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NKG2D-Deficient Mice Are Defective in Tumor Surveillance in Models of Spontaneous Malignancy

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SUMMARY

Ligands for the NKG2D stimulatory receptor are frequently upregulated on tumor lines, rendering them sensitive to natural killer (NK) cells, but the role of NKG2D in tumor surveillance has not been addressed in spontaneous cancer models. Here, we provided the first characterization of NKG2D-deficient mice, including evidence that NKG2D was not necessary for NK cell development but was critical for immunosurveillance of epithelial and lymphoid malignancies in two transgenic models of de novo tumorigenesis. In both models, we detected NKG2D ligands on the tumor cell surface ex vivo, providing needed evidence for ligand expression by primary tumors. In a prostate cancer model, aggressive tumors arising in NKG2D-deficient mice expressed higher amounts of NKG2D ligands than did similar tumors in wild-type mice, suggesting an NKG2D-dependent immunoediting of tumors in this model. These findings provide important genetic evidence for surveillance of primary tumors by an NK receptor.

INTRODUCTION

NKG2D is a stimulatory immunoreceptor expressed by natural killer (NK) cells and various T cell subsets, including activated CD8^{+} T cells, fractions of gamma-delta, NKT cells, and some activated CD4^{+} T cells (Groh et al., 2001; Groh et al., 1999; Jamieson et al., 2002; Raulet, 2003; Saez-Borderias et al., 2006). The receptor binds to several NKG2D ligands, including the MICA and MICB (MHC class I chain related) proteins expressed by humans but not mice (Bauer et al., 1999), and a family of proteins called Rae1 (mouse) or ULBP (human), shared by rodents and humans [(Cerwenka et al., 2000; Cosman et al., 2001; Diefenbach et al., 2000), reviewed in (Raulet, 2003)].

NKG2D ligands are poorly expressed by normal cells but are frequently upregulated in tumor cells (Groh et al., 1999), tumor cell lines (Cerwenka et al., 2000; Diefenbach et al., 2000; Pende

et al., 2001; Pende et al., 2002), and in some infected cells (Gourzi et al., 2006; Lodoen et al., 2003; Siren et al., 2004). The mechanisms leading to ligand upregulation are under investigation (Cerwenka et al., 2000; Gasser et al., 2005; Hamerman et al., 2005). One pathway that has been implicated is the DNA-damage-response pathway (Gasser et al., 2005), which is frequently activated in precancerous lesions as well as advanced tumors (Bartkova et al., 2005; Gorgoulis et al., 2005). Ligand-expressing cells can activate NKG2D-expressing NK cells or T cells in vitro (Bauer et al., 1999; Cerwenka et al., 2000; Diefenbach et al., 2000). Transfected tumor cell lines expressing NKG2D ligands are rejected in vivo in an NKG2D-dependent fashion (Cerwenka et al., 2001; Diefenbach et al., 2001). Whereas these findings are consistent with a role of NKG2D in tumor surveillance, there is little direct evidence for such a role. Indeed, at least some tumors may evade NKG2D surveillance (Coudert et al., 2005; Groh et al., 2002; Oppenheim et al., 2005). For instance, some cancers shed high amounts of soluble NKG2D ligands, which are believed to cause downregulation of NKG2D on the surface of lymphocytes (Groh et al., 2002).

In addition to a potential role in tumor surveillance, NKG2D has been implicated in pathogen immunity (Cosman et al., 2001; Groh et al., 2001), autoimmunity (Groh et al., 2003; Ogasawara et al., 2004), and graft rejection (Ogasawara et al., 2005). In order to address the role of NKG2D (encoded by Klrk1) in vivo, we have generated and characterized NKG2D-deficient mice. We showed that NKG2D-deficient NK cells developed normally and were defective in NKG2D recognition yet retained activity against MHC-deficient tumor cells and bone marrow grafts. We chose two models in which mice carrying transoncogenes develop spontaneous, autochthonous tumors. By using NKG2D-deficient TRAMP mice, a transgenic model of prostate adenocarcinoma, and NKG2D-deficient Eμ-myc mice, a transgenic model of B cell lymphoma, we demonstrated that NKG2D plays a critical role in tumor surveillance in vivo.

RESULTS

Generation of NKG2D-Deficient Gene-Targeted Mice

By gene targeting, we replaced six exons of the Klrk1 gene with a neo cassette in the Bruce-4 embryonic stem cell line derived

from inbred C57Bl/6 (B6) mice (Figure S1A available online). By targeting B6 ES cells, we ensured that the mice would have the well-characterized B6 NK gene complex, which encodes many key NK receptors, including the marker NK1.1. We generated an initial colony of mice in which the *neo* cassette was retained in the gene and subsequently deleted the *neo* cassette by crossing the mice to a B6 strain that expresses the Cre recombinase in the germline (Figure S1A). The *neo*-deleted mice were backcrossed to B6 mice, and *Klrk1* heterozygous offspring lacking the Cre transgene were intercrossed. The experiments shown compared *Klrk1*$^{-/-}$, *Klrk1*$^{+/+}$, and in some cases *Klrk1*$^{+/-}$ littermates derived from intercrosses of *Klrk1*$^{+/-}$ mice from the *neo*-deleted or *neo*-in colonies as indicated.

Klrk1$^{-/-}$ (NKG2D-deficient) mice were born in the expected Mendelian ratio (data not shown). The mice exhibited no visible alterations in major organs or overt pathology. Therefore, NKG2D plays a dispensable role in embryonic development, despite early data showing broad expression of Rae1 transcripts in midstage embryos, especially in the central nervous system (Nomura et al., 1996).

NK cells were present in normal numbers in the spleen, bone marrow, lymph node, lung, and liver of *Klrk1*$^{-/-}$ mice (Figures 1A and 1B, data not shown) but lacked NKG2D surface expression (Figure 1C), whereas cells from *Klrk1*$^{+/-}$ mice showed modestly reduced cell-surface expression of NKG2D compared to wild-type mice (Figure 1C).

Klrk1$^{-/-}$ mice had normal numbers and proportions of CD4^{+} and CD8^{+} T cells, TCRγδ T cells, NKT cells, and B cells in the spleen, bone marrow, and lymph nodes (Figure S2, and data not shown). The frequency of CD8^{+}CD44^{+} memory T cells in the spleen was also normal (data not shown). NK subsets defined by CD11b and CD27 were not substantially different, whereas various maturation markers including NK1.1, CD11b, DX5, CD122, and CD43 were expressed normally (Figures 1A and 1B). The mutant mice had normal or minor differences in the expression of various stimulatory and inhibitory receptors including NK1.1, 2B4, Ly49D, Ly49C, Ly49I, Ly49F, KLRG1, Ly49G2, Ly49A, NKp46, CD94, and NKG2A (Figure S3, Figure 1, data not shown). Together, these data indicate that NKG2D expression is dispensable for normal phenotypic development of NK cells, B cells, and T cells.

