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Effect of Ly49 Haplotype Variance on NK Cell Function and Education

Rajen Patel,* Simon Bélanger, † Lee-Hwa Tai, † Angela D. Troke,,* and Andrew P. Makrigiannis*†

The class I MHC-specific receptors expressed by murine NK cells exhibit remarkable variation. Specific activating killer Ig-related receptor/Ly49 have major effects on autoimmune and infectious disease induction and outcome in humans and mice. However, these studies are greatly affected by individual background genetics. Furthermore, the educational impact of variable inhibitory KIR/Ly49 gene numbers on NK cell development and the subsequent ability to survey for MHC class I (MHC-I) expression remain unknown. To address these questions, Ly49 congenic mice were generated that maintain a 129-derived Ly49 gene cluster on a C57BL/6 genetic background (B6.Ly49129 mice), and the in vitro and in vivo NK cell function of these mice was compared with their inbred parental 129S1 and C57BL/6 counterparts. Notably, target cell recognition directed by haplotype by inhibitory Ly49 receptors, B6.Ly49129 congenic mice exhibited robust NK cell activity, demonstrating efficient NK cell education by the 129S1 Ly49 cluster during development. The responsiveness of NK cells expressing 129S1 Ly49 was shown to be mediated by subsets expressing one or more self-MHC receptors, including Ly49I, Ly49O, Ly49V, and NKG2A. These findings demonstrate that the genetically segregating and diverse MHC-I and Ly49 loci in mice exhibit independent and epistatic effects on NK cell education that can be uncoupled during the intercrossing of inbred strains. The Journal of Immunology, 2010, 185: 4783–4792.

Natural killer cells play an essential role in the innate immune system. These lymphocytes target virally infected or malignant cells and play an important role in the rejection of cells with aberrant MHC class I (MHC-I) expression (1). NK cells kill target cells through contact-mediated cytotoxicity, and they also produce cytokines and chemokines to facilitate the recruitment and activation of other immune cells. NK cell response is determined by the integration of signals from activating and inhibitory receptors found on the surface of each NK cell. The sum of these two opposing signals determines whether the NK cell will mediate lysis against the encountered cell (2).

Rodent Ly49 receptors, which act as the functional homologs of the human killer Ig-related receptors (KIRs), can induce NK cell activation or inhibition, depending on the intracellular domain or adaptor associated with the receptor. Inhibitory Ly49 binds to MHC-I molecules found on normal self-cells to limit cytotoxicity via an ITIM located within the intracellular domain of the receptor. The ITIM recruits phosphatases, such as SHIP and Src homology region 2 domain-containing phosphatase 1 and 2, to diminish any activating signals triggered by tyrosine kinases (3). Activating Ly49 associates with the adaptor protein DAP12, which contains ITAMs essential for transducing an activating signal to the NK cell. Some activating Ly49 receptors recognize surface expression of pathogen-encoded molecules. For example, Ly49H can bind to the murine CMV (MCMV)-encoded MHC-like molecule m157 expressed on infected host cells (4), and Ly49P is also involved in recognition of MCMV-infected target cells through m04 (5). Allogeneic and xenogeneic host MHC-I binding by some activating Ly49 has also been reported (6, 7).

NK cells in normal mice are educated to recognize cells lacking normal expression of self–MHC-I molecules (termed “missing self” recognition) (8). However, NK cells from β2m−/− or TAP−/− mice are not able to recognize MHC-I–deficient cells because MHC-I molecules are absent during NK cell development (9–11). In contrast, the ectopic expression of an H-2Dβ transgene in C57BL/6J (B6) mice facilitates rejection of wild-type (WT) B6 cells by transgenic NK cells as a result of the introduction of a novel self–MHC-I allele during NK cell education (12). Consistent with this, a greater number of educating self–MHC-I molecules in the host correlates with enhanced NK cell function upon exposure to MHC-I–deficient targets (13). Thus, the environmental MHC-I alleles expressed during NK cell development have a major impact on determining the subsequent specificity and functional potential of mature NK cells.

In the NK cell licensing or disarming models, inhibitory Ly49 expressed by NK cells also plays a significant role in their education, because rare NK cells lacking self–MHC-I–specific inhibitory receptors appear to be hyporesponsive (14, 15). Functional licensing or disarming of NK cells is thought to be mediated by inhibitory Ly49 binding to self–MHC-I alleles during NK cell development in the bone marrow. In addition, the rheostat model proposes that
increasing numbers of expressed self-MHC-I–specific inhibitory recep-
tors cumulatively result in NK cells with an increased capacity for
MHC-dependent degranulation and cytokine production (13, 16).

