Aberrant DAP12 Signaling in the 129 Strain of Mice: Implications for the Analysis of Gene-Targeted Mice[1]

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NK cells are implicated in antiviral responses, bone marrow transplantation and tumor immunosurveillance. Their function is controlled, in part, through the Ly49 family of class I binding receptors. Inhibitory Ly49s suppress signaling, while activating Ly49s (i.e., Ly49D) activate NK cells via the DAP12 signaling chain. Activating Ly49 signaling has been studied primarily in C57BL/6 mice, however, 129 substrains are commonly used in gene-targeting experiments. In this study, we show that in contrast to C57BL/6 NK cells, cross-linking of DAP12-coupled receptors in 129/J mice induces phosphorylation of DAP12 but not calcium mobilization or cytokine production. Consistent with poor-activating Ly49 function, 129/J mice reject bone marrow less efficiently than C57BL/6 mice. Sequence analysis of receptors and DAP12 suggests no structural basis for inactivity, and both the 129/J and C57BL/6 receptors demonstrate normal function in a reconstituted receptor system. Most importantly, reconstitution of Ly49D in 129/J NK cells demonstrated that the signaling deficit is within the NK cells themselves. These unexpected findings bring into question any NK analysis of 129/J, 129Sv, or gene-targeted mice derived from these strains before complete backcrossing, and provide a possible explanation for the differences observed in the immune response of 129 mice in a variety of models. *The Journal of Immunology,* **2002, 169: 1721–1728.**

Natural killer cells are implicated in a variety of immune responses including antiviral responses, bone marrow transplantation, and tumor immunosurveillance (1). In addition, as a principal source of IFN-γ early in the immune response, NK cells are pivotal in directing the cellular immune response away from a Th2-type response and toward a Th1-type response. Because of their ability to affect multiple facets of immunity, a great deal of work has recently been directed at the regulation of NK cell function. Much of this work has focused on the biology and biochemistry of the class I MHC-binding receptors of NK cells. Human NK cells possess two major types of inhibitory receptors, the killer Ig-like receptors (KIR)[3] and the type II members of the NKG2 family of genes (2–4). These receptor families recognize classical MHC class I and nonclassical HLA-E or MIC, respectively. Murine NK cells have devised a different means to the same end. They use a large family of type II C-type lectin-like receptors known as Ly49s (5–7). In both mice and humans, the majority of class I binding receptors are involved in the inhibition of cellular signals. Ligation of these inhibitory receptors by the appropriate class I proteins leads to tyrosine phosphorylation of the receptor's immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Phosphorylated ITIMs then serve as the recruitment point for the protein tyrosine phosphatase, SHP, Src homology-2 containing phosphatase (SHP)-1 (6). Recruited and activated SHP-1 then acts to dephosphorylate tyrosine-containing substrates required for the propagation of the cytolytic signal. Although efficient recruitment and activation of SHP-1 is required for the inhibitory activity, the exact substrates of the phosphatase are as yet unknown. Regardless, the result is attenuation of target-induced signals within the NK cell preventing target lysis and target-induced cytokine production.

In addition to the inhibitory receptors of NK cells, there are members of the KIR, Ly49, and NKG2 families that lack ITIMs and generate activation signals rather than inhibitory signals (6, 8). Examples include the short-tailed KIR such as KIR2DS2, Ly49D, H, L, M, P, and W and NKG2C (5, 6, 8–10). Instead of ITIMs, these receptors contain a charged residue (arginine or lysine) within their transmembrane domains. The positive charge within the transmembrane domain facilitates specific interaction with one of two newly described signal transduction proteins, DAP12 or DAP10 (11–15). These chains become tyrosine phosphorylated following receptor engagement then recruit downstream effector molecules such as the Syk tyrosine kinase, or phosphatidylinositol 3 kinase (16–18). In most cases, the ultimate downstream effects of ligation of these activating receptors are still unknown, however, reports from various laboratories demonstrate increased cytotoxicity, cytokine production (including IFN-γ), and most recently, substantial induction of chemokine production (8, 19–21).

Due to the efficiency with which 129/Sv-derived embryonic stem cells colonize an embryo and contribute to the germline, 129/Sv-derived embryonic stem cells are commonly used in gene-targeting experiments. In most cases, these animals are then backcrossed to other

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[3] Abbreviations used in this paper: KIR, killer Ig receptor; ITIM, immunoreceptor tyrosine-based inhibitory motif; SHP, Src homology-2 containing phosphatase; HEK, human embryonic kidney; CHO, Chinese hamster ovary; YAC, yeast artificial chromosome; BMC, bone marrow cells; ^{125}IUdR, ^{125}I-labeled iododeoxyuridine.

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strains such as C57BL/6 (B6), improving breeding efficiency and facilitating analysis of the knockout phenotype. In more rare cases, the original chimeric mice are crossed back to 129/Sv. However, there are now several examples where the penetrance and/or severity of a given phenotype is affected by its genetic background (22–24). The study of immunity in gene-targeted mice is particularly dangerous due to genetically based differences in the immune response. In fact, a comparison of immune models in the most commonly used strains for gene targeting, B6 and 129, demonstrates significant differences. For example, 129 mice are highly susceptible to infection with Sendai virus (25). The high virus titers are observed despite high IgG2a responses and high levels of IFN-γ produced in these mice. Studies of bone marrow chimeras confirmed the association between virus titer, IFN-γ, IgG2a, and the genetic background of the immune cells (25). 129 mice are also highly susceptible to murine CMV (26) and the antiviral response of resistant strains is mediated by NK cells (27, 28). In addition, 129 mice are highly susceptible to the induction of experimental systemic lupus erythematosus, and there are marked differences in the susceptibility of B6 and 129 mice to experimental autoimmune encephalomyelitis (29, 30). Moreover, analysis of bone marrow graft rejection, a process regulated by Ly49-bearing cells (31), showed that 129/J mice failed to reject, or weakly rejected, marrow from a variety of donor strains that were rejected by B6 mice (32). All of these differences are observed despite the fact that B6 and 129/J mice share the same MHC haplotype, H-2^b.

