Ly49$^-$ precursor cells mirror our previous in vivo findings, in which splenic NK1.1$^+$Ly49$^-$ precursor cells, when transferred to sublethally irradiated mice, were capable of initiating expression of Ly49G2 and Ly49C/I but were incapable of initiating expression of Ly49A (Ly49F was not tested) (Dorfman and Raulet, 1998). Indeed, the present data with bone marrow populations argue that the earlier in vivo result did not reflect a limitation of splenic NK cells as compared to bone marrow NK cells, a possibility we had previously considered (Dorfman and Raulet, 1998).

The simplest interpretation of the different results with NK1.1$^+$DX5$^+$Ly49$^-$ and c-kit$^+$Sca-2$^+$Lin$^-$ precursor populations is that the potential to express different Ly49 receptors changes with the developmental stage. In this model, the potential to initiate Ly49A expression is diminished once a developing precursor cell expresses NK1.1, but the potential to initiate expression of Ly49G2 and Ly49C/I is maintained.

The notion that the potential to express different Ly49 receptors becomes successively restricted during development was strongly supported by the clonal analysis of NK cells sorted to express only Ly49A or only Ly49G2. Ly49A$^+$C/F/G2/I$^-$ NK cells gave rise to colonies containing Ly49C/I$^+$, Ly49F$^+$, and Ly49G2$^+$ cells, as might be expected for a receptor that is expressed early in the pathway. In contrast, Ly49G2$^+$A/C/F/I$^-$ NK cells were able to initiate Ly49C/I$^+$ expression but not Ly49A or Ly49F expression. The results suggest that the potential to initiate Ly49A expression is lost early. After cells have initiated Ly49G2 expression, the capacity to initiate expression of Ly49F is also lost, while the potential to initiate Ly49C/I expression is maintained. In recently reported bulk culture experiments, Ly49A expression was detected after Ly49G2 and Ly49C/I (and/or Ly49H) on developing NK cells (Williams et al., 1999). However, our clonal analysis of sorted Ly49G2$^+$A/C/F/I$^-$ cells did not yield any Ly49A$^+$ progeny, even though Ly49A expression was shown to be quite stable once initiated. Our results support the notion that Ly49 receptor expression potential becomes increasingly restricted as NK cells develop.

A possible model to explain the data is that the initiation of receptor expression occurs with a strict sequence in development, reflecting sequential waves of potential to express different receptors. Alternatively, there may be no specific preferred sequence of receptor initiation, but rather a process in which receptor genes become sequentially unavailable for de novo initiation. In the latter scheme, the earliest cells would have the potential to express all receptors with approximately equal likelihood, while later cells would be more restricted with respect to which receptors can be initiated. We have no information as to the molecular mechanisms underlying successively restricted Ly49 expression, though gene order on the chromosome must be considered a possibility (Dillon and Grosveld, 1993).

Implications of the Results for the Product Rule

We have previously pointed out that the frequency of NK cells expressing a given combination of Ly49 receptors approximately equals the product of the frequencies of cells in the total population expressing each receptor ("the product rule") and suggested on this basis that receptor gene expression is initiated randomly in NK cell progenitors (Raulet et al., 1997). While our new results suggest temporal restrictions on Ly49 receptor initiation, they do not contradict the proposal that the receptor initiation pattern in individual NK cells is a stochastic process. We propose that at an early stage where expression of one Ly49 receptor is allowed, only a fraction of the cells actually succeed in initiating expression. Stochastic initiation of a different receptor at a later stage can occur in some cells that do express the earlier receptor and in some cells that do not. Thus, expression of different receptors may be displaced in time yet still be independent and stochastic. Such a process would be expected to yield receptor expression frequencies fully consistent with the product rule.

Despite the indications that expression of different Ly49 receptors often occurs independently, in one instance we observed a possible deviation from this pattern. Initiation of Ly49F expression occurred at a high frequency (approximately 50%) in colonies derived from Ly49A$^+$Ly49C/F/G2/I$^-$ precursor cells, despite the fact that Ly49F is expressed by only approximately 12% of all NK cells in normal B6 mice. Initiation of Ly49F expression does not depend on previous initiation of Ly49A expression, however, since approximately two-thirds of Ly49F$^+$ NK cells in normal mice do not express

Ly49A (T. Hanke, H. T., and D. H. R., unpublished data). Nevertheless, the data suggest that expression of Ly49F is not wholly independent of expression of Ly49A, perhaps indicating commonalities in the regulation of the *Ly49f* and *Ly49a* genes.

