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Cre Recombinase-Mediated Inactivation of H-2D\textsuperscript{d} Transgene Expression: Evidence for Partial Missing Self-Recognition by Ly49A NK Cells\textsuperscript{1}

Vassilios Ioannidis,* Jacques Zimmer,* Friedrich Beermann, † and Werner Held\textsuperscript{2*}

We have established H-2D\textsuperscript{d}-transgenic (Tg) mice, in which H-2D\textsuperscript{d} expression can be extinguished by Cre recombinase-mediated deletion of an essential portion of the transgene (Tg). NK cells adapted to the expression of the H-2D\textsuperscript{d} Tg in H-2\textsuperscript{b} mice and acquired reactivity to cells lacking H-2D\textsuperscript{d}, both in vivo and in vitro. H-2D\textsuperscript{d}-Tg mice crossed to mice harboring an Mx-Cre Tg resulted in mosaic H-2D\textsuperscript{d} expression. That abrogated NK cell reactivity to cells lacking D\textsuperscript{b}. In D\textsuperscript{d} single Tg mice it is the Ly49A\textsuperscript{+} NK cell subset that reacts to cells lacking D\textsuperscript{d}, because the inhibitory Ly49A receptor is no longer engaged by its D\textsuperscript{d} ligand. In contrast, Ly49A\textsuperscript{+} NK cells from D\textsuperscript{d} × MxCre double Tg mice were unable to react to D\textsuperscript{d}-negative cells. These Ly49A\textsuperscript{+} NK cells retained reactivity to target cells that were completely devoid of MHC class I molecules, suggesting that they were not anergic. Variegated D\textsuperscript{d} expression thus impacts specifically missing D\textsuperscript{d} but not globally missing class I reactivity by Ly49A\textsuperscript{+} NK cells. We propose that the absence of D\textsuperscript{d} from some host cells results in the acquisition of only partial missing self-reactivity. The Journal of Immunology, 2001, 167: 6256–6262.

Expression of MHC class I molecules protects cells from NK cell-mediated killing. This is regulated via the interaction of incompletely defined receptors that activate NK cell effector function and inhibitory NK cell receptors specific for MHC class I molecules. The engagement of MHC class I receptors generates inhibitory signals that abort the induction of the NK cell’s lytic program. In the absence of MHC class I molecules on target cells NK cells fail to receive inhibitory signals, which allows target cell lysis to proceed.

Murine NK cells recognize classical MHC class I molecules via Ly49 family receptors. In general these receptors are specific for one and occasionally two class I isotypes, sometimes discriminating alleles thereof (1). The prototype receptor, Ly49A, inhibits NK cell function upon the interaction with the H-2D\textsuperscript{d} but not the D\textsuperscript{b} class I molecule (2). The specificity of Ly49 receptors together with their combinatorial distribution allows NK cells to react to subtle changes in a cell’s MHC class I make-up, such as the absence of a single class I allele (3).

The MHC class I environment in which NK cells mature or exist is an important determinant of their reactivity to target cells. The requirements for NK cell adaptation to class I were addressed using two experimental systems: MHC-deficient and MHC-different mouse models. First, although NK cells arise in the absence of MHC class I molecules in β\textsubscript{2}-microglobulin (β\textsubscript{2}m)-deficient mice, they were found to be impaired in their ability to lyse various target cells (4–6). In radiation bone marrow chimeras, the absence of class I molecules from either the radioresistant cells or some or all of the radiosensitive (hematopoietic) cells was sufficient to render NK cells hyporesponsive (4, 5, 7). In addition, MHC differences between mice were used to investigate NK cell function and tolerance. NK cells that developed in H-2\textsuperscript{b} mice expressing an H-2D\textsuperscript{d} transgene (Tg) acquired the ability to reject bone marrow grafts from H-2\textsuperscript{b} donors. Nontransgenic (non-Tg) and Tg mice were self-tolerant, as the respective syngeneic bone marrow grafts were not rejected (8). Thus, NK cells can adapt to the presence of MHC class I molecules. They acquire tolerance toward MHC-identical cells and reactivity to cells that lack them.

