Effect of Ly49 Haplotype Variance on NK Cell Function and Education

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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.Ly49^{129} 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 activating Ly49 receptors was profoundly affected by allelic variation in B6.Ly49^{129} congenic cells versus C57BL/6 NK cells. Furthermore, when assessing NK cell function based on education and subsequent recognition of the C57BL/6 MHC-I haplotype by inhibitory Ly49 receptors, B6.Ly49^{129} 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 $\beta2m^{-/-}$ 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-2D^d 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

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Abbreviations used in this paper: 129S1, 129S1/SvImJ; B6, C57BL/6; KIR, killer Ig-related receptor; LAK, lymphokine-activated killer; MCMV, murine CMV; MHC-I, class I MHC; NKC, NK gene complex; poly-IC, polyinosinic-polycytidylic acid; SNP, single nucleotide polymorphism; WT, wild-type.

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increasing numbers of expressed self–MHC-I–specific inhibitory receptors 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 polymorphic 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 experiments 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 target cells, respectively (4, 21); however, such *Ly49* haplotype deficiencies can be rescued by transgenes (22).

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 ligand 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), despite 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 evidenced 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 hyporesponsive NK cells.

The educational capacity of inhibitory Ly49 receptors in 129-strain mice remains unclear. It is also unclear whether *Ly49* haplotype 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 potential 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.Ly49^{129} 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 background were purchased from The Jackson Laboratory (Bar Harbor, ME). K$^{b-/-}$, D$^{b-/-}$, and K$^{b-/-}$D$^{b-/-}$ 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.Ly49^{129} congenic mice possessing the *Ly49*^{129S1} gene cluster on the B6 background were produced by initially crossing B6 and 129S1 mice, crossing the resulting F_1 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 *Ly49*^{129S1} gene cluster by PCR for *Ly49v*^{129} (forward: 5′-GTGTTCAGTCCCCTGGAAGT-3′; reverse: 5′-CTTGGTTTTATTATACAATCTGTTCTT-3′). After the final backcross generation, mice heterozygous for a 129S1 *Ly49* cluster were bred to produce homozygous B6.Ly49^{129} mice, which was confirmed by an additional PCR for the absence of Ly49g^{B6} (forward: 5′-GAAGCTCATTGTGATAGCTTG-3′; reverse: 5′-TGTGTGCTGTGAGGAATCTG-3′). B6.Ly49Q$^{lox/lox}$ were produced by crossing Ly49Q$^{lox/lox}$ mice (129S1 background) to B6 mice for 10 generations. Heterozygotes were identified in each generation by genomic PCR for the Ly49Q$^{lox/lox}$ allele, as previously described (29). All breeding and manipulations performed on animals were in accordance with university guidelines and approved by the University of Ottawa animal ethics committee. Animal care was provided in accordance with the procedures approved by the University of Ottawa Animal Care Committee.

Single nucleotide polymorphism and Southern genome analyses

To determine B6 versus 129S1 genome content, single nucleotide polymorphism (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 genome of B6.Ly49^{129} mice is of B6 origin, except for a region containing the NKC on chromosome 6 spanning nucleotides 79,759,628–138,203,431. All chromosomes of B6.Ly49Q$^{lox/lox}$ 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 *Ly49g*, *Ly49o*, and *Ly49e* cDNAs.

Cells and viruses

RMA (H-2^b thymoma) and RMA-S (MHC-deficient RMA variant) were a gift from Dr. A. Veillette (Clinical Research Institute of Montreal, Montreal, Quebec, Canada). YAC-1 (TIB-160; H-2^k lymphoma) was purchased from American Type Culture Collection (Manassas, VA). The YB2/0 rat-cell line was kindly provided by Dr. J. Ryan (University of California, Veterans 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 U/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 U/ml penicillin, and 100 μg/ml streptomycin). Plastic-adherent lymphokine (IL-2)–activated killer (LAK) cells were grown in culture and used as effector cells in [^{51}Cr]-release cytotoxicity assays, as previously described (20). For mAb blocking during CHO killing assays, effectors were pretreated for 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-CD94 (18d3), anti-Ly49A/D (12A8), anti-Ly49C/I/F/H (14B11), anti-Ly49H (3D10), and anti-CD107a (ebo1D48) were purchased from eBioscience (San Diego, CA). Anti-NKG2A/C/E (20d5), anti-NKRP1C^{B6} (PK136), anti-Ly49D (4E5), anti-Ly49G^{B6} (Cwy-3), anti-Ly49G (4D11), anti-Ly49C/I (5E6), and anti–IFN-γ (XMG1.2) were purchased from BD Biosciences (San Jose, CA). AT8 (anti-Ly49G^{129}) 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 serum (Sigma-Aldrich), and 2.4G2 (anti-FcγRIII). Dead cells were excluded using propidium iodide (BD Biosciences). Flow cytometry was performed on a FACSCalibur (BD Biosciences) and CyAn ADP (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 (β2M$^{-/-}$, K$^{b-/-}$, D$^{b-/-}$, or K$^{b-/-}$D$^{b-/-}$) mice were differentially labeled with 0.5 and 5 μM CFSE (Invitrogen), respectively. A total of 5×10^6 of each population were mixed at a ratio of 1:1 and administered by i.v. injection into untreated or pretreated (150 μg polyinosinic-polycytidylic acid [poly-IC] 24 h prior) recipient mice. After 16 h, recipient spleens were harvested, prepared, and analyzed by flow cytometry for the presence of CFSE-labeled donor cells. The amount of rejection was calculated as follows: percentage rejection = $1 - ([\text{CFSE}^{low}/\text{CFSE}^{high}]\text{input}/[\text{CFSE}^{low}/\text{CFSE}^{high}]\text{output}) \times 100\%$.

IFN-γ–induction assays

Splenocytes from poly-IC–treated mice (150 μg, 18 h prior) were incubated 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. $DX5^{+}TCR\beta^{-}$ 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^{129} 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. 1*A*), the *Ly49* gene cluster of 129S1 was bred onto a B6 genetic background. B6.Ly49^{129} 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^{129} 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^{129} 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^{129} mice using various *Ly49* cDNAs and restriction-enzyme combinations (Fig. 1*B*, 1*C*). Thus, RFLP analysis suggests that the 129S1 *Ly49* gene cluster in the B6.Ly49^{129} line is intact.

