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A Genetic Defect in Mice That Impairs Missing Self Recognition Despite Evidence for Normal Maturation and MHC Class I–Dependent Education of NK Cells

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In studies of a CD1d1-deficient mouse strain, we unexpectedly observed a severely impaired capacity for NK cell–mediated rejection of MHC class I–deficient (spleen or tumor) cells. Studies of another CD1-defective strain, as well as intercrosses with C57BL/6 mice, indicated that the impaired missing self rejection (IMSR) NK cell defect was a recessive trait, independent from the targeted CD1 locus. Studies with mixed bone marrow chimeras indicated that the defect is intrinsic to NK cells. The IMSR mice had normal proportions of NK cells, displaying a typical cell surface phenotype, as evaluated using a panel of Abs to developmental markers and known receptors. The impaired missing self recognition could not be overcome through cytokine stimulation. There was also an impaired capacity with respect to NKG2D-dependent cytotoxicity, whereas the mice exhibited normal Ly49D/DAP12-dependent responses in vivo and in vitro. The NK cell system of IMSR mice showed two hallmarks of MHC-dependent education: skewing of the Ly49 receptor repertoire and differential in vitro responsiveness between NK cells with and without inhibitory receptors for self-MHC (“licensing”). We conclude that these mice have a recessive trait that perturbs the missing self reaction, as well as NKG2D-dependent responses, whereas other aspects of the NK system, such as development, capacity to sense MHC molecules during education, and Ly49D/DAP12-dependent responses, are largely intact. The Journal of Immunology, 2014, 192: 1577–1586.

Natural killer cells are considered an integral part of the innate immune system, even if they display some adaptive immune system features (1). They respond to infections and tumors and are important in hematopoietic stem cell transplantation (2–4). NK cell recognition of target cells is based on the interplay between multiple activating and inhibitory receptors, which allows recognition of “dysregulated self” (e.g., missing MHC class I molecules, stress-induced cellular ligands, or combinations of such alterations) (5). Important inhibitory receptors in mice are the family of Ly49 receptors binding to different MHC class Ia alleles, as well as NKG2A, which binds to the class Ib molecule Qa-1 (6). Activating receptors can bind to induced or constitutively expressed ligands. NKG2D interacts with the mouse ligands H60, Rae-1, and MULT-1, which are all upregulated on infected and transformed cells. There are additional activating receptors, such as Nkp46, CD16, Ly49D, Ly49H, and NKRP1c (NK1.1) (7). Activating receptors signal through different adaptor molecules, such as DAP12, DAP10, CD3ζ, and FcεRIγ. These different proximal activation pathways converge downstream, leading to activation of the cellular machineries for cytotoxicity and cytokine secretion. The inhibitory receptors interfere with these processes by recruitment of phosphatases that dephosphorylate critical substrates within the activation pathways (7). In addition, the SLAM family receptors, such as 2B4 (CD244), Ly108, Ly9, CD84, and CRACC, have the capacity to submit both activating and inhibitory signals to the NK cell, depending on the presence or absence of SAP family adaptor molecules (8).

As a consequence of this regulation via inhibitory MHC class I–specific receptors, NK cells are efficient killers of cells with low expression (or the wrong type) of MHC class I molecules, a phenomenon known as “missing self recognition” (9, 10). This killing activity can be directed against infected or transformed cells, which, in addition to reduced MHC class I levels, also express stress-induced ligands for activating receptors, but it also extends to killing of cells that are otherwise normal (11, 12). The activating receptors and pathways involved in the latter scenario are largely unknown. To perform missing self recognition optimally, NK cells undergo an MHC class I–dependent education process. This process has two main influences: on the frequency of different NK subsets, as defined by their expression pattern of inhibitory receptors (“the NK repertoire”) (13, 14), and on functional responsiveness status within each such cellular subset (“licensing”) (15, 16). The exact mechanisms underlying these MHC influences are not known.