Due to the generalized effect on NK cell function in the absence of MHC class I, MHC-deficient mice were not suited to address the function of NK cell subsets that are defined by the expression of specific receptors for MHC class I. Indeed, the rejection of MHC-different but not MHC-deficient bone marrow cells could be prevented by the enforced expression of an appropriate inhibitory MHC receptor on all NK cells (9–11). Further, it was shown that NK cell self-tolerance was ensured via the inhibitory effect of MHC receptors (12–14); e.g., the inhibitory Ly49A receptor prevented H-2\textsuperscript{d} NK cells from attacking syngeneic cells. In contrast, self-tolerance of H-2\textsuperscript{b} Ly49A\textsuperscript{+} NK cells was independent of Ly49A function (12, 13). Because Ly49A\textsuperscript{+} NK cells from H-2\textsuperscript{b} mice killed β\textsubscript{2}m-deficient target cells it was concluded that self-tolerance was ensured by the inhibition through H-2\textsuperscript{b}-specific receptors (13). In agreement with this thesis, in a panel of human NK cell clones, each clone expressed at least one inhibitory receptor specific for self-MHC (15). These results suggest that autoaggressive clones are not present in the NK cell compartment. However, they did not rule out the existence of additional tolerance mechanisms. Autoaggressive NK cells may be refractory to cloning.

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3 Abbreviations used in this paper: β\textsubscript{2}m, β\textsubscript{2}-microglobulin; Tg, transgene/transgenic; loxD\textsuperscript{d}, H-2D\textsuperscript{d} gene flanked with loxP sites; bc, backcross; ER, estrogen receptor; CD3-Cy, CD3-CyChrome.
Consistent with this possibility, murine NK cell clones lacking known self-MHC-specific inhibitory receptors were detected using a single cell PCR assay (16). Evidence for the existence of potentially autoaggressive NK cells was also obtained using a class I MHC mosaic mouse, where H-2b cells coexpressed with H-2e cells expressing H-2D^d (17). NK cells in these mice were unable to react to H-2b/H-2^D^d cells in vitro and in vivo. However, upon the removal of H-2b/H-2^D^d cells in vitro the reactivity of H-2b/H-2^D^d NK cells to H-2b/H-2^D^d target cells was observed. Based on these findings it was proposed that NK cell tolerance may also be ensured by NK cell silencing (17, 18).

To further address the function of NK cells in relation to variable patterns of MHC class I expression we have engineered a genetic “off switch” for an H-2D^d Tg using Cre recombinase. The shut down of H-2D^d expression in a fraction of cells prevented NK cell reactivity to H-2b/H-2^D^d cells. This was associated with the inability of the Ly49A^+ NK cell subset to perform missing D^d recognition. However, these Ly49A^+ NK cells were able to react to target cells, which were completely lacking MHC class I molecules, suggesting that they were not silent but rather specifically unable to react to cells lacking D^d. These findings are discussed in the context of the adaptation of NK cells to self-MHC class I.

Materials and Methods

Transgene construction and transgenic mice

An oligo containing two loxP sites (GenBank accession no. M10494, nucleotides 14–47), an internal Spel site, and external Xbal sites was cloned into the Xbal site of pSK II (Stratagene, La Jolla, CA). The 1.8-kb Xbal fragment from pDd1 (19) was then inserted into the loxP-flanked Spel site, which destroyed both Spel and XbaI sites. The loxP-1.8-kb Dd1-loxP fragment was removed from the vector (using the external XbaI sites) and replaced by 2.2-kb loxD d fragment, which was ligated into pSK II (Stratagene, La Jolla, CA) with the XbaI site, and external XbaI sites. This resulted in a 283-bp fragment for the loxD d allele. Tg lines were established from C57BL/6J X SK (18) mice, and appropriate western blots were used for confirmation of transgene expression. In all experiments appropriate littermate mice were used as controls.

Bone marrow graft rejection

Recipient mice were lethally irradiated (960 rad from a 137 Cs source) and reconstituted 24 h later by i.v. inoculation of 10^6 (H-2b^+) bone marrow cells. To deplete NK cells some recipient mice received 100 μg of purified mAb PK136 i.p. 24 h before irradiation. Five days after irradiation the mice were injected with 3 μCi of ^51Cr (Amersham, Dübendorf, Switzerland) i.p. Twenty-four hours later the incorporation of radioactivity into the spleen was measured using a gamma counter.

