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Review

Granzymes in cancer and immunity

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Cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells are indispensable factors in the body's ongoing defence against viral infection and tumor development. CTL/NK cells recognize and kill infected or abberant target cells by two major pathways: either through introduction of a battery of proteases – called granzymes – to the target cell cytosol, or through TNF superfamily-dependent killing. During granzyme-dependent killing, target cell death is quick and efficient and is mediated by multiple granzymes, acting via redundant cell death pathways. Although granzyme-mediated cell death has been intensively studied, recent work has also hinted at an alternative, proinflammatory role for these enzymes. Thus, in addition to their well-established role as intracellular effectors of target cell death, recent data suggest that granzymes may have an extracellular role in the propagation of immune signals. In this study, we discuss the role of granzymes as central factors in antitumor immunity, as well possible roles for these proteases as instigators of inflammation.
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Cytotoxic T cells (CTL) and natural killer (NK) cells recognize and kill virus-infected or transformed cells through two main pathways. CTLs use ligands of the tumor necrosis factor superfamily on their cell surface to bind and eliminate target cells expressing the corresponding receptors.[1] In addition, CTL/NK cells also use the granule exocytosis pathway to target cell death, which operates through the delivery of the contents of cytotoxic granules to the surface of target cells. The granule protein, perforin, promotes granzyme delivery to the target cell cytosol and, on entry, these proteases cleave their cohort of substrates to promote rapid and efficient cell death.[2]

Although there are multiple granzymes, each with distinctive cell death-promoting activity, evidence implicating individual granzymes with protection from tumor formation has been relatively scarce, as mice lacking individual granzymes typically remain cancer free. Because perforin facilitates the delivery of all granzymes to target cells, CTL/NK cells from perforin-deficient mice are defective in granzyme-mediated cytotoxicity.[3,4] Importantly, perforin-knockout animals are much more susceptible to spontaneous tumor development than wild-type littermates and also succumb to chemically induced tumors at a higher rate, suggesting a redundancy of function between individual granzymes with regard to tumor control.[3,5–7]

Elevated levels of circulating extracellular granzymes A and B have previously been associated with various inflammatory diseases.[8–10] Although this could simply reflect elevated CTL/NK numbers in response to persistent inflammation – and associated spontaneous or inadvertent release of granzymes into the extracellular space – recent studies have suggested an alternative explanation for the presence of circulating granzymes.[11–13] In what could well turn out to be a landmark paper on the subject, Metkar *et al.*[12] have shown that extracellular granzyme A can promote the release of proinflammatory cytokines from human monocytic cells and murine peritoneal macrophages, the significance of which is underlined by the finding that granzyme A-deficient mice are resistant to the lethal effects of endotoxic shock. Coupled with observations that certain granzymes are expressed and secreted by B cells, mast cells, keratinocytes, basophils, as well as other cell types, in the absence of detectable perforin, this suggests that granzymes may have hitherto unsuspected roles in immunity.[11,14–20] Thus, in addition to acting as cell death effector molecules on delivery to target cells, granzymes may also possess activity on release into the extracellular space (Figure 1). The expanding role for granzymes as possible soluble mediators of inflammation will be discussed later in this review.

Cytotoxic Functions of Granzymes

CTL/NK granzymes are typically safely contained within cytotoxic granules, in which they are prevented from damaging the host cell. After a CTL/NK cell recognizes a target, cytotoxic granules move along microtubules to polarize at the plasma membrane, adjacent to the target, where they are secreted into the immunological synapse between the two cells.[2] The major constituents of cytotoxic granules are perforin and granzymes, which combine to promote rapid cell death when delivered to

