# Expression of Natural Killer Receptor Alleles at Different Ly49 Loci Occurs Independently and Is Regulated by Major Histocompatibility Complex Class I Molecules

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## Abstract

Ly49 receptor genes are expressed by subsets of natural killer (NK) cells in an overlapping fashion, accounting for the capacity of NK subsets to attack host cells that have selectively downregulated self–major histocompatibility complex (MHC) class I molecules. It was shown previously that most NK cells express only one or the other allele of a given Ly49 gene, while a smaller population expresses both alleles. However, the methods used to detect monoallelic and biallelic cells were nonquantitative. Here, new allele-specific antibodies were used to provide the first quantitative examination of biallelic and monoallelic expression of Ly49A and Ly49G2. The results demonstrate conclusively that most Ly49A$^+$ and Ly49G2$^+$ NK cells express the corresponding gene in a monoallelic fashion, with a smaller subset expressing both alleles. Unexpectedly, biallelic Ly49A$^+$ NK cells were more numerous than predicted by completely independent allelic expression, suggesting some heterogeneity among NK progenitors in the potential to express a given Ly49 gene. The data also show that cells expressing one allele of Ly49G2 may express Ly49A from the same or opposite chromosome with equal likelihood, indicating that the expressed allele is chosen independently for different Ly49 genes. Finally, the data demonstrate that biallelic expression of Ly49A or Ly49G2 occurs least frequently in mice that express ligands for these receptors (H-2$^d$ mice), and most frequently in class I–deficient mice. Thus, biallelic expression of Ly49 genes is regulated by interactions of NK cell progenitors with MHC class I molecules.

Key words: major histocompatibility complex • natural killer cells • monoallelic expression • allelic exclusion • Ly49

## Introduction

NK cells play an important role in the recognition and lysis of pathogen-infected or transformed target cells (1, 2). NK cells are regulated in part by the various MHC class I–specific receptors expressed on the cell surface (3, 4). Most of these receptors transduce an inhibitory signal upon ligand engagement, accounting for the tendency of NK cells to attack class I–deficient target cells while sparing normal cells. Other receptors, including some specific for MHC class I molecules, stimulate NK cells (5–7). The balance of inhibitory and stimulatory signaling determines the outcome of NK–target cell interactions.

In mice, two families of MHC class I–specific receptors have been described: the Ly49 family (8) and the NKG2/CD94 family (9, 10). The Ly49 family comprises at least nine members, seven of which contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in the cytoplasmic domains. Functional, genetic, and binding data defined D$^d$ and D$^k$, among other MHC molecules, as ligands for Ly49A, with no detectable binding to H-2$^b$ class I molecules (11–15). Ly49G2 is functionally inhibited by H-2$^d$ but not H-2$^b$ cells (16), and has been shown to specifically bind to soluble tetrameric D$^d$ molecules (14).

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MHC class I–specific inhibitory receptors, including Ly49 receptors, are expressed in a variegated pattern on NK cells, such that each receptor is expressed by 10–50% of NK cells. Receptor expression is stable once initiated, as suggested by experiments in which purified NK subsets are expanded in vitro or transferred in vivo (11, 17, 18). Coexpression of Ly49 family members occurs frequently (19), with an average of three to four receptors expressed by each NK cell (20). Different receptors are expressed with a considerable degree of independence, as indicated by the finding that the frequency of NK cells coexpressing a given Ly49 receptor pair or trio can be estimated by multiplying the frequencies of cells that express each of the receptors individually (the "product rule" [19]). These findings suggest that the initial choice of which receptors are to be expressed in a developing NK cell is governed by a stochastic mechanism. Additional evidence indicates that different receptors are expressed on developing NK cells in a sequential, cumulative fashion (18, 21).

Ly49 genes exhibit limited allelic polymorphism, allowing one to separately monitor the expression of each allele of the same gene using RNA analysis or allele-specific mAbs. Such analyses revealed that Ly49 genes are expressed in a predominantly monoallelic fashion (17, 22, 23). For example, costaining of NK cells from (B6 × BALB/c)F1 mice with a combination of the A1 mAb, specific for $Ly49A^{B6}$, and the JR9-318 mAb, reactive with both $Ly49A^{B6}$ and $Ly49A^{BALB}$, detected a $JR9^+A1^-$ population and an equally numerous $JR9^+A1^+$ population (17). Analysis of the mRNA in these subsets by reverse transcription (RT)[1]-PCR confirmed that the $JR9^+A1^-$ population consisted almost exclusively of cells expressing only the $Ly49A^{BALB}$ allele, whereas the $JR9^+A1^+$ population consisted predominantly of cells expressing the $Ly49A^{B6}$ allele. These data suggested that $Ly49A^+$ NK cells in the F1 mice consist primarily of two subsets of approximately equal size, each of which expresses only one of the two Ly49A alleles (17). Monoallelic expression of nonrearranging, non–X–linked genes has been observed in only a few other cases, such as the mouse odorant receptor genes (24), certain cytokine genes (25–27), and possibly the Pax-5 transcription factor (27, 28). These findings have spurred a great deal of interest in the mechanisms underlying monoallelic gene expression as well as its significance.

The population analysis described above demonstrated that Ly49A is expressed in a predominantly monoallelic fashion, but was insufficiently discriminating to determine whether a minor subset of cells expresses both Ly49A alleles. To address this question, a clonal analysis of Ly49A expression was undertaken. Single $Ly49A^+$ NK cells from F1 mice were expanded in culture into small colonies. RNA samples from the colonies were analyzed by RT-PCR to determine which Ly49A alleles were expressed. The analysis confirmed that most $Ly49A^+$ NK cells expressed only one or the other Ly49A allele. However, a smaller fraction of clones was also detected in which both Ly49A alleles were expressed (22). It appeared that the frequency of biallelic clones could be roughly estimated by multiplying the frequencies of NK cells expressing each allele independently.

