Ly49 family receptors are required for cancer immunosurveillance mediated by natural killer cells

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Running Title: Ly49 in cancer immunosurveillance

Keywords: natural killer cells, innate immunity, cancer immunosurveillance, Ly49, MHC-I

Financial Support: This work was supported by the Canadian Institutes of Health Research (MOP 62841). M.M.T. is supported by an Ontario Graduate Scholarship. A.P.M. is a Canada Research Chair in Innate Pathogen Resistance.

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Word count (excluding references): 4,971

Number of figures: 7

1
ABSTRACT

According to the missing-self hypothesis, natural killer (NK) cells survey for target cells which lack class I major histocompatibility complex (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\textsuperscript{KD}), in several models of carcinoma and metastasis. We found that NKC\textsuperscript{KD} mice exhibited uncontrolled tumor growth and metastases. Expression of two MHC-I alleles, H-2K\textsuperscript{b} and H-2D\textsuperscript{b}, was decreased in tumors from NKC\textsuperscript{KD} mice in support of the likelihood of NK-mediated tumor immunoediting. These tumor cells exhibit directed alterations to their cell surface expression in response to the genetically-altered immune environment in order to evade host recognition. Immunoediting in NKC\textsuperscript{KD} 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\textsuperscript{KD} 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\textsuperscript{KD} 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.
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 class I major histocompatibility complex (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, while 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 lab 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 down-regulated (19). NKC<sup>KD</sup> mice provide a genetically-based approach to study the importance of Ly49 receptors in cancer immunosurveillance. As previously described (19), NKC<sup>KD</sup> 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, B2m<sup>−/−</sup>, and E<sub>μ</sub>-myc transgenic [strain: B6.Cg-Tg(IghMyc)22Bri/J] mice were purchased from The Jackson Laboratory. Ly49<sup>Tg</sup> mice were kindly provided by Dr. Michael Bennett (University of Texas, Dallas) and backcrossed to the C57BL/6 background for at least six generations. B6.Ly49<sup>129</sup>, B6.NKC<sup>KD</sup> (Klra15<sup>tm1.1Apma</sup>) and NKC<sup>KD</sup>-Ly49<sup>Tg</sup> mice were previously described (19-21). Due to linkage disequilibrium, B6.NKC<sup>KD</sup> mice possess a 129-derived NKC. Therefore, the B6.Ly49<sup>129</sup> congenic mouse strain, which harbors a 129-derived NKC on the B6 background, serves as the genetically correct WT control. For brevity, B6.NKC<sup>KD</sup> and B6.Ly49<sup>129</sup> are referred to as NKC<sup>KD</sup> and WT.

E<sub>μ</sub>-myc transgenic mice were crossed with WT and NKC<sup>KD</sup> to produce WT.E<sub>μ</sub>-myc<sup>Tg</sup> and NKC<sup>KD</sup>.E<sub>μ</sub>-myc<sup>Tg</sup> mice, respectively. Genotyping for the E<sub>μ</sub>-myc transgene was performed by PCR using primers: forward 5ʿCTGGGTCACTCACATTTAAC and reverse 5ʿGTATATCAGTCACCGCAGGT to attain a product size of approximately 500bp. All <i>in vivo</i> 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.

Cells, Antibodies, and Flow Cytometry

B16F10.LacZ was kindly provided by Dr. Rebecca Auer (Ottawa Hospital Research Institute, Ottawa, Ontario). 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 were stably transfected with H-2K<sup>b</sup>-pEF6 and/or H-2D<sup>b</sup>-pEF6 expression vectors using Lipofectamine 2000 (Invitrogen), as per manufacturer’s guidelines, and selected in 4.5 μg/mL or 9 μg/mL blasticidin. Antibody staining was performed using anti-H-2K<sup>b</sup> (AF6-88.5.5.3) (eBioscience), anti-H-2D<sup>b</sup> (KH95) (BD Biosciences), anti-MULT1 (5D10) (eBioscience), and anti-pan Rae-1 (186107) (R&D Systems). Cells were acquired on a CyAN-ADP flow cytometer with Summit 4.3 software (Beckman Coulter), and analyzed using Kaluza 1.2 software (Beckman Coulter).