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Abbreviations: CTL, cytotoxic T lymphocyte; NK, natural killer; TNF, tumor necrosis factor; CAD, caspase-activated DNase; ICAD, inhibitor of caspase-activated DNase; RAG2, recombination-activating gene 2; MCA, Melamine Cyanurate; TCR, T cell receptor; TGF, transforming-growth factor; Treg, T regulatory; AICD, activation-induced cell death; IL, interleukin; MCMV, murine cytomegalovirus; HSP, heat-shock protein
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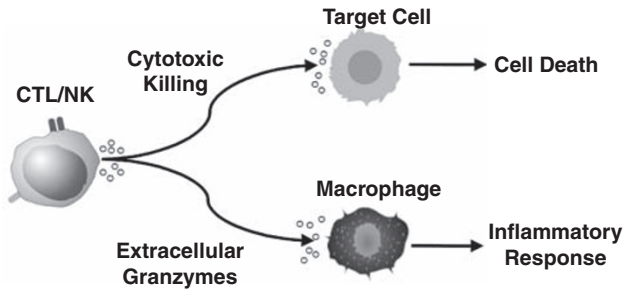

Figure 1 Cytotoxic *versus* inflammatory roles of granzymes. Although a large body of evidence implicates cytotoxic granule constituents in pathways resulting in target cell death, recent evidence suggests that these granzymes may also serve alternative functions as initiators or amplifiers of inflammatory responses. In the latter situation, granzymes released into the extracellular space may directly or indirectly provoke inflammatory reactions by acting on immune cells or through the actions of extracellular granzyme substrates on such cells

the target cell cytosol. The pore-forming protein, perforin, was originally thought to facilitate granzyme entry into target cells by physically forming holes in the cell membrane through which granzymes may pass.[21] Although the intervening years have thrown up alternative mechanisms for perforin-mediated granzyme delivery, studies using knockout mice have unequivocally demonstrated the crucial role that this protein has in granzyme-mediated cytotoxicity.[22,23] As effector cells lacking perforin cannot deliver granzymes to target cells, perforin deficiency translates into a complete loss of cytotoxic granzyme function, with perforin-deficient CTLs defective for target cell killing.[22,23] As we shall discuss later, this leads to a plethora of immune deficiencies, which highlight the important role that the perforin/granzyme system has in defending the body against disease.

Granzymes are a distinct family of serine proteases, with different members harbored by humans and mice, likely reflecting the diverse environmental challenges met by their respective immune systems, which has led to different evolutionary trajectories of their constituent granzymes. Along with granzyme A, granzyme B is one of the most abundant granzymes and, consequently, granzyme B-mediated cytotoxicity has been intensively studied.[2,24,25] Effector cells lacking granzyme B kill targets at a much slower rate than do wild-type cells, which demonstrates the important role that this protease has in executing the timely demise of infected or tumorigenic cells.[26,27] The efficiency of granzyme B-dependent killing is largely because of the ability of this protease to activate the target cell's intrinsic cell death proteases, the caspases, either directly or indirectly (Figure 2). Direct proteolysis and activation of caspases 3 and -7 by granzyme B leads to the caspase-mediated degradation of hundreds of cellular protein substrates, which promotes fast and efficient apoptosis.[2,28–30]

In addition, granzyme B can also promote caspase activation through the well-defined cytochrome c/Apaf-1 pathway in which proteolysis and activation of the BH3-only protein BID by granzyme B promotes BID-mediated opening of the BAX/BAK channel in the mitochondrial outer membrane (Figure 2). This important event leads to BAX/BAK-mediated release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol, where it binds to and activates a caspase-activating platform, called the apoptosome.

