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

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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.

N atural 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 respond to a small 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). Other cis binding sequesters inhibitory receptors and renders...
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^BALB16^)Whe (Ly49A Tg), B6.DBA/2Tg(Ly49-72A^BALB30^)Whe (Ly49-72A Tg), and B6.DBA/2Tg(Ly49A-Y8F^BALB15^)Whe (Ly49A-Y8F Tg) mice were all generated using the BALB allele of Ly49A (9).

**Cell lines**

The cell lines used in this paper, C1498 (H-2\(^b\)), RMA (H-2\(^b\)), RMA/S (MHC-I\(^0\)), and D\(^d\)-transfected C1498 and RMA cells (6 × 10\(^5\)) 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. For 4 h, cultures were stained for flow cytometry.

Spleen cells (6 × 10\(^5\)) from polyinosinic-polycytidylic acid (poly IC)–activated NK cells were exposed to RMA (H-2\(^b\)), RMA/S (MHC-I\(^0\)), or RMA D\(^d\)-transfected cells (6 × 10\(^5\)) 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. For 4 h, cultures were stained for flow cytometry.

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

**In vivo target cell rejection**

Mice were injected i.p. with 100 μg polyclonal (Sigma-Aldrich) 24 h before a 1:1 mixture of H-2\(^b\) (labeled 3.0 μM CFSE [Molecular Probes]) and H-2D\(^d\) splenocytes (labeled with 0.3 μM CFSE [2 × 10\(^6\) cells total]). In some experiments, CMTMR (2.5 μM; Invitrogen)-labeled β2m-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\(^{\text{final}}\)/percentage of D\(^d\) cells (CFSE\(^{\text{initial}}\))/percentage of H-2\(^b\) cells (CFSE\(^{\text{final}}\)))/percentage of D\(^d\) cells (CFSE\(^{\text{initial}}\))] × 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\(^d\)^t Tg) 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-cdx 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\(^d\) cells 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\(^b\) splenocytes was ablated.
splenocytes by Ncr-cre Dd and CD4-cre Dd 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-2Dd on either NK cells or T cells prevented the NK cell–mediated rejection of H-2b targets. In contrast, all mouse strains efficiently rejected splenocytes that lacked all MHC-I molecules (Fig. 1F), demonstrating that cell type–specific H-2Dd ablation does not globally impair NK cell function.

We further determined whether T cell and NK cell–specific H-2Dd deficiency impairs NK cell function. To this end, we limited the abundance of Dd-deficient T cells by reconstituting lethally irradiated Dd mice with various mixtures of Dd and CD4-cre Dd BM precursors. After stable engraftment for >8 wk, we first verified that recipient mice contained the expected percentage of Dd-negative T cells (Fig. 2A) and then determined the ability of NK cells to reject H-2b target cells in vivo. The presence of >10% of Dd-negative T cells in chimeric mice significantly impaired NK cell–mediated rejection of H-2b targets. However, once the fraction of H-2Dd-negative T cells was <10%, there was no longer a significant adverse effect on NK cell mediated rejection of H-2b targets (Fig. 2B). Of note, the absolute number of Dd-negative cells present in these latter mice was similar to that in Ncr-Cre Dd mice, which indicated that NK cell–specific Dd 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 Dd mice could not be explained by the presence of a small population of Dd-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 Dd-specific Ly49A and the H-2b–specific Ly49C, Ly49I, and NKG2A receptors (Fig. 3A). When comparing H-2b to Dd NK cells from poly IC–primed mice, only the NK cell subset from Dd mice that expressed Ly49A and lacked Ly49C, Ly49I, and NKG2A receptors (Ly49A+CIN2 NK cells) responded significantly to H-2b (RMA) target cells (Fig. 3B–D). Ly49A+CIN2 NK cells from Dd mice efficiently released LAMP-1 and produced IFN-γ in response to RMA (H-2b) (Fig. 3B–D) or RMA/S tumor target cells (MHC-I low) (Supplemental Fig. 1B) but...
from CD4-cre Dd (Fig. 1C). In contrast, Ly49A+CIN T cell–specific Dd deletion had a similar adverse effect on NK effector responses (Supplemental Fig. 1D). Thus, NK cell and stimulation using PMA plus ionomycin resulted in comparable mouse strains (Supplemental Fig. 2A). Moreover, pharmacological cells (H-2b) for 4 h (which had been primed with poly IC 24 h before, were exposed to RMA a specific NK cell subset. Splenocytes from the indicated strains of mice, significantly as shown (*p < 0.05) in seven of the nine receptors or subunits that we analyzed (78%). In contrast, Ly49A Tg expression in Ncr-cre Dd mice resulted in limited repertoire changes (three of eight receptors [38%]) (Fig. 4A). In contrast, Ly49A Tg expression in CD4-cre Dd mice resulted in abundant repertoire changes (six of eight receptors [75% changes]) (Fig. 4A), which essentially corresponded to those seen in Ly49A Dd mice. T cell–selective Dd deletion thus dissociated the phenotypic from the functional adaptation of NK cells, whereas NK cell–specific Dd deletion impaired both repertoire and functional adaptation. Cell type–specific Dd deficiency had no significant additional effects on the abundance (Supplemental Fig. 2B, 2C) and/or on the maturation of Ly49A+CIN+ NK cells.

