NKG2D function protects the host from tumor initiation

Mark J. Smyth,[1] Jeremy Swann,[1] Erika Cretney,[1] Nadeen Zerafa,[1] Wayne M. Yokoyama,[2] and Yoshihiro Hayakawa[1]

[1]Cancer Immunology Program, Trescowthick Laboratories, Peter MacCallum Cancer Centre, East Melbourne, Victoria 3002, Australia
[2]Howard Hughes Medical Institute, Washington University School of Medicine, St Louis, MO 63110

The activation NKG2D receptor has been shown to play an important role in the control of experimental tumor growth and metastases expressing ligands for NKG2D; however, a function for this recognition pathway in host protection from de novo tumorigenesis has never been demonstrated. We show that neutralization of NKG2D enhances the sensitivity of wild-type (WT) C57BL/6 and BALB/c mice to methylcholanthrene (MCA)–induced fibrosarcoma. The importance of the NKG2D pathway was additionally illustrated in mice deficient for either IFN-γ or tumor necrosis factor–related apoptosis-inducing ligand, whereas mice depleted of natural killer cells, T cells, or deficient for perforin did not display any detectable NKG2D phenotype. Furthermore, IL-12 therapy preventing MCA-induced sarcoma formation was also largely dependent on the NKG2D pathway. Although NKG2D ligand expression was variable or absent on sarcomas emerging in WT mice, sarcomas derived from perforin-deficient mice were Rae-1$^+$ and immunogenic when transferred into WT syngeneic mice. These findings suggest an important early role for the NKG2D in controlling and shaping tumor formation.

CORRESPONDENCE
Mark Smyth:
mark.smyth@petermac.org

NKG2D is a key homodimeric activation receptor expressed on the cell surface of almost all NK cells, γδ cells, some cytolytic CD8$^+$ αβ T cells and NKT cells, and a small subset of CD4$^+$ αβ T cells (1–5). Several ligands that bind to NKG2D are members of the MHC class Ib family (5, 6). In humans, the polymorphic MHC class I chain–related molecules (MIC) A and MICB can be recognized by NKG2D (3, 7). Although MIC molecules have not been found in mice, the *retinoic acid early inducible-1* (*Rae-1*) gene products UL16-binding protein-like transcript 1 (Mult1) and a distantly related minor histocompatibility Ag, H60, have been reported as NKG2D ligands in mice (5, 6, 8, 9).

The immune system responds to "stressed self," and stress signals include molecules released by dying cells such as putative endogenous ligands for TLRs (10, 11), uric acid (12), and surface molecules, including MHC Class Ib gene products, that are up-regulated by heat shock, retinoic acid, IFN-γ, TLR signaling, growth factors, viral infection, DNA damage, and UV irradiation (13–17). In particular, unlike conventional MHC class I, the MHC class Ib MIC proteins display up-regulated surface expression on stressed cells and are frequently overexpressed by tumors that are reportedly infiltrated by larger numbers of lymphocytes than are MIC$^-$ tumors (18, 19). In the mouse, the expression of the MHC Class Ib molecules, Rae-1, and H60 is negligible in normal skin but is strongly induced by skin painting with chemical carcinogens (20). Natural or induced expression of NKG2D ligands markedly enhances the sensitivity of tumor cells to NK cells in vitro (2–5, 8, 21). Expression of NKG2D ligands by tumor cells also results in immune destruction in vivo, and the ectopic expression of NKG2D ligands, Rae-1, and H60 in several tumor cell lines results in the rejection of the tumor cells expressing normal levels of MHC class I molecules (6, 22). Immune depletion and other experiments showed that rejection was dependent on NK cells and/or CD8$^+$ T cells and perforin (22, 23). Our more recent study has illustrated that some cytokines mediate their antitumor activity, largely via the NKG2D–NKG2D ligand pathway (24).

However, despite the assumption that NKG2D-mediated engagement of stress-induced

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ligands may be a key aspect of tumor immune surveillance (20, 25), no study has ever evaluated the importance of the pathway in de novo tumorigenesis. We show the importance of the NKG2D activation receptor in controlling the natural and activated host response to spontaneous malignancy.