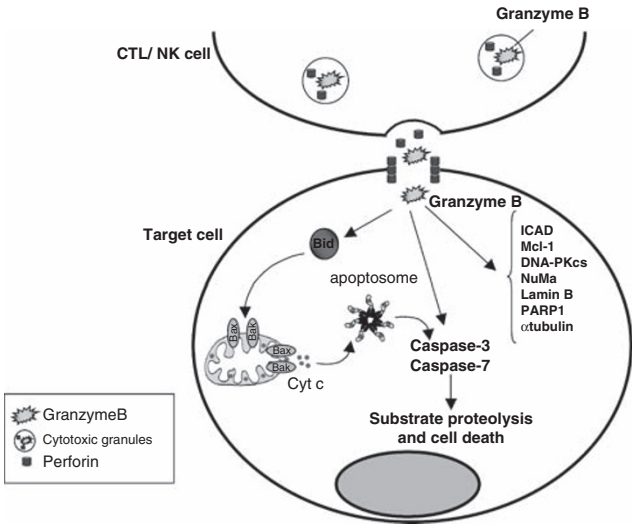

Figure 2 Pathways to granzyme B-mediated cell death. Granzyme B, together with other granzymes, enters the target cell by a perforin-dependent mechanism. On entry into the target cell cytosol, granzyme B promotes apoptosis through two main pathways, either through BID-dependent mitochondrial permeabilization or through direct caspase processing and activation. Granzyme B-mediated proteolysis of the BH3-only protein BID exposes a myristolyation signal in this protein, targeting it to mitochondria, in which it induces oligomerization of BAX and/or BAK in the mitochondrial outer membrane. The latter event facilitates cytochrome *c* release into the cytosol, assembly of the apoptosome, with subsequent caspase-9 activation and the ensuing caspase cascade. Note that antiapoptotic BCL-2 family members can inhibit cytochrome *c* release and block this pathway. Granzyme B can also directly process effector caspases 3 and -7 to promote apoptosis. Direct activation of the latter effector caspases leads to a caspase activation cascade and proteolysis of numerous caspase substrates, resulting in the efficient death of the target cell. Granzyme B can also directly cleave ICAD, the inhibitor of a DNase (CAD), which can promote internucleosomal DNA hydrolysis that is synonymous with this mode of killing. Granzyme B has also been shown to cleave a variety of other proteins implicated in the maintenance of nuclear integrity (Lamin B), as well as in protection against cell death (MCL-1), DNA repair (DNA-PK_{cs}), microtubule dynamics (α-tubulin) and a host of autoantigens (NuMa, Mi-2)

In turn, the apoptosome promotes downstream caspase activation and cell death.[31–33] Interestingly, human and mouse granzyme B show stark differences in their ability to access the BID pathway to cell death, with mouse granzyme B displaying little ability to process and activate BID as compared with its human counterpart.[29,34,35] Importantly, on experimental inactivation of caspases, granzyme B can still kill cells – although with much slower kinetics – and this most likely results from BID-mediated mitochondrial dysfunction, together with proteolysis of other substrates by granzyme B, such as ICAD and β-tubulin (Figure 2).[28,29,36]

As mentioned above, effector cells lacking granzyme B retain cytotoxic activity through the actions of the remaining granzymes, of which granzyme A is the most abundant. Whereas granzyme A may promote target cell death through multiple pathways, proteolysis of components of the endoplasmic reticulum-associated SET complex has been proposed to constitute the main mechanism for cell death by this granzyme (Figure 3).[37] Activation of the Dnase NM23-H1 within this complex, by proteolysis and inactivation of its inhibitor (SET) by granzyme A, is thought to promote the single-strand DNA degradation most commonly associated with killing by this granzyme.[37] In addition, proteolysis and

