Ly49 Family Receptors Are Required for Cancer Imunosurveillance Mediated by Natural Killer Cells

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Abstract

According to the missing-self hypothesis, natural killer (NK) cells survey for target cells that lack MHC-I molecules. The Ly49 receptor family recognizes loss of MHC-I and is critical for educating NK cells, conferring the ability to eliminate transformed or infected cells. In this study, we evaluated their requirement in innate immune surveillance of cancer cells using genetically manipulated mice with attenuated expression of Ly49 receptors (NKC×D) in several models of carcinoma and metastasis. We found that NKC×D mice exhibited uncontrolled tumor growth and metastases. Expression of two MHC-I alleles, H-2Kb and H-2Dd, was decreased in tumors from NKC×D mice in support of the likelihood of NK-mediated tumor immunoediting. These tumor cells exhibited directed alterations to their cell surface expression in response to the genetically altered immune environment to evade host recognition. Immunoediting in NKC×D mice was restricted to MHC-I molecules, which are ligands for Ly49 receptors, while expression of Rae-1 and Mult1, ligands for another NK cell receptor, NKG2D, were unaffected. Restoring NK cell education in NKC×D mice with a transgene for the inhibitory self-MHC-I receptor Ly49I restored suppression of cancer onset and growth. Interestingly, immune surveillance mediated by activating Ly49 receptors remained intact in NKC×D mice, as demonstrated by the ability to stimulate the NKG2D receptor with tumor cells or splenocytes expressing Rae-1. Together, our results genetically establish the integral role of Ly49 in NK cell-mediated control of carcinogenesis through MHC-I-dependent missing-self recognition. Cancer Res; 74(14); 3684–94. ©2014 AACR.

Introduction

Natural killer (NK) cells were originally identified by their unique killing of tumor cells without prior sensitization, which differed from the defined functions of T and B lymphocytes (1, 2). Rare individuals with reduced NK cell numbers, cytotoxicity, and/or cytokine production are characteristically more susceptible to certain viral infections, highlighting the necessity of NK cells in immunity (3). Low NK cell activity in cancer-diagnosed individuals is associated with poor prognosis, and those with advanced stage cancer often possess minimally cytotoxic NK cells (4).

NK cells recognize tumor targets by the action of various activating and inhibitory receptors on their surface (5). Members of the Ly49 receptor family, the murine functional homologue of the human killer-cell Ig-like receptor (KIR) family, can be either activating or inhibitory, and interact with MHC-I molecules (6). NK cells detect aberrant cells with reduced surface expression of MHC-I through the Ly49 receptors: successful engagement of MHC-I transmits an inhibitory signal, whereas an absence of MHC-I and/or the presence of activating ligands induces killing (7–9). Accordingly, target cells can express ligands that bind to a variety of activating and inhibitory receptors on NK cells, and the interplay between inhibitory and activating signals determines the NK cell response (5). NK cell functionality depends on earlier exposure to MHC-I, as proposed by the education hypothesis, in which a self-specific Ly49 receptor must interact with self-MHC-I in order for the NK cell to become functional (10, 11).

Cancer immunoediting describes how the immune system possesses a dual role in protecting the host, as well as in shaping the tumor environment (12). Tumors evade immune recognition through various mechanisms, such as alteration of the tumor microenvironment or changes in MHC-I expression. Reduced or complete loss of MHC-I expression has been seen in a wide array of human cancers, including colorectal (13, 14), breast (13, 15), bladder (16), ovarian (17), and cervical carcinoma (18), suggesting the presence of immunoediting.

Our laboratory has generated a mutant mouse strain in which expression levels of the Ly49 receptors—as well as the NKG2, CD94, and KLRI receptors—in the mouse natural killer gene complex (NKC) are downregulated (19). NKC×D mice

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provide a genetically based approach to study the importance of Ly49 receptors in cancer immunosurveillance. As previously described (19), NKCG\(^{KD}\) mice exhibit silenced Ly49 expression on approximately 80% of NK cells but with normal cell numbers and proportions, as well as unaffected expression levels of cell surface markers encoded outside the NKC. In this study, we show that Ly49 plays a major role in NK cell cancer immunosurveillance.