Flank tumor model

WT, NKC<sup>KD</sup>, and NKC<sup>KD</sup.Ly49<sup>Tg</sup> mice were challenged on the right flank by subcutaneous injection of 5x10<sup>5</sup> or 1x10<sup>6</sup> RMA, RMA-S, B16F10.LacZ, B16F10.H-2K<sup>b</sup> H-2D<sup>b</sup>, 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, NKC<sup>KD</sup>, and NKC<sup>KD</sup.Ly49<sup>Tg</sup> mice were intravenously challenged with 1x10<sup>5</sup> or 2x10<sup>5</sup> B16F10.LacZ cells in PBS. For experiments with poly(I:C) stimulation, 100 μg of poly(I:C) in PBS were injected intravenously 24 hours prior to 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 μm thickness and H&E stained.

**Carcinogen-induced model**

WT and NKC<sup>KD</sup> mice were injected subcutaneously into the hind flank with 100 μg 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<sup>2</sup>/day) was calculated from tumor area with respect to number of days post-MCA injection.

**Spontaneous lymphoma model**

E<sub>μ</sub>-myc<sup>Tg</sup> 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-2K<sup>b</sup> (AF6-88.5.5.3) (eBioscience), anti-H-2D<sup>b</sup> (KH95) (BD Biosciences), anti-Mult1 (5D10) (eBioscience), and anti-pan Rae-1 (186107) (R& D Systems). Mean fluorescence intensity for H-2K<sup>b</sup>, H-2D<sup>b</sup>, Rae-1 and Mult1 expression in the spleen and lymph nodes of E<sub>μ</sub>-myc<sup>Tg</sup> mice was standardized to levels in WT control mice.

**In vitro and in vivo NK cell assays**
Adherent lymphokine (IL-2)–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 IFN-γ was performed as previously described (19). NKp46+ TCRβ– cells were analyzed for IFN-γ 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 labelled with 0.5 μM, 3 μM and 8 μM CFSE, and combined at a 1:1:1 ratio for a total of 2x10^6 cells. Peritoneal cells were harvested 16 hours following injection and analyzed by flow cytometry for the presence of CFSE-labelled 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 NKC^{KD} mice

It was of interest to determine whether the lack of MHC-I immunosurveillance by NK cells in NKC^{KD} 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 NKC^{KD} mice than WT mice (Fig. 1A and C). Tumor incidence was more prevalent in the NKC^{KD} group, with 92% (dose of 5x10^5 cells/mouse) and 100% (dose of 1x10^6 cells/mouse) developing tumors after 30 days, compared to 65% and 85%, respectively, in the WT group (Fig. 1A and C). Tumor size at comparable dates was larger in NKC^{KD} mice (Fig. 1B and D); the overall growth rate of the tumors in NKC^{KD} mice is accelerated. Tumor development and growth in NKC^{KD} mice is comparable to that of B2m^{-/-} mice (Fig. 1C and D), which also possess hyporesponsive NK cells.

B16F10 are highly aggressive murine melanoma cells which lack MHC-I expression (24, 25). Subcutaneous injection of 1x10^6 B16F10.LacZ cells promoted solid tumor formation in both NKC^{KD} and WT mice. At day 6, 7% of WT mice developed tumors in contrast to 53% of NKC^{KD} mice (Supplementary Fig. S1A). Tumor size at comparable dates was larger in NKC^{KD} mice (Supplementary Fig. S1B). This defect in NKC^{KD} 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-2K^b and H-2D^b were similar between NKC^{KD} and WT mice (Fig. 1E and F, and Supplementary Fig. S1C and D).