Cytotoxicity assays

NK cell-mediated lysis of lymphoblastoid cells was done as described in Ref. 22. Briefly, erythrocyte-depleted spleen cells were passed over a nylon wool column. Nonadherent cells were cultured in complete DME plus 500 ng/ml recombinant human IL-2 (a gift from Glaxo Wellcome, Geneva, Switzerland). At day 3 adherent and nonadherent cells were harvested and used as effectors. T cell blasts were obtained by culturing spleen cells (2 × 10^6 cells/ml) in DME plus 2.5 μg/ml Con A (Sigma-Aldrich, Buchs, Switzerland). After 2 days dead cells were removed by Ficoll density gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden). Cells (3 × 10^6) were labeled with 50 μCi of ^51Cr for 1 h at 37°C. After three washes, 5 × 10^3 labeled target cells were mixed with effector cells in duplicate at various E:T ratios in 96-well U-bottom plates. Supernatants were harvested after 4 and 5 h and ^51Cr release into the supernatant was determined using a gamma counter. The percentage of NK1.1^+ CD5^+ cells in the effector cell population was estimated using flow cytometry.

Detection of intracellular IFN-γ

Total splenocytes were cultured (10 ml at 2 × 10^6 cells/ml) in the presence of human IL-2 as above. After 4 days, nonadherent cells were discarded and adherent cells (containing normally 30–50% NK cells) were detached using PBS/EDTA. After washing, 1.5 × 10^6 cells were plated in a 24-well culture plate in a final volume of 2 ml in the presence of IL-2. Stimulator cells (2 × 10^5) were added: C1498 (H-2^d, thymoma), D^d-transfected C1498 (kindly provided by W. Seaman, University of California, San Francisco, CA), Brefeldin A (10 μg/ml, Sigma-Aldrich) was added 3 h after the onset of cultures. After overnight culture, the cells were harvested, washed, and surface-labeled as described above using biotinylated anti-NK1.1 followed by a mixture of D3-Cy, Ly49A-FITC, and streptavidin-allophycocyanin to reveal the biotinylated mAb. After two washes in PBS 5% FCS, cells were fixed and permeabilized according to the supplier’s instructions (BD PharMingen). The cells were then stained intracellularly with the PE-conjugated anti-IFN-γ (XMG1.2) or an isotype-matched control mAb (BD PharMingen). After washing, the samples were analyzed using four-color flow cytometry as above.
Results
Modification of the H-2D\(^{d}\) gene

An 8-kb EcoRI fragment containing the entire H-2D\(^{d}\) gene (19) was modified by introducing two loxP sites into noncoding regions. The DNA sequence, which is flanked by two loxP sites, is deleted upon the action of the Cre recombinase. Because the two loxP sites in the H-2D\(^{d}\) gene flank the promoter region and the exons that encode the \(\alpha_1\) and \(\alpha_2\) domains, de novo expression of H-2D\(^{d}\) will be prevented following Cre-mediated recombination (Fig. 1).

Generation and characterization of D\(^{d}\)-Tg mice

The loxP-modified H-2D\(^{d}\) gene was injected into fertilized (B6 \(\times\) DBA/2) F2 oocytes to generate Tg mice. Five Tg lines were initially established by backcrossing to B6 mice. Offspring of H-2\(^{b}\) haplotype and with a NK gene complex of B6 origin were selected as detailed in Materials and Methods. The different lines expressed H-2D\(^{d}\) on lymphoid cells at levels slightly below to twice as high as compared with B10.D2 (H-2\(^{b}\)) mice (data not shown). One Tg line (28) termed hereafter D\(^{d}\)-Tg, that expressed H-2D\(^{d}\) at levels similar to B10.D2 (H-2\(^{b}\)) mice, was analyzed in more detail (Fig. 2A).

NK cells from D\(^{d}\)-Tg mice react to the absence of H-2D\(^{d}\)

Short-term (3–4 days) IL-2-activated NK cells from D\(^{d}\)-Tg (H-2\(^{b}\+D\(^{d}\)+) mice killed H-2\(^{b}\) lymphoblast targets. They did not kill syngeneic (H-2\(^{b}+D\(^{d}\)+) lymphoblast targets, indicating self-tolerance. Moreover, Tg and non-Tg NK cells killed MHC class I-deficient (due to a disruption of the \(\beta_2m\) gene) target cells equally well (Fig. 3A). These results suggest that D\(^{d}\)-Tg NK cells have adapted to the presence of the class I Tg and react to its absence in agreement with missing self-recognition. Thus, the introduction of two loxP sites into the H-2D\(^{d}\) Tg did not alter the expected effects of D\(^{d}\)-Tg expression on NK cell reactivity.