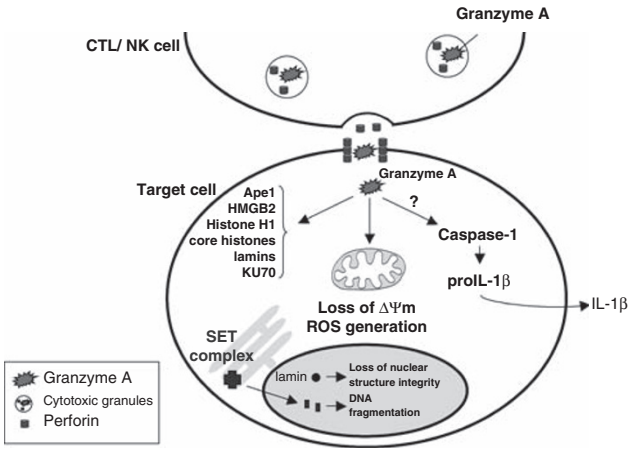

Figure 3 Granzyme A promotes cell death by targeting the nucleus and also mediates the release of mature IL-1β. On entry into the target cell, granzyme A promotes a decrease in mitochondrial transmembrane potential (deltaψ_m) and an increase in reactive oxygen species (ROS) through a poorly understood mechanism that might involve proteolysis of NDUFS3. This ROS increase leads to the translocation of the SET complex to the nucleus where it may be involved in the transcription of genes associated with oxidative stress responses. Granzyme A also directly targets three members of the SET complex for proteolysis: the nucleosome assembly protein, SET, the high mobility group protein 2 (HMG2) and the base-excision repair enzyme apurinic/apyrimidinic endonuclease 1 (Ape 1). Proteolysis of SET releases the inhibition of DNase NM23-HI, resulting in the single-strand DNA nicks most commonly associated with granzyme A-mediated cell death. Granzyme A also weakens the structural integrity of the nucleus by targeting Lamins A–C for proteolysis. A recent study suggests that granzyme A may also promote the release of the proinflammatory cytokine IL-1β through a mechanism dependent on caspase-1-mediated proteolysis of pro-IL-1β

activation of the 3′–5′ endonuclease, TREX1, by granzyme A, may inhibit DNA repair by removing bases from the free 3′ ends, thus preventing NM23-H1-cut strands from re-annealing.[38] Other pathways to granzyme A-mediated cell death have been described; however, recent work has questioned whether granzyme A has a cytotoxic role at physiological concentrations. This will be discussed later in the review when we consider possible non-cell death functions of this granzyme.[12,35,39]

The remaining granzymes have not been studied as comprehensively as granzyme A and B. However, recent work has begun to uncover a possible mode of action for some of these proteases. Similar to granzyme A, granzyme K shows tryptase-like activity and has been shown to process similar substrates.[40–42] Thus, it is possible that granzyme K may be important in situations in which granzyme A function has been compromised by viral inhibitors. Evidence of a role for granzyme C in promoting cell death is scant, although it may be cytotoxic in some contexts.[43,44] The Metase, granzyme M, has been shown to promote a caspase- and mitochondria-independent mechanism of cell death.[45] More recently, it was suggested that this granzyme may promote cell injury by disrupting the microtubule network through proteolysis of α-tubulin and also by cleavage and inactivation of the essential multifunctional protein, nucleophosmin.[46,47]

One of the persistent problems associated with studies relating to the cytotoxic activity of various granzymes is whether the concentrations of granzymes that have been used to demonstrate cytotoxicity are actually achievable under physiological settings. Whereas granzyme B displays cytotoxic activity at low nanomolar concentrations, many studies in this area have used granzymes at micromolar concentrations that may not be attainable *in vivo*. Because the concentration of granzymes that are delivered at the immunological synapse is unknown, it remains to be determined whether all granzymes are truly cytotoxic at physiological levels or have other roles in CTK/NK-mediated processes.

Role of the Immune System in Cancer Prevention

Immune surveillance describes the process whereby pre-cancerous and malignant cells are recognized by the immune system as damaged and are consequently targeted for elimination. In recent years, this model has been broadened to include the concept of immunoediting, in which transformed cells evolve under selective pressure from the immune system to give rise to tumors that are increasingly immune resistant.[48] However, although some tumors undoubtedly escape clearance in this way, evidence is mounting to suggest that cells of the immune system can prevent the growth of precancerous cells and also mediate regression of established tumors.[16] In early studies on this subject, the rate of spontaneous solid tumor formation in nude mice, which lack a thymus and therefore possess a greatly reduced number of T cells, was found to be the same as that of their wild-type littermates.[49,50] Although the growth of experimentally induced tumors was somewhat higher in these mice, the results cast doubt on the ability of the immune system to protect against tumor growth.[51] However, it was subsequently discovered that nude mice possess NK cells and a limited number of functional CTLs. More convincing evidence for tumor immune surveillance came from the observation that mice lacking RAG2, which is required for T-cell receptor gene rearrangement (such mice thus lack mature B or T cells), are much more susceptible to spontaneous tumor formation and also develop tumors at a greater rate on exposure to carcinogens or after injection of tumor cells.[52] Specifically, 60% of RAG2$^{-/-}$ mice developed tumors after treatment with the chemical carcinogen, MCA, whereas only 13% of wild-type mice developed MCA-induced tumors. In addition, all RAG2$^{-/-}$ mice spontaneously formed malignant lesions, half of which developed into carcinomas, whereas 81% of wild-type mice remained cancer free.[52] This study has since been reinforced by others that used mice deficient in different components of the TCR system to examine the important contribution of specific T-cell subsets to cancer prevention.[53] In addition, the importance of NK cells for cancer immunity has been illustrated by studies in which mice with gene-targeted or depleted NK and NKT cell populations were found to be 2–3 times more likely to develop MCA-induced tumors.[54,55] Therefore, aside from the eradication of cells infected with transforming viruses, CTL/NK cells have an important role in preventing the initiation and progression of cancer.

