Independent Control of *Ly49g* Alleles: Implications for NK Cell Repertoire Selection and Tumor Cell Killing[1]

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A novel murine NK cell-reactive mAb, AT8, was generated. AT8 recognizes Ly49G from 129/J, BALB/c, and related mouse strains, but does not bind to Ly49G^{B6}. Costaining with AT8 and a Ly49G^{B6}-restricted Ab (Cwy-3) provides the first direct evidence that Ly49G protein is expressed from both alleles on a significant proportion of NK cells from four different types of F_1 hybrid mice. The observed level of biallelic Ly49G expression reproducibly followed the product rule in both freshly isolated and cultured NK cells. Surprisingly, the percentage of NK cells expressing both Ly49G alleles could be dramatically increased in vitro and in vivo through IL-2R- and IFN receptor-dependent signaling pathways, respectively. Unexpectedly, Ly49G^{B6+} NK cells in an H-2^d, but not H-2^b, background were more likely to lyse D^{d+} and Chinese hamster ovary tumor cells than Ly49G$^{BALB/129+}$ NK cells. Furthermore, Ly49G^{B6+} NK cells also proliferated to a higher degree in response to poly(I:C) than NK cells expressing a non-Ly49G^{B6} allele in an H-2^d, but not H-2^b, background. These results suggest that Ly49G^{B6} has a lower affinity for H-2D^d than Ly49G$^{BALB/129}$, and the genetic background calibrates the responsiveness of NK cells bearing self-specific Ly49. Other H-2D^d receptors on the different Ly49G$^+$ NK cell subsets were unequally coexpressed, possibly explaining the disparate responses of Ly49G^{B6+} NK cells in different hybrid mice. These data indicate that the stochastic mono- and biallelic expression of divergent Ly49G alleles increases the range of MHC affinities and the functional potential in the total NK cell population of heterozygous mice. ***The Journal of Immunology,*** **2004, 172: 1414–1425.**

Natural killer cells express a complex array of target cell receptors controlling activation such as killer cell Ig-like receptor (KIR),[3] Ly49, 2B4, NKG2/CD94, NKG2D, NKp44, NKp46, and others (1). The ligands of these receptors are diverse and include MHC, viral MHC mimics, integrins, viral hemagglutinins, and MHC-related stress-inducible proteins. The importance of human NK cell receptors in innate immunity is illustrated by statistical correlations between the presence of a particular KIR (KIR2DS1) and corresponding MHC ligand in HIV control and progression to AIDS (2). In mice, the functional analogues to human KIR are the *Ly49* family of NK cell receptors. Although they represent structurally distinct protein families, the murine C-type lectin-related Ly49 and the human Ig-related KIR protein families are both expressed in a variegated fashion in NK cell subsets (3, 4). Furthermore, KIR and Ly49 associate with identical signaling molecules to achieve either activation or inhibition of NK cells in response to MHC class I ligands (5; http://stke.sciencemag.org/cgi/content/full/OC_sigtrans;2001/75/re1).

The role of at least one activating Ly49 appears to be viral recognition. The ability of some mouse strains to control mouse CMV (MCMV) titers in the spleen (*Cmv1^r*) has been found to correlate with the presence of the *Ly49h* gene, and depletion of Ly49H$^+$ NK cells leads to higher viral titers in infected organs (6–8). Ly49H signals for the activation of NK cells by binding to the MCMV-encoded protein m157 expressed on the surface of infected cells (9). Interestingly, the putative Ly49H allele in MCMV-susceptible 129/J mice, Ly49U, does not bind to m157 (9). Furthermore, the inhibitory Ly49I^{129}, but not Ly49I^{B6}, shows affinity for m157. Thus, variation in *Ly49* alleles may dramatically affect immunity to infectious pathogens. Resistance to other viruses, such as ectromelia and HSV, has also been mapped to the NK gene complex in which the *Ly49* gene cluster is found (10–12).

Human NK cells express between two and nine KIR and NKG2 receptors per cell; however, despite such heterogeneity, each NK cell possesses an inhibitory receptor for one or more self HLA Ags (4). In the general human population, the combination of *KIR* genes expressed among individuals of even the same ethnicity is highly variable due to a large number of distinctive KIR haplotypes (13). There are a minimum of 15 different KIR haplotypes, each with different numbers and types of *KIR* genes, which could account for hundreds of different diploid genotypes (14). This does not take into account the polymorphism between alleles of the same haplotype, suggesting that the number of human NK cell receptor repertoires is extremely large. To date, only two *Ly49* haplotypes have been characterized in inbred mice, although at least five are predicted to exist based on early RFLP studies (15). The C57BL/6 (B6) *Ly49* haplotype was mapped by a number of groups and confirmed by the B6 mouse genome-sequencing

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[3] Abbreviations used in this paper: KIR, killer cell Ig-like receptor; CHO, Chinese hamster ovary; MCF, mean channel fluorescence; MCMV, mouse CMV; RNK, rat natural killer.

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project (16–19). The Ly49 repertoire of 129/J mice was revealed through the characterization of 10 Ly49-related transcripts with complete open reading frames, including three activating Ly49 (20). In contrast, the B6 haplotype contains only two functional activating Ly49. Analysis of the 129 genome resulted in the discovery and mapping of 19 Ly49-related genes, some of which are known only from a few exons. The 129/J Ly49 gene cluster is approximately the same size as the B6 cluster (~600 kb), and contains sets of genes not recognizable in the B6 haplotype, as well as lacking some Ly49 gene blocks found in the B6 mouse (21). Therefore, like the KIR, Ly49 haplotypes can vary in the number and type of genes present.

Murine NK cells express up to six *Ly49* and *NKG2* genes, with an average of three different receptors per cell (3). The process by which *Ly49* gene expression is initiated is not understood, but early reports suggested that expression is stochastic, such that the frequency of NK cells expressing two or more *Ly49* genes is approximately the same as the product of the individual frequencies (the product rule) (22). The MHC class I background of the mouse affects the level of expression of Ly49s that interact with self class I, but not the frequency of NK cells that express a particular Ly49 (23). Also, the activating Ly49D and H proteins are coexpressed to an extent greater than predicted by the product rule (24). An early report using RT-PCR analysis of sorted NK cells from hybrid mice suggested that *Ly49a* and *c* gene expression are subject to monoallelic exclusion (25). However, subsequent RT-PCR studies detected single NK cells expressing mRNA from both *Ly49a* alleles (26). Similar studies have shown that this is also true for Ly49G (27). The probabilistic mechanism that generates variegated Ly49 expression may account for the low frequency coexpression of both alleles of a single gene. Using Ly49A allele-specific mAb, biallelic expression was detected at the protein level (28). Such evidence was also presented for Ly49G, but was complicated by a lack of allele-specific mAb. Stochastic mechanisms also appear to regulate KIR allele expression, because human NK cells can express one or both alleles for *KIR3DL1* and *3DL2*, with a single induced allele being more common than expression of both alleles (29).

Certain *Ly49* genes appear to be conserved between the two Ly49 haplotypes that have been mapped (B6 and 129/J), such as *Ly49q*, *e*, and *g* (21). Serologic evidence indicates that Ly49G is a common receptor gene because the 4D11 mAb binds to an NK cell subpopulation in all mouse strains tested (30). $Ly49G^{B6}$ binds to mouse class I MHC ($H\text{-}2D^d$) as determined by target cell lysis and soluble MHC tetramer binding (31, 32). The characterization of *Ly49g*-related cDNAs from several mouse strains revealed that the level of nucleotide sequence conservation of the coding region is from 98 to 100% (33). Surprisingly, Ly49G from B6 mice is actually the most divergent allele from all studied mouse strains, including CBA/J, C3H/HeJ, 129/J, and BALB/c. For example, 129/J and BALB/c Ly49G alleles only have 1 aa difference, while they both differ from B6 by 11 aa. While investigating the Ly49 repertoire of 129/J mice, we discovered that $Ly49G^{129}$ possesses different binding affinities for soluble class I MHC tetramers compared with those reported for $Ly49G^{B6}$ (20). Similarly, the ectodomain of $Ly49G^{BALB}$ (which is almost identical with $Ly49G^{129}$) more efficiently binds $H\text{-}2D^d$, $H\text{-}2L^d$, and xenogeneic MHC ligands than that of $Ly49G^{B6}$ (34). A study of Ly49A alleles from multiple mouse strains also found allelic variability in MHC binding (35). These studies show that, like KIR, different Ly49 alleles can have distinct MHC ligands.

In this study, we describe the production and characterization of a novel mAb (AT8) that is specific for the 129 and BALB/c alleles of Ly49G. Monoclonal AT8 was used in conjunction with the $Ly49G^{B6}$-specific mAb Cwy-3 to study Ly49G allele coexpression in F_1 hybrid mice and their role in NK cell-mediated tumor cell lysis and proliferation in vivo.