Generation and analysis of D\(^{d}\) \(\times\) Cre double Tg mice

The presence of two loxP sites in the D\(^{d}\) Tg offered the opportunity to manipulate H-2D\(^{d}\) expression in vivo using Cre-Tg mice. D\(^{d}\)-Tg mice were thus crossed with Mx-Cre-Tg mice. The Mx promoter allows the induction of Cre expression via an IFN-\(\alpha\)-\(\beta\)-inducible regulatory element (20). Second, D\(^{d}\)-Tg mice were crossed with CMV-CreER-Tg mice, which constitutively express a fusion protein of Cre recombinase with the ligand binding domain of the human ER driven by the CMV promoter (21). The activity of the fusion protein can be induced upon the administration of the synthetic ligand 4-hydroxytamoxifen.

Without deliberate induction of Cre expression or of its activity we have assessed H-2D\(^{d}\) expression in double Tg mice using flow cytometry. Approximately one-third of PBL or lymph node cells in young adult D\(^{d}\) \(\times\) Mx-Cre double Tg mice were completely H-2D\(^{d}\)-negative (Fig. 2B). A similar fraction of cells expressed H-2D\(^{d}\) at the same level as D\(^{d}\) single Tg mice while the remaining cells expressed intermediate H-2D\(^{d}\) levels. These latter cells may harbor a partial recombination of the multicopy Tg locus. No or very little H-2D\(^{d}\) loss (<1% of cells) had occurred in lymphoid cells of D\(^{d}\) \(\times\) CMV-CreER mice.
The cytotoxic activity of these preparations was tested in standard 51Cr release assays using Con A-activated lymphoblasts derived from β2m-deficient (open squares), H-2Dd/B6 (open circles), or H-2Dd/B6 (closed circles) spleen cells. A. Effector cells were derived from Dd-Tg (H-2Dd) or B6 (H-2b) mice. B. Effector cells were from Dd × Mx-Cre or Dd × CMV-CreER double Tg mice. The percentages of NK cells in the respective effector cell cultures were consistently 10–20% of total cells. Graphs show means (± SD) compiled from three to seven independent experiments. For β2m-deficient targets only the mean is shown.

Recombination of the loxDdTg was confirmed at the level of genomic DNA. Lymph node cell-derived DNA was subjected to PCR amplification using primers specific for the H-2Dd gene, which are normally separated by ~2.2 kb of intervening sequence (p2–p3, as indicated in Fig. 1). Following recombination of the H-2Dd gene these primers were expected to yield an amplification product of 420 bp. Indeed, a PCR product specific for a recombinant Dd-Tg was exclusively observed in lymphoid tissue of Dd × Mx-Cre double Tg but not of Dd single Tg or Dd × CMV-CreER double Tg mice (Fig. 4A), consistent with the cytometric analysis. This assay allowed us to determine whether Dd recombination has also occurred in nonlymphoid tissues. Indeed, Dd recombination was observed in tail DNA of both Dd × Mx-Cre and Dd × CMV-CreER mice (Fig. 4B). The two Dd × Cre double Tg mice thus displayed distinct patterns of H-2Dd expression. Mx-Cre led to H-2Dd loss on some but not all lymphocytes (hematopoietic origin) and in tail (nonhematopoietic origin). In contrast, lymphocytes in Dd × CMV-CreER mice were homogeneously H-2Dd positive while Dd recombination was evident in tail. These findings indicated considerable leakiness of the two Cre induction systems, yet they clearly established the functionality of the H-2Dd off switch.