The Perforin–Granzyme Pathway in Cancer Prevention

Although both granzymes A and B have been shown to be important for clearing viruses such as ectromelia, an

increased risk of cancer has yet to be conclusively associated with the loss of individual granzymes[56–61]. Nevertheless, perforin-deficient mice are much more likely to develop tumors in a number of different settings, which highlights the significant redundancy of function that exists among granzymes with regard to tumor eradication.[3,5–7]

As mentioned above, loss of perforin leads to a complete failure of effector cells to lyse targets *in vitro*.[3] In addition to a failure to clear lymphocytic choriomeningitis virus, perforin-deficient mice were initially observed to be partially defective in eradicating experimentally introduced fibrosarcoma tumor cells.[3] In a follow-up study, perforin-deficient mice developed fibrosarcomas – initiated by treatment with the carcinogen MCA – much more rapidly and with greater incidence than did wild-type mice, whereas sarcomas induced by the Maloney murine sarcoma virus were larger and less likely to regress.[7] NK cells were subsequently shown to mediate antitumor cytotoxicity in a perforin-dependent manner, which suggests that perforin/granzymes have a role in the innate immune response to tumors.[5,62,63]

With regard to spontaneous tumor formation, perforin-deficient mice were found to be at a much greater risk of developing malignancy in certain B, T and NKT cells. Indeed, over half of all perforin-deficient animals developed spontaneous B-cell lymphomas after 12 months of age.[55] The role of perforin in protecting against lymphoma was underlined by the finding that the combined loss of perforin and p53 resulted in earlier onset but not increased incidence of lymphoma when compared with perforin knockout alone, suggesting that perforin, rather than p53, is the main factor in lymphoma surveillance.[55] Significantly, tumor rejection was mediated by NK cells or γ–δ T cells in a perforin-dependent manner when lymphomas from perforin-null mice were transplanted into their wild-type littermates.[6] Therefore, the perforin/granzyme system seems to have an important role in lymphoma prevention, at least in some settings.

Tumor progression requires suitable growth-promoting conditions in the area surrounding the developing tumor and, as most cancer patients possess viable immune cells, a critical aspect of cancer progression entails inhibition or evasion of the immune system.[64] In normal cells, TGF-β blocks progression of the cell cycle to stop proliferation, promote differentiation or induce apoptosis. However, many cancer cells acquire resistance to the antiproliferative effects of TGF-β, which greatly aids cancer progression. In addition, tumors can escape from immune surveillance by secreting immunosuppressive TGF-β, which inhibits proliferation and activation of T cells in the tumor microenvironment, making TGF-β production one of the most potent mechanisms of immune avoidance by tumors.[65] It is therefore significant that an important mechanism through which TGF-β inhibits T-cell mediated tumor clearance is by the inhibition of the expression of five cytotoxic genes including perforin, granzyme A and granzyme B.[66] Importantly, inhibition of TGF-β in mice restored cytolytic gene expression in CTLs and promoted tumor clearance, further emphasizing the role of the perforin/granzyme pathway in tumor prevention.[66]

Regulatory T cells normally promote tolerance to self-antigens by eliminating self-reactive lymphocytes.[67] Unfortunately, Treg cells have also been shown to induce tolerance to tumor-associated antigens, thus inhibiting the T cell-mediated antitumor immune response.[67] Interestingly, $CD4^+Foxp3^+$ Treg-mediated inactivation of tumor-specific CTLs in the tumor microenvironment has been shown to be contact dependent and reliant on perforin and granzyme B to the extent that mice lacking this granzyme clear transplanted tumor cell lines more efficiently than do wild-type mice.[68] Therefore, in some settings, a tumor may turn the tables on the immune system by using the destructive power of the perforin/granzyme pathway to its advantage. However, more evidence is required before this concept can be fully embraced.[68]