Materials and Methods

**Mice**

C57BL/6 (B6), B2m\(^{-/-}\), and E\(\mu\)-myc transgenic [strain: B6. Cg-Tg(IghMyc)22Bri/J] mice were purchased from The Jackson Laboratory. Ly49ITg mice were kindly provided by Dr. Michael Bennett (University of Texas, Dallas, TX) and backcrossed to the C57BL/6 background for at least six generations. B6. Ly49\(^{129}\), B6.NKCG\(^{KD}\) (Klra15\(^{m1.LaPma}\)), and NKCG\(^{KD}\)-Ly49ITg mice were previously described (19–21). Because of linkage disequilibrium, B6.NKCG\(^{KD}\) mice possess a 129-derived NKC. Therefore, the B6.Ly49\(^{129}\) congenic mouse strain, which harbors a 129-derived NK on the B6 background, serves as the genetically correct wild-type (WT) control. For brevity, B6.NKCG\(^{KD}\) and B6.Ly49\(^{129}\) are referred to as NKCKD and WT, respectively. Genotyping for the E\(\mu\)-myc transgene was performed by PCR using primers: forward 5\('\)CTGGGTCACTCACATTTAAC and reverse 5\('\)GTATATCAGTCACCGCAGGT to attain a product size of approximately 500 bp. All \(in\) \(vivo\) experiments utilized mice between 6 and 9 weeks of age. Breeding and manipulations performed on animals were in accordance with and approved by the University of Ottawa Animal Ethics Committee (Ottawa, Ontario, Canada).

**Cells, antibodies, and flow cytometry**

B16F10.LacZ was kindly provided by Dr. Rebecca Auer (Ottawa Hospital Research Institute, Ottawa, Ontario, Canada). All cell lines have been tested and authenticated. MHC-I staining of all tumors cells are conducted every 6 to 12 months. All cell lines were confirmed to be mycoplasma free and showed appropriate pathologic morphology. B16F10. LacZ was stably transfected with 2\(\text{Kb}\)-pEF6 and/or 2\(\text{H}-2\text{Db}\)-pEF6 expression vectors using Lipofectamine 2000 (Invitrogen), as per manufacturer’s guidelines, and selected in 4.5 or 9 \(\mu\)g/mL G418. Antibody staining was performed using anti-E\(\mu\)-myc (AF6-88.5.5.3; eBioscience), anti-H-2\(\text{Db}\) (KH95; BD Biosciences), anti-MULT1 (5D10; eBioscience), and anti-pan Rae-1 (186107; R&D Systems). Mean fluorescence intensity (MFI) for H-2\(\text{Kb}\), H-2\(\text{Db}\), Rae-1, and Mult1 expression in the spleen and lymph nodes of E\(\mu\)-myc\(^{18}\) mice was standardized to levels in WT control mice.

**Flank tumor model**

WT, NKCG\(^{KD}\), and NKCG\(^{KD}\)-Ly49ITg\(^{16}\) mice were challenged on the right flank by subcutaneous injection of 5 \(\times\) 10\(^5\) or 1 \(\times\) 10\(^6\) RMA, RMA-S, B16F10.LacZ, B16F10.H-2\(\text{Kb}\)-H-2\(\text{Db}\), or B lymphoma cells in PBS. Mice were monitored daily for tumor development. Date of tumor appearance was recorded from when the tumor is first palpable. Tumor size was determined using an electronic caliper (Marathon) to measure the length and width. Mice were euthanized upon tumor length or width exceeding 12 mm.

