FUNCTIONAL SIGNIFICANCE
OF THE PERFORIN/GRANZYME
CELL DEATH PATHWAY

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Perforin/granzyme-induced apoptosis is the main pathway used by cytotoxic lymphocytes to eliminate virus-infected or transformed cells. Studies in gene-disrupted mice indicate that perforin is vital for cytotoxic effector function; it has an indispensable, but undefined, role in granzyme-mediated apoptosis. Despite its vital importance, the molecular and cellular functions of perforin and the basis of perforin and granzyme synergy remain poorly understood. The purpose of this review is to evaluate critically recent findings on cytotoxic granule-mediated cell death and to assess the functional significance of postulated cell-death pathways in appropriate pathophysiological contexts, including virus infection and susceptibility to experimental or spontaneous tumorigenesis.

Cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells are effector lymphocytes that share common cytotoxic pathways that are necessary for defence against virus-infected or transformed cells. Both types of cell kill their cellular targets by either of two mechanisms that require direct contact between the effector and target cells. In the first pathway, cytoplasmic granule toxins — predominantly a membrane-disrupting protein known as perforin — and a family of structurally related serine proteases (granzymes) with various substrate specificities are secreted by exocytosis and together induce apoptosis of the target cell. The granule-exocytosis pathway powerfully activates cell-death pathways that operate through the activation of apoptotic cysteine proteases (caspases), but it also leads to cell death in the absence of activated caspases. The second pathway involves the engagement and aggregation of target-cell death receptors, such as FAS (CD95), by their cognate ligands, such as FAS ligand (FASL), on the killer-cell membrane, which results in classical caspase-dependent apoptosis. The main function of the FAS–FASL pathway is to eliminate self-reactive lymphoid cells.

Studies in gene-disrupted mice indicate clearly that the perforin pathway is involved intimately in defence against viral pathogens and transformed cells. But, recently, unsuspected roles for perforin as a crucial mediator of the immune surveillance of spontaneously transformed cells and in regulating B-cell homeostasis and T-cell memory have also been shown in vivo. Paradoxically, perforin is instrumental in the pathogenesis of certain autoimmune disease models, such as insulin-dependent diabetes in non-obese diabetic (NOD) mice, but it might suppress other autoimmune diseases, including experimental autoimmune encephalomyelitis (EAE). The signalling pathways that regulate granule polarization and exocytosis are being determined; these pathways might be disrupted by the interaction of cancer cells with some cytotoxic lymphocytes. The recent findings that granzyme entry into target cells involves binding to specific membrane receptors and that some tumours synthesize serpins (serine protease inhibitors) that can neutralize granzymes have indicated potential new ways in which tumours might escape immune attack. In this review, we discuss the functional significance of recent advances in our understanding of the granule (perforin/granzyme) pathway.
Box 1 | Insights into the molecular basis of granule-mediated cell death

The most important recent findings in terms of the physiological function of the granule-exocytosis pathway are listed below.

- Perforin and granzymes induce target-cell apoptosis cooperatively. Granzymes are necessary for triggering apoptosis in target cells, but they depend on being appropriately delivered by perforin. The cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells of ashen mice — which lack functional Rab27a, a protein that is involved in vesicular docking and secretion — have reduced killing through the granule-mediated pathway, owing to defective granule exocytosis in effector lymphocytes. Although many reconstituted models of granule killing have used purified perforin and granzymes, the recent demonstration that these toxins might be co-delivered as part of a macromolecular complex with the chondroitin sulphate proteoglycan serglycin reminds us of the limitations of many in vitro experimental systems.

- Granzyme B, which cleaves target-cell proteins at specific aspartate residues, is the most potent activator of caspase-mediated, as well as caspase-independent, cell death. The uptake of granzyme B into target cells is now believed to be mediated (at least in part) by endocytosis through the cation-independent mannose 6-phosphate receptor.

- Granzyme B mainly triggers caspase activation indirectly, rather than by direct caspase processing. It achieves this by directly activating pro-apoptotic BH3-only members of the BCL-2 family, such as BH3-interacting domain death agonist (BID), which results in the leakage of pro-apoptotic mitochondria mediators, such as cytochrome c, into the cytosol. BID-independent pathways for granzyme-mediated apoptosis also seem to exist. Some viruses encode potent granzyme-B inhibitors, and the endogenous granzyme-B inhibitor P19 might be expressed aberrantly by some cancer cells.

- Despite its inability to activate caspases, granzyme A can kill cells by the direct cleavage of nuclear proteins, thereby facilitating the formation and accumulation of single-stranded DNA breaks.

- The molecular basis for the relative resistance of CTLs and NK cells to perforin-mediated lysis has been explained recently. Henk and colleagues have shown that cathepsin B, a lysosomal protease that is also present in cytolytic granules, becomes sequestered on the effector-cell membrane after degranulation and can inactivate perforin molecules that diffuse back to the effector cell.

Cell-death pathways and immunity

The cytoplasmic granules of CTLs and NK cells are complex organelles that combine specialized storage and secretory functions with the generic degradative functions of typical lysosomes. In these "secretory lysosomes" are packaged the mediators of a collection of diverse cell-death pathways that have evolved to kill cells harbouring intracellular pathogens, particularly viruses, rapidly. As viruses depend on the metabolic machinery of living cells, many of them have devised ways to prolong the life of an infected cell by blocking apoptotic cell death. Even when the suicide pathways are stalled, the death stimulus that is mediated by an activated CTL or NK cell remains rapid, powerful and multi-pronged, and cell death follows in minutes, thereby limiting viral replication and spread. As viral defences against apoptosis have evolved under adaptive pressure from the host’s immune system, it follows that some viruses might have developed strategies to delay or prevent cell death induced by cytotoxic lymphocytes. Therefore, an exquisite evolutionary balance might exist between some pathogens and the immune mechanisms that are designed to limit their spread.