Accelerated melanoma cell-induced metastatic growth in NKC^{KD} mice
The B16F10.LacZ cells were also used in an experimental pulmonary metastases model to determine the ability of NKC<sup>KD</sup> mice to control metastatic growth. The number of metastases was greater in NKC<sup>KD</sup> mice compared to WT mice regardless of incubation time and cell dose (Fig. 2A and B). Histologically, NKC<sup>KD</sup> 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 NKC<sup>KD</sup> mouse lungs (Fig. 2C). These data suggest an inability of NKC<sup>KD</sup> 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 NKC<sup>KD</sup> mice were directly activated by poly(I:C) injection. The lungs of untreated WT mice contained a mean of 45 metastases compared to a mean of 11 in the poly(I:C)-treated group (Fig. 2D). Comparatively, for NKC<sup>KD</sup> 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 NKC<sup>KD</sup> mice suggests that NK cells from NKC<sup>KD</sup> 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 NKC<sup>KD</sup> mice is due to defective missing-self recognition, the cells were stably transfected to express two MHC-I alleles, H-2K<sup>b</sup> and H-2D<sup>b</sup>, either individually or together (Fig. 3A). Transfection of the cells did not affect expression of the NKG2D ligands, Rae-1 and Mult1
Expression of a single MHC-I allele was insufficient to significantly protect these tumors from WT NK control compared to the NKC\textsuperscript{KD} (Fig. 3C and 3E). However, expression of both H-2K\textsuperscript{b} and H-2D\textsuperscript{b} (Fig. 3G) resulted in similar metastatic numbers in the lungs of WT and NKC\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 is MHC-I-dependent, since upon expression of both H-2K\textsuperscript{b} and H-2D\textsuperscript{b}, metastatic numbers in WT and NKC\textsuperscript{KD} mice are comparable. Similarly to the parental B16F10, poly(I:C) stimulation prior to tumor challenge greatly reduced the number of metastases in both WT and NKC\textsuperscript{KD} compared to untreated (Fig. 3D, F and H). These results support that the hyporesponsive nature of NK cells from NKC\textsuperscript{KD} mice is not due to a more generalized defect since stimulation restores function.

Inhibitory self-MHC-I-specific Ly49I restores NK cell cancer immunosurveillance in NKC\textsuperscript{KD} mice

To test the hypothesis that NK cells from NKC\textsuperscript{KD} mice are uneducated and hyporesponsive, and therefore unable to control tumor and metastatic growth, a transgene for Ly49I was introduced into NKC\textsuperscript{KD} mice. Introduction of the Ly49IT\textsuperscript{tg} significantly delayed RMA-S-induced flank tumor onset in NKC\textsuperscript{KD} mice, approaching WT levels (Fig. 4A). WT and NKC\textsuperscript{KD}-Ly49IT\textsuperscript{tg} mice presented with similar late period tumor growth, in contrast with NKC\textsuperscript{KD} mice (Fig. 4B). Similar results were also seen with the experimental lung metastases, wherein the number of metastases was reduced in NKC\textsuperscript{KD}-Ly49IT\textsuperscript{tg} mice (mean of 24) compared to NKC\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 NKC\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 NKC\textsuperscript{KD} mice with hyporesponsive NK cells would be more susceptible to MCA-induced tumors. NKC\textsuperscript{KD} mice exhibited earlier sarcoma onset than WT; by day 85, all NKC\textsuperscript{KD} mice had developed tumors, compared to approximately 50\% of WT mice (Fig. 5A). Tumor growth was accelerated in NKC\textsuperscript{KD} mice (Fig. 5B), with a significantly greater relative growth rate in NKC\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 NKC\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 immunoediting in NKC\textsuperscript{KD} mice

E\textsubscript{μ-}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 pathological importance of this cancer, especially with parallels to human Burkitt’s lymphoma, NKC\textsuperscript{KD}.E\textsubscript{μ-}myc\textsuperscript{Tg} and WT.E\textsubscript{μ-}myc\textsuperscript{Tg} mice were generated to study the effect of silenced Ly49 expression. NKC\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 to approximately 90\% of NKC\textsuperscript{KD} mice (Fig. 6A). While both groups possessed mice exhibiting delayed lymphoma onset, this lapse was more prominent in WT mice.
Since NKC\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 NKG2D ligands, Rae-1 and Mult1. NKC\textsuperscript{KD}.\textit{Eμ-myc}\textsuperscript{Tg} mice exhibited reduced MHC-I expression in both the mature and immature B cell populations compared to WT mice with and without the Eμ-\textit{myc}\textsuperscript{Tg} (Fig. 6B-H). In contrast, expression of NKG2D ligands were negligible or very low, with no statistically significant differences between WT.Eμ-\textit{myc}\textsuperscript{Tg} and NKC\textsuperscript{KD}.\textit{Eμ-myc}\textsuperscript{Tg} (Supplementary Fig. S2A-G). This highlights the significance of the observed downregulation of MHC-I in NKC\textsuperscript{KD}.\textit{Eμ-myc}\textsuperscript{Tg}.