**Experimental metastasis model**

WT, NKCG\(^{KD}\), and NKCG\(^{KD}\)-Ly49ITg\(^{16}\) mice were intravenously challenged with 1 \(\times\) 10\(^5\) or 2 \(\times\) 10\(^7\) B16F10.LacZ cells in PBS. For experiments with poly(I:C) stimulation, 100 \(\mu\)g of poly(I:C) in PBS were injected intravenously 24 hours before tumor injection. Animals were euthanized 5 or 14 days following tumor inoculation and lungs were stained with X-gal (Bioshop) as described previously (22). Representative tumor burden was determined on the largest lung (left) lobe, by the number of surface visible metastases. For histology, fresh-frozen lungs embedded in OCT were sectioned at 8 \(\mu\)m thickness and hematoxylin and eosin (H&E) stained.

**Carcinogen-induced model**

WT and NKCG\(^{KD}\) mice were injected subcutaneously into the hind flank with 100 \(\mu\)g methylcholanthrene dissolved in corn oil. Mice were monitored weekly for tumor development and scored as tumor positive upon tumor width exceeding 5 mm and exhibiting progressive growth. Tumor size was determined using an electronic caliper (Marathon) to measure length and width. Sarcoma growth rate (mm\(^2\)/day) was calculated from tumor area with respect to number of days post-MCA injection.

**Spontaneous lymphoma model**

E\(\mu\)-myc\(^{18}\) mice were monitored daily, and euthanized upon exhibiting progressively swollen lymph nodes upon palpation or respiratory distress. The spleen and lymph nodes were dissociated to attain a single cell suspension. Tumor cells were incubated with Fc block (anti-CD16/CD32), then stained with anti-IgM (eB121-15F9; eBioscience), anti-B220 (RA3-6B2; eBioscience), anti-H-2\(\text{Kb}\) (AF6-88.5.5.3; eBioscience), anti-H-2\(\text{Db}\) (KH95; BD Biosciences), anti-MULT1 (5D10; eBioscience), and anti-pan Rae-1 (186107; R&D Systems). Mean fluorescence intensity (MFI) for H-2\(\text{Kb}\), H-2\(\text{Db}\), Rae-1, and Mult1 expression in the spleen and lymph nodes of E\(\mu\)-myc\(^{18}\) mice was standardized to levels in WT control mice.

**In vitro and in vivo NK cell assays**

Adherent lymphokine (IL2)-activated killer (ALAK) cells were grown in culture and used as effector cells in \[^{3}\text{H}]\text{Thymidine incorporation and IFN-\(\gamma\) release cytotoxicity assays, as previously described (23). Intracellular staining for IFN-\(\gamma\) was performed as previously described (19). NKp46\(^{+}\)TCR\(^{+}\) cells were analyzed for IFN-\(\gamma\) by flow cytometry. Splenocyte and tumor cell rejection assays were performed as previously described (19, 21). Alternatively, MHC-I–expressing, MHC-I–deficient, and MHC-I–deficient Rae-1–expressing tumor cells were differentially labeled with 0.5, 3, and 8 \(\mu\)mol/L carboxyfluorescein diacetate succinimidyl ester (CFSE), and combined at a 1:1:1 ratio for a total of 2 \(\times\) 10\(^6\) cells. Peritoneal cells were harvested 16 hours following injection and analyzed by flow cytometry for the presence of CFSE-labeled tumor cells.
Statistical analysis

Statistical comparisons were performed on Kaplan–Meier plots depicting tumor onset using the log-rank test with Prism (GraphPad Software). For the remainder, statistical significance was determined by a two-tailed t test with cutoff P value of 0.05. *, P < 0.05; **, P < 0.01; ***, P < 0.001; n.s., not significant.

Results

Accelerated tumor cell-induced flank tumor growth in NKCKD mice

It was of interest to determine whether the lack of MHC-I immunosurveillance by NK cells in NKCKD mice affects long-term tumor control. Onset of MHC-I–deficient RMA-S–induced flank tumor formation was detected at least 3 days earlier in NKCKD mice than WT mice (Fig. 1A and C). Tumor incidence was more prevalent in the NKCKD group, with 92% (dose of $5 \times 10^5$ cells/mouse) and 100% (dose of $1 \times 10^6$ cells/mouse) developing tumors after 30 days, compared with 65% and 85%, respectively, in the WT group (Fig. 1A and C). Tumor size at comparable dates was larger in NKCKD mice (Fig. 1B and D); the overall growth rate of the tumors in NKCKD mice is accelerated. Tumor development and growth in NKCKD mice is comparable with that of $B2m^{−/−}$ mice (Fig. 1C and D), which also possess hyporesponsive NK cells.