In the process of evaluating bone marrow rejection in various strains of mice, we noted that 129/J mice are much less efficient at rejecting bone marrow under conditions where rejection is largely mediated by NK cells. In addition, our analyses of gene-targeted mice confirmed that the 129 NK cell Ly49 repertoire can segregate along with the mutant allele resulting in comparison of mice differing not only in the targeted gene but in the genetic composition of their NK cells. Detailed analysis determined that the NK cells of 129/J mice fail to signal properly through the DAP12 signal transduction apparatus. This effect is not due to genetic abnormalities of the receptor or signaling chain themselves but instead appears to be the result of an inherent abnormality within the NK cells of 129/J mice. These unexpected findings bring into question the analysis of Ly49 and/or NK cell function in 129/J, 129/Sv, or gene-targeted mice before substantial backcrossing.

Materials and Methods

Abs, NK cells, and cell lines

Rat mAbs recognizing the various Ly49 family members, 4E5 (Ly49D), 12A8 (Ly49D/A), and 4D11 (Ly49G2) have been previously described (8, 33, 34). Similarly, anti-DAP12 antiserum (Rb20) has been described (16). Biotinylated antiphosphotyrosine Ab, clone 4G10 was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-Syk Abs were the gift of DNAX (Palo Alto, CA) and/or were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary Abs used for cross-linking studies were from Kirkegaard and Perry Laboratories (Gaithersburg, MD).

Primary murine NK cells were prepared and cultured for 4–8 days in recombinant human IL-2 (Chiron, Emoryville, CA) as described (35). In some cases, mice were pretreated with IL-2 to increase NK cell infiltration into the liver. Livers were harvested and leukocytes were purified, stained with specific mAbs, and sorted using a high speed MoFlo cell sorter (Cytomation, Fort Collins, CO) (36). After sorting, specific cell populations were expanded in culture medium containing rIL-2.

Human embryonic kidney (HEK) 293 T cells and Chinese hamster ovary (CHO) cells were maintained in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. YAC-1 cells were maintained in RPMI 1640 containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Cell stimulations, immunoprecipitation, and Western blotting

Mouse NK cells were washed once in RPMI 1640 medium supplemented with 10% FCS and suspended in 1 ml of the medium at a concentration of 1–10 $\times$ 10^6/ml. The cells were incubated at 37°C with shaking. The cells were stimulated for the indicated times with the specified Abs cross-linked with goat anti-rat IgG. After stimulation, the cells were pelleted, the supernatant was removed, and they were lysed on ice for 10 min in 1 ml Triton X-100 lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, and 10 μg/ml each leupeptin and aprotinin). The cell lysate was clarified by centrifugation at 15,000 $\times$ *g* in a refrigerated microcentrifuge. The clarified lysates were incubated for 1–2 h at 4°C with anti-DAP12 Ab that had been prebound to protein G-Sepharose. The sepharose beads were then collected and washed three times with wash buffer buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 1 mM Na_3VO_4, 1 mM PMSF, and 10 μg/ml each leupeptin and aprotinin). For immunoblotting, immunoprecipitates were eluted with sample buffer, separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 5% BSA in PBS/0.1% Tween. The membranes were probed with anti-phosphotyrosine as described. The bound Abs were detected with HRP-conjugated secondary Abs and developed using the ECL system (Amersham Pharmacia, Piscataway, NJ) according to the manufacturer's protocol.

Vaccinia infections

Vaccinia virus stocks were maintained and propagated as described (37). For vaccinia infections, cells were suspended in Dulbecco's PBS at a concentration of 4 $\times$ 10^6 cells/ml. Infection was with a multiplicity of infection of 20 for 1.5 h at 37°C. After the initial infection, the cells were diluted in complete IL-2-containing medium to a concentration of 0.4 $\times$ 10^6 cells/ml and incubated for an additional 5–6 h. Cells were washed, an aliquot was removed for analysis of Ly49 expression, and the remaining cells were loaded with calcium dyes as described below.

Calcium mobilization

Analysis of the changes in intracellular Ca^{2+} concentration was conducted using a FACSort flow cytometer (BD Biosciences, Mountain View, CA) and the calcium-sensitive fluorochromes Fluo-3 and Fura Red (Molecular Probes, Eugene OR). Cells (5 $\times$ 10^6/ml) were incubated at 37°C in complete medium containing 5 μg/ml fluo-3-acetoxymethyl ester and 5 μg/ml Fura Red-acetoxymethyl ester. After 30 min, cells were washed in serum-free DMEM containing 50 mM Tris (pH 7.5) and held at room temperature in the dark until analysis. The Ca^{2+} was monitored with the loaded cells (40 μl) diluted to 500 μl with Dulbecco's PBS containing Ca^{2+} (130 μg/ml), Mg^{2+} (100 μg/ml), glucose (1 mg/ml), and sodium pyruvate (36 μg/ml) at 37°C. Cells were kept at 37°C during analysis. Baseline data was collected for 20–30 s, then cells were stimulated with primary mAb (10 μg/ml; BD PharMingen, San Diego, CA) followed 20–25 s later by goat anti-rat Ab (10 μg/ml; Kirkegaard and Perry Laboratories) as described in the figure legends. Data were analyzed using the MultiTime kinetic experiment analysis software (Phoenix Flow Systems, San Diego, CA) and are expressed as the percent-responding cells relative to unstimulated baseline measurements.

Plasmids and expression studies

The cDNA for Ly49D has been described. The cDNA for *Ly49r* was cloned and has been reported elsewhere (38). Briefly, a cDNA library from IL-2-stimulated 129/J liver NK cells was constructed in the pBK-CMV vector (Stratagene, La Jolla, CA) by Cell and Molecular Technologies (Philipsburg, NJ). The library was screened using *Ly49o* as the probe. The resulting 129/J Ly49R (Ly49R^{129}) clone shows 94.7% identity to B6 Ly49D (Ly49D^{B6}) at the amino acid level. *Ly49r*^{129} and *Ly49 day*^{B6} were then both PCR subcloned into pEF6/V5-His (pEF) using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Similarly, this library was screened using our DAP12 cDNA as a probe and the resulting clones were sequenced and found to be identical over the coding region to the reported B6 DAP12 sequence (data not shown).

