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Perforin Is a Major Contributor to NK Cell Control of Tumor Metastasis[1]

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We provide the first demonstration, using experimental and spontaneous models of metastasis in C57BL/6 (B6) (RM-1 prostate carcinoma) and BALB/c (DA3 mammary carcinoma) mice, that tumor metastasis is primarily controlled by perforin-dependent cytotoxicity mediated by NK1.1$^+$ cells. MHC class I^{low} RM-1 and DA3 tumor cells were sensitive in vitro to Fas-mediated lysis or spleen NK cells in a perforin-dependent fashion. Perforin-deficient NK cells did not lyse these tumors, and perforin-deficient mice were 10–100-fold less proficient than wild-type mice in rejecting the metastasis of tumor cells to the lung. Fas ligand mutant *gld* mice displayed uncompromised protection against tumor metastasis. Depletion of NK subsets resulted in greater numbers of metastases than observed in perforin-deficient mice, suggesting that perforin-independent effector functions of NK cells may also contribute to protection from tumor metastasis. ***The Journal of Immunology*****, 1999, 162: 6658–6662.**

Although suitable mouse models of tumor metastasis have existed for a considerable time (1, 2) and immunotherapy has been long proven in such models (3), there is no information regarding the effector molecules responsible for innate immunity against tumor metastasis. NK cells mediate spontaneous cytotoxicity against class I^{low} tumor cells and their metastases (4), and tumor escape variants that have low or no MHC class I expression are efficiently controlled in vivo by NK cells (5). While perforin-deficient mice and mice treated with NK cell-depleting mAbs have been used to demonstrate that NK cell-mediated tumor protection is perforin dependent (6–8), all of these studies have examined the primary growth of tumors implanted in the peritoneum or s.c. Where tumor metastasis has been evaluated, the cytotoxic effector molecules responsible for innate or acquired protection from metastasis have not been defined (9–13). Herein, we utilized mAb-treated wild-type mice and gene-deficient/mutant mice to evaluate the role of NK cell effector molecules in the control of the metastasis of two different syngeneic MHC class I^{low} tumors. In both these experimental and spontaneous models of lung metastasis, perforin was demonstrated to play a key role in NK1.1$^+$ cell-mediated immunity.

Materials and Methods

Mice

Inbred C57BL/6 and BALB/c mice were purchased from The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia. C57BL/6 perforin-deficient (B6.P^0)[3] mice derived as described (14) and BALB/c.perforin-deficient (BALB/c.P^0) mice bred by backcrossing and microsatellite analysis ($n = 7$) were maintained at the Austin Research Institute Biological Research Laboratories. Microsatellite analysis for production of the BALB/c.P^0 mice was conducted using fluorescently labeled or ^{32}P-labeled microsatellite PCR primers as described previously (15). C57BL/6 *gld* mice were obtained from the Centenary Institute of Cancer Medicine and Cell Biology, Sydney, Australia. C57BL/6 RAG-1-deficient (RAG-1^0) mice (16) were obtained from Dr. Lynn Corcoran, The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia. Mice 4–8 wk of age were used according to animal experimental ethics committee guidelines.

Cell culture and reagents

The mouse lymphomas Yac-1 (H-2^a), RMA-S (H-2^b) (17), RMA (H-2^b), and EL4 (H-2^b), the mouse mastocytomas P815 (H-2^d) and P388D1 (H-2^d), and the DA3 (H-2^d) mammary carcinoma (18) were grown in RPMI medium supplemented with additives as described (8). The mouse RM-1 (H-2^b) prostate carcinoma (19) and 3T3 (H-2^d) fibroblast cell line were maintained in Dulbecco's modified Eagle's medium and additives as above. Recombinant human IL-2 was a gift from Chiron, Emeryville, CA. IL-2-activated adherent mouse spleen NK cells were prepared as described (20).