$B16F10$ are highly aggressive murine melanoma cells that lack MHC-I expression (24, 25). Subcutaneous injection of $1 \times 10^6 B16F10.LacZ$ cells promoted solid tumor formation in both NKCKD and WT mice. At day 6, 7% of WT mice developed tumors in contrast with 53% of NKCKD mice (Supplementary Fig. S1A). Tumor size at comparable dates was larger in NKCKD mice (Supplementary Fig. S1B). This defect in NKCKD tumor control is MHC-I dependent, as flank tumor challenge outcomes with MHC-I–expressing RMA, and transfected $B16F10$ expressing both MHC-I molecules H-2Kb and H-2Dd were similar between NKCKD and WT mice (Fig. 1E and F and Supplementary Fig. S1C and S1D).

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Figure 1. Accelerated tumor cell-induced flank tumor growth in NKCKD mice. A and B, formation of solid flank tumors following subcutaneous injection of $1 \times 10^6$ MHC-I–deficient RMA-S. WT n = 13. NKCKD n = 11. C and D, $5 \times 10^5$ RMA-S. WT n = 17. NKCKD n = 13. $B2m^{−/−}$ n = 5. E and F, $5 \times 10^5$ MHC-I–expressing RMA. WT n = 16. NKCKD n = 16. Date of tumor appearance (A, C, and E) and mean tumor size ± SEM (B, D, and F). Data are pooled from three independent experiments.
Accelerated melanoma cell-induced metastatic growth in NK CKD mice

The B16F10.LacZ cells were also used in an experimental pulmonary metastases model to determine the ability of NK CKD mice to control metastatic growth. The number of metastases was greater in NK CKD mice compared with WT mice regardless of incubation time and cell dose (Fig. 2A and B). Histologically, NK CKD mice exhibited increased metastatic nodules in the lung parenchyma, and loss of morpho-functional structures such as open and wide pulmonary alveoli, instead having alveoli obstructed with hyperemic areas (Fig. 2C). Leukocyte infiltration can also be noted more frequently in NK CKD mouse lungs (Fig. 2C). These data suggest an inability of NK CKD mice to eliminate B16F10.LacZ tumor cells before they are able to seed in the lungs.

To demonstrate that the observed hyporesponsiveness is not due to a cytotoxic defect, the NK cells in WT and NK CKD mice were directly activated by poly(I:C) injection. The lungs of untreated WT mice contained a mean of 45 metastases compared with a mean of 11 in the poly(I:C)-treated group (Fig. 2D).

Comparatively, for NK CKD mice, metastases were also significantly reduced from a mean of 118 to 39 with poly(I:C) stimulation (Fig. 2D). The efficacy of poly(I:C) treatment in both WT and NK CKD mice suggests that NK cells from NK CKD mice are still responsive if activated in a Ly49-independent manner.

Defective recognition of melanoma cell-induced experimental pulmonary metastases is MHC-I dependent