NKG2D Deficiency Results in a Higher Incidence of Highly Malignant Prostate Adenocarcinomas

We investigated tumor surveillance in vivo by using the well-studied transgenic adenocarcinoma of the mouse prostate (TRAMP) model of autochthonous prostate cancer, which mimics human clinical disease (Kaplan-Lefko et al., 2003). In these mice, the rat probasin promoter directs the expression of the SV40 early genes (T and t antigen) to the prostatic epithelium at puberty. In male TRAMP mice, mild to severe prostate hyperplasia develops by 12 weeks of age, and was followed by the appearance of severe hyperplasia and adenocarcinoma by 18 weeks of age and metastatic disease by 30 weeks of age (Huss et al., 2001; Kaplan-Lefko et al., 2003). Previous studies demonstrated heterogeneity of TRAMP tumors, which varies with genotype (Degrassi et al., 2007; Gingrich et al., 1999). Aggressive early-arising carcinomas were large and progressed rapidly to poorly differentiated (PD) lesions as defined histologically, which is indicative of poor prognosis in human prostate cancer patients.
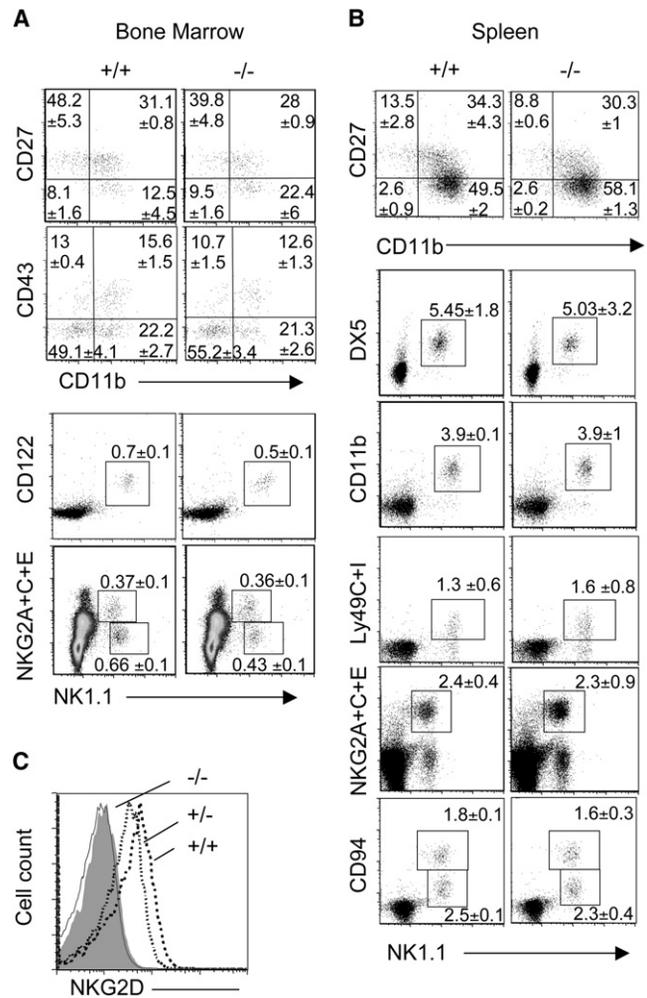
Figure 1. NK Cell Development Is Preserved in NKG2D-Deficient Mice

(A and B) Analysis of NK subsets in the bone marrow (BM, n = 3, [A]) and spleen (n = 3–5, [B]) of NKG2D-deficient *Klrk1*$^{-/-}$ (*neo* cassette deleted) mice and *Klrk1*$^{+/+}$ littermate controls. The top groups in (A) and (B) represent gated NK1.1^{+}CD3^{-} cells, whereas the remaining panels represent gated CD3^{-} cells. The numbers represent mean percentages (±SD) of cells expressing the indicated markers.

(C) Representative NKG2D staining on freshly isolated NK1.1^{+}CD3^{-} splenic NK cells is shown for *Klrk1*$^{-/-}$ mice with the *neo* cassette deleted and littermate controls. Isotype control stain is shown as shaded histogram. The bone marrow analysis was repeated in one additional independent experiment, and the spleen cell analysis was repeated in two additional independent experiments, with similar results.

A late-arising form exhibited persistent well differentiated (WD) and moderately differentiated (MD) lesions and progressed less rapidly to PD lesions (Degrassi et al., 2007). The aggressive form was generally substantially rarer in B6 TRAMP mice than in mice of mixed FvB-B6 genotype (Gingrich et al., 1999).

For our studies, B6-*Klrk1*$^{-/-}$ mice (*neo* cassette deleted) were crossed with B6-TRAMP mice, and the pups were intercrossed to generate male *Klrk1*$^{-/-}$ TRAMP mice (n = 43) as well as wild-type (*Klrk1*$^{+/+}$) TRAMP littermates (n = 33) for comparison. Male mice were monitored after puberty for development of prostate tumors. Animals were euthanized when they developed

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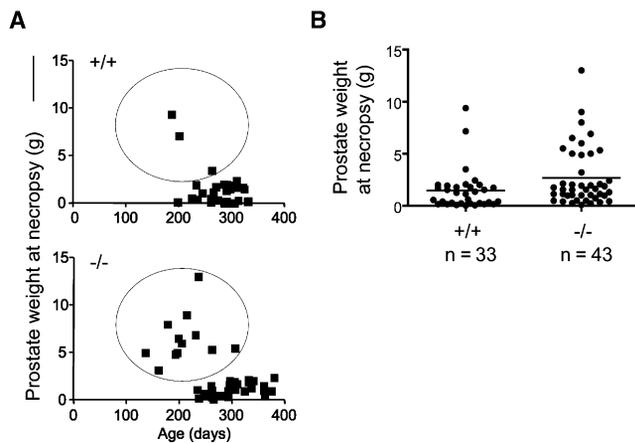
Figure 2. Increased Incidence of Large, Early Prostate Carcinomas in *Klrk1*$^{-/-}$ TRAMP Mice

(A) Each square represents the weight of the prostate tissue and age at necropsy of individual *Klrk1*$^{+/+}$ TRAMP (n = 33) (upper panel) or *Klrk1*$^{-/-}$ TRAMP littermates (n = 43) (lower panel) mice. Large, early tumors (>2 SEM greater than the mean weight, circled) were more frequent in *Klrk1*$^{-/-}$ mice (p = 0.029 by Fisher's exact test).