Although it is often vital for survival, the direct killing of infected cells is just one part of the total response to viruses. The importance of innate immune responses mediated through interferon-γ (IFN-γ) by NK cells is now appreciated, both to contain the initial infection and to promote an appropriate adaptive response, which takes several days to mature. Antigen-presenting dendritic cells (DCs) and subsets of T cells, such as CD1d-restricted NKT cells, regulate responses to peptide antigens that are presented on MHC class I molecules and to glycolipids, respectively. As-yet undefined events involving these cells establish a balance between innate responses and adaptive responses mediated by antigen-specific T and B cells. The role of perforin and granzymes in the pathophysiology of many diseases is now being dissected using gene-targeted mice that lack these molecules and in humans who have perforin mutations. Also, the involvement of death-receptor-mediated killing has been assessed in humans and mice with mutations in Fas (gld mice) or Fas (lpr mice), and in mice deficient in tumour-necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL). Much of the work that is discussed in this review is based on these powerful experimental settings.

New insights into granule-mediated cell death

The molecular pathways that underpin granule-mediated cell death have been reviewed recently. Perforin (described originally for its pore-forming properties) and the granzymes were purified, characterized biochemically and cloned many years ago. But, only recently has it been understood that the granule toxins cooperate to bring about the demise of targeted cells and do so by inducing apoptosis. Most importantly, the mediators of many cell-death pathways are packaged in the granules, but they all seem to depend on perforin for their effective delivery. The original notion that granzymes and other toxins diffuse into the cell through perforin pores in the plasma membrane is now being seriously questioned. More recent models take into account observations such as perforin-independent receptor-mediated uptake of granzymes by the target cell and the many interrelated death pathways that are activated by granzymes A and B (Box 1). Although these findings are clearly of great potential importance, the cellular and molecular functions of perforin remain contentious and continue to be fiercely debated.

New insights into granzymes and viral defence

Granzyme functions in vivo. The functions of granzymes A and B in inducing target-cell apoptosis have been investigated extensively in vitro, and they are better understood than the role of perforin at the molecular level. The fatal immunodeficiency that is observed in perforin gene-targeted mice infected with lymphoproliferative choriomeningitis virus (LCMV) indicates both the physiological relevance of the granule pathway for viral defence and the irreplaceable role of perforin in that process. The importance of perforin has been supported by studies in perforin-deficient mice that are infected with other viruses, including the natural poxvirus pathogen ectromelia. By contrast, individual granzymes show considerable functional redundancy, so that mice that are deficient in one or even several
Serpins
A serine protease inhibitor. Serpins are a large family of intracellular and extracellular protease inhibitors, with many diverse functions. Some serpins show 'cross-class inhibition' and are, therefore, effective inhibitors of other protease families, such as the cysteine proteases. PP9 is a recently described serpin that is synthesized by cytotoxic T lymphocytes (CTLs). It is postulated to neutralize granzyme B molecules that are misdirected to the cytosol, thereby protecting the CTL from accidental suicide. Many viruses encode serpins that block caspases, the enzymes that are responsible for apoptotic death.

Granules have more-focal immune deficits than perforin-deficient mice on the same genetic background (TABLE 1). The CTLs of mice that lack granzyme A induce morphologically normal apoptosis in vitro, and those of mice that are deficient in granzyme B induce the nuclear features of apoptosis (particular DNA fragmentation) more slowly than do wild-type CTLs. This is despite the fact that granzyme-B-deficient mice have since been found to lack granzyme C also, and to be defective possibly for the expression of other neighbouring granzyme genes. The genes encoding all of these granymes are structurally related and tightly linked on chromosome 14. Evidence for an indispensable role for granzymes in viral immunity has emerged only in the past few years, with the finding that mice that lack both granzyme A and the ‘granzyme B cluster’ are as susceptible as perforin-deficient animals to cytomegalovirus infection. The most important progress with granzyme-deficient mice and viral infection has come from the joint work of Mullbacher, Simon and co-workers. The susceptibility of granzyme-deficient mice to cytomegalovirus was not associated with an inability to mount a CTL response to other pathogens or any additional susceptibility to related viruses. This raises the interesting possibility that granymes might either have additional extracellular functions or have evolved specifically to restrict poxvirus replication. Serpins that are elaborated by this family of viruses are far less efficient at blocking the granule-mediated, perforin-dependent cell death that is mediated by MHC-restricted antiviral CTLs than the purely caspase-dependent death that follows Fas ligation or cytokine processing through non-apoptotic caspases such as caspase-1 (REF 22). These data show that in cytomegalovirus infection, the perforin-mediated cytolytic pathway is not the primary target of serpins. These findings reiterate the relative importance of the perforin pathway for defence against viruses in vivo and point to the fact that granyme pathways do not depend solely on their capacity to activate caspases. Further study of cytomegalovirus in mice that are gene-targeted for granyme B only or other individual specific granymes (granymes C–M) will be very interesting.

Granymes. GVHD and tumour immunity. The transfer of allogeneic cells with cytotoxic capacity can result in tissue damage in the recipient. Although many graft-versus-host disease (GVHD) models indicate that the Fas–FasL pathway is of primary importance, granyme A and the ‘B-cluster’ granymes were shown to be essential for the lethal effects of GVHD induced by transferred alloseective CD8+ T cells. By contrast, alloseective CTLs from granyme-A- and/or ‘B-cluster’-deficient mice rejected melanomas in severe combined immunodeficient (SCID) mice as efficiently as did wild-type alloseective CTLs after adoptive transfer. It is possible that alloseective CTLs raised ex vivo might have a different dependence on granymes for perforin-mediated cytotoxicity to those arising in situ in GVHD.

