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

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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 NK.1+ cells. MHCI class Ilow 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 Ilow tumor cells and their metastases (4), and tumor escape variants that have low or no MHCI 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 Ilow 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.P0) mice derived as described (14) and BALB/c perforin-deficient (BALB/c.P0) mice bred by backcrossing and microsatellite analysis (n = 7) were maintained in the Austin Research Institute Biological Research Laboratories. Microsatellite analysis for production of the BALB/c.P0 mice was conducted using fluorescently labeled or 32P-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–/–) 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-2a), RMA-S (H-2b) (17), RMA (H-2b), and ELA (H-2b), the mouse mastocytomas P815 (H-2b) and P388D1 (H-2b), and the DA3 (H-2d) mammary carcinoma (18) were grown in RPMI medium supplemented with additives as described (8). The mouse RM-1 (H-2b) prostate carcinoma (19) and 3T3 (H-2d) 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**

MHCI class I expression was analyzed by flow cytometry (FACScaliber, Becton Dickinson, Mountain View, CA) using the anti-mouse mAbs anti-H-2Kd (Dr. P. Xing, Austin Research Institute, Heidelberg, Australia) or anti-H-2Dd (43-5-8S, PharMingen, San Diego, CA) and a FITC rat anti-mouse Ig (Silenus, Hawthorn, Australia) secondary.

**51Cr 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 51Cr 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–/–, B6.gld, or B6.P0 mice were inoculated s.c. between the shoulder blades with RM-1

3 Abbreviations used in this paper: B6.P0, C57BL/6 perforin-deficient; BALB/c.P0, BALB/c perforin-deficient; B6.RAG-1–/–, C57BL/6 RAG-1–/–; Fasl, Fas ligand.
tumor cells \((2 \times 10^6)\), 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 \(\mu\)g): 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.P0 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-GM1 Ab (Wako Chemicals, Richmond, VA) or mAbs as above (100 \(\mu\)g), on days 2-4, day 0 (day of tumor inoculation), and weekly thereafter. The anti-asialo-GM1 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-2d expression on RM-1 tumor cells were determined by flow cytometry and compared with RMA-S (TAP-2-deficient) and P388D1 (H-2d-positive) cells (Fig. 1A). DA3 cells displayed very low levels of H-2d expression. RM1 prostate carcinoma cells expressed little or no H-2d when compared with EL4 (H-2d-positive) and negative control RMA-S cells (Fig. 1B). 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. 1C). DA3 tumor cells were somewhat less sensitive to a combination of perforin and granzyme B (Fig. 1C). 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. 1D). 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.
anti-asialo-G M1 mice were poorly cytolytic (Fig. 1, A, 0.002) than in untreated B6 mice (Fig. 2, A0.004). While 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.P0 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.P0 mice, p < 0.002) than in untreated B6 mice (Fig. 2A). 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+ 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. 2A). 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. 2B). By contrast, in anti-NK1.1 mAb-treated B6 mice, the number of metastases following tail vein challenge was similar irrespective of the presence of a s.c. RM-1 tumor (compare Fig. 2A and Fig. 2B). Significantly, again there were increased numbers of metastases in B6.P0 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.P0 mice, p < 0.0009) (Fig. 2B). Interestingly, B6.RAG-1+ 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. 2B). 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 and lymph nodes following inoculation of BALB/c mice with a primary s.c. tumor. A number of different DA3 tumor doses (105 to 106) were inoculated s.c., and after 42 days lung metastases were counted. Significantly, there were increased numbers of metastases in BALB/c.P0 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.P0 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,
Additional immune mechanisms. We have already observed that remains to be tested whether these tumors up-regulate class I or was observed in IFN-γ-induced prevention of tumor metastasis (29). NK1.1+ T cell-deficient mice were demonstrated to no longer mediate IL-12-induced prevention of tumor metastasis. The RM-1 tumor 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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References