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

**Figure 3.** Cell type–specific H-2Dd 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-2d) 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-2Dd-transfected RMA cells (Supplemental Fig. 1C). In contrast, Ly49A+CIN− NK cells from Ncr-cre Dd and from CD4-cre Dd 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 Dd deletion had a similar adverse effect on NK cell function.

**Figure 4.** Modifications of the endogenous MHC-I receptor repertoire upon cell type–specific H-2Dd deletion

We assessed whether cell type–selective Dd 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-2Dd 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 Dd mice resulted in limited repertoire changes (three of eight receptors [38%]) (Fig. 4A). In contrast, Ly49A Tg expression in CD4-cre Dd mice resulted in abundant repertoire changes (six of eight receptors [75% changes]) (Fig. 4A), which essentially corresponded to those seen in Ly49A Dd mice. T cell–selective Dd deletion thus dissociated the phenotypic from the functional adaptation of NK cells, whereas NK cell–specific Dd deletion impaired both repertoire and functional adaptation. Cell type–specific Dd deficiency had no significant additional effects on the abundance (Supplemental Fig. 2B, 2C) and/or on the maturation of Ly49A+CIN− NK cells.
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 D4-binding properties. We have previously analyzed an Ly49A variant (A72), containing a rigid stalk, which failed to bind H-2Dd 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-2b) and to H-2Dd Tg B6 backgrounds, respectively. Primary NK cells were used to confirm that this receptor variant failed to productively interact with H-2Dd expressed on other cells (Fig. 5C). We have previously shown that a minimal length of the stalk, and not some other function of the α1s 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-2Dd in cis. Δα1 Tg NK cells bind soluble H-2Dd ligand, and this binding was reduced (2-fold) when NK cells were from H-2Dd 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-2Dd significantly increased following acid-mediated disruption of MHC-I complexes on living NK cells from H-2Dd 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-2Dd in cis but fails to functionally bind in trans.

Our previous analyses have shown that, unexpectedly, Wt Ly49A suppressed cytokine production in H-2b mice (i.e., when H-2Dd 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 (Ly49AΔα1, 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 Ly49AΔα1 (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-2Dd 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 agonists, 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 D4 deletion.

We also analyzed NK cells from mice expressing a Tg Ly49A receptor with a mutated ITIM (Ly49A ΔY8F). This receptor retained the ability to bind H-2Dd 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 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 spleens of mice expressing a Wt or the Δα1 Ly49A receptor Tg on a H-2b background. Histograms show Ly49A expression in gated NK cells. Numbers indicate the MFI of Ly49A staining. (C) Inhibitory capacity of the Ly49A Δα1 receptor was tested in lysis assays with cytokine-activated effectors against C1498 (top panel) and C1498 D4 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 β2m or Ly49A or with D4 multimer. Filled gray histograms depict the staining before and open histograms after acid stripping. Numbers indicate the MFI of staining with D4 tetramer.
We further show that MHC-I expressed by NK cells plays an essential role for their function. These results contrast with adoptive