**Table 1 | Mice with deficiencies or mutations in CTL death pathways**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
<th>Apoptotic response</th>
<th>Gene function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>gld (FasL mutant)</td>
<td>Autoimmune nephritis, lymphadenopathy, lymphoproliferation, hypergammaglobulinaemia</td>
<td>CTLs do not kill via the Fas–FasL pathway</td>
<td>Crucial for lymphoid homeostasis</td>
<td>124</td>
</tr>
<tr>
<td>lpr (Fas mutant)</td>
<td>Upragulation of Fasl; otherwise similar to gld</td>
<td>Target cells resistant to FasL-mediated apoptosis</td>
<td>Crucial for lymphoid homeostasis</td>
<td>125</td>
</tr>
<tr>
<td>Granzyme A deficient</td>
<td>Healthy, fertile; somewhat susceptible to cytomegalovirus infection</td>
<td>Unaltered</td>
<td>Defence against poxviruses</td>
<td>16, 20</td>
</tr>
<tr>
<td>Granzyme B deficient</td>
<td>Healthy, fertile; somewhat susceptible to cytomegalovirus infection</td>
<td>Delayed nuclear apoptotic changes in target cells</td>
<td>Mediates rapid cell death; defence against poxviruses</td>
<td>17, 26</td>
</tr>
<tr>
<td>Granzyme A and B deficient</td>
<td>Healthy, fertile; markedly susceptible to cytomegalovirus infection</td>
<td>Delayed nuclear apoptotic changes in target cells</td>
<td>Granyme A or B essential for controlling cytomegalovirus</td>
<td>19, 26</td>
</tr>
<tr>
<td>Perforin deficient</td>
<td>Healthy, fertile; reduced ability to clear many viral and other intracellular pathogens; highly susceptible to spontaneous B-cell lymphoma, and to many transplanted tumours and their metastases</td>
<td>Total lack of granyme-mediated apoptosis</td>
<td>Granyme-mediated death is important for tumour surveillance and defence against viruses</td>
<td>14, 61, 126</td>
</tr>
<tr>
<td>Perforin and FasL deficient</td>
<td>Female infertility; early death due to severe pancreatitis; hystero-salpingitis; massive expansion of CD4+ T-cell and macrophage populations</td>
<td>Defective granyme-mediated and Fas-mediated apoptosis</td>
<td>Granymes and Fas constitute a homeostatic response</td>
<td>43</td>
</tr>
<tr>
<td>Cathepsin C (dipeptidyl-peptidase I) deficient</td>
<td>Healthy, fertile</td>
<td>Failure to produce active granymes and some other haematopoietic serine proteases</td>
<td>Unknown</td>
<td>29</td>
</tr>
</tbody>
</table>

*Mice that were described originally as being deficient in granyme B only were subsequently found to lack the products of several linked genes also, including granyme C. CTL, cytotoxic T lymphocyte; FasL, Fas ligand; GVHD, graft-versus-host disease.
Notably, granzymes were also unnecessary for NK-cell-mediated rejection of MHC class I–deficient tumours or methylcholanthrene-induced sarcomas, whereas perforin clearly was required. Further studies must now be undertaken in other tumour, transplantation and immunoregulation models to determine whether granzymes have key functions in the absence of a specific pathogen challenge. Granzyme-deficiency states have not been identified yet in humans; however, patients who are deficient in dipetidyl peptidase I (DPPI; cathepsin C) should lack active (processed) granzymes A and B, and might, therefore, provide some clues.

**Perforin and granzyme synergy**

On the basis of ultrastructural analyses during the 1980s of purified perforin added to inert membrane preparations, such as lysed erythrocytes, the initial consensus was that polyperforin pores induce osmotic instability and target-cell lysis (necrosis) (Fig. 1a). The antigenic similarity between perforin and complement component 9 (C9) reinforced this view. When the pro-apoptotic activity of granzymes was discovered in the 1990s, the ‘textbook’ model was modified, so that polyperforin pores acted as passive conduits for granzymes to cross the cell membrane (Fig. 1b). In fact, there is virtually no evidence for
**Box 2** | Familial haemophagocytic lymphohistiocytosis (FHL)

**Clinical presentation**
Familial haemophagocytic lymphohistiocytosis (FHL) is a rare, congenital immune deficiency that occurs in infants and young children. The presentation is usually with unexplained fever, malaise and failure to thrive, in the context of marked hepatosplenomegaly, blood-clotting disturbances and neurological symptoms. The presentation might be preceded by overt viral infection, but there is no direct relationship with any specific pathogen. The condition is fatal, unless treated by bone-marrow transplantation.

**Pathology**
This involves the infiltration of lymphoid tissues and other organs by activated lymphocytes and macrophages, and rampant haemophagocytosis in the spleen. The patients have hypersecretion of inflammatory cytokines, such as tumour-necrosis factor and interleukin-1, and severely reduced or absent natural killer (NK)-cell activity. About one third of patients have little or no detectable perforin in their NK cells or activated CD8+ T cells.

**Genetics and pathogenesis**
The disorder is inherited as an autosomal recessive trait. At least three loci on chromosomes 9 and 10 have been implicated in mapping studies. About one in five cases is due to a structural mutation of the perforin protein, which is encoded on chromosome 10 (REF. 48). Missense and nonsense mutations (encoding perforin truncations) have been identified in many parts of the perforin gene. The molecular basis of the remaining cases is unknown.

**Mouse model**
Perforin-deficient mice remain generally healthy if maintained in a clean facility, but about half of the animals develop spontaneous B-cell lymphoma as they age (almost invariably beyond one year of age) 49. Mice that are infected experimentally with lymphocytic choriomeningitis virus have a remarkably similar disease course to FHL patients, with absent NK-cell responses and anti-viral cytotoxic T-lymphocyte activity, hepatosplenomegaly and elevation of circulating cytokine levels in the setting of uncontrolled viraemia.

