Cutting Edge: Granzymes A and B Are Not Essential for Perforin-Mediated Tumor Rejection

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Controversy still exists regarding the biological function of granzyme serine proteases released with perforin from the cytotoxic granules of NK cells and CTLs. In particular, it is not clear whether the major granzymes, A and B, play an essential role in tumor rejection mediated by the perforin pathway. We have now examined the relative importance of perforin and granzyme A and B clusters in five different tumor models that stringently distinguish their importance. We conclude that granzyme A and B clusters are not essential for CTL- and NK cell-mediated rejection of spontaneous and experimental tumors, raising the likelihood that either perforin alone or in combination with an additional granzyme or granule component(s) mediates cytotoxicity of tumor cells in vivo. The Journal of Immunology, 2003, 171: 515–518.

Cytotoxic T lymphocytes and NK cells play an important role in the immune surveillance of tumors (1–3). The granule exocytosis pathway used by NK cells and CTL predicts that perforin (pfp) released from cytotoxic granules facilitates the entry of serine proteases (granzymes) and other cytotoxic molecules into the target cell, probably through a process of endosomal disruption (4–6). The key role of pfp in CTL-mediated and NK cell-mediated control of tumor growth in vivo has been firmly established in a number of mouse experimental tumor models (7–10) and with the demonstration that B cell lymphoma surveillance is reduced in pfp-deficient (pfp<sup>−/−</sup>) mice (1, 11). There is considerable evidence from in vitro studies and granzyme B (grzB)-deficient (grzB<sup>−/−</sup>) mice to support the concept that CTL/NK cell-mediated DNA fragmentation in tumor cells requires pfp and grzB (12–15). GrzB<sup>−/−</sup> mice also do not express granzymes C, D, F, and G (16), but despite the lack of these granzymes the defect in inducing nuclear damage in vitro is kinetic and not absolute (17). Granzyme A (grzA) and grzB, which express highly restricted but distinct substrate specificities (18, 19), trigger apoptotic nuclear damage in the presence of pfp (14) and can synergize to mediate target cell death (20). More recently, grzA has been demonstrated to have a caspase-independent role in generating ssDNA nicks, rather than causing oligonucleosomal fragmentation (21–23), and provocatively this appears to be mediated by a known tumor suppressor, NM23-H1 (24). Nevertheless, only two studies have previously addressed the role of grzA and grzB in tumor control by NK cells and CTL and these reported inconsistent results (25, 26). Two potential problems existed with these previous studies: first, the use of grzB cluster-deficient mice not fully back-crossed on a C57BL/6 background (25); and second, the RMA-S tumor used in both studies was different in its tumorigenicity and its growth only weakly distinguished between wild-type (WT) and granzyme-deficient mice (25, 26). We have now addressed these major issues by assessing all tumor models on an equivalent C57BL/6 background and examining tumor models in which the pfp<sup>−/−</sup> mice are markedly susceptible to tumor growth or metastasis compared with WT mice. In examining this important question our new data unequivocally indicate that grzA and the B cluster granzymes are not critical for pfp-mediated tumor rejection.

**Materials and Methods**

**Mice**

Inbred C57BL/6 (B6) mice were purchased from The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia. C57BL/6 (pfp<sup>−/−</sup>) mice (targeted in C57BL/6 embryonic stem cells and kindly provided by Dr. Kagi, Ref. 27), C57BL/6 granzyme A-deficient (B6 grzA<sup>−/−</sup>, Ref. 28), C57BL/6 granzyme B cluster-deficient (B6 grzB<sup>−/−</sup>, Ref. 15), and C57BL/6 recombinating-activating gene (RAG)-1-deficient (B6 RAG-1<sup>−/−</sup>) mice were bred at the Peter MacCallum Cancer Institute (Victoria, Australia). Granzyme A and B cluster-deficient (B6 grzAB<sup>−/−</sup>) mice were created and bred at the Peter MacCallum Cancer Institute, and all mice used were genotyped using the PCR screening protocol previously described (29). All gene-targeted mice were derived from C57BL/6 embryonic stem cells or had been back-crossed over 10 generations to C57BL/6. Mice of 4–10 wk of age were used in all experiments that were performed according to animal experimental ethics committee guidelines.