Materials and Methods

Mice, NK cells, and cell lines

C57BL/6, 129/J, CBA/J, SWR/J, C3H/HeJ, BALB/c, SJL inbred mice, and $(CBA \times B6)F_1$, $(C3H \times B6)F_1$, $(129 \times B6)F_1$, and $(BALB \times B6)F_1$ hybrid mice were purchased from The Jackson Laboratory (Bar Harbor, ME). $(BALB \times B6)F_1$ were also purchased from Harlan Sprague-Dawley (Chicago, IL). 129Sv/J, CBA/NCr, and C3H/HeNCr were purchased from the National Cancer Institute (Frederick, MD). All mice were kept under pathogen-free conditions until use between 5 and 20 wk of age. All cell culture reagents were purchased from Invitrogen (Burlington, Ontario, Canada), unless otherwise indicated. Mouse NK cells were generated by culturing splenocytes depleted of RBC by ACK lysis for 3 days in RPMI 1640 medium containing 1000 Cetus U/ml human IL-2 (Hoffmann-LaRoche, Nutley, NJ) or 25% v/v of supernatant from the murine IL-2-producing cell line X360 (36), 10% FCS, 100 μM nonessential amino acids, 1 mM sodium pyruvate, 10 mM HEPES, 5×10^{-5} M 2-ME, 2 mM L-glutamine, and 100 U/ml penicillin plus 100 μg/ml streptomycin, followed by removal of plastic-nonadherent cells and culturing for a further 2–4 days. Routinely, 75–90% of the resulting cells were $NK1.1^+$. YB2/0, $YB2/0.D^b$, $YB2/0.D^d$, and $YB2/0.K^k$ rat cell lines were kindly provided by J. Ryan and M. Nakamura (University of California, Veterans Affairs Medical Center, San Francisco, CA). The X360 cell line was a kind gift of A. Veillette (Institut de Recherches Cliniques de Montreal). YB2/0 and X360 cells were grown in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, and 100 U/ml penicillin plus 100 μg/ml streptomycin (plus 1 mg/ml G418 for YB2/0 derivatives). Chinese hamster ovary (CHO) cells were grown in complete DMEM (DMEM supplemented with 10% FCS, 2 mM L-glutamine, and 100 U/ml penicillin plus 100 μg/ml streptomycin). Hybridomas were produced using the FO myeloma (American Type Culture Collection, Manassas, VA). FO was maintained in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin plus 100 μg/ml streptomycin, 10 mM HEPES, and 20 μg/ml azaguanine. Hybridomas were grown in hypoxanthine/aminopterin/thymidine medium (DMEM supplemented with 20% FCS, 2 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 1.6×10^{-5} M thymidine (Sigma-Aldrich, St. Louis, MO), 1×10^{-4} M hypoxanthine, 10% NCTC 109 medium, 1× antibiotic-antimycotic, 8×10^{-7} M aminopterin). The 129/J-Ly49 stable transfectants were generated with previously described pEF6 (Invitrogen) expression constructs. The 293- and L929-Ly49 or empty vector transfectants were grown in complete DMEM supplemented with 5 μg/ml blasticidin. Rat natural killer (RNK) stable transfectants were maintained in NK cell medium (without IL-2) plus 5 μg/ml blasticidin.

Abs, flow cytometry, and cell sorting

The following mAb were purchased from BD PharMingen (Mississauga, Ontario, Canada), unless listed otherwise, and used in multicolor flow cytometry (FACSort or FACSCalibur; BD Biosciences, Mountain View, CA) and cell sorting (MoFlo; Cytomation, Fort Collins, CO): FITC-labeled 4E5 ($Ly49D^{B6}$, $R/O/V^{129}$), PE- and FITC-4D11 (reacts with Ly49G from all strains tested and $Ly49T^{129}$), FITC-5E6 ($Ly49C/I^{B6}$), FITC-A1 ($Ly49A^{B6}$), purified and FITC-Cwy-3 ($Ly49G^{B6}$), APC-DX5 (pan NK cell), and PE-NK1.1 (B6 NK cells). The 1F8 ($Ly49C/I/H^{B6}$, I/U^{129}) was a generous gift from V. Kumar (University of Chicago, Chicago, IL). Hybridoma AT8 (mouse IgG2a, κ) was isotyped with IsoStrip (Roche, Basel, Switzerland), used as hybridoma supernatant, and detected by FITC-conjugated goat anti-mouse IgG (H + L) (Cedarlane Laboratories, Hornby, Ontario, Canada) or PE-conjugated goat anti-mouse Ig. For staining of fresh splenocytes, AT8 and purified Cwy-3 were detected by FITC rat anti-mouse IgG2a/2b and PE rat anti-mouse IgG1 mAbs, respectively, to avoid B cell cross-reactivity. Before addition of further Abs, unoccupied binding sites of secondary Ab were blocked with mouse serum (Sigma-Aldrich). AT8 was also purified from depleted cell-free culture supernatant by affinity purification on protein A-Sepharose (Amersham Biosciences, Baie d' Urfe, Quebec, Canada) using standard protocols. Purified AT8 was biotinylated with biotin-7 normal human serum (Roche), according to the manufacturer's instructions, and detected with streptavidin-PE.

mAb production

Five to ten million IL-2-activated 129/J NK cells were injected i.p. into B6 mice every 2 wk for a total of four challenges over 2 mo. Serum reactivity from tail-bled mice was assessed against 129/J NK cells before sacrifice. Spleens were removed and single cell suspensions were prepared before fusion to FO myeloma cells, essentially as described by Lerner (37).

Briefly, cells were mixed in a spleen-myeloma ratio of 3:1 and fused by slow addition of polyethylene glycol 1500 (Roche), slowly diluted with hypoxanthine/aminopterin/thymidine, seeded into 96-well flat-bottom plates, and cultured for 7–10 days. Supernatants from wells with macroscopic colonies were tested against 129/J NK cells for reactivity and detected with a FITC-conjugated mouse Ig-specific polyclonal Ab on a FACScan. NK cell-reactive hybridomas were single-cell cloned twice, expanded, isotyped, and used for analysis.

Cytotoxicity assay

IL-2-activated NK cells were washed with PBS, resuspended in complete RPMI medium, and seeded into wells of a 96-well, round-bottom, microtiter plate in graded dilutions to obtain the desired E:T ratios. In some cases, nonspecific rat IgG2a or 4D11 mAb was added for a final concentration of 1 μg/well. Target cells were labeled with 100 μCi $Na_2{}^{51}CrO_4$ (ICN Biomedicals, Toronto, Ontario, Canada) for 1 h at 37°C, washed three times, resuspended in complete RPMI medium, and added to the microtiter plate at a concentration of 5×10^3 cells/well. The microtiter plate was then incubated for 4 h at 37°C and 5% CO_2 in a 95% humidified atmosphere. Following centrifugation of the microtiter plate, 100 μl of supernatant was collected from each well, and ^{51}Cr release (in cpm) was ascertained by gamma counting. Percent lysis was determined by the following equation: percent lysis = $(E - S)/(M - S) \times 100$, in which E is the release from experimental samples, S is the spontaneous release, and M is the maximum release upon lysis with 10% SDS.

In vivo NK cell proliferation

Mice were injected i.p. with 200 μg of poly(I:C) (Sigma-Aldrich) in 200 μl of sterile Dulbecco's PBS on day 0. On day 4, mice were sacrificed by cervical dislocation, and the spleen was removed for analysis. After RBC lysis, the number of total splenocytes was determined for day 0 and 4 spleens, and individual samples of 10^6 splenocytes were stained with PE-NK1.1 and FITC-5E6, AT8 plus FITC anti-murine IgG2a, or FITC-Cwy3, and analyzed on a FACScan. Day 0 values were calculated as follows: (percentage of NK1.1$^+$Ly49$^+$ splenocytes) $\times$ (total number of splenocytes), and set at 100%. Day 4 percentage increases for the indicated NK cell subset were calculated as follows: ((percentage of NK1.1$^+$Ly49$^+$ splenocytes $\times$ total number of splenocytes)/(number of NK1.1$^+$Ly49$^+$ splenocytes from day 0)) $\times$ 100%.

Results

Production of the Ly49G allele-specific mAb AT8

In an effort to generate novel NK cell-specific mAbs, IL-2-activated NK cells from 129/J mice were injected i.p. into B6 recipients on a biweekly basis until anti-129/J NK cell reactivity was detected in the sera of immunized mice. Splenic B cells from immunized mice were fused to make hybridomas, and supernatants from these were tested for reactivity against 129/J NK cells with a labeled anti-mouse Ig secondary Ab by flow cytometry. One positive clone, AT8 (isotype: mouse IgG2a, κ), displayed a bimodal reactivity with ~50% of 129/J NK cells staining negative and 50% staining positive (Fig. 1*A*). Because variegated expression is a hallmark of the Ly49 family of class I MHC receptors, 129/J NK cells were costained with AT8 (plus a FITC-conjugated goat anti-mouse IgG-specific secondary Ab) and a panel of directly labeled Ly49-specific mAb and analyzed by two-color flow cytometry. All Ab combinations separated NK cells into double-negative, single-positive for either mAb, or double-positive populations with the exception of the 4D11-AT8 combination in which only double-negative and double-positive populations were evident (data not shown, and Fig. 1*C*). This strongly suggests that the Ag on 129/J NK cells recognized by mAb AT8 is Ly49G^{129}.

To confirm the identity of the receptor recognized by AT8, a series of cell lines stably transfected with 129/J-derived Ly49 expression constructs were analyzed by flow cytometry after staining with AT8. Like 4D11, AT8 induced a peak shift in fluorescence for L929-Ly49G^{129} cells (Fig. 1*B*). Cells expressing Ly49I, O, P, T, or V^{129} were not stained by AT8 in contrast to control mAb. These experiments formally prove that AT8 binds to Ly49G^{129}.

Other mAbs that can detect Ly49G expression have been produced, including 4D11, which binds to NK cells from numerous mouse strains (30). In contrast, some Ly49-specific mAb are also strain specific. To determine the strains with NK cells that react with AT8, IL-2-activated NK cells were prepared from different inbred mice and analyzed by two-color flow cytometry using 4D11 and AT8. L929-Ly49G^{129} and L929-pEF6 were used as a control. As shown in Fig. 1*C*, L929-Ly49G^{129} appeared as double-positive events, while L929 cells transfected with empty vector showed no staining with either mAb. NK cells from 129/J mice were separated into double-positive (Ly49G$^+$), or double-negative (Ly49G$^-$) populations showing that 4D11 and AT8 bind to the same Ag. Similarly, NK cells from C3H/HeJ, BALB/c, A/J, SJL, and FVB/N also were stained by both mAb (Fig. 1*C*, and data not shown). Only two strains were found not to react with AT8; one was B6, the host strain in which AT8 was produced, and SWR. In addition to the above listed mouse strains, several others were stained with AT8 and other Ly49G-specific mAb. Strain reactivities are shown in Table I. These results show that AT8 is a mouse strain-specific mAb, reacting with NK cells from many, but not all inbred lines. Also, as all NK cells in the strains tested were either double positive or double negative for 4D11 and AT8, it suggests that by flow cytometry these two mAb detect only Ly49G in NK cells.