The Ly49 receptors are encoded by a family of highly polymor-
phic and variably polygenic genes (17). In contrast, other NK gene
complex (NKC)-encoded receptors, such as the Nkrp1 gene
family, appear to be relatively stable (18). Early RFLP experi-
ments led to the observation that different inbred mouse strains
contain variable Ly49 haplotypes (19). For instance, NOD/ShiLtJ
mice have the largest Ly49 repertoire with 21 genes, 129S1/SvImJ
(129S1) mice possess 19 Ly49 genes, B6 mice possess 15 Ly49
genes, and BALB/c mice have the smallest known Ly49 repertoire
with only 8 genes (17, 20). The consequences to innate immunity
of Ly49 haplotype variation are most obviously manifested in the
absence of activating Ly49 receptor function. Inbred mouse strains
lacking genes encoding Ly49D and Ly49H are deficient in the
ability to kill CHO cells and MCMV-infected m157-bearing tar-
genotypes can be rescued by transgenes (22).

The Ly49 ability to kill CHO cells and MCMV-infected m157-bearing tar-
genomes can be rescued by transgenes (22). For Ly49D and Ly49H to
be effective killers, the target cells must lack Ly49-homologous
molecules. In the absence of activating Ly49 receptor function, the
lower cytotoxicity of 129-strain NK cells is reminiscent of hyporesponsive
target cells compared with B6-strain NK cells. The lower cyto-
xotoxicity of 129-strain NK cells is reminiscent of hyposensitive
NK cells.

The functional consequences of inhibitory Ly49 repertoire
variation for NK cells are not as well understood, although allelic
variation in Ly49A and/or Ly49G results in different MHC-I li-
gand affinities, as assessed by soluble MHC-I tetramer binding
and killing of MHC-I–transfected target cells (23, 24). It has long
been known that different inbred mouse strains exhibit variation in
NK cytotoxicity and cytokine production (25). For example, B6
NK cells appear to be more active than 129S1 NK cells (20), de-
spite possessing a smaller Ly49 haplotype (26). One explanation is
that activating Ly49 from the B6 strain deliver stronger DAP12-
derived signals than similar Ly49 from the 129S1 strain, as evi-
denced in studies of Ly49D function (27). However, 129-strain NK
cells also exhibit lower Ly49D-independent killing of tumor target
cells compared with B6-strain NK cells. The lower cytotoxicity of
129-strain NK cells is reminiscent of hyposensitive NK cells.

The educational capacity of inhibitory Ly49 receptors in 129-
strain mice remains unclear. It is also unclear whether Ly49 haplo-
type variation affects NK cell activity in different inbred mouse
strains. To determine whether the Ly49 receptors expressed by 129-strain NK cells possess altered functional or educational po-
tential versus B6-strain Ly49 receptors and to assess whether Ly49 haplotype variation affects NK cell functional potential on a
given genetic background, a congenic mouse strain expressing the
129S1 Ly49 gene cluster on the B6 genetic background (B6.
Ly49129 mice) was generated and assessed for NK cell activity
relative to inbred B6 and 129S1 mice in vitro and in vivo.

Materials and Methods

Mice

Mice were maintained in the Animal Care Veterinary Service of the
University of Ottawa in a specific pathogen-free environment. B6 mice,
129S1 mice, and β2-microglobulin−/− (β2m−/−) mice on a B6 back-
ground were purchased from The Jackson Laboratory (Bar Harbor, ME).
Kb−/−, Dp−/−, and Kd−/− Dp−/− mice on a B6 background were purchased
from Taconic Farms (Germantown, NY) under the National Institute of
Allergy and Infectious Diseases Exchange Program, NIH:004215 (28). B6.
Ly4920 congenic mice possessing the Ly49129S1 gene cluster on the B6
background were produced by initially crossing B6 and 129S1 mice,
crossing the resulting F1 hybrids to a B6 mouse, and screening the progeny
for the presence of 129-specific Ly49 genes. This was repeated for 10
 generations. Each generation was verified for the presence of Ly4920S1
 gene cluster by PCR for Ly4920 (forward: 5′-TTGTTGCAGCCCTTGACCTTGTTATATACTAGTCTT-3′). Af-
 ter the final backcross generation, mice heterozygous for a 129S1 Ly49
cluster were bred to produce homozygous B6.Ly49129 mice, which was
confirmed by an additional PCR for the absence of Ly49b6 (forward: 5′-
GAAAGCTCATTGTGATAGCTTT-3′; reverse: 5′-TTGTTGGTCTGGAGAATCGT-3′). B6.Ly49Qlox/lox mice were produced by crossing Ly49Qlox/lox mice
(129S1 background) to B6 mice for 10 generations. Heterozygotes were
identified in each generation by genomic PCR for the Ly49Qlox allele.

Single nucleotide polymorphism and Southern genome
analyses

To determine B6 versus 129S1 genome content, single nucleotide poly-
orphism (SNP) analysis was performed using an Illumina Beadstation
500G mouse medium density linkage panel (The Center for Applied
Genomics-Sick Kids Hospital, Toronto, Ontario, Canada). The whole ge-
ome of B6.Ly49129 mice is of B6 origin, except for a region containing
the NKC on chromosome 6 spanning nucleotides 79,759–138,203,431.
All chromosomes of B6.Ly49Qlox/lox mice were B6 derived, except for
chromosome 6 (nucleotides 118,022,343–149,214,236). RFLP analysis
was performed on 10 μg DNA isolated from the thymus and digested with
EcoRI, KpnI, or BamHI (New England Biolabs, Beverly, MA) before gel
electrophoresis and transfer to nylon membranes (Amersham Biosciences,
Piscataway, NJ). Blots were probed with a mixture of Ly49b, Ly49o, and
Ly49e cDNAs.