RESULTS AND DISCUSSION

NKG2D neutralization enhances methylcholanthrene (MCA)–induced sarcoma formation in mice

Neutralizing anti-NKG2D mAbs have the ability to enhance the growth and metastasis of tumors that ectopically or endogenously express NKG2D ligands (22, 23). To evaluate the importance of the NKG2D pathway in de novo tumorigenesis, B6 mice were inoculated with increasing doses of MCA and treated for 8 wk with control or anti-NKG2D mAb (Fig. 1 A). At all MCA doses examined, mice treated with anti-NKG2D mAb had a greater incidence of fibrosarcoma than control Ig–treated mice. Similar increased incidence was obtained when treating mice for more extended periods of time with anti-NKG2D mAb (day 0–140; unpublished data). Sarcoma formation was also assessed in BALB/c mice inoculated with MCA. In concert with the findings in B6 mice, neutralization of NKG2D increased sarcoma formation in BALB/c mice (Fig. 1 B). Thus, in two different strains of mice, the activation receptor NKG2D controls the initiation of MCA-induced sarcoma.

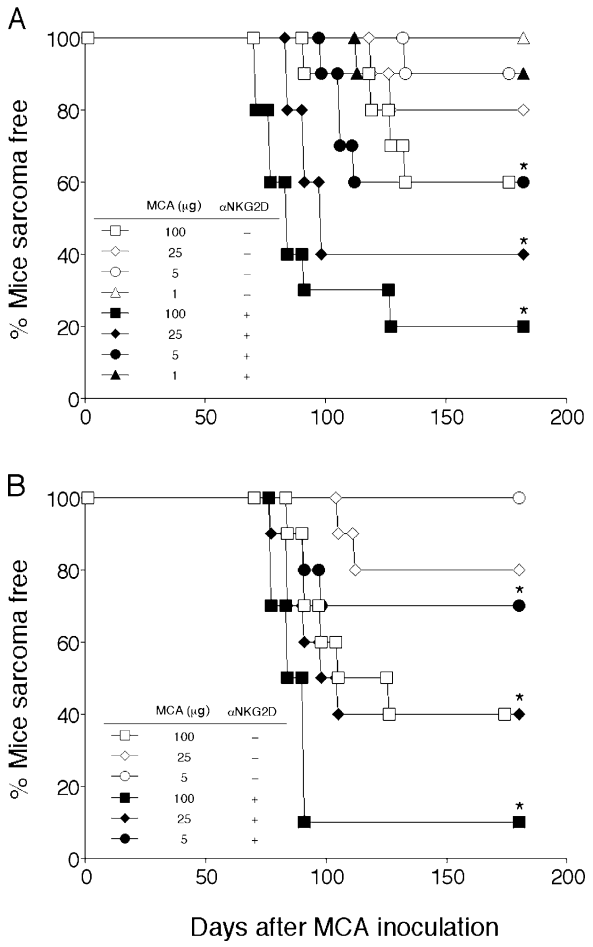

Figure 1. NKG2D neutralization enhances MCA initiation of sarcoma. Groups of 10 B6 WT (A) or BALB/c WT (B) mice were injected s.c. into the hind flank with 1–100 μg MCA as indicated. Mice were treated i.p. twice weekly from day 0 (the day of MCA inoculation) with 250 μg of control Ig (cIg) or anti-NKG2D mAb for 8 wk. Palpable sarcomas (>10 mm^2) were recorded, and tumor-free mice were monitored for 200 d. The asterisks denote a significant effect of anti-NKG2D as determined by Fisher's exact test ($P < 0.05$).

