

PATHOBIOLOGY IN FOCUS

Intracellular *versus* extracellular granzyme B in immunity and disease: challenging the dogma

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The cytotoxic granzyme B (GrB)/perforin pathway has been traditionally viewed as a primary mechanism that is used by cytotoxic lymphocytes to eliminate allogeneic, virally infected and/or transformed cells. Although originally proposed to have intracellular and extracellular functions, upon the discovery that perforin, in combination with GrB, could induce apoptosis, other potential functions for this protease were, for the most part, disregarded. As there are 5 granzymes in humans and 11 granzymes in mice, many studies used perforin knockout mice as an initial screen to evaluate the role of granzymes in disease. However, in recent years, emerging clinical and biochemical evidence has shown that the latter approach may have overlooked a critical perforin-independent, pathogenic role for these proteases in disease. This review focuses on GrB, the most characterized of the granzyme family, in disease. Long known to be a pro-apoptotic protease expressed by cytotoxic lymphocytes and natural killer cells, it is now accepted that GrB can be expressed in other cell types of immune and nonimmune origin. To the latter, an emerging immune-independent role for GrB has been forwarded due to recent discoveries that GrB may be expressed in nonimmune cells such as smooth muscle cells, keratinocytes, and chondrocytes in certain disease states. Given that GrB retains its activity in the blood, can cleave extracellular matrix, and its levels are often elevated in chronic inflammatory diseases, this protease may be an important contributor to certain pathologies. The implications of sustained elevations of intracellular and extracellular GrB in chronic vascular, dermatological, and neurological diseases, among others, are developing. This review examines, for the first time, the multiple roles of GrB in disease pathogenesis.

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INTRODUCTION

Granzymes

Granzymes are a family of conserved serine proteases stored within the cytotoxic granules of cytotoxic lymphocytes (CLs) whose functions were once believed to be primarily involved in immune-targeted cell death. There are 5 granzymes expressed in humans: granzymes A, B, H, K, and M, and 11 in mice (A, B, C, D, E, F, G, K, L, M, and N).¹ Granzymes A and B are the most abundant granzymes and for this reason have been the most studied. Granzyme B (GrB), which is the primary focus of this review, has received much attention over the past two decades.

GrB was first discovered in the mid-1980s, where several groups reported the presence of the protease in granules within CLs.^{2–5} Also known as cytotoxic T lymphocyte-associated serine esterase 1 or granzyme 2, GrB is a 32-kDa

serine protease resembling chymotrypsin, and has homologues expressed in a number of different species. The gene product encoding *GrB* is ~3500 bp long, contains five exons and four introns, and maps to chromosome 14 on the human genome.⁶ Similar to caspases, GrB has a preference for cleaving peptides immediately adjacent to aspartate (Asp) residues.^{7,8} This specificity is due to the structure of the GrB active site, which contains an arginine (Arg) residue positioned at the side of the active site pocket.⁹ An interaction between an Asp residue at the P1 position of the substrate and the Arg residue within the active site is key for enzyme–substrate interaction.⁹

Although once believed to be expressed exclusively by natural killer (NK) cells and cytotoxic T cells (CTLs), recent reports have shown that GrB can be expressed by various additional cell types. Under certain pro-inflammatory

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conditions, GrB can be expressed by CD4⁺ cells, mast cells, activated macrophages, neutrophils, basophils, dendritic cells (DCs), T regulatory cells, and nonimmune cell types such as smooth muscle cells (SMCs), chondrocytes, spermatocytes, type II pneumocytes, Sertoli cells, primary spermatocytes, granulosa cells, and syncytial trophoblasts.^{10–20}

Granzyme expression is regulated at both the transcriptional and translational levels, and is influenced by many of the same factors that stimulate immune cell activation. Transcriptional activation of GrB within T lymphocytes involves activation of the T cell receptor and co-stimulation with cytokines.²¹ The promoter region upstream of the GrB transcription start site contains binding sites for two transcription factors, activating transcription factor/cyclic AMP-responsive element-binding protein (ATF/CREB) and activator protein-1 (AP-1), and two lymphoid specific-factors, Ikaros and core-binding factor (CBF/PEBP2).^{22–25} All transcription factors act together to regulate GrB expression, and mutations to any of the transcription factor-binding sites will abrogate GrB expression.^{24,26} Most lymphocytes constitutively express GrB transcripts and upregulate transcription when the lymphocyte has been activated. In T lymphocytes and NK cells, many of the extracellular factors that stimulate T cell activation will also augment GrB expression, including the composition of the cytokine milieu, the nature of receptor engagement, and the presence of helper or regulatory CD4⁺ T cell populations.²¹