(B) The average weights of prostate tumors at necropsy is depicted for both cohorts (p = 0.0184 by Mann-Whitney test).

tumor masses as detected by palpation or were moribund. Because all TRAMP mice develop tumors, the question was whether NKG2D deficiency results in more aggressive adenocarcinomas or alters the kinetics of tumorigenesis. Strikingly, early-arising, massive prostate tumors were rare in the wild-type mice and three times more frequent in the NKG2D-deficient littermates. Defined as those that exceeded the mean mass by more than 2 standard errors of the mean (SEMs) (i.e., > 2.7 g), the large tumors arose in 12 of the 43 *Klrk1*$^{-/-}$ mice (27.9%) compared to only 3 of the 33 wild-type mice (9.1%) (p = 0.029 by Fisher's exact test) (Figure 2A). As a result, the mean weight of prostate tissue at necropsy in the *Klrk1*$^{-/-}$ cohort significantly exceeded that of *Klrk1*$^{+/+}$ TRAMP mice (p = 0.0184 by Mann-Whitney test) (Figure 2B). Prostate carcinomas were not detected in the nontransgenic *Klrk1*$^{-/-}$ males under survey (data not shown).

Histological examination of some of the tumors that arose in this analysis demonstrated that all ten of the large tumors that

we examined, eight from *Klrk1*$^{-/-}$ mice and two from wild-type mice, exhibited nearly uniform PD lesions, whereas only a minority of the late-arising tumors (4/25) exhibited PD lesions, in most cases multifocally (Table 1, Figure S4, data not shown). WD lesions were detected in most of the late-arising tumors (14/25), whereas more than a quarter of the late-arising tumors from mice of either genotype were classified as phylloides-like tumors, a nonmalignant epithelial-stromal tumor (Tani et al., 2005) (Table 1). In conclusion, the histology data confirm the highly malignant status of the early-arising tumors. Thus, NKG2D-deficient mice are three times more susceptible than wild-type littermates to highly malignant, early-arising prostate adenocarcinoma, demonstrating that NKG2D-mediated immune mechanisms limit the development of the most aggressive form of prostate cancer in B6-TRAMP mice.

Consistent with the role of NKG2D in surveillance of prostate adenocarcinomas, an analysis of dissociated prostate tumors from a separate cohort of *B6-Klrk1*$^{+/+}$ TRAMP mice showed common, though sporadic, expression of NKG2D ligands on the cell surface, whereas normal prostate dissociations did not express ligands (Figures 3A and 3B). Interestingly, however, ligand expression was largely restricted to the smaller late-arising tumors and absent from the large early-arising ones in these wild-type mice (Figure 3B). The results raise the possibility that loss of NKG2D ligands by large aggressive TRAMP tumors reflects NKG2D-dependent immunoselection or editing (Dunn et al., 2002). Consistent with this possibility, we observed substantially higher amounts of Rae1 transcripts in large *Klrk1*$^{-/-}$ tumors as compared to large *Klrk1*$^{+/+}$ tumors (mean 2.12 versus 0.14 relative units, respectively, n = 4 each, p = 0.028) (Figure 3C, left panel). Furthermore, both of the dissociated large *Klrk1*$^{-/-}$ tumors we examined showed cell-surface expression of Rae1 and MULT1 (Figure 3D) whereas those from *Klrk1*$^{+/+}$ mice did not. These data suggest that NKG2D-dependent immunoselection (or editing) favors loss of NKG2D ligands on early-arising, aggressive tumors. The smaller late-arising tumors, in contrast, expressed similar amounts of transcripts for NKG2D ligands whether they arose in *Klrk1*$^{-/-}$ or wild-type mice (Figure 3C, right panel, and data not shown) and displayed the ligands at the cell surface (Figure 3B and data not shown). Despite expressing NKG2D ligands, these tumors were apparently refractory to NKG2D-mediated surveillance, suggesting that they were

Table 1. Histological Analysis of Prostate Tumors from *Klrk1*$^{+/+}$ TRAMP and *Klrk1*$^{-/-}$ TRAMP Mice

Tumor Size and Histology	Genotype of TRAMP Mice		
	Klrk1$^{+/+}$ (n = 13)	*Klrk1*$^{-/-}$ (n = 22)	Combined
Large tumors[a]	2/13[b]	8/22	10/35
PD adenocarcinoma	2/2, 100%	8/8, 100%	10/10, 100%
Smaller tumors	11/13	14/22	25/35
PD adenocarcinoma	1/11, 9.1%	3/14, 21.4%	4/25, 16.0%
WD adenocarcinoma	7/11, 63.4%	7/14, 50.0%	14/25, 56.0%
Phylloides (nonmalignant)	3/11, 27.2%	4/14, 28.6%	7/25, 28.0%

[a] Large tumors were defined as those exceeding the mean by more than 2 standard errors of the mean (i.e., > 2.7 g).
[b] The entries represent the number of large or smaller tumors over the total number of tumors of each genotype examined histologically followed by the percentage of large or smaller tumors that exhibit the indicated histology. Histological classification was based on the maximal histological grade observed for a given animal. Poorly differentiated (PD) lesions are most aggressive, whereas well differentiated (WD) lesions less aggressive and Phylloides tumors are nonmalignant.
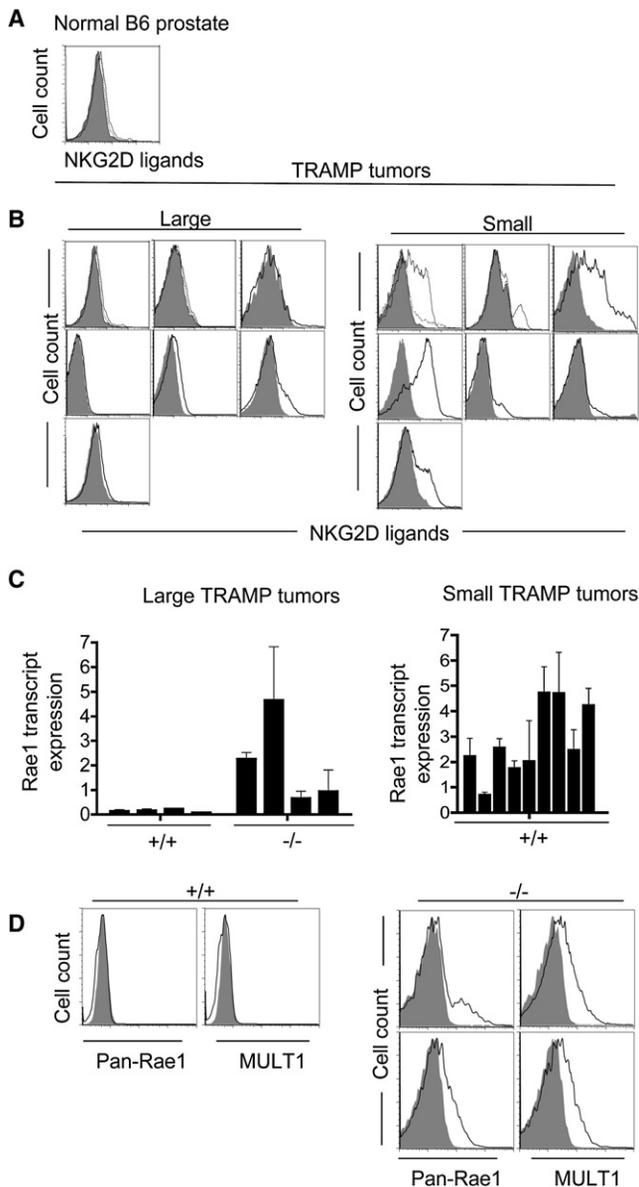
Figure 3. Expression of NKG2D Ligands by Prostate Carcinomas Ex Vivo

(A and B) Reduced cell-surface expression of NKG2D ligands on large, early-arising B6-TRAMP prostate tumors as compared to smaller, late-arising ones. Freshly dissociated prostate tissue from a representative nontransgenic $Klrk1^{+/+}$ B6 mouse (A) and sets of large and smaller tumors isolated from $Klrk1^{+/+}$ B6-TRAMP mice (B) were compared. Labeled NKG2D tetramers (plain line) were used to detect all NKG2D ligands, and streptavidin-PE served as a negative control (shaded histogram). In a few cases, the specificity of staining was proved by inhibition with unlabeled NKG2D tetramers (dotted line).