Disparate mono- and biallelic expression of Ly49G on NK cells from various mouse strains

Previous studies using pan-Ly49G mAb 4D11 concluded that Ly49G was a commonly expressed receptor, but that the level of expression varied dramatically between different mouse strains (30). Furthermore, the extent to which both Ly49G alleles are expressed in the total NK cell population is unclear. Cwy-3 is a mAb developed by the Kane laboratory that is specific for the B6 allele of Ly49G (38). Using Cwy-3 in conjunction with AT8 (which does not bind Ly49G^{B6}), the allelic usage of Ly49G was analyzed in several different B6-containing F_1 hybrid mice. Interestingly, the Ly49G alleles in two different types of hybrid mice were expressed in different percentages of IL-2-activated NK cells (Fig. 2*A*). In (129 $\times$ B6)F_1 mice, Ly49G^{129} was expressed on more NK cells than Ly49G^{B6} (28% vs 22%, respectively). In contrast, more (BALB $\times$ B6)F_1 NK cells expressed Ly49G^{B6} than Ly49G^{BALB} (22 vs 17%, respectively).

Prior reports have shown that Ly49A mRNA and protein can be expressed from neither, one, or both alleles in any given NK cell (26, 28). Attempts to ascertain Ly49G biallelic expression at the protein level have been hampered by a lack of allele-specific mAb. Fresh NK cells from F_1 mice were sorted into Ly49G$^+$ and Ly49G$^-$ populations and then expanded in vitro using IL-2. During expansion, sorted populations maintained their Ly49G expression status as determined by 4D11 staining. Using Ly49G allele-specific mAbs, AT8 and Cwy-3, Ly49G$^+$ NK cells from (129 $\times$ B6)F_1 and (BALB $\times$ B6)F_1 mice clearly displayed biallelic and monoallelic expression of Ly49G (Fig. 2, *B* and *C*, respectively). Greater Ly49G biallelic expression was evident in (129 $\times$ B6)F_1 vs (BALB $\times$ B6)F_1 NK cells. This is the first clear verification at the protein level that both *Ly49g* alleles are expressed in the same cell in a significant percentage of the total NK cell population.

Interestingly, Ly49G^{129} and Ly49G^{BALB} expression in Ly49G biallelic positive NK cells displayed lower fluorescence intensity than on Ly49G monoallelic positive NK cells (Fig. 2, *B* and *C*). This effect seemed more pronounced in (BALB $\times$ B6)F_1 NK cells, with biallelic cells having a *y*-axis (AT8) mean channel fluorescence (MCF) of 48 and Ly49G^{BALB+} monoallelic cells having an MCF of 142. Similarly, Ly49G^{B6} expression on Ly49G biallelic

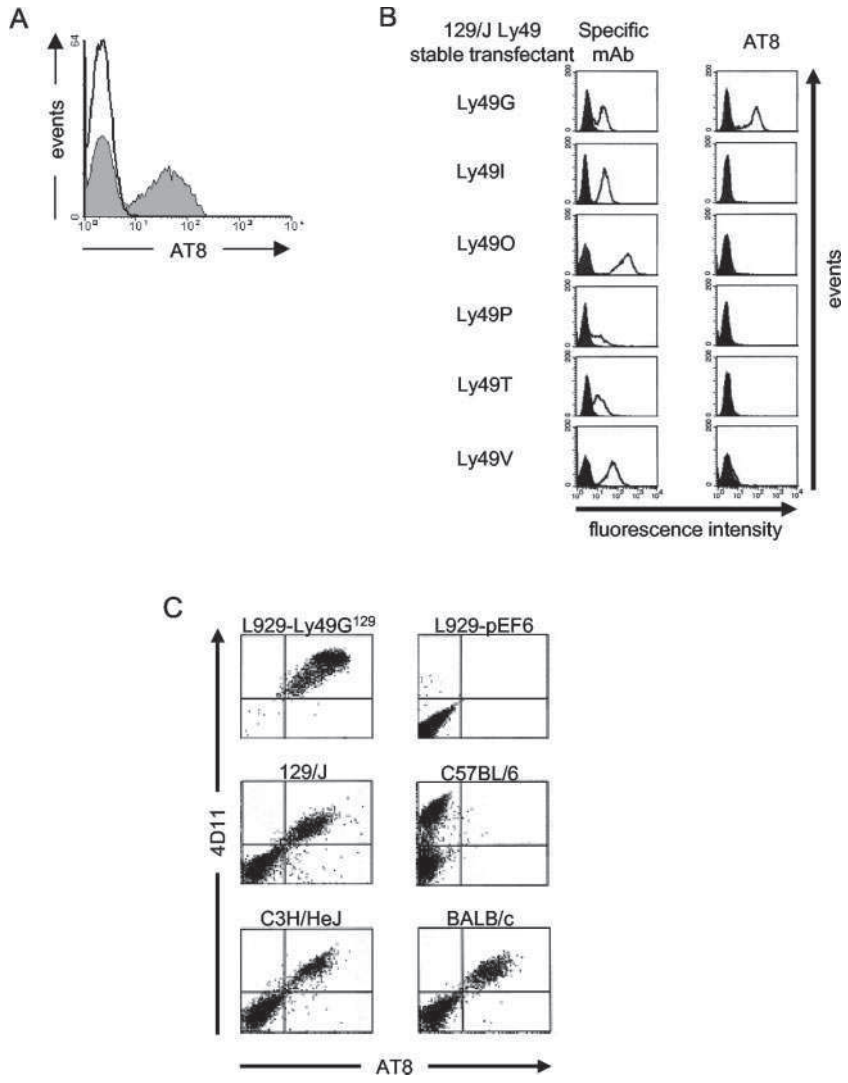

FIGURE 1. mAb AT8 binds to Ly49G in an allele-specific manner. *A*, Supernatant of hybridoma AT8 was used in a two-step staining (with PE-conjugated goat anti-mouse IgG) of 129/J IL-2-activated NK cells. The open histogram shows staining by PE secondary alone, and the filled histogram is staining with AT8, followed by PE secondary. *B*, Stable transfectants expressing *Ly49* cDNAs (open) or empty vector controls (filled; L929-pEF6 and RNK-pEF6) were stained with PE-4D11 (L929-Ly49G^{129}, L929-Ly49T^{129}), FITC-1F8 (L929-Ly49I^{129}), A1 plus FITC goat anti-mouse IgG (293-Ly49P^{129}), PE-4E5 (RNK-Ly49O^{129}, RNK-Ly49V^{129}), or AT8 plus PE or FITC goat anti-mouse Ig. Control staining for 293-Ly49P^{129} was the FITC secondary alone. *C*, IL-2-activated NK cells from the indicated mouse strains or L929 stable transfectants were stained with AT8, FITC goat anti-mouse IgG, blocked with mouse serum, followed by PE-4D11. All cells were analyzed using a FACScan.

positive NK cells was also noticeably lower in (BALB × B6)F_1 NK cells compared with monoallelic Ly49G^{B6+} NK cells (Cwy-3/*x*-axis MCF of 53 and 78, respectively). Ly49G^{B6} expression levels did not appear modulated in (129 × B6)F_1 mono- vs biallelic Ly49G$^+$ NK cells. Biallelic expression of Ly49G and lower expression of individual Ly49G alleles in biallelic expressing NK cells were confirmed using biotinylated AT8 and directly labeled (FITC) Cwy-3 to rule out cross-reactivity with secondary Abs. These data show unequivocally that both Ly-49G alleles can be expressed on a single NK cell and that expression levels of individual alleles are calibrated differentially on Ly49G mono- vs biallelic positive NK cells.

Strain-, age-, and activation-dependent biallelic Ly49G expression in fresh splenic NK cells

Biallelic Ly49G expression was readily observed in NK cell cultures described above; however, it is possible that this may have been a consequence of the prolonged stimulation through the IL-2R complex. To determine the frequency of activation of individual *Ly49g* alleles in normal unstimulated NK cells, splenocytes from (129 × B6)F_1 and (BALB × B6)F_1 mice were isolated and analyzed by flow cytometry directly ex vivo. Eight percent of DX5$^+$ gated splenocytes from 5-wk-old (129 × B6)F_1 mice were Ly49G^{129+}Ly49G^{B6+} (Fig. 3*A*). Surprisingly, DX5$^+$ splenocytes from 5-wk-old (BALB × B6)F_1 mice contained Ly-49G^{BALB+}Ly49G^{B6-} and Ly49G^{BALB-}Ly49G^{B6+} populations, but very few double-positive cells (<1%). This is in complete contrast to IL-2-activated (BALB × B6)F_1 (Fig. 2*C*) and unstimulated, age-matched (129 × B6)F_1 NK cells, which have a significant subset expressing both Ly49G alleles. Also, Ly49G^{BALB} was expressed at much lower frequencies than Ly49G^{129} in 5-wk-old hybrid mice, despite being almost identical (1 aa difference). According to the product rule, the observed (8%) level of Ly49G biallelic expression in (129 × B6)F_1 NK cells was slightly higher than expected (6%), while in (BALB × B6)F_1 NK cells the product rule correctly predicted the observed level of Ly49G allele coexpression.

The age of the mice used was considered as a factor affecting the presence of biallelic Ly49G$^+$ expression; the age of the (BALB × B6)F_1 mice used in Fig. 2*C* was 20 wk. Ly49G expression is detected in NK cells of newborn mice and plateaus after 6–8 wk of age (39). Fresh splenocytes from 8-wk-old (BALB × B6)F_1 mice (littermates of the 5-wk-old mice) were analyzed for expression of Ly49G alleles. In contrast to 5-wk-old (BALB × B6)F_1 mice, 8-wk-old littermates clearly displayed 2% of NK cells positive for both Ly49G alleles (Fig. 3*A*). Ly49G biallelic expression frequencies went up from 8 to 12% in (129 × B6)F_1 NK cells. These data show that NK cells from older mice are more likely to express both Ly49G alleles than younger mice.