The post-transcriptional regulation of GrB is evident in many cell types, although the mechanisms involved in this regulation are not fully understood. Comparable levels of GrB transcripts are detected in resting and activated plasma DCs, but significantly higher levels of GrB protein is evident in the activated cells.¹⁵ Resting mouse NK cells have an abundance of GrB transcripts, but not of GrB protein or cytotoxicity.²⁷ However, once the cells have been activated, there is a significant increase in GrB protein levels, with relatively little change in GrB transcript levels.²⁷ In contrast, human mast cells express high levels of GrB transcripts and relatively low levels of GrB protein.²⁸

The post-translational regulation of GrB is accomplished through several mechanisms that include the synthesis of GrB as a propeptide requiring proteolytic cleavage for activation and the tagging of GrB with a mannose-6-phosphate receptor (MPR) used to target the protease to the acidic lytic granule.²⁹ These mechanisms will be discussed in more detail in the Granzyme synthesis, storage and exocytosis section of this review.

Specific inhibitors regulate the activity of GrB to minimize accidental GrB-mediated apoptosis. The only known endogenous inhibitor of GrB in humans is protease inhibitor-9 (PI-9), which is a potent inhibitor of GrB and is expressed by immune cells as protection against accidental cytosolic GrB leakage.^{30,31} Endothelial cells, vascular SMCs, and hepatocytes have also shown an ability to express PI-9 as a means of protection from GrB-mediated cytotoxicity.^{32–35}

High levels of PI-9 expression can be found in DCs, T cells, and endothelial cells of lymphoid and non-lymphoid tissue, as well as in cells of immune-privileged tissues, including the eyes, testes, ovaries, and placenta.³⁶ In mice, serine protease inhibitor 6 shares homology with human PI-9, in which it functions to regulate GrB activity.³⁷ Recently, Sipione *et al*³⁸ discovered an inhibitor of mouse and human GrB, *serpina3n*, expressed by mouse Sertoli cells. *Serpina3n* shares homology with the human α -1-antitrypsin, which interestingly does not appear to show any inhibitory effect on GrB in humans. GrB activity may also be indirectly influenced by the actions of granzyme H (GrH) and granzyme M (GrM). GrM may inhibit the action of human PI-9, thereby promoting GrB activity,³⁹ whereas GrH has the ability to cleave the adenoviral protein Ad5-100K in an effort to counter viral defense against GrB.⁴⁰

Given its role in immune-mediated cytotoxicity, increased attention has been devoted toward GrB in an effort to elucidate the mechanisms of CTLs and NK cell-mediated elimination of cells. Traditional views on the function of GrB have, for the most part, been limited to its intracellular pro-apoptotic role. However, as discussed below, recent reports suggest that there may also be perforin- and/or apoptosis-independent roles for GrB in disease progression. Apoptosis and extracellular matrix (ECM) modification are hallmarks of many, if not most, chronic inflammatory disorders and the involvement of GrB in a number of inflammatory diseases is becoming evident and will be the focus of this review.

GrB-INDUCED CELL DEATH

Granzyme Synthesis, Storage and Exocytosis

A wide variety of immune cells possess cytotoxic capabilities, which are generally mediated by two pathways: the death receptor-mediated pathway involving the engagement of cell surface receptors with death ligands^{41–45} and the granule pathway mediated by the granzyme family of proteins.^{21,46} The granule pathway is the primary pathway used for the clearance of pathogen-infected cells and the eradication of tumors (reviewed in Russell and Ley⁴⁷). The key effectors of the granule pathway are the granzymes. Granzymes are expressed with a signal sequence that directs them to the endoplasmic reticulum. Cleavage of this signal peptide produces an inactive pro-enzyme that contains an N-terminal dipeptide and requires cleavage to produce an active protease.^{48,49} In the golgi, granzymes are tagged with a mannose 6-phosphate used to target the granzymes to the lytic granule.²⁹ Once inside the granule, granzymes are activated by removal of the N-terminal dipeptide by cathepsin C and stored on a scaffold of the chondroitin sulfate proteoglycan, *serglycin*.^{48–54} Storage on this scaffolding, in combination with the acidic pH of the lytic granules, acts to minimize the proteolytic activity of granzymes⁵⁵ (reviewed in Chowdhury and Lieberman²¹).

