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Education of Murine NK Cells Requires Both *cis* and *trans* Recognition of MHC Class I Molecules

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Although NK cells use invariant receptors to identify diseased cells, they nevertheless adapt to their environment, including the presence of certain MHC class I (MHC-I) molecules. This NK cell education, which is mediated by inhibitory receptors specific for MHC-I molecules, changes the responsiveness of activating NK cell receptors (licensing) and modifies the repertoire of MHC-I receptors used by NK cells. The fact that certain MHC-I receptors have the unusual capacity to recognize MHC-I molecules expressed by other cells (*trans*) and by the NK cell itself (*cis*) has raised the question regarding possible contributions of the two types of interactions to NK cell education. Although the analysis of an MHC-I receptor variant suggested a role for *cis* interaction for NK cell licensing, adoptive NK cell transfer experiments supported a key role for *trans* recognition. To reconcile some of these findings, we have analyzed the impact of cell type–specific deletion of an MHC-I molecule and of a novel MHC-I receptor variant on the education of murine NK cells when these mature under steady-state conditions in vivo. We find that MHC-I expression by NK cells (*cis*) and by T cells (*trans*), and MHC-I recognition in *cis* and in *trans,* are both needed for NK cell licensing. Unexpectedly, modifications of the MHC-I receptor repertoire are chiefly dependent on *cis* binding, which provides additional support for an essential role for this unconventional type of interaction for NK cell education. These data suggest that two separate functions of MHC-I receptors are needed to adapt NK cells to self–MHC-I. ***The Journal of Immunology*****, 2013, 191: 5044–5051.**

Natural killer cells can rapidly respond to infection, to transformed cells, and to bone marrow grafts based on the recognition of aberrations in the expression of "self." Detectable aberrations include both the loss of inhibitory ligands (indicating infection or cellular transformation) and the upregulation of activating ligands (indicating cell stress). Most normal host cells do also activate NK cells, but these are not killed because of the inhibitory effect of MHC-I molecules. Consequently, reduced MHC-I expression may be sufficient to render host cells susceptible to NK cell–mediated attack, which is known as "missing–self-recognition" (1).

Despite the use of innate recognition receptors to detect diseased host cells, NK cells can adapt both phenotypically and functionally to their environment. Most prominently, NK cells adapt to the presence of MHC-I molecules, which is globally referred to as NK cell education. Murine NK cells recognize MHC-I using Ly49 family receptors and CD94/NKG2A, which bind certain classical MHC-I alleles and the invariant Qa-1b, respectively. Individual MHC-I receptors are expressed randomly and define subsets of 5–50% of NK cells that overlap partially (2). A first aspect of NK cell education is that the expression of MHC-I molecules, or the enforced expression of a transgenic MHC-I receptor, modifies MHC-I receptor usage by NK cells (3–5). The precise role of these repertoire modifications is still unclear. Indeed, repertoire changes do not guarantee that each NK cell expresses an inhibitory receptor specific for self–MHC-I. Consequently, NK cells lacking inhibitory receptors for autologous MHC-I represent a significant fraction of the peripheral NK cell pool (6). A second aspect of NK cell education is the functional adaptation to MHC-I.

Self-tolerance of the above NK cells lacking inhibitory receptors for autologous MHC-I is ensured by their hyporesponsiveness to stimulation (6). In contrast, NK cells that recognize autologous MHC-I respond efficiently to stimulation via activation receptors (6–9). The MHC-I–dependent effect on the function of NK cell activation receptors is termed licensing (10). Although licensing depends on ITIM present in the intracellular portion of inhibitory MHC-I receptors (7, 9) (this study), the precise basis for licensing is debated (11, 12). A "disarming" model suggests that the absence of inhibitory interactions results in the continuous stimulation of NK cells, which eventually induces a state of hyporesponsiveness (13). This model requires only that inhibitory receptors inhibit NK cells via their ITIMs. Alternatively, an "arming" model proposes that engaged MHC-I receptors instruct NK cell activation receptors to become responsive (7, 8). This model implies that ITIMs have two distinct functions: arming and effector inhibition.

Members of the murine Ly49 and the human leukocyte Ig–like receptor families have the highly unusual ability to bind MHC-I expressed by other cells (*trans*) and by the NK cell itself (*cis*) (14–17). Although the former mediates NK cell inhibition, there is no evidence that *cis* binding inhibits the effector response (14). Rather *cis* binding sequesters inhibitory receptors and renders

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Abbreviations used in this article: B6, C57BL/6; bc, backcrossing; BM, bone marrow; KIR, killer Ig–related receptor; LAMP-1, lysosome-associated membrane protein; LILRB, leukocyte Ig–like receptor; β_2m, β_2-microglobulin; MFI, mean fluorescence intensity; MHC-I, MHC class I; poly IC, polyinosinic-polycytidylic acid; Tg, transgenic; Wt, wild-type.

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them unavailable for *trans* binding, thereby facilitating effector responses (9). To address the importance of the two types of interactions for NK cell education, we previously generated a variant of the Ly49A receptor, which retained binding to its high-affinity H-2D^d ligand in *trans* but failed to bind in *cis*. This receptor variant inhibited the NK cell effector response but failed to license NK cells (9). This outcome was inconsistent with the disarming model and suggested a role for *cis* interaction for NK cell licensing. However, adoptive transfers of functionally mature NK cells into MHC-I–deficient hosts resulted in a rapid loss of NK cell function (18), which strongly supported the disarming model. Moreover, adoptive transfers of MHC-I–deficient NK cells into MHC-I–sufficient hosts showed that *MHC-I* gene expression by NK cells was not required to improve the function of NK cells (18, 19), which called into question a role of *cis* recognition for NK cell education.

To reconcile some of these findings, we analyzed the impact of NK cell– and T cell–specific H-2D^d deficiency and of a novel Ly49A receptor variant on the function of NK cells that mature under steady-state conditions in vivo. These systems revealed that MHC-I expression by NK cells and by other cells, and MHC-I recognition in *cis* and in *trans*, are all essential for the normal function of NK cells. The finding that modifications of the MHC-I receptor repertoire are chiefly dependent on *cis* binding provides further support for an essential role for this unconventional type of interaction for the education of NK cells.