Cells and viruses

RMA (H-2b thymoma) and RMA-S (MHC-deficient RMA variant) were
gifted from Dr. A. Veillette (Clinical Research Institute of Montreal, Mon-
real, Quebec, Canada). YAC-1 (TIB-160; H-2b lymphoma) was purchased
from American Type Culture Collection (Manassas, VA). The YB/2 rat
cell line was kindly provided by Dr. J. Ryan (University of California,
Veterners Affairs Medical Center, San Francisco, CA). These cell lines were
grown in complete RPMI medium (RPMI 1640 supplemented with 10% 
FBS, 2 mM L-glutamine, 100 μU/ml penicillin, and 100 μg/ml streptomycin).
CHO cells (American Type Culture Collection) were grown in complete
DMEM (DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 
μU/ml penicillin, and 100 μg/ml streptomycin). Plastic-adherent lympho-
kine (IL-2)–activated killer (LAK) cells were grown in culture and used as
effecter cells in 51Cr-release cytotoxicity assays, as previously described
(20). For mAb blocking during CHO killing assays, effectors were pre-
 pared to 20 min with 10 μg/ml of the anti-Ly49 mAb or isotype control
before target cells were added. MCMV preparation, injection, and plaque
 assay were performed as previously described (30).

Abs and flow cytometry

Anti-CD49b (DX5), anti-TCR-β (H57-597), anti-CD9 (18D3), anti-
Ly49A/D (12A8), anti-Ly49C/F/H (14B11), anti-Ly49H (3D10), and
anti-CD107a (e601D48) were purchased from eBioscience (San Diego,
CA). Anti-NK2CA/CE (2D65), anti-NKRP1g (PK6), anti-NK9D1
(4E5), anti-Ly49C (Cy3), anti-Ly49G (4D11), anti-Ly49Cl (5E6),
and anti-IFN-γ (XMGI,1.2) were purchased from BD Biosciences (San Jose,
CA). AT8 (anti-Ly49G25) was produced in our laboratory (24). Various
isotype controls were purchased from eBioscience or BD Biosciences.
FcRs were blocked using rat serum (Sigma-Aldrich, St. Louis, MO), mouse se-
rum (Sigma-Aldrich), and 2.4G2 (anti-FcγRII) (Beckman Coulter,
Fullerton, CA). Acquisition and analysis were performed using CellQuest
Pro or Kaluza software.

CFSE splenocyte-rejection assay

Splenocytes from syngeneic or MHC-deficient (B2M−/−, Kb−/−, Dp−/−, or
Kd−/− Dp−/−) mice were differentially labeled with 0.5 and 5 μM CFSE
(Invitrogen), respectively. A total of 5 × 105 of each population were
injected i.v. into untreated or pretreated (150 μg/kg Pro or Kaluza software.

IFN-γ–induction assays

Splenocytes from poly-I:C–treated mice (150 μg, 18 h prior) were in-
cubated for 5 h with tumor cells, at a ratio of 1:1, in flat-bottom 96-well
plates coated with 5 μg/ml anti-NKG2D or anti-NKp46 mAbs or with 5 ng/ml PMA and 1 μg/ml ionomycin. GolgiPlug (BD Biosciences) was added after 1 h of incubation. Cells stained for CD49b (DX5) and TCR-β were fixed and permeabilized with the Cytofix/Cytoperm kit (BD Biosciences) and stained for IFN-γ after 4 h of incubation with target cells upon GolgiPlug treatment. DX5⁻/TCR-β⁻ cells were analyzed for IFN-γ by flow cytometry.

Statistical analysis

Statistical significance was determined by a two-tailed unpaired Student t test, with a cutoff p value of 0.05. Data are presented as mean ± SEM.

Results

Ly49 expression and genetic organization of B6.Ly49<sup>129</sup> mice

To determine whether the function of NK cells in 129-strain mice relative to B6-strain mice is impacted by their divergent Ly49 haplotype (Fig. 1A), the Ly49 gene cluster of 129S1 was bred onto a B6 genetic background. B6.Ly49<sup>129</sup> congenic mice were produced by serially backcrossing hybrid pups onto the B6 genetic background and selecting the resulting progeny by genomic PCR for Ly49v (an Ly49 present in only 129S1 and other 129-related strains). The selection process was repeated for 10 successive generations to obtain B6.Ly49<sup>129</sup> mice, as verified by SNP analyses (see Materials and Methods).

RFLP analysis was performed to verify the integrity of the Ly49 region in the B6.Ly49<sup>129</sup> mice and to rule out the generation of a B6/129 hybrid Ly49 gene cluster as a result of an intracluster chromosome exchange during the backcrossing process that would not be detected by SNP analyses. Genomic DNA from the three strains was prepared, and Southern blot analysis was performed using various Ly49 cDNA probes. The pattern of Ly49-containing DNA fragments was identical for 129S1 and B6.Ly49<sup>129</sup> mice using various Ly49 cDNAs and restriction-enzyme combinations (Fig. 1B, 1C). Thus, RFLP analysis suggests that the 129S1 Ly49 gene cluster in the B6.Ly49<sup>129</sup> line is intact.