It would be highly useful to have mice that possess selective defects in certain NK reactivities but that are normal with respect to NK cell development and other functions. The present study investigates a mouse strain in which we initially, and by chance, observed such a phenotype (i.e., a profound defect in the ability to reject normal cells of the “missing self” phenotype, despite normal proportions of NK cells). We present a characterization of the NK system in this CD1d1−/− mouse strain, which is one of the published gene-targeted strains that has been constructed to study CD1-restricted NKT cells (17).
We initially studied missing self recognition in this mouse because CD1d1 is a nonclassical MHC molecule and, therefore, might be involved in the education of NK cells. It is a crucial molecule for positive selection of NKT cells; thus, CD1d1−/− mice lack CD1-restricted NKT cells (17, 18). CD1d1 is associated with β2-microglobulin (β2m), although its expression is independent of TAP function, and it binds and presents lipid and glycolipid Ags to NKT cells (19). Furthermore, CD1d1 was reported to bind to and inhibit IL-2–activated NK cells (20, 21). Multiple lines of CD1-deficient mice have been generated (17, 18), all of which display developmental and functional defects in NKT cells due to the lack of CD1-mediated positive selection in the thymus. The defect in rejection of target cells displaying a “missing self” phenotype that we observed in one of the CD1 mutant mice was not due to the lack of NKT cells or the CD1d1 molecule itself. Our data suggest that this defect, which we term “impaired missing self rejection” (IMSR), is caused by one or more recessive gene(s) that leaves NK cell maturation and several NK cell functions relatively intact.

Materials and Methods

Mice

Mice were bred and maintained under specific pathogen–free conditions at the Department of Microbiology, Tumor and Cell Biology, Karolinska Institute. Experiments were performed according to governmental and institutional guidelines and regulations and were approved by the local ethical committee (North Stockholm District Court). Animals were 6–10 wk of age at the start of the experiments. C57BL/6 (B6) (H-2b) mice and BALB/c (H-2d) mice were bred for ≥10 y at the Department of Microbiology, Tumor and Cell Biology, Karolinska Institute. β2m-deficient mice were obtained from the femur, tibia, pelvis, and spine by disruption in PBS supplemented with 1% FCS and 2 mM EDTA) using a mortar and pestle. Erythrocytes were lysed, and 10⁷ cells (from one of the donor strains or as a 1:1 mixture from both strains) were injected i.v. into the tail veins of lethally irradiated (9 Gy) B6 (Ly5.2+), CD1d1−/−, or B6.Ly5.1+ mice. Transplanted mice received antibiotics (Tribissen 400/80; Schering-Plough Animal Health) in their drinking water for 3 wk following transplantation. Chimerism was assessed by flow cytometry 8–9 wk after reconstitution using Abs specific for Ly5.1 and Ly5.2. In bone marrow chimeras, in vivo rejection of MHC class I−/− target cells was tested, and in mixed bone marrow chimeras, the responsiveness at the single-cell level was tested between 8 and 10 wk after reconstitution.

Abs and FACS analysis

Spleen cells were obtained as described above. All surface staining was performed on ice and in PBS complemented with 0.5% FCS. FcRs were blocked with anti-FcγRII/III (2.4G2). The following mAbs were used: anti-CD3 (145-2C11), anti-CD122 (PK136), anti-NKp46 (29A1.4), anti-2B4 (m2B4/By6458.1), anti-KLRG1 (2F1/KLRG1), anti-Ly-49G2 (4D11), and anti-Ly49A (IELY48) (all from BioLegend); anti-CD1d1 (1B1), anti-CD49b (Dx5), anti-Ly49D (4E5), anti-NKG2D (CX5), and anti-CD27 (LG.3A10) (all from BD Pharmingen); and anti-NKG2A/C/E (2D5) (AdB Serotec). The anti-Ly49C (4L0331) hybrid-oma was a kind gift from Suzanne Lemieux (Institut Armand Frappier) (27, 28) and was used in combination with anti-mouse IgG3 (Southern Biological Technology). Dead cells were excluded using the Live/DEAD Fixable Aqua Dead cell stain kit (Invitrogen Molecular Probes). Cells were acquired using a FACSscan flow cytometer or LSR II SORP (BD Bioscience) and analyzed using CellQuest Pro or FlowJo (TreeStar) software.

CFSE labeling and analysis of in vivo rejection

Splene cells were mechanically disrupted into single-cell suspensions and erythocytes were depleted by osmotic lysis. Cells were then labeled with low or high concentrations of CFSE (Invitrogen Molecular Probes). Target cells and control cells were mixed in a 1:1 ratio and coinjected i.v. into mice. The injection mix was analyzed for reference. At the indicated time points, the spleens were harvested, erythocytes were depleted, and the relative percentages of cells in each population were measured by flow cytometry (25). For kinetic experiments, blood was drawn at indicated time points after injection, and erythocytes were depleted before flow cytometric analysis. The relative survival of target cells was calculated: (% control cells in sample/% control cells in inoculate)/(% target cells in sample/% target cells in inoculate) (25). The results are expressed as the ratio of remaining cells, target cells versus control. At least 3000 CFSE− cells were acquired in each sample.