Using the clonal system, monoallelic expression was extended to two additional Ly49 genes, Ly49C and Ly49G2. As was observed for Ly49A, biallelic expression in a minority of clones was documented in the case of Ly49G2 (23). Consistent with earlier evidence generated with NK cell populations (17), the clonal analysis also suggested that individual NK cells that coexpress two or more Ly49 genes may express alleles from the same or opposite chromosomes (23). Based on these findings, we proposed that the initial choice of NK receptor genes for expression is imposed by mechanisms that act locally to stably regulate cis-acting control sequences associated with each Ly49 allele, independent of neighboring Ly49 genes or of the Ly49 allele on the opposite chromosome (17, 19).

Previous data has indicated that the MHC molecules of the host impact the Ly49 receptor repertoire. Specifically, the frequencies of NK cells coexpressing various combinations of Ly49 receptors were lower in class $I^+$ mice than in MHC class I–deficient mice (29). Data suggest that expression by an NK cell of a Ly49 receptor specific for self-MHC reduces the likelihood that the cell will express others. For example, expression of a Ly49A transgene in all NK progenitor cells resulted in a reduced frequency of NK cells expressing endogenous Ly49G2 receptors (30). The effect was observed in mice expressing $H\text{-}2^d$, to which the receptors bind, but not in MHC class I–deficient mice. Given that Ly49 receptors are expressed sequentially during development, we proposed that the interaction of an expressed Ly49 receptor with self-MHC ligands could inhibit subsequent expression of additional Ly49 receptors by the same NK cell (19). Why regulate coexpression of multiple Ly49 receptors? Evidence suggests that two Ly49 receptors expressed by the same NK cell independently inhibit the cell when engaged separately (31), and, when both are engaged, function cumulatively to cause greater inhibition than either receptor alone (31a). By regulating the coexpression of multiple self-MHC–specific receptors, the NK cell may optimize its capacity to attack target cells that selectively or only partially downregulate class I molecules. An interesting question is whether this mechanism also limits the coexpression of both alleles of the same Ly49 gene. Consistent with this possibility, clonal analysis suggested that mice lacking MHC class I molecules contained a higher frequency of biallelic $Ly49A^+$ NK cells than normal mice (22).

The clonal analyses suffer from the limited number of clones that could be analyzed, and the fact that the clones were expanded in vitro before analysis, possibly introducing experimental bias. Here we describe new allele-specific mAbs that allow the direct detection of Ly49 monoallelic and biallelic populations in ex vivo NK cells. With the use of these Abs, we provide the first accurate quantitation of monoallelic and biallelic populations, and demonstrate that

[1]*Abbreviations used in this paper:* CHO, Chinese hamster ovary; RT, reverse transcription.

host MHC molecules determine the extent of biallelic expression of two different Ly49 receptors. Furthermore, the mAbs were used to demonstrate directly that coexpression of two different Ly49 loci can occur from the same or opposite chromosomes with equal likelihood. This finding suggests that each Ly49 locus is independently regulated with respect to which allele is expressed.

## Materials and Methods

*Mice.* B10.D2nSnJ, C57Bl/6J (B6), BALB.B mice (all from The Jackson Laboratory), and B6-$\beta2m^{-/-}$ mice backcrossed five times to B6 (22, 32) were bred in the animal facility at the University of California at Berkeley. BALB/cJ mice were purchased from The Jackson Laboratory. To generate "BALB.B-$\beta2m^{-/-}$ mice", B6 $\beta2m^{-/-}$ mice were crossed to BALB.B mice, and the hybrids were backcrossed twice more to BALB.B. Backcross progeny were selected that harbored one $\beta2m$ knockout allele (based on Southern blotting with a $\beta2m$ probe) and that only expressed the NK complex (NKC) of the BALB strain (based on lack of staining of PBLs with PK136 mAb specific for NK1.1 and genomic Southern blotting of EcoRI-digested tail DNA with a NKRP1A partial cDNA probe). Backcrossed animals were intercrossed, selecting $\beta2m^{-/-}$ progeny. The resulting mice were $\beta2m^{-/-}$ $NKC^{BALB/BALB}$ and mostly homozygous for BALB alleles at other loci, and were maintained by intercrossing.

The Ly49 heterozygous mice compared in Fig. 5 were generated as follows: $\beta2m^{-/-}$-F1 mice by crossing B6-$\beta2m^{-/-}$ and BALB.B-$\beta2m^{-/-}$ mice; H-$2^b$-F1 mice by crossing B6 (H-$2^b$) and BALB.B (H-$2^b$) mice; and the H-$2^d$-F1 mice by crossing B10.D2nSnJ (H-$2^d$) and BALB/cJ (H-$2^d$) mice. Comparisons indicated that it made no difference whether one strain or the other was used as the mother.

*Production of $Ly49A^{BALB}$-specific TNTA mAb.* $Ly49A^{BALB}$ cDNA (17) was cloned into the pIRES vector (CLONTECH Laboratories, Inc.) and stably transfected into Chinese hamster ovary (CHO) cells. B6 mice were immunized intraperitoneally six times with between 1 and $10 \times 10^6$ $Ly49A^{BALB}$-expressing CHO cells in PBS at 2–3-wk intervals. 3 d after the final boost, splenocytes were harvested and fused as described (33), except P3×63-Ag8.653 (American Type Culture Collection) was used as the fusion partner. Hybridoma supernatants were screened by flow cytometry for reactivity with CHO-$Ly49A^{BALB}$ transfectants (but not untransfected CHO cells) and a subset of BALB/c NK cells. Of three $Ly49A^{BALB}$-specific hybridomas, TNTA (IgG2a, κ) was subcloned and used for this study. TNTA mAb was purified from hybridoma supernatants by affinity chromatography over protein A-agarose columns.

