Ly49 Family Receptors Are Required for Cancer Immunosurveillance 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(XD)) in several models of carcinoma and metastasis. We found that NKC(XD) mice exhibited uncontrolled tumor growth and metastases. Expression of two MHC-I alleles, H-2Kb and H-2Dd, was decreased in tumors from NKC(XD) 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(XD) 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(XD) 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(XD) 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 NKG2D, CD94, and KLRI receptors—in the mouse natural killer gene complex (NKC) are downregulated (19). NKC(XD) mice...
provide a genetically based approach to study the importance of Ly49 receptors in cancer immunosurveillance. As previously described (19), NKc–/– 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 Eμ-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. Ly49ITg, B6.NKC–/– (Kla15tm1.Pam4), and NKc–/–Ly49ITg mice were previously described (19–21). Because of linkage disequilibrium, B6.NKC–/– mice possess a 129-derived NKC. Therefore, the B6.Ly49ITg congenic mouse strain, which harbors a 129-derived NKC on the B6 background, serves as the genetically correct wild-type (WT) control. For brevity, B6.NKC–/– and B6.Ly49ITg are referred to as NKc–/– and WT, respectively.

Eμ-myc transgenic mice were crossed with WT and NKc–/– to produce WT.Eμ-mycTg and NKc–/–Eμ-mycTg mice, respectively. Genotyping for the Eμ-myc transgene was performed by PCR using primers: forward 5’TACATGATAAGTCTGCATAGATCC and reverse 5’TATGCTACCTTCTTATACAC to obtain 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 verified to be mycoplasma free and showed appropriate pathologic morphology. B16F10. LacZ was stably transfected with H-2Kb-pEF6 and/or H-2Db-pEF6 expression vectors using Lipofectamine 2000 (Invitrogen), as per manufacturer’s guidelines, and selected in 4.5 or 9 μg/mL blasticidin. Antibody staining was performed using anti-H-2Kb (AF6-88.5.53; eBioscience), anti-H-2Db (KH95; BD Bioscience), anti-MULT1 (5D10; eBioscience), and anti-pan Rae-1 (186107; R&D Systems). Mean fluorescence intensity (MFI) for H-2Kb, H-2Db, Rae-1, and MULT1 expression in the spleen and lymph nodes of Eμ-mycTg 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 [51Cr]-release cytotoxicity assays, as previously described (23). Intracellular staining for IFNγ was performed as previously described (19). NKp46+/TCRγδ+ cells were analyzed for IFNγ 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-1β–expressing tumor cells were differentially labeled with 0.5, 3, and 8 μmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE), and combined at a 1:1:1 ratio for a total of 2 × 106 cells. Peritoneal cells were harvested 16 hours following injection and analyzed by flow cytometry for the presence of CFSE-labeled tumor cells.

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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 × 10⁵ cells/mouse) and 100% (dose of 1 × 10⁶ 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 × 10⁶ 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-2Db were similar between NKCKD and WT mice (Fig. 1E and F and Supplementary Fig. S1C and S1D).
Accelerated melanoma cell-induced metastatic growth in NKCKD mice

The B16F10.LacZ cells were also used in an experimental pulmonary metastases model to determine the ability of NKCKD mice to control metastatic growth. The number of metastases was greater in NKCKD mice compared with WT mice regardless of incubation time and cell dose (Fig. 2A and B). Histologically, NKCKD 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 NKCKD mouse lungs (Fig. 2C). These data suggest an inability of NKCKD 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 NKCKD 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 NKCKD 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 NKCKD mice suggests that NK cells from NKCKD 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 NKCKD 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 NKCKD (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 × 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 NKCK\textsuperscript{KD} 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-2K\textsuperscript{b} and H-2D\textsuperscript{b}, metastatic numbers in WT and NKCK\textsuperscript{KD} 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 NKCK\textsuperscript{KD} compared with untreated (Fig. 3D, F, and H). These results support that the hyporesponsive nature of NK cells from NKCK\textsuperscript{KD} 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 NKCK\textsuperscript{KD} mice**

To test the hypothesis that NK cells from NKCK\textsuperscript{KD} are uneducated and hyporesponsive, and therefore unable to control tumor and metastatic growth, a transgene for Ly49I\textsuperscript{K} was introduced into NKCK\textsuperscript{KD} mice. Introduction of the Ly49I\textsuperscript{K} significantly delayed RMA-S–induced flank tumor onset in NKCK\textsuperscript{KD} mice, approaching WT levels (Fig. 4A). WT and NKCK\textsuperscript{KD}–Ly49I\textsuperscript{K} mice presented with similar late period tumor growth, in contrast with NKCK\textsuperscript{KD} mice (Fig. 4B). Similar results were also seen with the experimental lung metastases, wherein the number of metastases was reduced in NKCK\textsuperscript{KD}–Ly49I\textsuperscript{K} mice (mean of 24) compared with NKCK\textsuperscript{KD} (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 NKCK\textsuperscript{KD} mice**