Lack of missing Dd recognition by NK cells from Dd × Cre double Tg mice in vivo

The partial loss of H-2Dd expression had no significant effects on the abundance of NK cells or the representation of several MHC class I-specific Ly49 receptors (Fig. 2C and data not shown). The impact of the H-2Dd expression patterns on NK cell reactivity was tested using bone marrow graft rejection experiments. Lethally irradiated Dd × Cre double Tg mice were challenged with bone marrow cells from H-2b (Dd-negative) mice. The proliferation of bone marrow stem cells (indicating acceptance of the graft and thus the absence of a NK cell reaction against it) was read out by [125I]UdR incorporation into spleens of recipient mice. As shown in Fig. 5, Dd (H-2b-Dd) single Tg mice rejected H-2b bone marrow grafts, whereas Dd-Tg mice were able to accept H-2b-Dd grafts (left). Twenty-four hours later the incorporation of radioactivity into the spleen was measured. Open circles represent mean values (± SD) from three or more mice. Values for individual mice are shown when fewer recipients were used. High incorporation of label indicates acceptance of the graft and thus the absence of a NK cell-mediated rejection. Low values are indicative of graft rejection.
grants judged by the low incorporation of $^{[125]}$UdR into the recipients’ spleens. This was not due to the inability of the grafted cells to expand, as a high $^{[125]}$UdR incorporation was observed in B6 (H-2$^b$) recipient mice. In contrast to D$^d$ single Tg mice, both types of D$^d$ × Cre double Tg mice were unable to reject the H-2$^b$ graft (5). The extent of H-2D$^d$ loss in the two types of double Tg mice was thus sufficient to prevent missing D$^d$ recognition. The nonreactive state was specific for H-2$^b$ grafts, as all mice rejected MHC class I-deficient bone marrow grafts. While the data recapitulate those obtained in another model of H-2D$^d$ mosaic mice (17), they further establish that H-2D$^d$ loss from some nonlymphoid cells was sufficient to abolish missing D$^d$ recognition.

**NK cells from D$^d$ × CMV-CreER mice acquire missing D$^d$ recognition in vitro**

We next determined whether H-2D$^d$ recombination in double Tg mice influenced NK cell reactivity in vitro. Short-term (3 days) IL-2-activated NK cells from D$^d$ × Mx-Cre mice were unable to kill H-2$^b$ lymphoblasts (Fig. 3B). MHC class I-deficient targets were readily killed, consistent with the bone marrow rejection experiments. However, in contrast to the in vivo data, NK cells from D$^d$ × CMV-CreER-Tg mice killed H-2$^b$ target cells as efficiently as NK cells from D$^d$ single Tg mice (Fig. 3B). The in vitro culture may thus enable missing D$^d$ reactivity perhaps due to an IL-2-dependent reactivation of silenced NK cells, as suggested before (23). In addition, the separation from nonlymphoid cells, some of which lack H-2D$^d$, may be required to restore reactivity in vitro. Indeed, missing D$^d$ reactivity by NK cells from mosaic mice was restored upon the removal of hematopoietic cells lacking H-2D$^d$ (17).

**Ly49A$^+$ NK cells from D$^d$ × Mx-Cre mice perform missing class I but not missing D$^d$ recognition**

Missing D$^d$ recognition by NK cells from H-2$^b$D$^d$ mice is mediated by the Ly49A$^+$ NK cell subset (12). This suggested that in D$^d$ × Mx-Cre-Tg mice, the inability to perform missing D$^d$ reactions was due to a malfunction of the Ly49A$^+$ NK cell subset. This further raised the issue of whether these Ly49A$^+$ cells were completely nonreactive (anergic) or whether they were selectively nonreactive to cells lacking D$^d$. To address these questions we crossed D$^d$ × Mx-Cre mice to mice expressing a Tg Ly49A on all their NK cells. Like D$^d$ × Mx-Cre double Tg (Fig. 3), NK cells from Ly49A$^+$ × D$^d$ × Mx-Cre triple Tg mice showed minimal cytotoxic activity to H-2$^b$ lymphoblasts (Fig. 6). However, NK cells from triple Tg mice efficiently killed β2m-deficient lymphoblasts or YAC-1 target cells (Fig. 6 and data not shown). Ly49A$^+$ NK cells in D$^d$ × Mx-Cre mice are thus not anergic but rather selectively impaired in their reactivity to cells lacking D$^d$.