*Production of $Ly49G2^{B6}$-specific 3/25 mAb.* BALB/c mice were immunized repeatedly with CHO cells stably transfected with the $Ly49G2^{B6}$ cDNA (34) in the pIRES expression vector. Spleen cells were fused to P3×63Ag.8653 cells with polyethylene glycol (PEG), and hybridomas were selected in HAT medium. Supernatants of candidate clones were screened for staining of CHO-$Ly49G2^{B6}$ (but not CHO-$Ly49F^{B6}$) transfectants and $Ly49G2^{B6}$ transgenic thymocytes (Hanke, T., and D.H. Raulet, unpublished observations). Out of two $Ly49G2^{B6}$-specific mAbs, 3/25 (IgG1, κ) was subcloned and used throughout this study. Depending on the experiment, 3/25 mAb was used directly from hybridoma supernatants, or purified from ascites by ammonium sulfate precipitation followed by size exclusion chromatography.

*Other mAbs.* Anti-$Ly49A^{B6}$ mAb A1 (35), anti-Ly49A (non-allele-specific) mAb JR9-318 (36), anti-Ly49C mAb 5E6 (37), anti-Ly49D mAb SED-85 (38), anti-Ly49F mAb HBF-719 (38), and the anti-FcR mAb 2.4G2 (39) have been described previously. The hybridoma producing 4D11 (40) was purchased from American Type Culture Collection. Abs were conjugated to FITC (Boehringer) or biotin (Pierce Chemical Co.). Anti–NK1.1-PE and DX5-PE conjugates were purchased from BD PharMingen. Anti–CD3-Tricolor, anti–CD8-Tricolor, streptavidin-Tricolor, goat anti–mouse IgG, and mouse IgG were purchased from Caltag Labs. Streptavidin-RED613 was purchased from GIBCO BRL. Anti-His tag rabbit polyclonal IgG was purchased from Santa Cruz Biotechnology, Inc. Anti–rabbit–Ig-FITC was purchased from Dako.

*Transient Transfection of COS Cells.* Ly49 cDNAs (17, 41) cloned into the pME18S expression vector were transiently transfected into COS cells with Lipofectamine Plus (GIBCO BRL). For Ly49D and Ly49H transfections, a DAP12 (42) expression vector was cotransfected. 1 d later the cells were trypsinized and replated. On day 2, the COS cells were harvested and stained with TNTA mAb, 3/25 mAb, a positive control Ly49-specific mAb, or an anti-His polyclonal Ab as indicated.

*Flow Cytometry and Cell Sorting.* Nylon wool nonadherent splenocytes were preincubated with 2.4G2 mAb to block FcR. To discriminate Ly49A alleles, cells were costained with A1-FITC, TNTA-biotin, DX5-PE, anti–CD3-Tricolor, and anti–CD8-Tricolor followed by straptavidin-613. To discriminate Ly49G2 alleles, cells were stained in four steps: 3/25 mAb; goat anti–mouse IgG-biotin; mouse IgG; and 4D11-FITC, DX5-PE, anti–CD3-Tricolor, anti–CD8-Tricolor, and streptavidin-RED613. In Fig. 3, NK cells were sorted for expression of different Ly49G2 alleles before analysis and subsequent culturing. Nylon wool nonadherent splenocytes from 10–15 B6 mice were stained in four steps: 3/25 mAb; goat anti–mouse-biotin; and mouse IgG; 4D11-FITC, anti–NK1.1-PE, and streptavidin-Tricolor. Stained splenocytes were sorted on an ELITE flow cytometer (Beckman Coulter). Aliquots of the sorted populations were used immediately for RT-PCR analysis and the remaining cells were cultured in RPMI supplemented with 10% FCS, antibiotics, glutamine, βME, and 800 ng/ml IL-2 (Chiron Corp.). After 10 d, an aliquot of cells was used for RT-PCR analysis and the remaining cells were stained for Ly49A (A1-FITC and TNTA-biotin; streptavidin-613) or for Ly49G2 (3/25 mAb; goat anti–mouse IgG-biotin; mouse IgG; 4D11-FITC and streptavidin-613).

*RT-PCR.* RT-PCR was performed on $5–7 \times 10^4$ freshly sorted cells or on 10-d IL-2–cultured sorted cells as described previously (23).

## Results

*Independent Expression of Ly49A Alleles.* The A1 mAb binds Ly49A encoded by the B6 Ly49A allele ($Ly49A^{B6}$), but not the BALB Ly49A allele ($Ly49A^{BALB}$; reference 17). A mAb with reciprocal specificity for $Ly49A^{BALB}$, but not $Ly49A^{B6}$, was generated by immunizing B6 mice with CHO cell transfectants expressing $Ly49A^{BALB}$. The resulting allele-specific mAb, TNTA, stained transiently transfected COS cells expressing $Ly49A^{BALB}$ cDNA, but did not stain COS cells expressing any of nine different B6 Ly49 cDNAs, including $Ly49A^{B6}$ (Fig. 1). Furthermore, TNTA stained 10–15% of freshly isolated NK cells from BALB

mice, but did not stain B6 NK cells (Fig. 2 A). It is highly likely that TNTA recognizes only Ly49A in BALB mice, because the staining pattern exactly overlaps with that of the well-characterized nonallele-specific Ly49A mAb, JR9-318. Furthermore, TNTA and JR9-318 reciprocally compete for binding of Ly49A$^{BALB}$ on NK cells from BALB.B mice (data not shown).