(C) Reduced amounts of Rae1 transcripts in large (>2.7 g) $Klrk1^{+/+}$ TRAMP tumors as compared to large $Klrk1^{-/-}$ TRAMP tumors (p = 0.0286 with the Mann-Whitney test) (left panel) or smaller $Klrk1^{+/+}$ TRAMP tumors (right panel). Values determined by quantitative reverse transcriptase (RT)-PCR with primers that detect all Rae1 isoforms were normalized to HPRT transcript amounts, and these data were normalized to the amount in nontransgenic B6 prostates (average of five independent B6 mice). Graphs show the mean ± SD of two to six separate quantitative PCR (qPCR) assays for each sample.

(D) Cell-surface staining of Rae1 and MULT1 (solid line) on two dissociated large $Klrk1^{-/-}$ TRAMP tumors and a large tumor from an $Klrk1^{+/+}$ TRAMP litter-

sequestered from NKG2D-dependent effector mechanisms or evaded them (Groh et al., 2002; Lee et al., 2004).

Upon necropsy, the $Klrk1^{+/+}$ and $Klrk1^{-/-}$ mice were also screened for macroscopic metastases in liver, lung, and kidney. The percentage of mice with such visible metastases was similar in $Klrk1^{-/-}$ and wild-type mice (approximately 21% in each), raising the possibility that NKG2D functions primarily at an early stage of tumorigenesis rather than at the stage of metastasis.

NKG2D Deficiency Accelerates the Progression of Eµ-Myc-Induced Lymphomas

In order to study the role of NKG2D in the surveillance of lymphoid tumorigenesis, we used Eµ-myc transgenic mice (Adams et al., 1985), in which constitutive c-*myc* oncogene expression in the B cell lineage results in selective formation of B, pre-B, or mixed pre-B and B lymphomas (Harris et al., 1988; Langdon et al., 1986). Genetic crosses were used to generate B6-$Klrk1^{-/-}$ Eµ-myc transgenic mice (n = 24) and B6-$Klrk1^{+/+}$ Eµ-myc transgenic littermates (n = 34), which were monitored twice weekly after birth for palpable tumors and signs of illness as manifested by lymphadenopathy, tachnypea, and hunched posture. Only mice showing enlarged lymphoid organs at necropsy were designated as having lymphomas. Myc-driven lymphomas arose significantly earlier in $Klrk1^{-/-}$ mice than in $Klrk1^{+/+}$ mice, with the median time of onset occurring more than 7 weeks earlier in the $Klrk1^{-/-}$ mice (p = 0.005 with the log-rank test) (Figure 4A). None of the nontransgenic $Klrk1^{-/-}$ or $Klrk1^{+/+}$ mice developed lymphoma during a comparable period of monitoring (data not shown).

Light-scatter analysis of spleen and lymph node cell samples of all the affected mice showed increased frequencies of lymphoblasts compared to nontransgenic samples, suggesting the presence of tumor cells. Tumors arising in both $Klrk1^{+/+}$ (n = 7) and $Klrk1^{-/-}$ (n = 5) Eµ−myc transgenic mice expressed B220 but were heterogeneous with respect to the amount of B220 expression and IgM expression, suggesting that B, pre-B, and mixed pre-B and B cell lymphomas were represented (Figure 4B and data not shown). We detected no differences in the distribution of these tumor types when comparing this small sample of tumors from $Klrk1^{+/+}$ versus $Klrk1^{-/-}$ mice. NKG2D ligands Rae1 and MULT1 were not expressed on nontransgenic B cells but were detected at varying amounts on all the tumor samples (Figure 4B and data not shown). The amounts of both ligands varied from tumor to tumor in mice of both genotypes, with MULT1 expression being more common. There was no indication in this survey that expression of NKG2D ligands was selected against in $Klrk1^{+/+}$ mice, despite the clear evidence that NKG2D-mediated surveillance is operative for these lymphomas. These data suggest that evasion of NKG2D-mediated surveillance by Eµ-myc-induced lymphomas occurs by mechanisms that do not depend on loss of NKG2D ligands.

NKG2D Deficiency Does Not Affect the Incidence of Carcinogen-Induced Sarcomas

We addressed the role of NKG2D in the well-studied 3-methylcholanthrene (MCA)-induced carcinogenesis model. $Klrk1^{-/-}$

mate. Isotype control stains are shown as shaded histograms. Gated CD45-negative (nonhematopoietic), PI-negative (live) cells were examined.