Materials and Methods

Mice

B6.DBA/2Tg(Ly49A^{BALB}16)Whe (Ly49A Tg), B6.DBA/2Tg(Ly49-72A^{BALB}30)Whe (Ly49-72A Tg), and B6.DBA/2Tg(Ly49A-Y8F^{BALB}15)Whe (Ly49A-Y8F Tg) mice were all generated using the BALB allele of Ly49A (9). B6.DBA/2Tg(H-2D^{d}flox28)Whe (H-2D^d) transgenic (Tg) mice, containing a floxed *H-2D^d* gene, B6.Cg-Tg(Cd4-cre)1Cwi (CD4-Cre Tg), and B6 Ncr1tm1.1(icre)Viv (Ncr-Cre knockin mice) have been described previously (20–22). C57BL/6 (B6) and β_2-microglobulin (β_2m)-deficient B6 mice (B6.129P2-*B2m^{tm1Unc}*/J) were purchased from The Jackson Laboratory. Ly49A $\Delta\alpha$1 Tg mice were generated by removing aa 70–90 of the stalk region (α1s segment) of Ly49A^{BALB} (23), subcloning the cDNA into a modified pHSE cassette (24), and injecting the construct into fertilized (B6 $\times$ DBA/2)F$_1$ $\times$ B6) oocytes. A Tg line (number 9) was established by backcrossing (bc) to B6 (H-2^b) mice. For the experiments shown in this paper, Ly49A $\Delta\alpha$1 Tg mice were B6 bc $>$ 5, Ly49A wild-type (Wt) and Ly49-72A Tg mice were B6 bc 7-9, and Y8F Tg mice were B6 bc 5-6. All mice used were homozygous for NK and H-2 complexes of B6 origin. Animal experimentation followed protocols approved by the Service Vétérinaire de l'Etat de Vaud.

Generation of mixed bone marrow chimeras

D^d mice were injected i.p. with 200 μg mAb PK136 (anti-NK1.1) to deplete NK1.1$^+$ cells. They were irradiated (two doses of 450 rad 4 h apart from a ^{137}Cs source) 1 d later and reconstituted with various mixtures of T cell–depleted D^d and CD4-Cre D^d bone marrow (BM) (4 $\times$ 10^6 cells total) the following day. Recipients were allowed to engraft for $>$8 wk before analysis.

Cell lines

The cell lines used in this paper, C1498 (H-2^b), RMA (H-2^b), RMA/S (MHC-I^{low}), and D^d-transfected C1498 and RMA cells, have been described previously (14).

NK cell culture and lysis assays

Nylon wool nonadherent splenocytes were cultured in complete DMEM plus 0.5 μg/ml recombinant human IL-2 (a gift from N. Rufer, University of Lausanne). After 5 d, plastic–adherent cells were harvested and used as effectors. C1498- and C1498 D^d–transfected tumor cells (14) were used as target cells in standard Cr release assays.

Flow cytometry

Nylon wool nonadherent splenocytes were incubated with mAb 2.4G2 (CD16/32) hybridoma supernatant before staining with mixtures of biotin, FITC, or PE-labeled Ly49 or CD94/NKG2 mAbs: A1 (Ly49A^{B6}); JR9-318 (Ly49A); 4D12 (Ly49C/E) (note: Ly49E is expressed by $<$1% of adult NK cells) (25); 4E5 (Ly49D), HBF (Ly49F); 4D11 (Ly49G2); YLI90 (Ly49I); 18D3 (CD94); 20D5 (NKG2A/C/E); 16A11 (NKG2A^{B6}); and 14B11 (Ly49C, F,I,H), followed by streptavidin-PECy7/CD3-allophycocyanin Alexa780 and NK1.1-PerCP-Cy5.5. Alternatively, NK1.1-PerCPCy5.5 and CD3-APC were used in conjunction with PE-labeled H-2D^d multimer.

For IFN-γ assays, spleen cells (5 $\times$ 10^6/ml) from unprimed mice were stimulated with plastic-bound NK1.1 (5–10 μg/ml) (7) or PMA (50 ng/ml) and ionomycin (1 μg/ml). After 1 h, GolgiPlug and GolgiStop (BD Biosciences) were added. After 9 h, the cells were harvested and stained for flow cytometry.

Spleen cells (6 $\times$ 10^6) from polyinosinic-polycytidylic acid (poly IC)–activated NK cells were exposed to RMA (H-2^b), RMA/S (MHC-I^{low}) or RMA D^d cells (6 $\times$ 10^6) or to mAb-coated plates for 4 h. Anti–lysosome-associated membrane protein (LAMP-1) mAb was added to the cultures, and GolgiPlug and GolgiStop were added after 1 h. After 4 h, cultures were stained for flow cytometry.

Cells were analyzed on a FACSCanto flow cytometer with CellQuest Pro Software (BD Biosciences). Acid treatment of primary NK cells was done as described previously (14).

In vivo target cell rejection

Mice were injected i.p. with 100 μg poly IC (Sigma-Aldrich) 24 h before a 1:1 mixture of H-2^b (labeled 3.0 μM CFSE [Molecular Probes]) and H-2^{b}D^d splenocytes (labeled with 0.3 μM CFSE [2 $\times$ 10^7 cells total]). In some experiments, CMTMR (2.5 μM; Invitrogen)-labeled β_2m-knockout splenocytes were added. Recipient spleens were analyzed 24 h later for the presence of transferred cells. The percentage of specific rejection was calculated as follows: 100 $-$ [(percentage of H-2^b cells (CFSE^{hi})final/percentage of D^d cells (CFSE^{low})final)/(percentage of H-2^b cells (CFSE^{hi})initial/percentage of D^d cells (CFSE^{low})initial)] $\times$ 100.

Statistical analysis

Statistical significance was determined using ANOVA or a two-tailed Student *t* test with equal sample variance. Data sets were considered significantly different when $p < 0.05$.