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**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 (Ly49A−NKG2: Ly49A [mAb A1]), C, D, F, G2, H, I, and NKG2A/CD94). The NK cell subset expressing endogenous Ly49A86 (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 the expression of the Wt or the Δα1 (cis only) Ly49A Tg receptor among "null" NK cells from non-Tg mice. Open histograms depict Ly49A expression among "null" NK cells from non-Tg mice. Numbers indicate the percentage of cells in the respective gate. (B) Total spleen cells from the indicated types of mice on a H-2Db background were stimulated with immobilized NK1.1 mAb (anti-NK1.1) or left unstimulated (No) in the absence of cytokine addition. The bar graph depicts the mean percentage (±SD) of IFN-γ-positive null NK cells using the Wt, the A72 (trans only), the Δα1 (cis only), or the Y8F (ITIM mutant) Ly49A Tg on a H-2Db and on a H-2Dd (Dd) background. (C) 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. 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 modulation 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 Dd 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 Dd 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 Dd 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 repertoire receptor. 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 MF1 (28, 36). It was proposed that this process may improve NK cell function in the transfer setting (19). Uptake of H-2D\textsuperscript{b} from surrounding cells was also observed in Ncr-Cre Dd 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 NK cells should harbor specific receptors at the plasma membrane controls natural killer cell tolerance. Sci. Signal. 4: ra21.


Supplemental Figure Legends

Figure S1 Effector responses of Ly49A+CIN- NK cells

(A) Recipient mice were primed with polyIC and some recipients were depleted of NK1.1+ cells using mAb PK136. Twenty four h later, mixtures of H-2\(^b\) and D\(^d\) splenocytes were labelled with a high and a low concentration of CFSE, respectively, were injected i.v. into recipient mice. 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.

Total spleen cells from the indicated types of mice, injected with poly(IC) 24 h before, were exposed to RMA/S cells (B), D\(^d\)-transfected RMA cells (C) PMA plus ionomycin (D) or left unstimulated (E). After 4h we determined IFN\(\gamma\) production and LAMP-1 release by gated Ly49A+CIN- NK cells. The bar graphs depict the mean percentage (±SD) of IFN\(\gamma^+\) (left) or LAMP-1+ cells (right) among Ly49A+CIN- NK cells. Significance of differences relative to the respective littermate mice was calculated using t-test whereby ** p<0.01, *p<0.05 and ns (non significant) (p>0.05).

Figure S2 Priming and abundance of Ly49A+CIN- NK cells

(A) Mice were injected with polyIC 24 h before analysis of NK cells. Histograms show CD69 (top) and intracellular Granzyme B expression (bottom) in Ly49A+CIN- NK cells from naive (blue) and from poly IC primed mice (red) of the indicated type.

Further, Ly49A+CIN- NK cells showed normal expression of activation receptors including NK1.1, NKp46 and NKG2D. The expression of maturation markers (including KLRG1, CD11b and CD27) was not informative, since there was no significant difference between Ly49A+CIN- NK cells from H-2\(^b\) compared to D\(^d\) mice.
(B) Mean number (±SD) of NK cells in the spleen of non-primed mice of the indicated type. There are no significant differences between data sets (p>0.05) (n= 8-16).

(C) Mean percentages (±SD) of NK cells expressing Ly49A and lacking Ly49C, Ly49I and NKG2A (Ly49A+CIN-) in the spleen of non-primed mice of the indicated type mice. Significant differences between pairs are indicated as * (p<0.05), ns non significant (p>0.05) (n= 6-14).

**Figure S3 Abundance of NK cells in Ly49A Tg mice**

Mean number (±SD) of NK cells in the spleen (A) and the bone marrow (B) of the indicated strains of Ly49A Tg mice (n=10-15). None of the differences reach statistical significance based on a one way Anova test.
Figure S1

A

Recipient H-2b Dd Dd

Cell number

CFSE

LAMP1+ of Ly49A+CIN- NK cells (%)

IFNγ+ of Ly49A+CIN- NK cells (%)

B

RMA/S

C

RMA Dd

D

PMA + Ionomycin

E

Not stimulated

Recipient H-2b Dd Dd

CFSE
Figure S2

A

Total NK cells

Ly49A+/CIN-

Cell number

CD69

GzmB

B

Spleen

Number of NK cells (x10^6)

C

Ly49A+/CIN-

% of NK cells

H-2b  Dd  Ncr-Cre  CD4-Cre

H2b  Dd  Ncr-Cre  CD4-Cre

ns

ns

ns
Figure S3

(A) Spleen

(B) Bone marrow

Number of NK cells (x 10^6)

H-2^b  D^d

Non Tg  WT Tg  Δx1 Tg  Δx1 Δy Tg

Non Tg  WT Tg  Δx1 Tg  Δx1 Δy Tg