Tumor-growth experiments

A total of 10⁶ ascites-grown RMA and RMA-S cells were inoculated s.c. into opposite flanks of the same mouse. The growth of solid tumors was followed and measured by palpations twice weekly. Tumor size was calculated using the formula (π/6) × L × W × H (26). All mice were sacrificed 2 wk after injection.

Bone marrow chimeras

Bone marrow cells from CD1d1−/− (Ly5.2+) and B6.Ly5.1+ mice were obtained from the femur, tibia, pelvis, and spine by disruption in PBS supplemented with 1% FCS and 2 mM EDTA) using a mortar and pestle. Erythrocytes were lysed, and 10⁷ cells (from one of the donor strains or as a 1:1 mixture from both strains) were injected i.v. into the tail veins of lethally irradiated (9 Gy) B6 (Ly5.2+), CD1d1−/−, or B6.Ly5.1+ mice. Transplanted mice received antibiotics (Tribissen 400/80; Schering-Plough

Results

CD1d1−/− mice fail to reject spleen cells lacking cell surface MHC class I/β2m molecules

NK cells from B6 mice rapidly eliminate MHC class I−/− cells in vivo (25). Because we were interested in whether the
CD1d molecule and/or CD1-restricted T cells expressing NK cell markers were important for this elimination, we analyzed the rejection of β2m−/− cells by mice with a nonfunctional CD1d gene (CD1d1−/−). The mice were challenged with a mixture of β2m−/− and control B6 spleen cells labeled with different concentrations of CFSE. At 48 h after injection, the ratio of β2m−/−/B6 spleen cells was analyzed by flow cytometry (Fig. 1) (25). As shown in Fig. 1A, B6 mice efficiently rejected β2m−/− cells through a mechanism that is dependent on NK1.1 cells, whereas CD1d1−/− mice lacked the ability to reject these cells. To determine whether this could simply be explained by a slower rejection rate by the CD1d1−/− NK cells, kinetic studies were performed in which rejection was assessed over a period of 4 d (Fig. 1C). These studies revealed a profound defect, rather than just a delay, in missing self rejection in CD1d1−/− mice.

To determine whether NKTs, other T cells or B cells, or indeed the CD1d1 molecule per se was involved in the defective missing self rejection, we tested a broader panel of mutant mouse strains. These included an independently derived CD1-deficient mouse strain (CD1−/−) (18); Jc281−/− mice, which lack the gene segment used by the invariant TCR on type 1 NKT cells (24); and RAG-deficient mice, which lack all mature T and B cells (23). All of these data suggest that the deficiency in missing self rejection evident in CD1d1−/− mice is not due to the absence of the CD1d1 molecule, NKT cells, or other T or B cells.

The defective rejection of β2m−/− cells in CD1d1−/− mice is a recessive trait not linked to the CD1 locus

To investigate the genetic control of this NK cell defect in CD1d1−/− mice, we generated and tested different crosses with B6 mice. (CD1d1−/− × B6)F1 mice rejected β2m−/− spleen cells as efficiently as did B6 mice, indicating a recessive trait (Fig. 1D). There was a dichotomy in experiments with F2 mice: 28 of 35 (80%) F2 mice rejected β2m−/− cells efficiently, whereas 7 of 35 accepted the graft. Both responders and nonresponders were noted among CD1d1−/− F2 mice, as well as CD1+ F2 mice, as assessed by flow cytometry. Although these data indicate that the trait is not linked to the CD1 locus, they do not permit any conclusions as to whether one or multiple recessive genes are involved. There are at least two possibilities for the origin of this deficiency. First, a spontaneous mutation may have occurred, either in the embryonic stem cell line or during backcrosses to B6 (38). Secondly, the genetic defect may represent an allelic variation that has been carried over from the 129S6 strain that has been associated with defects in NK cell function (39).

To investigate this latter possibility, we generated (CD1d1−/− × 129S6)F0 offspring and tested the ability of these mice to reject β2m−/− cells (Fig. 1E). 129S6 mice, similar to CD1d1−/− mice failed to reject β2m−/− cells. However, (B6 × CD1d1−/−)F1 mice rejected equally well as did B6 mice (Fig. 1D), whereas (B6 × 129S6)F0 mice had an intermediate rejection capacity. The (CD1d1−/− × 129S6)F0 mice also displayed an intermediate rejection. If the same gene(s) gave rise to the defective rejection in CD1d1−/− and 129S6 mice, (CD1d1−/− × 129S6)F0 mice should display an equally defective rejection as the parental strains. In contrast, our results suggest that the defect in CD1d1−/− mice could be complemented by 129S6 allele(s), although other 129S6-derived genes contribute to a less efficient rejection response in F1 crosses to CD1d1−/− mice, as well as to B6 mice. This argues against the genetic defect in CD1d1−/− mice being transmitted from the 129S6 strain.