Upon engagement, activated granzymes are delivered to target cells after the rapid polarization of lytic granules

toward the immunological synapse (IS).⁵⁶ The IS functions as a conduit for the transfer of lytic granules and other soluble factors between the CLs and the target cell.⁵⁷ The movement of lytic granules from CLs to the target cell is directional and depends on an underlying microtubule cytoskeleton, known as the microtubule-organizing center.^{58–60} Once delivered to the site of secretion, lytic granules fuse with the plasma membrane and release their contents into a secretory cleft formed between the CL and the target cell.

Granzyme Entry into Target Cells

Delivery of granzymes into the cytoplasm of target cells induces cell death. The major lytic proteins packaged within the granules are granzymes and the pore-forming protein perforin. Traditional models indicate that GrB delivery into a target cell is mediated by perforin; however, the mechanism by which this is accomplished remains an active area of investigation. Perforin is a Ca^{2+} -dependent pore-forming protein that multimerizes in the target cell's plasma membrane, forming 5–20 nm pores.^{61,62} Early models suggested that perforin facilitates the movement of granzymes into the cytoplasm of the target cell through the formation of pores; however, this model was challenged when it was discovered that granzymes could be endocytosed without perforin.^{63–66} More recent models show that GrB binds to the MPR and is rapidly endocytosed by the target cell.⁶⁷ This process is likely dynamin-dependent and results in the formation of endosomes containing GrB and perforin; however, a dynamin-independent role has been proposed.^{67–71} GrB is then released from the endosomes into the cytosol, using a perforin-dependent mechanism where it can target cellular substrates and initiate cell death.^{69,72,73} However, although increasing evidence supports a role for MPR in GrB uptake, controversy does exist with regard to the dynamin/MPR mechanism as some evidence suggests this may not be required for GrB entry. It should also be noted that transfer of GrB to the endosome can occur in a perforin-independent manner.^{69,71,72,74} Although a role beyond facilitating granzyme entry into cells is not known, perforin is required for CL-driven GrB-mediated apoptosis, as perforin deficiencies are associated with impaired lymphocyte-mediated cytotoxicity. Perforin-deficient mice are highly susceptible to viral infection^{75,76} and cancer.^{77,78} In humans, perforin deficiencies are associated with familial hemophagocytic lymphohistocytosis, an autosomal-recessive disorder resulting in uncontrolled T and NK cell activation and proliferation.^{79–81}

Although MPR and perforin are believed to be important for GrB delivery, several groups have reported alternative mechanisms for GrB entry. First, serglycin–GrB complexes, which interact with perforin, can incorporate into target cell membranes *in vitro* and deliver GrB to the cytoplasm without the formation of perforin-induced pores.^{52,53,68,82} This process was shown by Veugelers *et al*⁶⁷ to be enhanced by the cell surface receptor, heparan sulfate. GrB entry may also be facilitated by other cell-surface-bound proteins, such as heat

shock protein-70 (Hsp-70).⁸³ Hsp-70 present on the surface of tumor cells not only facilitates the movement of GrB into the tumor cell but also stimulates the production and delivery of GrB by NK cells.⁸⁴

GrB-induced Apoptosis

GrB delivery and substrate identification in apoptotic cell death has been the subject of much research. Once released into the cytoplasm, GrB can target substrates in the cytosol and the nucleus, and induce apoptosis through multiple pathways, as illustrated in Figure 1.

GrB shows broad substrate specificity, but preferentially cleaves after Asp residues. One of the first substrates to be identified for GrB was pro-caspase-3.⁸⁵ GrB-activated caspase-3 results in the processing of several cellular substrates integral to eliciting the apoptotic phenotype, including the inhibitor of caspase-activated deoxyribonuclease, the DNA damage sensor, poly(ADP ribose) polymerase (PARP), nuclear lamins, and many others (reviewed in Hengartner⁸⁶). Several other caspases, including caspase-2, -6, -7, -8, and -10, are reported to serve as direct substrates for GrB *in vitro*; however, only cleavage of caspase-3, -7 and -8 has been established *in vivo*.^{87–89}