Regulation of Inhibitory Ly49 Receptor Expression by Stromal Class I MHC

Our results demonstrate a significant effect of stromal class I MHC expression in determining the sequential expression of inhibitory Ly49 receptors by clonally differentiating NK cells. Importantly, the in vitro assay fully recapitulates previous in vivo studies (Held and Raulet, 1997; Raulet et al., 1997), showing that transgenic expression of Ly49A results in reduced numbers of Ly49G2$^+$ NK cells in H-2^d mice, has less effect in H-2^b mice, and has no effect in class I–deficient mice. As noted previously, the intermediate results obtained with H-2^b stromal cells suggest that Ly49A interacts weakly with H-2^b class I molecules, sufficient to observe developmental effects but insufficient to inhibit NK cell effector activity (Held and Raulet, 1997). The results with the in vitro clonal assay go beyond the in vivo studies by demonstrating that MHC expression influences both the proportion of clones that contain Ly49G2$^+$ cells and the average percentage of Ly49G2$^+$ cells found within each clone. The results are consistent with the notion that engagement of class I ligands by Ly49A reduces the likelihood per unit time that the developing NK cell will initiate expression of Ly49G2. Thus, fewer clones will ultimately contain cells expressing Ly49G2, and those that do may tend to initiate expression later in the clonal expansion phase. Alternatively, MHC expression may impact the rate of proliferation or death of Ly49G2$^+$ cells that arise in each clone. The in vitro system should be advantageous in discriminating these possibilities.

An equally significant conclusion is that the effects of class I MHC expression in the in vitro system are mediated primarily by stromal cell MHC molecules, rather than by MHC molecules on the NK cells themselves. A caveat is that these clonal cultures contain a low density of hematopoieitic cells for the first week of culture, which may minimize NK cell–hematopoieitic cell interactions. In this respect, we cannot rule out that additional MHC effects may occur at the high cell densities that occur in vivo. Nevertheless, the results suggest minimally that repertoire formation is primarily determined by interactions in *trans* between Ly49 receptors on precursor cells and MHC ligands expressed by other cells (stromal or hematopoietic), rather than through *cis* interactions of Ly49 receptors and MHC ligands on the same cell. Such cell-autonomous interactions were suggested by the X-ray crystal structure of Ly49A complexed with H-2D^d, which raised the possibility that Ly49A and D^d may interact in the same plasma membrane (Tormo et al., 1999).

It is interesting to consider the implications of the collective results for the development of the repertoire of Ly49 receptors, especially if Ly49 receptor expression is initiated in a defined order. Consider the early developing NK cells in H-2^d mice that have just initiated expression of Ly49A. Engagement of D^d by Ly49A should decrease the probability of subsequent Ly49G2 expression. This effect should lead not only to a decreased number of Ly49A$^+$G2$^+$ cells, as previously noted, but to an increased number of Ly49A$^+$G2$^-$ cells. As predicted, we previously observed an increased percentage of Ly49A$^+$G2$^-$ NK cells in B10.D2 mice compared to B6 or B6-$\beta_2m^{-/-}$ mice (Held et al., 1996). On the other hand, if Ly49G2 expression can only be initiated after expression of Ly49A, we would not expect to observe a similar increase in Ly49G2$^+$A$^-$ NK cells. Again, the previous data fit with this prediction, because the percentage of Ly49G2$^+$A$^-$ NK cells did not differ in B10.D2 mice as compared to B6 and B6-$\beta_2m^{-/-}$ mice (Held et al., 1996). The Ly49G2$^+$A$^-$ NK cells were presumably derived from cells that failed to express Ly49A at the earlier stage and were, therefore, not influenced by expression of Ly49A ligands. As indicated by this example, an ordered sequence of Ly49 receptor expression is expected to impact the final repertoire considerably.

Finally, we note that MHC molecules from both the stromal and the hematopoietic cells in the cultures reduce the cell surface expression levels of Ly49G2 and Ly49A. Previous studies have shown that the MHC-dependent reduction in Ly49A cell surface expression levels is a posttranscriptional event and may reflect ligand-induced receptor modulation (Held and Raulet, 1997).