The NKG2D pathway is perforin mediated and only partially responsible for host protection from sarcoma by NK cells or T cells

To determine whether NKG2D neutralization could account for all host NK cell– and T cell–mediated protection from MCA sarcoma, B6 RAG-$1^{-/-}$ and B6 WT mice depleted of NK cells were additionally treated with control or anti-NKG2D mAbs. At a dose of 25 μg MCA, both WT mice depleted of NK cells and RAG-$1^{-/-}$ mice had a similar time of onset and number of sarcomas when treated with anti-NKG2D compared with control Ig (Fig. 2 A). Similar data was obtained at lower doses of MCA (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20050994/DC1). In each case, both WT mice depleted of NK cells and RAG-$1^{-/-}$ mice also demonstrated more sarcomas than WT mice neutralized with anti-NKG2D (Fig. 2 A and Fig. S1). Because control of MCA-induced sarcoma is mediated by a combination of perforin, TRAIL, and IFN-γ effector molecules, we next examined whether NKG2D neutralization in mice deficient in each of these pathways was effective (Fig. 2 B). Clearly, the importance of the NKG2D pathway was additionally illustrated in mice deficient for either IFN-γ or TRAIL, whereas mice deficient for perforin did not display any detectable NKG2D phenotype. A very similar pattern of response was also observed at lower doses of carcinogen (unpublished data). These data suggest that the NKG2D pathway was operating primarily to activate host perforin-mediated cytotoxicity.

Established MCA-induced sarcomas do not generally express the Rae-1 NKG2D ligands

Given the potential of the immune system to edit tumors, including sarcomas (26, 27), it might be expected that various gene-targeted mice might have an increased proportion of tumors displaying an unedited phenotype and expressing Rae-1. We derived fibrosarcoma cell lines from several WT and gene-targeted mice treated with MCA (Fig. 3 A and not depicted). Evaluation of up to 16 sarcomas derived from WT mice demonstrated Rae-1. expression was variable (6/16, 3 high and 3 low) or absent (10/16), and similar frequencies were observed in sarcomas derived from TRAIL$^{-/-}$ mice (5/8 negative for Rae-1; Fig. 3 A). In contrast, sarcomas derived from perforin-deficient mice were shown to universally