A major mechanism by which GrB is believed to induce cell death is through the cleavage of the BH3-only protein Bid into a truncated form, gtBid, which then translocates to the mitochondrion and disrupts mitochondrial membrane integrity through interactions with the pro-apoptotic proteins Bax and/or Bak.^{90–92} Disruption of the mitochondrial membrane integrity increases membrane permeability, leading to the release of apoptogenic factors, such as cytochrome *c*, smac/DIABLO, Omi/HtrA2, and apoptosis-inducing factor (AIF).^{93,94} Cytochrome *c* release stimulates the formation of a macromolecular complex consisting of cytochrome *c*, dATP, apaf-1, and pro-caspase-9 known as the apoptosome, which results in the activation of caspase-9 and the subsequent activation of caspases-3 and -7. GrB also cleaves the anti-apoptotic protein Mcl-1, which results in the release of the pro-apoptotic Bcl-2 family member Bim, followed by mitochondrial outer membrane permeabilization and cytochrome *c* release.⁹⁵

Several other intracellular substrates have been identified including of PARP, the nuclear mitotic apparatus protein, cytoskeletal components such as α -tubulin, the nuclear-envelope intermediate filament protein (lamin B), and ROCKII.^{96–104} GrB has also been shown to target proteins involved in cellular homeostasis and the stress response, including Hsp-70 and Hsp-90 from the heat shock family of proteins, and also the heat-shock-associated proteins Hip, Hop, and Bag1-L.^{105–108}

Although the mechanisms of CL/NK-mediated GrB-induced apoptosis have been studied for some time, the relative contribution of these pathways to disease pathogenesis is less understood. With the discovery of GrB expression in non-immune cell types such as chondrocytes and keratinocytes, the effect of apoptosis (if any) mediated by these cells in

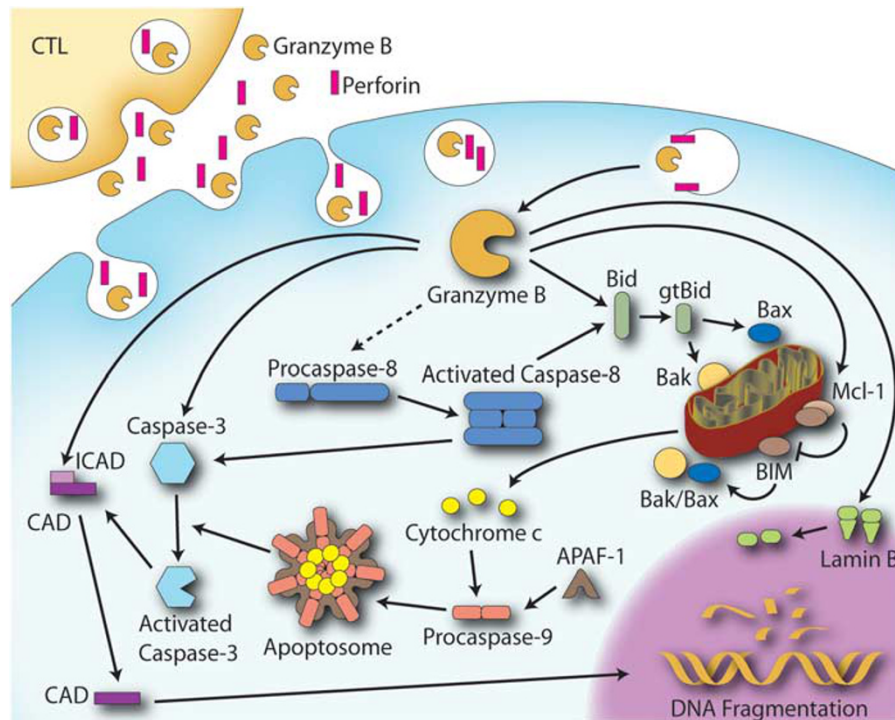


Figure 1 Classical granzyme B (GrB)/perforin-mediated apoptosis pathway. GrB internalization is facilitated by perforin. Upon internalization, GrB initiates apoptosis primarily through the cleavage of Bid into a truncated form (gtBid) that triggers mitochondrial cytochrome c release and apoptosis formation leading to caspase activation and manifestation of the apoptosis phenotype. GrB can also bypass the mitochondrial pathway and initiate caspase activation directly and/or cleave caspase substrates such as the inhibitor of caspase-activated deoxyribonuclease (ICAD), thereby allowing CAD to translocate to the nucleus to fragment DNA. GrB also cleaves the nuclear membrane protein lamin B, resulting in a loss of integrity of the nuclear membrane.

disease has yet to be determined and presents a new role for GrB in disease pathogenesis. However, as several GrB-expressing cells may not co-express perforin and/or form IS with target cells, it is likely that these cells would exert more of an extracellular impact on disease. A role of GrB in chronic inflammation and disease will be described in more detail in the following sections of this review.