To examine monoallelic versus biallelic expression of Ly49A, (B6 × BALB.B)F1 mice (H-2$^{b/b}$) were generated. Costaining with A1 and TNTA on freshly isolated, gated NK cells revealed that each mAb stains 10–15% of NK cells (Fig. 2 A). Replicate measurements showed that an average of 8.1% of the (B6 × BALB.B)F1 NK cells expressed Ly49A$^{BALB}$ only, 7.5% expressed Ly49A$^{B6}$ only, and 4.4% expressed both alleles. Therefore, NK cells express Ly49A predominately from one allele or the other with approximately equal likelihood, but a small subset of cells coexpresses both Ly49A alleles.

*Independent Expression of Ly49G2 Alleles.* To distinguish the expression of individual Ly49G2 alleles, a mAb specific for the Ly49G2 receptor expressed on B6 NK cells was generated. BALB/c mice were immunized with CHO cell transfectants expressing the B6 allele of Ly49G2 (Ly49G2$^{B6}$). A resulting mAb, 3/25, stained Ly49G2$^{B6}$-transfected COS cells, but not transfectants expressing any of the other eight B6 Ly49 family members (Fig. 1). The 3/25 mAb stained an average of 47.1% of freshly isolated NK cells from B6 mice, and did not stain BALB NK cells (Fig. 2 B). The staining pattern in B6 mice exactly overlapped the staining pattern of the nonallele-specific mAb 4D11, arguing that 3/25 reacts only with Ly49G2$^{B6}$ in B6 mice (Fig. 2 B).

To examine allelic expression of Ly49G2, NK cells from (B6 × BALB.B)F1 mice were co-stained with 3/25 and the nonallele-specific Ab 4D11 (Fig. 2 B). Because 3/25 recognizes Ly49G2$^{B6}$, and 4D11 recognizes both the B6 and BALB alleles of Ly49G2, there are no single positive 3/25$^+$ 4D11$^-$ NK cells. Unexpectedly, however, two distinct populations were observed in the double-positive 3/25$^+$ 4D11$^+$ quadrant. We hypothesized that the 3/25$^+$4D11$^{high}$ population expressed both Ly49G2$^{B6}$ and Ly49G2$^{BALB}$, whereas the 3/25$^+$4D11$^{low}$ population represented NK

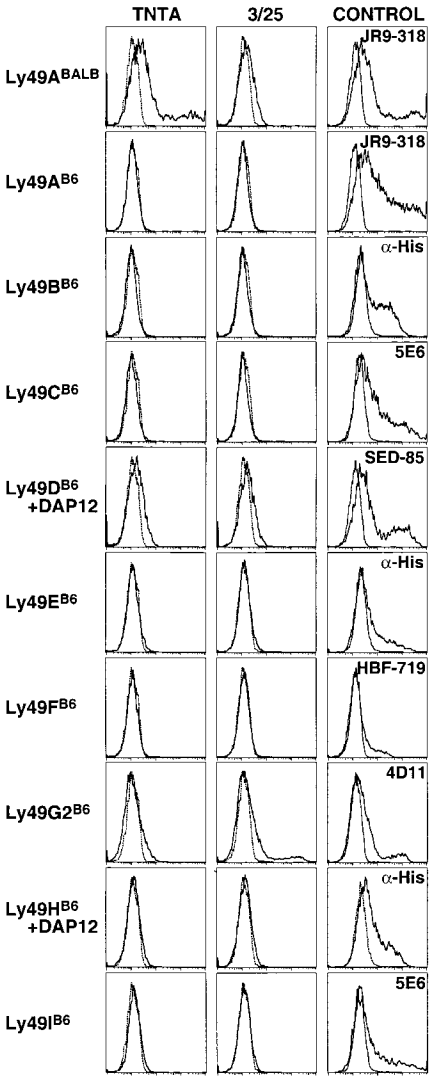


**Figure 1.** Specificity of TNTA and 3/25 mAbs. TNTA mAb (first column) or 3/25 mAb (second column) was used to stain COS cells transiently transfected with the indicated Ly49 expression constructs (solid lines) or untransfected COS cells (dashed lines). Ly49D and Ly49H constructs were cotransfected with a DAP12 expression construct. Ly49B, Ly49E, and Ly49H cDNAs were epitope (6x-His) tagged. Transiently transfected cells varied in transfection efficiency, as shown by staining with the appropriate Ly49-specific mAb or an anti-his rabbit polyclonal IgG in the case of tagged receptors (third column).