Flow cytometry

MHC class I expression was analyzed by flow cytometry (FACScaliber, Becton Dickinson, Mountain View, CA) using the anti-mouse mAbs anti-H-2K^{b}D^b (Dr. P. Xing, Austin Research Institute, Heidelberg, Australia) or anti-H-2D^d (34-5-8S, PharMingen, San Diego, CA) and a FITC rat anti-mouse Ig (Silenus, Hawthorn, Australia) secondary.

^{51}Cr release assays

The cytotoxicity of mouse spleen NK cells, IL-2-activated adherent NK cells, soluble Fas ligand (FasL) (provided by Dr. David Lynch, Immunex, Seattle, WA), or a combination of perforin and granzyme B were assessed by 4-h ^{51}Cr release assays against labeled targets as described (8). Each experiment was performed at four (only 100:1 shown) different E:T ratios twice using duplicate samples.

Experimental metastases: RM-1 lung colonization

Tail vein inoculum challenges were performed to ascertain whether systemic antimetastatic activity existed after primary RM-1 tumor growth. These were conducted using the s.c tumor as a primary as described by Eastham et al. (21). Briefly, groups of five B6, B6.RAG-1^0, B6.*gld*, or B6.P^0 mice were inoculated s.c. between the shoulder blades with RM-1

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[3] Abbreviations used in this paper: B6.P^0, C57BL/6 perforin-deficient; BALB/c.P^0, BALB/c perforin-deficient; B6.RAG-1^0, C57BL/6 RAG-1-deficient; FasL, Fas ligand.

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tumor cells (2×10^6), and tumors were allowed to establish for 9 days. At this point, under induced anesthesia, s.c tumors were surgically resected and RM-1 cells were injected via the dorsolateral tail vein. Mice were euthanized 14 days later, the lungs removed and fixed in Bouin's solution, and individual surface lung metastases were counted with the aid of a dissecting microscope. Control experiments were performed by inoculating mice with RM-1 cells via the tail vein and 14 days later counting lung metastases. Some groups of B6 mice were depleted of T lymphocytes and/or NK cells in vivo, by treatment with mAb (100 μg): anti-CD4 (GK1.5, rat IgG2b) and anti-CD8 (53-6.7, rat IgG2a, Sigma); anti-NK1.1 (PK136, mouse IgG2a, American Type Culture Collection); anti-CD3 (KT3, rat IgG2a) with or without anti-NK1.1 on days -2, 0 (day of i.v. tumor inoculation), 2, and 9. These schedules have previously been shown to effectively deplete T cell or NK cell subsets following analysis using FITC-labeled mAbs as described (8). The data were recorded as the mean ($n = 5$) number of lung colonies $\pm$ SE. The excised tumor was weighed wet, and the data were recorded as the mean weight (grams) $\pm$ SE. Significance was determined by an unpaired *t* test to determine a two-tailed *p* value.

Spontaneous metastases: DA3 lung colonization

DA3 mammary tumor cells were injected s.c. on the left flank into groups of five BALB/c and BALB/c.P^0 mice. Mice were euthanized 42 days later and lung metastases counted as above. Some groups of BALB/c mice were depleted of NK cells in vivo by treatment with rabbit anti-asialo-G_{M1} Ab (Wako Chemicals, Richmond, VA) or mAbs as above (100 μg), on days -4, day 0 (day of tumor inoculation), and weekly thereafter. The anti-asialo-G_{M1} antisera was shown to effectively deplete DX5$^+$ (rat IgM, PharMingen) NK cells in BALB/c mice (data not shown). Data were recorded as above.