To determine whether defective rejection of the parental, MHC-I-negative B16F10.LacZ lung metastases by NK CKD mice is due to defective missing-self recognition, the cells were stably transfected to express two MHC-I alleles, H-2Kb and H-2Db, either individually or together (Fig. 3A). Transfection of the cells did not affect expression of the NKG2D ligands, Rae-1 and Mult1 (Fig. 3B). Expression of a single MHC-I allele was insufficient to significantly protect these tumors from WT NK control compared with the NK CKD (Fig. 3C and E). However, expression of both H-2Kb and H-2Db (Fig. 3G) resulted in similar metastatic numbers in the lungs.
Figure 3. Defective recognition of melanoma cell-induced experimental pulmonary metastases is MHC-I dependent. Pulmonary metastases following intravenous injection of MHC-I-deficient B16F10.LacZ transfected with H-2Kb, H-2Db, or both. Flow cytometry results showing H-2Kb and H-2Db (A), and Rae-1 and Mult1 expression in transfected cells compared with parental B16F10.LacZ (B). Quantification of metastases 5 days following injection of $2 \times 10^5$ B16F10.LacZ cells expressing H-2Kb (C and D), H-2Db (E and F), or H-2Kb (G and H), and H-2Db with poly(I:C) stimulation (D, F, and H) of recipient mice before tumor injection. Horizontal lines represent mean. Each symbol represents a single mouse. Data are pooled from two to three independent experiments.
of WT and NKCKD mice, suggesting a loss of NK-mediated control due to MHC-I inhibition. These results suggest that NK cell recognition and elimination of B16F10.LacZ tumor cells are MHC-I dependent, because upon expression of both H-2Kb and H-2Db, metastatic numbers in WT and NKCKD mice are comparable. Similarly to the parental B16F10, poly (I:C) stimulation before tumor challenge greatly reduced the number of metastases in both WT and NKCKD compared with untreated (Fig. 3D, F, and H). These results support that the hyporesponsive nature of NK cells from NKCKD mice is not due to a more generalized defect because stimulation restores function.

**Inhibitory self-MHC-I–specific Ly49I restores NK cell cancer immunosurveillance in NKCKD mice**

To test the hypothesis that NK cells from NKCKD mice are uneducated and hyporesponsive, and therefore unable to control tumor and metastatic growth, a transgene for Ly49I was introduced into NKCKD mice. Introduction of the Ly49I transgene significantly delayed RMA-S–induced flank tumor onset in NKCKD mice, approaching WT levels (Fig. 4A). WT and NKCKD-Ly49ITg mice presented with similar late period tumor growth, in contrast with NKCKD mice (Fig. 4B). Similar results were also seen with the experimental lung metastases, wherein the number of metastases was reduced in NKCKD-Ly49ITg mice (mean of 24) compared with NKCKD (mean of 89; Fig. 4C). These observed differences in the numbers of metastases suggest that Ly49I-mediated education is necessary to engender an NK cell response against tumors.

**Accelerated onset and growth of MCA-induced sarcoma in NKCKD mice**

Previous studies show a role for NK cells in controlling methylcholanthrene (MCA)-induced tumors (26–28). It was of interest to determine whether NKCKD mice with hyporesponsive NK cells would be more susceptible to MCA-induced tumors. NKCKD mice exhibited earlier sarcoma onset than WT; by day 85, all NKCKD mice had developed tumors, compared with approximately 50% of WT mice (Fig. 5A). Tumor growth was accelerated in NKCKD mice (Fig. 5B), with a significantly greater relative growth rate in NKCKD (mean of 1.97) than WT mice (mean of 0.65; Fig. 5C). This suggests that control of MCA-induced tumors is impaired in NKCKD mice, and that the defect is not restricted to MHC-I–deficient tumor cell recognition.