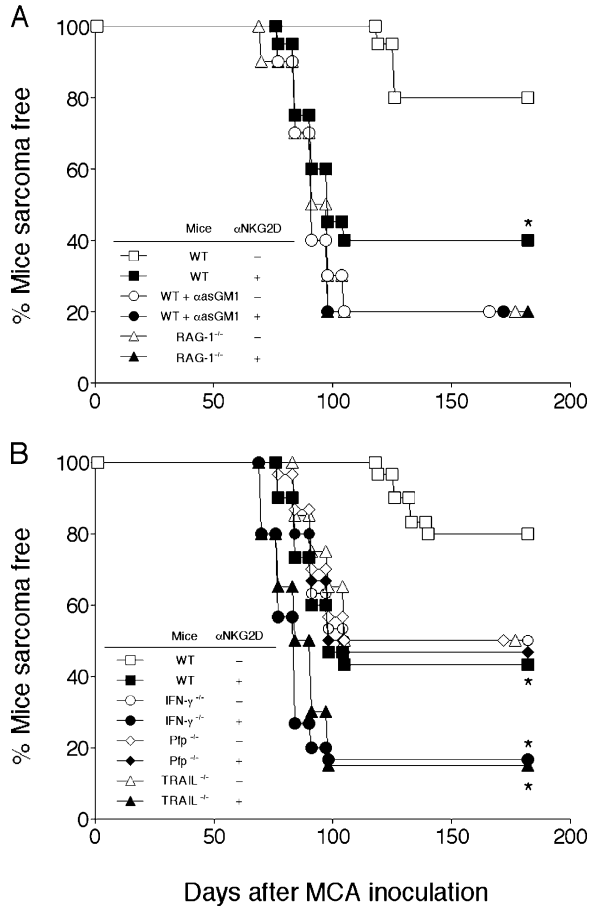

Figure 2. The NKG2D pathway is perforin mediated and only partially responsible for host protection from sarcoma by NK cells or T cells. (A) Groups of 20 B6 WT or 10 B6 RAG-1$^{-/-}$ mice were injected s.c. in the hind flank with 25 μg MCA as indicated. Some WT mice received weekly treatments with anti-asGM1 for 8 wk to deplete NK cells. (B) Groups of B6 WT mice ($n = 20$) or gene-targeted B6 mice deficient for perforin (pfp$^{-/-}$; $n = 30$), TRAIL ($n = 20$), or IFN-γ ($n = 30$) were injected s.c. into the hind flank with 25 μg MCA as indicated. In both A and B, all mice were treated i.p. twice weekly from day 0 (the day of MCA inoculation) with 250 μg of control Ig (cIg) or anti-NKG2D mAb for 8 wk. Palpable sarcomas (>10 mm^2) were recorded, and tumor-free mice were monitored for 200 d. The asterisks denote a significant effect of anti-NKG2D as determined by Fisher's exact test ($P < 0.05$).

express medium to high levels of Rae-1 (7/7; Fig. 3 A). Interestingly, when the Rae-1$^+$ sarcomas were injected at low doses into WT recipients, their growth was retarded in a host NKG2D- and perforin-dependent manner (Fig. 3 B). Not surprisingly, the growth of Rae-1$^-$ sarcomas from WT or TRAIL$^{-/-}$ mice was similar in WT and pfp$^{-/-}$ mice and unaffected by NKG2D neutralization (Fig. 3 B). Therefore, it is possible that the NKG2D–NKG2D ligand pathway only plays a tumor suppressive role very early in the host response to transformation. Once established, some tumors may express NKG2D ligands and yet continue to grow, perhaps by inducing immune suppression by directly down-modulating NKG2D on local immune effector cells or by other means of tumor escape. In a similar manner, it will now be important to completely evaluate Rae-1 expression on sarcomas derived from WT mice neutralized for NKG2D and H60 on all sarcomas derived from BALB/c mice.

IL-12 therapy is only partially effective in mice neutralized for NKG2D

IL-12 plays an essential role in the interaction between the innate and adaptive arms of immunity (28) produced by APCs and acting on T cells and NK cells to generate cytotoxic lymphocytes. IL-12 is also the major cytokine responsible for Th1 cell differentiation, allowing potent production of IFN-γ. Recently, we established that IL-12–mediated antimetastatic activity in part via the NKG2D–NKG2D ligand recognition (24). Because it had been shown that IL-12 effectively suppressed the development of MCA-induced sarcomas (29), we next wished to assess whether the efficacy of IL-12 was NKG2D dependent. WT mice given a lethal dose of MCA were largely protected by repetitive cycles of IL-12 treatment; however, those mice additionally receiving anti-NKG2D mAb failed to effectively respond to therapy and displayed a greater incidence of sarcomas (Fig. 4).

Conclusion

This is the first report to illustrate the importance of NKG2D in controlling natural and activated host immunity to tumor initiation. Previous studies have illustrated the importance of this pathway in the host immune response to s.c. or i.v. injection of experimental tumors either naturally or ectopically expressing various NKG2D ligands, but these studies told us nothing about the role of NKG2D in tumorigenesis (6, 22–24). In the absence of any NKG2D gene–targeted mouse or, more particularly, a conditional NKG2D gene–targeted mouse, it is not possible to determine which effector cell is using NKG2D to control tumor formation. However, experiments performed here and previously using the MCA-induced sarcoma model suggest that NK cells, invariant NKT cells, and T cells (26, 30, 31) may all play a role in host response to the initiation of these tumors. Of note, NKG2D neutralization did not further sensitize WT mice depleted of NK cells, perforin-deficient mice, or RAG-1$^{-/-}$ mice deficient in both T cells and B cells. In contrast, the role of NKG2D was independent of host IFN-γ and TRAIL effector function, and these data collectively demonstrate that NKG2D is only one recognition pathway by which effector lymphocytes control tumor initiation.