To verify that B6.Ly49<sup>129</sup> NK cells express Ly49 belonging to the 129S1 haplotype, flow-cytometric analysis was carried out on fresh DX5⁻/TCR-β⁻ splenocytes from B6, 129S1, and B6.Ly49<sup>129</sup> mice, using a panel of mAbs known to be specific for B6- and/or 129-derived Ly49 (Fig. 2). B6.Ly49<sup>129</sup> and 129S1 NK cells displayed no binding to Cwy-3, 3D10, and 5E6 mAbs, which bind to the B6 alleles of Ly49G, Ly49H, and Ly49Cl, respectively. In contrast, a subset of B6.Ly49<sup>129</sup> and 129S1 NK cells were stained by AT8, a mAb produced in our laboratory that binds to Ly49G<sup>129</sup> but not Ly49G<sup>B6</sup> (24). Anti-Ly49 mAbs 4E5 and 12A8 bound to a larger NK cell subset in B6.Ly49<sup>129</sup> and 129S1 mice versus B6 mice (Fig. 2). Unexpectedly, although 14B11 (anti-Ly49C/IF/FH) also bound to NK cell subsets in all three strains, the percentage of positive NK cells in B6.Ly49<sup>129</sup> mice was significantly different from that observed for 129S1 or B6 mice, with 14B11 staining an intermediate-sized subset of B6.Ly49<sup>129</sup> NK cells. This hybrid Ly49-expression pattern may be a result of an internal cross-over within the Ly49 cluster between B6 and 129S1 chromosomes during meiosis or an epistatic effect of the B6 genome; however, the former possibility was ruled out by the RFLP analyses described above (Fig. 1B, 1C). For independent confirmation, the recently described Ly49Q-deficient mouse was also backcrossed onto the B6 background for 10 generations (B6.129-Ly49Q<sup>lox/lox</sup> mice); like the B6.Ly49<sup>129</sup> mice, these mice carry the 129-strain Ly49 gene cluster but with a defective Ly49q gene. The 14B11 staining, and all other Ly49 staining, of B6.129-Ly49Q<sup>lox/lox</sup> NK cells was similar to that of B6.Ly49<sup>129</sup> mice (Fig. 2). These data suggest that genetic background can significantly impact the expression pattern of Ly49 gene products.

To determine the background in the B6.Ly49<sup>129</sup> mouse from which non-Ly49 NK cell surface markers are encoded, especially those of the NKC for which SNP analysis was not informative, two groups (NKC and non-NKC) of surface markers were examined by flow cytometry. All NKC-associated markers (CD94, NKG2A, NK1.1) examined displayed a 129-like pattern for B6. Ly49<sup>129</sup> NK cells (Supplemental Fig. 1A). In contrast, flow cytometry for markers not found in the NKC (CD11a, CD62L, CD2) was roughly the same for the three strains (CD11a and CD2), or it demonstrated a B6-strain-expression pattern in B6.Ly49<sup>129</sup> NK cells when a strain difference was evident (CD62L; Supplemental Fig. 1B). Although all of the markers examined were present on B6 and 129S1 NK cells, except for the strain-specific NK1.1 mAb (PK136, anti-NKRP1C<sup>B6</sup>), the surface levels and/or subset percentage (in the case of bimodal expression) of these cell-surface proteins sometimes differed on NK cells from the two inbred strain backgrounds. Specifically, the NK subsets expressing NKG2A/C/E, CD94, and CD62L were different in terms of the positively staining proportion and/or surface density (Supplemental Fig. 1). Strain-specific differences in the expression of these NK cell markers may be due to genetic background influences and/or allelic sequence divergence affecting mAb binding. Collectively, these results suggest that the altered Ly49-expression patterns observed in the B6.Ly49<sup>129</sup> congenic mice are limited to epistatic influences of the B6 genetic background imposed upon 129-derived Ly49 gene products.

B6.Ly49<sup>129</sup> congenic NK cells possess cytotoxicity similar to B6-derived NK cells