A

B
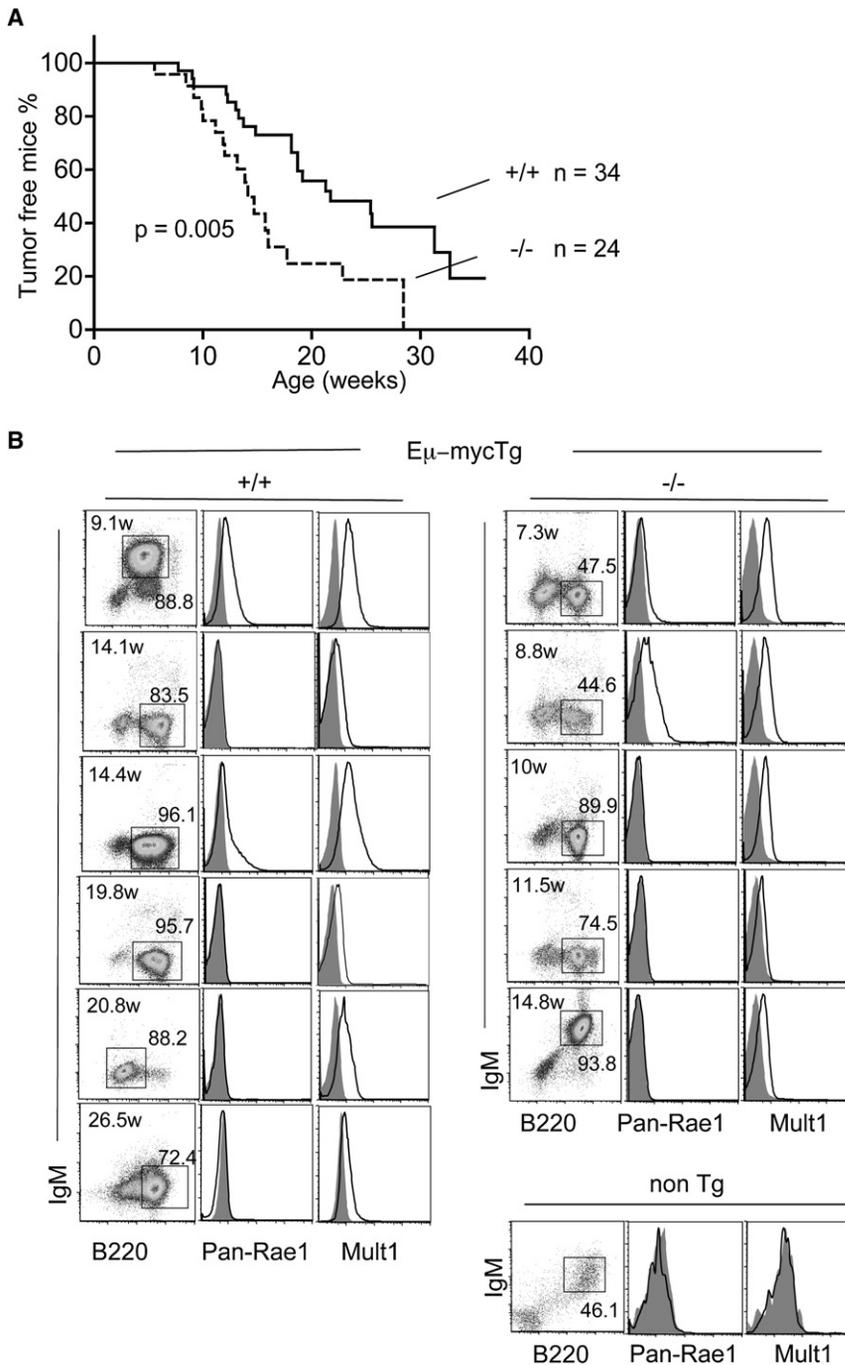
Figure 4. Accelerated Onset of Myc-Driven Lymphomas in *Klrk1*$^{-/-}$ Mice

(A) Kaplan-Meier representation of tumor progression among *Klrk1*$^{-/-}$ *Eμ-myc* transgenic (n = 24) and *Klrk1*$^{+/+}$ *Eμ-myc* transgenic mice (n = 34). p = 0.005 by the log-rank test.

(B) Ex vivo analysis of lymph node cell suspensions isolated from *Klrk1*$^{+/+}$ *Eμ-myc*, *Klrk1*$^{-/-}$ *Eμ-myc*, and nontransgenic *Klrk1*$^{+/+}$ mice, as indicated. Dot plots show B220 and IgM expression on the blast (high forward scatter) population from independent mice that were examined after the onset of illness. The age (weeks) at necropsy is specified for each mouse, as well as the percentage of cells included in the gate. The histograms show pan-Rae1 and MULT1 expression (solid line) on the gated populations indicated in each dot plot. The shaded histograms represent the staining with an isotype control antibody.

foundly repress the expression of neighboring genes (Kim et al., 1992; Pham et al., 1996; Xu et al., 1996), and many of the genes near NKG2D encode NK receptors, the results with *neo*-in mice are clearly unreliable compared to results in mice where the *neo* cassette is deleted.

Analysis of numerous freshly dissociated advanced fibrosarcomas from the *neo*-in or *neo*-deleted mice showed a great deal of heterogeneity in ligand expression regardless of *Klrk1* genotype, with some tumors expressing ligands at a very low level or not at all (data not shown). Hence, there was no evidence from these studies that NKG2D selected for loss of NKG2D ligands by tumor cells in this model. In conclusion, the present data do not support an important role for NKG2D in surveillance of MCA-induced sarcomas in vivo.

Klrk1$^{-/-}$ NK Cells Are Defective in NKG2D Function but Retain Other NK Cell Functions

The deficient tumor surveillance in *Klrk1*$^{-/-}$ mice could arise if the mutation resulted in impaired development of NK cells, an NK cell subset, or NK cell effector function, as opposed to a selective deficiency in NKG2D function. As expected, sorted interleukin-2 (IL-2)-activated NK cells from *Klrk1*$^{-/-}$ mice were devoid of lytic activity against RMA target cells transfected with the Rae1ε NKG2D ligand (Figure 6A). *Klrk1*$^{+/-}$ NK cells, which had lower expression of NKG2D than wild-type cells, mediated reduced lysis of these target cells. In contrast, *Klrk1*$^{-/-}$ NK cells were only partially impaired in their capacity to lyse tumor cell lines that naturally express NKG2D ligands, such as YAC-1 and C1498, comparable to the activity of wild-type NK cells in the presence of NKG2D antibody (Figures 6B and 6C).

mice (in which the *neo* cassette was deleted) and *Klrk1*$^{+/+}$ littermates were injected with two doses of MCA, and fibrosarcomas arose at the site of subcutaneous carcinogen application after a latency period. With a dose of 25 μg MCA, fibrosarcoma incidence was identical in *Klrk1*$^{-/-}$ and *Klrk1*$^{+/+}$ mice (Figure 5A, p = 0.69), whereas with 5 μg MCA, the incidence was, if anything, lower in *Klrk1*$^{-/-}$ mice, though not significantly (Figure 5B, p = 0.1). Surprisingly, a separate initial analysis of *Klrk1*$^{-/-}$ mice that retained the *neo* cassette showed an increased incidence of MCA-induced tumors (data not shown). However, because the PGK-*neo* cassette inserted in a locus can pro-

Figure 5. Comparable Incidence of MCA-Induced Fibrosarcomas in NKG2D-Deficient Mice and Wild-Type Littermates

$Klrk1^{-/-}$ (dashed line) and wild-type littermates (solid line) (*neo* cassette deleted) were injected subcutaneously (s.c.) with 25 μg (A) or 5 μg (B) of MCA) and monitored for tumor development. Tumor-bearing mice were defined by the presence of a mass of at least 7 mm diameter growing upon two consecutive measurements. These data were compiled from two independent experiments. p values are based on the log-rank test.

These data validate the findings of earlier studies suggesting that recognition by NKG2D accounts for only part of the activity of NK cells against YAC-1 and C1498 tumor cells (Jamieson et al., 2002). Moreover, NKG2D-deficient NK cells showed no defect in lysis of the MHC class I-low RMA-S cell line, which lacks NKG2D ligands and is recognized by an unknown stimulatory NK receptor other than NKG2D (Figure 6D). These data support the conclusion that the *Klrk1* mutation selectively disables recognition by NKG2D and does not alter functional recognition by other NK receptors.