FIGURE 1. The failure of CD1d1−/− mice to reject spleen cells lacking cell surface MHC class I β2m molecules is a recessive trait that segregates independently of the CD1d1 locus. CFSE-labeled β2m−/− and B6 spleen cells were coinjected i.v. into mice that were untreated or NK depleted with anti-NK1.1 (A–C) or anti–TMβ1-1 (D, E). Abs. (A, B, D, and E) Splenic lymphocytes were analyzed using flow cytometry at 48 h after injection. (C) Blood lymphocytes were analyzed at 24–96 h after injection. Rejection of spleen cells is depicted as a ratio of recovered β2m−/−/B6 spleen cells and corrected for percentages in the cell inoculate. Plots represent an average of six mice/group (A), at least two mice/group (B), six mice/group and at least three mice/NK-depleted control group (C), individual mice (D), and an average of eight mice/group and at least four mice/control group (E) and are from six mice (A), at least two mice (B), and two (C–E) independent experiments. (D) There was no significant linkage between CD1d1 deficiency and the defect in missing self rejection. p = 1.0, Fisher exact test. (E) There was a statistically significant difference between the following comparisons: *129S6 versus (B6 × 129S6)F1, ***B6 versus either CD1d1−/− or 129S6 mice, ***B6 versus (B6 × 129S6)F1, ***CD1d1−/− versus (B6 × CD1d1−/−)F1, ***129S6 or CD1d1−/− versus (129S6 × CD1d1−/−)F1. Error bars denote SD. *p < 0.05, ***p < 0.001, 129, 129S6 mice.

Taken together, our genetic studies indicated that the impaired missing self rejection is a recessive trait not linked to the CD1 locus. We denote this trait and the mice that carry it IMSR, to avoid the risk of creating a misunderstanding concerning the role of the targeted CD1 locus in the mice in which the trait was observed.

IMSR mice exhibit defective missing self triggered resistance to tumor outgrowth

To investigate whether the defect also affected long-term growth of MHC class I–deficient tumor cells, 106 RMA (cell surface MHC class I) and 105 RMA-S (cell surface MHC class II) tumor cells were inoculated i.p. in different flanks, and outgrowth was assessed for up to 2 wk. No RMA-S tumors formed in the B6 mice.
unless they were pretreated with anti-NK1.1 Ab (Fig. 2A) (10). However, in 7 of 8 IMSR mice, RMA-S established progressively growing tumors, indicating that the defect also persisted during a longer exposure to MHC-deficient cells. Importantly, RMA-S tumors grew faster in NK-depleted IMSR mice, resulting in larger tumor sizes at 2 wk (Fig. 2B). Because this difference between nontreated and NK-depleted mice was also observed with the MHC class I–expressing tumor RMA in IMSR mice, as well as in B6 mice, we conclude that there is an additional NK cell–mediated rejection pathway that is unrelated to the MHC status of the tumor cells and that is not affected in mice with the IMSR trait.

Mice with the IMSR trait have normal numbers of NK cells, without any major defect in maturation or the expression of activating receptors

There are several possibilities to explain the functional defect observed in this study (e.g., an arrest in NK cell development, a defect affecting a distinct activating receptor and/or pathway, or a perturbed MHC-dependent education for missing self recognition). The total number of spleen cells was reduced on average in mice with the IMSR trait, but the percentage of NK1.1+CD3− NK cells in the spleen was normal, excluding an absolute arrest in early NK cell development. To study the NK cell population in more detail, we analyzed their surface marker profiles. β2m−/− mice were included as a control strain in which no MHC-dependent NK cell development, this skewing might be absent, resembling β2m−/− mice in this respect. To address this hypothesis, we used polychromatic flow cytometry to analyze expression of Ly49C, Ly49L, Ly49A, Ly49G2, and NK2A in IMSR, B6, and β2m−/− NK cells. We first observed that the frequencies of NK cells expressing each of these inhibitory receptors (analyzed individually) were somewhat reduced compared with B6 and β2m−/− mice (Fig. 3C). With respect to the receptor repertoire, we observed that skewing of NK cell subsets in CD1d1−/− mice was even more pronounced than in B6 mice; there was a markedly increased frequency of NK cells expressing none or only one self-specific inhibitory receptor and a dramatic decrease in the frequency of NK cells expressing five receptors in CD1d1−/− mice (Fig. 3D).