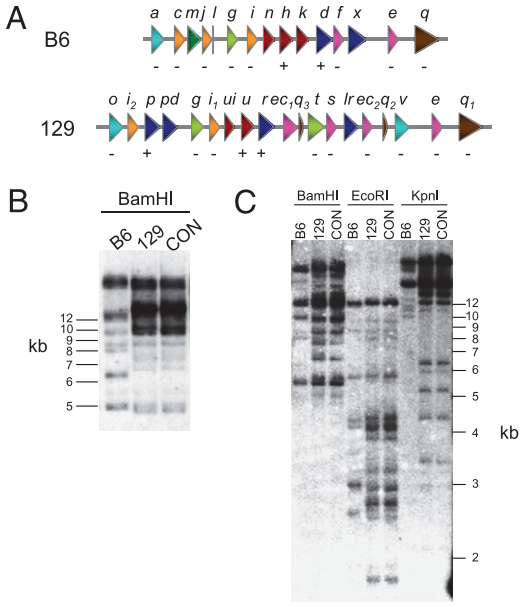

FIGURE 1. 129S1 and B6.Ly49^{129} mice exhibit identical *Ly49* gene organization. *A*, The *Ly49* gene clusters of B6 and 129 mice. Arrowheads indicate *Ly49* gene location, orientation, and size. Genes with the same colors indicate relation by descent or by duplication. Functional genes encoding inhibitory (ITIM-containing) Ly49 are indicated with a minus (−) sign, whereas genes encoding functional activating Ly49 are indicated with a plus (+) sign. *B* and *C*, RFLP analysis of the *Ly49* gene cluster was performed on B6.Ly49^{129}, 129S1, and B6 mice. Thymus DNA was isolated from the indicated mouse strains and analyzed by Southern blot using the indicated restriction enzymes for digestion. The blots were hybridized with *Ly49o* and *Ly49e* (*B*) and *Ly49g* and *Ly49e* (*C*) cDNA probe cocktails.

To verify that B6.Ly49^{129} NK cells express Ly49 belonging to the 129S1 haplotype, flow-cytometric analysis was carried out on fresh $DX5^{+}TCR\beta^{-}$ splenocytes from B6, 129S1, and B6.Ly49^{129} mice, using a panel of mAbs known to be specific for B6- and/or 129-derived Ly49 (Fig. 2). B6.Ly49^{129} and 129S1 NK cells displayed no binding to Cwy-3, 3D10, and 5E6 mAbs, which bind to the B6 alleles of Ly49G, Ly49H, and Ly49C/I, respectively. In contrast, a subset of B6.Ly49^{129} and 129S1 NK cells were stained by AT8, a mAb produced in our laboratory that binds to Ly49G^{129} but not Ly49G^{B6} (24). Anti-Ly49 mAbs 4E5 and 12A8 bound to a larger NK cell subset in B6.Ly49^{129} and 129S1 mice versus B6 mice (Fig. 2). Unexpectedly, although 14B11 (anti-Ly49C/I/F/H) also bound to NK cell subsets in all three strains, the percentage of positive NK cells in B6.Ly49^{129} mice was significantly different from that observed for 129S1 or B6 mice, with 14B11 staining an intermediate-sized subset of B6.Ly49^{129} 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. 1*B*, 1*C*). For independent confirmation, the recently described Ly49Q-deficient mouse was also backcrossed onto the B6 background for 10 generations (B6.129-Ly49Q$^{lox/lox}$ mice); like the B6.Ly49^{129} 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$^{lox/lox}$ NK cells was similar to that of B6.Ly49^{129} 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^{129} 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^{129} NK cells (Supplemental Fig. 1*A*). 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^{129} NK cells when a strain difference was evident (CD62L; Supplemental Fig. 1*B*). 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^{B6}), 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^{129} congenic mice are limited to epistatic influences of the B6 genetic background imposed upon 129-derived *Ly49* gene products.

B6.Ly49^{129} 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^{129} mice were used as effectors against a panel of target cells in [^{51}Cr]-

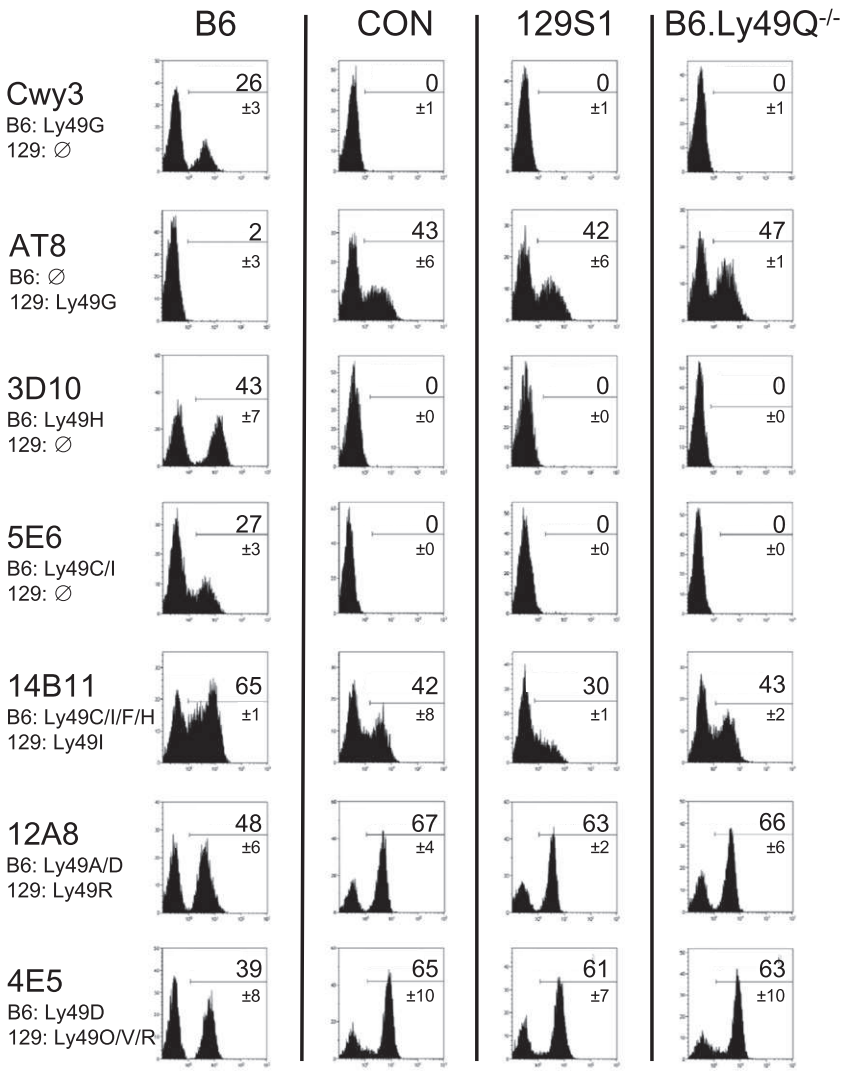

FIGURE 2. 129- and non–129-like Ly49 expression on B6.Ly49^{129} NK cells. Splenocytes from the indicated mouse strains were stained with allophycocyanin–anti-DX5, PE-anti–TCR-β, and the indicated FITC–anti-Ly49 mAbs, followed by multi-parameter flow-cytometric analysis. The anti-Ly49–staining profiles are shown after gating on NK cells (DX5^{+}TCR-β^{-}). The Ly49 reactivity in B6 and 129S1 mice is shown below the name of each mAb. The mean percentage (± SD) of positively stained NK cells is indicated in each graph. A representative experiment is shown from at least four separate experiments for each mAb.