Table I. *Reactivity of NK cells from different inbred mouse strains to Ly49G-specific mAbs*

Inbred Mouse Strain[a]	AT8[b]	Cwy-3	4D11
129/J, 129Sv/J	+[c]	−	+
C57BL/6	−	+	+
BALB/c	+	−	+
C3H/HeJ, C3H/HeNCr	+	−	+
SJL	+	−	+
CBA/J, CBA/NCr	+	−	+
SWR	−	−	+
FVB/N	+	−	+
A/J	+	−	+

[a] Plastic-adherent NK cells were generated from spleens of the indicated strains by culture in IL-2 for 5–6 days.
[b] NK cells were stained with AT8, 4D11, or Cwy-3, as described in *Materials and Methods*, and analyzed on a FACScan.
[c] +, Positive staining; −, negative staining.

Next, the possibility that signals emanating from the IL-2R can induce expression of silent *Ly49g* alleles was tested. Aliquots of the same 5-wk-old F_1 splenocytes described above were cultured in IL-2 for 6 days and analyzed for Ly49G allele expression. Surprisingly, in addition to single-positive populations, an unambiguous population (3%) was evident that expressed both Ly49G alleles in (BALB × B6)F_1 NK cells (Fig. 3*A*). IL-2-cultured NK cells from 5-wk-old (129 × B6)F_1 mice did not increase biallelic Ly49G expression significantly. In agreement with Fig. 2, *B* and *C*, IL-2 stimulation of NK cells from older (12-wk) F_1 mice resulted in elevated levels of biallelic Ly49G$^+$ NK cells (Fig. 3*A*). For the most part, actual biallelic Ly49G expression was similar to the predicted levels using the product rule.

To test the possibility that Ly49G biallelic expression can be stimulated by IL-2R-independent pathways, 5-wk-old F_1 mice were challenged with poly(I:C), which induces the production of type I IFN driving NK cell proliferation (40). Very few (<1%) DX5$^+$ splenocytes analyzed directly ex vivo from poly(I:C)-challenged 5-wk-old (BALB × B6)F_1 mice expressed both Ly49G alleles. In complete contrast, biallelic Ly49G expression in (129 × B6)F_1 NK cells more than doubled (8–19%). Collectively, these results show for the first time that IL-2R and IFN receptor signals can induce the expression of silent Ly49G alleles in NK cells depending on the strain background. It is also possible that cytokine receptor signaling can preferentially induce proliferation of NK cells expressing Ly49G from both alleles.

To directly test the possibility that IL-2 can turn on expression of silent Ly49 alleles, splenocytes from (BALB × B6)F_1 mice were stained with AT8 and Cwy3, sorted into $G^{BALB+}G^{B6-}$ and $G^{BALB-}G^{B6+}$ populations, and then grown in IL-2. After expansion in IL-2, the $G^{BALB+}G^{B6-}$ sorted population kept its phenotype. Surprisingly, 25% of the IL-2-expanded $G^{BALB-}G^{B6+}$ populations now expressed Ly49G^{BALB} (Fig. 3*B*). Similar experiments with (129 × B6)F_1 mice yielded similar results; $G^{129+}G^{B6-}$cells kept their phenotype after IL-2 stimulation, while 20–25% of $G^{129-}G^{B6+}$ became Ly49G^{129+} (data not shown). These experiments were very reproducible, and identical results were obtained whether cells were analyzed with directly labeled mAb or using a labeled secondary with unlabeled primary mAb. This is the first report of Ly49 expression being inducible by IL-2.

The variability of Ly49G allele usage was investigated in IL-2-activated NK cells from (CBA × B6)F_1 and (C3H × B6)F_1 hybrid mice. Biallelic Ly49G expression was present in NK cells from both types of mice, with the Ly49G^{B6} allele being expressed on a larger percentage of NK cells than Ly49G^{CBA} or Ly49G^{C3H} (Fig. 3*C*). Furthermore, NK cells expanded in IL-2 from three individual sex- and age-matched mice of each hybrid strain were compared, and the mono- and biallelic percentages of Ly49G were found to be very similar. Interestingly, biallelic Ly49G expression levels conformed to the product rule, i.e., the observed percentage of biallelic Ly49G$^+$ NK cells was almost the same as the product of the percentages of the total individual Ly49G allele percentages. As found in the other two hybrid strains, Ly49G expression levels on NK cells expressing both Ly49G alleles were lower than monoallelic Ly49G$^+$ cells. These results show that in addition to monoallelic expression, Ly49G is also expressed from both alleles in significant and reproducible subsets of NK cells from four different hybrid strains.

Differential tumor cell cytotoxicity of NK cells expressing Ly49G^{B6}, but not Ly49G$^{BALB/129}$, to ligand-bearing target cells is strain dependent

Ly49G is not expressed on the same percentage of NK cells in different inbred mouse strains (30). Similarly, in hybrid mice, the two Ly49G alleles are unequally expressed on total NK cells (Fig. 2*A*). To determine whether the different Ly49G allele-positive subsets also have functional differences, splenocytes from (129 × B6)F_1 and (BALB × B6)F_1 hybrid mice were sorted into AT8$^+$ (Ly49G$^{129/BALB+}$) or Cwy-3$^+$ (Ly49G^{B6+}) populations, expanded in IL-2, and then used in cytotoxicity assays against ^{51}Cr-labeled YB2/0 or stable transfectants expressing various mouse MHC class I proteins. Sorted NK cells were typically 80–95% Ly49G$^+$ after expansion. The (129 × B6)F_1 sorted and unsorted (bulk) NK cells were able to efficiently kill parental YB2/0 to a similar extent (Fig. 4*A*). Likewise, all (129 × B6)F_1 subsets displayed the same high cytotoxicity toward YB2/0.D^b and YB2/0.K^k target cells. In contrast, target cells expressing the Ly49G ligand (H-2D^d) were lysed differentially by sorted vs unsorted cells. The (129 × B6)F_1 NK cells sorted for expression of Ly49G^{129} or Ly49G^{B6} displayed ~50% of unsorted NK cell cytotoxicity toward YB2/0.D^d (Fig. 4*A*). This is in agreement with the findings of several groups that Ly49G^{B6} and Ly49G^{129} recognize H-2D^d (20, 31). These data further suggest that Ly49G^{129+} and Ly49G^{B6+} NK cell subsets have comparable affinity for H-2D^d.

NK cell subsets expressing different Ly49G alleles in (BALB × B6)F_1 mice were next assessed for their response to target cells bearing different class I MHC ligands. Like (129 × B6)F_1, sorted and unsorted (BALB × B6)F_1 NK cells efficiently lysed parental YB2/0, YB2/0.D^b, and YB2/0.K^k transfectants to an almost equal extent (Fig. 4*B*). Also, like (129 × B6)F_1, both Ly49G$^+$ subsets from (BALB × B6)F_1 mice displayed significantly lower cytotoxicity toward YB2/0.D^d compared with unsorted NK cells. Surprisingly, in contrast to (129 × B6)F_1, Ly49G^{B6+} NK cells from (BALB × B6)F_1 mice displayed reproducibly higher cytotoxicity toward H-2D^{d+} target cells compared with Ly49G^{BALB+} NK cells (Fig. 4*B*). These results show that Ly49G^{B6+} NK cells from (BALB × B6)F_1, but not (129 × B6)F_1 mice are less sensitive to target cells expressing H-2D^d.

To rule out that the differential response of Ly49G^{B6} from Ly-49G^{129} and Ly49G^{BALB} to cognate ligand is a consequence of using YB2/0 cells as targets, CHO cells were next used in cytotoxicity assays. CHO cells express a ligand for Ly49G (41). When CHO cells were used as targets for sorted and unsorted NK cells from (BALB × B6)F_1 and (129 × B6)F_1 mice, the pattern of cytotoxicity paralleled that of YB2/0.D^d very closely (Fig. 4, *A* and *B*). Ly49G^{B6+} and Ly49G^{129+} NK cells from (129 × B6)F_1 achieved only 25% of the lysis attained by unsorted NK cells. However, Ly49G^{B6+} NK cells from (BALB × B6)F_1 mice displayed 75% of the lysis of unsorted NK cells, while Ly49G^{BALB+} NK cells showed CHO lysis levels comparable to Ly49G$^+$ cells