The stable formation of transmembrane pores in vivo. Such lesions can be formed in living cells in vitro by high concentrations of purified perforin or by contact with CTL or NK-cell clones that are continuously stimulated with high concentrations of interleukin-2 (IL-2), but the physiological relevance of this finding is unclear. Ring lesions have been identified only tentatively in one paper that investigated the immunopathology of viral myocarditis 50. Although perforin is clearly an essential enabler of granyme-mediated apoptosis, there is now considerable and diverse evidence that its role is not simply to provide transmembrane pores. As has been shown by many groups, the uptake of granzymes into cells does not require perforin and is mediated efficiently and rapidly by receptor-mediated endocytosis 51–54 (FIG. 1c). Also, nucleated cells must respond rapidly to osmotic stress, by internalizing or shedding ‘leaky’ patches of plasma membrane, so it is not surprising that static membrane pores are hard to find. It is well accepted that ‘lytic’ concentrations of perforin that are insufficient for significant osmotic injury can deliver pro-apoptotic granzyme B into a cell effectively 55, but there is also evidence that cells that are exposed to high concentrations of perforin can die an apoptotic, rather than necrotic, death. Cells that are exposed to perforin concentrations that cause >95% loss of intracellular [3H]Cr pre-loaded into their cytoplasm (chromium-release assay) continue to exclude spherical molecules of as little as 9 kDa for more than 90 minutes, whereas they avidly take up fluorescein isothiocyanate (FITC)-labelled 32-kDa granzyme B, which results in cell shrinkage and death by apoptosis 56. This important observation clearly shows the selectivity of the membrane-disrupting activity of perforin. The pro-apoptotic function of perforin could not be replicated by adding other membrane-disrupting proteins with granzyme B, even when high concentrations of the complement membrane-attack complex were used, the pores of which have strong structural and antigenic similarities to polyperforin pores 57. The recent discovery that granzymes and perforin both bind to the target-cell surface as part of a single macromolecular complex associated with serglycin 58 further diminishes the probability of passive diffusion of granzymes. Rather, we favour a role for perforin in disrupting endosomal trafficking after granzyme uptake into the target cell, although it has not been possible to show the entry of perforin into the target-cell cytoplasm 59 (FIG. 1d).

Recently, it has been shown that the mannose 6-phosphate receptor (MPR) can act as a receptor for granzyme-B uptake when it is overexpressed on the surface of certain cells 60. Normally, most MPR is found in the trans-Golgi network and in late endosomes, whereas little is found on the cell surface. It has been known for many years that mannose 6-phosphate residues on granymes A and B enable the accurate intracellular trafficking of granymes to CTL/NK granules through the MPR pathway 61, and the new findings are an extrapolation of the previous ones. The findings of Motyka et al. 62 are particularly provocative as they showed that the absence of MPRs prevented the rejection of tumour allografts across a complete H-2 mismatch. If corroborated, this finding would indicate an essential role for granzyme B in rejection across a complete H-2 mismatch, which has yet to be shown in granzyme-B- or perforin-deficient mice. Also, the findings potentially raise a new mechanism of tumour immune evasion, which would have consequences for CTL vaccine approaches to cancer.

**Functional consequences of perforin deficiency**
Host survival depends on the immune system for protection against pathogens and malignant cells, and on regulatory mechanisms that prevent immune-mediated tissue damage. Studies in perforin-deficient mice have confirmed the essential in vivo role of perforin in target-cell apoptosis induced by CTLs and NK cells 63, in immune responses to cancer 64 and in certain infections 65. In vivo studies have also indicated an important immunoregulatory role for perforin 66–68. A widespread role for perforin in immunosurveillance and immunoregulation has been strengthened by the observed high incidence of spontaneous malignancy, particularly B-cell lymphoma, in perforin-deficient mice 69 and the mapping of perforin mutations in a lethal, inherited human disorder of immune dysregulation known as familial haemophagocytic lymphohistiocytosis (FHL) 70 (Box 2). The implications of these and other recent developments in perforin biology are discussed below.
Perforin and tumour immunity. The generation of gene-targeted mice has enabled investigators to define more accurately the importance of single effector molecules for tumour immunity. Natural41 and IL-12-induced42 anti-metastatic activities of NK cells against various non-lymphoid tumours — including melanomas, and prostate and breast carcinomas — have been shown to be perforin mediated. In several tumour models that are controlled by innate immunity involving NKT cells and/or NK cells, host IFN-γ and direct cytotoxicity mediated by perforin independently function as anti-tumour effectors that together control the initiation, growth and spread of tumours in mice. Nevertheless, perforin-deficient NK cells can upregulate the expression of Fas on some tumour cells directly and then kill the cells in a Fas-dependent manner43. Furthermore, inhibitors of apoptosis can block the rejection of tumours mediated by NK cells through the FasL pathway13,14, which indicates that death-receptor-mediated apoptosis has a more prominent role in the clearance of NK-cell-sensitive tumours than was suggested previously. In addition to perforin and FasL, NK cells also use TRAIL to control the initiation, growth and metastasis of tumours in mice15,16.

Granule-mediated killing is also of paramount importance for CTL-mediated lysis in vitro, and one of the most successful CTL vaccines against aggressive mouse tumours requires perforin-mediated effector function17. Nevertheless, some CTL-mediated tumour rejection is clearly independent of perforin18,19. This is true for r2 CTLs20, in that the transfer of perforin-deficient T2 cells showed no difference in therapeutic efficacy compared with wild-type T2 cells. It should be noted that many of these studies involved the adoptive transfer of large numbers of perforin-deficient CTLs to sites at which the secretion of cytokines, such as IFN-γ and TNF, might be sufficient to inhibit tumour growth.

Most of the experimental models that have been studied so far have been of limited relevance to tumours that arise de novo as a result of environmental mutagens or genetic defects. Immunosuppression clearly predisposes to malignancy, particularly in the lymphoepithelial compartment. In modern clinical practice, this is best recognized in the post-transplant setting and in AIDS-related immunodeficiency, both of which are strongly associated with malignancy, particularly tumours that have a clear viral aetiology (such as those induced by Epstein–Barr virus [EBV] and herpes simplex virus). Immune surveillance by cytotoxic lymphocytes against cancer has been postulated for decades, but direct evidence of a protective role against spontaneous malignancy has been lacking. Recent studies in mice have clearly shown immunosurveillance of various spontaneous malignancies45–48. In particular, these studies have highlighted an important role for perforin expressing cells — including CTLs, NK cells, NKT cells and γδ T cells — in preventing spontaneous tumour formation. Ageing perforin-deficient mice from two genetic backgrounds (C57BL/6 and BALB/c) have been shown to be more susceptible to spontaneous B-cell lymphoma and myeloma than wild-type ageing mice. Experiments with murine leukaemia virus have also indicated an important role for perforin in regulating the onset of lymphoma44. Together, these findings raise the issue of whether malignancy in the B-cell compartment of ageing perforin-deficient mice is caused by the aberrant and sustained proliferation of these cells after microbial challenge or retrovirus activation. At present, it is difficult to rule out an effect of endogenous retroviruses that are activated with age. An alternative explanation for spontaneous lymphomas of B-cell origin is the dysregulated control of B-cell activation and survival in perforin-deficient mice. So far, the development of lymphoma in two mouse strains has been documented under pathogen-free conditions, but it is difficult to eliminate a role for chronic B-cell stimulation by commensal organisms, as this would require housing the animals in totally germ-free conditions for their entire life. However, so far, a role for perforin in immunoregulation has been observed only after microbial challenge — for example, LCMV infection in perforin-mut mice, or in children with FHL — or in the absence of another main regulatory pathway such as FAS–FASL. It remains to be tested whether the perforin pathway normally compensates for FAS, in that mutations of FAS and other oncogenic mutations rarely occur together in B cells. Regardless of the aetiology of these spontaneous lymphomas, they are strongly rejected when transplanted into wild-type mice, but not perforin-deficient mice, thereby confirming the role of perforin in immunosurveillance and the impressive immunogenicity of the spontaneous lymphomas.