We examined the responsiveness of $Klrk1^{-/-}$ NK cells to direct crosslinking of stimulatory receptors other than NKG2D by using plate-bound antibodies specific for stimulatory receptors. Crosslinking of NK1.1 or Ly49D resulted in similar interferon-γ

(IFN-γ) production by $Klrk1^{-/-}$ and $Klrk1^{+/+}$ NK cells. $Klrk1^{-/-}$ and $Klrk1^{+/+}$ NK cells also responded similarly to pharmacological stimuli provided by phorbol 12-myristate 13-acetate (PMA) plus ionomycin (Figure 6E). These data provide direct evidence that the activating functions of stimulatory NK receptors other than NKG2D are unaffected by the *Klrk1* mutation.

As a measure of NK functions in vivo in NKG2D-deficient mice, we tested whether NKG2D participates in "missing self" recognition of bone marrow cells in vivo. Irradiated $Klrk1^{+/+}$ or $Klrk1^{-/-}$ B6 mice were challenged with class I-deficient ($B2m^{-/-}$), B6 strain bone marrow cells, mixed in the same inoculum with wild-type bone marrow cells that served as an internal negative control (Fernandez et al., 2005). $Klrk1^{+/+}$ and $Klrk1^{-/-}$ recipient mice rejected the class I-deficient bone marrow grafts similarly,
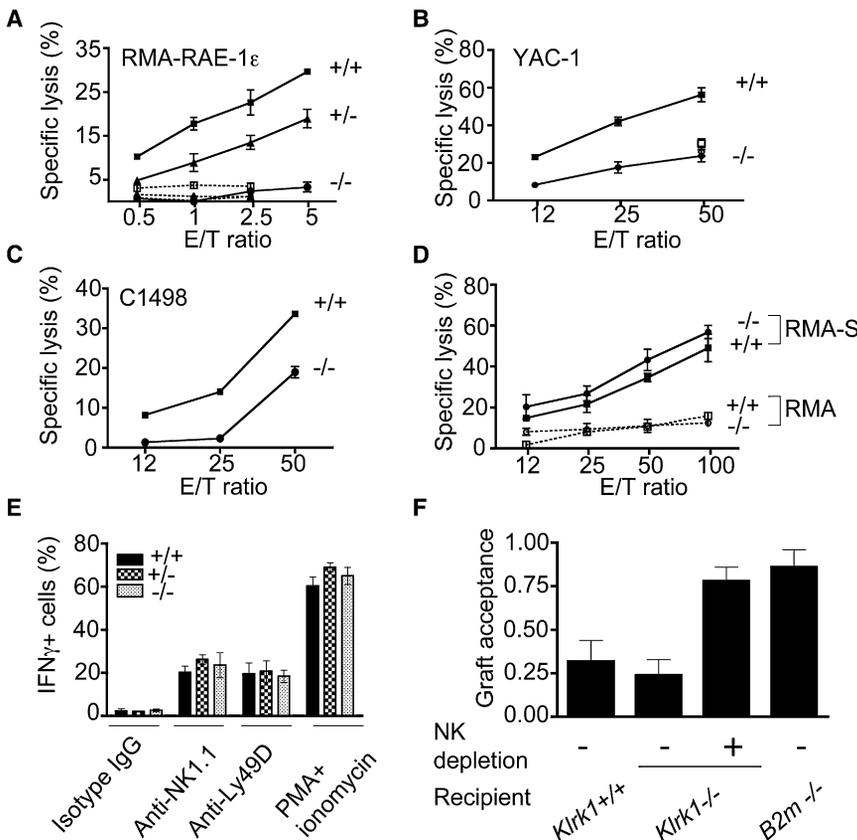
Figure 6. NKG2D Deficiency Does Not Impair NK Cell Functions In Vitro or In Vivo

(A–C) IL-2-activated splenic NK cells (sorted NK1.1+CD3− cells in [A], unsorted cells in [B] and [C]) from $Klrk1^{-/-}$ mice fail to lyse RMA-Rae1ε target cells, and show reduced lysis of YAC-1 and C1498 target cells. Lysis in the presence of NKG2D mAb is shown for $Klrk1^{+/+}$ (open squares) and $Klrk1^{-/-}$ (open circles) effector cells in (A) and (B). Results represent means ± SD.

(D) Normal lysis of class I-low RMA-S cells by $Klrk1^{-/-}$ IL-2-activated NK cells. Results represent means ± SD obtained with $Klrk1^{-/-}$ mice (*neo* cassette retained) and littermate controls.

(E) Splenocytes from $Klrk1^{-/-}$ mice (*neo* cassette deleted) and littermate controls (n = 5 for each genotype) were stimulated in vitro for 5 hr on plates coated with NK1.1 mAb (PK136, 40 μg/ml), Ly49D mAb (SED85, 10 μg/ml), or control mouse immunoglobin G (IgG) (40 μg/ml) in the presence of Golgi plug before staining and analysis. In parallel, NK cells were stimulated with or without a mixture of PMA and ionomycin. Intracellular IFN-γ was detected by flow cytometry on gated NK1.1+CD3− cells, except for the anti-NK1.1-stimulated cells, which were gated on DX5+CD3− cells. Results represent means ± SD (n = 5).

(F) In vivo rejection of $B2m^{-/-}$ bone marrow cells by $Klrk1^{-/-}$ mice. A mixture of CFSE- (5 μM) labeled BM cells from C57Bl/6-Ly5.2 and $B2m^{-/-}$ C57Bl/6-Ly5.1 mice was injected i.v. in irradiated $Klrk1^{+/+}$ (n = 4), $Klrk1^{-/-}$ (n = 6), negative control C57Bl/6 $B2m^{-/-}$ (n = 2), or NK-depleted $Klrk1^{-/-}$ (n = 3) recipients. Similar results were obtained in one additional independent experiment.

and depletion of NK1.1^{+} cells with PK136 monoclonal antibody (mAb) prevented rejection by $Klrk1^{-/-}$ mice (Figure 6F). As expected, $B2m^{-/-}$ recipient mice ($Klrk1^{+/+}$) failed to reject class I-deficient bone marrow grafts (Bix et al., 1991). These data indicate that NKG2D is not required for rejection of class I-deficient, B6 strain bone marrow grafts, in line with other studies suggesting no role for NKG2D in rejection of B6 bone marrow grafts by F1 mice (Ogasawara et al., 2005). Thus, the present data demonstrate that NKG2D deficiency does not globally impair NK cell functions in vivo.

DISCUSSION

By using $Klrk1$ gene-targeted mice, we have provided direct genetic evidence for a role of NKG2D in surveillance of primary prostate adenocarcinoma and B lymphoma. Transgenic models of spontaneous malignancy, such as those used here, are usually considered reliable models of human cancer in comparison to high-dose carcinogen models, which may not accurately mimic spontaneous human tumors in many respects (Prehn, 1975). The effect on tumor incidence is likely to be a direct effect of NKG2D deficiency because we found no substantial alterations in development of NK cells or other cells or in NK cell functions other than those mediated by NKG2D.