Previous studies show a role for NK cells in controlling methylcholanthrene (MCA)-induced tumors (26–28). It was of interest to determine whether NKCK\textsuperscript{KD} mice with hyporesponsive NK cells would be more susceptible to MCA-induced tumors. NKCK\textsuperscript{KD} mice exhibited earlier sarcoma onset than WT; by day 85, all NKCK\textsuperscript{KD} mice had developed tumors, compared with approximately 50% of WT mice (Fig. 5A). Tumor growth was accelerated in NKCK\textsuperscript{KD} mice (Fig. 5B), with a significantly greater relative growth rate in NKCK\textsuperscript{KD} (mean of 1.97) than WT mice (mean of 0.65; Fig. 5C). This suggests that control of MCA-induced tumors is impaired in NKCK\textsuperscript{KD} 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 immuonoediting in NKCK\textsuperscript{KD} mice**

Eµ-\textit{myc} transgenic mice possess the \textit{myc} oncogene coupled to the immunoglobulin \( \mu \) 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, NKCK\textsuperscript{KD}Eµ-\textit{myc}\textsuperscript{Tg} and WT.Eµ-\textit{myc}\textsuperscript{Tg} mice were generated to study the effect of silenced Ly49 expression. NKCK\textsuperscript{KD} 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 NKCK\textsuperscript{KD} mice (Fig. 6A). Although both groups possessed mice exhibiting delayed lymphoma onset, this lapse was more prominent in WT mice.

Because NKCK\textsuperscript{KD} mice lack MHC-I–educated NK cells, it was of interest to determine whether this would lead to cancer immunoediting. To test this hypothesis, immature (IgM\textsuperscript{+}) and mature (IgM\textsuperscript{−}B) lymphoma cells from the spleen and lymph nodes were analyzed for expression of the MHC-I molecules, H-2K\textsuperscript{b} and H-2D\textsuperscript{b}, as well as the NK ligands, Rae-1 and Mult1. NKCK\textsuperscript{KD}Eµ-\textit{myc}\textsuperscript{Tg} mice exhibited reduced MHC-I expression in both the mature and immature B-cell populations compared with WT mice with and without the Eµ-\textit{myc}\textsuperscript{Tg} (Fig. 6B–H).
B lymphoma cells were isolated from a Nkcc3−/−myc−/− mouse that exhibited reduced MHC-I expression and low-level expression of NKG2D ligands (Fig. 6I). This decrease in MHC-I expression rendered the tumor cells more susceptible to rejection by WT mice compared with Nkcc3−/− mice (Fig. 6J). 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 Nkcc3−/−myc−/−, but not NKG2D ligand expression, suggests that silenced Ly49 expression in Nkcc3−/− mice leads to MHC-I-directed tumor immunoediting.

**Signaling through NKG2D is unaffected in Nkcc3−/− mice**

To study the effects of activating ligand expression on MHC-I-expressing and −deficient target cells in Nkcc3−/− mice, the rejection of splenocytes from B6 or B2m−/− mice transgenically expressing Rae-1 was studied. Activation through NKG2D is intact in Nkcc3−/− mice because rejection of splenocytes from Rae-1e78 mice were similar between WT and Nkcc3−/− (Fig. 7A). Although there is lower rejection of B2m−/− Rae-1e78 splenocytes by Nkcc3−/− 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-1E8 cells (Fig. 7B). Similarly, in vivo rejection of RMA and RMA-S ectopically expressing Rae-1B by WT and Nkcc3−/− mice showed parallel results. Rejection of both Rae-1Bhigh and Rae-1Blow cells were similar (Fig. 7C–E), suggesting that NKG2D signaling is fully intact in Nkcc3−/− mice. Although rejection of RMA-S-Rae-1B was lower in Nkcc3−/− 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 Nkcc3−/− mice. These results suggest that although Nkcc3−/− 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 Nkcc3−/− mice**