**Cell culture and reagents**

The spontaneous B cell lymphomas, PN53H-1 and PNK-15, were derived from B6 pfp<sup>−/−</sup> mice as described (1, 11). The spontaneous B cell lymphomas β<sub>2</sub>μ<sup>−/−</sup>NPN-2 and β<sub>2</sub>μ<sup>−/−</sup>NPN-8 were similarly derived from B6 pfp<sup>−/−</sup> β<sub>2</sub>μ<sup>−/−</sup> microglobulin-deficient (β<sub>2</sub>μ<sup>−/−</sup>) mice (30) and are deficient for MHC class I and CD1d, but lack NKG2D ligands (S. E. A. Street, M. J. Smyth, and J. A. 2 Address correspondence and reprint requests to Dr. Mark J. Smyth, Cancer Immunology Program, Peter MacCallum Cancer Institute, Locked Bag 1, A’Beckett Street, 8006 Victoria, Australia. E-mail address: m.smyth@pmci.unimelb.edu.au

3 Abbreviations used in this paper: pfp, perforin; grz, granzyme; RAE, retinoic acid early inducible; WT, wild-type; β<sub>2</sub>μ, β<sub>2</sub>μ-microglobulin; RAG, recombination-activating gene.

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leukemia (31, 32). All cells were grown in DMEM or RPMI 1640 medium supplemented with 10% (v/v) FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Grand Island, NY). Recombinant human IL-2 was a kind gift from Chiron (Emeryville, CA) and recombinant mouse IL-12 from the Genetics Institute (Cambridge, MA).

Tumor control in vivo

Three different experimental systems were employed. The B cell lymphomas, PNS3H-1, PNK-15, βμNPN-2, and βμNPN-8 were transplanted directly from B6 pfp−/− RAG-1−/− mice into B6 WT mice or B6 mice gene-targeted for RAG-1, pfp, grzA and/or grzB. Groups of five B6 WT or B6 gene-targeted mice were injected i.p. with increasing numbers of lymphoma cells and observed daily for tumor growth for 150 days. Some groups of B6 WT mice were depleted of CD8+ T cells as previously described (1). Mice were sacrificed upon abdominal swelling and disseminated lymphoma development was confirmed.

Groups of five untreated B6 WT or gene-targeted mice were injected s.c. with vector alone-infected or Rae-1+-infected RMA tumor cells (5 × 104 cells) in 0.2 ml PBS as indicated. Mice were observed every 2 days for tumor growth using a caliper square measuring along the perpendicular axes of the tumors (the product of two diameters ± SE) and sacrificed when tumors reached a size >12 mm diameter. Mice without any signs of tumor growth were kept under observation for at least 100 days. B16F10 melanoma cells or 3LL Lewis lung carcinoma cells were inoculated i.v. at a dose of 5 × 105 cells previously shown in groups of five WT or gene-targeted mice to result in similar numbers of lung metastases (31). Some groups of mice were treated with either recombinant IL-2 or with IL-12 as described (9, 33). Mice received IL-2, 100,000 U/200 μl PBS i.p. daily, on days 3–7 (where day 0 was the day of tumor inoculation) or 250 U IL-12 i.p. on days −5, −4, −3, −2, −1, 1, 2, 3, 4, and 5 (where day 0 was the day of i.v. tumor inoculation). Mice were sacrificed 14 days later, the lungs removed, and surface metastases counted with the aid of a dissecting microscope. In all metastasis models, the data were recorded as the mean number of metastases ± SEM. Significance was determined by a Mann-Whitney rank sum U test.