We still know relatively little about what controls the expression of NKG2D ligands in vivo, although various stresses such as heat, retinoids, carcinogens, and other agents that induce DNA damage may all be effective. The expression of Rae-1 and H60 was induced by skin painting with the carcinogens 7,12-dimethylbenz[*a*]anthracene (DMBA)/12-*O*-tetradecanoylphorbol 13-acetate (TPA), and the importance of the NKG2D pathway in host protection from skin carci-

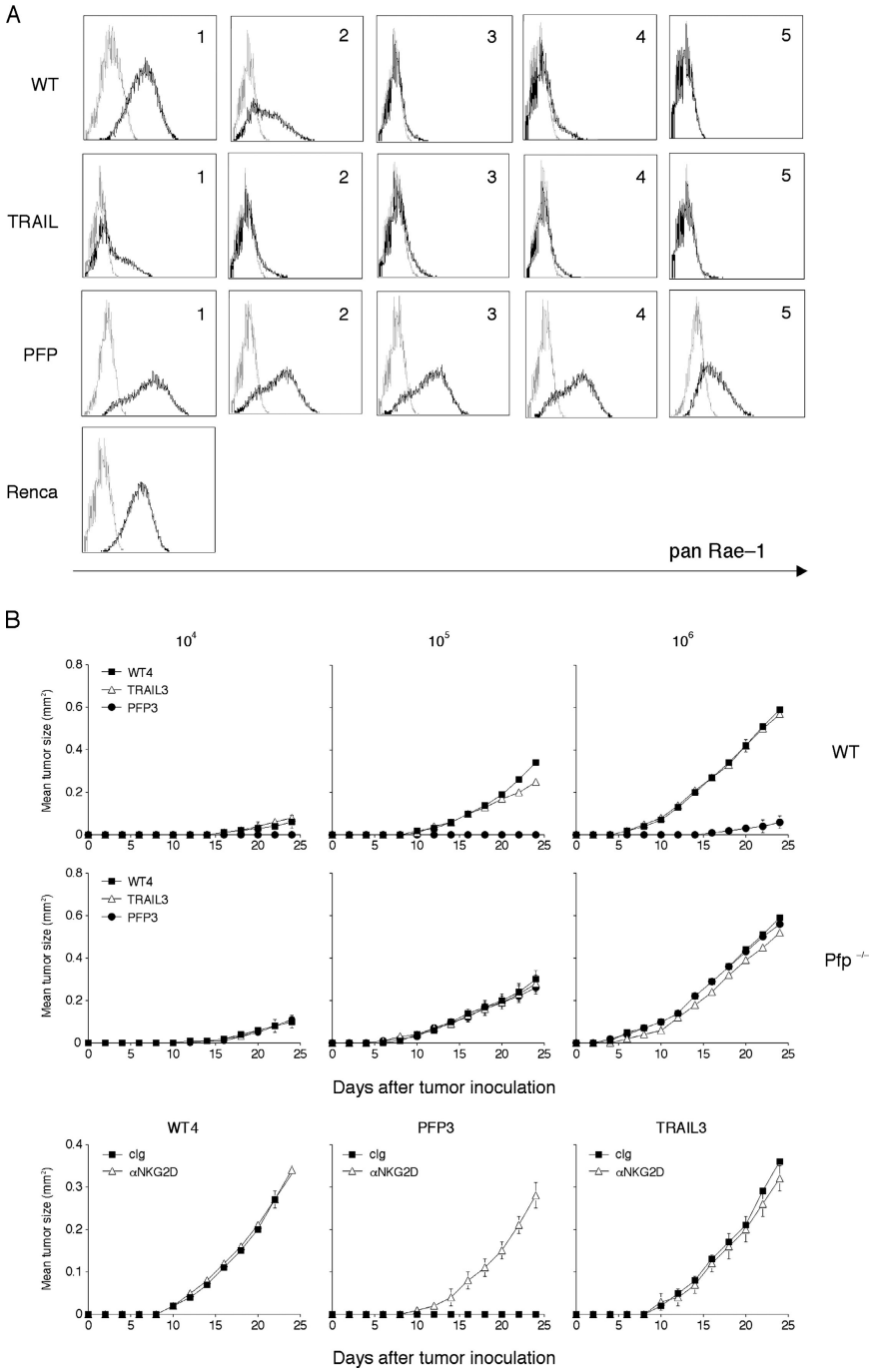

Figure 3. Expression of NKG2D ligands indicates immunoediting. (A) MCA-induced sarcoma cell lines were derived from various mice and assessed for Rae-1 expression as indicated in Materials and methods. Five representative sarcoma cell lines derived from WT, TRAIL$^{-/-}$, and pfp$^{-/-}$ mice (1–5) are shown with Rae-1^{+} Renca renal carcinoma as a positive control. Gray lines, isotype control; black lines, Rae-1. (B) Representative MCA-induced sarcoma cell lines derived from WT, TRAIL$^{-/-}$, and pfp$^{-/-}$ mice were assessed for s.c. growth in groups of five WT or pfp$^{-/-}$ mice at the doses indicated (tumor cell number). As indicated, WT, TRAIL$^{-/-}$, and pfp$^{-/-}$ sarcoma cell lines were also injected s.c. (10^5) into groups of five WT mice in the presence of control Ig (clg) or neutralizing anti-NKG2D mAb (αNKG2D). Anti-NKG2D and clg were administered twice weekly i.p. for 3 wk. The data is representative of two independent experiments with these tumor lines, and four tumors from each strain have been transplanted with similar results.