The selective failure of Ly49A$^+$ NK cells to react to missing D$^d$ may be related to a failure of the Ly49A receptor to inhibit NK cells in a D$^d$ mosaic situation. To address this issue we have exposed NK cells to tumor cells and determined the production of IFN-γ at the single cell level using intracellular flow cytometry (Fig. 7) (24). A significant fraction of Ly49A$^+$ NK cells from B6 mice (25.6 ± 6.0%) produced IFN-γ following overnight exposure to the syngeneic (H-2$^b$) tumor cell line C1498 (Table I). Even though values were variable between experiments, significantly more Ly49A$^+$ NK cells (36.7 ± 7.3% vs. p < 0.02) from D$^d$-Tg mice produced IFN-γ in response to C1498 (Table I). This assay thus appropriately indicates a contribution of missing D$^d$ reactivity to IFN-γ production by Ly49A$^+$ NK cells from H-2$^b$D$^d$ mice. Ly49A$^+$ NK cells from D$^d$ × Mx-Cre mice behaved essentially like those from B6; i.e., a similarly small fraction (26.0 ± 9.6%) of Ly49A$^+$ NK cells produced IFN-γ (Table I). These data are compatible with the above lysis assays and suggest that Ly49A$^+$ NK cells from Mx-Cre mice behave similarly to those from B6 mice: they do not react to missing D$^d$, yet are able to produce IFN-γ in response to C1498 target cells. The exposure to D$^d$-transfected C1498 cells reduced the percentage of IFN-γ-expressing Ly49A$^+$ D$^d$ × Mx-Cre NK cells ~4-fold as compared with C1498 cells (Table I). The capacity of Ly49A to function as an inhibitory receptor is thus intact in D$^d$ × Mx-Cre mice. It is interesting to note that while Ly49A reduced the fraction of IFN-γ-expressing cells in response to C1498 D$^d$ targets, the mean fluorescence intensity of IFN-γ staining in the residual Ly49A$^+$ IFN-γ$^+$ NK cells was not significantly lowered (data not shown). Therefore, this single cell assay suggests that the engagement of the inhibitory Ly49A receptor affects NK cell effector function in an all-or-none fashion. IFN-γ production by Ly49A$^+$ NK cells was independent from the coexpression of the D$^d$-specific activating Ly49D receptor (data not shown), which is able to initiate IFN-γ production (25).

The responsiveness to C1498 and C1498 D$^d$ cells was not significantly different among Ly49A-negative NK cells or NK cells expressing Ly49C/I (Table I), which are inhibitory receptors specific for H-2$^b$ MHC class I molecules (1). Variegated D$^d$ expression thus impacts specifically the NK cell subset expressing the D$^d$-specific inhibitory Ly49A receptor and prevents selectively missing D$^d$ reactivity. However, the failure to acquire missing D$^d$ reactivity is not related to an inability of Ly49A to function as an inhibitory receptor.

**FIGURE 6.** Reactivity of NK cells from Ly49A × D$^d$ × Mx-Cre triple Tg mice. Nylon wool nonadherent spleen cells from the indicated mouse strains were cultured for 3 days in the presence of IL-2. The cytotoxic activity of these preparations was tested against Con A-activated target cells derived from H-2$^b$ (B6) (□) or H-2$^b$D$^d$ (●) or β2m-deficient spleen cells (■). Graphs show mean percentage of specific lysis (± SD) compiled from four values obtained in two independent experiments. For β2m-deficient targets only the mean is shown.
Table I.  Production of IFN-γ by NK cell subsets

<table>
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<tr>
<th>Mouse Strain</th>
<th>NK Cell Subtype</th>
<th>% intracellular IFN-γ+ Cells</th>
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<tr>
<td></td>
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<tr>
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</tr>
<tr>
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<td>Ly49C/I</td>
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</tr>
<tr>
<td>Dd Mx-Cre</td>
<td></td>
<td>4.2 ± 1.8 16.4 ± 7.3</td>
</tr>
</tbody>
</table>

a  Data are derived from six to eight independent experiments.

b Significantly different from B6 (p < 0.02) or B6 Dd Mx (p < 0.05).

c Significantly different (p < 0.01) from corresponding value obtained with C1498. Data evaluation is based on the two-tailed Student t test.

Discussion

We describe in this report the construction of a molecular switch to irreversibly extinguish a Tg MHC class I molecule. The Tg is ubiquitously expressed and its down-regulation reflects present or past expression of Cre recombinase. This mouse strain provides a novel tool to establish various patterns of Dd expression using available Cre Tgs that are under the control of different cis-acting regulatory elements. Importantly, these patterns can be established using a Tg founder line with a proven NK cell phenotype. Phenotypes in Dd × Cre Tg mice can thus be accurately compared with those of founder mice. This is of importance in light of our observation that not all Dd-Tg founder mice were equally capable of rejecting H-2Dd-negative bone marrow grafts (our unpublished observation). Differences in the capacity to reject grafts may be related to the site of Tg insertion. Indeed, several of the founder mice initially displayed a mosaic Dd expression, raising the possibility that mosaic Dd expression, which affects NK cell reactivity, persisted in some tissues of some Tg lines.