293T cells were transfected with FuGene 6 in accordance with the manufacturer's directions (Roche Molecular Biochemicals, Indianapolis, IN). Briefly, 300,000 cells were plated in six-well plates 1 day before transfection. Cells were transfected in the presence of medium containing 2% FBS with 0.25 μg/well each of pEF-Ly49D^{B6} or R^{129}, Syk (the gift of Dr. R. Geahlen, Purdue University, West Lafayette, IN), pSport2-DAP12, and an AP-1 luciferase reporter construct (39). DNA/FuGene 6 complexes were prepared in bulk for transfection of multiple wells in a ratio of 1–1.5 μg DNA to 5 μl FuGene 6. Six hours later, stimulating Abs (8 μg/ml 4E5 or 4D11 plus 1.7 μg/ml goat anti-rat IgG) was added. After an overnight culture, the cells were harvested and 10% of the cells were removed and tested for Ly49D expression using flow cytometry. The remaining cells

were lysed and assayed for luciferase activity using a luciferase assay system (Promega, Madison WI) and a Moonlight 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI). Data are reported as the fold induction in light units compared with the control stimulations and are adjusted for protein concentration.

Cytotoxicity assays and cytokine assays

The induction of IFN-γ by Ly49D/R cross-linking and cytotoxicity assays against the YAC-1 and CHO targets was done as described. Ab-dependent killing used 721.221 targets coated with anti-HLA-DR Ab (8).

Bone marrow transplantation

These procedures were performed as described (40). Briefly, irradiated mice (8 Gy) were infused with 2.5 or 3.5×10^6 donor bone marrow cells (BMC). Proliferation of transplanted BMC in recipients was judged in terms of splenic uptake (%) of ^{125}I-labeled iododeoxyuridine (^{125}IUdR), a specific DNA precursor and thymidine analog 5 days after cell transfer (40). The statistics used are described in detail (40). The percentage of injected ^{125}IUdR incorporated into each spleen was calculated and converted to $\log_{10}$ values. Geometric mean (95% confidence limits) values of groups (four to six mice) are presented. The significance of differences between any two groups was calculated by parametric and nonparametric methods using the Vax computer UTSTAT NGROUP program provided by the Academic Computing Service at the University of Texas (Austin, TX). Animal care was provided in accordance with the procedures outlined in "A Guide for the Care and Use of Laboratory Animals" (National Institutes of Health Publication No. 86-23).

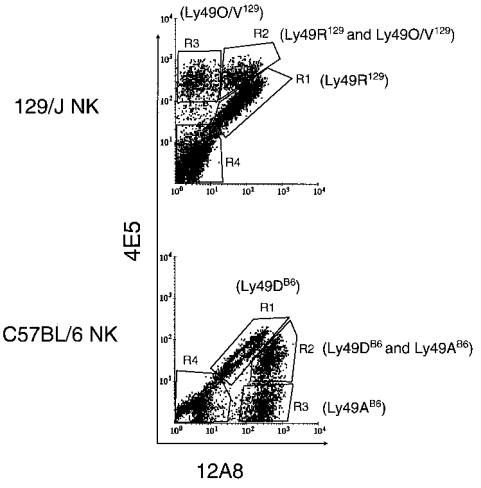

FIGURE 1. Delineation of NK populations of 129/J and C57BL/6 mice. IL-2-propagated NK cells were stained using mAbs 12A8 and 4E5. Cells were then analyzed using two-color flow cytometry. The populations sorted and analyzed in this study are denoted by the region markers.

Results

In the process of analyzing NK cell activation in gene-targeted mice, we found apparent defects in selected NK cell functions consistent with a block in the Ly49D/DAP12 signal transduction pathway. However, careful analysis of the phenotype of these NK cells suggested that they carried the 129/J repertoire of Ly49s, not that of B6, mice (38). This suggested that our observed differences might be due, in part, to the genetic background of these mice. In fact, an exhaustive study of the 129/J Ly49 repertoire demonstrated dramatic differences between the Ly49s expressed in 129/J and those seen in B6 mice (38, 41). For clarity, we use the nomenclature X^Y, where X = the Ly49 gene and Y = the strain of origin of the particular Ly49. Therefore, in 129/J mice, mAb 4E5 reacts not only with the activator Ly49R^{129}, but also with the inhibitory receptors Ly49O^{129} and Ly49V^{129} as well (38). These experiments also demonstrated that in B6 mice, mAb 12A8 recognizes both an activator (Ly49D^{B6}) and an inhibitor (Ly49A^{B6}), whereas in 129/J it reacts only with the activator, Ly49R^{129} (38). The resulting populations are demonstrated in Fig. 1. In a two-color analysis of NK cells with 4E5 and 12A8, the 4E5 and 12A8 double-bright population (Fig. 1, R1) identifies a population of NK cells where 4E5 or 12A8 reacts only with activating receptors. In NK cells of B6 mice, the 4E5/12A8 reactive activator in this population is Ly49D, whereas in 129 mice the 4E5/12A8 reactive receptor is the Ly49D-like activator, Ly49R. Immunoprecipitation of these NK cells with 4E5 shows no substantial phosphorylated receptor, indicating that there are no 4E5 reactive inhibitory receptors in this population (38). In contrast, region 2 represents a much smaller population that coexpresses the 4E5/12A8 reactive activators (Ly49D in B6 and Ly49R in 129) along with inhibitory receptors that are recognized by 12A8 or 4E5. Therefore, in 129 mice region 2 represents cells that are Ly49R^{129}-positive and coexpress the 4E5-reactive inhibitors, Ly49O^{129} and/or Ly49V^{129} (Fig. 1, R2). In B6 mice, region 2 contains cells that express Ly49D^{B6} and the 12A8 reactive inhibitor, Ly49A^{B6}. In contrast, Region 3 (Fig. 1, R3) contains cells that are negative for the 4E5/12A8-reactive activators Ly49R^{129} and Ly49D^{B6}. Instead, in 129 mice these cells express the inhibitors Ly49O^{129} and/or Ly49V^{129}, and in B6 these cells express Ly49A^{B6}. Details in the elucidation of these populations can be found elsewhere (38). Using this information, it was then possible to analyze DAP12 function in B6, 129/J, or chimeric mice without the interference of 4E5-cross-reactive inhibitory receptors. Thus, two-color sorting was used to isolate R1 (when cell numbers permitted), or a combination of R1 and R2, NK cells from 129/J or B6 mice. In these populations, the vast majority of cells should be activated by 4E5 without substantial interference from any 4E5-reactive inhibitory receptors. After sorting, the cells were expanded in culture medium containing IL-2 then harvested for biochemical and/or functional analysis. The activating Ly49, Ly49D, has been shown to facilitate the cell-mediated cytotoxicity of the CHO cell line (42). Therefore, we tested the ability of 129 NK cells, either Ly49R-positive or Ly49R-negative, to kill CHO cells in vitro. Surprisingly, sorted 129/J-derived NK cells failed to efficiently lyse CHO cells or to produce IFN-γ upon cross-linking with 4E5 (Fig. 2, *A* and *B*). In contrast, B6-derived NK cells killed CHO efficiently and produced significant levels of IFN-γ. Significantly, the lack of response we observed was not due to a global defect of 129/J-derived NK cells to kill targets or produce IFN-γ, as they killed YAC-1 targets just as well as B6-derived cells (Fig. 2*C*), and stimulation of the 129/J-derived NK cells with the combination of phorbol ester and ionomycin led to substantial production of IFN-γ (Fig. 2*B*). Further confirmation of the specificity of the deficit in activating Ly49 signaling came from analysis of the ability of NK cells of these two strains to produce IFN-γ in response to stimulation with IL-12 and IL-2 or IL-2 and IL-18. In these assays, the 129/J-derived cells made as much, if not more IFN-γ, than B6 NK cells (Fig. 2*D*). In addition, 129 NK cells mediate Ab-dependent cellular cytotoxicity of anti-HLA-DR-coated 721.221 cells at a level comparable to that of C57BL/6 mice (Fig. 3). In the absence of Ab, 721.221 cells are killed poorly.