A Role for Granzymes in Immunomodulation

Although the perforin/granzyme pathway has an important role in CTL-mediated clearance of tumorigenic and infected cells, it may also have a role in regulating immune system homeostasis during infection. In humans, loss of perforin function leads to a syndrome called familial hemophagocytic lymphohistiocytosis, caused by the inability of activated CTLs to efficiently clear antigen-presenting targets.[69] This has dire consequences for immune system function, as both T cells and macrophages expand to meet a persistent pathogen challenge, leading to harmful levels of proinflammatory cytokines in circulation. In addition, overactivated macrophages phagocytose blood cells, leading to severe anemia.[69]

Although failure to clear certain viruses can lead to deregulated immune system homeostasis, evidence is emerging to suggest that granzymes may also be involved in the elimination of clonally expanded $CD4^+$ T cells, which is required to control the size of the lymphocyte pool during and after infection. One of the major mechanisms of $CD4^+$ T-cell eradication is by activation-induced cell death (AICD), which is normally mediated through the Fas–Fas ligand death pathway. However, granzymes have recently been found to promote AICD in patients with nonfunctioning Fas. Autoimmune lymphoproliferative syndrome is caused by a loss of function mutation in the *CD95/FAS* gene, leading to lymphoproliferation and autoimmunity, presumably through a failure of T cells to undergo AICD. Interesting recent evidence suggests that the perforin/granzyme pathway can promote AICD of T lymphocytes purified from these patients. However, the extent to which this can compensate for loss of the Fas pathway has not been determined.[70,71] Intriguingly, activated B cells have also been found to express and secrete granzyme B, leading to the possibility that this granzyme may participate in B cell-dependent immune responses. However, the role that granzyme B has in this context remains unclear.[11,15]

Extracellular Role of Granzymes in Immune Reactions

Since their discovery over 20 years ago, granzymes have been largely understood to function in an intracellular context, promoting the death of virus-infected or transformed cells by proteolysis of substrates (such as BID and caspases) within such cells. Apart from sporadic reports proposing other roles for these proteases, the vast majority of granzyme research has focused on elucidating the cell death-inducing properties

of these enzymes (Figure 1). However, recent work has uncovered a novel, noncytotoxic role for granzymes in the propagation of immune signals, which may open the door for a new area of granzyme research.

Granzyme A. It has long been known that patients presenting with infectious disease and certain proinflammatory conditions have elevated levels of granzyme A in the extracellular space. For example, rheumatoid arthritis patients have increased levels of granzyme A in the synovial fluid of swollen joints.[72] In addition, high levels of this protease have been noted in the bronchoalveolar lavage of asthma sufferers and in patients with chronic obstructive pulmonary disease.[73,74] The idea that high levels of circulating granzyme A are associated with overactivation of the immune system is also borne out by studies that have identified increased levels of this protease in patients with persistent HIV infection or acute cytomegalovirus and Epstein–Barr virus infection, and this could reflect increased CTL/NK activity and impaired clearance of dead cytotoxic cells.[26,75,76] Extracellular substrates for granzyme A are only beginning to emerge (Table 1); however, it is possible that proteolysis of cell surface proteins by circulating granzyme A may mediate some of the damage seen in proinflammatory disease states associated with this enzyme.[77]