EXTRACELLULAR GrB ACTIVITY

Until recently, GrB was largely studied in its intracellular capacity, specifically in the context of apoptosis. However, the granzymes were originally identified as both intracellular and extracellular proteases, and over the past few years, increased research has focused on extracellular GrB activity.^{109–113} Several groups have reported that GrB is present in the ECM of tissue and can be found extracellularly in bodily fluids.^{54,111,114} Several novel extracellular substrates for GrB have been identified, potential implications of ECM cleavage have been described, and extracellular GrB activity has been linked to arthritis, vascular pathologies, and other diseases. Although cell types such as keratinocytes, chondrocytes, and neutrophils can express both GrB and perforin simultaneously, other cell types such as mast cells express GrB in the absence of perforin and GrA, suggesting that GrB may act exclusively extracellularly in these cells.^{11,20,115–117} This is probably due to the GrB gene localization to a cluster of

genes along with mast cell proteases (separate from the perforin and GrA genes). As a result, GrB can be expressed by myeloid cells (DCs, granulocytes) and others upon activation, independently of perforin.^{15,17,118}

GrB is released from cytotoxic granules upon target cell recognition. Upon reaching the neutral pH of the extracellular environment, GrB is instantly active and can readily cleave susceptible extracellular substrates. The stimuli involved in GrB release have not been fully elucidated; however, several mechanisms have been described to date. GrB may leak into the ECM from the IS during target cell engagement, it may be released non-specifically upon TCR signaling or after prolonged IL-2 stimulation, and it is likely released after other unidentified stimuli.^{119,120} Recently, Prakash *et al*¹²¹ found that GrB is constitutively released from CTLs and NK cells *in vivo* and that GrB release can occur in the absence of target cell engagement. GrB is released in both active and inactive forms, suggesting that there may be an extracellular GrB activator for the zymogen form of the enzyme.¹²¹ The pro-form of GrB may be regulated outside cells in a process similar to the extracellular regulation of other ECM proteases, such as the pro-forms of matrix metalloproteases (MMPs), although this has yet to be defined. Besides NK cells and CTLs, other immune and nonimmune cell types also express and secrete GrB; however, the stimuli and signaling pathways regulating GrB release are largely unknown in these cell types.

GrB is present in the plasma of healthy individuals with median levels of approximately 20–40 pg/ml reported in the literature.^{54,111} Serum levels of GrB are elevated in several diseases such as human immunodeficiency virus-1 infection, Epstein–Barr virus infection, arthritis, and others.^{54,111,114} Apart from the potential blood clotting implications that will be described later, it is worth noting that although circulating GrB may be useful as a biomarker for several diseases, it may not have a large effect on disease progression and may be present in the serum due to leakage from tissues where it is more abundant. In diseased tissues, particularly in areas of inflammation, extracellular granzyme concentration would be expected to be much higher than that in the blood. In these focal areas, GrB will cause the most damage due to the abundance of ECM substrates and the associated network of susceptible cells in tissue. In addition to plasma, GrB is also present in the synovial fluid (SF) of rheumatoid arthritis (RA) patients, the cerebrospinal fluid (CSF) of multiple sclerosis (MS) and Rasmussen encephalitis patients, as well as the bronchoalveolar lavage (BAL) in chronic obstructive pulmonary disease (COPD) and lung inflammation.^{122–125} Although the GrB inhibitor PI-9 is present in normal human plasma, GrB retains 70% of its activity in the plasma, suggesting that PI-9 does not efficiently inhibit GrB activity in the blood.^{126,127} There is a lack of evidence for an endogenous extracellular GrB inhibitor that is physiologically effective, thus its extracellular activity may be largely unregulated in contrast to other ECM proteases such as MMPs, which are tightly regulated by the tissue inhibitors of metalloproteases. This lack of extracellular regulation of GrB activity may have important implications with respect to a potential degenerative role for GrB in disease.