Results and Discussion

Tumor rejection by CTL and NK cells does not require grzA and the B cluster granzymes

Pfp-deficient mice spontaneously develop lymphomas of B cell origin (1, 11), and a number of these lymphomas have previously been characterized in vitro and in vivo following transplantation into WT or pfp−/− mice. Strikingly, these C57BL/6 pfp−/− mouse-derived lymphomas are avidly rejected by CD8+ T cells in WT mice even when as many as 107–108 tumor cells are given, yet can still grow and kill pfp−/− mice at doses as low as 105 cells (1, 11). We transplanted two such MHC class I expressing B cell lymphomas independently into cohorts of WT, RAG-1−/−, grzA−/−, grzB−/−, grzAB−/−, and pfp−/− mice (all on a C57BL/6 background). Although as many as 107 lymphoma cells were rejected by WT, grzA−/−, grzB−/−, and grzAB−/− mice, pfp−/−, and RAG-1−/− and WT mice depleted of CD8+ cells succumbed to disseminated lymphoma at doses as low as 103 tumor cells (Fig. 1, a and b). This represents at least a 10,000-fold difference in sensitivity to these lymphomas between grzAB−/− and pfp−/− mice. In a similar manner the same strains of mice were inoculated with B cell lymphomas originally derived from B6 pfp−/− βμ−/− mice (Fig. 1, c and d). These lymphomas lack MHC class I and are rejected in WT mice by NK cells (S. E. A. Street, M. J. Smyth, and J. A. Trapani, unpublished data). Although rejection was naturally mediated by a different subset of effector cells, again only pfp, but not grzA and grzB, was essential for tumor control. Collectively, these data illustrate that regardless of the effector cell, spontaneous B cell lymphomas are exquisitely sensitive to pfp-mediated control, even in the absence of grzA and the grzB clusters.

FIGURE 1. Pfp-mediated rejection of spontaneous B cell lymphomas does not require grz A and/or B cluster. Groups of mice (n = 5) of various strains were injected i.p. with increasing numbers of PNS3H-1 (a), PNK-15 (b), βμNPN-2 (c), or βμNPN-8 (d) tumor cells (103–107) as indicated. Mice were observed daily for tumor growth for 150 days by monitoring abdominal swelling and development of disseminated lymphoma. Tumor-free mice are represented in the right column inserted and individual mice are represented by each symbol.
To further substantiate any requirement for grzA and grzB in tumor control, we examined the pfp-mediated rejection of RMA lymphoma cells expressing the NKG2D activating ligand, Rae-1β. We have previously demonstrated that RMA-Rae-1β cells can be powerfully rejected in WT mice by NK and CD8 T cells in a manner strictly dependent upon pfp (32). In concert with our previous lymphoma rejection data (Ref. 25 and Fig. 1), s.c. RMA-Rae-1β tumors were rejected in WT, grzA−/−, grzB−/−, and grzAB−/− mice, but not pfp−/− mice (Fig. 2a), whereas RMA control tumors grew equivalently in all strains of mice (Fig. 2b). Arguably, tumor rejection mediated by a natural immune response may not represent that delivered by highly activated effector cells and consequently we further examined the role of grzA and grzB in tumor rejection mediated by cytokine-activated effector cells. We have previously demonstrated that both IL-12 and high-dose IL-2 mediate rejection of B16F10 lung metastases in an NK cell- and pfp-dependent manner (9, 33). In this study we have confirmed the role of pfp and demonstrated that a lack of grzA or the B cluster granzymes does not affect the efficacy of these IL-2- or IL-12-dependent responses against B16F10 metastases (Fig. 3a) or the effect of IL-12 administration on 3LL Lewis lung metastases (Fig. 3b). This metastasis experiment with melanoma and lung carcinoma cells further confirms that tumor cells of many origins, including epithelial, melanomas, sarcomas, and lymphomas, and in multiple sites (lymphoid organs, lungs, i.p., and s.c.) appear to be rejected independently of grzA and grzB cluster proteases (25).

FIGURE 2. Pfp-mediated rejection of Rae-1β-expressing tumors does not require grz A and/or B cluster. The s.c. inoculation of RMA-Rae-1β (a) or RMA (5 × 10⁴) (b) cells developed into groups of five WT, pfp−/−, grzA−/−, grzB−/−, and grzAB−/− mice or RAG-1−/− mice depleted of NK cells. Tumor growth was examined every second day and data recorded are the means ± SEM. Multiple symbols at the x-axis represent no tumor growth.