Although granzyme A was observed to promote cytokine release from monocytes and to cleave and possibly activate the proinflammtory cytokine IL-1β over 20 years ago, a causal link between high levels of circulating granzyme A and the propagation of proinflammatory conditions was not fully appreciated until recently.[78,79] In addition to the previously described role in monocyte activation, Metkar *et al*.[12] have shown that granzyme A, whether purified or supplied by CTL, can promote the release of IL-1β from primary mouse macrophages. Importantly, granzyme A-null mice are protected from the lethal effects of LPS-induced toxicity, which supports a role for this granzyme in proinflammatory cytokine signaling during infection.[12] The role of the inflammation-associated protease, caspase-1, in promoting the maturation of IL-1β during infection has long been known.[80,81] Granzyme A-mediated release of IL-1β from macrophages seems to be dependent on caspase-1 activity, which suggests a mechanism whereby granzyme A can alert the immune system to disease by tapping into this important cell intrinsic proinflammatory pathway.[12] Together with recent data indicating that native granzyme A may not be cytotoxic at physiological concentrations, this work suggests that the primary role of granzyme A in the response of the immune system to infection and disease is only beginning to emerge.[12,35] Indeed, the recent observation that granzyme A may contribute to the lethal hypersecretion of TNFα during MCMV infection of perforin-null mice supports the notion of this granzyme as a mediator of immune signaling.[82]

Granzyme B. In addition to granzyme A, high levels of circulating extracellular granzyme B have also been observed in patients presenting with various infections and disease states, the most well documented being rheumatoid arthritis.[8–10,76] Granzyme B has been shown to cleave many extracellular matrix components including fibronectin, vitronectin and laminin (Table 1), which may promote detachment-induced cell death, or anoikis.[83,84] Indeed, targeting of the extracellular matrix has been proposed as a mechanism of granzyme B-mediated joint erosion during rheumatoid arthritis, which may explain the proinflammatory activites of this granzyme in some contexts.[9] However, the observation that granzyme B knockout mice are even more resistant to the lethal effects of LPS-induced endotoxic shock than mice deficient in granzyme A suggests that granzyme B may have a more central, noncytotoxic role in the immune response to disease than previously appreciated.[12] Indeed, ECM proteins such as hyaluronan may also act as danger signals on proteolysis by extracellular proteases and, in this form, may function to promote immune cell activation.[85–87]

Table 1 Extracellular granzyme substrates

Granzyme	Enzymatic activity	Substrates	Proposed functions
A	Tryptase	Pro-urokinase plasminogen activator[96]	Lymphocyte migration.
		Fibronectin[77]	Fibrin clots.
		Collagen IV[77]	Lymphocyte migration.
		Myelin basic protein[97]	Lymphocyte migration.
		Basement membrane proteoglycans[98]	Pathogenesis of multiple sclerosis.
		Thrombin-like receptor or neurites[99]	Monocyte activation, cytokine production by fibroblasts and epithelial cells.
		Platelet thrombin receptor	Induction of neurite retraction.
		Proteinase-activated receptor 2[100]	Desensitization of platelets to thrombin-induced aggregation.
			Infectious colitis pathogenesis
B	Aspase	Aggrecan, cartilage proteoglycans[101]	Joint destruction in rheumatoid arthritis.
		Vitronectin, fibronectin and laminin[83]	Extracellular matrix remodeling, induction of anoikis of endothelial cells.
		Fibronectin, smooth muscle cells ECM[102]	Possibly lymphocyte migration. Limitation of virus infectivity. Inhibition of tumour cell migration.
		Neuronal glutamate receptor[103]	Perforin-independent death (atherosclerosis).
		Plasminogen[104]	Autoantigens generation in Rasmussen's encephalitis.
			Vascular defects (scleroderma)
K	Tryptase	Unknown	Substrates and function are unknown but elevated levels are detected in sepsis and human lung diseases[73,88]

Other granzymes. Elevated levels of granzyme K have been found in plasma from septic patients while this granzyme was also upregulated in acute airway inflammation, suggesting that other granzymes may also have proinflammatory roles. In addition, both granzyme H and granzyme B have been shown to limit viral replication by directly targeting viral proteins for proteolytic degradation, which suggests a noncytotoxic role for granzymes in protecting against infection.[88–91]

Thus, a new paradigm for granzyme action is beginning to emerge in which granzymes released or actively secreted into the extracellular space may promote cytokine activation/secretion, either directly or indirectly, thereby amplifying immune responses to infected or transformed cells (Figure 1).

How are Granzymes Released During Inflammation?