B lymphoma cells were isolated from a NKC\textsuperscript{KD}.\textit{Eμ-myc}\textsuperscript{Tg} mouse which 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 to NKC\textsuperscript{KD} 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 NKC\textsuperscript{KD}.\textit{Eμ-myc}\textsuperscript{Tg}, but not NKG2D ligand expression, suggests that silenced Ly49 expression in NKC\textsuperscript{KD} mice leads to MHC-I-directed tumor immunoediting.

Signaling through NKG2D is unaffected in NKC\textsuperscript{KD} mice

To study the effects of activating ligand expression on MHC-I-expressing and –deficient target cells in NKC\textsuperscript{KD} mice, the rejection of splenocytes from B6 or \textit{B2m}\textsuperscript{−/−} mice transgenically expressing Rae-1 was studied. Activation through NKG2D is intact in NKC\textsuperscript{KD} mice since rejection of splenocytes from Rae-1\textit{e}\textsuperscript{Tg} mice were similar between WT and NKC\textsuperscript{KD} (Fig. 7A).
While there is lower rejection of $B2m^{-/}$ Rae-1ε$^Tg$ 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 $B2m^{-/}$ (Fig. 7B). Similarly, in vivo rejection of RMA and RMA-S ectopically expressing Rae-1β by WT and NKC$^{KD}$ mice showed parallel results. Rejection of both Rae-1β$^{high}$ and Rae-1β$^{low}$ cells were similar (Fig. 7C-E), suggesting that NKG2D signaling is fully intact in NKC$^{KD}$ mice. While rejection of RMA-S-Rae-1β was lower in NKCKD 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 NKCKD mice. These results suggest that while NKCKD 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 IFN-γ production in NKC$^{KD}$ mice**

It was of interest to determine whether loss of Ly49 expression affects cytotoxicity or IFN-γ production by NK cells. NK cells from NKC$^{KD}$ mice exhibited normal production of IFN-γ following stimulation with PMA+ionomycin (Supplementary Fig. S3A), as well as various tumor cells: RMA, RMA-Rae-1β$^{low}$, RMA-Rae-1β$^{high}$, RMA-S, and RMA-S-Rae-1β (Supplementary Fig. S3B), suggesting that loss of Ly49 does not affect IFN-γ production. A direct comparison of the killing between WT and NKCKD 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-1β (Supplementary Fig. S3D) by NKCKD-derived ALAK cells is impaired, while killing of MHC-I-expressing RMA and RMA-Rae-1β$^{high}$ and RMA-Rae-1β$^{low}$ is comparable between WT and NKCKD, suggesting a cytotoxicity defect with regards to missing-self recognition. While 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 NKCl mice. Overall, these results suggest that loss of Ly49 affects the cytotoxicity of the NK cells in response to missing-self signals.
DISCUSSION