A second, most important influence of host MHC is to regulate the responsiveness of NK cells at the single-cell level in a process that has been coined “licensing” (15, 16). To determine whether the NK cells in IMSR mice are licensed, we used an in vitro responsiveness assay measuring degranulation via the marker CD107 in response to stimulation with plate-bound Abs against NKp46 (35). To separate the response of licensed versus unlicensed NK cells, we designed an Ab panel that allowed us to gate on cells expressing one or several of the receptors Ly49C, Ly49L, or NK2A (CIN+ cells) versus cells expressing none of those receptors (CIN−), independently of the expression of other inhibitory receptors. NK cells from IMSR mice exhibited a clear general reduction in their ability to respond to stimulation through the NKp46 receptor compared with B6 mice (Fig. 3E). However, there still was a significantly increased response by the CIN+ subpopulation compared with the CIN− subpopulation, similar to the differences in B6 NK cells. These data, and the observation that CIN+ and CIN− subpopulations from β2m−/− mice responded equally (Fig. 3E) (15), argue against a pure educational defect in NK cells from IMSR mice. Additionally, NK cells from all three mouse strains responded similarly to PMA/ionomycin, indicating that all NK cells retained the capability to respond (Fig. 3F).

There is no known cell surface marker that can indicate whether an NK cell has gone through licensing and achieved responsiveness. However, there is a correlation between licensing and expression of the killer cell lectin-like G1 (KLRG1) receptor, because it is expressed more frequently on licensed NK cells than on unlicensed NK cells (15). NK cells from IMSR mice expressed KLRG1 to a similar extent as did B6 mice (Fig. 3B) (i.e., markedly higher

**FIGURE 2.** Defective missing self triggered resistance to RMA-S tumor outgrowth in IMSR mice. Mice were injected with RMA and RMA-S tumor cells (10⁷ each) s.c. on opposite flanks, and tumor outgrowth was monitored until one or both tumors reached a diameter of 20 mm; the figure shows growth after 14 d. At least six mice from two independent experiments are included in each group. (A) Percentages of B6 and IMSR mice with palpable tumors of RMA-S and RMA. (B) Mean tumor sizes (mm³) for RMA-S and RMA. Error bars denote SD. **p < 0.01. ns, Not significant.
than NK cells in the education/licensing-defective β2m−/− mice). In conclusion, we did not obtain any evidence to support defective MHC-dependent education in IMSR mice.

The IMSR trait is intrinsic to NK cells
The IMSR trait could be due to alteration(s) in the nonhematopoietic environment rather than being intrinsic to NK cells or other bone marrow–derived cells. To address this issue, we generated bone marrow–chimeric mice using both IMSR and B6 mice as donors and recipients, respectively. Rejection of bone marrow–derived cells was observed less efficiently in the B6-to-B6 control (Fig. 4A, 4B). Furthermore, reconstitution of IMSR hosts with B6 bone marrow resulted in rejection of β2m−/− target cells as efficiently as in the B6-to-B6 control (Fig. 4A, 4B).

These data indicated that the missing self recognition defect is manifested in the bone marrow cells and is not due to the host environment. To test whether the defect was intrinsic to NK cells, we constructed mixed hematopoietic chimeras by reconstitution of irradiated B6 or IMSR mice with a mixture of bone marrow from B6 (Ly5.1+) and IMSR (Ly5.2+) mice. At 8–10 wk after reconstitution, splenic NK cells from mice pretreated with the IFN-α/β inducer Tilorone were tested in the single-cell responsiveness assay using YAC-1 tumor targets as stimulus. As depicted in Fig. 4C and 4D, the NK cells of IMSR origin always yielded a poor degranulation response compared with NK cells of B6 origin, even if they had developed in the same host environment. This demonstrated that the defect is intrinsic to IMSR NK cells.

IL-2–activated NK cells from IMSR mice cannot distinguish MHC class I− cells from MHC class I+ cells in vitro
We next investigated whether the defect would remain in vitro following cytokine stimulation, a treatment that can induce functional responses in unlicensed NK cells (46). Although IL-2–activated B6 NK cells killed RMA-S cells significantly better than did RMA cells in an in vitro assay, NK cells from IMSR mice displayed considerably less cytotoxicity and, importantly, did not significantly distinguish RMA-S from RMA cells (Fig. 5A). Thus the deficient recognition of missing self observed in vivo extends to killing of RMA-S tumor cells in vitro and was not overcome by cytokine preactivation.