release cytotoxicity assays. Killing of MHC-sufficient RMA targets was low for NK cells from all three strains (Fig. 3*A*), whereas the killing of MHC-deficient RMA-S targets was significantly greater (Fig. 3*A*). The killing of RMA-S targets by B6 LAK effector cells was much stronger than that displayed by 129S1 LAK cells, whereas B6.Ly49^{129} 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.Ly49^{129} LAK cells, whereas the cytotoxicity displayed by 129S1 LAK cells was significantly reduced in comparison (Fig. 3*A*). Interestingly, the killing of CHO cells by B6.Ly49^{129} LAK cells was intermediate between that of B6 and 129S1 LAK cells (Fig. 3*A*); 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.Ly49^{129} 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.Ly49^{129} NK cells. Ab-blocking experiments showed that the 129S1 alleles in B6.Ly49^{129} LAK cells for Ly49D and Ly49G mediate induction and inhibition of cytotoxicity toward CHO cells, respectively (Fig. 3*B*). Overall, the cytotoxicity mediated by B6.Ly49^{129} NK cells is much more comparable to that of B6 NK 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.Ly49^{129} NK cells when stimulated with YAC-1 cells (Fig. 4*A*), 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.Ly49^{129} NK cells were induced at twice the levels of 129S1 NK cells, B6.Ly49^{129} IFN-γ induction was slightly lower than the level shown by B6 NK cells (Fig. 4*A*). Activation via plate-bound mAb to NKG2D resulted in roughly equal percentages of IFN-γ^{+} NK cells from the three strains of mice (Fig. 4*B*), 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. 4*B*), 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.Ly49^{129} NK cells.

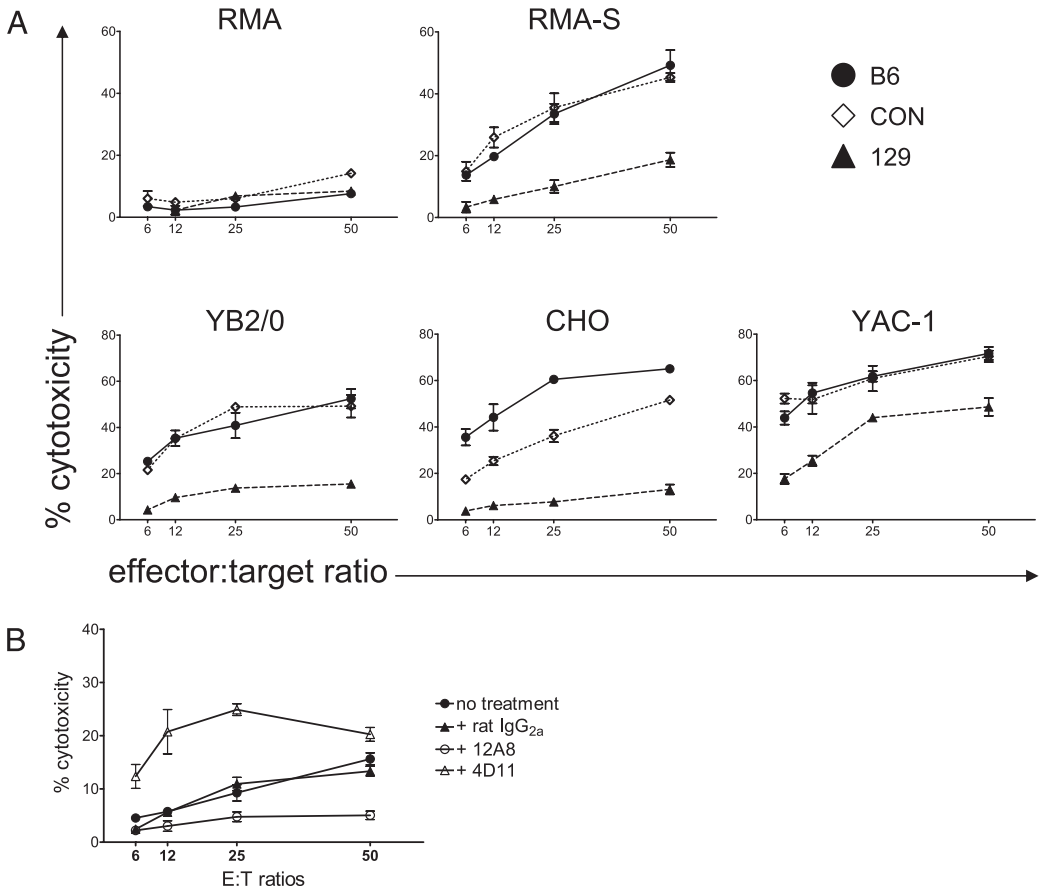

FIGURE 3. B6.Ly49^{129} NK cells display strong, B6-like cytotoxicity toward tumor target cells. *A*, Plastic-adherent IL-2–activated NK (LAK) cells were prepared from B6, 129S1, and B6.Ly49^{129} splenocytes and used in cytotoxicity assays against ^{51}Cr-labeled RMA, RMA-S, YB2/0, YAC-1, and CHO tumor target cells at the indicated E:T ratios. *B*, LAK cells were prepared from B6.Ly49^{129} mice and used in cytotoxicity assays against CHO cells in the presence of the indicated mAbs. mAb 4D11 binds to Ly49G^{129}, whereas mAb 12A8 binds to Ly49R^{129}. The mean percentage of lysed tumor cells (± SD) from triplicate wells is indicated. A representative experiment from three to five separate experiments for each target cell is shown.