NK cells are known to play an important role in the in vivo recognition and control of tumors (30). Our genetic NKC<sup>KD</sup> 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 NKC<sup>KD</sup> mice; however, those induced with MHC-I-expressing tumor cells were comparable between WT and NKC<sup>KD</sup> mice. This suggests the uncontrolled tumor growth in NKC<sup>KD</sup> 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 NKC<sup>KD</sup> mice than WT mice. However, the number of metastases induced by MHC-I-expressing transfectants in WT mice approached NKC<sup>KD</sup> levels, since 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 NKC<sup>KD</sup> 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 NKC<sup>KD</sup> background develop lymphoma earlier than their WT counterparts also possessing the oncogene. The defect in NKC<sup>KD</sup> mice is due to reduced cytotoxicity of the NK cells since decreased killing is observed in response to missing-self. Signaling through NKG2D is fully intact in NKC<sup>KD</sup> mice; in vitro killing and in vivo rejection of RMA Rae-1-expressing target cells is similar between WT and NKC<sup>KD</sup> mice. The observed differential killing and rejection of RMA-S-Rae-1-expressing target cells by WT and NKC<sup>KD</sup> mice may be attributable to defective missing-self recognition in NKC<sup>KD</sup> mice; though there is evidence that signaling through NKG2D may compensate for such defects (19).
Studies have reported that NK cells must undergo an education process in which interaction between inhibitory self-specific Ly49 and MHC-I molecules during development results in the acquisition of NK cell function (10, 11). In our studies, following Ly49I\textsuperscript{Tg} restoration in NKC\textsuperscript{KD} mice, overall increased tumor control was observed, confirming that \textit{in vivo} education is mediated by inhibitory self-specific Ly49. As well, the ability of Ly49I to rescue hyporesponsive NK cells supports that 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). While flank tumor size in the NKC\textsuperscript{KD}-Ly49I\textsuperscript{Tg} mice is comparable to WT, suggesting near complete restoration of NK cell control, NKC\textsuperscript{KD}-Ly49I\textsuperscript{Tg} 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-2K\textsuperscript{b}, and Ly49V, G2, and O bind to H-2D\textsuperscript{b} (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 non-classical 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 NKC\textsuperscript{KD}.E\textmu-myc\textsuperscript{Tg} exhibit marked down-regulation of both H-2K\textsuperscript{b} and H-2D\textsuperscript{b}, in comparison to WT.E\textmu-myc\textsuperscript{Tg} mice as well as normal B cells from non-E\textmu-myc\textsuperscript{Tg} mice. The hyporesponsive state of NK cells in our NKC\textsuperscript{KD} 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 NKG2D, 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 towards 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 different immune evasion mechanisms are undertaken by different cancers. In our analysis of B cell lymphomas from NKC^KD and WT mice, no difference in NKG2D ligand expression was observed, which further highlights the differential MHC-I expression seen between NKC^KD.Eμ-myc^Tg and WT.Eμ-myc^Tg. 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 employing 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. While 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 towards 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.
ACKNOWLEDGMENTS

The authors thank Dr. Lee-Hwa Tai and Christiano de Souza for guidance with the pulmonary metastases model and helpful discussion.
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FIGURE LEGENDS

Figure 1. Accelerated tumor cell-induced flank tumor growth in NKC<sup>KD</sup> mice. Formation of solid flank tumors following subcutaneous injection of (A and B) 1 x 10<sup>6</sup> MHC-I-deficient RMA-S. WT <i>n</i>=13. NKC<sup>KD</sup> <i>n</i>=11. (C and D) 5 x 10<sup>5</sup> RMA-S. WT <i>n</i>=17. NKC<sup>KD</sup> <i>n</i>=13. <i>B2m</i><sup>−/−</sup> <i>n</i>=5. (E and F) 5 x 10<sup>5</sup> MHC-I-expressing RMA. WT <i>n</i>=16. NKC<sup>KD</sup> <i>n</i>=16. (A, C and E) Date of tumor appearance and (B, D and F) mean tumor size ± SEM. Data are pooled from three independent experiments.

Figure 2. Accelerated melanoma cell-induced metastatic growth in NKC<sup>KD</sup> mice. Pulmonary metastases induced by intravenous injection of MHC-I-deficient B16F10.LacZ melanoma cell (A) 2 x 10<sup>5</sup> cells injected and harvested 5 days later, and (B) 1 x 10<sup>5</sup> cells injected and harvested 14 days later. Data are pooled from three independent experiments. (C) Representative whole lung image and cross-section H&E staining of lungs harvested 14 days following intravenous injection of 1 x 10<sup>5</sup> B16F10.LacZ cells. (D) Stimulation with 100 μg poly(I:C) prior to injection of 2 x 10<sup>5</sup> B16F10.LacZ cells. Number of metastases quantified on largest lung lobe after 5 days. Data are pooled from two independent experiments. Each symbol represents a single mouse. Horizontal line represents mean.