FIGURE 3. Cytokine immunotherapy of metastases is pfp-dependent, but not grz A and B cluster-dependent. Groups of five mice as indicated were inoculated i.v. with 5 × 10⁴ B16F10 tumor (a) or 3LL tumor (b) cells. Groups of mice were untreated (□) or treated with either recombinant IL-2 (■) or IL-12 (■). Mice received IL-2, 100,000 U/200 μl PBS i.p. daily from days 3 through 7 (where day 0 was the day of tumor inoculation) or 250 U IL-12 i.p. on days −5, −4, −3, −2, −1, 1, 2, 3, 4, 5 (where day 0 was the day of i.v. tumor inoculation). Mice were euthanized 14 days later, the lungs removed, and surface metastases counted. Data was recorded as the mean number of metastases ± SEM. Significance was determined by a Mann-Whitney Rank sum U test, *p < 0.05.

Conclusions

In vitro analysis of the importance of grzA and the grzB cluster proteases is of little physiological significance to tumor rejection. We have graphically demonstrated in all tumor models examined thus far that grzA and grzB are not necessary for tumor rejection in vivo. In this study, compelling evidence was provided in five different settings, including potent CTL- and NK cell-mediated rejection of spontaneous disseminated B cell lymphomas, NKG2D-mediated rejection of lymphoma, and IL-2 or IL-12 immunotherapy of melanoma and lung carcinoma metastases. Of particular note was the finding that spontaneous disseminated B cell lymphomas arising in pfp−/− mice were rejected avidly in grzAB−/− mice like WT mice, despite their growth in pfp−/− mice at a 10,000-fold smaller tumor cell dose. These experiments clarify any doubt raised by two previous studies that reported conflicting conclusions based on the RMA-S lymphoma tumor model alone. We reiterate that, although grzB−/− or grzAB−/− effector cells are clearly defective in their ability to induce DNA fragmentation (15, 25), grzA and grzB are not necessary for tumor cell death in vitro as measured by clonogenicity, the most stringent measure of cell death (25). Therefore, it is very likely that effector lymphocytes lacking grzA and/or the grzB cluster proteases mediate tumor rejection by a pfp-dependent mechanism that involves alternative
granule components, or through pathways reliant on perforin alone. Apart from grzA and grzB cluster proteases, cytotoxic granules contain at least two other serine proteases, grzM and grzK, FasL, and a number of other potentially cytotoxic molecules. Dipeptidyl peptidase I-deficient mice that have inactive granymes or mice deficient in all granymes, including M and K, will be useful tools in which to study NK cell- and CTL-mediated tumor rejection. The tumors used in this study and those previous are Fas-insensitive, suggesting that FasL is not the granule component delivered by pf. Our study does not eliminate the possibility that some tumor cells or normal cells might be particularly sensitive to grzA and grzB, however there are few, if any, examples in which tumor or virus-infected target cells have been shown to be resistant to effector cells specifically lacking grzA and grzB. The true role of grzA and grzB in pf-p-mediated cell death remains an intriguing mystery, but one imagines viruses such as ectromelia and CMV will provide important clues. Despite contending that there are no convincing in vivo studies to date confirming a functional role for granymes in cancer immune surveillance or rejection, there is no doubt that granymes are essential for defense against a number of important viral pathogens (34). From an evolutionarily viewpoint, there can be little doubt that the primary raison d’être for granymes is viral defense, and examples, such as the equivalent susceptibility of grzAB/−− and pf/−− mice to the poxvirus ectromelia, support this idea (35). Recently, a human grzB polymorphism was reported to lead to defective apoptosis induction (36). This allele was surprisingly frequent in the populations studied, with up to 29% of individuals found to be homozygous carriers of the mutation. This important revelation may soon permit an evaluation of whether grzB, at least, predisposes to defects in viral immunity and/or cancer susceptibility in humans.

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References