noma was implicated but not directly proven (20). The signaling events that are responsible for the up-regulation of Rae-1 expression on MCA-induced sarcoma tumor cells are not yet clear, and it will now be important to determine exactly at what stage Rae-1 molecules are expressed after MCA inoculation. Clearly, NKG2D ligand expression by

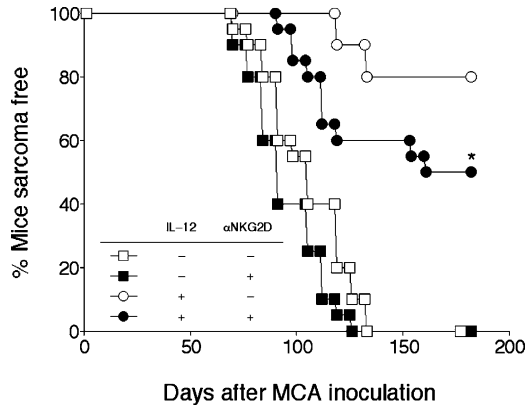

Figure 4. IL-12 therapy is only partially effective in mice neutralized for NKG2D. Groups of 20 B6 WT mice were injected s.c. into the hind flank with 400 μg MCA. Mice were treated i.p. twice weekly from day 0 (the day of MCA inoculation) with 250 μg of control Ig (clg) or anti-NKG2D mAb for 8 wk. Mice also received either PBS or 100 ng IL-12 daily in 5-d courses for 3 wk on and 1 wk off in two 4-wk cycles from the day of MCA inoculation. Palpable sarcomas (>10 mm^2) were recorded, and tumor-free mice were monitored for 200 d. The asterisks denote a significant effect of anti-NKG2D as determined by Fisher's exact test ($P < 0.05$).

tumor cells may not be a barrier to tumor growth because many primary tumors and tumor cell lines naturally express NKG2D ligands, and in some cases these ligands may be secreted (32, 33). Direct experimentation has shown that less tumor rejection occurred when tumors only expressed intermediate levels of Rae-1 (22), so it may be that a threshold level of ligand expression can be maintained on several tumor cells in the population to effectively down-regulate immunity. It now remains to be determined whether NKG2D plays an important role in other models of tumor immune surveillance and whether there is some survival advantage for some tumors to express NKG2D ligands at later stages of tumor development.