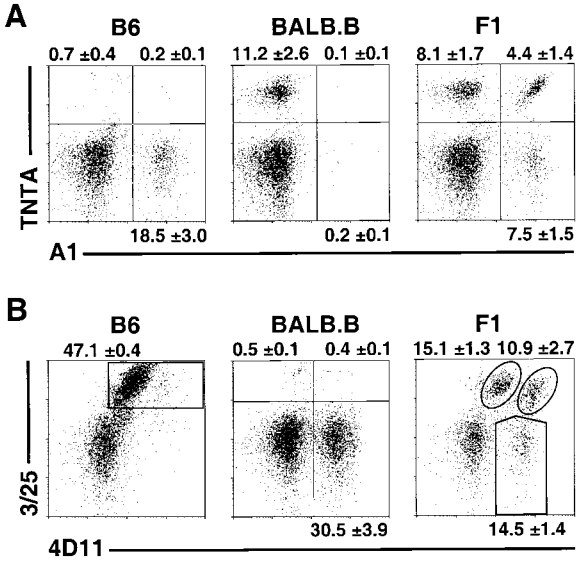


**Figure 2.** Predominantly monoallelic expression of Ly49A and Ly49G2. Freshly isolated, nylon wool nonadherent splenic cells from B6, BALB.B, and (B6 × BALB.B)F1 mice (all H-2$^b$) were harvested. Gated DX5$^+$CD3$^-$CD8$^-$ NK cells were analyzed with (A) the Ly49A$^{B6}$ specific mAb, A1, versus the Ly49A$^{BALB}$ specific mAb, TNTA, or (B) with Ly49G2$^{B6}$-specific mAb 3/25 versus 4D11, which recognizes both Ly49G2$^{B6}$ and Ly49G2$^{BALB}$ alleles. Data represent means ± SD of three or four determinations on cells from individual animals.

cells expressing only Ly49G2$^{B6}$. The dim 4D11 staining of the latter population might occur because 3/25, which was added first in the staining experiments, partially blocks 4D11 binding. Indeed, a control experiment demonstrated that 3/25 blocks binding of Ly49G2$^{B6}$ by 4D11 (data not shown).

To test allelic expression at the mRNA level, we sorted freshly isolated 3/25$^{+}$4D11$^{low}$, 3/25$^{+}$4D11$^{high}$, and 3/25$^{-}$4D11$^{+}$ populations to >90% purity (Fig. 3 A). mRNA was prepared directly from these cells, and RT-PCR was performed with primers that amplify the transcripts of both Ly49G2 alleles. The resulting PCR products were then digested with the diagnostic restriction enzyme, Taq$\alpha$I, which only cleaves the Ly49G2$^{BALB}$ cDNA (23). The analysis confirmed that the 3/25$^{+}$4D11$^{low}$ population contained only Ly49G2$^{B6}$ transcripts, the 3/25$^{+}$4D11$^{high}$ population contained both Ly49G2$^{B6}$ and Ly49G2$^{BALB}$ transcripts, and the 3/25$^{-}$4D11$^{+}$ population contained only Ly49G2$^{BALB}$ transcripts (Fig. 3 B). These expression patterns are stable, as similar results were obtained with sorted cells that had been expanded for 10 d in IL-2 (Fig. 3 B). Based on the staining patterns (Fig. 2 B), we conclude that an average of 15.1% of F1 NK cells express Ly49G2$^{B6}$ only, 14.5% express Ly49G2$^{BALB}$ only, and 10.9% express both Ly49G2 alleles. Thus, Ly49G2, like Ly49A, is expressed predominantly in a monoallelic fashion but with a distinct population of biallelic cells.

*Independent Allelic Expression of Ly49A and Ly49G2.* By using the new allele-specific mAbs in conjunction, it was possible to determine directly whether cells that coexpress Ly49A and Ly49G2 do so coordinately from the same chromosome, from opposite chromosomes, or whether there is a bias for one configuration or the other. The three Ly49G2$^{+}$ populations described above were expanded for 10 d in medium containing IL-2 before reanalysis. Costaining with the 3/25 and 4D11 mAbs confirmed that the populations had largely retained their phenotype with respect to allelic expression of Ly49G2 alleles (Fig. 4 A). Costaining with TNTA and A1 mAbs was employed to determine allelic expression of Ly49A (Fig. 4 B). The analysis revealed that cells expressing Ly49G2$^{B6}$ were equally likely to express Ly49A$^{B6}$ or Ly49A$^{BALB}$, and a small fraction of such cells expressed both Ly49A alleles. Similarly, cells expressing Ly49G2$^{BALB}$ were approximately equally likely to express Ly49A$^{B6}$ or Ly49A$^{BALB}$, though there may be a small bias for Ly49A$^{B6}$ in this instance. Finally, NK cells expressing both Ly49G2 alleles can also express either Ly49A allele, and a small fraction of cells expresses all four alleles. These data suggest that in the case of coexpression of different Ly49 receptor genes, there is little or no coordination in terms of which chromosome is used to direct synthesis of the two receptors.

*MHC Class I Molecules Control the Frequency of Ly49 Biallelic NK Cells.* The new mAbs were employed to investigate the effect of MHC background on the frequencies of cells expressing Ly49A or Ly49G2 in a monoallelic versus biallelic fashion. For this purpose, we compared three MHC-different Ly49 heterozygous crosses: H-2$^{d}$-F1, H-2$^{b}$-F1, or class I–deficient $\beta$2m$^{-/-}$-F1 (see Materials and Methods). All three were hybrids between BALB and B6 or B10 background mice, and therefore were of similar genetic background. In each case we compared F1 mice to the parental Ly49 homozygous strains. Costaining with A1 and TNTA mAbs revealed predominant monoallelic expression of Ly49A in all three F1s, as well as a smaller biallelic population (Fig. 5 A). Similarly, costaining with 3/25