It was of interest to determine whether loss of Ly49 expression affects cytotoxicity or IFNγ production by NK cells. NK cells from Nkcc3−/− mice exhibited normal production of IFNγ following stimulation with PMA/ionomycin (Supplementary Fig. S3A), as well as various tumor cells: RMA, RMA-Rae-1Bhigh, RMA-Rae-1Blow, RMA-S, and RMA-S-Rae-1B (Supplementary Fig. S3B), suggesting that loss of Ly49 does not affect IFNγ production. A direct comparison of the killing between WT and Nkcc3−/− 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-1B (Supplementary Fig. S3D), by Nkcc3−/−-derived ALAK cells is impaired, while killing of MHC-I−expressing RMA and RMA-Rae-1Bhigh and RMA-Rae-1Blow is comparable between WT and Nkcc3−/−, suggesting a cytotoxicity defect with regards to missing-self recognition. Although there is observed killing of RMA-S by Nkcc3−/− 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 Nkcc3−/− 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\textsuperscript{KD} mice. A, B-cell lymphoma progression in NKC\textsuperscript{KD}Eµ-myc\textsuperscript{Tg} (n = 35) and WT.Eµ-myc\textsuperscript{Tg} (n = 24) mice. B–H, ex vivo analysis of spleen and lymph node cell suspensions isolated from NKC\textsuperscript{KD}Eµ-myc\textsuperscript{Tg} (n = 30) and WT.Eµ-myc\textsuperscript{Tg} (n = 21) mice. B cells (B220\textsuperscript{+}) were analyzed separately as immature (IgM\textsuperscript{−}/IgM\textsuperscript{+}) or mature (IgM\textsuperscript{+}) for expression of H-2K\textsubscript{b} and H-2D\textsubscript{b} by flow cytometry. MFI was standardized to that of B cells from non-Eµ-myc\textsuperscript{Tg} transgenic mice (dotted line). I, MHC-I and NKG2D ligand expression in B lymphoma cells cultured from a NKC\textsuperscript{KD}Eµ-myc\textsuperscript{Tg} (black histogram) compared with B cells from a non-Eµ-myc\textsuperscript{Tg} mouse (gray histogram). J, in vivo rejection of B lymphoma cells originating from an NKC\textsuperscript{KD}Eµ-myc\textsuperscript{Tg} 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\textsuperscript{5} B lymphoma cells from an NKC\textsuperscript{KD}Eµ-myc\textsuperscript{Tg} injected into the hind flank of WT (n = 19) and NKC\textsuperscript{KD} (n = 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 NKCKD 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 NKCKD mice; however, those induced with MHC-I–expressing tumor cells were comparable between WT and NKCKD mice. This suggests the uncontrolled tumor growth in NKCKD 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 NKCKD mice than WT mice. However, the number of metastases induced by MHC-I–expressing transfectants in WT mice approached NKCKD 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 NKCKD 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 NKCKD background develop lymphoma earlier than their WT counterparts also possessing the oncogene. The defect in NKCKD 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 NKCKD mice; in vitro killing and in vivo rejection of RMA Rae-1εTg mice (A) and B2m−/− and B2m−/− Rae-1εTg mice (B) relative to B6 mice. C, Rae-1 expression levels in parental RMA cells, and RMA cells ectopically expressing Rae-1εhigh and Rae-1εlow. In vivo rejection of MHC-I-expressing RMA Rae-1εhigh (D) and Rae-1εlow (E) relative to RMA. F, in vivo rejection of MHC-I-deficient RMA-S and RMA-S-Rae-1ε 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 NKCKD mice. In vivo rejection of CFSE-labeled splenocytes from Rae-1εTg mice (A) and B2m−/− and B2m−/− Rae-1εTg mice (B) relative to B6 mice. C, Rae-1 expression levels in parental RMA cells, and RMA cells ectopically expressing Rae-1εlow and Rae-1εhigh. In vivo rejection of MHC-I-expressing RMA Rae-1εlow (D) and Rae-1εhigh (E) relative to RMA. F, in vivo rejection of MHC-I-deficient RMA-S and RMA-S-Rae-1ε 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 Ly49T6 expression 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 cell populations that the phenomenon observed is the result of a loss of Ly49 expression and not due to the partial silencing of two or other adjacent gene families, encoding the CD94/NKG2 and KLR1/E molecules (19). Although flanks tumor size in the NKcKd-Ly49T6 mice is comparable with WT, suggesting near complete restoration of NK cell control, NKcKd-Ly49T6 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-mycT6 exhibit marked downregulation of both H-2Kb and H-2Dd, in comparison with WT.Eμ-mycT6 mice as well as normal B cells from non-Eμ-mycT6 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 NGK2D, with respect to expression of its ligands H60 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 NKGD2-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 NKcKd and WT mice, no difference in NKGD2 ligand expression was observed, which further highlights the differential MHC-I expression seen between NKcKd-Eμ-mycT6 and WT.Eμ-mycT6. Some cancers secrete NKGD2 ligands as a way to nullify the function of NKGD2+ 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 immunosurveillance 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.

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Conception and design: M.M. Tu, A.P. Makrigiannis
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Study supervision: A.P. Makrigiannis
Monitored NKcKd-Eμ-mycT6 and WT.Eμ-mycT6 mice B-cell lymphoma progression and conducted experiments associated with these mice: A. Mottashed
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