Although the role of NKG2D in such models of tumorigenesis has not been previously investigated, it was previously reported that methylcolanthrene-induced tumors were more frequent when mice were treated long-term with blocking NKG2D antibodies (Smyth et al., 2005). In our studies of the MCA carcinogenesis model, however, we failed to observe an increased incidence of MCA-induced sarcomas in $Klrk1^{-/-}$ mice from which the neo cassette was deleted, with both standard and limiting MCA doses. The basis of the discrepancy is unknown, but one possibility is that sustained exposure to high doses of NKG2D antibodies in vivo, like exposure of NK cells to NKG2D ligands in vivo (Coudert et al., 2005; Oppenheim et al., 2005), causes generalized NK cell defects that could impair NK cell-mediated rejection of target cells recognized via distinct NK receptors. Alternatively, long-term antibody treatment, including the presence of complexes of injected antibodies and host anti-antibodies, could alter the immune response in unpredictable ways.

Previous studies provided evidence for expression of NKG2D ligands on primary human tumors (Farag et al., 2002; Groh et al., 1999; Salih et al., 2003), but there was little data on ligand expression by primary mouse tumors. The data herein include valuable evidence that NKG2D ligands are expressed at the surface of freshly isolated primary tumor cells ex vivo in two mouse tumor systems. It was interesting that at the time of necropsy, there was considerable heterogeneity in the amounts and identity (Rae1 versus MULT1) of ligands expressed by different tumors. The heterogeneity cannot be attributed mainly to selection by NKG2D-dependent mechanisms because it was prevalent among the tumors arising in NKG2D-deficient mice. It appears likely, therefore, that the signals that regulate ligand expression are heterogeneous in advanced tumor cells or that ligands are in some cases spontaneously downregulated or shed from the surface of advanced tumor cells, as has been reported elsewhere for human tumors (Eisele et al., 2006; Holdenrieder et al., 2006; Kaiser et al., 2007).

Despite this heterogeneity of ligand expression in advanced tumors, it is likely that nascent tumors express ligands in a more consistent fashion in at least some of the tumor types we studied. It seems probable that this is true in the case of early-arising prostate carcinomas and Eμ-myc lymphomas because in both models, NKG2D surveillance had a substantial effect on the incidence or time of onset of palpable tumors, which would not be expected if many emerging tumors expressed no ligands or low amounts of ligands.

Early surveillance would be in accord with published evidence that NKG2D ligands are induced by the DNA-damage response (Gasser et al., 2005), which is known to be activated at an early stage in tumorigenesis (Bartkova et al., 2005; Gorgoulis et al., 2005). Furthermore, it has been shown that oncogene-induced stress, such as that which occurs as a result of myc overexpression, activates the DNA-damage-response pathway (Dominguez-Sola et al., 2007; Reimann et al., 2007; Vafa et al., 2002). Upregulation of NKG2D ligands as a consequence of these and other relatively early events in tumorigenesis may sensitize emerging tumor cells to elimination by NKG2D-dependent mechanisms.

Immunoediting, also called immunoselection, is a process where immune responses select for variant tumor cells that have lost expression of specific molecules or antigens that are targeted by immune effector cells (Dunn et al., 2004). Immunoediting is therefore a likely explanation for our finding that early arising tumors from $Klrk1^{+/+}$ mice lacked NKG2D ligands, whereas tumor cells from $Klrk1^{-/-}$ mice did not. We lack evidence, however, that the Rae1-negative tumor cells that arise in $Klrk1^{+/+}$ mice are the progeny of Rae1^{+} tumor cells. A possible prediction of the immunoediting hypothesis is that transfer to wild-type mice of Rae1^{+} or MULT1^{+} tumors from $Klrk1^{-/-}$ mice should lead to rejection, whereas transfer of tumors devoid of Rae1 and MULT1 expression from $Klrk1^{+/+}$ mice should not. Such a study is not currently possible, however, because we have so far been unable to successfully and reproducibly engraft primary prostate tumors procured from TRAMP mice into syngeneic hosts (N.R.G., unpublished data).

In contrast to the results with early-arising TRAMP tumors, expression of NKG2D did not influence NKG2D ligand expression by late-arising TRAMP tumors or Eμ-myc lymphomas, at least at the advanced stages examined. Eμ-myc lymphomas differed from late-arising TRAMP tumors, however, in that Eμ-myc lymphomas were subject to NKG2D-dependent immune surveillance, whereas the late-arising prostate carcinomas were neither delayed nor less frequent in hosts that expressed NKG2D as compared to $Klrk1^{-/-}$ hosts, suggesting that they were not subject to NKG2D-dependent immune surveillance.

Taken together, these data suggest that the role of NKG2D-dependent surveillance differs in the three types of tumors studied here. In the case of early-arising prostate carcinomas in TRAMP mice, many of the tumors are eliminated, and the few that are not eliminated evade surveillance by extinguishing expression of NKG2D ligands. In the case of Eμ-myc lymphomas, it appears that the emerging tumors are mostly NKG2D sensitive, but a fraction of tumors escape NKG2D surveillance without losing NKG2D ligands. Several possible evasion mechanisms can be envisaged, including shedding of NKG2D ligands from tumor cells or other mechanisms that inactivate NKG2D^{+} effector cells (Groh

et al., 2002; Lee et al., 2004); the loss of adhesion molecules or properties necessary for these cells to be recognized by NK cells and T cells; or rapid tumor growth that outpaces elimination by NKG2D^{+} cells, and which may ultimately cause local or systemic desensitization of NKG2D^{+} cells (Coudert et al., 2005; Oppenheim et al., 2005). The final category is represented by late-arising prostate carcinomas, which appear to be generally refractory to NKG2D-dependent surveillance. It is possible that this type of tumor is sequestered from NKG2D-dependent immune responses, locally activates inhibitory processes or regulatory cells that inactivate NKG2D^{+} cells (Lee et al., 2004), or expresses NKG2D ligands in a delayed fashion compared to the early-arising ones.

The data herein represent genetic evidence for a role of the NKG2D receptor in tumor surveillance. The findings add to a growing number of studies with gene-targeted mice that have re-energized the immune surveillance theory, by showing roles for specific immune cells or functions in protection from cancer (Girardi et al., 2001; Shankaran et al., 2001; Smyth et al., 2000; van den Broek et al., 1996). The present findings are unique in showing the role of a specific innate immunity receptor, NKG2D. Considering that NKG2D is expressed by numerous T cell subsets, the relative roles of NKG2D expressed by NK cells or T cells is an important issue that will be addressed in the future. Regardless of the outcome, the data suggest that interventions to enhance the NKG2D axis of immunity and/or prevent its deregulation may have applications in cancer immunotherapy (Diefenbach et al., 2001; Jinushi et al., 2006; Zhang et al., 2006; Zhou et al., 2006). Conversely, the observation that different subtypes of prostate tumors vary in their susceptibility to NKG2D-dependent surveillance suggests that such applications will need to be tailored to specific cancer subtypes.