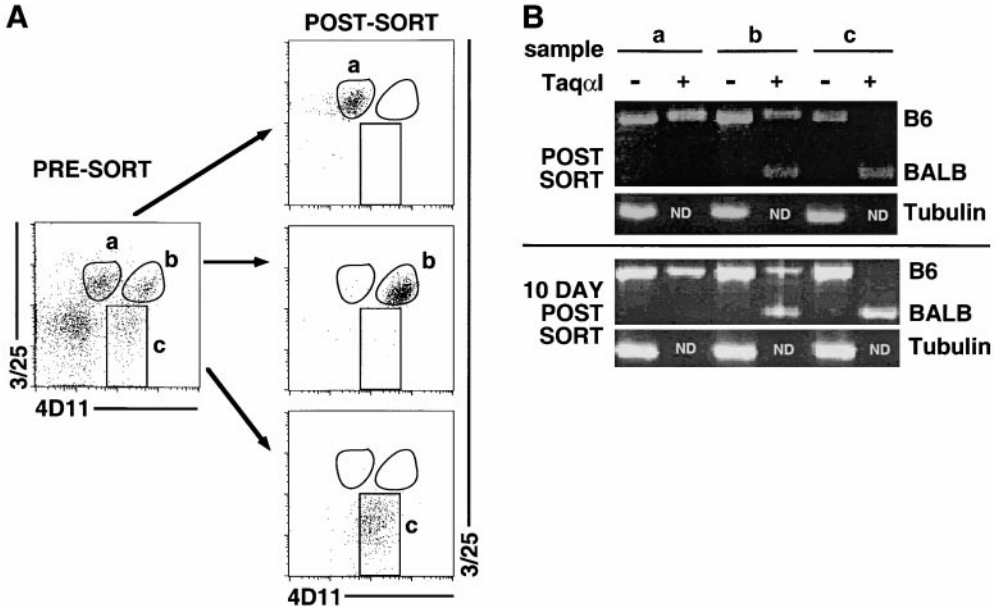


**Figure 3.** Cell sorting and identification of Ly49G2 populations. Pooled, freshly isolated nylon wool nonadherent spleen cells from (B6 × BALB.B)F1 mice were stained with NK1.1, 3/25, and 4D11. (A) Three populations of NK1.1$^{+}$ cells were separated by cell sorting: (a) 3/25$^{+}$4D11$^{low}$; (b) 3/25$^{+}$4D11$^{high}$; and (c) 3/25$^{-}$4D11$^{+}$. As tested by postsort analysis, the populations were each of >90% purity. (B) The Ly49G2 alleles expressed by each population were determined by RT-PCR with Ly49G2 specific primers. Before electrophoresis, PCR products were digested with Taq$\alpha$1, a diagnostic restriction enzyme that cleaves only the Ly49G2$^{BALB}$ cDNA. RT-PCR analysis was performed on cells immediately postsort (top) and after in vitro expansion for 10 d in the presence of IL-2 (bottom). Because the tubulin PCRs were tested only without digestion, the Taq$\alpha$1 lanes are empty (ND). The experiment was repeated with NK cells from class I–deficient mice with identical results.

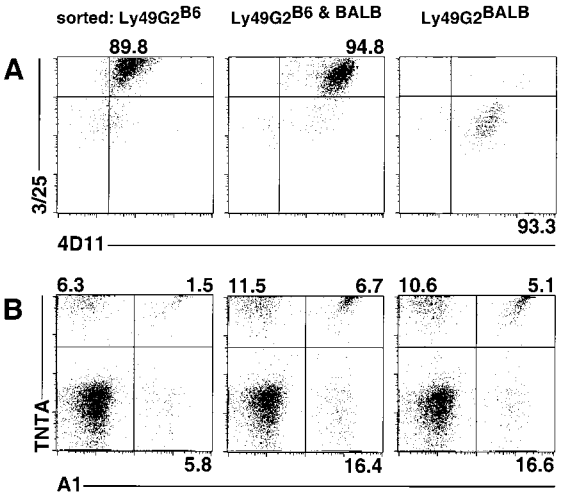


**Figure 4.** Independent allelic expression of Ly49A and Ly49G2. NK cells expressing Ly49G2$^{B6}$ only, Ly49G2$^{BALB}$ only, or both Ly49G2$^{B6}$ and Ly49G2$^{BALB}$ were sorted and expanded for 10–15 d in medium containing IL-2. (A) Reanalysis of the sorted populations with Ly49G2 mAbs demonstrated that they remained >89% pure after the culture. (B) Analysis of the sorted populations using allele-specific Ly49A mAbs. The experiment was repeated with NK cells from class I–deficient mice with similar results.