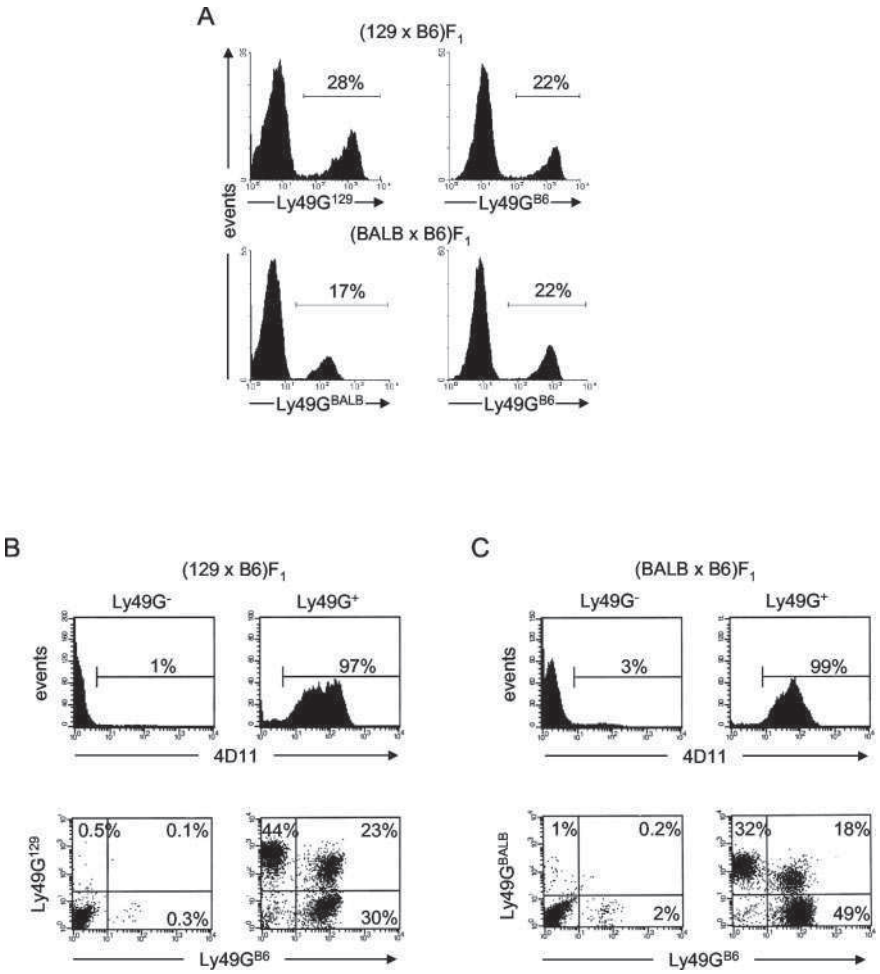

FIGURE 2. Ly49G can be expressed from one or both alleles in NK cells. *A*, IL-2-activated NK cells from adult mice of the indicated F_1 hybrid strains were stained with biotinylated AT8 plus streptavidin-PE ($Ly49G^{129/BALB}$) or FITC-Cwy-3 ($Ly49G^{B6}$). The percentage of cells staining positively is indicated. *B* and *C*, Fresh splenocytes from F_1 mice were sorted for or against Ly49G expression (4D11) and then expanded in IL-2 for 6 days before receptor analysis. *Upper panel*, Shows total Ly49G expression after in vitro expansion; *lower panel*, shows Ly49G allele expression in the sorted populations. The percentage of NK cells staining positively for one or both mAb is shown in the relevant quadrant. Stained cells were analyzed on a FACScan.

from (129 × B6)F_1 mice (Fig. 4, *A* and *B*). CHO killing by $Ly49G^{B6+}$ NK cells could be rescued to control levels by the addition of 4D11, but not control Ig (Fig. 4*C*), suggesting that most, if not all, of the inhibition seen with this subset from either mouse strain is mediated through $Ly49G^{B6}$ and not other coexpressed inhibitory receptors that also bind CHO ligands. Collectively, these data demonstrate a previously unappreciated characteristic of NK cell-mediated cytotoxicity, namely that the level of inhibition toward cognate ligand by NK cells bearing $Ly49G^{B6}$ is strain dependent.

Increased proliferation of $Ly49G^{B6+}$ NK cells after poly(I:C) challenge in (BALB × B6)F_1, but not (129 × B6)F_1 mice

During infection of B6 mice with MCMV or lymphocytic choriomeningitis virus, $Ly49G^{+}$ NK cells proliferate to a significantly higher degree than $Ly49G^{-}$, $Ly49C/I^{+}$, or bulk NK cells (42). In addition, $Ly49G^{B6+}$ cells are detected in close association with MCMV-infected cells in the spleen and liver in situ (43). Mimicking viral infection by i.p. injection of poly(I:C) also induces a preferential increase in $Ly49G^{B6+}$ NK cell numbers (40). To determine whether NK cells expressing different Ly49G alleles in heterozygous mice also display a higher proliferation capability, (129 × B6)F_1 and (BALB × B6)F_1 mice were injected with poly(I:C). Ly49 subsets were analyzed at days 0 and 4 postchallenge, and the fold increase in NK cell number was determined. As shown in Fig. 5, $Ly49G^{B6+}$ and $Ly49G^{129+}$ NK cell subsets from (129 × B6)F_1 mice displayed similar levels of proliferation to each other, and to a greater extent than bulk or $Ly49C/I^{+}$ NK cells. However, in (BALB × B6)F_1 mice, the $Ly49G^{B6+}$ subset consistently displayed a significantly higher level of proliferation compared with $Ly49G^{BALB+}$ NK cells in response to poly(I:C). Although both $Ly49G^{+}$ subsets attained 2- to 3-fold higher levels than day 0 NK cells, only the $Ly49G^{B6+}$ NK cells proliferated to a proportionally greater extent than bulk or $Ly49C/I^{+}$ NK cells in (BALB × B6)F_1 mice (Fig. 5). These in vivo proliferation results suggest that, like target cell killing, $Ly49G^{B6+}$ NK cells have a lower threshold for activation than $Ly49G^{BALB+}$ NK cells in (BALB × B6)F_1 mice, but that this phenomenon is strain dependent, as it is not observed when comparing $Ly49G^{B6+}$ with $Ly49G^{129+}$ NK cells in (129 × B6)F_1 mice. The lack of reactivity of $Ly49G^{B6+}$ NK cells from (BALB × B6)F_1 mice toward cognate ligand is possibly a result of NK cell development in an $H\text{-}2D^{d+}$ host.

Differential expression of Ly49 family members in NK cells with different Ly49G allele expression

Unequal expression of other Ly49 family members that are also receptors for $H\text{-}2D^{d}$ in $Ly49G^{B6}$ vs $Ly49G^{BALB}$ allele-positive NK cells of (BALB × B6)F_1 mice may be one mechanism for their apparent differential activation threshold to target cells bearing $H\text{-}2D^{d}$ and in vivo proliferation in an $H\text{-}2D^{d+}$ host. To evaluate this possibility, splenic NK cells from (BALB × B6)F_1 and (129 × B6)F_1 were sorted for $Ly49G^{B6}$ or $Ly49G^{BALB/129}$ expression using mAb Cwy-3 or AT8, respectively, and expanded in IL-2. Coexpression of different $H\text{-}2D^{d}$ receptors with Ly49G was determined by costaining NK cells with AT8 or Cwy-3 plus mAb specific for other Ly49. Interestingly, the levels of coexpression for the different Ly49 proteins were fairly different between the

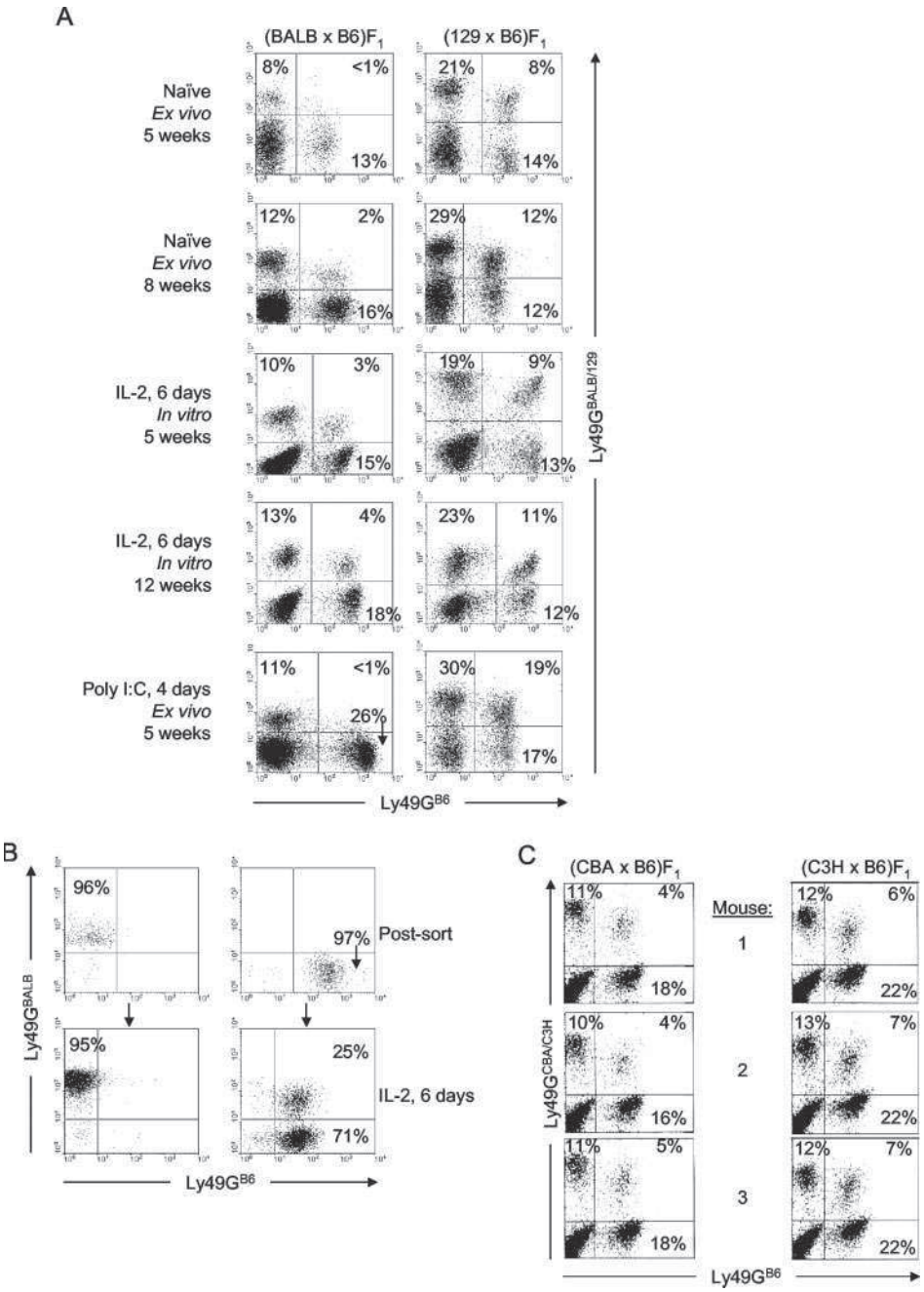

FIGURE 3. Biallelic Ly49G expression is a consequence of age, strain, and activation status. *A*, Fresh splenocytes from control or poly(I:C)-challenged F_1 hybrid mice of the indicated ages were stained with FITC-Cwy-3, APC-DX5, biotinylated AT8, and streptavidin-PE. Data are presented after gating on $DX5^+$ cells. Alternatively, fresh splenocytes from mice of various ages were cultured in IL-2 for 6 days and then stained with AT8 plus FITC anti-mouse IgG2a and Cwy-3 plus PE anti-mouse IgG1. Staining with FITC-Cwy-3 and biotinylated AT8 gave similar results. *B*, RBC-depleted splenocytes from (BALB × B6)F_1 mice were stained with AT8 plus FITC anti-mouse IgG2a and Cwy3 plus PE anti-mouse IgG1 and sorted into $AT8^+Cwy3^-$ and $AT8^-Cwy3^+$ populations. Sorted cells were expanded for 6 days in IL-2 and then stained with AT8 and Cwy3. One-step and two-step staining gave identical results. *C*, NK cells from three individual age- and sex-matched (CBA × B6)F_1 and (C3H × B6)F_1 mice were expanded in IL-2 and analyzed after staining with AT8, followed by PE goat anti-mouse IgG, blocked with mouse serum, and FITC-Cwy-3. The percentages of cells staining positively with either mAb are indicated in the appropriate quadrant. Stained cells were analyzed on a FACSCalibur.