Results

RM-1 prostate carcinoma and DA3 mammary carcinoma cells have low class I expression and are sensitive to NK cell perforin-mediated lysis

The levels of H-2^d expression on DA3 tumor cells were determined by flow cytometry and compared with RMA-S (TAP-2-deficient) and P388D1 (H-2^d-positive) cells (Fig. 1*A*). DA3 cells displayed very low levels of H-2^d expression. RM1 prostate carcinoma cells expressed little or no H-2^b when compared with EL4 (H-2^b-positive) and negative control RMA-S cells (Fig. 1*B*). RM-1 tumor cells were relatively sensitive to a sublytic concentration of perforin combined with increasing concentrations of granzyme B when compared with a classical NK-sensitive target, RMA-S, and insensitive 3T3 cells (Fig. 1*C*). DA3 tumor cells were somewhat less sensitive to a combination of perforin and granzyme B (Fig. 1*C*). Both DA3 and RM-1 tumor cells were lysed when exposed to soluble FasL, although neither was as sensitive as FasL-sensitive Yac-1 target cells (Fig. 1*D*). Similar sensitivity of these target cells was demonstrated using cytotoxic anti-Fas mAbs or d12S effector cells (22) that exclusively lyse via a Fas-dependent mechanism

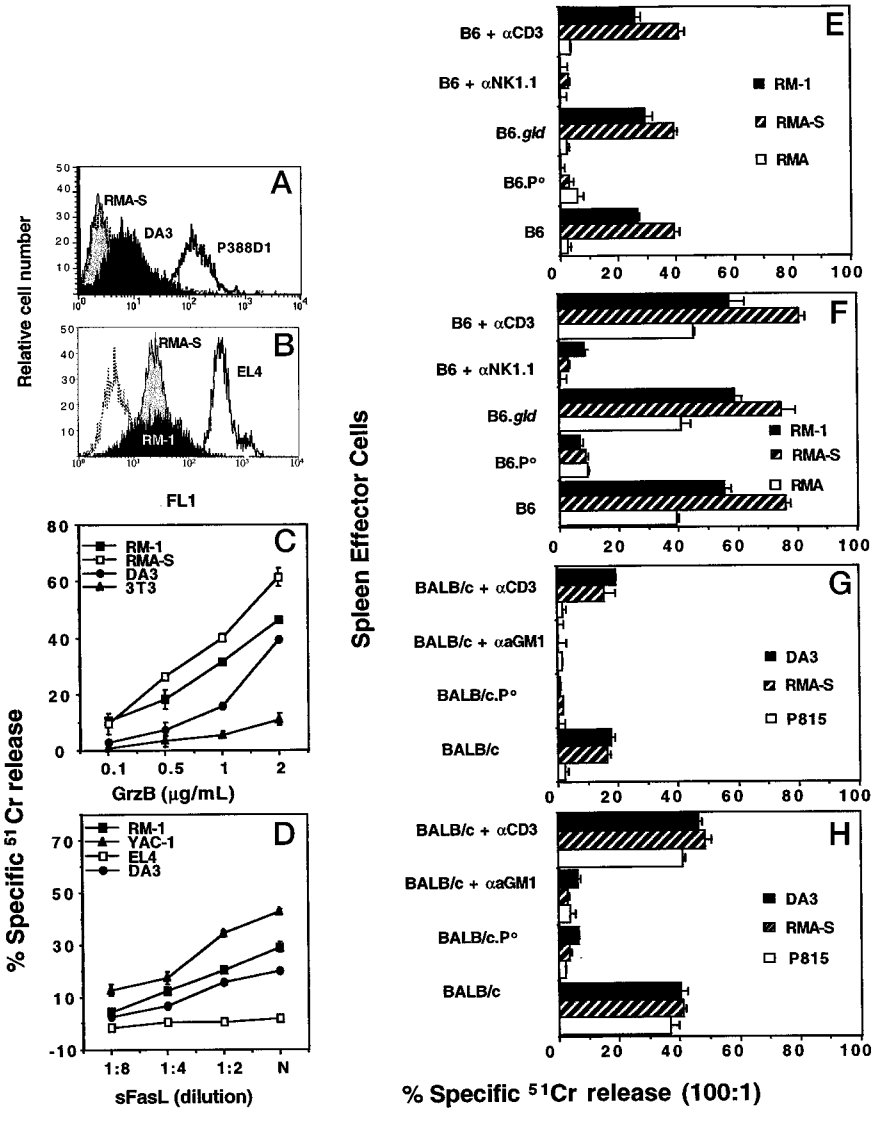

FIGURE 1. Class I expression and sensitivity of RM-1 and DA3 tumor cells to NK cell-mediated cytotoxicity. *A*, Levels of H-2^d expression on DA3 cells (black) were determined by flow cytometry and compared with RMA-S (gray) and P388D1 (white) cells. *B*, Levels of H-2^b expression on RM-1 cells (black) were determined by flow cytometry and compared with RMA-S (gray) and EL4 (white) cells. Isotype controls for *A* and *B* are denoted by the dotted lines. The cytotoxicity of perforin (30 U) and increasing concentrations of granzyme B (GrzB) (0 to 2 μg/ml) (*C*) or dilutions of recombinant soluble FasL (sFasL) (*D*) were assessed by ^{51}Cr release assays against labeled targets as indicated. The cytotoxicity of resting (*E*, *G*) and IL-2-activated adherent (*F*, *H*) spleen NK cells from B6 or BALB/c mice were assessed by ^{51}Cr release assays against labeled RM-1, RMA-S, and RMA (*E*, *F*) or DA3, RMA-S, and P815 (*G*, *H*) targets as indicated. An E:T ratio of 100:1 is shown. In *C*--*H*, the spontaneous release of ^{51}Cr was always $<15\%$, and each experiment was performed twice using duplicate samples. α, anti-. FL1, fluorescence 1.