To further characterize NK cells of IMSR mice, we investigated killing mediated via defined activating receptors. We first tested whether CD1d1−/− NK cells were capable of performing ADCC via the CD16 receptor pathway. We used RMA cells preincubated with Thy-1.2 Ab as target cells. IL-2–activated B6 NK cells exhibited increased killing of Ab-coated target cells, whereas the increase was considerably less impressive with NK cells from IMSR mice (Fig. 5B). We next tested killing of the prototype NK cell target YAC-1 cells, known to be highly sensitive to NK cell–mediated killing mainly via NKG2D (47). IL-2–stimulated B6 NK cells killed YAC-1 target cells significantly better than did NK cells from IMSR mice (Fig. 5C). Altogether, the cytotoxicity data indicated that NK cells from IMSR mice are also profoundly defective in missing self dependent killing in vitro following cytokine activation and that they have at least a partial impairment of NK killing mediated via CD16 and NKG2D receptors.

NK cells from IMSR mice degranulate and produce IFN-γ after Ab stimulation via the activating receptor Ly49D
We next measured the IFN-γ and degranulation (CD107a) responses in NK cells after stimulation through the activating receptors Ly49D and NK1.1. NK cells were preactivated in vivo with Tilorone and then stimulated with plate-bound Abs in vitro. In NK cells from IMSR mice, we observed a normal capacity with

FIGURE 3. IMSR NK cells exhibit no major defects in maturation or MHC-dependent education. Flow cytometry phenotypic analysis of NK cells; data shown are based on events gated on singlet, live, NK1.1+/CD3− lymphocytes. (A) Plots represent Mac-1 versus CD27 expression. (B) Overlay graph shows KLRG1 expression. (C) Bar graph depicts the total frequency of NK1.1+/CD3− cells expressing a certain inhibitory receptor, independently of the expression of other receptors. (D) Bar graph depicts the frequency of NK cells expressing one specific individual, all, or none of five receptors analyzed simultaneously. Spleen NK cells from IMSR and B6 mice were stimulated with plate-bound Ab against anti-NKp46 (E) or PMA/ionomycin (F) for 4 h, and the percentages of CD107a−responding cells of the denoted subset (gated on singlet, live, NK1.1+/CD3− Ly49C/I/NKG2A+, or NK1.1+/CD3− Ly49C/I/NKG2A−) were quantified. Responses of unstimulated cells are subtracted from the data shown. Plots show one representative mouse of each type out of six from three experiments (A) or at least four mice from at least two experiments (B). Bar graphs show a mean of at least four mice of each type from at least two experiments (C, D) or at least three mice from two experiments (E, F). Error bars denote SD. *p < 0.05, **p < 0.01, ***p < 0.001. ns, Not significant.
FIGURE 4. Impaired NK cell function in IMSR mice is intrinsic to NK cells. IMSR and B6 bone marrow were harvested and injected separately (A, B) or in a 1:1 ratio mixture (C, D) into irradiated hosts. B6 mice congenic for the Ly5.1 marker were used as recipients or donors, where appropriate, to allow assessment of chimerism after 8–10 wk. (A and B) In all mice, PBLs consisted of ≈80% donor cells. After 8–10 wk, CFSE-labeled β2m−/− and B6 spleen cells were coinjected i.v. into untreated (A) or anti–NK1.1-treated (B) chimeras or mice. The graphs show the ratio of recovered β2m+/−/B6 spleen cells, corrected for percentages in the cell inoculate, at 48 h after injection. Spleen NK cells from Tilorone-pretreated B6, IMSR and β2m−/− mice, as well as from mixed bone marrow chimeras in B6 or IMSR hosts were stimulated with YAC-1 tumor cells (C) or PMA/ionomycin (D) for 4 h, and the percentages of CD107a-responding cells were quantified by flow cytometry. Responses of unstimulated cells are subtracted from the data shown. Plots show one representative experiment out of three with two to four mice/group (A, B) or six individual mice from three independent experiments (C, D). (A and B) *p < 0.05, **p < 0.01, ***p < 0.001, versus B6→B6. (C) p = 0.007, B6 versus IMSR. p = 0.01, B6 versus β2m−/−. p = 0.01, B6 versus IMSR NK cells in either B6 or IMSR host.

Discussion

We explored an unexpected defect in NK cell–mediated rejection of cells representing “missing self” in a previously described CD1d−/− mice. We reported previously that rejection of allogeneic BALB/c cells by B6 NK cells is dependent on intact function of the signaling molecule DAP12, whereas rejection of MHC-deficient cells is not (25). This most likely reflects the action of the DAP12-dependent activating receptor Ly49D, for which H-2Dd (present on BALB/c cells) is a strong ligand. There was the expected in vivo rejection of BALB/c cells in IMSR mice (Fig. 7A). This result, together with the in vitro data presented in Fig. 6, indicates that activation via Ly49D/DAP12 is intact in IMSR NK cells. Our in vitro cytotoxicity results suggested an impaired activation via NKG2D in CD1d−/− NK cells (Fig. 5C), which prompted us to test NKG2D-dependent NK cell rejection of RMA–Rae1−γ tumor cells in vivo (33). In accordance with the in vitro results, IMSR mice displayed a significantly reduced in vivo rejection of RMA–Rae1−γ cells compared with B6 mice (Fig. 7B).