Concluding Remarks

The clonogenic assay presented here furnishes evidence that bone marrow stromal cells provide signals necessary for efficient clonal growth of NK cells, as well as for initiation of Ly49 expression. Further studies will be necessary to define the nature of these signals, which could be cytokine(s) and/or cell–surface counterreceptors. An interesting possibility is that stromal cells provide stimulatory signals to developing NK cells akin to the signals that activate NK cell lytic activity. Activation of stimulatory receptors on developing NK cells may be necessary to initiate the Ly49 receptor expression program. Obviously, the in vitro system can be employed as an assay to identify the relevant signals. Since the genotype of the stroma and progenitor can be varied independently of each other, the system also holds promise for dissecting the genetic mechanisms responsible for shaping the repertoire of developing NK cells. Such an analysis will require extensive examination of the effects of genetic mutations on the developing repertoire in the context of varying the MHC molecules expressed by stromal cells and NK progenitors.

Experimental Procedures

Animals

C57Bl/6NCR (B6, H-2^b), B10.D2 (H-2^d), and B6-$\beta_2m^{-/-}$ mice were bred in the animal facility of the University of California (Berkeley, CA) and used at 7-12 weeks of age. Congenic B6-Ly5.1 mice were purchased from the National Cancer Institutes (Frederick, MD), where they are listed as B6-Ly5.2 according to the previous nomenclature. Ly49A transgenic mice on the B10.D2 and B6-$\beta_2m^{-/-}$ background were described (Held and Raulet, 1997).

Antibodies and Staining

The following mAbs and reagents were used: JR9–318 (anti-Ly49A; kindly provided by J. Roland), HBF-719 (anti-Ly49F; H. T., unpublished data), 4D11 (anti-Ly49G2 [Mason et al., 1995]), and SW5E6 (anti-Ly49C/I; generously provided by Drs Vinay Kumar and Michael

Bennett [Sentman et al., 1989; Brennan et al., 1996]). TMβ1 (anti-IL-2Rβ), PK136 (anti-NK1.1), DX5 (anti-pan-NK marker), 2B8 (anti-c-kit), Sca-2 (anti-TSA-1), anti-Mac-1, anti-TER-119, 500A2 (anti-CD3), and anti-CD19 were purchased from Pharmingen. Anti-B220, anti-Gr-1, and Streptavidin-Tricolor were purchased from Caltag. Staining procedures were previously described (Held et al., 1996). Nonspecific staining was prevented by preincubating the cells with anti-FcγRII/III receptor antibody 2.4G2 (Unkeless, 1979). Cells were washed twice between staining reactions and once before final analysis. The samples were analyzed with an EPICS XL flow cytometer (Coulter, Hialeah, FL), gating for live cells based on the forward and side light scatter profile.

Sorting of Bone Marrow Precursor Cells

Bone marrow cells were isolated from 7- to 12-week-old B6 mice by irrigation of the femurs and tibias, followed by purification over stepwise Percoll (Pharmacia) gradients. Cells recovered between the 44% Percoll and 60% Percoll layers were recovered, stained, and sorted using an ELITE flow cytometer (Coulter Immunology, Hialeah, FL) using the following combinations: (CD3ϵ, CD19, Ly49A, F, G2, C/I)-FITC, NK1.1-PE, and DX5-biotin (for $NK1.1^+DX5^+Ly49^-$ cells); Sca-2-FITC, c-kit-PE, and Lin (NK1.1, CD3ϵ, B220, Gr-1, Mac-1, TER-119)-biotin (for $c\text{-}kit^+Sca\text{-}2^+Lin^-$ cells); (CD3ϵ, CD19, Ly49F, G2, C/I)-FITC, NK1.1-PE, and Ly49A-biotin (for $Ly49A^+C/F/G2/I^-$ cells); (CD3ϵ, CD19, Ly49A, F, C/I)-FITC, NK1.1-PE, Ly49G2-biotin (for $Ly49G2^+A/C/F/I^-$ cells).

Limiting Dilution Culture of Bone Marrow Precursor Cells

Bone marrow stromal cells were prepared from B6, B10.D2, or B6-$\beta_2m^{-/-}$ mice by plating 2.5×10^5 cells/well in 96-well flat bottom plates (Falcon 3077), in RPMI 1640 medium containing 10% fetal calf serum (Delfino et al., 1996). After 3 weeks, the plates were exposed to 20 Gy, and the adherent remaining stroma was used as a feeder layer for NK progenitors. OP9 cells (Kodama et al., 1994), an H-2^k murine stromal cell line (Williams et al., 1999), were used as stromal cells in some experiments. OP9 cells were seeded at 5×10^4 cells/well in 96-well flat bottom plates, cultured for 4–6 hr, and irradiated with 25 Gy before adding NK progenitors.