two $Ly49G^+$ subsets in both hybrid strains (Fig. 6). Ab A1 will only detect $Ly49A^{B6}$ in either hybrid strain, as it does not react with BALB/c or 129/J NK cells (25). $Ly49A^{B6}$ coexpression in (BALB × B6)F_1 mice was approximately equal (6–8%) in $Ly49G^{B6+}$ vs $Ly49G^{BALB+}$ cells, suggesting it does not play a role in differential responses to H-$2D^d$ (Fig. 6*A*). Surprisingly, $Ly49G^{B6+}$ NK cells from (129 × B6)F_1 mice displayed more than twice the surface levels of $Ly49A^{B6}$ compared with $Ly49G^{129+}$ NK cells (30 vs 15%, respectively). These results suggest that the equal responses of $Ly49G^+$ NK cells from this hybrid strain toward H-$2D^d$ are products of very different contributions from $Ly49A^{B6}$ (Fig. 6*B*).

mAb 4E5 detects the expression of $Ly49D^{B6}$ alone in (BALB × B6)F_1 mice, as 4E5 displays no reactivity to BALB/c NK cells (30). Like Ly49A, Ly49D will bind H-$2D^d$, but results in NK cell activation leading to increased cytotoxicity and cytokine production (44). Forty percent of $Ly49G^{BALB+}$ NK cells express $Ly49D^{B6}$, compared with 25–30% of $Ly49G^{B6+}$ NK cells (Fig. 6*A*), indicating that a H-$2D^d$-specific activating receptor is more likely to be expressed with the allele of Ly49G that binds more strongly to H-$2D^d$. The 4E5 staining of (129 × B6)F_1 NK cells followed the same pattern as (BALB × B6)F_1: lower expression on $Ly49G^{B6}$ vs $Ly49G^{129}$ (Fig. 6*B*). However, 4E5 staining in this hybrid strain is less informative, as this mAb will detect the inhibitory H-$2D^d$ receptors $Ly49O^{129}$ and $Ly49V^{129}$ in addition to the activating $Ly49D^{B6}$ and $Ly49R^{129}$ (20).

Inhibitory H-$2D^d$ receptors, $Ly49C^{B6}$ and $Ly49I^{B6}$, can be detected using mAb 5E6 in (129 × B6)F_1 mice, as 5E6 was produced in a 129/J mouse and does not react with 129/J NK cells (25). In contrast to $Ly49A^{B6}$, $Ly49C/I^{B6}$ was more highly expressed on $Ly49G^{129+}$ NK cells than $Ly49G^{B6+}$ NK cells (17 vs 7%, respectively; Fig. 6*B*). This suggests a complex array of differing contributions of inhibitory signals, eventually leading to equal inhibition toward $H\text{-}2D^{d+}$ targets by $Ly49G^{129+}$ and $Ly49G^{B6+}$ NK

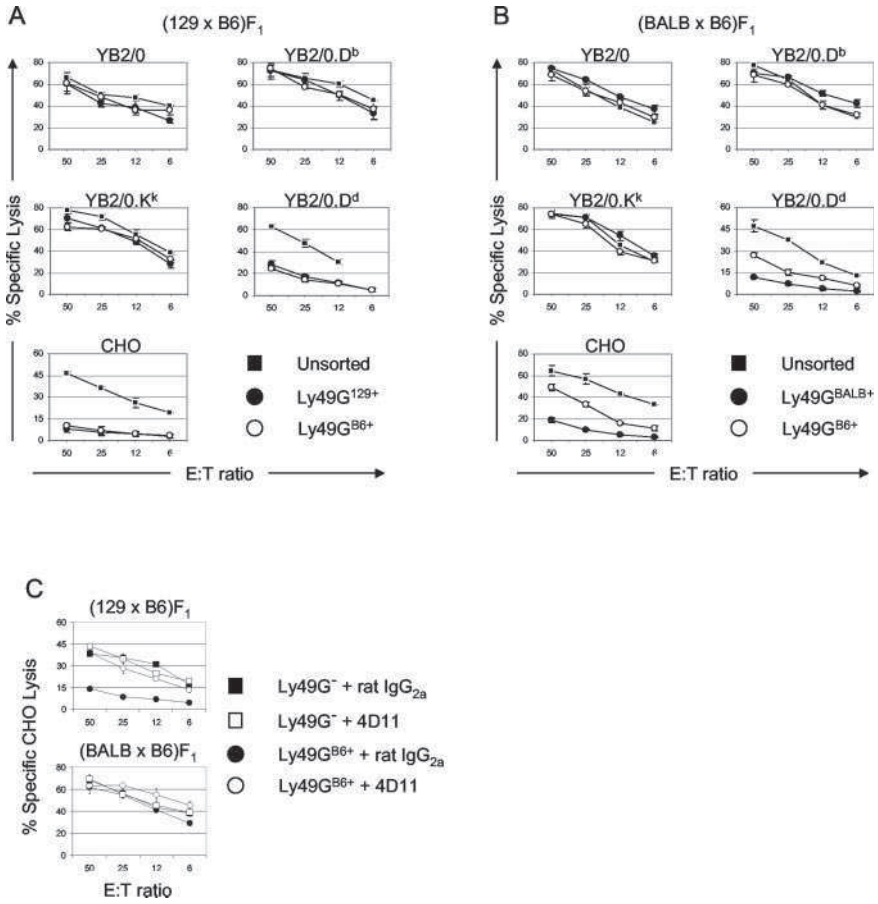

FIGURE 4. Sensitivity of Ly49G^{B6+}, but not Ly49G^{129+} or Ly49G^{BALB+} NK cells to ligand is dependent on genetic background. Fresh splenocytes from (129 × B6)F$_1$ (*A*) or (BALB × B6)F$_1$ (*B*) mice were sorted with AT8 (Ly49G$^{129/BALB}$) or Cwy-3 (Ly49G^{B6}) and expanded separately along with unsorted cells in IL-2. Activated NK cells were then used in 4-h ^{51}Cr release cytotoxicity assays against YB2/0, YB2/0.D^b, YB2/0.D^d, YB2/0.K^k, or CHO target cells at the indicated E:T ratios. *C*, The indicated IL-2-activated NK cells from fresh splenocyte sorting were used in a CHO killing assay in the presence of control rat IgG2a or 4D11. Representative data from four similar experiments are shown. The data represent the mean of triplicate wells ± SD.

cells from (129 × B6)F$_1$ mice. Overall, 5E6 staining of Ly49G$^+$ NK cells from (BALB × B6)F$_1$ mice was much greater than (129 × B6)F$_1$ mice, with Ly49G^{BALB+} NK cells expressing slightly higher levels than Ly49G^{B6+} NK cells (Fig. 6*A*). As all Ly49 that react with 5E6 in BALB/c NK cells are not known, the analysis is more complex, but Ly49C^{BALB} is most likely among the 5E6$^+$ population (45). These data suggest that the greater inhibition displayed by Ly49G^{BALB+} vs Ly49G^{B6+} NK cells to H-2D^d may be due in small part to greater expression of Ly49C alleles on Ly49G^{BALB+} NK cells. In addition, the expression levels of NKG2A/C/E were analyzed and found to be equal between the two Ly49G allele-positive subsets in both (BALB × B6)F$_1$ and (129 × B6)F$_1$ NK cells (data not shown). Collectively, these data demonstrate that other Ly49 that can bind H-2D^d are not equally coexpressed in the two Ly49G allele-positive NK subsets of (BALB × B6)F$_1$ and (129 × B6)F$_1$ mice, and that these differences may affect responses such as cytotoxicity toward H-2D^{d+} target cells and proliferation in H-2D^{d+} mice.