Results

NK cell function in mice with cell type–specific H-2D^d ablation

To address the roles for *cis* versus *trans* recognition for NK cell education, we specifically ablated H-2D^d expression in *cis* (NK cells) or in *trans* (T cells). T cells were chosen as the *trans*-presenting cell type because they are known NK cell targets in vivo (26, 27). To generate cell type–selective D^d deficiency, we combined a floxed H-2D^d (*D^d*) Tg (20) with NK cell or T cell–specific Cre expression using *Ncr-Cre* knockin and *CD4-Cre* Tg mice, respectively (21, 22). As expected, surface expression of H-2D^d was absent from essentially all T cells in *CD4-Cre D^d* mice (Fig. 1A), whereas B cells, all myeloid cells, dendritic cells, and NK cells faithfully expressed H-2D^d (Fig. 1A). In *Ncr-Cre D^d* mice, H-2D^d was present on all T cells, B cells, and myeloid cells but absent from most NK cells. However, ~15% of NK cells were H-2D^d positive (Fig. 1A). Most of these NK cells expressed the H-2D^d–specific Ly49A receptor (Fig. 1B). On the basis of our earlier findings (28), we considered the possibility that these NK cells had recombined the floxed *D^d* gene but were H-2D^d positive because of Ly49A-mediated uptake from surrounding cells. Indeed, NK cell culture at a low density for 3 d resulted in the complete loss of H-2D^d when Ly49A NK cells were derived from *Ncr-cre D^d* mice. In contrast, H-2D^d expression was stably maintained on NK cells from *CD4-cre D^d* mice (Fig. 1C).

We determined the function of NK cells by assessing the rejection of target cells in vivo. Mixtures of H-2^b and D^d splenocytes, which had been labeled with a high and a low concentration of CFSE, respectively, were injected i.v. into poly IC–primed recipient mice. As expected, *D^d* mice efficiently rejected H-2^b (CFSE high) splenocytes (Fig. 1D, 1E), and this depended on the presence of NK1.1$^+$ cells (Supplemental Fig. 1A). In contrast, rejection of H-2^b

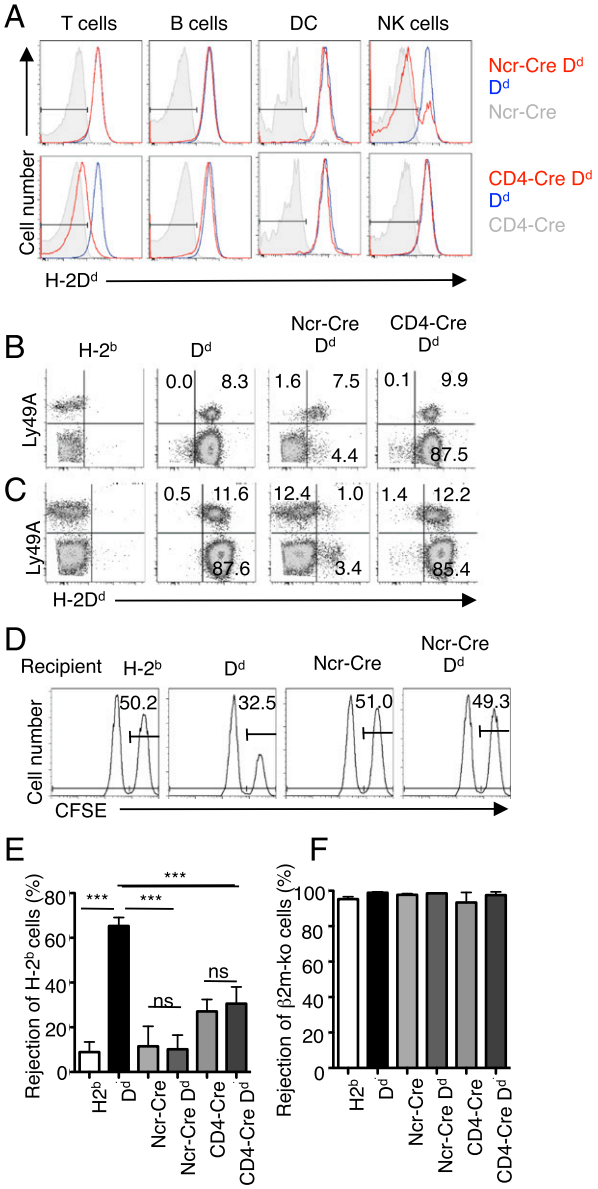

FIGURE 1. Both NK cell and T cell–specific H-2D^d deficiency impairs NK cell function. (**A**) H-2D^d expression was analyzed on T cells, B cells, dendritic cells (DC), and NK cells from *D^d* mice and compared with those of *Ncr-Cre D^d* (*top panel*) and *CD4-Cre D^d* mice (*bottom panel*). The expression of H-2D^d relative to Ly49A was analyzed on freshly isolated NK cells (**B**) and following a 3-d culture in IL-2 (**C**). Numbers indicate the percentage of cells in the respective quadrant. (**D**) Mixtures of H-2^b and D^d splenocytes, which were labeled with a high and a low concentration of CFSE, respectively, were injected i.v. into recipient mice, which had been primed with poly IC 24 h before. Numbers in histograms depict the relative abundance of CFSE^{hi} (H-2^b) cells in spleens of the indicated recipient mice, 24 h after cell injection. (**E**) Specific rejection of H-2^b splenocytes by the indicated recipient mice. The bar graphs show the mean percentage of rejection ± SD from 3 to 15 mice per strain. ***$p < 0.001$. ns, Not significant ($p > 0.05$). (**F**) Specific rejection of β_2m-knockout (ko) splenocytes by the indicated recipient mice. The bar graphs show the mean percentage of rejection ± SD from two to five mice per strain.

splenocytes by *Ncr-cre D^d* and *CD4-cre D^d* mice was inefficient and not different from that by littermate mice (Fig. 1D, 1E). Despite the presence on all other cell types, the lack of H-2D^d on either NK cells or T cells prevented the NK cell–mediated rejection of H-2^b targets. In contrast, all mouse strains efficiently rejected splenocytes that lacked all MHC-I molecules (Fig. 1F), demonstrating that cell type–specific H-2D^d ablation does not globally impair NK cell function.