Although the requirement of perforin for granzyme delivery to target cells has been well described, little is known about how granzymes may escape into the extracellular space to promote inflammation. One obvious mechanism of granzyme release is through the leakage of these proteases from the immunological synapse during CTL/NK killing, although a possible escape of granzymes in this way has not been rigorously investigated. However, it is also possible that granzymes may be actively released into the extracellular space under certain circumstances, and their actions may be completely perforin independent in this context.

In this regard, it is noteworthy that several groups have independently reported that non-CTL/NK cells express granzymes in response to stimuli that activate such cells. For example, transformed and activated primary B cells, mast cells, keratinocytes, basophils, macrophages and blood polymorphonuclear neutrophils have all been shown to express granzyme B.[11,14–20] Mast cells are especially abundant at the boundary between the internal environment and the outside world, for example, in the skin and lungs, and are thus perfectly situated to coordinate an immune response against a nascent infection. Granzyme B, but not granzyme A or perforin, is expressed by mast cells and secreted in an active form after ligation of the FcεR1 receptor.[17] Basophil-secreted granzyme B promoted the death of adherent target cells and degraded endothelial cell–cell contacts and this has been suggested to mediate increased vascular permeability and extravasation by basophils at points of infection.[17] Furthermore, keratinocytes have been shown to express granzyme B and perforin on irradiation, with UVB-treated keratinocytes acquiring cytotoxicity toward various transformed cell lines in a perforin/granzyme B-dependent manner.[92] Recently, primary B cells were found to secrete active granzyme B, but not perforin or granzyme A, in the presence of IL-21 after B-cell receptor stimulation with either viral antigens or activating antibodies.[11] B cells from patients previously vaccinated against certain viruses produced more granzyme B when subsequently challenged with specific viral antigen, whereas granzyme B-expressing B cells lived longer and were more active than nongranzyme B-expressing memory B cells.[11] B cell-secreted granzyme B may therefore augment the immune response to a nascent infection and may account for elevated granzyme B levels in serum from virus-infected patients.[76] Thus, evidence is accumulating to indicate that CTL/NK cells are not the only cells capable of producing and releasing active granzyme B.

Danger signals, or alarmins, are endogenous molecules that normally reside within cells but are released into the extracellular milieu during necrosis, generally as a result of trauma-induced cell lysis. Release of these proteins is followed by binding to and activation of immune cells, which is thought to alert the immune system to potentially harmful situations.[93] A large body of evidence implicates heat-shock protein 70 (HSP70) as a potential danger signal and it is interesting to note that HSP70 has been shown to mediate the uptake of granzyme B in a perforin-independent manner.[94] It is therefore intriguing to speculate that high levels circulating HSP70, released during situations of nonphysiological cell death such as chronic viral infection, may mediate the uptake of serum granzyme B by cells of the immune system and thus propagate the emerging proinflammatory properties of this protease. However, it is clear that investigations into the uptake of granzymes in noncytotoxic scenarios will require many more studies before a clearer picture begins to emerge.

Conclusions

The importance of the perforin/granzyme pathway in the prevention of cancer, as well as in immune homeostasis, has been clearly demonstrated by studies that use mice deficient in the crucial granzyme delivery agent, perforin. In addition, the recent study by Metkar *et al*.[12], which demonstrated a profound resistance to LPS-induced shock in both granzyme A and granzyme B-deficient mice, has hinted at a new mode of action of these important proteases as effectors of inflammation. Apoptosis is characterized by the removal of unwanted cells from the body without giving rise to unnecessary immune responses, making this form of cell death immunologically silent. A recent study has identified a role for apoptosis-inducing caspases as repressors of immune system activation through proteolysis and inactivation of the proinflammatory cytokine, IL-33.[95] Thus, it is intriguing to speculate that, whereas caspases and granzymes were once considered to have equivalent functions in promoting cell death, their actions may diverge with regard to immune system activation, and this may ultimately reflect the different scenarios (physiological for caspases, disease-associated for granzymes) in which these enzymes become activated.

Going forward, the challenge will be to identify extracellular substrates for granzymes that on proteolysis, can explain the apparent proinflammatory effects of these proteases. Identification of such substrates may open up new perspectives on the underlying causes of persistent inflammation in chronic inflammatory conditions.

Conflict of interest

The authors declare no conflict of interest.

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