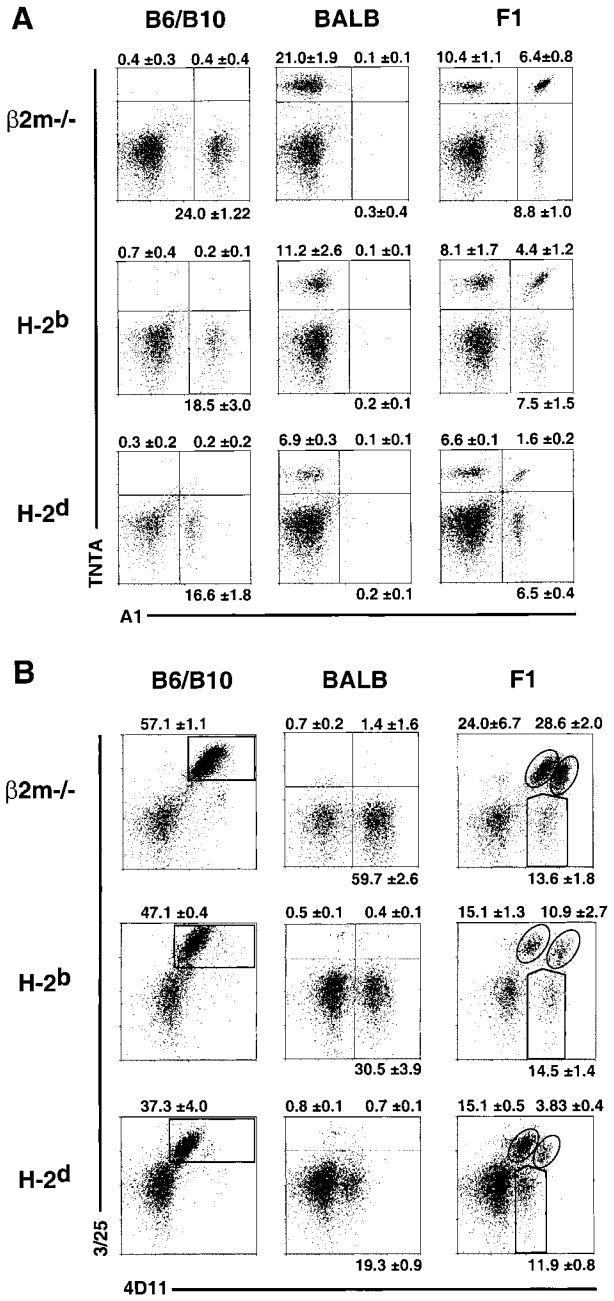


**Figure 5.** MHC class I molecules determine the extent of biallelic Ly49 expression. Freshly isolated, nylon wool nonadherent, gated DX5$^+$CD3$^-$CD8$^-$ NK cells from MHC-different mice were examined for mono- and biallelic expression of (A) Ly49A and (B) Ly49G2. In each case, Ly49 heterozygous (F1) mice were compared with the parental strains. Data represent means ± SD of three or four determinations on cells from individual animals.

and 4D11 demonstrated predominant monoallelic expression of Ly49G2, with a smaller biallelic population in each case (Fig. 5 B). As reported previously (29), the frequencies of NK cells expressing Ly49A or Ly49G2, regardless of which allele, were marginally lower in H-2$^b$ mice than in β2m$^{-/-}$ mice, and lower yet in H-2$^d$ mice (which express the strong D$^d$ ligand for both Ly49A and Ly49G2; Fig. 5 and Table I). More significantly, the size of the biallelic population varied substantially depending on MHC expression. Ligand-expressing H-2$^d$ mice contained substantially fewer biallelic Ly49A$^+$ cells than either H-2$^b$ or class I–deficient mice (Fig. 5 A and Table I). A similar picture emerged when Ly49G2 expression was examined. H-2$^d$ F1 mice contained substantially fewer Ly49G2 biallelic cells than H-2$^b$ F1 mice, which in turn had significantly fewer biallelic cells than class I–deficient mice (Fig. 5 B, and Table I). These data indicate that expression of a strong Ly49 ligand results in restricted biallelic expression of both Ly49A and Ly49G2.

## Discussion

The new mAbs allow the direct detection of NK populations expressing Ly49A and Ly49G2 in a monoallelic or biallelic fashion. The data confirm earlier findings suggesting that Ly49 genes are expressed in a predominantly, but not exclusively monoallelic fashion. Most NK cells that express Ly49A or Ly49G2 express one or the other allele of the respective gene, with approximately equal likelihood. A smaller fraction of cells expresses both alleles. Biallelic Ly49G2$^+$ NK cells are more frequent than biallelic Ly49A$^+$ NK cells, consonant with the higher overall frequency of Ly49G2$^+$ NK cells. Consistent with previous studies of Ly49A, our data suggest that monoallelic expression of Ly49G2 is a stable phenotype that is maintained as NK cells divide in culture.

The mAbs also allow direct cell surface detection of cells that coexpress Ly49A and Ly49G2 alleles in various combi-

**Table I.** *Monoallelic and Biallelic Ly49 Expression Varies with MHC*

| | Percentage of NK cells expressing indicated Ly49 alleles* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Ly49A$^{B6/BALB}$ | | | | Ly49G2$^{B6/BALB}$ | |
| Heterozygote | Ly49A$^{B6}$ | Ly49A$^{BALB}$ | Predicted‡ | Observed | Ly49G2$^{B6}$ | Ly49G2$^{BALB}$ | Predicted‡ | Observed |
| $\beta 2m^{-/-}$-F1 | 15.2 ± 1.3 | 16.8 ± 1.5 | 2.6 | 6.4 ± 0.8 | 52.6 ± 6.6 | 42.2 ± 3.1 | 22.1 | 28.6 ± 2.0 |
| H-2$^b$-F1 | 11.9 ± 2.6 | 12.4 ± 3.0§ | 1.5 | 4.4 ± 1.4§ | 26.0 ± 3.9‖ | 25.4 ± 3.8‖ | 6.7 | 10.9 ± 2.7‖ |
| H-2$^d$-F1 | 8.1 ± 0.4§ | 8.2 ± 0.3§ | 0.7 | 1.6 ± 0.2‖ | 19.0 ± 0.1¶ | 15.7 ± 1.2¶ | 3.0 | 3.8 ± 0.4¶ |

*NK cells from the indicated F1 mice were stained as shown in Fig. 5. Data represents mean ± SD of results from three or more individual mice.
‡The frequency of biallelic cells predicted by assuming completely independent allelic expression, calculated by multiplying the frequency of cells expressing one allele by the frequency of cells expressing the other allele.
§$P < 0.05$ versus group above by Student's *t* test.
¶$P < 0.02$.
‖$P < 0.01$.

nations. The data indicate that every possible assortment of the four Ly49A and Ly49G2 alleles is represented in the NK cell population. Notably, an NK cell that expresses a given Ly49G2 allele can coexpress a Ly49A allele from the same or opposite chromosome with equal likelihood. These data represent strong evidence that the mechanism that chooses a Ly49 allele for expression acts highly locally on the chromosome, without enhancing or diminishing the probability that another gene nearby in the closely linked gene complex will be expressed. It can be concluded that these mechanisms do not "open" or "close" the entire array of Ly49 genes on a given chromosome, but rather act individually on each Ly49 allele. Together with studies suggesting that the average NK cell expresses approximately three Ly49 receptors (20), the data suggest that each cell expresses a patchwork of expressed Ly49 genes selected from one or the other or both chromosomes.