**Earlier onset of B-cell lymphoma and evidence for MHC-I–directed tumor immunoeediting in NKCKD mice**

Eμ–myc transgenic mice possess the myc oncogene coupled to the immunoglobulin μ enhancer, resulting in spontaneous B-cell lymphoma development, a lethal malignancy starting at 6 weeks of age (29). Considering the pathologic importance of this cancer, especially with parallels to human Burkitt lymphoma, NKCKD.Eμ–mycITg and WT.Eμ–mycITg mice were generated to study the effect of silenced Ly49 expression. NKCKD mice developed lymphomas significantly earlier than WT mice, and exhibited reduced tumor control; following 100 days of age, approximately 60% of WT mice were lymphoma positive, compared with approximately 90% of NKCKD mice (Fig. 6A). Although both groups possessed mice exhibiting delayed lymphoma onset, this lapse was more prominent in WT mice. Because NKCKD mice lack MHC-I–educated NK cells, it was of interest to determine whether this would lead to cancer immunoeediting. To test this hypothesis, immature (IgM+) and mature (IgM−) B lymphoma cells from the spleen and lymph nodes were analyzed for expression of the MHC-I molecules, H-2Kb and H-2Db, as well as the NKG2D ligands, Rae-1 and Mult1. NKCKD.Eμ–mycITg mice exhibited reduced MHC-I expression in both the mature and immature B-cell populations compared with WT mice with and without the Eμ–mycITg (Fig. 6B–H).
B lymphoma cells were isolated from a NKc^KD,El-myc^Tg mouse that exhibited reduced MHC-I expression and low-level expression of NKG2D ligands (Fig. 6f). This decrease in MHC-I expression rendered the tumor cells more susceptible to rejection by WT mice compared with NKc^KD mice (Fig. 6f). Furthermore, WT mice were able to better control the growth of B lymphoma cell-induced flank tumors (Fig. 6k). Overall, our observation of decreased MHC-I expression in NKc^KD,El-myc^Tg, but not NKG2D ligand expression, suggests that silenced Ly49 expression in NKc^KD mice leads to MHC-I-directed tumor immunoeediting.

**Signaling through NKG2D is unaffected in NKc^KD mice**

To study the effects of activating ligand expression on MHC-I--expressing and -deficient target cells in NKc^KD mice, the rejection of splenocytes from B6 or B2m^−/− mice transgenically expressing Rae-1 was studied. Activation through NKG2D is intact in NKc^KD mice because rejection of splenocytes from Rae-1e^Tg mice were similar between WT and NKc^KD (Fig. 7a). Although there is lower rejection of B2m^−/− Rae-1e^Tg splenocytes by NKc^KD mice than WT mice, this can be attributed to the loss of MHC-I expression on these cells, as seen in the rejection of B2m^−/− Rae-1e^Tg (Fig. 7b). Similarly, in vivo rejection of RMA and RMA-S ectopically expressing Rae-1^β by WT and NKc^KD mice showed parallel results. Rejection of both Rae-1^β^high and Rae-1^β^low cells were similar (Fig. 7c–f), suggesting that NKG2D signaling is fully intact in NKc^KD mice. Although rejection of RMA-S-Rae-1^β was lower in NKc^KD mice (Fig. 7f), this is attributed to the MHC-I deficiency of these cells, and is evidenced by the differential rejection of RMA-S by WT and NKc^KD mice. These results suggest that although NKc^KD mice exhibit defective missing-self recognition, signaling through NKG2D, independent of MHC-I expression, is able to induce NK cell killing.