These novel mouse strains allowed us to study the function of NK cells, which develop and exist in the presence of two types of host cells. Some cells express and others lack the strong Ly49A ligand H-2Dd, whereas all host cells express H-2b class I molecules. The use of two distinct Cre Tgs (Mx- and CMV-Cre) resulted in Dd loss in different compartments. The reactivity of NK cells arising in these situations was compared with NK cells that develop in an environment where all or no cells express H-2Dd, respectively. Our data revealed functional differences among NK cells from the different types of mice. Both Dd mosaic mouse strains failed to reject H-2b bone marrow grafts, similar to mice completely lacking Dd. In contrast to NK cells from Dd × CMV-CreER mice, those from Mx-Cre mice were further unable to kill H-2b lymphoblasts. This prompted us to specifically investigate the NK cell subset expressing the relevant inhibitory MHC receptor Ly49A. We found that Ly49A+ NK cells in mosaic mice were indeed unable to perform missing Dd recognition; however, these cells were not completely unreactive, as MHC class I-deficient targets were readily killed.

Two distinct models may account for our data as well as previous findings (17). In the first one, the absence of Dd from some host cells may have a negative effect and silence NK cell clones that would normally perform missing Dd recognition. The concept of NK cell silencing is primarily supported by the observation that missing Dd reactivity is restored when Dd-positive NK cells are cultured separately from Dd-negative hematopoietic (17) or non-hematopoietic host cells (Fig. 3). However, because these Ly49A+ NK cells perform missing class I recognition and are thus not silent, we propose an alternative interpretation of our observations. The encounter with Dd-deficient host cells during NK cell development may not allow complete adaptation to self-MHC class I. Whereas these NK cells acquire missing H-2b recognition, they do not acquire proper missing Dd reactivity. This scenario is in line with a number of recent observations. Mature NK cells are inhibited in a cumulative fashion when NK cells express multiple self-specific Ly49 receptors (26, 27). This suggests that self-specific inhibitory MHC receptors function independently, each contributing to NK cell inhibition. The acquisition of missing self-reactivity during NK cell development may thus similarly occur independently, such that NK cells in a Dd mosaic mouse acquire missing H-2b reactivity (using H-2b-specific inhibitory receptors) but fail to acquire missing Dd reactivity (using Ly49A). However, the selective failure to acquire missing Dd reactivity was not due to a defective inhibitory function of Ly49A (Table I). We have recently provided evidence that Ly49A-H-2b interaction during NK cell development has a positive impact on NK cell development (28). This positive effect was only observed when H-2d was present on both the radiosensitive and radiosensitive compartments. In addition, the expression of a human Ig-like killer inhibitory receptor may enhance the survival of memory T cells in Tg mice (29).

These findings are consistent with the idea that MHC-specific receptors, in addition to the well-established inhibitory role, may perform additional functions. Indeed, besides SHP-1, the inhibitory motif in the Ly49A cytoplasmic tail recruited also SHP-2 (30). Finally, killer inhibitory receptors were shown to recruit phosphatidylinositol 3-kinase and thus potentially couple to signaling pathways that provide growth and/or survival signals (32, 33). It is thus possible that negative and putative positive functions of Ly49A are dissociated in Dd mosaic mice. Finally, the fact that NK cells react to missing Dd once they are cultured separately from Dd-negative host cells could also be interpreted as a rapid acquisition of missing Dd reactivity as soon as Ly49A is continuously engaged by neighboring cells. Although we currently favor the second hypothesis, additional experiments will be required to discriminate between the two models.

The acquisition of missing self-reactivity seems to be completely prevented by ligand deficiency in some cells of the radiosensitive (hematopoietic) compartment (Refs. 7, 17, and 34 of this study). NK cells in all these mice encountered ligand-deficient host cells throughout the entire body, including the bone marrow, where NK cell adaptation to the MHC class I environment may take place. Therefore, it will be of interest to refine the analysis and to assess the function of NK cells that develop in a homogeneously Dd+ bone marrow yet encounter Dd-deficient host cells in the periphery. That can now be achieved using Tg mice, which express Cre in an organ-specific fashion. It will also be important to determine the minimal number of H-2Dd-deficient host cells required to prevent missing Dd recognition. In this context it has been known for a long time that NK cells have a limited capacity to eliminate grafted tumor cells (103–105 cells) even if these cells lack MHC class I partially or completely (35, 36). The relative inefficiency of NK cells has primarily been attributed to their limited capacity for clonal expansion. The data obtained in mosaic mice thus raise the possibility that the presence of sufficient numbers of class I-deficient tumor cells may at some stage disable NK cell reactions.
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