(data not shown). Resting (Fig. 1*E*) and IL-2-activated NK cells (Fig. 1*F*) from the spleens of B6 mice were cytolytic to RM-1 tumor cells. As observed for many other tumor targets in vitro (8, 14), NK cells from B6.P^0 or B6 mice treated with anti-NK1.1 mAb were poorly cytolytic (Fig. 1, *E* and *F*), whereas those NK cells from B6.*gld* mice or B6 mice treated with anti-CD3 mAb were as cytolytic toward RM-1 as NK cells from B6 mice (Fig. 1, *E* and *F*). Resting (Fig. 1*G*) and IL-2-activated NK cells (Fig. 1*H*) from the spleens of BALB/c mice were cytolytic to DA3 tumor cells, while again NK cells from BALB/c.P^0 or BALB/c mice treated with anti-asialo-G_{M1} mice were poorly cytolytic (Fig. 1, *G* and *H*). These data further supported a role for NK cell perforin in direct lysis of RM-1 and DA3 in vitro.

Control of experimental RM-1 tumor colonization in the lung by NK1.1$^+$ cells is perforin dependent

Initially untreated B6 (wild-type or mutant) mice or B6 mice treated with anti-CD3, anti-CD8, anti-CD4, or anti-NK1.1 mAb were inoculated i.v. with increasing numbers of RM-1 tumor cells, and 14 days later the lung metastases were counted. At the lower inoculated doses of RM-1 cells, a significantly greater number of lesions were observed in B6.P^0 mice ($p < 0.004$) and, more evidently, in B6 mice treated with anti-NK1.1 mAb (B6 anti-NK1.1 mAb-treated mice had more metastases than B6.P^0 mice, $p < 0.002$) than in untreated B6 mice (Fig. 2*A*). A combination of anti-NK1.1 and anti-CD3 mAb did not compromise tumor control more than anti-NK1.1 mAb alone. B6.*gld* mice, B6.RAG-1^0 mice (lacking functional T and B cells), or B6 mice depleted of T cell subsets did not have a significantly greater number of RM-1 tumor metastases than untreated B6 mice (all $p > 0.052$) (Fig. 2*A*). These mice served as controls for those prior given a s.c. tumor 9 days before resection and systemic challenge with RM-1 tumor cells.