We further determined whether T cell and NK cell–specific H-2D^d ablation exerted a comparable negative effect on NK cell function. To this end, we limited the abundance of D^d-deficient T cells by reconstituting lethally irradiated *D^d* mice with various mixtures of *D^d* and *CD4-Cre D^d* BM precursors. After stable engraftment for >8 wk, we first verified that recipient mice contained the expected percentage of D^d-negative T cells (Fig. 2A) and then determined the ability of NK cells to reject H-2^b target cells in vivo. The presence of >10% of D^d-negative T cells in chimeric mice significantly impaired NK cell–mediated rejection of H-2^b targets. However, once the fraction of H-2D^d–negative T cells was <10%, there was no longer a significant adverse effect on NK cell mediated rejection of H-2^b targets (Fig. 2B). Of note, the absolute number of D^d-negative cells present in these latter mice was similar to that in *Ncr-Cre D^d* mice, which indicated that NK cell–specific D^d deletion had a more profound negative effect on NK cell function than T cell–specific deletion. Moreover, the data showed that the negative effect seen in *Ncr-Cre D^d* mice could not be explained by the presence of a small population of *D^d*-negative cells.

We next sought to corroborate the in vivo rejection results at the single-cell level and to determine which NK cell subsets were affected. We compared the response of NK cell subsets that were defined by the expression of the D^d-specific Ly49A and the H-2^b–specific Ly49C, Ly49I, and NKG2A receptors (Fig. 3A). When comparing H-2^b to D^d NK cells from poly IC–primed mice, only the NK cell subset from D^d mice that expressed Ly49A and lacked Ly49C, Ly49I, and NKG2A receptors (Ly49A$^+$CIN$^-$ NK cells) responded significantly to H-2^b (RMA) target cells (Fig. 3B–D). Ly49A$^+$CIN$^-$ NK cells from *D^d* mice efficiently released LAMP-1 and produced IFN-γ in response to RMA (H-2^b) (Fig. 3B–D) or RMA/S tumor target cells (MHC-I^{low}) (Supplemental Fig. 1B) but

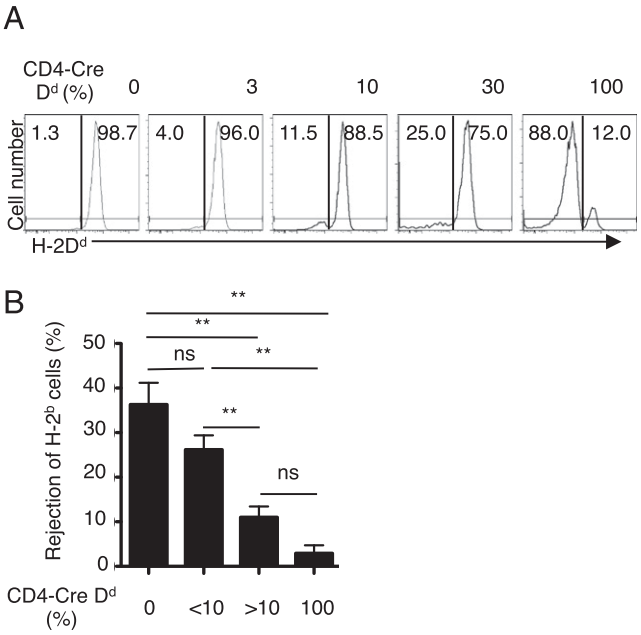

FIGURE 2. NK cells are not tolerized by a small fraction of D^d-deficient T cells. (**A**) Lethally irradiated *D^d* mice were reconstituted with variable mixtures of *D^d* and *CD4-Cre D^d* BM precursors as indicated. After stable reconstitution, the percentage of D^d-negative cells was determined among CD4 T cells. (**B**) The reactivity of NK cells in recipient mice was assessed using in vivo lysis assays as described in Fig. 1F. The bar graphs show the mean percentage of rejection ± SD from three to five mice per strain. **$p < 0.01$.