NK cells of IMSR mice show normal in vivo rejection capacity via Ly49D/DAP12 but severely impaired NKG2D-induced rejection

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independent experiment. *p < 0.05, **p < 0.01. ns, Not significant.

Bone marrow rejection capacity (49). The difference between these results and the current study, in which 129S6 mice did not reject β2m-deficient or TAP-deficient spleen cells, may be due to differences in the assays used (Fig. 1E, data not shown). Although 129/J mice have a nonfunctional DAP12-signaling pathway (50), the DAP12-dependent pathway appeared normal in IMSR mice. 129S6 mice differ in their Ly49 gene cluster compared with B6 mice (51), but IMSR mice express NK1.1 and B6 Ly49 receptors, indicating that the NK cell gene complex in these mice is derived from B6. Therefore, it appears likely that a mutation has occurred during the generation or the backcrossing of the mutant mice. In experiments designed to delete the Zfa gene, only expressed in adult testis, an unexpected brain-behavior phenotype was observed in the resulting mice, and this was due to an unrelated single gene mutation in the embryonic stem cells used (38).

With regard to the nature of the functional NK cell defect in IMSR mice, one attractive explanation is impairment of the MHC-dependent education process, similar to mice with targeted deletion of the Zfa gene (36, 37). The NK cells in IMSR mice seemed to undergo normal maturation. All four stages in the developmental program (40, 42) were represented, and they expressed a number of activating receptors. Further studies of NK cell phenotypic markers did not support a defect in education, similar to β2m−/− mice, as an explanation for the phenotype of the IMSR mice. One hallmark of MHC class I–dependent education is skewing of the inhibitory MHC receptor repertoire (13, 14). This skewing was even more accentuated in IMSR mice than in B6 mice, as compared to β2m−/− mice, indicating that host MHC molecules had indeed imposed an imprint on the repertoire. The NK population of MHC class I–deficient mice has fewer KLRG1+ cells than that of MHC class I–expressing mice; within the latter, the NK cells expressing self-receptors are also more likely to express KLRG1 than are those cells that do not have such receptors (15). In contrast, NK cells from IMSR mice expressed KLRG1 with a similar frequency and geometric mean fluorescence intensity as did B6 mice.

The most widely used criterion for education of NK cells is the functional responsiveness (“licensing”) assay. The NK cells of the subset presumed to be licensed in IMSR mice (CIN+ NK cells expressing receptors for MHC class I molecules of the H-2b haplotype) showed the same reduced responsiveness as the corresponding subset in β2m−/− mice. However, CIN+ NK cells of IMSR mice were still significantly more responsive than was the CIN− subset devoid of self-receptors, in contrast to what was observed in β2m−/− mice. This indicates that NK cells in IMSR mice can sense and be influenced by MHC class I molecules during education, although the responsiveness to anti-NKp46 stimulation of all subsets is impaired.

Altogether, the studies of NK cell phenotypic markers and in vitro responsiveness did not support the notion that IMSR mice have a global education defect similar to that in β2m−/− mice. This
implied the possibility of a broader functional defect; therefore, we investigated pathways for other cognate cytotoxic NK interactions. The Ly49D pathway, acting via DAP12, appeared normal, as judged by in vivo rejection of ligand-expressing cells, as well as responses to in vitro stimulation of Ly49D. Importantly, the latter argues against any global defect in cytokine or degranulation responses in NK cells of IMSR mice. However, in vivo and in vitro cytotoxicity triggered via the NKG2D pathway was impaired, as evaluated by NK cell–dependent in vivo rejection of RMA tumor cells expressing the NKG2D ligand Rae-1γ, as well as in vitro cytotoxicity against YAC-1 targets. The CD16-dependent ADCC activity was also reduced, although not totally abolished. These results indicate that NK function triggered via at least two different receptors is affected in IMSR mice. We considered the possibility that IMSR mice may have increased levels of NKG2D ligands, inducing downregulation of NKG2D expression and functional impairment of NK cells, similar to the situation in Rae-1γ-transgenic mice (52). However, although NKG2D cell surface levels were slightly reduced on IMSR NK cells, IMSR spleen cells were not rejected by NK cells in B6 mice, arguing against a functionally significant increase in NKG2D ligands. 