To determine whether the lack of CHO killing and IFN-γ production in 129 NK cells might be associated with a lack of biochemical signaling events, we purified Ly49R or Ly49D-positive (Fig. 1, R1 or a combination of R1 and R2) NK cells from 129 and B6 mice and monitored their intracellular calcium levels during stimulation with mAb 4E5 (Fig. 4). These data clearly show that 129/J NK cells failed to mobilize calcium via the Ly49R^{129}/DAP12 pathway. Similar results were seen with 12A8 stimulation (data not shown). It should be noted that this finding is not due to

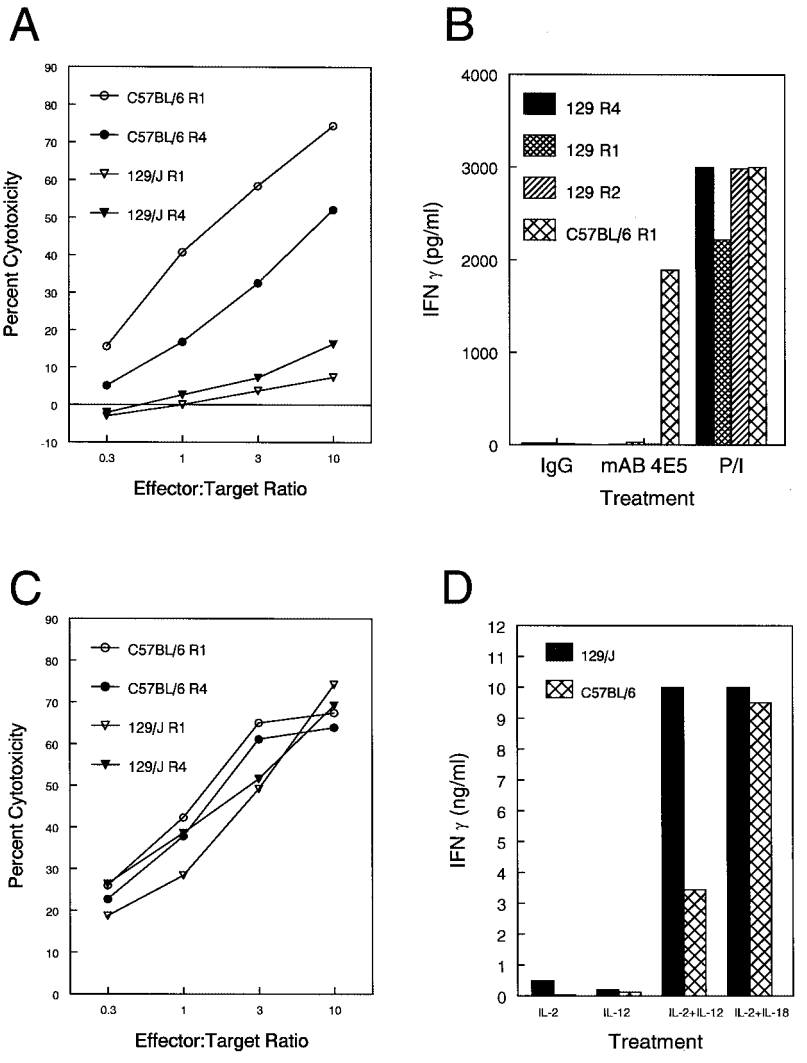

FIGURE 2. Ly49R^{129} fails to mediate cytolysis or cytokine production in 129/J NK cells. *A* and *C*, Sorted populations of 129/J or C57BL/6 NK cells were propagated in IL-2 then used in cytolytic assays with CHO targets (*A*) or YAC-1 targets (*C*) at the indicated ratios. *B*, Sorted and propagated NK cells were precoated with 4E5 (4E5) or control IgG (IgG) on ice, then incubated overnight on plates coated with goat anti-rat Ab, or a combination of 10 ng/ml PMA and 1 μg/ml ionomycin (P/I). Cell-free supernatants were assayed for IFN-γ by ELISA. *D*, Sorted and propagated NK cells were cultured overnight in the indicated combinations of IL-2, IL-12, and/or IL-18. Cell-free supernatants were assayed for IFN-γ by ELISA.