Discussion

The differential cytotoxicity and proliferation of Ly49G^{B6+} NK cells from (129 × B6)F$_1$ vs (BALB × B6)F$_1$ mice was a surprising finding. It is possible that Ly49G^{B6+} NK cells that develop in an H-2^d background have lower reactivity to H-2D^d and are less likely to be inhibited by binding of this ligand. Another explanation is coexpression or lack thereof of additional receptors for H-2^d ligands. NK cell responses to specific MHC ligands on target cells are additive effects of multiple Ly49 receptors as different Ly49 specific for the same MHC can be coexpressed on a single NK cell (3). In activated NK cells from (BALB × B6)F$_1$ mice, Ly49A^{B6} and Ly49C/I^{B6} are expressed almost equally on Ly49G^{B6+} and Ly49G^{BALB+} subsets (Fig. 6*A*), but Ly49G^{B6+} NK cells are clearly less inhibited by H-2D^{d+} target cells (Fig. 4*B*). These data suggest that Ly49G^{BALB} has greater affinity for H-2D^d than Ly49G^{B6}. This agrees with a recent report that RNK cells stably transfected with a chimeric activating Ly49 bearing the extracellular domain of Ly49G^{BALB} lysed H-2D^{d+} target cells to a greater extent than RNK expressing a chimeric activating Ly49G^{B6} (34). The same differential in cytotoxicity between RNK cells expressing the two Ly49G alleles was also seen for CHO cells. This agrees with our data that Ly49G^{BALB+} NK cells were inhibited to a greater degree than Ly49G^{B6+} NK cells by CHO targets (Fig. 4*B*). Furthermore, the selective inhibition of Ly49G^{129+} NK cell cytotoxicity to H-2D^{d+} target cells independently confirms earlier studies measuring the binding of soluble MHC tetramers to cells transiently transfected with various 129/J *Ly49* cDNAs (20). Tetramers of H-2D^b and H-2K^k did not bind to Ly49G^{129}-transfected 293T cells; similarly, Ly49G^{129+} NK cells lysed YB2/0.D^b and YB2/0.K^k as efficiently as the YB2/0 parental cell line (Fig. 4*A*). Therefore, the MHC specificities of Ly49G^{129} predicted by soluble tetramer binding appear to be correct.

Interestingly, in (129 × B6)F$_1$ NK cells, Ly49A^{B6} is expressed on twice as many Ly49G^{B6+} vs Ly49G^{129+} NK cells, yet cytotoxicity to H-2D^{d+} target cells is equally inhibited in both Ly49G$^+$ subsets. As Ly49G^{129} and Ly49G^{BALB} only differ in the carboxyl-terminal amino acid, their MHC affinities are likely to be very similar, if not identical. It is possible that the weaker affinity for H-2D^d by Ly49G^{B6} is compensated by the higher expression of Ly49A^{B6} on Ly49G^{B6+} NK cells and may account for the equal inhibition of the two Ly49G$^+$ subsets in (129 × B6)F$_1$ mice in response to H-2D^d. The equal responses of Ly49G^{B6+} and Ly49G^{129+} NK cells from (129 × B6)F$_1$ mice to CHO cells were

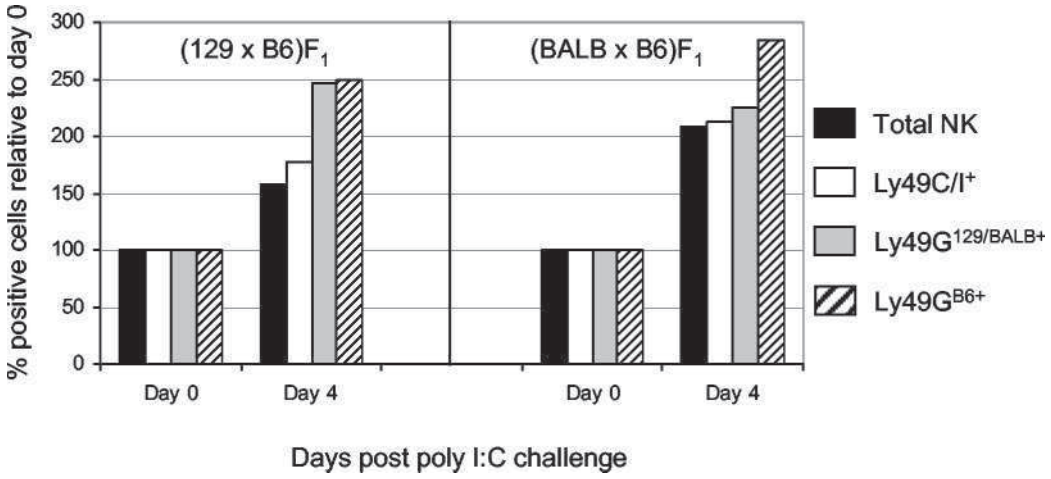

FIGURE 5. Greater proliferation of Ly49G^{B6} in (BALB × B6)F$_1$, but not (129 × B6)F$_1$ mice, in response to poly(I:C) challenge. The (129 × B6)F$_1$ and (BALB × B6)F$_1$ hybrid mice were injected i.p. with 200 μg of poly(I:C) in sterile PBS on day 0. Splenocytes were isolated and counted at days 0 and 4 postinjection, stained with PE-NK1.1 (total NK cells), FITC-5E6 (Ly49C/I), FITC-Cwy-3 (Ly49G^{B6}), or AT8 plus FITC anti-mouse IgG2a (Ly49G$^{129/BALB}$), and analyzed on a FACScan. For all subsets analyzed, only NK1.1$^+$ populations were considered. Data are presented as the percentage increase in cell numbers relative to day 0. These results are representative of four experiments.

possibly a consequence of higher Ly49A expression in Ly49G^{B6+} NK cells, although Ly49A has not been formally shown to bind any CHO ligands. The different responses to H-2D^{d+} target binding by NK cells expressing different Ly49G alleles in this study are analogous to a study of Ly49A alleles from various inbred mice and their differing capacity to bind soluble H-2D^d tetramers (35).

Evidence is provided for the first time with two specific and noncross-reacting mAb that most Ly49G$^+$ NK cells express this gene from only one allele; however, Ly49G protein can be expressed in a biallelic fashion on a significant proportion of NK cells. The frequency of NK cells coexpressing both Ly49G alleles was very different in (BALB × B6)F$_1$ vs (129 × B6)F$_1$ mice (Fig. 3*A*). Higher Ly49G biallelic expression in (129 × B6)F$_1$ may be a consequence of the lack of H-2D^d being present during NK cell maturation. Held and Kunz (27) first presented data that *Ly49g* mRNA is expressed from both alleles on some NK cells. The frequency of Ly49G$^+$ NK cells from (BALB.B × B6)F$_1$ mice (H-2^b) expressing both alleles was ~16%. In contrast, there was very little Ly49G biallelic expression in NK cells from 5-wk-old (BALB × B6)F$_1$ mice (H-2$^{b/d}$) (Fig. 3*A*). Also, unlike 129 and B6 mice, the total Ly49 repertoire from BALB/c mice has not been elucidated, and so it is possible that unknown receptors exist in this strain that cross-react with AT8 and 4D11.

The presence of the ligand H-2D^d in the hybrid mice used in the present study was also a factor, as hybrid mice in H-2^b and MHC-deficient backgrounds had significantly higher levels of Ly49G biallelic expression (28). Also, the coexpression of two H-2D^d receptors such as Ly49A and Ly49G is much lower in H-2^d vs H-2^b NK cells (40). Similarly, biallelic Ly49A expression is much higher in MHC-deficient mice compared with normal (H-2^b or H-2^d) mice (26). Finally, age was also clearly a factor in (BALB × B6)F$_1$ mice, as 8-wk-old littermates had larger and more clearly defined Ly49G$^+$ biallelic subsets. All these findings suggest that lower Ly49G biallelic expression in (BALB × B6)F$_1$ vs (129 × B6)F$_1$ is most likely MHC dependent.

Interestingly, the level of Ly49G protein was significantly less on biallelic vs monoallelic positive cells (Figs. 2, *B* and *C*; 3, *A* and *B*). The lower levels of Ly49G on cell expressing both Ly49G alleles was seen with both direct and indirect flow cytometry, and so was not a consequence of using secondary Abs. The effect was more pronounced with the non-Ly49G^{B6} alleles, but was evident for Ly49G^{B6} as well. These data indicate that Ly49G levels are calibrated depending on whether one or both alleles are transcribed. The factors controlling Ly49 expression levels are not well understood. If the modulation is a feedback mechanism in response to inhibitory signal from ligand, then this suggests that Ly49G^{129} has ligands in H-2^b mice, and Ly49G$^{CBA/C3H}$ have ligands in H-2$^{b/k}$ mice. The ligands for Ly49G$^{CBA/C3H}$ have not been reported, but Ly49G^{129} did not have any detectable affinity for H-2D^b or H-2K^b (20).

IL-2R signal transduction results in increased transcription of many types of genes, including those involved in cell cycle progression, cytotoxicity, and those coding for cell surface proteins. It has previously been shown that some splenic NK cells have the potential to express new Ly49 proteins after reintroduction into a host mouse (39). Fig. 3*A* illustrates an interesting and novel aspect of Ly49G expression, specifically, that in addition to the age of mice, NK activation by IL-2 in vitro or poly(I:C) challenge in vivo leads to previously undetected biallelic Ly49G expression, suggesting that silent *Ly49g* genes are inducible. This is a completely novel finding. It is widely thought that *Ly49* gene expression is stable during in vitro IL-2 expansion of NK cells. Indeed, the small, but obvious, increase in the number of biallelic Ly49G$^+$ cells in response to IL-2 would probably be unnoticed, as it would only increase the percentage of seemingly monoallelic Ly49G expression by a small percentage.

It appears that IL-2 in Ly49G monoallelic positive NK cells is activating the silent allele of Ly49G. This is supported by the data in Fig. 2 showing that IL-2 stimulation of NK cells sorted for or against Ly49G expression results in biallelic Ly49G expression only in previously Ly49G$^+$ cells. Definitive proof that IL-2 is turning on silent Ly49G alleles was shown in Fig. 3*B* with sorted single Ly49G$^+$ cells expanded in IL-2. Very surprisingly, unlike Ly49G$^{129/BALB+}$Ly49G^{B6-} NK cells that never turned on Ly49G^{B6} expression, a significant proportion of Ly49G$^{129/BALB-}$Ly49G^{B6+} turned on Ly49G$^{129/BALB}$. This suggests that the promoters of the different Ly49G alleles may be differentially regulated by IL-2 or that the lower affinity of Ly49G^{B6} for class I MHC results in a weaker inhibitory signal that allows the expression of additional Ly49 receptors. This implies that Ly49G$^{129/BALB}$ provides sufficient inhibitory signal to prevent the aquisition of additional Ly49 receptors, but Ly49G^{B6} does not.