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-2K<sup>b</sup>, H-2D<sup>b</sup> or both. Flow cytometry results showing (A) H-2K<sup>b</sup> and H-2D<sup>b</sup>, and (B) Rae-1 and Mult1 expression in transfected cells compared to parental B16F10.LacZ. Quantification of metastases 5 days following injection of 2 x 10<sup>5</sup> B16F10.LacZ cells expressing (C-D) H-2K<sup>b</sup>, (E-F) H-2D<sup>b</sup> or (G-H) H-2K<sup>b</sup> and H-2D<sup>b</sup>
with (D, F and H) poly(I:C) stimulation of recipient mice prior to tumor injection. Horizontal lines represent mean. Each symbol represents a single mouse. Data are pooled from two to three independent experiments.

**Figure 4. Inhibitory self-MHC-I-specific Ly49I restores NK cell function in NKC\textsuperscript{KD} mice.** Formation of solid flank tumors following subcutaneous injection of MHC-I-deficient RMA-S. (A) Date of tumor appearance and (B) mean tumor size ± SEM. WT \( n = 15 \). NKC\textsuperscript{KD} \( n = 16 \). NKC\textsuperscript{KD}-Ly49ITg \( n = 18 \). (C) Quantification of pulmonary metastases induced by 2x10\(^5\) B16F10.LacZ melanoma cells harvested 5 days later. Each symbol represents a single mouse. Horizontal line represents mean. Data are pooled from three independent experiments.

**Figure 5. Accelerated onset and growth of MCA-induced sarcoma in NKC\textsuperscript{KD} mice.** Sarcoma development following subcutaneous injection of 100 μg MCA into the hind flank of WT (\( n = 10 \)) and NKC\textsuperscript{KD} (\( n = 10 \)) mice. Mice were observed weekly for tumor development. (A) Date of tumor appearance. (B) Individual tumor growth was measured weekly with each line representing tumor growth in a single mouse. (C) Sarcoma growth rate calculated from tumor size with respect to time. Upper and lower limits of the box represents 25\(^{\text{th}}\) and 75\(^{\text{th}}\) percentiles with whiskers indicating minimum and maximum values. Data are pooled from two independent experiments.

**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}.\textit{Eμ-myc}\textsuperscript{Tg} (\( n = 35 \)) and WT.\textit{Eμ-myc}\textsuperscript{Tg} (\( n = 24 \)) mice. (B-H) \textit{Ex vivo} analysis of spleen and lymph node cell suspensions isolated from NKC\textsuperscript{KD}.\textit{Eμ-myc}\textsuperscript{Tg} (\( n = 30 \)) and WT.\textit{Eμ-myc}\textsuperscript{Tg} (\( n = 21 \)) mice. B cells (B220\(^+\)) were analyzed.
separately as immature (IgM⁻) or mature (IgM⁺) for expression of H-2Kᵇ and H-2Dᵇ by flow cytometry. MFI was standardized to that of B cells from non-Eμ-\textit{myc} transgenic mice (dotted line). (I) MHC-I and NKG2D ligand expression in B lymphoma cells cultured from a NKC^{KD}.Eμ-\textit{myc}^{Tg} (black histogram) compared to B cells from a non-Eμ-\textit{myc}^{Tg} mouse (grey histogram). (J) \textit{In vivo} rejection of B lymphoma cells originating from an NKC^{KD}.Eμ-\textit{myc}^{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 5x10⁵ B lymphoma cells from an NKC^{KD}.Eμ-\textit{myc}^{Tg} injected into the hind flank of WT \((n=19)\) and NKC^{KD} \((n=18)\) mice. Mean ± SEM. Data are pooled from four independent experiments.