ECM Substrates

Granzymes were initially discovered as both intracellular and extracellular proteases, and since early the 1990's, several ECM substrates for GrB such as aggrecan, fibronectin, vitronectin, and laminin have been identified (Table 1).^{109,110,113} In terms of cleavage site identification, the lone cleavage site described thus far is for vitronectin.¹³⁴ The cleavage site is in the RGD domain of vitronectin, which is an integrin-binding motif. GrB-mediated cleavage of this domain disrupts cellular–vitronectin interactions and influences cell adhesion and migration properties.¹³⁴

In addition to ECM proteolysis, GrB can act on extracellular substrates involved in the clotting cascade such as plasmin, plasminogen, von Willebrand Factor (VWF), and the matrix form of fibrinogen.^{129,130} GrB cleaves VWF in domains that are necessary for platelet interaction and cleavage prevents platelet aggregation, spreading, tethering, and adhesion to the VWF multimer.¹²⁹ As GrB has a high affinity for ECM binding, Buzza *et al*¹²⁹ suggest that it would accumulate in areas of inflammation and prevent/delay thrombosis in these areas. GrB-dependent cleavage of plasmin and

Table 1 Extracellular GrB substrates and receptors: implications of proteolysis

Protein	Implications
<i>Proteoglycans</i>	
Aggrecan ¹²⁸	Disruption of structural integrity in cartilage
Cartilage proteoglycans ¹²⁸	Disruption of structural integrity in cartilage
<i>Blood proteins/clotting</i>	
von Willebrand factor ¹²⁹	GrB cleavage site in the domain of platelet interaction, prevention/delay of thrombosis
Plasminogen ¹³⁰	Cleavage yields angiostatin, which is anti-angiogenic. Implications in angiogenesis
Plasmin ¹³⁰	As plasmin is pro-angiogenic, cleavage results in the reduction of angiogenesis
<i>Cell receptors</i>	
Neuronal glutamate receptor ¹³¹	GrB cleaves the non-glycosylated form of the receptor into an autoantigenic fragment
FGFR1 ¹³²	Cleavage activates pro-cell death functions as well as inactivates pro-growth signals
Notch1 ¹³²	Cleavage results in cell signaling affecting tumor survival and antiviral activities
Acetylcholine receptor ¹³³	Cleavage results in a reduction of the receptor in neuromuscular junctions and yields an autoantigenic fragment
<i>Other ECM components</i>	
Vitronectin ¹³⁴	GrB cleavage site in integrin-binding domain, implications in cell adhesion, migration, and anoikis
Fibronectin ^{134,135}	Cell adhesion, migration, and anoikis
Fibrinogen ¹²⁹	Matrix form of fibrinogen is cleaved. The uncleaved protein responsible for platelet adhesion and thrombus growth. Cleavage results in anti-thrombosis implications
Laminin ¹³⁴	Cell adhesion, anoikis
Smooth muscle cell matrix ¹³⁵	Cell adhesion, anoikis

plasminogen has implications outside clotting as degradation of these proteins may also inhibit angiogenesis.¹³⁰

GrB can cleave cell surface receptors such as the neuronal glutamate receptor, Notch1, and FGFR1.^{131,132} The cleavage of these cell surface receptors may have implications in cell

signaling affecting tumor survival and antiviral activities. Cleavage fragments of receptors can also have physiological activities such as seen with the FGFR1 cleavage fragment, which can activate pro-cell death functions and inactivate pro-growth signals facilitated by FGFR1 in cancer.

Consequences of ECM Degradation

GrB-mediated ECM cleavage may contribute to disease not only through mechanical damage but also through other mechanisms (Figure 2). One consequence of ECM cleavage is anoikis, a form of cell death similar to apoptosis that is caused by a loss of cell–matrix interaction. Fibronectin, laminin, and vitronectin are adhesive proteins that are involved in connecting cells to the surrounding insoluble matrix. As all of these proteins are substrates for GrB, GrB-mediated cleavage may induce anoikis in various cell types. Choy *et al*¹³⁵ described anoikis in cultured SMCs when treated with extracellular GrB that cleaved fibronectin in the matrix. They¹³⁵ also showed that ~30% of SMC death induced by CLs was perforin-independent but GrB-dependent, pointing toward anoikis as the mechanism of cell death. Buzza *et al*¹³⁴ further investigated this by seeding endothelial cells on pure fibronectin, laminin, and vitronectin matrices and reported cell detachment and death by anoikis.

GrB-dependent cleavage of vitronectin and fibronectin can also inhibit cell motility and migration.¹³⁴ UVA stimulates keratinocyte motility; however, GrB expression during exposure inhibits this process, possibly protecting against carcinogenesis and the normal skin structure disruption that results from keratinocyte migration.¹³⁶ An area poorly understood is whether GrB-generated ECM fragments can elicit chemotactic properties. GrB ECM cleavage fragments could possess chemotactic properties, leading to the recruitment of immune cells and promotion of inflammation.¹³⁷ Indeed, fibronectin fragments can attract both neutrophils and monocytes.^{137–140} Fibronectin fragments also have other properties such as inducing MMP expression by chondrocytes.¹⁴⁰ ECM fragments may also act as signaling molecules in neighboring resident cells, as mentioned previously with the FGFR1 fragment.¹³² As the ECM has affinity for and serves as a reservoir for many growth factors and cytokines, the disruption of the ECM by GrB could induce the release of these growth factors and influence surrounding cells in an indirect manner.