To determine whether Ly49 haplotype divergence affects NK cytotoxic potential, LAK cells from B6, 129S1, and B6.Ly49<sup>129</sup> mice were used as effectors against a panel of target cells in [3<sup>11</sup>]Cr-
release cytotoxicity assays. Killing of MHC-sufficient RMA targets was low for NK cells from all three strains (Fig. 3A), whereas the killing of MHC-deficient RMA-S targets was significantly greater (Fig. 3A). The killing of RMA-S targets by B6 LAK effector cells was much stronger than that displayed by 129S1 LAK cells, whereas B6.Ly49129 LAK cells showed vigorous RMA-S killing identical to that of B6 LAK cells. The killing of the NKG2D-ligand–bearing YAC-1 and the xenogeneic YB2/0 targets was similarly high for B6 and B6.Ly49129 LAK cells, whereas the cytotoxicity displayed by 129S1 LAK cells was significantly reduced in comparison (Fig. 3A). Interestingly, the killing of CHO cells by B6.Ly49129 LAK cells was intermediate between that of B6 and 129S1 LAK cells (Fig. 3A); this suggests that extrinsic influences of the B6 background and intrinsic influences of the allelically divergent B6 and 129S1 Ly49D/R gene products may impact the overall cytotoxicity of the congenic B6.Ly49129 NK cells. However, unknown 129S1 genes closely linked to the Ly49 cluster cannot be formally excluded as contributors to the decreased killing of CHO cells by B6.Ly49129 NK cells. Ab-blocking experiments showed that the 129S1 alleles in B6.Ly49129 LAK cells for Ly49D and Ly49G mediate induction and inhibition of cytotoxicity toward CHO cells, respectively (Fig. 3B). Overall, the cytotoxicity mediated by B6.Ly49129 NK cells is much more comparable to that of B6 LAK cells. This suggests that the 129S1 Ly49 cluster can sufficiently educate NK cells and that the relative hyporesponsiveness of 129S1 NK cells is likely due to extrinsic background influences independent of Ly49 gene cluster variation.

The 129S1 Ly49 repertoire can efficiently educate NK cells on distinct genetic backgrounds

To further test the function of B6 NK cells bearing a 129S1 Ly49 receptor repertoire, the production of IFN-γ after tumor cell, receptor-mediated, and pharmacological stimulation was determined. 129S1 NK cells consistently produced less IFN-γ than B6 or B6.Ly49129 NK cells when stimulated with YAC-1 cells (Fig. 4A), and although this defect was reproducible using RMA-S cells as stimulators, the relatively low IFN-γ induction by RMA-S was subject to background because of the poly-IC pretreatment of mice. It should be noted that although B6.Ly49129 NK cells were induced at twice the levels of 129S1 NK cells, B6.Ly49129 IFN-γ induction was slightly lower than the level shown by B6 NK cells (Fig. 4A). Activation via plate-bound mAb to NKG2D resulted in roughly equal percentages of IFN-γ+ NK cells from the three strains of mice (Fig. 4B), suggesting that the YAC-1 IFN-γ induction defect shown by 129S1 NK cells is not due to defective NKG2D signaling. Pharmacological stimulation resulted in comparable levels of IFN-γ in NK cells from the three strains of mice (Fig. 4B), suggesting that the NK cells from the three strains have roughly the same potential to produce IFN-γ, although 129S1 NK cell induction via PMA/ionomycin was consistently slightly lower that that of B6 or B6.Ly49129 NK cells.
Recent reports demonstrated that NK cells expressing inhibitory Ly49 or KIR specific for self–MHC-I produce more IFN-γ than NK cells expressing no self–MHC-I–specific inhibitory receptors (14, 15). To determine whether the NK cell licensing/disarming hypotheses hold true for NK cells bearing a 129-derived Ly49 receptor repertoire, NK cells were coincubated with YAC-1 targets and then multi-parametric flow cytometry was used to identify the NK subsets producing IFN-γ. The percentages of the Ly49 +IFN-γ+ and Ly49−IFN-γ+ subsets (from total cells) after stimulation are shown in Fig. 4C. The lectin-like inhibitory NKG2A/CD94 heterodimer is expressed on a subset of NK cells (Supplemental Fig. 1A) that recognizes Qa-1b, a ubiquitously expressed MHC-Ib molecule that can present the H-2Db leader peptide. Like Ly49, NKG2A confers educational specificity in B6 mice (15). Interestingly, NKG2A+ B6.Ly49129 NK cells produced 2-fold more IFN-γ than NKG2A− cells after YAC-1 stimulation (Figs. 4C, 5A). In addition, Ly49I, Ly49O, and Ly49V from 129-strain mice were shown to functionally bind Kdb and/or Ddb (31). In agreement with this, B6.Ly49129 NK cells expressing these inhibitory self–MHC-I–specific receptors produced more IFN-γ than the corresponding negative subset (Figs. 4C, 5A). The percentage of IFN-γ+ NK cells in the Ly49 + and Ly49− subsets after YAC-1 stimulation are shown in Fig. 5A and 5B. The expression of self–MHC-I–specific receptors endows that specific NK cell subset with the ability to respond more vigorously. Similarly, the expression of multiple self–MHC-I–specific receptors leads to greater responsiveness than does the expression of a single self-MHC receptor, as shown by the fold-induction of the positive subset over the negative subset (Fig. 5B). Similar results were found with less complex stimuli, such as plate-bound anti-Nkp46 mAb (Fig. 5C, 5D), which further supports the hypothesis that increased IFN-γ production is a general characteristic of NK cells expressing self-MHC receptors, regardless of Ly49 haplotype.