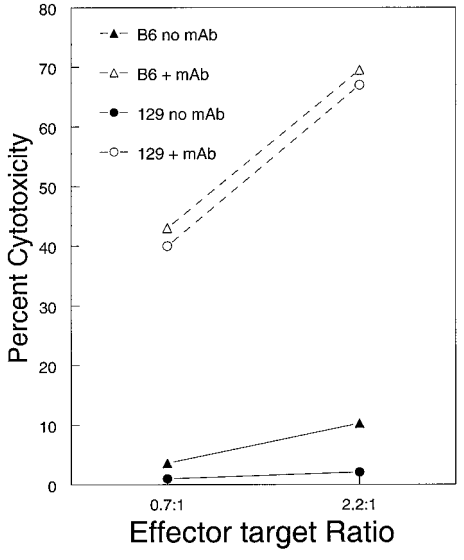

FIGURE 3. Ab-dependent cellular cytotoxicity is intact in 129 NK cells. Purified 4E5-NK cells from 129/J and B6 mice were used as effectors in a 4-h chromium release assay with 721.221 target cells at the indicated effector-target ratios. The target cells were either coated or not with anti-HLA-DR before addition to the assay. Each condition was assayed in triplicate and the means from a representative experiment are presented.

a global lack of calcium mobilization in 129/J mice, because comparable TCR-mediated calcium mobilization was observed in the thymocytes of 129/J and B6 (data not shown).

If the activating receptors of 129/J mice fail to signal, one would expect biological functions dependent on these receptors to be diminished. One of the few defined immunological systems requiring activating Ly49s is the rejection of bone marrow (31). In these systems, H-2^{b} mice will reject H-2$^{b/d}$ heterozygous bone marrow grafts. A general depletion of NK cells, or the specific depletion of 4E5 reactive cells, results in acceptance of the graft. To test the engraftment of heterozygous bone marrow into 129/J and B6 mice, we transplanted radiolabeled BMCs from D8 mice (C57BL/6 mice transgenic for H2-D^{d} and therefore, H-2$^{d/b}$; Ref. 43) into irradiated D8 (H-2$^{d/b}$), 129/J (H-2^{b}), or B6 (H-2^{b}) recipients. The results showed that D8 marrow efficiently repopulated D8 mice but failed to engraft in B6 mice. In contrast to B6, the D8 marrow was only partially rejected by 129/J mice (Table I). Although there are alternative explanations for these findings, they are consistent with ineffective signaling via the activating Ly49s of 129/J mice.

Mechanistically, the lack of Ly49R^{129}-mediated calcium mobilization in 129 NK cells could be due to either gross genetic abnormalities in Ly49R^{129}, DAP12, or both. However, cloning of Ly49R^{129} confirmed that it was intact and 94% identical to Ly49D^{B6} at the amino acid level (38). Interaction with DAP12, and the subsequent signaling, is mediated via the transmembrane and cytoplasmic domains of the receptors. In these regions

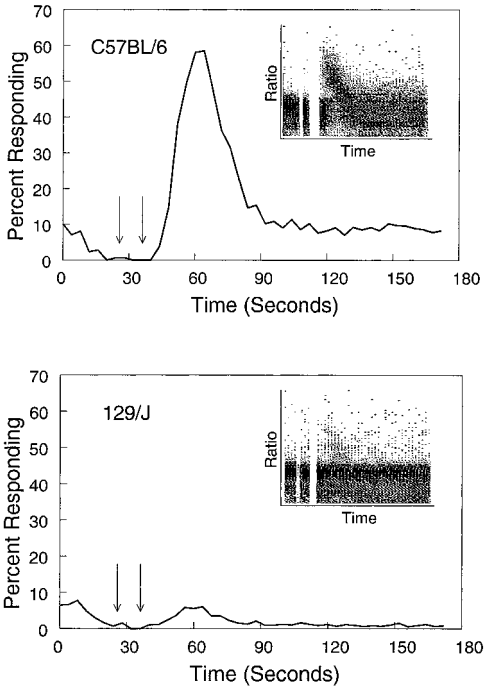

FIGURE 4. Ly49R^{129} fails to mobilize calcium in 129/J NK cells. Sorted and propagated NK cells were loaded with the calcium dyes fluo-3 and Fura Red. Intracellular calcium was monitored by flow cytometry as the cells were stimulated with 10 μg/ml 4E5 (first event arrow) followed by 10 μg/ml goat anti-rat IgG (second event arrow). Data are presented as the percent of responding cells. Raw data depicting the changes in ratio between bound and free dyes is presented in the inset.

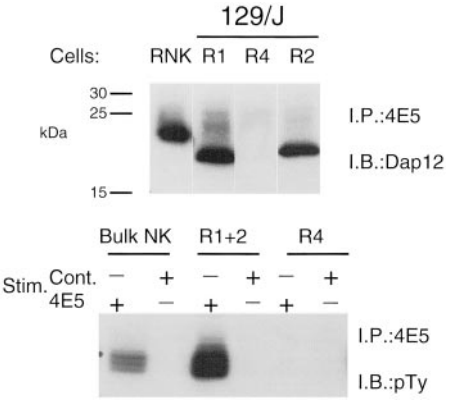

FIGURE 5. Ly49R^{129} is physically and functionally coupled to DAP12 in NK cells. *Upper panel,* Sorted and propagated NK cells from the indicated regions of Fig. 1 (10^7/lane) from 129/J mice or RNK-D cells were lysed and immunoprecipitated with mAb 4E5. Coimmunoprecipitated DAP12 was detected by immunoblotting with DAP12-specific antiserum. Rat DAP12 migrates slower than mouse DAP12. *Lower panel,* Cells similar to *upper panel* were stimulated with 4E5 or not for 1 min where indicated. Phosphorylation of coimmunoprecipitated DAP12 was detected by anti-phosphotyrosine immunoblotting. The filter was stripped and reprobed with DAP12-specific Ab to show equal loading of DAP12.