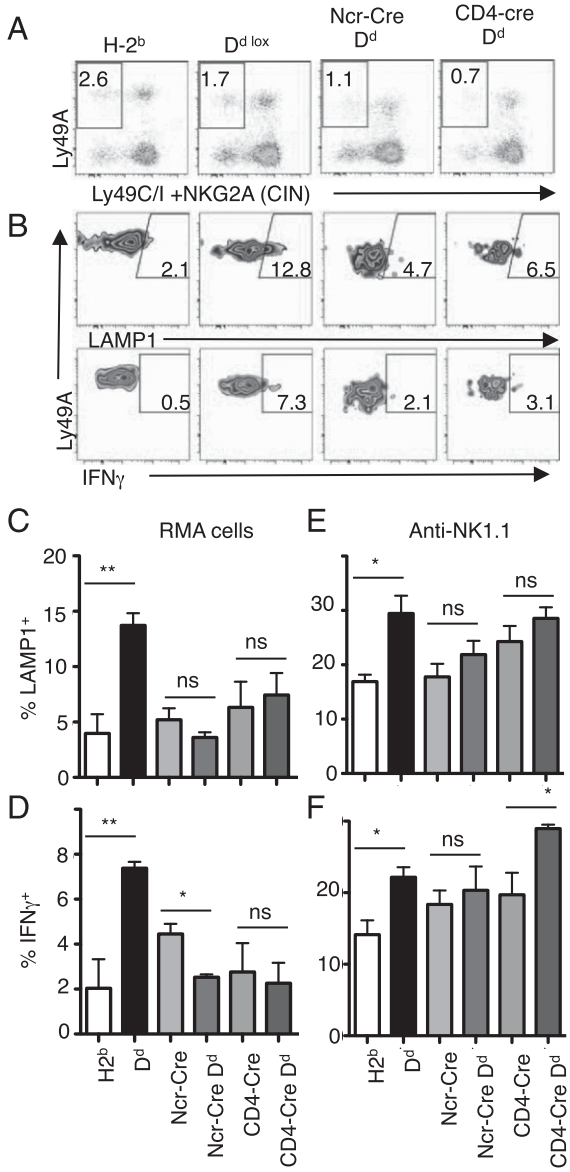

FIGURE 3. Cell type–specific H-2D^d ablation impacts the function of a specific NK cell subset. Splenocytes from the indicated strains of mice, which had been primed with poly IC 24 h before, were exposed to RMA cells (H-2^b) for 4 h (**A**–**D**) or simulated with anti-NK1.1 (**E**, **F**). NK cells (NK1.1$^+$CD3$^-$) expressing Ly49A but lacking Ly49C, Ly49I, and NKG2A receptors (Ly49A$^+$CIN$^-$) (A) were analyzed for the surface expression of LAMP-1 and for intracellular IFN-γ (B–F). Each panel represents the mean ± SD of at least three independent experiments. Groups differed significantly as shown (*$p < 0.05$, **$p < 0.01$).

not in response to H-2D^d–transfected RMA cells (Supplemental Fig. 1C). In contrast, Ly49A$^+$CIN$^-$ NK cells from *Ncr-cre D^d* and from *CD4-cre D^d* mice responded poorly to stimulation with RMA (Fig. 3B–D) or with RMA/S cells (Supplemental Fig. 1B). In contrast Ly49A$^+$CIN$^+$ NK cells from these mice responded to RMA/S cells, indicating a specific impairment of the Ly49A$^+$CIN$^-$ population. The functional deficit was not due to deficient priming, because poly IC injection resulted in a comparable upregulation of CD69 and granzyme B expression by Ly49A$^+$CIN$^-$ NK cells of all mouse strains (Supplemental Fig. 2A). Moreover, pharmacological stimulation using PMA plus ionomycin resulted in comparable effector responses (Supplemental Fig. 1D). Thus, NK cell and T cell–specific D^d deletion had a similar adverse effect on NK cell function.

To address the basis for the defect, we stimulated NK cells via a defined activation receptor. As expected, Ly49A$^+$CIN$^-$ NK cells from *D^d* mice responded significantly better than those from *H-2^b* mice (Fig. 3E, 3F). In contrast, when *D^d* was ablated from NK cells (in *Ncr-Cre D^d* mice), the effector response was not increased compared with littermate mice (Fig. 3E, 3F). When *D^d* was ablated from T cells (*CD4-Cre D^d* mice), LAMP-1 release was also not increased, but IFN-γ production was increased compared with littermate mice (Fig. 3E, 3F). Although the defect of *CD4-Cre D^d* NK cells may not be completely explained by deficient licensing, the defect of *Ncr-Cre D^d* NK cells can be.

Modifications of the MHC-I receptor repertoire upon cell type–specific H-2D^d deletion

We assessed whether cell type–selective D^d deletion impacted additional aspects of the NK cells adaption to self–MHC-I such as the modification of the MHC-I receptor repertoire. The introduction of a Wt Ly49A Tg into H-2D^d mice significantly modified the endogenous MHC-I receptor repertoire (Fig. 4A, first row). Specifically, the abundance of NK cells expressing the inhibitory Ly49G2 and Ly49I and the activating Ly49D and Ly49H receptors were reduced, whereas that of CD94 and NKG2A, which form the inhibitory CD94/NKG2A heterodimer, was expanded (Fig. 4A). Overall, we noted significant changes ($p < 0.05$) in seven of the nine receptors or subunits that we analyzed (78%). In contrast, Ly49A Tg expression in *Ncr-cre D^d* mice resulted in limited repertoire changes (three of eight receptors [38%]) (Fig. 4A). In contrast, Ly49A Tg expression in *CD4-cre D^d* mice resulted in abundant repertoire changes (six of eight receptors [75% changes]) (Fig. 4A), which essentially corresponded to those seen in Ly49A Tg *D^d* mice. T cell–selective D^d deletion thus dissociated the phenotypic from the functional adaptation of NK cells, whereas NK cell–specific D^d deletion impaired both repertoire and functional adaptation. Cell type–specific D^d deficiency had no significant additional effects on the abundance (Supplemental Fig. 2B, 2C) and/or on the maturation of Ly49A$^+$CIN$^-$ NK cells.

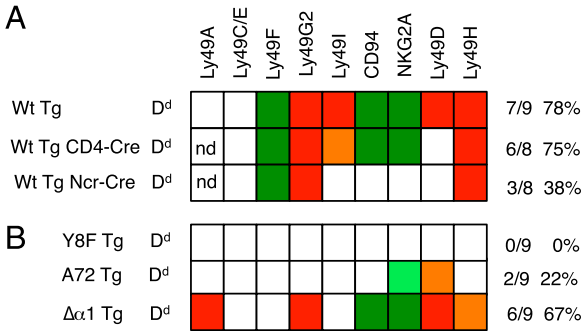

FIGURE 4. Modifications of the endogenous MHC-I receptor repertoire. Splenic CD3$^-$NK1.1$^+$ NK cells from the indicated mice were analyzed for the expression of inhibitory MHC-I receptors (endogenous Ly49A, Ly49C/E, Ly49F, Ly49G2, Ly49I, and CD94/NKG2A) and activating Ly49 receptors (Ly49D and Ly49H). Data are shown as heat maps, which depict differences in the size of specific NK cell subsets between non-Tg D^d mice and mice expressing a Wt Ly49A Tg without or with cell type–specific D^d ablation (**A**) or expressing the (*trans* only) A72, the (*cis* only) Δα1, or the (ITIM mutant) Y8F Ly49A Tg (**B**). Data are from 4 to 20 independent determinations. Open squares indicate no difference ($p > 0.05$). Orange and red depict significantly decreased frequencies of $p < 0.05$ and $p < 0.01$, respectively. Light green and dark green depict significantly increased frequencies of $p < 0.05$ and $p < 0.01$, respectively. nd, Not determined.