Do cytotoxic lymphocytes protect epithelial tissues from cancer? This issue is crucial, because carcinomas of breast, lung, colon and prostate are common and among the most difficult to treat. The ability to augment any cytotoxic response would potentially provide a valuable adjunct to the therapy that is available at present. Past studies have indicated that at least some chemically induced sarcomas (non-haematological) are controlled in a perforin- and IFN-γ-dependent manner21, and more recently, the late onset of lung adenocarcinomas in 15–20% of ageing perforin- and IFN-γ-deficient mice has been reported. These studies are a prelude to examining spontaneous tumour formation in larger groups (>100) of ageing perforin-deficient mice. Exciting improvements in genetic techniques and mouse models of epithelial cancer will now enable further studies in which perforin-deficient mice are back-crossed to mouse models of cancer — such as TRAMP-transgenic (prostate carcinoma), Her2/Neu-transgenic (mammary carcinoma), k-Ras-mutant (spontaneous lung cancer) or Apc-mutant (colon adenoma) mice. These models should also allow an assessment to be made of the stage (such as hyperplasia, carcinoma in situ or invasive malignancy) at which perforin-mediated surveillance exerts its effects on the tumour and its ability to metastasize.

Perforin and transplantation. Perforin-mediated cytotoxicity also has an important role in the mechanisms of GVHD and graft-versus-tumour (GVT) effects after

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**T**<sub>1/2</sub> A designation that is used to describe subsets of CD8<sup>+</sup> cytotoxic T lymphocytes. T<sub>1</sub> cells typically secrete IFN-γ and GM-CSF and have strong cytotoxic capacity, whereas T<sub>2</sub> cells secrete IL-4 and IL-10 and are less effective killers.
indicate that graft-versus-host disease (GVHD) can be mediated mainly through the Fas–Fas ligand (Fasl) pathway, which is activated upon recognition of host MHC by the T-cell receptor (TCR). By contrast, transformed cells that are induced by local interferons (IFNs) to upregulate their surface expression of peptide–MHC complexes mainly activate the granule cell–death pathway in CD8+ cytotoxic T lymphocytes (CTLs). This functional dichotomy might be exploited to maximize graft-versus-tumour (GVT) effects and minimize GVHD by specifically inhibiting the Fas–FasL pathway. IFNR, IFN receptor.

GVHD is an important complication of allogeneic BMT and is dependent on both donor T-cell perforin- and FASL-mediated pathways. FASL-defective donor T cells have diminished GVHD activity, but have intact GVT activity. The FASL pathway is important for the GVHD activity of both CD4+ and CD8+ T cells. By contrast, perforin-deficient donor T cells have intact GVHD activity but diminished CD8+ T-cell-mediated GVT activity. These and other data indicate that blockade of the FAS–FASL pathway could be used to ameliorate GVHD without impairing the GVT effect in allogeneic BMT (FIG. 2). NK cells also mediate the acute rejection of incompatible bone-marrow cell (BMC) grafts. The perforin and FASL pathways have been shown to have an important role in NK-cell-mediated rejection of allogeneic and MHC class-I-deficient BMCs. The relative roles of donor T-cell and NK-cell perforin, FASL, TNF and TRAIL in GVHD and GVT effects after allogeneic BMT remain to be determined. EBV-specific CD8+ T cells in patients who suffer from EBV-associated lymphoproliferative disease after BMT contain intracellular perforin and have cytotoxic activity against autologous EBV-derived lymphoblastoid cell lines that is perforin dependent.

Determining the expression of perforin in EBV-specific lymphocytes, together with a quantification of EBV DNA, might be useful for predicting the clinical course of patients who have post-transplant lymphoproliferation.

Perforin, immunoregulation and autoimmunity. Perforin-deficient mice have essentially normal immune homeostasis. For example, perforin deficiency impairs neither the rejection of grafted lymphocytes nor the in vivo homeostasis of DCs. However, considerable evidence indicates a pivotal role for perforin in immunoregulation in situations of immune–system disturbance caused by microbial infection, autoimmunity or loss of other cell–death pathways. For example, as in perforin-deficient gld (Fasl-defective) mice, several studies have shown recently that perforin regulates the elimination of CD8+ T cells after an acute exposure to foreign antigen. Notably, perforin-deficient mice have an increased clonal expansion and persistence of superantigen- and virus-specific T cells that could not be reproduced by inhibiting the elimination of antigen-presenting cells in perforin-sufficient mice. These findings, and the increased clonal expansion of alloreactive perforin-deficient T cells after their transfer into irradiated scid/scid mice (which lack T and B cells), strongly support a role for perforin in activation-induced cell death. In a GVHD model, perforin had an important regulatory role in the prevention of humoral autoimmunity through the elimination of both autoreactive B cells and antigen-specific T cells. Following on from previous studies by Binder et al., Matloubian et al. showed that perforin is involved in downregulating T-cell responses during chronic LCMV infection. Together, these studies support the notion of an intrinsic role for perforin in regulating the expansion, and then the contraction, of CD8+ T-cell populations after infection. In summary, the data support the existence of a perforin-dependent mechanism to regulate the extent of CD8+ T-cell clonal expansion in models as diverse as acute bacterial infection, chronic and acute viral infection, GVHD and DC immunization (reviewed in ref. 72). However, this regulatory process might depend on the nature of the stimulus, including pathogen virulence or strength of stimulation, and might be influenced by other genetic factors.