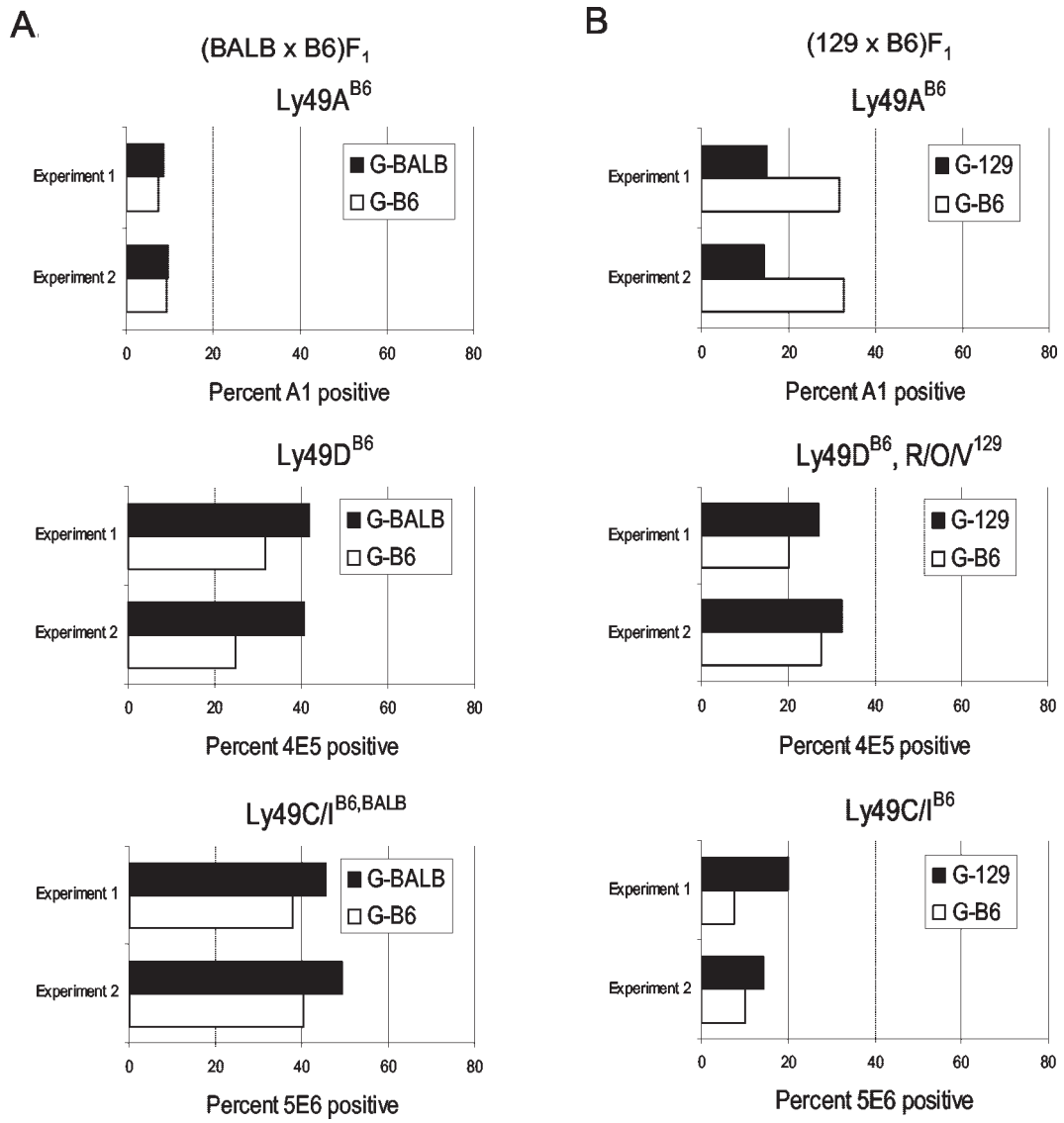

FIGURE 6. Unequal coexpression of H-2D^d receptors on different Ly49G allele-positive NK cells. NK cells from (BALB × B6)F$_1$ (*A*) or (129 × B6)F$_1$ (*B*) mice were sorted with AT8 (Ly49G$^{BALB/129}$) or Cwy-3 (Ly49G^{B6}) and expanded in IL-2. NK cells were then stained with AT8 plus PE anti-mouse IgG2a or Cwy-3 plus PE anti-mouse IgG1, followed by mouse serum, and then FITC-A1, FITC-5E6, or FITC-4E5. Two-color analysis on stained cells was performed on a FACScan, and data are presented as the percentage of coexpression relative to the total number of Ly49G$^+$ events. Shown are the results of two independent experiments that are representative of five experiments.

It is also possible that stimulation through the IL-2R or the response to poly(I:C) challenge is causing the preferential proliferation of NK cells expressing both Ly49G alleles. The preferential proliferation of Ly49G$^+$ NK cells in response to poly(I:C) has been recently reported (40). Also, in contrast to Ly49A, C, or D$^+$ NK cells, Ly49G$^+$ NK cells preferentially proliferate 3 days after infection with MCMV or LCMV. Thus, Ly49G$^+$ NK cells may be inherently better at responding to viral (or viral mimic) challenge. Alternatively, the high level of proliferation of Ly49G$^+$ cells can be explained by the lack of ligand (H-2D^d) in B6 and (129 × B6)F$_1$ mice. In (BALB × B6)F$_1$ mice, H-2D^d is present and inhibits Ly49G^{BALB+} NK cells to a greater extent than Ly49G^{B6+} NK cells. The lower proliferation of the Ly49C/I$^+$ NK cells in both types of hybrid mice correlates with the affinity of Ly49C/I for both H-2^b and H-2^d MHC ligands (32, 46).

No effect of close linkage on Ly49 coexpression was observed. Ly49A^{B6} was twice as likely to be expressed on Ly49G^{B6+} vs Ly49G^{129+} NK cells, while Ly49C/I^{B6} showed the opposite expression pattern (Fig. 6*B*). Therefore, it appears that local transcription in the Ly49 cluster does not influence induction of neighboring genes. This agrees with findings in (BALB × B6)F$_1$ NK cell cultures started from single clones that *Ly49 a*, *c*, and *g* allelic mRNA can be expressed from either chromosome in an independent fashion (27). In this respect, it is interesting to note that Ly49D^{B6} was always expressed on a higher percentage of Ly49G^{BALB+} NK cells than on Ly49G^{B6+} NK cells (Fig. 6*A*). All three receptors have affinity for H-2D^d, but Ly49G^{BALB} binding is stronger than Ly49G^{B6}. This suggests that sufficient inhibitory signal is more likely to be generated from Ly49G^{BALB} than Ly49G^{B6} to prevent autoimmune responses by Ly49D^{B6} expression in H-2D^{d+} mice.

Although differences in the expression of individual alleles will not increase the complexity of the NK cell receptor repertoire in inbred mouse strains, it is surely a factor in human populations that are likely to be heterozygous for most loci, including NK cell receptor genes. Logically, heterozygosity at genomic loci that code

for NK cell receptors involved in pathogen recognition would greatly benefit the innate immune system by increasing the number of pathogens against which NK cells could quickly respond, thus allowing the adaptive immune system time to expand specific T and B cells.

Whereas heterozygosity is obviously an advantage with respect to activating NK cell receptors, the advantages from diversity at loci coding for inhibitory NK cell receptors are less clear. Due to the large number of *Ly49* genes in a given haplotype, the diversity of receptor repertoires on individual NK cells is impressive; this diversity is greatly increased if the mouse is a hybrid (heterozygous) between two disparate *Ly49* gene cluster haplotypes (such as 129/J and B6). This is due to the fact that haplotype-specific genes are present; for example, 129/J mice express Ly49V, T, and P, but no alleles are present in B6 mice (21). Also, even when alleles of a given gene are present in Ly49 heterozygous mice, they often have different MHC specificities and so are functionally distinct genes. Therefore, biallelic expression in heterozygous, but not homozygous mice further increases the complexity of NK clones with the ability to respond to various types of self MHC.

Because NK cells express Ly49 (and KIR) receptors stochastically, one possible advantage of diverse inhibitory *Ly49* alleles (with different MHC specificities) is that individual NK cells in a heterozygous mouse have more Ly49 receptors with different MHC specificities to choose from during development compared with a homozygous mouse. Likewise, in the heterozygous mouse, there would be two different sets of *MHC* genes, so that individual NK cells have a wider selection of inhibitory ligands, and two different sets of *Ly49* genes to select a receptor to recognize self MHC. If *Ly49* genes are initiated only until there is sufficient self-recognition and inhibition, as has been proposed (39), then there will be more NK cells in heterozygous mice that are inhibited by different self MHC ligands compared with homozygous mice. Infection with certain viruses and some malignancies result in the down-regulation of specific *MHC* genes, leaving expression of other MHC unaltered (47). Thus, one would predict that NK cells in heterozygous mice would be less tolerant of down-regulation of individual *MHC* genes compared with NK cells from homozygous mice, because Ly49 allele diversity and the associated differences in MHC binding generate a greater array of NK cells with differing MHC reactivity. The analysis of the reactivity of *Ly49i* and *Ly49h* alleles with the MCMV class I mimic m157 demonstrates the importance of variegated expression of the Ly49 family (9). Ly-49I^{129} is inhibited by m157, but Ly49I^{B6} is not. The converse is true for the activating receptors: Ly49H^{B6} recognizes m157, and Ly49U^{129} does not. In heterozygous mice, only NK cells that express the B6 *Ly49i* allele or no Ly49I would be capable of recognizing virus-infected cells via the Ly49H-activating receptor. Therefore, the independent control of *Ly49* alleles produces an array of NK cells that can detect the complex changes in class I expression produced by pathogens that attempt to evade detection by the immune system.

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