Collectively, in vitro analyses of B6.Ly49129 NK cell function suggest that 129S1 Ly49 gene cluster-mediated education is sufficient to confer normal NK cell cytotoxicity and cytokine production. Therefore, the relative functional NK hyporesponsiveness observed in 129S1 mice, compared with B6 mice, is not due to intrinsic differences in the Ly49 genes themselves. In turn, extrinsic effects of the genetic background, including educating influences of the MHC-I haplotype, can efficiently modulate NK cell function independently of Ly49 gene cluster content.
Enhanced MHC-I surveillance by NK cells with 129-strain Ly49

The ability of NK cells to kill otherwise normal cells deficient in MHC-I in vivo is well established (32) and is hypothesized to be a consequence of education via MHC–Ly49 interactions during NK cell development (14, 15, 33, 34). To determine whether 129-strain Ly49 receptors can satisfactorily educate NK cells to reject MHC-I–deficient cells, an in vivo splenocyte-rejection assay was performed. Splenocytes from Kb$^{2/2}$, Db$^{2/2}$, or Kb$^{2/2}$Db$^{2/2}$ mice were fluorescently labeled and injected into B6, 129S1, or B6.Ly49 mice along with differentially labeled WT splenocytes as an internal rejection control. B6 and 129S1 mice were able to reject Kb$^{2/2}$ splenocytes as determined by flow cytometry on the spleens of recipient mice, but the rejection by B6 mice was ∼20% greater than that of 129S1 mice (Fig. 6A). The rejection of Kb$^{2/2}$Db$^{2/2}$ splenocytes by B6 and 129S1 mice was similar to the rejection of Kb$^{2/2}$ splenocytes. Interestingly, B6.Ly49 mice rejected Kb$^{2/2}$Db$^{2/2}$ splenocytes significantly better than did B6 or 129S1 mice.

To amplify possible differences in rejection potential by NK cells, the experiment was repeated with NK cell priming via injection of recipient mice with poly-IC before MHC-I–deficient splenocyte introduction. Poly-IC challenge results in IFN-α/IL-12 secretion and IL-15 trans-presentation by dendritic cells and subsequent NK cell activation (35). As expected, with poly-IC priming, rejection of MHC-deficient splenocytes was significantly greater in all cases (Fig. 6B). Interestingly, although 129S1 mice...
rejected less well than either of the B6 background mice, rejection of K1−/− cells by B6.Ly49129 mice was consistently and significantly greater than that of B6 mice (Fig. 6B). Rejection of D1−/− cells was also dramatically increased by poly-IC in all three mouse strains, with B6 and B6.Ly49129 mice rejecting equally well and more strongly than 129S1 mice (Fig. 6B). Thus, D1− is also an educating MHC-I for NK cells in all three strains, but this is only apparent when NK cells are primed. The preconditioning of B6 and B6.Ly49129 mice resulted in almost total rejection of K1−/−D1−/−-splenocytes (Fig. 6B), again showing the contribution of D1 to NK cell education. In summary, NK cells from B6.Ly49129 mice contain genes other than Ly49 genes cannot be formally excluded.

Ly49 allelic polymorphism and haplotype size vary dramatically in mice, and this situation mirrors KIR variability in humans (36). For the same strategy to have evolved in two evolutionary distinct gene families that encode structurally unrelated proteins, there must be a significant advantage or necessity to NK cell education and function. Specific MHC-I receptor haplotypes have advantages in specific situations (e.g., haplotypes containing specific activating receptors involved in resistance to infectious diseases) (37, 38). Similarly, disorders, such as autoimmune diseases, can also be attributed to the association of certain KIR/Ly49 genes with some haplotypes (39). However, these population studies are complicated by individual differences in genetic background. Whether different MHC-I receptor haplotypes on the same genetic background can differentially affect NK cell education during specific circumstances. Similarly, the MCMV susceptibility of 129-strain mice is presumed to be due to the inability of the 129-strain allele of Ly49H mice (Ly49U; Fig. 1A) to efficiently recognize the MCMV-encoded m157 protein on the surface of infected cells (4). However, Ly49P in MA/My (H-2k) mice provides resistance to MCMV via a host MHC-I–dependent mechanism (30). MCMV-susceptible 129S1 mice express Ly49P on NK cells (Fig. 1A); however the relative DAP12-signaling deficiency of 129-strain mice complicates studies of the activating receptors (e.g., Ly49P or Ly49U) on 129-strain NK cells. B6.Ly49129 mice provide a controlled genetic background, including normal DAP12 signaling, on which to test the MCMV responsiveness of the 129S1 Ly49 repertoire. B6.Ly49129 mice infected with MCMV displayed high splenic viral titers, similar to those seen in 129S1 mice, and significantly greater than B6 mice (Fig. 7). These data formally demonstrate that there are no potent activating Ly49 receptors in the 129S1 Ly49 repertoire that can induce specific NK cell-mediated killing of Smith-strain MCMV-infected cells on an H-2b background.