Ly49R^{129} and Ly49D^{B6} differ by only 1 aa. The arginine residue critical for interactions with DAP12 is present in both. In addition, we cloned DAP12 from a 129/J cDNA library and found this gene to be identical to its B6 counterpart (data not shown). Therefore, genetic abnormalities of Ly49R^{129} and/or DAP12 are not the cause of the lack of responsiveness in 129/J NK cells.

We next assessed the ability of Ly49R^{129} to physically and functionally couple to DAP12 in 129/J NK cells. In these experiments, we sorted 4E5$^+$/12A8$^+$ NK cells (Fig. 1, R1 or R1 + R2) from 129/J mice, expanded the cells in culture with IL-2, then immunoprecipitated Ly49R^{129} and the associated DAP12 using 4E5. Coimmunoprecipitated DAP12 was detected using DAP12 specific antiserum (Fig. 5*A*). The rat NK cell line expressing murine Ly49D (RNK-D; Ref. 16) cells were used as a positive control and the 4E5 negative population (Fig. 1, R4) served as a negative control. Note that although rat DAP12 migrates more slowly than mouse DAP12, consistent with their genetic identity, DAP12 of 129 origin migrates the same as B6 DAP12 (D. W. McVicar, unpublished observations). The ability of Ly49R^{129} to functionally couple to DAP12 was assayed by cross-linking Ly49R^{129} with 4E5 or control Ab, immunoprecipitating with 4E5 and immunoblotting the associated DAP12 with anti-phosphotyrosine. Fig. 4*B* shows efficient functional coupling of DAP12 to Ly49R^{129}. These data suggest that the failure of the Ly49R^{129} occurs downstream of its association with DAP12.

To differentiate between a failure of Ly49R^{129} to signal properly as opposed to a failure of 129/J-derived NK cells to receive DAP12-mediated signals correctly, we assessed Ly49R^{129} and Ly49D^{B6} signaling in a model that is independent of their strain of origin. Our model system uses reconstitution of the receptor complex in HEK293T cells. We have shown that reconstitution of the Ly49D^{B6} receptor system facilitates mAb 4E5-mediated increases in AP-1 reporter activity in a DAP12- and Syk-dependent manner (L. S. Taylor and D. W. McVicar, manuscript in preparation). Therefore, we transfected HEK293T cells with DAP12, Syk, an AP-1 reporter and either Ly49D^{B6} or Ly49R^{129}. After 6 h, the receptors were cross-linked with mAb 4E5, and 18 h later the cells were harvested, tested for Ly49 expression, and then assayed for AP-1 activity. In this system, both Ly49D^{B6} and Ly49R^{129} induced a 3- to 4-fold increase in AP-1 activity (Fig. 6). These data definitively demonstrate that Ly49R^{129} is capable of signaling to the same extent as its B6 counterpart, Ly49D^{B6}, and lend strong support to the possibility that the deficit in Ly49R^{129} signaling lies within the 129/J NK cells themselves.

We next addressed the possibility that the lack of Ly49R^{129} signaling was due to an inherent problem within 129/J NK cells by reconstituting Ly49D^{B6} expression in NK cells from either 129/J or B6 mice. In these experiments, 4E5-negative NK cells were sorted from 129/J or B6 mice and expanded in IL-2 containing medium. After 3–5 days of culture, the cells were harvested and infected with a recombinant vaccinia virus expressing Ly49D^{B6}. Infection for 6–7 h resulted in 55–67% expression of Ly49D^{B6} (Fig. 7*A*). These cells were then harvested, washed, and intracellular calcium was analyzed following cross-linking with mAb 4E5. The data presented in Fig. 6*B* demonstrate that reconstituted Ly49D^{B6} signaled well in B6 NK cells. In contrast, although >50% of the 129/J NK cells expressed Ly49D^{B6}, cross-linking

Table I. *Growth of D8 BMC in 129 vs C57BL/6 host mice*

Group	BMC Donor (H2)	Host Strain (H2)	Splenic ^{125}IUdR Uptake (%)[a]
1	D8 (b, D^d)	D8 (b, D^d)	2.17 (1.53–3.07)[b]
2	D8 (b, D^d)	129/J (b)	0.94 (0.35–2.54)
3	D8 (b, D^d)	C57BL/6 (b)	0.11 (0.02–0.78)

[a] Host mice were exposed to 8 Gy ^{137}Cs gamma rays and infused with inocula of 2.5×10^6 BMC. The isotope assay was performed 5 days after BMC transfer. Values are expressed as geometric means (95% confidence limits).

[b] Geometric mean values of groups 1 and 3 and 2 and 3 were significantly different ($p = 0.009$ and 0.024, respectively), while values of 1 and 2 were not different ($p = 0.05$).

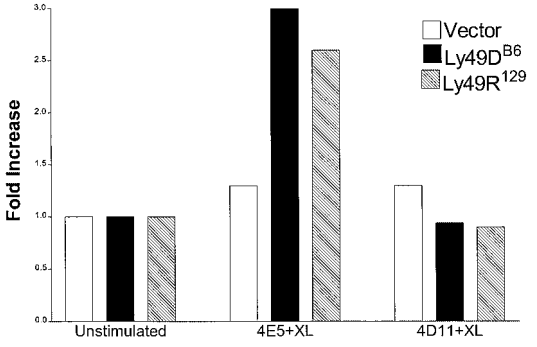

FIGURE 6. Ly49R^{129} signals normally as part of a reconstituted receptor complex. HEK293T cells were transfected with DAP12, Syk, an AP-1 luciferase reporter construct, and either Ly49D^{B6} or Ly49R^{129}. After transfection, the cells were stimulated with antiLy49D/R (4E5) or control (4D11) followed by cross-linking (XL) with goat anti-rat IgG. Equal expression of Ly49D^{B6} and Ly49R^{129} was confirmed using flow cytometry. Data are presented as fold increase in AP-1 activity detected 18 h later. The figure shows data representative of two independent experiments.

with 4E5 failed to induce any calcium mobilization (Fig. 7*B*, *lower panel*). NK cells of both strains were responsive to ionomycin (data not shown). A subsequent experiment confirmed this finding. Together these data demonstrate that the lack of Ly49R^{129} responsiveness is not due to the receptor configuration or DAP12, but rather is due to an apparent inherent defect in the 129/J signaling apparatus.