Sorted bone marrow precursor cells were seeded at various concentrations onto irradiated stromal cells in RPMI-1640 (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum, 2 mM glutamine, 50 μM 2-mercaptoethanol, 10 mM HEPES, and antibiotics. The following cytokines were added at the initiation of culture: recombinant murine c-kit ligand (100 U/ml final concentration; gift of F. Melchers, Basel Institute for Immunology), recombinant murine IL-7 (100 U/ml; gift from A. Cumano, Institut Pasteur, Paris), recombinant murine flt-3 ligand (100 U/ml; gift of DNAX Inc., Palo Alto, CA), and recombinant human IL-15 (100 ng/ml) or recombinant murine IL-15 (20 ng/ml), both gifts of Immunex (Seattle, WA). Human rIL-15 was employed in only one experiment (Table 1, first experiment), with mouse rIL-15 used for all other experiments. No difference in results was observed with mouse versus human rIL-15, although we used the mouse rIL-15 at a lower concentration. Cultures were incubated at 37°C in humidified air with 7% CO_2. Most clones were tested at day 9–11 by flow cytometry for expression of CD19, CD3, NK1.1, and Ly49A, F, G2, and C/I.

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References

Antica, M., Wu, L., Shortman, K., and Scollay, R. (1994). Thymic stem cells in mouse bone marrow. Blood *84*, 111–117.

Brennan, J., Lemieux, S., Freeman, J., Mager, D., and Takei, F. (1996). Heterogeneity among Ly49C NK cells: characterization of highly related receptors with differing functions and expression patterns. J. Exp. Med. *184*, 2085–2090.

Brown, M.G., Fulmek, S., Matsumoto, K., Cho, R., Lyons, P.A., Levy, E.R., Scalzo, A.A., and Yokoyama, W.M. (1997). A 2-MB YAC contig and physical map of the natural killer gene complex on mouse chromosome 6. Genomics *42*, 16–25.

Daniels, B.F., Karlhofer, F.M., Seaman, W.E., and Yokoyama, W.M. (1994). A natural killer cell receptor specific for a major histocompatibility complex class I molecule. J. Exp. Med. *180*, 687–692.

Delfino, D.V., Patrene, K.D., Lu, J., Deleo, A., Deleo, R., Herberman, R.B., and Boggs, S.S. (1996). Natural killer cell precursors in the CD44neg/dim T-cell receptor population of mouse bone marrow. Blood *87*, 2394–2400.

Dillon, N., and Grosveld, F. (1993). Transcriptional regulation of multigene loci: multilevel control. Trends Genet. *9*, 134–137.

Dorfman, J.R., and Raulet, D.H. (1998). Acquisition of Ly49 receptor expression by developing natural killer cells. J. Exp. Med. *187*, 609–618.

Hackett, J., Jr., Tutt, M., Lipscomb, M., Bennett, M., Koo, G., and Kumar, V. (1986). Origin and differentiation of natural killer cells. II. Functional and morphologic studies of purified $NK\text{-}1.1^+$ cells. J. Immunol. *136*, 3124–3131.

Hanke, T., Takizawa, H., McMahon, C.W., Busch, D.H., Pamer, E.G., Miller, J.D., Altman, J.D., Liu, Y., Cado, D., Lemonnier, F.A., et al. (1999). Direct assessment of MHC class I binding by seven Ly49 inhibitory NK cell receptors. Immunity *11*, 67–77.

Held, W., and Raulet, D.H. (1997). Ly49A transgenic mice provide evidence for a major histocompatibility complex-dependent education process in NK cell development. J. Exp. Med. *185*, 2079–2088.

Held, W., Dorfman, J.R., Wu, M.-F., and Raulet, D.H. (1996). Major histocompatibility complex class I–dependent skewing of the natural killer cell Ly49 receptor repertoire. Eur. J. Immunol. *26*, 2286–2292.

Hoglund, P., Sundbäck, J., Ollson-Alheim, M.Y., Johansson, M., Salcedo, M., Öhlén, C., Ljunggren, H.G., Sentman, C., and Karre, K. (1997). Host MHC class I gene control of NK cell specificity in the mouse. Immunol. Rev. *155*, 11–28.

Jaleco, A.C., Blom, B., Res, P., Weijer, K., Lanier, L.L., Phillips, J.H., and Spits, H. (1997). Fetal liver contains committed NK progenitors, but is not a site for development of $CD34^+$ cells into T cells. J. Immunol. *159*, 694–702.