Generation and analysis of Ly49A Δα1 transgenic mice

To confirm the roles for *cis* versus *trans* recognition for NK cell education, we used the transgenic expression of Ly49A receptor variants with distinct D^d-binding properties. We have previously analyzed an Ly49A variant (A72), containing a rigid stalk, which failed to bind H-$2D^d$ in *cis* but retained *trans* binding. Even though this receptor variant inhibited NK cell effector function, it was unable to license NK cells (9), suggesting that *cis* binding was necessary to improve the responsiveness of NK cells. To independently test this hypothesis, we took advantage of another Ly49A variant with a deletion of the α1 element (aa 70–90) in the stalk region (Ly49A Δα1) (Fig. 5A), which interacted with MHC-I in *cis* but failed to inhibit effector function based on the analysis of transfectants (29). We generated Ly49A Δα1 Tg mice (Fig. 5B) that were backcrossed to B6 (H-2^b) and to H-$2D^d$ Tg B6 backgrounds, respectively. Primary NK cells were used to confirm that this receptor variant failed to productively interact with H-$2D^d$ expressed on other cells (Fig. 5C). We have previously shown that

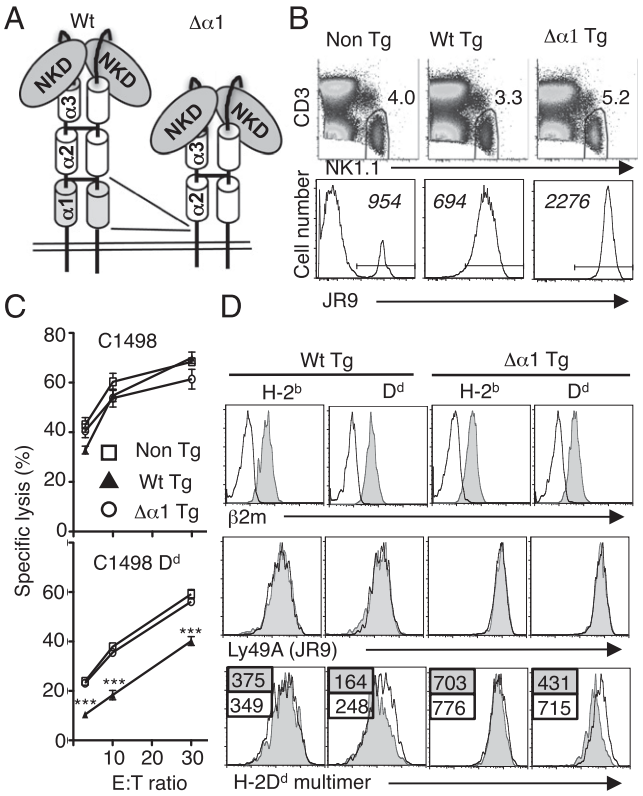

FIGURE 5. Generation of mice expressing a transgenic Ly49A Δα1 receptor. (**A**) Schematic representation of the Wt and Ly49A Δα1 receptors, highlighting the ligand binding Ly49A NK domains (NKD) (dark gray) and the known (α3) and predicted (α2 and α1) α-helical segments of the stalk. The membrane proximal α1 segment (light gray) was deleted to generate the Ly49A Δα1 receptor. (**B**) Density plots show the abundance of $CD3^-$ $NK1.1^+$ NK cells in the spleen of mice expressing a Wt or the Δα1 Ly49A receptor Tg on a H-2^b background. Histograms show Ly49A expression (mAb JR9) in gated NK cells. Numbers indicate the MFI of Ly49A staining. (**C**) The inhibitory capacity of the Ly49A Δα1 receptor was tested in lysis assays with cytokine-activated effectors against C1498 (*top panel*) and C1498 D^d tumor targets cells (*bottom panel*). Data represent means ± SD of triplicate determinations at the indicated E:T cell ratios. (**D**) Splenocytes from the indicated types of mice were exposed to an acidic buffer to disrupt MHC-I–peptide complexes. Gated $CD3^-NK1.1^+$ NK cells were further stained with mAbs to β_2m or Ly49A or with D^d multimer. Filled gray histograms depict the staining before and open histograms after acid stripping. Numbers indicate the MFI of staining with D^d tetramer.

a minimal length of the stalk, and not some other function of the $\alpha 1_S$ segment, was critical for inhibitory Ly49A receptor function (29). It is thought that inhibitory receptors must be coengaged with activating NK cell receptors in submicroscopic clusters to antagonize activation signaling (30). According to the concept of size-based segregation (31, 32), activating and inhibitory receptor–ligand pairs need to fit into the synaptic cleft of 100–150 Å between the NK cell and the target cell membrane. Activating and inhibitory interactions may become mutually exclusive when the Ly49A stalk is too short. We next confirmed that the Δα1 receptor retained the capacity to bind H-$2D^d$ in *cis*. Δα1 Tg NK cells bind soluble H-$2D^d$ ligand, and this binding was reduced (2-fold) when NK cells were from H-$2D^d$ as compared with B6 mice (mean fluorescence intensity [MFI] of gray histograms) (Fig. 5D). This was due to receptor masking, because the binding of soluble H-$2D^d$ significantly increased following acid-mediated disruption of MHC-I complexes on living NK cells from H-$2D^d$ mice (compare the MFI of gray to the open histograms) (Fig. 5D) (14). Thus, in agreement with our previous analyses (29), these data confirm that the Ly49A Δα1 receptor binds H-$2D^d$ in *cis* but fails to functionally bind in *trans*.

Our previous analyses have shown that, unexpectedly, Wt Ly49A suppressed cytokine production in H-2^b mice (i.e., when H-$2D^d$ was absent (9)), indicating that unengaged receptors have a suppressive effect. Because the coexpression of MHC-I receptors modified the NK cell response (9), we determined the effect of the different Ly49 Tgs in the absence of endogenous MHC-I receptors. To this end, we focused on the small NK cell subset, which lacks all known MHC-I receptors in B6-background mice ($Ly49A^{B6}$, C, D, F, G2, H, I, and NKG2A/CD94), termed null NK cells (1–2% of NK cells) (Fig. 6A). The use of the BALB allele of Ly49A to generate Tg mice allowed the exclusion of NK cells expressing endogenous $Ly49A^{B6}$ (mAb $A1^+$). Null NK cells expressed the various Tg Ly49A receptors (Fig. 6A), allowing us to determine the impact of Tg expression on NK cell responsiveness.