Prior exposure of B6 mice to a primary s.c. RM-1 tumor reduced the number of lung metastases following tail vein challenge, suggesting a protective effect in B6 mice (Fig. 2*B*). By contrast, in anti-NK1.1 mAb-treated B6 mice, the number of metastases following tail vein challenge was similar irrespective of the pregrowth of a s.c. RM-1 tumor (compare Fig. 2*A* and Fig. 2*B*). Significantly, again there were increased numbers of metastases in B6.P^0 mice compared with wild-type B6 mice ($p < 0.0001$), but even more were again detected in anti-NK1.1 mAb-treated mice (B6 anti-NK1.1 mAb-treated mice had more metastases than B6.P^0 mice, $p < 0.0009$) (Fig. 2*B*). Interestingly, B6.RAG-1^0 mice or B6 mice treated with anti-CD3 displayed significantly more lung colonies than untreated B6 mice ($p < 0.0001$), while B6.*gld* mice or those B6 mice depleted of CD4$^+$ and CD8$^+$ cells did not (Fig. 2*B*). Importantly, again a combination of anti-NK1.1 and anti-CD3 did not display additive effects, suggesting that CD3$^+$ cells are not responsible for systemic protection from lung colonization of RM-1 tumor cells over and above NK1.1$^+$ cells. The mean wet weight of the s.c. RM-1 tumors excised from all mice was not significantly different (data not shown). Overall, these data suggested that 1) prior s.c. growth of RM-1 provided some protection to further i.v. challenge with RM-1 tumor cells, 2) CD3$^-$ NK1.1$^+$ cells were responsible for the protection from lung colonization by RM-1 cells, and 3) perforin was a mediator, but not the only one, of protection to RM-1 tumor cells by NK1.1$^+$ cells.

Control of spontaneous DA3 tumor colonization in the lung by NK1.1$^+$ cells is perforin dependent

Since the RM-1 tumor is spontaneously metastatic only in an orthotopic setting (23), we decided to examine the general importance of NK cell perforin in another spontaneous model of metastasis. DA3 is a mammary carcinoma that metastasizes to the lung