MATERIALS AND METHODS

Mice. Inbred C57BL/6 and BALB/c WT mice were purchased from the Walter and Eliza Hall Institute of Medical Research. The following gene-targeted mice were bred at the Peter MacCallum Cancer Centre: C57BL/6 perforin-deficient (B6 $pfp^{-/-}$) mice; C57BL/6 TRAIL-deficient (B6 $TRAIL^{-/-}$) mice (a gift from J. Peshcon, AMGEN, Seattle, WA) (34); and C57BL/6 IFN-γ–deficient (B6 IFN-$\gamma^{-/-}$) mice. All mice that were originally generated on a 129 background have been backcrossed between 10–12 times onto the C57BL/6 background. Male mice of 6–12 wk of age were used in all experiments that were performed according to animal experimental ethics committee guidelines.

MCA-induced sarcoma. Groups of 10–30 male mice were inoculated s.c. in the right hind leg with 0.1 ml maize oil containing 1–400 μg MCA (Sigma-Aldrich), and mice were monitored weekly for the development of fibrosarcoma as previously described (30). The growth of some MCA-induced fibrosarcomas initiated in B6 and BALB/c or PBS- or IL-12–treated B6 WT mice was monitored weekly and measured by a caliper square along the perpendicular axes of the tumors. Palpable tumors >10 mm^2 with progressive growth for two successive weeks were recorded. Tumor-free mice did not develop sarcomas beyond 200 d. Some 100-μg MCA-induced sarcomas that had reached a size of 0.5 cm^2 were excised aseptically. Tumors were cut into small pieces and treated with 1 mg/ml collagenase (type II; Sigma-Aldrich) and 40 μg/ml DNase I at 37°C for 1 h, clumps were removed, and single cells were cultured in RPMI 1640 with 10% FCS and 2 mM L-glutamine. The cells were split with 0.1% EDTA when they were confluent. All tumor cell lines were kept in culture for at least 3 mo to minimize cellular contamination. Some sarcoma lines were injected s.c. into WT or gene-targeted mice as indicated in the figures.

IL-12 and anti-NKG2D treatment protocols. Recombinant mouse IL-12 (Genetics Institute) was suspended in PBS and administrated i.p. as described previously (35). Groups of mice received 5-d courses of either PBS or 100 ng IL-12 for 3 wk on and 1 wk off in two 4-wk cycles from the day of MCA inoculation. Some groups of B6 or BALB/c mice were treated i.p. with either 250 μg hamster anti–mouse NKG2D mAb (C7 clone) (36) or 250 μg hamster control Ig mAb twice weekly for 8 wk after tumor inoculation. It should also be noted that $NKG2D^+$ NK effector cells are not depleted by anti-NKG2D mAb treatment (24). This twice-weekly treatment regimen completely suppresses NKG2D-mediated rejection of RMA-S–Rae-1β tumor cells (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20050994/DC1). NK cells were specifically depleted in B6 mice using 100 μg i.p. rabbit anti-asialo GM1 antibody (Wako Chemicals) on days 0, 1, and 7 and weekly thereafter for 9 wk (after tumor inoculation) as described previously (37).

Flow cytometric analysis. MCA-induced sarcoma cell lines were derived from various mice and assessed for NKG2D ligand expression as follows. Staining was performed in PBS with 0.5% BSA and 0.04% sodium azide on ice using the anti–pan Rae-1–PE or PE-conjugated rat IgG2a isotype control. Anti–pan Rae-1 mAb (clone 186107, rat IgG2a isotype; provided by L. Lanier, University of San Francisco, San Francisco, CA) reacts with Rae-1α, β, γ, δ, and ε, as previously described (38). The stained cells were analyzed on a flow cytometer (LSR II; Becton Dickinson), and the data were processed using an FCS-Express 2 program (De Novo Software).

Statistical analysis. Significant differences in sarcoma-free mice were determined by the Fisher's exact test. $P < 0.05$ was considered significant.

Online supplemental material. Fig. S1 shows that anti-NKG2D mAb is without effect in NK cell– or T cell–deficient mice. Fig. S2 depicts anti-NKG2D mAb as effectively neutralizing NKG2D. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20050994/DC1.

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