**Decreased cytotoxicity but not IFNγ production in NKc^KD mice**

It was of interest to determine whether loss of Ly49 expression affects cytotoxicity or IFNγ production by NK cells. NK cells from NKc^KD mice exhibited normal production of IFNγ following stimulation with PMA+ionomycin (Supplementary Fig. S3a), as well as various tumor cells: RMA, RMA-Rae-1^β^low, RMA-Rae-1^β^high, RMA-S, and RMA-S-Rae-1^β (Supplementary Fig. S3b), suggesting that loss of Ly49 does not affect IFNγ production. A direct comparison of the killing between WT and NKc^KD mice was studied through an in vitro cytotoxicity assay. The direct killing of the MHC-I--deficient tumor cells, RMA-S (Supplementary Fig. S3c) and RMA-S-Rae-1^β (Supplementary Fig. S3d), by NKc^KD-derived ALAK cells is impaired, while killing of MHC-I--expressing RMA and RMA-Rae-1^β^high and RMA-Rae-1^β^low is comparable between WT and NKc^KD, suggesting a cytotoxicity defect with regards to missing-self recognition. Although there is observed killing of RMA-S by NKc^KD cells, which is not expected, the levels are lower than that of WT and may be attributable to residual Ly49 expression on approximately 20% of NK cells in NKc^KD mice. Overall, these results suggest that loss of Ly49 affects the cytotoxicity of the NK cells in response to missing-self signals.
Figure 6. Earlier onset of B-cell lymphoma and MHC-I-directed tumor immunoediting in NKC<sup>KD</sup> mice. A, B-cell lymphoma progression in NKC<sup>KD,Eµ-myc</sup> (<em>n</em> = 35) and WT,Eµ-myc (<em>n</em> = 24) mice. B–H, ex vivo analysis of spleen and lymph node cell suspensions isolated from NKC<sup>KD,Eµ-myc</sup> (<em>n</em> = 30) and WT,Eµ-myc (<em>n</em> = 21) mice. B cells (B220<sup>+</sup>) were analyzed separately as immature (IgM/C<sub>0</sub><sup>+</sup>) or mature (IgM<sup>+</sup>) for expression of H-2K<sup>b</sup> and H-2D<sup>b</sup> by flow cytometry. MFI was standardized to that of B cells from non-Eµ-myc transgenic mice (dotted line). I, MHC-I and NKG2D ligand expression in B lymphoma cells cultured from a NKC<sup>KD,Eµ-myc</sup> (black histogram) compared with B cells from a non-Eµ-myc<sup>+</sup> mouse (gray histogram). J, in vivo rejection of B lymphoma cells originating from an NKC<sup>KD,Eµ-myc</sup> relative to the MHC-I-expressing RMA. Each symbol represents a single mouse. Horizontal line represents mean. Data are pooled from two independent experiments. K, tumor development following subcutaneous injection of 5 × 10<sup>5</sup> B lymphoma cells from an NKC<sup>KD,Eµ-myc</sup> injected into the hind flank of WT (<em>n</em> = 19) and NKC<sup>KD</sup> (<em>n</em> = 18) mice. Mean ± SEM. Data are pooled from four independent experiments.
Discussion

NK cells are known to play an important role in the in vivo recognition and control of tumors (30). Our genetic NK CKD mouse model provides long-term Ly49 downregulation, allowing a study of NK cell targeting in cancer immunosurveillance. Flank tumor growth induced with MHC-I–deficient tumor cells is accelerated in NK CKD mice; however, those induced with MHC-I–expressing tumor cells were comparable between WT and NK CKD mice. This suggests the uncontrolled tumor growth in NK CKD mice is a result of defective missing-self recognition. In the pulmonary metastasis model, there are a greater number of metastases on the lungs of NK CKD mice than WT mice. However, the number of metastases induced by MHC-I–expressing transfectants in WT mice approached NK CKD levels, because the functional NK cells are now inhibited by MHC-I on the tumor cells. Flank tumors induced with the carcinogen, MCA, exhibited accelerated onset and growth in NK CKD mice, in accordance with previous work demonstrating control of MCA-induced tumors by NK cells (26, 27). Finally, in the oncogene-driven B-cell lymphoma model, we observed that mice on the NK CKD background develop lymphoma earlier than their WT counterparts also possessing the oncogene. The defect in NK CKD mice is due to reduced cytotoxicity of the NK cells because decreased killing is observed in response to missing-self. Signaling through NKG2D is fully intact in NK CKD mice; in vitro killing and in vivo rejection of RMA Rae-1ε Tg are similar between WT and NK KD mice. The observed differential killing and rejection of RMA-S-Rae-1β relative to RMA. Each dot represents a single mouse. Mean ± SEM. Data are pooled from two to four independent experiments.