**Figure 7. Signaling through NKG2D is unaffected in NKC^{KD} mice.** \textit{In vivo} rejection of CFSE-labelled splenocytes from (A) Rae-1ε^{Tg} mice and (B) \(B2m^{-/-}\) and \(B2m^{-/-}\) Rae-1ε^{Tg} mice relative to B6 mice. (C) Rae-1 expression levels in parental RMA cells, and RMA cells ectopically expressing Rae-1β^{low} and Rae-1β^{high}. \textit{In vivo} rejection of MHC-I-expressing RMA (D) Rae-1β^{low} and (E) Rae-1β^{high} relative to RMA. (F) \textit{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.
Figure 1

A. 1x10^6 RMA-S

B. 1x10^6 RMA-S

C. 5x10^5 RMA-S

D. 5x10^5 RMA-S

E. 5x10^5 RMA

F. 5x10^5 RMA

% tumor-free mice vs. Days post injection

Tumor size (mm^2) vs. Days post injection

WT vs. NKCKD vs. B2m-/-
Figure 2

A. $2 \times 10^5$ B16F10.LacZ

B. $1 \times 10^5$ B16F10.LacZ

C.

WT

NKC$^{KD}$

D. $2 \times 10^6$ B16F10.LacZ

WT

WT+poly(I:C)

NKC$^{KD}$

NKC$^{KD}$+poly(I:C)
Figure 3

A


H-2K^b  H-2D^b

Transfected  Parental

B


Pan Rae1  Mult1

C

B16F10.H-2K^b

Number of lung tumor metastases at day 5

WT  NKC^KD

D

B16F10.H-2K^b

Number of lung tumor metastases at day 5

WT  WT+poly(I:C)  NKC^KD  NKC^KD poly(I:C)

E

B16F10.H-2D^b

Number of lung tumor metastases at day 5

WT  NKC^KD

F

B16F10.H-2D^b

Number of lung tumor metastases at day 5

WT  WT+poly(I:C)  NKC^KD  NKC^KD poly(I:C)

G

B16F10.H-2K^b H-2D^b

Number of lung tumor metastases at day 5

WT  NKC^KD

H

B16F10.H-2K^b H-2D^b

Number of lung tumor metastases at day 5

WT  WT+poly(I:C)  NKC^KD  NKC^KD poly(I:C)

n.s.
Figure 4

A 5x10^5 RMA-S

% tumor-free mice

Days post injection

B 5x10^5 RMA-S

Tumor size (mm^2)

Days post injection

C 2x10^5 B16F10.LacZ

Number of lung tumor metastases at day 5

WT NKC^KD NKC^KD-Ly49^Tg

n.s. *** ***
Figure 5

A

Days post injection

% tumor-free mice

B

Days post injection

Tumor size (mm²)

C

Tumor growth rate (mm²/day)

WT

NKC<sup>KD</sup>

**
Figure 6

A

% tumor-free mice

Age (Days)

0 50 100 150 200 250 300

WT Eμ-myc Tg

- NKC KD Eμ-myc Tg

B

Spleen

Relative MFI (%)

H-2K0

H-2D0

IgM+

IgM-

C

Axillary LN

Relative MFI (%)

H-2K0

H-2D0

IgM+

IgM-

D

Brachial LN

Relative MFI (%)

H-2K0

H-2D0

IgM+

IgM-

E

Cervical LN

Relative MFI (%)

H-2K0

H-2D0

IgM+

IgM-

F

Inguinal LN

Relative MFI (%)

H-2K0

H-2D0

IgM+

IgM-

G

Mesenteric LN

Relative MFI (%)

H-2K0

H-2D0

IgM+

IgM-

H

Renal LN

Relative MFI (%)

H-2K0

H-2D0

IgM+

IgM-

I

MHC-I NKG2D ligands

H-2K0

H-2D0

Eμ-myc WT

Pan Rae1

MULT1

J

% rejection

Days post injection

WT

NKC KD

K

Tumor size (mm2)

Days post injection

WT

- NKC KD

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Ly49 family receptors are required for cancer immunosurveillance mediated by natural killer cells

Megan M Tu, Ahmad Bakur Mahmoud, Andrew Wight, et al.

Cancer Res  Published OnlineFirst May 6, 2014.