Discussion
Ly49 allelic polymorphism and haplotype size vary dramatically in mice, and this situation mirrors KIR variability in humans (36). For the same strategy to have evolved in two evolutionary distinct gene families that encode structurally unrelated proteins, there must be a significant advantage or necessity to NK cell education and function. Specific MHC-I receptor haplotypes have advantages in specific situations (e.g., haplotypes containing specific activating receptors involved in resistance to infectious diseases) (37, 38). Similarly, disorders, such as autoimmune diseases, can also be attributed to the association of certain KIR/Ly49 genes with some haplotypes (39). However, these population studies are complicated by individual differences in genetic background. Whether different MHC-I receptor haplotypes on the same genetic background can differentially affect NK cell education during
development and subsequent NK cell function in the periphery of the host is a question that has not been directly addressed. The present study attempted to answer this question by backcrossing a non-B6 (129S1) \(\text{Ly49}\) gene cluster onto the B6 genetic background and directly comparing NK cell function in these congenic mice in vitro and in vivo relative to 129S1 and B6 inbred mice. NK cells from 129-strain mice possess inferior cytokine and cytotoxicity responses compared with other inbred mouse strains, such as B6. In contrast, B6.Ly49 129 mice displayed strong, B6-like in vitro killing of tumor target cells and IFN-\(\gamma\) production (Figs. 3A, 4A). Furthermore, the in vivo rejection of MHC-I–deficient cells by B6.Ly49 129 mice was equal to, if not better than, B6 mice (Fig. 6A, 6B). Thus, the Ly49 gene cluster of 129-strain mice can efficiently educate NK cells, and the NK cell functional defect of 129-strain mice is not due to \(\text{Ly49}\) gene cluster variation. Furthermore, the data also suggest that the in vivo cytotoxic potential of NK cells toward normal MHC-I–deficient cells is modulated by Ly49 haplotype variation, perhaps as a result of differing numbers of inhibitory self-MHC receptors. In contrast, in vitro cytotoxic responses toward tumor cells were very similar in B6-background NK cells with different Ly49 haplotypes, although additional activating NK cell receptors, such as NKG2D, also contribute to antitumor cell responses and possibly obscure any Ly49 haplotype-derived functional differences. Although B6.Ly49 129 NK cells killed RMA-S equally well compared with B6 NK cells (Fig. 3A), RMA-S induced less IFN-\(\gamma\) in B6.Ly49 129 NK cells versus B6 NK cells (Fig. 4A). This can be explained by the finding that degranulation (killing) is easier to induce or requires less education in terms of multiple self-MHC receptors expressed per NK cells than does IFN-\(\gamma\) production (13, 16).

Despite an intact and complete 129S1 Ly49 gene cluster in B6. Ly49 129 congenic mice, as assessed by RFLP analysis, the surface-expression patterns of Ly49I (as detected by 14B11) on B6.Ly49 129 NK cells differed from that of 129S1 NK cells (Fig. 2). The reason for this difference is not clear. It is known that the presence or absence of MHC-I, the number of MHC-I molecules, and the MHC-I haplotype can affect the overall Ly49 repertoire and receptor-expression levels (33, 34, 40, 41). However, B6, 129S1, and B6.Ly49 129 mice have identical MHC-Ia protein sequences (\(K^b\) and \(D^b\)). Although MHC-Ib sequences and gene numbers differ between 129-strain and B6 mice (42), there is no evidence that Ly49 receptors can recognize these proteins. Furthermore,
similar 14B11 staining results were independently observed in a second congenic mouse line (B6.129-Ly49Q<sup>lox/lox</sup>), with a smaller 129-donor segment on chromosome 6 (Fig. 2). Collectively, these data suggest that NKC/MHC-I–independent genes on the B6 background may modulate Ly49 expression levels.

As previously shown for 129 inbred strains, the MCMV susceptibility of B6.Ly49<sup>129</sup> mice (Fig. 7) is likely a direct consequence of defective recognition of m157 by activating Ly49U (the 129-strain Ly49H allele), combined with intact recognition of m157 by inhibitory Ly49I alleles (4). In contrast, B6.Ly49<sup>129</sup> NK cells killed RMA-S and YAC-1 target cells equally well compared with B6 NK cells (Fig. 3A). Data from a previous study using NOD.NK.1<sup>B6</sup> congenic mice agree with our finding that B6.Ly49<sup>129</sup> NK cell-mediated killing of RMA-S cells is independent of the NKC (43). Interestingly, the cytotoxicity toward CHO cells displayed by B6.Ly49<sup>129</sup> NK cells was intermediate between that of B6 (high) and 129S1 (low) NK cells (Fig. 3A). Thus, B6.Ly49<sup>129</sup> NK cells possess an overall high, B6-like cytotoxicity potential for prototypic MHC-I–deficient NK targets, but they display an inherent susceptibility to MCMV infection and a partial deficiency in CHO cell recognition. The CHO cell MHC-I molecule, Hml-1-C4, is a ligand for Ly49D on B6 NK cells, and blockade of Ly49D with specific mAb decreases CHO cell killing dramatically (6, 44). However, the 129-strain allele of Ly49D (Ly49R) has a significantly divergent coding sequence, including several amino acid substitutions in the extracellular domain (31); thus, it is possible that Ly49R does not efficiently bind to CHO Hml-1-C4, which, in turn, leads to decreased CHO cytotoxicity. In addition, possible stronger binding by the inhibitory Ly49G<sup>129</sup>, which was also shown to recognize a CHO ligand (45), may contribute to lower CHO killing by B6.Ly49<sup>129</sup> NK cells. The effects of mAb blocking of Ly49R and Ly49G on congenic NK cells during CHO cytotoxicity assays shown in Fig. 3B are consistent with this hypothesis.