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. 4*C*. The lectin-like inhibitory NKG2A/CD94 heterodimer is expressed on a subset of NK cells (Supplemental Fig. 1*A*) that recognizes Qa-1^b, a ubiquitously expressed MHC-Ib molecule that can present the H-2D^b leader peptide. Like Ly49, NKG2A confers educational specificity in B6 mice (15). Interestingly, NKG2A$^+$ B6.Ly49^{129} NK cells produced 2-fold more IFN-γ than NKG2A$^-$ cells after YAC-1 stimulation (Figs. 4*C*, 5*A*). In addition, Ly49I, Ly49O, and Ly49V from 129-strain mice were shown to functionally bind K^b and/or D^b (31). In agreement with this, B6.Ly49^{129} NK cells expressing these inhibitory self–MHC-I–specific receptors produced more IFN-γ than the corresponding negative subset (Figs. 4*C*, 5*A*). The IFN-γ production in the negative subsets may be due to the expression of the other self–MHC-I–specific receptors. In agreement with this, when combinations of self–MHC-I–specific receptors were examined, the negative subset produced the least IFN-γ relative to the corresponding Ly49/NKG2A$^+$ subset (Fig. 4*C*). As a control for self-specificity, the same experiment was performed with Ly49G$^+$ and Ly49G$^-$ NK cells from B6.Ly49^{129} mice. Ly49G^{129} has little or no specificity for K^b or D^b compared with Ly49I/O/V (31). Similar to Ly49A in B6 mice (14), after YAC-1 stimulation, the proportion of IFN-γ$^+$Ly49G$^+$ NK cells was not larger than the IFN-γ$^+$ Ly49G$^-$ NK cell subset (Figs. 4*C*, 5*A*).

The percentage of IFN-γ$^+$ NK cells in the Ly49$^+$ and Ly49$^-$ subsets after YAC-1 stimulation are shown in Fig. 5*A* and 5*B*. 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. 5*B*). Similar results were found with less complex stimuli, such as plate-bound anti-Nkp46 mAb (Fig. 5*C*, 5*D*), 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.Ly49^{129} 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.

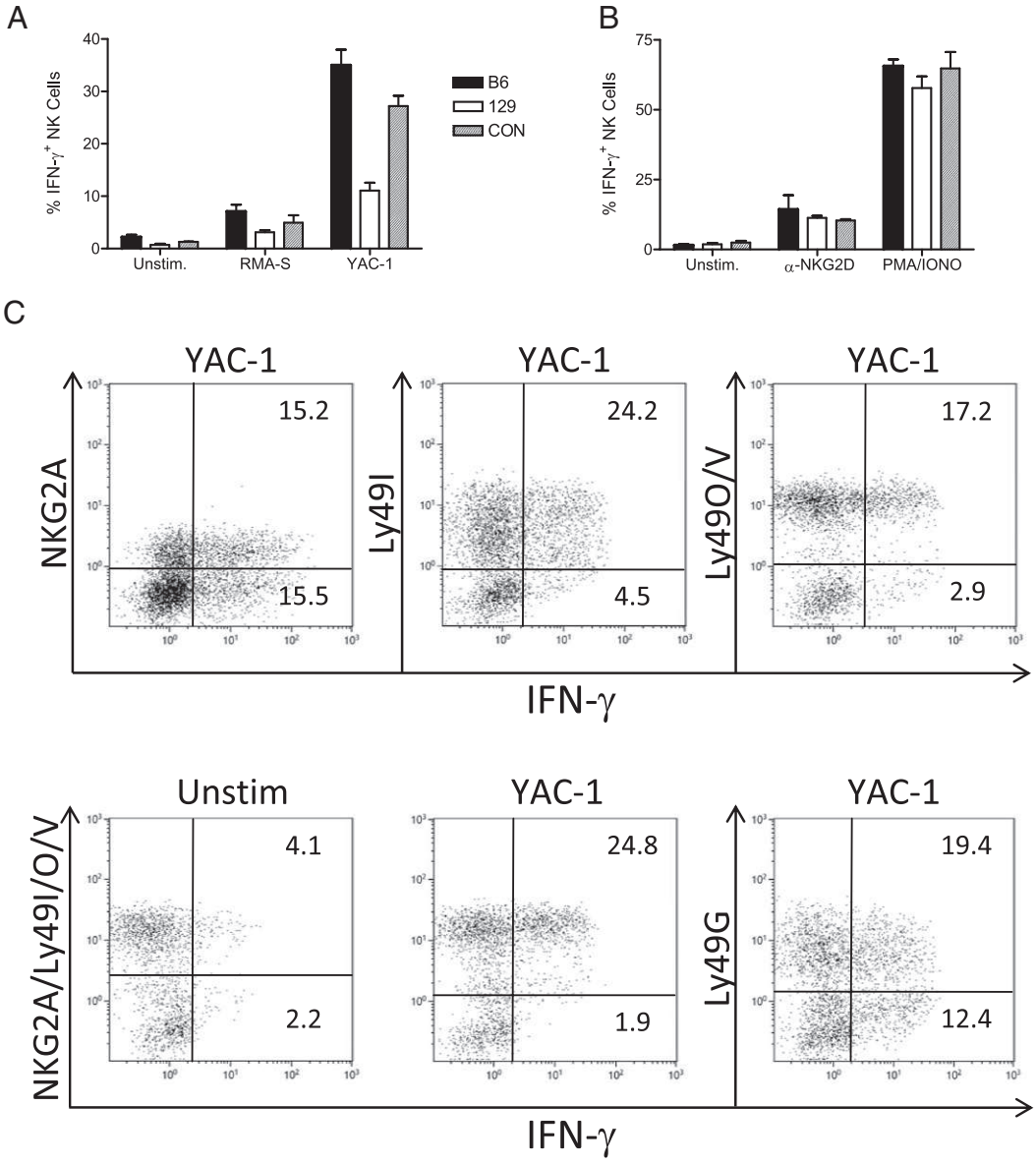

FIGURE 4. Activation-induced IFN-γ production in B6.Ly49^{129} NK cells is dependent on expression of self-MHC receptors. Splenocytes from poly-IC–injected B6, 129S1, or B6.Ly49^{129} mice were incubated with RMA-S or YAC-1 tumor cells in a 1:1 ratio (*A*) or on plates coated with anti-NKG2D mAb or with PMA/ionomycin (*B*), followed by intracellular staining for IFN-γ and analysis of DX5^{+}TCR-β^{-}–gated cells by flow cytometry. The mean (± SD) of three mice for each strain is shown. *C*, The same experiment was performed as in *A* with YAC-1 stimulation, but NK cells were also stained for Ly49I, Ly49O, Ly49V, Ly49G, or NKG2A to reveal NK cell subset-specific contribution to IFN-γ production. The numbers in each quadrant indicate the percentage of total cells.