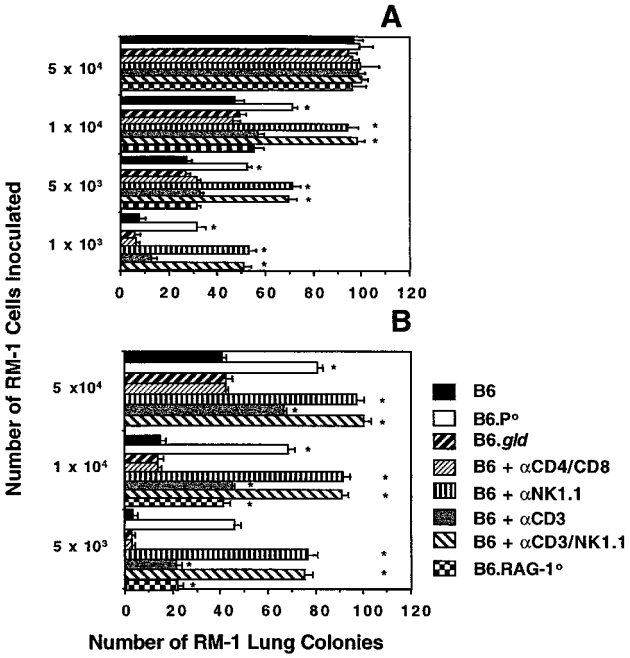

FIGURE 2. Lung colonization of RM-1 tumor is partially controlled by NK cell perforin. *A*, B6, B6.P^0, B6.RAG-1^0, and B6.*gld* mice, or B6 mice treated with anti (α)-CD4 and anti-CD8, anti-CD3, anti-NK1.1, or a combination of anti-NK1.1 and anti-CD3, on days -2, 0 (the day of RM-1 tumor inoculation), 2, and 9, were inoculated i.v. with increasing numbers of RM-1 tumor cells (10^3–5×10^4) as indicated (B6.RAG-1^0, not receiving 10^3 cells). Fourteen days after tumor inoculation, the lungs of these mice were harvested and fixed, and colonies were counted and recorded as the mean number of colonies $\pm$ SE. These mice served as controls for those in *B*. *B*, B6, B6.*gld*, B6.RAG-1^0, or B6.P^0 mice were inoculated s.c. between the shoulder blades with RM-1 tumor cells (2×10^6), and tumors were allowed to establish for 9 days. Subcutaneous tumors were then resected, and a dose range of RM-1 cells (as indicated) were injected via the tail vein. Mice were euthanized 14 days later, the lungs were removed and fixed, and colonies were counted. Some groups of B6 mice were depleted of CD4$^+$ and CD8$^+$, CD3$^+$, and/or NK1.1$^+$ cells in vivo by treatment mAb on days -2, 0 (day of i.v. tumor inoculation), 2, and 9. Asterisks indicate the groups that are significantly different from B6 untreated mice (*, $p < 0.0005$).

and lymph nodes following inoculation of BALB/c mice with a primary s.c. tumor. A number of different DA3 tumor doses (10^5 to 10^6) were inoculated s.c., and after 42 days lung metastases were counted. Significantly, there were increased numbers of metastases in BALB/c.P^0 mice compared with wild-type B6 mice ($p < 0.0019$), but fewer than found in anti-asialo-G_{M1} Ab-treated mice (Fig. 3). Although it was not practical to accurately quantify metastasis to the lymph nodes, it was noted that both BALB/c.P^0 mice and BALB/c mice treated with anti-asialo-G_{M1} Ab had more lymph node involvement. There were an increased number of lung metastases in anti-CD3- but not anti-CD4- and anti-CD8-treated mice, but this was not determined to be statistically significant (Fig. 3). The mean wet weight of the s.c. DA3 tumors excised from the mice was again not significantly different (data not shown). These data in a spontaneous model supported the major findings in the experimental metastasis RM-1 tumor model that 1) NK cells were responsible for the protection from lung colonization by metastases and 2) perforin was a mediator, but not the only effector mechanism used by NK cells.

Discussion

Despite the fact that perforin-deficient mice have previously been observed to display increased susceptibility to many syngeneic,

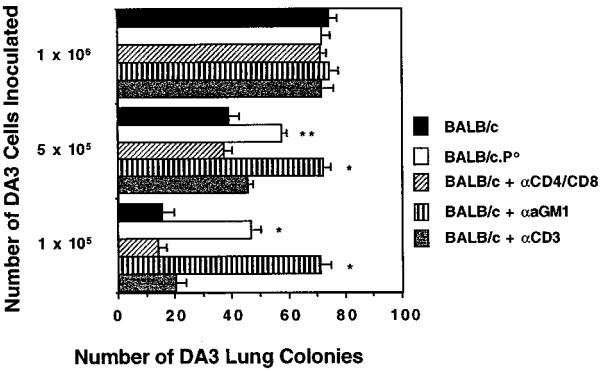

FIGURE 3. Control of spontaneous DA3 tumor colonization in the lung by $NK1.1^+$ cells is perforin dependent. *A*, BALB/c, BALB/c.P^0, or BALB/c mice treated with anti (α)-asialo-G_{M1}, anti-CD3, or anti-CD4 and anti-CD8, on day −4, day 0 (the day of DA3 tumor inoculation), and weekly thereafter, were inoculated s.c. with increasing numbers of DA3 tumor cells (10^5 to 10^6) as indicated. Forty-two days after tumor inoculation, the lungs of these mice were harvested and fixed, and colonies were counted and recorded as the mean number of colonies ± SE. Asterisks indicate the groups that are significantly different from BALB/c untreated mice (*, $p < 0.0005$; **, $p < 0.002$).