Familial haemophagocytic lymphohistiocytosis. The excessive accumulation of CD8+ T cells in perforin-deficient mice, which results in immune-mediated damage, mimics the clinical picture in perforin-deficient children who suffer from FHL. FHL is a rare, rapidly fatal, autosomal recessive immune disorder that is characterized by the uncontrolled activation of T cells and macrophages and the overproduction of inflammatory cytokines. Non-malignant activated T cells and macrophages infiltrate the bone marrow, spleen, liver, lymph nodes and central nervous system, where they produce fever, hepatomegaly, pancytopenia, coagulation and neurological abnormalities. Serum levels of inflammatory cytokines derived from activated T cells and macrophages (such as IFN-γ, TNF, IL-1 and IL-6)
are markedly elevated. Indeed, FHL occurs in previously healthy infants, and is only curable by BMT. FHL is inherited as an autosomal recessive single gene defect that can be mapped to any one of at least three loci. Initial analysis of the perforin gene of unrelated 10q21–22-linked FHL patients showed that there are genetic and functional defects of perforin and established perforin as an important negative regulator of human cellular immune activation\(^4\). Mutations that have been characterized include homozygous nonsense mutations, missense mutations and single amino-acid deletions. A reported mutation in codon 374, which results in a premature stop codon, is the most common perforin mutation to have been identified so far in FHL patients\(^5\). Between 20\% and 40\% of FHL patients are estimated to carry perforin mutations of both alleles. CTL and NK-cell activity is markedly reduced or absent in these patients, and immunostaining or flow cytometry of their cultured lymphocytes showed little or no perforin in the cytotoxic granules\(^6\). A thorough examination of individual mutations in FHL patients and their relationship to perforin expression and function should indicate much about the key structural residues and domains in this protein. Although a causative infectious agent for FHL has not been defined yet, it is likely to be a microorganism, the control of which would be strictly perforin dependent.

**Perforin and autoimmunity.** Perforin has also been shown to contribute to or suppress autoimmune disease in a limited number of models. Autoimmune diabetes in NOD mice involves initial insulitis — which requires CD4\(^+\) T cells operating through the Fas–FasL pathway — and the final destruction of pancreatic \(\beta\)-cells by CD8\(^+\) CTLs\(^7\). Perforin-deficient NOD mice were shown to develop diabetes rarely, despite the fact that they express FasL and have fully developed insulitis\(^8\). Interestingly, mice that lack only one perforin allele were also less susceptible to diabetes, which indicates the importance of gene dosage in this disease setting. Surprisingly, however, perforin-deficient K3-TCR transgenic NOD mice that express an oligoclonal or monoclonal T-cell repertoire had a higher frequency of developing diabetes than their perforin-competent littermates\(^9\). Other transgenic models of diabetes give contrasting results, with some showing that disease is completely independent of the expression of perforin and Fas\(^10\) and others showing that diabetes requires both pathways\(^11\). The overexpression of Bel-2 by \(\beta\)-cells does not prevent autoimmune diabetes\(^12\), which is consistent with previous observations that Bel-2 can block perforin/granzyme-B-mediated cell death efficiently, but is ineffective against intact cytotoxic granules\(^13\). Strategies to prevent perforin-mediated apoptosis have potential merit, but we predict that they will necessarily have to target perforin specifically, rather than the granzymes. In contrast to mice with diabetes, in which perforin might be an effector molecule for the disease, perforin-deficient mice develop chronic relapsing myelin oligodendrocyte glycoprotein (MOG)-induced autoimmune encephalomyelitis\(^14\). This phenotype is similar to that observed in NK-cell-depleted mice that are injected with MOG\(^15\) and, so, it is possible that perforin suppresses T-cell autoimmunity when expressed by NK cells.

**Perforin and infectious disease.** The resolution of infections with many intracellular pathogens requires the effector functions of both NK cells and CD8\(^+\) CTLs. Direct cytotoxicity mediated by perforin is essential for the control of both non-cytopathic\(^16\) and cytopathic viruses\(^17\). Of the viruses that are controlled by perforin, only cytomegalovirus also requires granzymes for its elimination, although studies in many other virus models have yet to be reported. Perforin is also important for the control of intracellular bacterial infections, such as *Mycobacterium tuberculosis*, for which the combined action of perforin and the antibacterial agent granulysin — both of which are expressed in the granules of CTLs and NK cells — influences the outcome of infection\(^18\). Granulysin reduces the viability of a broad spectrum of pathogenic bacteria, fungi and parasites in vitro\(^19\). More recently, perforin-deficient mice were shown to have accelerated mortality after *Histoplasma capsulatum* infection\(^20\). The adoptive transfer of CD8\(^+\) T cells from perforin-deficient mice indicated that CD8\(^+\) T cells can use perforin-dependent and -independent mechanisms to control histoplasmosis, which is consistent with a role for IFN-\(\gamma\) and/or TNF in limiting fungal growth. Similar findings have been reported also for *Trypanosoma cruzi* infection\(^21\). In a model of *Histoplasma* and LCMV co-infection (where LCMV induces immunosuppression), it was shown that CD8\(^+\) T cells could suppress immunity by different mechanisms, and that immunopathology was perforin dependent, whereas lethality was perforin independent\(^22\).