GrB IN DISEASE

GrB has the capacity to act on both intracellular and extracellular substrates. Recently, with the discovery of new immune and nonimmune cell sources of the protease, its importance in infection and cancer is evolving while its contribution to other chronic diseases is emerging. Infiltrating immune cells during chronic inflammation results in elevated levels of GrB to diseased tissue and induces apoptosis in damaged and inflamed areas. Extracellular concentrations of GrB in bodily fluids are elevated

in various diseases, and the extracellular activity of this protease in chronic inflammation is an emerging area of research. The remainder of the review will summarize and critically review the current knowledge pertaining to the intracellular and extracellular roles for GrB in inflammatory diseases. As there have been many excellent reviews written pertaining to the role of GrB activity in infection and cancer,^{21,141,142} we will focus on chronic diseases where the pathogenic role of GrB is emerging (Table 2 and Figure 3).

GrB in Autoantigen Production

Autoimmunity results when the body does not recognize its own proteins as self, and as a result, it mounts an immune response against itself. The process of apoptosis has been intricately described as key initiator of autoimmune responses and autoimmune disease. During apoptosis, autoantigens from various subcellular compartments are clustered in surface blebs and apoptotic bodies of dead cells, in which they are highly accessible to the immune system.²⁶² These peptides are captured and presented to immune cells by antigen-presenting cells, which drive the autoimmune response.

This process may occur during immune cell-mediated apoptosis, in which enzymes such as caspases and GrB are responsible for uncovering cryptic epitopes of intracellular proteins from the proteolytic cleavage of cellular substrates. Some of these hidden epitopes are not present during immune cell development and as a result the immune system may not always tolerate them (ie, recognize them as self-proteins), thus they become autoantigenic and are identified as foreign by the immune system.

Recently, it has been shown that some of these intracellular-derived autoantigens are cleaved by GrB during CTL-induced apoptosis. Furthermore, many of these cleavage fragments are unique to GrB and are not synthesized in other forms of cell death. Casciola-Rosen *et al*²⁰⁵ have described some of these GrB-specific autoantigens in great detail and several disease-specific examples are provided in this review. Of 29 well-defined autoantigens, 21 in systemic lupus erythematosus (SLE), scleroderma (SSc), Sjögren syndrome (SS), and myositis are directly cleaved by GrB into unique autoantigenic fragments. Non-autoantigens were either not cleaved by GrB or cleaved into fragments identical to those cleaved during other forms of apoptosis and as a result are not unique. This suggests these non-unique fragments were already tolerized during immune cell development and are not autoantigenic, whereas the uniquely cleaved autoantigens would have escaped tolerance.²⁰⁵ As GrB appears to selectively and uniquely generate a wide variety of autoantigens in several autoimmune diseases and as there is a high-titer autoantibody response to most of these antigens in autoimmune patients, GrB has been identified as a critical enzyme in autoimmune disease pathogenesis.^{205,263}