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 $K^{b-/-}$, $D^{b-/-}$, or $K^{b-/-}D^{b-/-}$ mice were fluorescently labeled and injected into B6, 129S1, or B6.Ly49^{129} mice along with differentially labeled WT splenocytes as an internal rejection control. B6 and 129S1 mice were able to reject $K^{b-/-}$ 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. 6*A*). The rejection of $K^{b-/-}$ cells by B6.Ly49^{129} mice was as efficient as that of B6 mice. In contrast, the rejection of $D^{b-/-}$ cells by all three mouse strains was detectable, but low, with no significant differences (Fig. 6*A*). This result suggests that D^{b} contributes less than K^{b} to NK cell education during development and is in agreement with previous studies (34). The rejection of $K^{b-/-}D^{b-/-}$ splenocytes by B6 and 129S1 mice was similar to the rejection of $K^{b-/-}$ splenocytes. Interestingly, B6.Ly49^{129} mice rejected $K^{b-/-}D^{b-/-}$ 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. 6*B*). Interestingly, although 129S1 mice

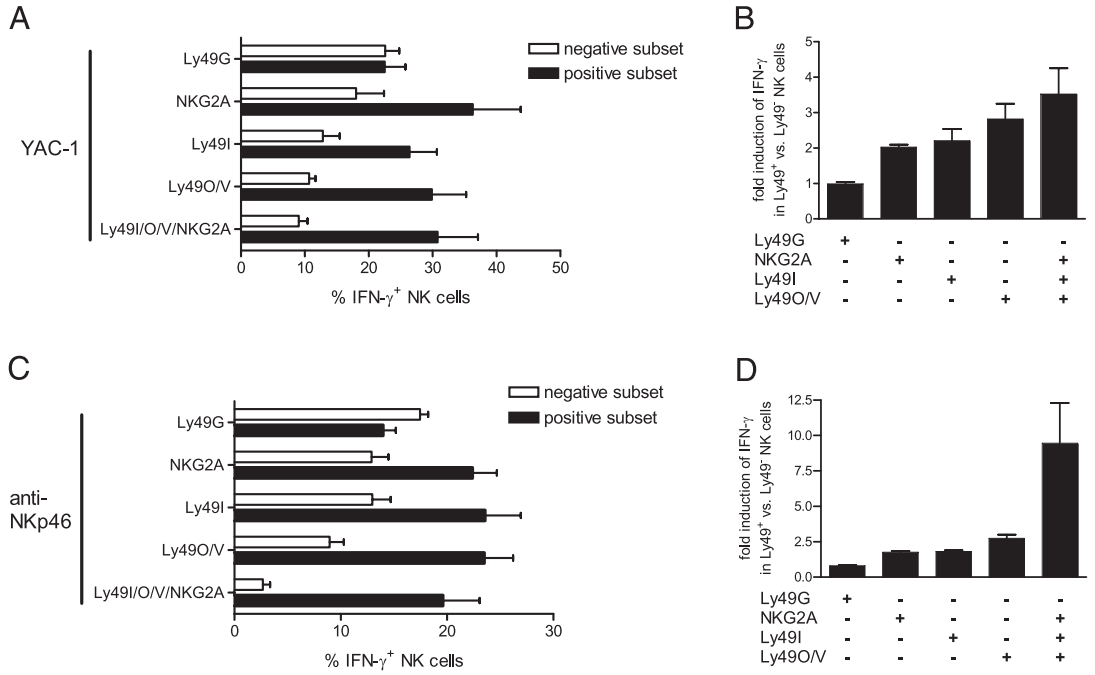

FIGURE 5. Relative responsiveness of NK cells expressing 129-derived self-MHC receptors. *A*, B6.Ly49^{129} splenocytes were coincubated with YAC-1 and then stained for IFN-γ and various Ly49 haplotypes and/or NKG2A as in Fig. 4*B*. The mean percentage (± SD) of IFN-γ$^+$ NK cells in the Ly49$^+$ and Ly49$^-$ subsets from one representative experiment using three mice is shown. *B*, The fold difference in IFN-γ$^+$ NK cells by Ly49/NKG2A$^+$ versus Ly49/NKG2A$^-$ subsets after YAC-1 stimulation is depicted as an index. *C* and *D*, The results of similar analyses and calculations that were performed for B6.Ly49^{129} NK cells stimulated with plate-bound anti-NKp46 mAb.

rejected less well than either of the B6 background mice, rejection of K$^{b-/-}$ cells by B6.Ly49^{129} mice was consistently and significantly greater than that of B6 mice (Fig. 6*B*). Rejection of D$^{b-/-}$ cells was also dramatically increased by poly-IC in all three mouse strains, with B6 and B6.Ly49^{129} mice rejecting equally well and more strongly than 129S1 mice (Fig. 6*B*). Thus, D^b 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.Ly49^{129} mice resulted in almost total rejection of K$^{b-/-}$D$^{b-/-}$ splenocytes (Fig. 6*B*), again showing the contribution of D^b to NK cell education. In summary, NK cells from B6.Ly49^{129} mice are effective at discriminating normal from MHC-I–deficient cells and, thus, the 129S1 Ly49 haplotype is sufficient to educate NK cells for the rejection of K^b-deficient and/or D^b-deficient cells. Moreover, the 129S1 Ly49 repertoire appears to exhibit a stronger impact on NK education than the B6 Ly49 repertoire in the recognition of specific self–MHC-I alleles.

Because the 129S1 donor region on chromosome 6 of B6.Ly49^{129} mice may contain genes other than *Ly49* that can affect NK cell-mediated killing of MHC-deficient cells, we repeated the rejection experiment using B6.Ly49Q$^{lox/lox}$ mice, which are also congenic for the 129S1 *Ly49* gene cluster. B6.Ly49Q$^{lox/lox}$ mice possess roughly half the 129S1 genetic contribution of B6.Ly49^{129} mice, as assessed by SNP analysis (see *Materials and Methods*). B6.Ly49Q$^{lox/lox}$ mice, like B6.Ly49^{129} mice, rejected K$^{b-/-}$D$^{b-/-}$ splenocytes more strongly than did pure B6 mice (Fig. 6*C*). These results are consistent with the hypothesis that the 129S1 *Ly49* gene cluster provides superior NK cell education compared with the B6 *Ly49* haplotype; however, the contribution of closely linked 129S1-derived non-*Ly49* genes cannot be formally excluded.