As expected, NK1.1 mAb-induced IFN-γ production by null NK cells from non-Tg control mice was inefficient (Fig. 6B, 6C). The coexpression of H-$2D^d$ and the Wt Ly49A receptor significantly improved IFN-γ production (Fig. 6B, 6C). In contrast, the A72 receptor (*trans* only) failed to improve IFN-γ production (Fig. 6C), in agreement with our lysis data (9). Similarly, the Δα1 receptor (*cis* only) failed to improve IFN-γ production by null NK cells (Fig. 6B, 6C). NK cell stimulation by pharmacological means, rather than via cell surface receptors, resulted in comparable IFN-γ production by all types of transgenic NK cells (Fig. 6D). These data suggested that exclusive *cis* and *trans* interactions were both not sufficient for the functional adaptation of NK cells. This result was consistent with the outcomes of NK cell and T cell type–specific D^d deletion.

We also analyzed NK cells from mice expressing a Tg Ly49A receptor with a mutated ITIM (Ly49A Y8F). This receptor retained the ability to bind H-$2D^d$ in *cis* and in *trans* but did not inhibit NK cell effector function (9). However, the expression of this receptor did not improve IFN-γ production (Fig. 6D) in agreement with (7). Thus, the functional adaptation of NK cells to MHC-I depended on an intact ITIM.

Modifications of the MHC-I receptor repertoire by transgenic Ly49A receptors

We next investigated whether Ly49A Tg expression impacted NK cell development and maturation. Ly49A Tg expression did not significantly alter the number of splenic or BM NK cells (Supplemental Fig. 3) or the expression of markers of NK cell differentiation and maturation (including KLRG1, CD11b, and CD27)

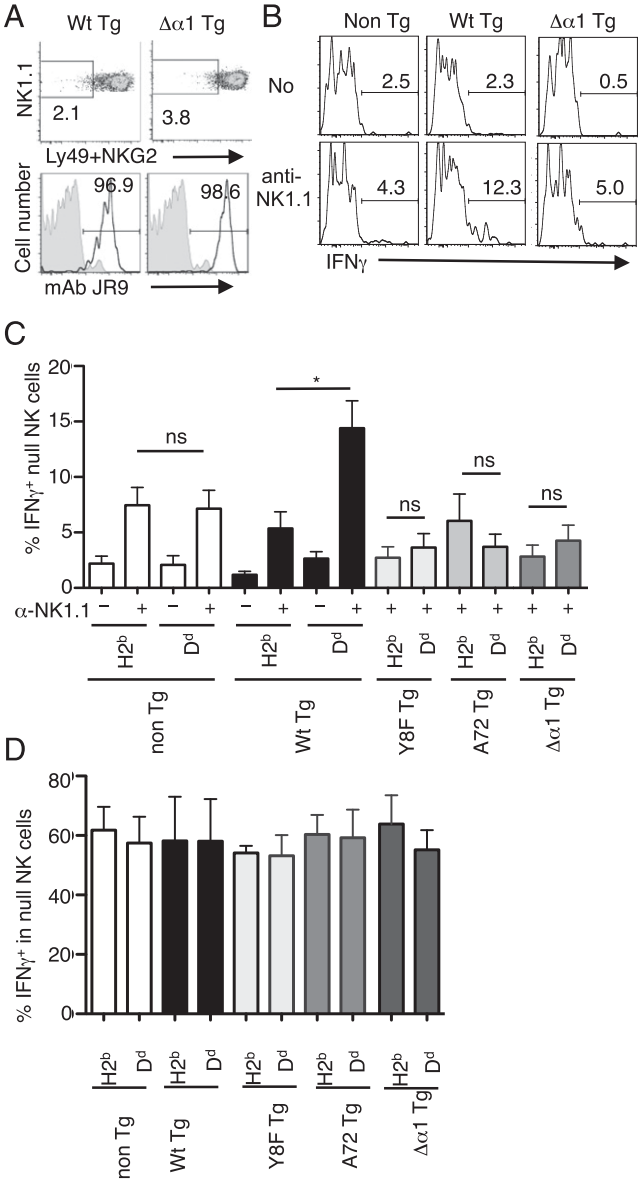

FIGURE 6. Responsiveness of Ly49A Tg NK cells. (**A**) Identification of "null" NK cells ($CD3^{-}NK1.1^{+}$) that lack all known MHC-I receptors in B6-background mice ($Ly49^{+}NKG2$: Ly49A [mAb A1], C, D, F, G2, H, I, and NKG2A/CD94). The NK cell subset expressing endogenous $Ly49A^{B6}$ (mAb $A1^{+}$) could be excluded from the analysis because of the use of the BALB allele for all Ly49A transgenic receptors. Filled gray histograms depict Ly49A expression among "null" NK cells from non-Tg mice. Open histograms depict the expression of the Wt or the Δα1 (*cis* only) Ly49A Tg receptor in null NK cells. Numbers indicate the percentage of cells in the respective gate. (**B**) Total spleen cells from the indicated types of mice on a $H2^{b}D^{d}$ background were stimulated with immobilized NK1.1 mAb (anti-NK1.1) or left unstimulated (No) in the absence of cytokine addition. Numbers indicate the percentage of null NK cells producing IFN-γ. (**C**) The bar graph depicts the mean percentage (±SD) of IFN-γ–positive null NK cells from mice expressing the Wt, the A72 (*trans* only), the Δα1 (*cis* only), or the Y8F (ITIM mutant) Ly49A Tg on a $H\text{-}2^{b}$ and on a $H\text{-}2^{b}$ D^{d} (D^{d}) background. (**D**) The bar graph shows the mean percentage (±SD) of IFN-γ–positive null NK cells from the indicated type of mice after stimulation with PMA plus ionomycin. $*p < 0.05$. ns, Not significantly different ($p > 0.05$) (n = 3–7).