Previous studies in our laboratory directly compared Ly49G<sup>B6+</sup> and Ly49G<sup>129</sup> NK cells from (129X1 × B6) F<sub>1</sub> mice and found that they exhibited equal allele-specific inhibition with regard to CHO cytotoxicity (24). However, Ly49D/R expression levels were not equal on the two subsets, complicating the interpretation of these data. The present study supports and extends a previous report showing that the 129-strain allele of Ly49D (Ly49R) can initiate a mAb-dependent stimulatory signal equivalent to that of Ly49D<sup>B6</sup> in reconstitution experiments (27). The conclusion from the previous report was that the defect in Ly49R-mediated cytotoxicity/cytokine production displayed by 129P3 NK cells, relative to B6 Ly49<sup>D</sup><sup>+</sup> NK cells, was downstream of the receptor. Our current data using B6.Ly49<sup>129</sup> mice is in agreement with this conclusion.

Whether characterized by the licensing, disarming, or rheostat models, we present evidence that the education of NK cells expressing 129-strain Ly49 receptors that recognize self–MHC-I alleles (K<sup>B</sup> and/or D<sup>B</sup>) results in significantly greater functional responses compared with NK cell subsets lacking these self-MHC receptors (Figs. 4C, 5A). However, the weaker overall functionality of inbred 129S1 versus congenic B6.Ly49<sup>129</sup> NK cells demonstrates that NK cell education via self–MHC-I ligands only partially governs NK cell responsiveness. For example, signals emanating from DAP12-coupled receptors are clearly defective in 129-strain mice (27). Interestingly, a recent report indicates that unlicensed or armed NK cells that express the m157-specific Ly49H activating receptor respond best to MCMV infection in vivo (46). Similarly, the unlicensed Ly49D<sup>+</sup> NK cells make more IFN-γ than do licensed Ly49D<sup>+</sup> NK cells in response to ligand-bearing cells (46).

Unexpectedly, the rejection of MHC-I–deficient cells by B6. Ly49<sup>129</sup> mice was significantly greater than that mediated by B6 mice in some experiments, depending on the number of MHC-I molecules missing from the splenocyte graft and the activation status of the NK cells (Fig. 6A, 6B). Our analysis of B6.Ly49Q<sup>lox/lox</sup> mice, which are also congenic for the Ly49<sup>129</sup> complex with a significantly smaller 129S1 donor region, is consistent with the hypothesis that 129S1-derived Ly49 are responsible for the increased NK cell function in congenic mice (Fig. 6C). These conclusions must be prefaced with the caveat that we cannot formally exclude the possibility of closely linked 129S1-derived non-Ly49 genes as the cause for increased rejection of MHC-I–deficient cells, decreased CHO tumor cell killing, and MCMV susceptibility of congenic mice relative to B6 mice.

The rheostat or tuning models of NK cell education state that the degranulation and cytokine-production potentials of NK cells are directly related to the number of self–MHC-I–specific inhibitory receptors expressed by the NK cell (13, 16). The 129S1 Ly49 cluster contains more genes encoding inhibitory Ly49 and activating Ly49 receptors compared with the B6 cluster (Fig. 1A). The number of genes encoding inhibitory self-MHC receptors, in particular, is also larger in the 129S1 haplotype. Specifically, B6 mice can use Ly49C, Ly49I, and NKG2A for licensing/education of NK cells, whereas 129S1 and B6.Ly49<sup>129</sup> mice can use Ly49I, Ly49O, Ly49V, and NKG2A (Figs. 4C, 5A). Thus, it is possible that the increased in vivo rejection of MHC-I–deficient cells by B6.Ly49<sup>129</sup> mice is a consequence of B6.Ly49<sup>129</sup> NK cells being more highly tuned, perhaps by more inhibitory Ly49 receptors per NK cell on average. The equal killing of tumor cells by B6. Ly49<sup>129</sup> and B6 NK cells may be due to tuning differences being masked by activating tumor cell ligands, such as Rae1 on YAC-1 cells, whereas RMA-S is derived from a pre-existing lymphoma (RMA) that was subsequently mutated by repeated exposure to carcinogens (1). Thus, RMA-S killing and MHC-deficient splenocyte rejection assays are complementary but not interchangeable. Although final proof of tuning by 129-strain Ly49 receptors will require the generation of more single Ly49 allele-specific mAbs, the 129-strain Ly49 gene complex clearly possesses the capacity to educate NK cells for immunosurveillance against missing self, and the NK cell functional deficiency of 129-strain mice is not due to a divergent Ly49 cluster of these mice relative to B6 mice.

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Disclosures
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References