B6.Ly49^{129} mice are relatively susceptible to MCMV

The strongest effects of Ly49/KIR haplotype divergence are apparent for genes encoding activating receptors, in which the absence of a specific gene results in the loss of NK cell activation under 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.1*A*) to efficiently recognize the MCMV-encoded m157 protein on the surface of infected cells (4). However, Ly49P in MA/My (H-2^k) mice provides resistance to MCMV via a host MHC-I–dependent mechanism (30). MCMV-susceptible 129S1 mice express Ly49P on NK cells (Fig. 1*A*); 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.Ly49^{129} mice provide a controlled genetic background, including normal DAP12 signaling, on which to test the MCMV responsiveness of the 129S1 Ly49 repertoire. B6.Ly49^{129} 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-2^b 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

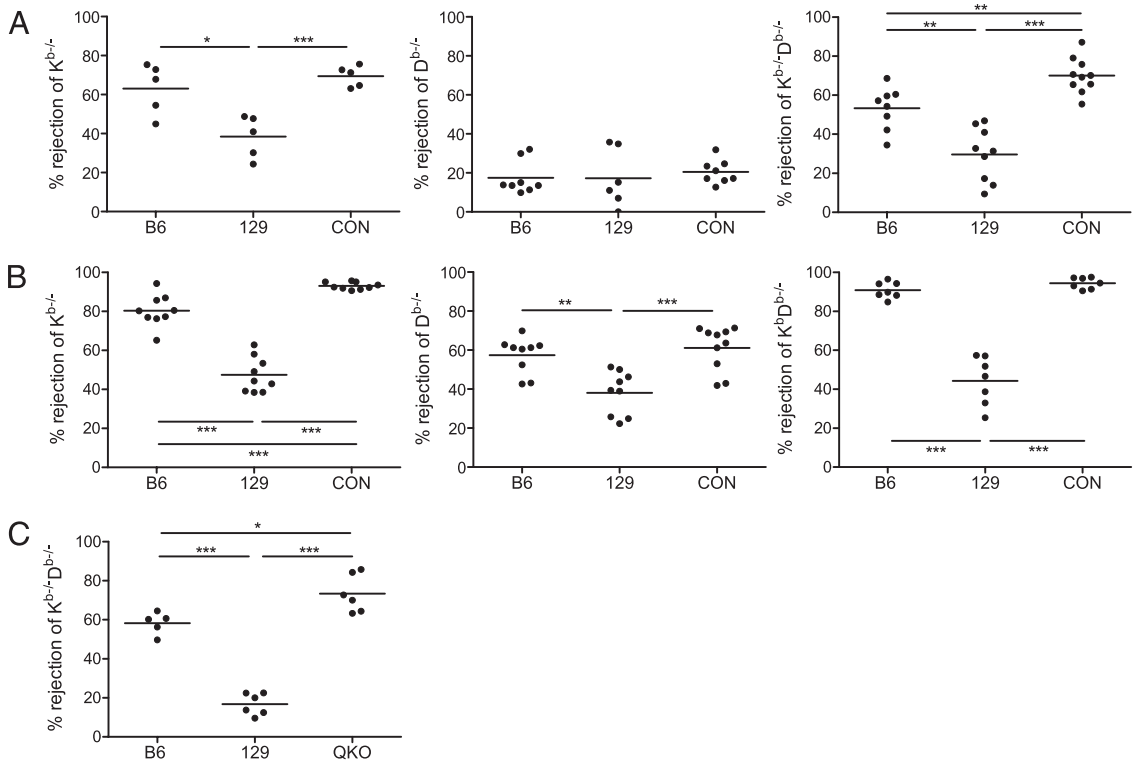

FIGURE 6. Efficient rejection of MHC-deficient cells by mice possessing a 129S1 Ly49 haplotype. Untreated mice (*A*) or poly-IC–pretreated B6, 129S1, and B6.Ly49^{129} mice (*B*) were injected i.v. with a mixture of WT and MHC-deficient ($K^{b-/-}$, $D^{b-/-}$, or $K^{b-/-}D^{b-/-}$) splenocytes that were differentially labeled with CFSE. After 16 h, splenocytes from recipient mice were analyzed by flow cytometry for the presence of CFSE-high and CFSE-low cells. The relative proportion was calculated to obtain the percentage of rejection. Horizontal bars indicate the mean, and each symbol represents a single mouse. The results are presented as aggregate data of at least three separate experiments. *C*, Untreated B6.Ly49Q$^{lox/lox}$ (QKO) mice were tested for their ability to reject $K^{b-/-}D^{b-/-}$ splenocytes, as described above, in comparison with B6 and 129S1 mice. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

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) *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-γ production (Figs. 3*A*, 4*A*). Furthermore, the in vivo rejection of MHC-I–deficient cells by B6.Ly49^{129} mice was equal to, if not better than, B6 mice

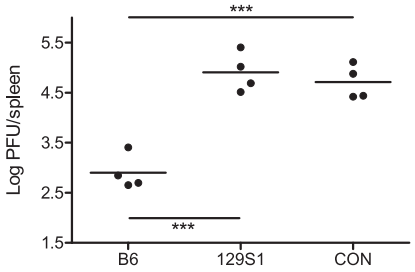

FIGURE 7. B6.Ly49^{129} mice are susceptible to MCMV. B6, 129S1, and B6.Ly49^{129} mice were injected i.p. with 5000 PFU of Smith strain MCMV. After 3 d, the splenic viral titer was evaluated by plaque assay on BALB/c murine embryonic fibroblasts. Horizontal bars indicate the mean total PFU per spleen, and each symbol represents a single mouse. Data are representative of two experiments with three or four mice per experimental group. $***p < 0.001$.

(Fig. 6*A*, 6*B*). 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 *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. 3*A*), RMA-S induced less IFN-γ in B6.Ly49^{129} NK cells versus B6 NK cells (Fig. 4*A*). 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-γ 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 by14B11) 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$^{lox/lox}$), with a smaller 129-donor segment on chromosome 6 (Fig. 2). Collectively, these data suggest that NKC/MHC-Ia–independent genes on the B6 background may modulate Ly49 expression levels.

As previously shown for 129 inbred strains, the MCMV susceptibility of B6.Ly49^{129} 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^{129} NK cells killed RMA-S and YAC-1 target cells equally well compared with B6 NK cells (Fig. 3*A*). Data from a previous study using NOD.NK1.1^{B6} congenic mice agree with our finding that B6.Ly49^{129} NK cell-mediated killing of RMA-S cells is independent of the NKC (43). Interestingly, the cytotoxicity toward CHO cells displayed by B6.Ly49^{129} NK cells was intermediate between that of B6 (high) and 129S1 (low) NK cells (Fig. 3*A*). Thus, B6.Ly49^{129} 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, Hm1-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 Hm1-C4, which, in turn, leads to decreased CHO cytotoxicity. In addition, possible stronger binding by the inhibitory Ly49G^{129}, which was also shown to recognize a CHO ligand (45), may contribute to lower CHO killing by B6.Ly49^{129} NK cells. The effects of mAb blocking of Ly49R and Ly49G on congenic NK cells during CHO cytotoxicity assays shown in Fig. 3*B* are consistent with this hypothesis.

Previous studies in our laboratory directly compared Ly49G^{B6+} and Ly49G^{129+} NK cells from (129X1 $\times$ B6) F_1 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^{B6} 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 Ly49D^{+} NK cells, was downstream of the receptor. Our current data using B6.Ly49^{129} 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^{b} and/or D^{b}) results in significantly greater functional responses compared with NK cell subsets lacking these self-MHC receptors (Figs. 4*C*, 5*A*). However, the weaker overall functionality of inbred 129S1 versus congenic B6.Ly49^{129} 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^{+} NK cells make more IFN-γ than do licensed Ly49D^{+} NK cells in response to ligand-bearing cells (46).