(data not shown), in agreement with our previous analyses (9). Finally, we addressed whether Tg Ly49A receptors modified the repertoire of endogenous MHC-I receptors used by NK cells. As shown above, the expression of the Wt Ly49A Tg resulted in abundant repertoire changes (78% of analyses) (Fig. 4A). In contrast, the expression of the ITIM mutant Y8F Tg did not modify the receptor repertoire (zero of nine analyses) (Fig. 4B). Thus, signaling via the ITIM is essential for the functional (Fig. 6D) (7) and for the phenotypic adaptation of NK cells. The expression of the 72A Tg (*trans* only) had very limited effects on endogenous MHC-I receptor usage (two of nine analyses, 22%) (Fig. 4B). This outcome corresponded to the impact of NK cell–specific D^{d} deletion (Fig. 4A). Thus, *trans* interactions do neither adapt the repertoire nor the function of NK cells. In contrast the (*cis* only) Ly49A Δα1 Tg receptor significantly skewed the endogenous MHC-I receptor repertoire (67% of analyses) (Fig. 4B). This result corresponded to that obtained in *CD4-Cre* D^{d} mice (Fig. 4A), where *cis* interactions occurred and *trans* interactions were intermittent. These data suggest that *cis* interaction was sufficient to shape the receptor repertoire but that both *cis* and *trans* interactions were needed to produce functional NK cells.

Discussion

The finding that both *cis* and *trans* binding are necessary for the functional adaptation of NK cells raises the possibility that the two types interactions serves distinct functions. An important role for *trans* interaction has been established based on the adoptive transfer of fully mature NK cells. *Trans* interactions protect mature NK cells from the adverse effects of chronic stimulation (33). A similar effect may explain the functional deficit of NK cells that mature in CD4-Cre D^{d} mice. The precise role of *cis* interaction remains to be established. However, because there is no evidence that it is inhibitory, it may not serve to protect NK cells from chronic stimulation. Further along these lines, it may be interesting to consider the modifications of the MHC receptor repertoire. These generally correlate with, and may thus be a by-product, or the consequence of functional maturation. If repertoire modifications are indeed a by-product of functional adaptation, then, based on its prominent role to skew the repertoire, *cis* interaction may render NK cell activation receptors responsive. The enhanced NK cell activation levels may lead to adaptations of the receptor repertoire. Then, if MHC-I receptors interacted only in *cis* but could not productively or continuously interact in *trans*, NK cells would not be protected from the adverse effects of chronic stimulation and activation receptors may eventually be desensitized. According to this model, *cis* engagement of MHC-I receptors would instruct NK cell activation receptors to become responsive and *trans* engagement would protect NK cells from exhaustion. Consistent with this notion, individual NK cells can at the same time recognize MHC-I in *cis* and in *trans*, because not all MHC-I receptors are engaged in *cis*. Although available data are consistent with such a scenario, additional experimentation will be required to rigorously test this model.

With regard to the role of MHC-I expression in *trans*, our experimental setup provides evidence that the absence of MHC-I from surrounding T cells is sufficient to impair NK cell fitness in vivo. These data confirm and extend findings based on mixed bone marrow chimeras (33, 34) (Fig. 2; our unpublished data), MHC-I mosaic mice (20, 35) and the adoptive transfer of mature NK cells (18). The novel information provided by our analyses is that the absence of MHC-I from a single, defined cell type, as opposed to a fraction of all hematopoietic cell types, is sufficient to impair NK cell fitness. It will be of interest to address whether all cell types have this capacity or whether T cells are particularly potent to stimulate NK cells.

We further show that MHC-I expressed by NK cells plays an essential role for their function. These results contrast with adoptive

NK cell transfer studies, which found that the function of MHC-I–deficient NK cells improved following their transfer into a MHC-I–sufficient environment (18, 19). These data suggested that MHC-I expression by NK cells was not essential to improve NK cell function. However, there are important differences between the experimental approaches, which may account for the distinct outcomes. Cell transfers generated transient states and addressed the behavior of peripheral NK cells that, to our knowledge, encountered MHC-I for the first time. In contrast, we assessed the function of peripheral NK cells under steady-state conditions, and these cells were exposed to MHC-I during their development in the bone marrow. Thus, the developmental stage may impact how NK cells adapt to MHC-I encounter or loss. Consistent with this notion, we observed significant changes in MHC-I receptor usage (percentage of positive cells), which was not evident upon adoptive transfer of peripheral NK cells (18). Whatever the precise reason for the distinct outcomes, we can at least exclude one possible mechanism for the functional adaptation in a MHC-I mosaic environment: MHC-I–deficient NK cells can acquire significant quantities of MHC-I molecules (up to 15%) from surrounding cells via their Ly49 receptors, which reduces the Ly49 MFI (28, 36). It was proposed that this process may improve NK cell function in the transfer setting (19). Uptake of H-2D^d from surrounding cells was also observed in *Ncr-Cre D^d* mice; however, the respective NK cells were not functional. Thus MHC-I uptake from surrounding cells does not seem to be sufficient to establish functional competence. Rather, MHC-I gene expression by NK cells is important for their functional maturation.

The analysis of NK cell function in MHC-I mosaic situations is of relevance for the understanding of the behavior of donor-derived NK cells that arise in leukemia patients receiving allogeneic stem cell transplantation. Recipients of HLA haploidentical stem cell transplants reportedly benefit from a graft-versus-leukemia effect mediated by NK cells when leukemia cells express HLA class I alleles that are not engaged by killer Ig–related receptor (KIR) of the donor (KIR ligand mismatch) (37–39). However, because of the HLA mosaic situation in the recipient, it is not obvious how donor-derived NK cells become competent to kill leukemic cells. According to the disarming model, recipient HLA should exert a dominant-negative role on NK cell function. One possible explanation for the positive role of donor HLA is that donor-type HLA molecules play an instructive role for NK cell licensing. If so, KIR may also educate NK cells based on the HLA molecules expressed by the NK cells themselves.

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Disclosures

E.V. is a cofounder and shareholder of Innate Pharma. The other authors have no financial conflicts of interest.

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