**Effector-pathway selection influences pathology**

**Effector pathways can dictate viral immunopathology.** There is considerable evidence now emerging that the effector-cell response induced by a pathogen can influence immunopathology, often in an organ-specific manner. Recent evidence shows that effector molecules such as perforin and IFN-\(\gamma\) regulate some aspects of CD8\(^+\) T-cell homeostasis by mechanisms that might or might not be dependent on pathogen clearance. Naive CD8\(^+\) T cells do not express perforin or IFN-\(\gamma\), and mice that lack these molecules can mount CD8\(^+\) T-cell responses to infection, which indicates that these effector molecules are not required for the survival of naive CD8\(^+\) T cells. However, after viral challenge, the balance between perforin cytotoxicity and IFN-\(\gamma\) secretion can dictate survival and influence immune homeostasis and immunopathology. Augmented perforin-mediated immunopathology is observed in IFN-\(\gamma\)-deficient mice infected with LCMV\(^23\), and conversely, a large, fatal overproduction of inflammatory cytokines occurs in perforin-deficient mice that are infected with the same virus. After LCMV infection, viral clearance depends mainly on perforin, but virus-induced liver damage occurs only when both the Fas–FasL and perforin pathways, including granzymes A and B, are activated simultaneously\(^24\). Similarly, perforin is required for the
effective clearance of Theiler's virus, and in perforin-deficient C57BL/6 mice, the virus induces a severe immune-mediated demyelinating disease similar to EAE\(^{29}\). By contrast, in other viral infections, perforin might not be crucial for clearance, but it does impact severely on pathology. For example, although perforin-deficient mice can clear an ocular infection with herpes simplex virus normally, perforin-dependent cytotoxicity is the main determinant of chronic corneal inflammation and stromal keratitis\(^{40}\). In some infections, the apoptotic pathway that is used can influence life-threatening immunopathology. Both the perforin and Fas–FasL pathways are crucial for the invasion of encephalitic flavivirus into the brain, and mice that are deficient in either the granule–exocytosis or Fas pathways are resistant to a low-dose peripheral infection with this neurotropic virus\(^{41}\).

**Effectors pathways and T-cell homeostasis after infection.** In situations of pathogen challenge, antigen-specific T-cell homeostasis also seems to be regulated by perforin, although the details of this process are unclear. A key study with *Listeria monocytogenes* — a pathogen that is cleared, in part, by a perforin-dependent pathway — indicated that perforin dictates T-cell memory by controlling the clonal expansion of CD8\(^{+}\) T cells in response to antigen, whereas IFN-γ regulates immunodominance (that is, which epitope is preferentially recognized) and the death of CD8\(^{+}\) T cells\(^{42}\). These data supported an earlier study in which perforin was shown to downregulate T-cell responses during chronic LCMV infection\(^{43}\). In another study, a high LCMV burden drove virus-specific T cells to functional unresponsiveness (anergy) and eventual elimination. The absence of perforin, Fasl, or TNF receptor 1 (TNFR1) had no significant effect on T-cell proliferation at the onset of LCMV infection\(^{44}\). However, these molecules did reduce, in an additive fashion, the longevity of the virus-specific CD8\(^{+}\) T-cell population once it had become anergic. It is possible that the expansion of antigen-specific T cells in perforin-deficient mice might simply be the result of reduced clearance and increased antigen load. If perforin downregulates CD8\(^{+}\) T-cell immune responses directly, it remains to be determined whether this occurs by CTL FRATRICIDE or the elimination of antigen-presenting DCs. Extended DC survival could be an important factor given that the availability of antigen-presenting cells in vivo might limit immune responses\(^{45,47}\). Analysis of this regulation is complicated by the fact that immature DCs might be targets for perforin-expressing NK cells\(^{64}\), so that perforin-deficient NK cells might skew the innate immune response. Furthermore, some pathogens might influence the pool of perforin-expressing effector T cells by influencing CTL maturation directly\(^{49}\).

**Organ specificity of effector responses.** The various apoptotic pathways of cytotoxic lymphocytes have organ specificity. For example, adenovirus-infected hepatocytes are more sensitive to killing through Fas–FasL than through the perforin pathway, but the delayed clearance of adenovirus in Fasl-mutant mice from the liver has not been observed in other organs\(^{50}\). These data support previous observations in murine cytomegalovirus infection, in which perforin could control viral titres in the spleen more effectively than in the liver\(^{51}\). This organ specificity might not be explained only by the differential sensitivity of different cell types to perforin, but also by the local cytokine environment. Cytokines such as IL-4 can both enhance FasL expression on CD8\(^{+}\) T cells\(^{102}\) and induce the development of T-cell populations that express very little perforin and granzymes\(^{103}\).

**Escape from CTL/NK-cell-mediated cell death.** Tumour and pathogen immune-escape mechanisms that control lymphocyte-mediated apoptosis have been well documented and debated\(^{104,105}\). Most attention has been focused on the ability of tumours and viruses to subvert the death-receptor pathways. However, it has become apparent recently that some granule-exocytosis pathways can be inhibited by viral proteins and endogenous intracellular serpins. The adenovirus L4–100 kDa assembly protein (100K) is a granzyme-B substrate that prevents cytotoxic-lymphocyte-mediated granule-induced apoptosis of infected target cells by potently inhibiting granzyme B\(^{106}\). This inhibition is absolutely dependent on residue Asp48 in 100K, which is found in a classic granzyme-B consensus cleavage motif. Herpes simplex virus 1 (HSV-1) inhibits perforin/granzyme-B-mediated apoptosis by expression of the Us5 gene, which encodes glycoprotein J\(^{107}\), although its mechanism of action is unknown. The discovery of endogenous serpins such as P19 that regulate the activity of granzymes in cytotoxic lymphocytes\(^{108}\) has given rise to the idea that they might be overexpressed by tumours to block granzyme B\(^{109}\) (Fig. 3). Although various human and mouse tumours do express serpins, there is no evidence so far that granzyme B is a key weapon of the immune system to eradicate tumours. Perhaps more interesting is that serpins are expressed by some antigen-presenting cells, and might therefore prolong their survival. Mature DCs become more resistant to CTL-mediated apoptosis by virtue of the expression of serpins\(^{110}\).