Discussion

Our finding that the DAP12 complex fails to signal in 129/J mice is intriguing given the significant differences that have been observed in the immune responses of 129 mice in a variety of models. These include the high susceptibility of 129/J mice to infection with Sendai and CMV viruses and the induction of systemic lupus erythematosus as well as differences in their susceptibility to experimental autoimmune encephalomyelitis (25, 26, 29, 30). 129/J mice are also more susceptible to infection with *Schistosoma mansoni* than are B6, and when infected with the intraerythrocytic *Babesia*-like organism, WA-1, 129/J mice exhibit higher levels of peak parasitemia and a 5-fold higher mortality rate (43, 44). The role for NK cells in general, or DAP12-coupled receptors specifically, in these processes is poorly defined. However, 129/J mice also show defects in bone marrow graft rejection, a process known to be dependent on NK cells (32). In murine models, F_1 hybrid (H-2^b/H-2^d) marrow is normally rejected by parental H-2^b or H-2^d recipients. Not only is this rejection dependent on NK cells, but specific removal of Ly49D$^+$ NK cells from B6 mice results in acceptance of H-2^b/H-2^d bone marrow (31). Therefore, our data demonstrating the relatively poor rejection of hybrid bone marrow by 129/J mice is consistent with poor signaling through activating Ly49s in 129/J mice.

Low stringency Southern blotting using Ly49-derived probes has suggested the potential existence of unique repertoires of receptors in various mouse strains (45). These findings became even more significant when genetic dissection of the KIR locus in humans defined the existence of multiple KIR haplotypes (46). Thus, a possible explanation for the lack of function documented in 129/J NK cells could be the presence of 4E5-reactive inhibitory receptors. Prompted by these possibilities, and the extensive use of 129 substrains in knockout experiments, genetic and biochemical analyses of the 129/J Ly49 repertoire have been conducted (38). These studies demonstrated dramatic differences between the repertoires

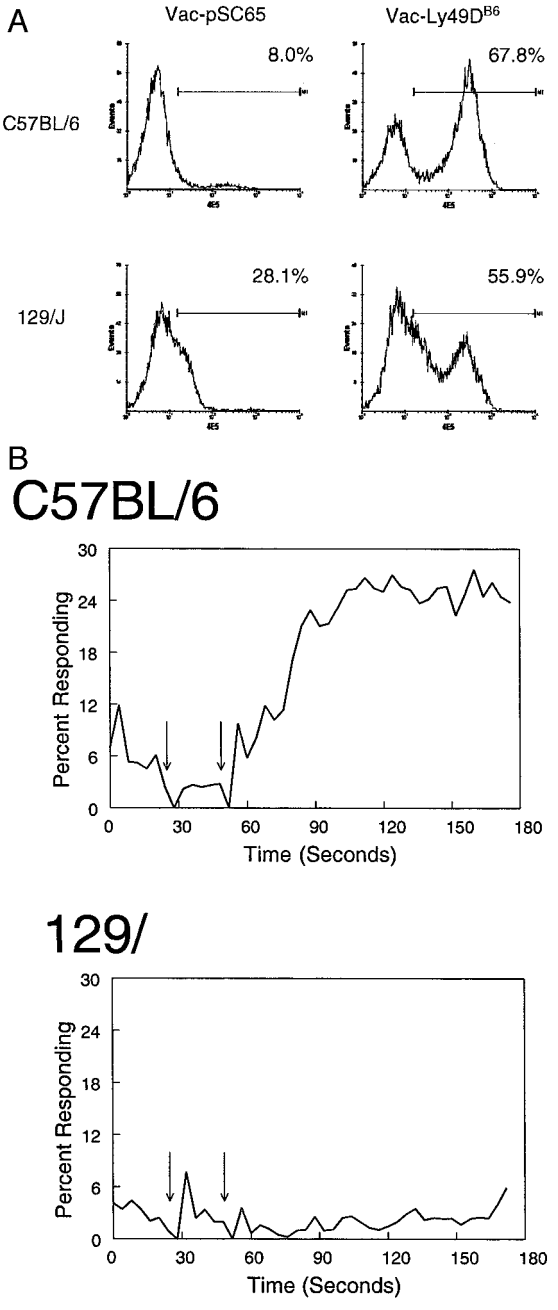

FIGURE 7. Ly49D^{B6} fails to signal when expressed on 129/J NK cells. Sorted R4 NK cells were infected with recombinant vaccinia virus expressing Ly49D^{B6} or control vaccinia. After infection for 6 h, an aliquot of cells was assayed for Ly49D^{B6} expression using 4E5 (*A*). The remaining cells were loaded with fluo-3 and intracellular calcium was monitored as they were stimulated with 4E5 (first event) and goat anti-rat IgG (second event) (*B*). Data are expressed as the percent of responding cells.

of 129/J and B6 mice. Of the 10 Ly49 genes identified in 129/J, only one, Ly49E, had the same primary amino acid sequence as a B6 Ly49. In fact, this analysis showed the recognition of inhibitory receptors by 4E5 in 129/J mice. However, biochemical analysis allowed the identification of subpopulations of 129/J NK cells where the vast majority of 4E5-reactive receptors were activating (populations R1 and R2), and these were nonresponsive. In addition, coligation of inhibitory receptors with activators has been shown to inhibit the phosphorylation of DAP12 normally seen when activators are engaged alone. In contrast, our data clearly show that cross-linking of activation receptors in 129/J mice does

lead to efficient phosphorylation of DAP12. Together with our analysis of sorted cell populations, we can therefore rule out coexpressed inhibitory receptors as the cause of our observation. Still, the lack of biological responses we detected upon cross-linking of DAP12-coupled Ly49s in 129/J mice might have been explained by small genetic differences between the 129/J versions of the activating receptors and or the DAP12 signaling chain itself, and those of B6 mice. However, we have eliminated this possibility by cloning the mAb 4E5-reactive activating receptor from 129/J mice, confirming its high homology to those of B6 mice, and its ability to signal when expressed ectopically (38). Together with the lack of signaling even when 129/J NK cells are reconstituted with the B6 receptor $Ly49D^{B6}$, we can only conclude that the DAP12 pathway in 129/J NK cells fails somewhere between DAP12 phosphorylation and calcium mobilization.