Unexpectedly, the rejection of MHC-I–deficient cells by B6.Ly49^{129} 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. 6*A*, 6*B*). Our analysis of B6.Ly49Q$^{lox/lox}$ mice, which are also congenic for the *Ly49*^{129S1} 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. 6*C*). 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. 1*A*). 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^{129} mice can use Ly49I, Ly49O, Ly49V, and NKG2A (Figs. 4*C*, 5*A*). Thus, it is possible that the increased in vivo rejection of MHC-I–deficient cells by B6.Ly49^{129} mice is a consequence of B6.Ly49^{129} 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^{129}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

The authors have no financial conflicts of interest.

References

1. Kärre, K., H. G. Ljunggren, G. Piontek, and R. Kiessling. 1986. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature* 319: 675–678.
2. Lanier, L. L. 2005. NK cell recognition. *Annu. Rev. Immunol.* 23: 225–274.
3. Ravetch, J. V., and L. L. Lanier. 2000. Immune inhibitory receptors. *Science* 290: 84–89.
4. Arase, H., E. S. Mocarski, A. E. Campbell, A. B. Hill, and L. L. Lanier. 2002. Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. *Science* 296: 1323–1326.
5. Kielczewska, A., M. Pyzik, T. Sun, A. Krmpotic, M. B. Lodoen, M. W. Munks, M. Babic, A. B. Hill, U. H. Koszinowski, S. Jonjic, et al. 2009. Ly49P recognition of cytomegalovirus-infected cells expressing H2-Dk and CMV-encoded m04 correlates with the NK cell antiviral response. *J. Exp. Med.* 206: 515–523.
6. Furukawa, H., K. Iizuka, J. Poursine-Laurent, N. Shastri, and W. M. Yokoyama. 2002. A ligand for the murine NK activation receptor Ly-49D: activation of