Taken together, these results can be interpreted as a defect that mainly influences the killing pathway used to sense “missing self” and the NKG2D-signaling pathway, while leaving other NK cell functions normal or slightly impaired. The impaired function downstream of NKG2D stimulation might simply be explained by the lower cell surface expression. However, this appears less likely because β2m−/− mice have even lower NKG2D expression but still reject tumor cells expressing ligands for NKG2D (RMA–Rae-1γ) in vivo, almost as well as B6 mice (data not included). Therefore, one possible interpretation of our results is that the defect lies downstream of the critical inhibitory MHC class I interaction during education. This could lead to NK cells receiving the signal needed for skewing of the NK cell repertoire and to achieve responsiveness (become licensed), but something is lacking for the response at the effector cell level to “missing self,” as well as in NKG2D-triggered function. Another possibility is that the IMSR NK cells instead have a genetic alteration that leads to increased activating signaling during education. This would lead to licensing and skewing of the Ly49 repertoire, but according to current ideas on NK cell education, also to a higher activation threshold to maintain tolerance to normal autologous cells. This higher threshold could also explain why we see reduced or impaired function through other activating receptors. In this context, it should be noted that there is evidence for this principle based on the reverse situation. In some strains with known defects in activating NK receptors, the NK cells actually display a lower activation threshold (53, 54). 

Although several activation receptors and pathways for NK cells are known, none has been able to fully explain how NK cells are activated when they sense “missing self” of normal cells or tumor cells without strong expression of stress or virus-induced ligands. Several gene-targeted mice are known to have functional NK cell defects, and it is interesting to compare these with the defects reported in this study. DAP12-deficient B6 mice fail to kill allogeneic BALB/c cells while missing self rejection is intact (25, 55), which is the opposite of what we observed in IMSR mice. Vav1−/− NK cells show no or only weak killing of various tumor cells, including targets with low MHC class I expression, such as RMA-S, as well as YAC-1 and other targets recognized via NKG2D/DAP10 (56–58). Interestingly, killing via DAP12 functions normally in these mice, just as in the mice that we studied. In addition, Vav1-deficient mice have a severe defect in T cell development (59), generating reduced numbers of peripheral T cells, whereas the defective mice used in this study show no T cell abnormalities apart from NKT cell deficiency (17, 19). Vav2- and Vav3-deficient mice, which also have normal T cell development, show severe defects in ITAM signaling, resulting in defects in killing via Ly49H/DAP12 and ADCC (56, 59). Fyn−/− mice display low killing of RMA-S and β2m−/− Con A blasts (60). The functional deficiency observed in the IMSR NK cells is most similar to the defect displayed by Fyn−/− mice, whereas they differ in one or more respects from the other gene-targeted mice described above. Western blot analysis confirmed the presence of Fyn protein in IMSR mice (data not shown), but we cannot exclude mutations that affect the function of this molecule. 

The Genista mice identified in a mutagenesis screen displayed a reduced killing against β2m-deficient targets in vivo as well as a reduced IFN-γ response after in vitro stimulation through the activating receptors NK1.1, Nkp46, NKG2D, and Ly49D (61). These mice have a maturation arrest leading to fewer fully mature NK cells caused by lack of neutrophils. NK cells from “so-called” NKC-knockdown mice with severely impaired Ly49 receptor expression, showed a reduced killing of β2m−/− targets but normal CD16 (ADCC) and NKG2D pathways (62). SAP-knockout and SAP R78A (binding site for Fyn) mutant NK cells show a dramatic decrease in killing of RMA-S, YAC-1, and MHC class I–Con A (63). These data demonstrate that 2B4 signaling through the SAP–Fyn pathway is important for the killing of hematopoietic cells, but there was no selective defect in missing self recognition. Two recently described mutant mouse strains, Ace and Chip, displayed defective rejection of β2m−/− spleen cells. The Chip mutation was mapped to one of the immune-receptor tyrosine-based switch motifs of 2B4 (64). However, most other NK cell functions have not been characterized in these strains. 

The conclusion from this comparative analysis is that there are several mouse strains with NK functional defects; although some of them include a severely impaired missing self recognition, none of them resulted in a phenotype identical to the one that we described in this article. Further studies of these mice, based either on candidate genes/molecules or on a more unbiased global (genomic or transcriptomic) approach will be required to map it. Meanwhile, one lesson from this study is to apply caution when interpreting results generated from gene-targeted mice: the observed phenotype may be caused by other genes than the one intended. More importantly, the mice described in this study may be useful to identify genes and proteins involved in the response to missing self.

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