A novel feature of the data is the finding that expression of one Ly49A allele is not completely independent of expression of the other. This conclusion is suggested by deviations between the observed percentages of biallelic NK cells and the percentages calculated under the assumption of allelic independence. The latter value corresponds to the frequency of Ly49A$^{B6+}$ cells multiplied by the frequency of Ly49A$^{BALB+}$ cells. Regardless of MHC haplotype, biallelic expression of Ly49A was two- to threefold more common than predicted by the product rule (Table I). Biallelic expression of Ly49G2 also deviated from the predictions of the product rule, but to a much smaller extent (Table I). As noted before (43), the fact that the Ly49 repertoire is controlled to some extent by interactions with host MHC molecules predicts that receptor coexpression is not completely independent, and should lead to deviations from the product rule. However, as deviations from the product rule were observed even in class I–deficient mice (Table I), they are unlikely to result solely from interactions with MHC molecules. The fact that biallelic cells were always more frequent than predicted by the product rule suggests instead that heterogeneity exists in developing NK cells in factors that control expression of Ly49A, with some cells exhibiting a generally higher likelihood of initiating Ly49A expression than others. There are many possible explanations for such variations, but one possibility is that the transcription factor TCF-1, previously reported as necessary for Ly49A expression (44), is not expressed equally in all NK progenitors.

An important finding of the study is that MHC class I genes determine the extent of biallelic Ly49 receptor gene expression. This conclusion was hinted at in a previous clonal analysis, which indicated a greater extent of biallelic expression in class I–deficient mice than in class I$^+$ mice (22). However, the small number of clones analyzed precluded quantitative comparisons, and could not assess whether biallelic expression differs in mice that express different MHC haplotypes. In this study, a strong deficit in biallelic Ly49A and Ly49G2 NK cells was clearly evident in H-2$^d$ mice, with H-2$^b$ mice intermediate and class I–deficient mice containing the highest percentage of biallelic cells. The control of biallelic expression is thus similar to the control of bigenic Ly49 expression. Previous studies showed that NK cells expressing multiple different Ly49 receptors are less frequent in class I$^+$ mice than in class I–deficient mice (29, 45). In the case of Ly49A and Ly49G2, two receptors with strong reactivity with H-2$^d$ and not H-2$^b$, the percentage of Ly49A/G2 double-positive cells was lowest in H-2$^d$ mice, intermediate in H-2$^b$ mice, and highest in class I–deficient mice, similar to the pattern observed in this report with respect to biallelic expression (29; Fig. 5). These data support the conclusion that the MHC-specific education mechanisms that regulate coexpression of different Ly49 genes similarly regulate coexpression of the two alleles of a given Ly49 gene.

The regulation of biallelic expression by MHC molecules is consistent with a previously proposed model of NK cell education (19, 21). The regulated sequential expression model proposed that as Ly49 receptor genes are stochasti-

cally switched on during NK cell development, engagement of an expressed receptor with host MHC class I molecules initiates feedback inhibition that reduces the likelihood that additional Ly49 receptor genes will be activated. This mechanism should prevent individual NK cells from expressing an excess of self-MHC–specific Ly49 receptors, and thus could optimize the sensitivity of NK cells to changes in host cell class I expression. Our data indicates that this form of regulation controls not only expression of different Ly49 genes, but also different alleles of a given Ly49 gene. A virtue of the model is that it can account for the reduced frequencies of biallelic Ly49A or Ly49G2 populations, as well as of the Ly49A/G2 coexpressing subset, in H-$2^b$ mice versus $\beta 2m^{-/-}$ mice, neither of which have strong ligands for these receptors. This is because some NK cells may express NK receptors specific for H-$2^b$ class I molecules before they express Ly49A or Ly49G2. The model predicts that in H-$2^b$ but not $\beta 2m^{-/-}$ mice, such cells will be inhibited from expressing additional Ly49 receptors, including Ly49A and Ly49G2. The reduced overall frequencies of NK cells expressing Ly49A or Ly49G2 should result in a lower percentage of cells coexpressing these receptor alleles, as was observed (Fig. 5 and Table I).

As the two alleles of a given Ly49 gene do not necessarily exhibit significant differences in specificity (14), it is interesting to ask why these genes are expressed in a predominantly monoallelic fashion. One possibility is based on the findings herein that each Ly49 allele is expressed mostly independently of the opposite allele and of alleles at other Ly49 loci. The mechanism that evolved to switch on a given Ly49 gene in only a fraction of progenitor cells apparently acts on cis-acting elements in each gene, and thus may activate one allele without activating the other. The result is a variegated, overlapping repertoire. A consequence of this is that some NK cells will express receptors specific for one self-MHC molecule and not others. These NK cells can attack self-cells that downregulate that class I molecule selectively. In this view, monoallelic expression is simply a by-product of the mechanism that evolved to ensure variegated expression of Ly49 genes, which happens to act independently on each Ly49 allele. Alternatively, the finding herein that biallelic expression is strongly regulated by MHC class I molecules presents another possibility. By allowing NK cells to express one or two doses of the same gene, the sensitivity of developing NK cells to inhibition by a given class I ligand may be adjusted more precisely. With a weaker ligand, biallelic expression may yield a greater amount of receptor on the cell surface, resulting in a greater inhibitory response in the NK cell, whereas monoallelic expression may be more beneficial in the case of a strong class I ligand. The reagents reported in this paper may be useful in testing the latter model.

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