tolerized NK cells from beta 2-microglobulin-deficient mice. *J. Immunol.* 169: 126–136.
7. Kane, K. P., E. T. Silver, and B. Hazes. 2001. Specificity and function of activating Ly-49 receptors. *Immunol. Rev.* 181: 104–114.
8. Ljunggren, H. G., and K. Kärre. 1990. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol. Today* 11: 237–244.
9. Ljunggren, H. G., L. Van Kaer, H. L. Ploegh, and S. Tonegawa. 1994. Altered natural killer cell repertoire in Tap-1 mutant mice. *Proc. Natl. Acad. Sci. USA* 91: 6520–6524.
10. Liao, N. S., M. Bix, M. Zijlstra, R. Jaenisch, and D. Raulet. 1991. MHC class I deficiency: susceptibility to natural killer (NK) cells and impaired NK activity. *Science* 253: 199–202.
11. Höglund, P., C. Ohlén, E. Carbone, L. Franksson, H. G. Ljunggren, A. Latour, B. Koller, and K. Kärre. 1991. Recognition of beta 2-microglobulin-negative (beta 2m-) T-cell blasts by natural killer cells from normal but not from beta 2m- mice: nonresponsiveness controlled by beta 2m- bone marrow in chimeric mice. *Proc. Natl. Acad. Sci. USA* 88: 10332–10336.
12. Ohlén, C., G. Kling, P. Höglund, M. Hansson, G. Scangos, C. Bieberich, G. Jay, and K. Kärre. 1989. Prevention of allogeneic bone marrow graft rejection by H-2 transgene in donor mice. *Science* 246: 666–668.
13. Brodin, P., T. Lakshmikanth, S. Johansson, K. Kärre, and P. Höglund. 2009. The strength of inhibitory input during education quantitatively tunes the functional responsiveness of individual natural killer cells. *Blood* 113: 2434–2441.
14. Kim, S., J. Poursine-Laurent, S. M. Truscott, L. Lybarger, Y. J. Song, L. Yang, A. R. French, J. B. Sunwoo, S. Lemieux, T. H. Hansen, and W. M. Yokoyama. 2005. Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature* 436: 709–713.
15. Fernandez, N. C., E. Treiner, R. E. Vance, A. M. Jamieson, S. Lemieux, and D. H. Raulet. 2005. A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules. *Blood* 105: 4416–4423.
16. Joncker, N. T., N. C. Fernandez, E. Treiner, E. Vivier, and D. H. Raulet. 2009. NK cell responsiveness is tuned commensurate with the number of inhibitory receptors for self-MHC class I: the rheostat model. *J. Immunol.* 182: 4572–4580.
17. Carlyle, J. R., A. Mesci, J. H. Fine, P. Chen, S. Bélanger, L. H. Tai, and A. P. Makrigiannis. 2008. Evolution of the Ly49 and Nkrp1 recognition systems. *Semin. Immunol.* 20: 321–330.
18. Carlyle, J. R., A. Mesci, B. Ljutic, S. Belanger, L. H. Tai, E. Rousselle, A. D. Troke, M. F. Proteau, and A. P. Makrigiannis. 2006. Molecular and genetic basis for strain-dependent NK1.1 alloreactivity of mouse NK cells. *J. Immunol.* 176: 7511–7524.
19. Yokoyama, W. M., L. B. Jacobs, O. Kanagawa, E. M. Shevach, and D. I. Cohen. 1989. A murine T lymphocyte antigen belongs to a supergene family of type II integral membrane proteins. *J. Immunol.* 143: 1379–1386.
20. Belanger, S., L. H. Tai, S. K. Anderson, and A. P. Makrigiannis. 2008. Ly49 cluster sequence analysis in a mouse model of diabetes: an expanded repertoire of activating receptors in the NOD genome. *Genes Immun.* 9: 509–521.
21. Idris, A. H., K. Iizuka, H. R. Smith, A. A. Scalzo, and W. M. Yokoyama. 1998. Genetic control of natural killing and in vivo tumor elimination by the Chok locus. *J. Exp. Med.* 188: 2243–2256.
22. Lee, S. H., A. Zafer, Y. de Repentigny, R. Kothary, M. L. Tremblay, P. Gros, P. Duplay, J. R. Webb, and S. M. Vidal. 2003. Transgenic expression of the activating natural killer receptor Ly49H confers resistance to cytomegalovirus in genetically susceptible mice. *J. Exp. Med.* 197: 515–526.
23. Mehta, I. K., J. Wang, J. Roland, D. H. Margulies, and W. M. Yokoyama. 2001. Ly49A allelic variation and MHC class I specificity. *Immunogenetics* 53: 572–583.
24. Makrigiannis, A. P., E. Rousselle, and S. K. Anderson. 2004. Independent control of Ly49g alleles: implications for NK cell repertoire selection and tumor cell killing. *J. Immunol.* 172: 1414–1425.
25. Kiessling, R., E. Klein, and H. Wigzell. 1975. "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *Eur. J. Immunol.* 5: 112–117.
26. Makrigiannis, A. P., D. Patel, M. L. Goulet, K. Dewar, and S. K. Anderson. 2005. Direct sequence comparison of two divergent class I MHC natural killer cell receptor haplotypes. *Genes Immun.* 6: 71–83.
27. McVicar, D. W., R. Winkler-Pickett, L. S. Taylor, A. Makrigiannis, M. Bennett, S. K. Anderson, and J. R. Ortaldo. 2002. Aberrant DAP12 signaling in the 129 strain of mice: implications for the analysis of gene-targeted mice. *J. Immunol.* 169: 1721–1728.
28. Pérarnau, B., M. F. Saron, B. R. San Martin, N. Bervas, H. Ong, M. J. Soloski, A. G. Smith, J. M. Ure, J. E. Gairin, and F. A. Lemonnier. 1999. Single H2Kb, H2Db and double H2KbDb knockout mice: peripheral CD8+ T cell repertoire and anti-lymphocytic choriomeningitis virus cytolytic responses. *Eur. J. Immunol.* 29: 1243–1252.
29. Tai, L. H., M. L. Goulet, S. Belanger, N. Toyama-Sorimachi, N. Fodil-Cornu, S. M. Vidal, A. D. Troke, D. W. McVicar, and A. P. Makrigiannis. 2008. Positive regulation of plasmacytoid dendritic cell function via Ly49Q recognition of class I MHC. *J. Exp. Med.* 205: 3187–3199.
30. Desrosiers, M. P., A. Kielczewska, J. C. Loredo-Osti, S. G. Adam, A. P. Makrigiannis, S. Lemieux, T. Pham, M. B. Lodoen, K. Morgan, L. L. Lanier, and S. M. Vidal. 2005. Epistasis between mouse Klra and major histocompatibility complex class I loci is associated with a new mechanism of natural killer cell-mediated innate resistance to cytomegalovirus infection. *Nat. Genet.* 37: 593–599.
31. Makrigiannis, A. P., A. T. Pau, A. Saleh, R. Winkler-Pickett, J. R. Ortaldo, and S. K. Anderson. 2001. Class I MHC-binding characteristics of the 129/J Ly49 repertoire. *J. Immunol.* 166: 5034–5043.
32. Bix, M., N. S. Liao, M. Zijlstra, J. Loring, R. Jaenisch, and D. Raulet. 1991. Rejection of class I MHC-deficient haemopoietic cells by irradiated MHC-matched mice. *Nature* 349: 329–331.
33. Roth, C., J. R. Carlyle, H. Takizawa, and D. H. Raulet. 2000. Clonal acquisition of inhibitory Ly49 receptors on developing NK cells is successively restricted and regulated by stromal class I MHC. *Immunity* 13: 143–153.
34. Johansson, S., M. Johansson, E. Rosmaraki, G. Vahlne, R. Mehr, M. Salmon-Divon, F. Lemonnier, K. Kärre, and P. Höglund. 2005. Natural killer cell education in mice with single or multiple major histocompatibility complex class I molecules. *J. Exp. Med.* 201: 1145–1155.
35. Lucas, M., W. Schachterle, K. Oberle, P. Aichele, and A. Diefenbach. 2007. Dendritic cells prime natural killer cells by trans-presenting interleukin 15. *Immunity* 26: 503–517.
36. Wilson, M. J., M. Torkar, A. Haude, S. Milne, T. Jones, D. Sheer, S. Beck, and J. Trowsdale. 2000. Plasticity in the organization and sequences of human KIR/ILT gene families. *Proc. Natl. Acad. Sci. USA* 97: 4778–4783.
37. Martin, M. P., X. Gao, J. H. Lee, G. W. Nelson, R. Detels, J. J. Goedert, S. Buchbinder, K. Hoots, D. Vlahov, J. Trowsdale, et al. 2002. Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. *Nat. Genet.* 31: 429–434.
38. Brown, M. G., A. O. Dokun, J. W. Heusel, H. R. Smith, D. L. Beckman, E. A. Blattenberger, C. E. Dubbelde, L. R. Stone, A. A. Scalzo, and W. M. Yokoyama. 2001. Vital involvement of a natural killer cell activation receptor in resistance to viral infection. *Science* 292: 934–937.
39. Martin, M. P., G. Nelson, J. H. Lee, F. Pellett, X. Gao, J. Wade, M. J. Wilson, J. Trowsdale, D. Gladman, and M. Carrington. 2002. Cutting edge: susceptibility to psoriatic arthritis: influence of activating killer Ig-like receptor genes in the absence of specific HLA-C alleles. *J. Immunol.* 169: 2818–2822.
40. Held, W., and D. H. Raulet. 1997. Ly49A transgenic mice provide evidence for a major histocompatibility complex-dependent education process in natural killer cell development. *J. Exp. Med.* 185: 2079–2088.
41. Hanke, T., H. Takizawa, and D. H. Raulet. 2001. MHC-dependent shaping of the inhibitory Ly49 receptor repertoire on NK cells: evidence for a regulated sequential model. *Eur. J. Immunol.* 31: 3370–3379.
42. Kumánovics, A., A. Madan, S. Qin, L. Rowen, L. Hood, and K. Fischer Lindahl. 2002. QUOD ERAT FACIENDUM: sequence analysis of the H2-D and H2-Q regions of 129/SvJ mice. *Immunogenetics* 54: 479–489.
43. Johansson, S. E., H. Hall, J. Björklund, and P. Höglund. 2004. Broadly impaired NK cell function in non-obese diabetic mice is partially restored by NK cell activation in vivo and by IL-12/IL-18 in vitro. *Int. Immunol.* 16: 1–11.
44. Idris, A. H., H. R. Smith, L. H. Mason, J. R. Ortaldo, A. A. Scalzo, and W. M. Yokoyama. 1999. The natural killer gene complex genetic locus Chok encodes Ly-49D, a target recognition receptor that activates natural killing. *Proc. Natl. Acad. Sci. USA* 96: 6330–6335.
45. Mason, L. H. 2000. Recognition of CHO cells by inhibitory and activating Ly-49 receptors. *J. Leukoc. Biol.* 68: 583–586.
46. Orr, M. T., W. J. Murphy, and L. L. Lanier. 2010. 'Unlicensed' natural killer cells dominate the response to cytomegalovirus infection. *Nat. Immunol.* 11: 321–327.