Kodama, H., Nose, M., Niida, S., and Nishikawa, S. (1994). Involvement of the c-kit receptor in the adhesion of hematopoietic stem cells to stromal cells. Exp. Hematol. *22*, 979–984.

Kubota, A., Kubota, S., Lohwasser, S., Mager, D.L., and Takei, F. (1999). Diversity of NK cell receptor repertoire in adult and neonatal mice. J. Immunol. *163*, 212–216.

Ljunggren, H.G., and Karre, K. (1990). In search of the "missing self": MHC molecules and NK cell recognition. Immunol. Today *11*, 237–244.

Lodolce, J.P., Boone, D.L., Chai, S., Swain, R.E., Dassopoulos, T., Trettin, S., and Ma, A. (1998). IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. Immunity *9*, 669–676.

Mason, L.H., Ortaldo, J.R., Young, H.A., Kumar, V., Bennett, M., and Anderson, S.K. (1995). Cloning and functional characteristics of murine large granlar lymphocyte-1: a member of the Ly-49 gene family (Ly-49G2). J. Exp. Med. *182*, 293–303.

McQueen, K.L., Freeman, J.D., Takei, F., and Mager, D. (1998). Localization of five new Ly49 genes, including three closely related to Ly49C. Immunogenetics *48*, 174–183.

Mingari, M.C., Vitale, C., Cantoni, C., Bellomo, R., Ponte, M., Schiavetti, F., Bertone, S., Moretta, A., and Moretta, L. (1997). Interleukin-15-induced maturation of human natural killer cells from early thymic

precursors: selective expression of CD94/NKG2-A as the only HLA class I-specific inhibitory receptor. Eur. J. Immunol. *27*, 1374–1380.

Mrózek, E., Anderson, P., and Caligiuri, M.A. (1996). Role of interleukin-15 in the development of human $CD56^+$ natural killer cells from $CD34^+$ hematopoietic progenitor cells. Blood *87*, 2632–2640.

Raulet, D.H. (1999). Development and tolerance of natural killer cells. Curr. Opin. Immunol. *11*, 129–134.

Raulet, D.H., Held, W., Correa, I., Dorfman, J., Wu, M.-F., and Corral, L. (1997). Specificity, tolerance and developmental regulation of natural killer cells defined by expression of class I-specific Ly49 receptors. Immunol. Rev. *155*, 41–52.

Sentman, C.L., Hackett, J., Jr., Kumar, V., and Bennett, M. (1989). Identification of a subset of murine natural killer cells that mediates rejection of $Hh\text{-}1^d$ but not $Hh\text{-}1^b$ bone marrow grafts. J. Exp. Med. *170*, 191–202.

Takei, F., Brennan, J., and Mager, D.L. (1997). The Ly49 family: genes proteins and recognition of class I MHC. Immunol. Rev. *155*, 67–77.

Tormo, J., Natarajan, K., Margulies, D.H., and Mariuzza, R.A. (1999). Crystal structure of a lectin-like natural killer cell receptor bound to its MHC class I ligand. Nature *402*, 623–631.

Trinchieri, G. (1989). Biology of natural killer cells. Adv. Immunol. *47*, 187–376.

Unkeless, J.C. (1979). Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors. J. Exp. Med. *150*, 580–596.

van den Brink, M.R., Boggs, S.S., Herberman, R.B., and Hiserodt, J.C. (1990). The generation of natural killer (NK) cells from NK precursor cells in rat long-term bone marrow cultures. J. Exp. Med. *172*, 303–313.

Williams, N.S., Moore, T.A., Schatzle, J.D., Puzanov, I.J., Sivakumar, P.V., Zlotnik, A., Bennett, M., and Kumar, V. (1997). Generation of lytic natural killer 1.1^+, $Ly\text{-}49^-$ cells from multipotential murine bone marrow progenitors in a stroma-free culture: definition of cytokine requirements and developmental intermediates. J. Exp. Med. *186*, 1609–1614.

Williams, N.S., Klem, J., Puzanov, I.J., Sivakumar, P.V., Bennett, M., and Kumar, V. (1999). Differentiation of $NK1.1^+$, $Ly49^+$ NK cells from flt3+ multipotent marrow progenitor cells. J. Immunol. *163*, 2648–2656.

Yokoyama, W.M. (1995). Natural killer cell receptors specific for major histocompatibility complex class I molecules. Proc. Natl. Acad. Sci. USA *92*, 3081–3085.