EXPERIMENTAL PROCEDURES

Mice

All mice were bred at the University of California, Berkeley, in compliance with institutional guidelines. C57BL/6J (B6, H-2[b]), B6-Ly5.1 mice (catalog name, B6-Ly5.2/Cr), and Eμ-myc transgenic mice [catalog name, C57BL/6J-Tg (IghMyc)22Bri/J] were purchased from the Jackson Laboratories (Bar Harbor, ME). B6-$B2m^{-/-}$-Ly5.1 mice were derived in our facilities from B6-$B2m^{-/-}$ mice (Zijlstra et al., 1990). C57BL/6 mice harboring a CMV-Cre transgene were generated from a CD1-Cre transgenic strain provided by Dr. A. Nagy (Samuel Lunenfeld Research Institute, Toronto, Canada) by backcrossing to B6. C57BL/6 Tg-TRAMP mice were screened for the presence of the transgene by polymerase chain reaction (PCR) as previously described (Greenberg et al., 1995). C57BL/6 Tg-Eμ-myc mice were screened for the presence of the transgene by PCR with the following primers: forward 5′-CAGCTGGCGTAAT AGCGAAGAG-3′ reverse 5′-CTGTGACTGGTGAGTACTCAACC-3′. Mice were euthanized by CO_2 inhalation in accord with the policies of the Office of Laboratory Animal and Care (OLAC) at the University of California, Berkeley.

Generation of NKG2D-Deficient Mice

Bruce-4 embryonic stem (ES) cells derived from C57BL/6 mice (Lemckert et al., 1997) were kindly provided by Dr. J.D. Sedgwick (Schering-Plough Biopharma, Palo Alto, CA) and transfected with a targeting vector (pKSTKNeoLoxP) designed to delete the exons 1b to 6, encoding the cytoplasmic, transmembrane domains and partial extracellular domain of NKG2D (Figure S1A). The vector contained the neo cassette and was flanked by a thymidine kinase (TK) cassette. Clones resistant to G418 and Gancyclovir were screened by Southern blotting with a probe flanking the 3′ short arm (Figure S1B), and two positive clones were confirmed by blotting with a 5′ probe (which hybridizes to the long arm) and a neo probe (data not shown). One targeted clone, BB9, when injected into BALB/c blastocysts, gave rise to viable chimeras that trans-

mitted the disrupted Klrk1 allele when crossed with C57BL/6J (B6/J) females. The $Klrk1^{+/-}$ B6 progeny of the chimeras were crossed again back to B6/J and then intercrossed to produce mice of all three genotypes. The same $Klrk1^{+/-}$ mice were separately crossed to a B6 transgenic strain that expresses Cre recombinase in the germline. Cre-transgene[+], $Klrk1^{+/-}$ offspring were bred back to B6/J mice, and Cre-transgene-negative, $Klrk1^{+/-}$ offspring that had deleted the neo cassette were identified by PCR and Southern blot and interbred to produce neo-deleted, Cre-transgene-negative mice of all three Klrk1 genotypes (+/+, +/−, and −/−). NKG2D-deficient mice were screened for the Klrk1 deletion by PCR with the following primers in the same PCR reaction: WT3 5′-CAGAGCAAGCTTCCTGTTTGTCTCA-3′; L1 5′-CAAGTAGTGTGCATTTCATT CAG-3′; and p3 5′-ATTGCTCCCTGTCTCATTGTCTT-3′. The amplification was carried out as follows: 94°C for 4 min, 36 cycles for 94°C for 50 s, 55°C for 1 min 20 s, 72°C for 1 min, and a final step at 72°C for 10 min. When resolved on a 2% agarose gel, the PCR yielded bands of 422 bp for the wild-type allele and 317 bp for the mutant allele.

Carcinogenesis

Aged-matched males were shaved and injected subcutaneously with 25 μg or 5 μg of MCA (Sigma Chemical, St. Louis, MO) dissolved completely in olive oil by heating in boiling water. Mice were examined twice weekly for at least 230 days for palpable tumors at the site of injection. Progressively growing masses of 7 mm in diameter or larger were scored as tumors.

Tumor Dissociation and Cell Staining

Solid tumors (MCA-induced fibrosarcomas and the prostate tissues of TRAMP mice) were dissected and minced in CO_2-independent medium (GIBCO) containing collagenase D (1 mg/ml, Roche Applied Science, Indianapolis) and DNase (1 μg/ml, Roche Applied Science). The tissue was incubated for 1 hr with shaking at 37°C before being passed through a strainer. The suspended cells were washed twice with Dulbecco's modified Eagle's medium (DMEM) 10% fetal calf serum (FCS) and part of the single-cell suspension was used for ex vivo staining. Enlarged spleen and lymph nodes from Eμ-myc Tg mice were dissected and dissociated in RPMI 10% FCS medium, and red blood cells and dead cells were removed by lympholyte treatment (Lympholyte M, Cederlane laboratories, Ontario, Canada). Surface staining was performed on freshly isolated tumor cells after a preincubation step with CD16 mAb to block the Fc receptors. Staining reagents included CD45-PECY5 mAb (eBioscience), pan Rae1-PE mAb (R&D Systems, Minneapolis, MN), anti-MULT1 (R&D Systems), and a distinct anti-MULT-1, kindly provided by Dr. S. Jonjic (University of Rijeka, Croatia) that was biotinylated in our lab (used for studies in Figure 4). NKG2D tetramer-biot-SAPE has been described (Diefenbach et al., 2000). Flow histograms obtained from solid tumors are shown for live cells by gating out the cells labeled with propidium iodide (PI).

Bone Marrow Graft Rejection

Donor bone marrow graft rejection assays were performed as previously described (Fernandez et al., 2005). In brief, a mixture of 5×10^6 cells each of CFSE-labeled B6-$B2m^{-/-}$-Ly5.1 and B6 (Ly5.2) bone marrow cells was injected intravenously (i.v.) into mice that had been irradiated earlier in the day with 9.5 Gy from a ^{137}Cs source. Recipient spleen cells, harvested 3 days later, were analyzed for CFSE[+] Ly5.1[+] (class I deficient) and CFSE[+] Ly5.2[+] (nonrejection control B6 cells) by flow cytometry. Some mice were predepleted of NK cells by intraperitoneal (i.p.) injection of NK1.1 antibody (Fernandez et al., 2005). Graft acceptance corresponds to the mean (± standard deviation [SD]) ratio of $B2m^{-/-}$ (Ly5.1[+]) to B6 (Ly5.1^{−}) cells among CFSE[+] recipient spleen cells.

Statistical Analysis

Statistical comparisons were performed with the log-rank test on Kaplan-Meier curves depicting lymphomas progression, the Fisher's exact test to evaluate prostate tumor incidence, and the two-tailed Mann-Whitney test assuming unequal variance. $p < 0.05$ denotes significance.

SUPPLEMENTAL DATA

Additional Experimental Procedures and four figures are available at http://www.immunity.com/cgi/content/full/28/4/571/DC1/.

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