Figure 7. Signaling through NKG2D is unaffected in NK KD mice. In vivo rejection of CFSE-labeled splenocytes from Rae-1ε Tg mice (A) and B2m−/− and B2m−/− Rae-1ε Tg mice (B) relative to B6 mice. C, Rae-1 expression levels in parental RMA cells, and RMA cells ectopically expressing Rae-1ε high and Rae-1β high. In vivo rejection of MHC-I-expressing RMA Rae-1ε high (D) and Rae-1β high (E) relative to RMA. F, in vivo rejection of MHC-I-deficient RMA-S and RMA-S-Rae-1β relative to RMA. Each dot represents a single mouse. Mean ± SEM. Data are pooled from two to four independent experiments.
results in the acquisition of NK cell function (10, 11). In our studies, following Ly49ITg restoration in NKCKD mice, overall increased tumor control was observed, confirming that in vivo education is mediated by inhibitory self-specific Ly49. As well, the ability of Ly49 to rescue hyporesponsive NK cells supports the phenomenon observed is the result of a loss of Ly49 expression and not due to the partial silencing of two other adjacent gene families, encoding the CD94/NKG2 and KLRI/E molecules (19). Although flank tumor size in the NKCKD-Ly49ITg mice display slightly faster tumor growth and greater number of metastases, possibly due to the absence of other self-specific Ly49 receptors. In a 129-strain Ly49 repertoire, Ly49I binds to H-2Kb, and Ly49V, G2, and O bind to H-2Db (31), and so these three other Ly49 receptors may also be important for NK cell education. As well, the contribution of other non-self Ly49s remains unclear; there is evidence that Ly49A recognizes the nonclassical MHC-I molecule H2-M3 and mediates NK cell education (32, 33). H2-M3-deficient mice exhibit defective cytotoxicity and tumor control (32, 33).

MHC-I downregulation in various mouse and human cancers has been shown to be a common mechanism for tumor escape (34, 35). B lymphoma cells isolated from NKCKD-Eμ-mycITg exhibit marked downregulation of both H-2Kb and H-2Db, in comparison with WT-Eμ-mycITg mice as well as normal B cells from non-Eμ-mycITg mice. The hyporesponsive state of NK cells in our NKCKD model removes any benefit of a tumor retaining MHC-I expression to evade NK cells, permitting these tumors to more readily downregulate MHC-I to escape effector T-cell recognition.

NK-mediated immunoediting has been reported in the context of the strong activating receptor NKGD2, with respect to expression of its ligands H60a and Rae-1 (36, 37). Expression of H60a was reduced following passage in Rag2−/− mice, which lack mature T and B lymphocytes, suggesting pressures from innate immune cells toward reduced ligand expression (36). Increased expression of Rae-1 was observed in NKG2D-deficient mice with prostate adenocarcinoma but not in mice with B-cell lymphoma (37), suggesting that different immune evasion mechanisms are undertaken by different cancers. In our analysis of B-cell lymphomas from NKCITg and WT mice, no difference in NKG2D ligand expression was observed, which further highlights the differential MHC-I expression seen between NKCITg-Eμ-mycITg and WT-Eμ-mycITg. Some cancers secrete NKG2D ligands as a way to nullify the function of NKG2D+ effector cells, and in such circumstances, MHC-I expression may be the determining factor in tumor clearance by NK cells (38). Understanding the effects of MHC-I expression on clinical outcomes could help to improve current treatments using Ly49 and KIR receptor signaling (39, 40). Low MHC-I expression is highly correlated with increased tumor-associated necrosis and poor prognosis (41, 42); however, complete loss of MHC-I expression is an indicator of good prognosis (43, 44). Such observations suggest that total loss of MHC-I renders the tumor sensitive to NK cells. In contrast, partially reduced MHC-I allows them to potentially evade both NK and T cells; the allele for antigen presentation is lost thus eluding T-cell recognition, and the remaining allele can inhibit NK cells. Although cancer therapy clinical trials at present do not include tumor MHC-I expression analysis as part of treatment monitoring, such practice could potentially improve the success of current therapies with targeted regimens toward specific MHC-I level and allele alterations.

This work contributes to a growing understanding of the role and importance of the Ly49 family in NK cells, not only as inhibitory receptors, but also as necessary mediators of NK cell function. This study provides in vivo support for the importance of Ly49 in NK cell-mediated tumor immuno surveillance and MHC-I–directed tumor immunoediting as a result of loss of Ly49 expression.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
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