class I-defective, chemical- or viral-induced tumors (6–8), this study herein is the first to demonstrate that perforin plays an important role in NK cell-mediated protection from tumor metastasis. Importantly, this fact has been verified in both experimental and spontaneous models of tumor metastasis using two different mouse strains where NK cells are critical. In addition, the data provide important additional information about innate immunity to prostate carcinoma which until recently had been lacking (13). By contrast, despite the sensitivity of RM-1 tumor cells to FasL-mediated lysis, there was no evidence that NK cell FasL was an important effector molecule in protection from RM-1 metastasis. The RM-1 tumor data further support previous experiments with FasL-sensitive RMA-S tumor cells that indicated that NK cell FasL played no role in tumor surveillance (8), and together these data question the antitumor relevance of NK cell FasL-mediated killing observed in vitro (24).

A further important observation was that depletion of NK cells compromised tumor protection significantly more than perforin deficiency. This finding was in contrast to previous experiments with RM-1 (unpublished data) and RMA-S (8) tumor cells inoculated into the peritoneum, where depletion with anti-NK1.1 mAb did not further compromise tumor protection in perforin-deficient mice. These data suggest that additional as yet undefined effector mechanisms of NK cells may control tumor metastasis to the lung but may not be important in the rejection of peritoneal tumor by NK cells. This effector mechanism could potentially involve another death-inducing TNF superfamily molecule such as TNF-related apoptosis-inducing ligand, which has recently been demonstrated to be used by some immature human NK cells (25). Alternatively, the ability of NK cells to make cytokines, such as IFN-γ, that affect T cell and other leukocyte responses may be important. The levels of class I on RM-1 was not enhanced by prolonged in vitro culture in IFN-γ or TNF, although a minor increase in class I (~2-fold) was observed in DA3 cells cultured in IFN-γ (data not shown). It remains to be tested whether these tumors up-regulate class I or Fas expression in vivo, thus making them targets for attack by additional immune mechanisms. We have already observed that the recruitment and accumulation of NK cells in response to class I-deficient tumors may require TNF depending on the site of tumor growth (8), and thus cytokine networks may also determine the types of effector mechanisms that the NK cell eventually uses. Supporting this notion is the apparently organ-specific effect of perforin in viral infection (26).

Data from the RM-1 experimental metastasis model indicated that $CD3^-$ $NK1.1^+$ cells were responsible for protection, but suggested that $CD3^+$ $NK1.1^+$ cells may play an accessory role. In particular, there was a significant increase in RM-1 metastases in B6.RAG-1^0 mice, mice depleted of $CD3^+$ cells, or mice depleted of Thy-1^+ cells (data not shown) compared with that observed in untreated- or anti-CD4/CD8-treated wild-type mice. It remains difficult to accurately account for the subpopulation(s) of $CD3^+$ $NK1.1^+$ cells that may be contributing to tumor protection since NKT cells are generally heterogeneous for many surface Ags and are found in very small proportions in most tissues (27). Previously, Takeda et al. (28) demonstrated that IL-12-activated $NK1.1^+$ T cells from the liver can inhibit liver and lung metastases following i.v. injection of tumors. In addition, Vα14 $NK1.1^+$ T cell-deficient mice were demonstrated to no longer mediate IL-12-induced prevention of tumor metastasis (29). $NK1.1^+$ T cells are exquisitely sensitive to IL-12 (30), and experiments currently in progress in Vα14 $NK1.1^+$ T cell-deficient, IL-12-deficient, and IFN-γ-deficient mice should be informative.

In summary, this study has highlighted that perforin plays an important role but is not totally responsible for NK cell-mediated protection from tumor metastasis. The future use of these experimental and spontaneous models of tumor metastases in various gene knockout and mutant mice will enable many new questions to be addressed. In particular, which other effector arms of innate immunity protect the host from tumor metastasis, and when and where do the these act?

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