In T cells, the biochemical components required for the mobilization of calcium via the TCR are rapidly becoming well-defined. However, in the case of DAP12-mediated receptors much less is known. We have demonstrated the activation of the Syk tyrosine kinase following cross-linking of the Ly49D/DAP12 complex (16). Moreover, we have shown that expression of dominant-negative Syk blocks the calcium mobilization associated with Ly49D/DAP12 cross-linking (16). Preliminary data suggest that 129/J mice do express Syk as indicated by immunoprecipitation and Western blotting (data not shown). Unfortunately, due to the difficulty in obtaining the large numbers of purified cells required for the analysis of Syk phosphorylation in response to cross-linking with mAb 4E5, we cannot yet conclude that Syk is activated normally in sorted subsets of NK cells of 129/J mice. However, it should be noted, that despite the apparent dependence of the DAP12 pathway on Syk, NK cells derived from $Syk^{-/-}$ mice do signal through Ly49D (47). This finding is likely the result of an adaptation to the lack of Syk as the NK cells develop because these cells also express the Syk-family kinase, Zap70. Further evidence for excluding Syk as the cause for 129/J nonresponsiveness is the general health of 129/J mice. $Syk^{-/-}$ mice exhibit severe hemorrhaging and die perinatally (48, 49).

Additional potential sites of the signaling blockade in 129/J mice include phospholipase Cγ, the Tec family kinases, and other proteins defined in Fc or TCR studies. Although it is possible that Syk, Zap-70, linker for activation of T cells, or one of these other potential targets could be harboring a significant mutation in 129/J mice, mutations in these proteins would be expected to have dramatic effects on TCR signal transduction. However, we have tested TCR-induced calcium mobilization in 129/J or 129/Sv mice and found it to be fully intact (data not shown). Perhaps, through the generation of cell lines from B6 and 129/J, and continued study of the NK cells of 129/J mice, it will be possible to identify the exact site where the DAP12 pathway fails.

Surprisingly, the $Ly49R^{129}$/DAP12 pathway may not be the only aberrant signaling pathway in 129/J mice. Corcoran and Metcalf (50) demonstrated a severely reduced response of 129/Sv-derived B cells to IL-5 treatment or Rp105 cross-linking. However, the former defect was attributed to an inability of 129/Sv cells to up-regulate the IL-5R. In contrast, they found normal levels of surface expression of Rp105, an orphan Toll-like receptor, but cross-linking failed to induce B cell proliferation even though 129/Sv B cells proliferated in response to LPS (50). These findings are in apparent contradiction with recent data demonstrating reduced LPS-mediated proliferation of B cells in mice lacking Rp105 (51). Perhaps, Corcoran and Metcalf's (50) data is a reflection of a polymorphism in 129-derived Rp105 that renders the stimulating Ab ineffective in this strain. Regardless, to our knowledge, our study is the first example of a specific defect in a well-characterized receptor system with the potential to effect several immune parameters of 129 mice.

The immunological basis for coexpression of MHC class I binding receptors capable of delivering activation signals with those that transmit negative signals is a continuing mystery in NK cell biology. One method of understanding their roles would be to study the immune response of individuals with divergent repertoires. However, due to obvious limitations in the study of humans, any association of the recently described KIR repertoires, or individual inhibitory or activating KIR, with immune status, susceptibility to disease, or other biological parameter has yet to be described. In contrast to humans, the study of various strains of mice should allow for the rapid analysis of multiple individuals with different inhibitory and activating receptor repertoires over a variety of well-defined disease models. Our data clearly show that even though the 129/J repertoire is now well-defined, the fact that various activating receptors may be nonfunctional in select strains of mice makes strain-to-strain comparisons difficult but critical for complete understanding of the activating, class I-binding, receptors of NK cells. In fact, two independent groups have recently shown that susceptibility to mouse CMV is associated with a deletion of the activating Ly49, Ly49H in BXD-8 mice (52, 53). 129/J mice carry a potential Ly49H allele (Ly49U) suggesting that susceptibility of this strain may be due to the lack of signaling we report in this study. This possibility is under investigation.

In summary, we have defined a profound deficit in the DAP12-mediated signaling capability of the NK cells of 129/J mice. This observation is not due to the coexpression of inhibitory receptors recognized by the stimulating Abs, nor is it due to the receptor itself. Reconstitution studies showed that the 129/J receptor worked well when expressed in human cells. Most importantly, expression of the B6 receptor on the NK cells of 129/J mice failed to reconstitute signaling, thereby defining the signal transduction cascade of 129/J NK cells as the site of the deficit. DAP12 phosphorylation, the most proximal event associated with receptor cross-linking, is intact in 129/J mice, but calcium mobilization is not, narrowly delineating the location of the defect in DAP12 signaling. The discovery of a defect in DAP12 signaling in 129 mice suggests that analysis of gene-targeted mice which have not been substantially backcrossed is subject to error caused by the lack of signaling in NK cells. Because the biological role of the DAP12-coupled receptors in NK cells and the potential impact of NK cells on a variety of immune responses is still poorly defined, extreme care must be taken when interpreting immunological data derived from mice with any significant 129 genetic content. Until the locus or loci responsible for this lack of signaling is identified, independent analysis of immune parameters of 129 mice should be studied in parallel with gene-targeted mice to eliminate the possible impact of the lack of DAP12 signaling in the immune phenotype.

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