An emerging, new mechanism of tumour immune escape is the ability to inhibit the microtubule-organizing centre (MTOC) of tumour-infiltrating lymphocytes (TILs), thereby precluding granule exocytosis and accounting for defective TIL lytic function\(^{111}\). Interestingly, the recovery of TCR-mediated MTOC mobilization requires proteasome function, which indicates the existence of a protein inhibitor of MTOC mobilization. Normally, a SYK/ZAP70–phosphatidylinositol 3-kinase (PI3K)–dependent signal cascade is triggered in CTLs and NK cells by target-cell recognition, which mobilizes the lytic granules towards the contacted target cell\(^{112}\). The inhibition of PI3K in NK cells blocks downstream kinase activation and interferes with the movement of perforin and granzyme B towards target cells\(^{113}\). By contrast, FASL–mediated killing is not dependent on the PI3K pathway\(^{114}\). These data imply that tumours, viruses and, indeed, normal cells could potentially inhibit death pathways selectively, independent of TCR ligation.
Figure 3 | Putative tumour immune-escape mechanisms operating at the level of CTL/NK-cell-mediated apoptosis. Hypothetically, cancer cells might inhibit apoptosis by failing to express mannose 6-phosphate receptor (MPR) on the cell surface, by overexpressing an endogenous serpin, such as the granzyme-B-specific inhibitor P19, or by expressing large amounts of FAS ligand (FASL) on their cell surface, leading to the death of cytotoxic lymphocytes (CTLs and NK cells). Putative (so far unidentified) proteases that cleave and inactivate perforin are also a possibility. The interaction of T-cell receptor (TCR) on CTLs with tumour peptide-MHC class I complexes might lead to faulty signalling through cascades that include the phosphoinositide 3-kinase (PI3K) pathway, the function of which is required normally for granule polarization and exocytic release of granule toxins. None of these modes of immune inhibition has yet been shown to operate in human cancer. The many ways in which cancer cells evade the immune system at the level of antigen presentation and/or expression of co-stimulatory molecules have not been considered in this figure. CTL, cytotoxic T lymphocyte; NK cell, natural killer cell; ZAP70, ζ-chain-associated protein kinase, 70 kDa.

**Future perspectives**

Immunologists, and cellular and molecular biologists are finally coming to grips with the molecular intricacies of the diverse apoptotic pathways that are induced by cytotoxic granules. With the recent explosion in our knowledge of caspase-based (intrinsc) apoptotic mechanisms, the spotlight has been on granzyme B because of its caspase-like proteolytic activity. But, we believe that it is a vast and potentially misleading over-simplification to functionally equate granzyme and caspase functions, and many biological questions remain about the essential role of granzyme B in immunity. Why are granzyme-B-deficient mice so healthy, and how do they deal so competently with so many viruses? Moreover, how do granzyme-B-deficient CTLs induce rapid and efficient cell death despite lacking the one granzyme that cleaves proteins at aspartate residues? What are the physiological roles of the ‘orphan’ granzymes, such as granzymes C, M and H, for which pro-apoptotic activity has not been shown? Could it be that each granzyme has evolved to protect the host against a specific dangerous virus, and that some of the protective mechanisms involve more than pro-apoptotic effects? Despite intense work over more than two decades, the complexity of cytotoxic granules is only now becoming appreciated, and many granule constituents remain undefined. Modern proteomic approaches will no doubt be brought to bear on these areas of research in the near future, and the spectrum of recognized granule-bound toxins and immunomodulatory agents might grow rapidly.

Our analysis of the available in vivo data shows that only one molecule is indispensable for granule-mediated cell death — perforin. It is remarkable that so little is known about how perforin functions at the molecular and cellular level, and yet, the clear lack of functional redundancy in perforin-deficient animals makes it clear that the best target for reducing CTL-mediated tissue damage in settings such as graft rejection and certain autoimmune diseases. The fact that NOD mice that are hemizygous for perforin expression have a reduced life-time incidence of diabetes compared with wild-type NOD mice offers hope that even a partial inhibition of the perforin pathway might lead to a therapeutic benefit. With an understanding of perforin structure and function will come a massive opportunity for generating focused immunosuppressive agents targeted specifically at effector CTLs. The recent discovery of an immune-deficiency state (FHL) that arises specifically from mutations in perforin function opens the door to a far deeper understanding of the individual domains and residues of perforin, provided that strategies can be developed to express mutated perforin molecules efficiently in suitable cells, and ideally also as purified, recombinant proteins.
Finally, we believe that it is becoming increasingly essential that wherever possible, experimental observations that are made in vitro are tested for their biological importance in vivo. The advent of gene-targeted mice offers many such opportunities, and several important observations have been made already, particularly concerning the role of cytotoxic lymphocytes in cancer immunosurveillance and viral immunity. The putative immunoregulatory roles of effector molecules such as perforin, IFN-γ and TRAIL, in particular, are only just becoming appreciated, and these complex interactions involving many cell types can only be studied meaningfully in intact animals. When combined with the sophistication of new cancer and infectious-disease models, and the promise of tissue-specific and conditional genetic knockout animals, transgenesis and a powerful technological platform for testing in vitro findings in whole animals. These approaches are likely to become particularly relevant to the immunological research of cancer when immunologically informative mouse models are interbred with genetically engineered cancer-prone mice.

4. References 2 and 3 were the first to show that granzyme B (reference 2) and CTLs (reference 3) can kill target cells independently of caspase activation.
15. A seminal paper showing the marked deficiency of perforin-deficient mice.

This study establishes that both granzyme A and ‘B-cluster’ granzymes are indispensable effector molecules that act in concert with perforin in host defense against the natural viral pathogen endocytosis. It describes the most convincing phenotype for a mouse lacking the 17q21–22-linked (1998).
28. CTLs kill Mycobacterium tuberculosis in infected cells in a perforin-dependent manner that requires the action of the granule-bound and evolutionarily ancient toxin granulysin.
34. The first description of perforin-mutant humans (10q21–22-linked familial haemophagocytic lymphohistiocytosis, FHL) confirms that perforin-based effector systems are involved not only in the apoptosis of abnormal cells, but also in the down-regulation of cellular immune activation.

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This study presents clear evidence that perforin- and Fasl-independent mechanisms contribute to the regulation of tumor immunity. The findings have implications for the development of immunotherapies targeting cancer and highlight the importance of understanding the interplay between these different effector mechanisms in vivo.


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