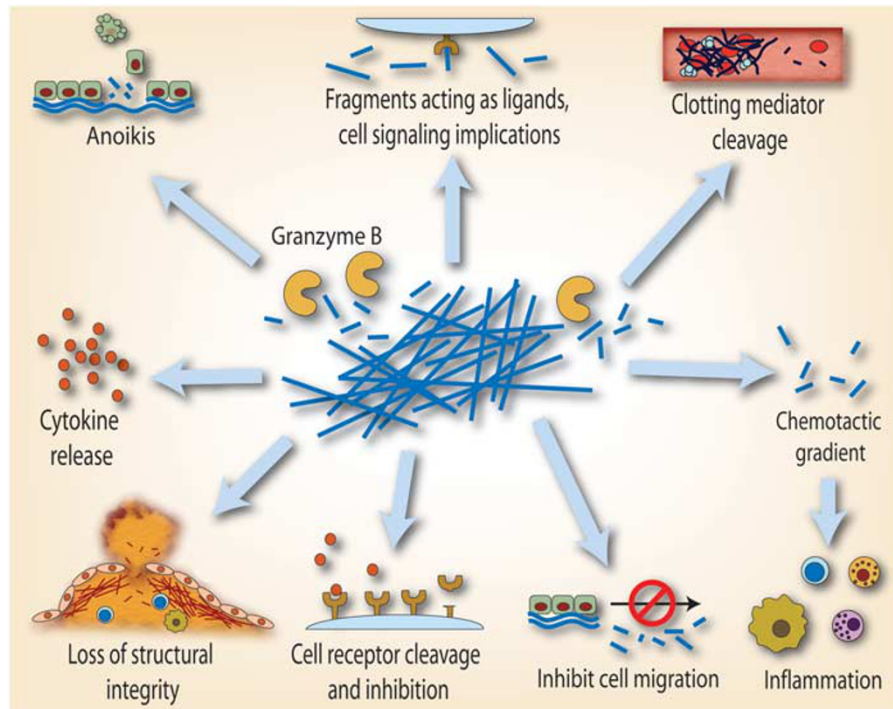


Figure 2 Putative extracellular (perforin-independent) roles for GrB in age-related chronic inflammatory disorders. During a number of chronic inflammatory conditions, GrB accumulates extracellularly in the tissues, blood stream, and other bodily fluids. GrB retains its activity in the blood, suggesting that, unlike MMPs and cathepsins, extracellular inhibitors of GrB activity may be limited. GrB can cleave proteins involved in structural integrity and wound healing such as fibronectin. GrB can also cleave proteins related to clotting (fibrinogen, vWF, plasminogen). GrB can induce detachment-mediated cell death (anoikis) through the cleavage of ECM. Although yet to be shown for granzymes, MMP-mediated fragments of fibronectin and elastin show chemotactic properties and may enhance the immune response in atherosclerosis. Fragments may also exhibit bioactive properties and may be able to release cytokines from the matrix. Granzymes may also have a role in the cleavage of cell surface receptors as seen with Notch1 and FGFR1.

SLE

SLE is a disease involving a specific autoimmune response toward nuclear autoantigens. High amounts of soluble nucleosomes have been found in SLE serum and SLE macrophages have reduced phagocytic activity, suggesting there is a defect in the clearance of apoptotic cell material.^{264,265} This may allow for a high availability of autoantigens for DCs to process and present, as this cell type is believed to be a driving force in SLE pathogenesis.²⁶⁶ In addition to DCs, CTLs are an important cell type in SLE autoimmunity. Effector CTLs from patients with SLE disease flares show greater perforin/GrB immunopositivity and the frequency of these cells coincides with disease progression. These CTLs also generate soluble nucleosomes and GrB-specific autoantigens, suggesting they contribute to the initiation of autoimmunity. Interestingly, in the presence of SLE serum, DCs can facilitate CTL differentiation into active effector cells, which are no different from those in SLE patients, providing further evidence that DCs are capable of presenting autoantigens to CTLs and inducing their activation in an autoimmune response.²⁰⁶

GrB has been implicated as a key protease in autoantigen processing in SLE and generates fragments unique to those of

other autoimmunity-associated proteases such as the caspases. For example, GrB is capable of producing autoantigens from the non-homologous end-joining pathway of DNA double-strand break repair. Sera from SLE patients contained antibodies against XRCC4, and three of these patients recognized the specific GrB cleavage epitope, suggesting GrB may produce these fragments *in vivo*.²⁰⁷ However, in a mouse model of autoimmunity in which GrB-deficient mice were injected with the mineral oil pristane, GrB knockout mice still produced autoantibodies and had a higher mortality than mice expressing GrB, suggesting that GrB may not be a critical enzyme in autoantigen synthesis. However, the latter study would not rule out the possibility that the other granzymes may be contributing to autoantigen formation in this model. In addition, the authors suggest that GrB may be protective because of its role in viral clearance, as viral infection is believed to promote autoimmunity.^{208,267}

SSc

SSc or systemic sclerosis is an inflammatory connective tissue disease that affects the vasculature and results in blood flow reduction, tissue ischemia, and wound healing defects. GrB cleaves several SSc autoantigens including topoisomerase I,

Table 2 GrB in disease

Condition	Intracellular <i>versus</i> extracellular	Description
<i>Lung diseases</i>		
Chronic obstructive pulmonary disease (COPD) ^{18,143,144}	Intracellular/ extracellular	Increased CTLs and NK cells expressing GrB in the blood and BAL of patients with COPD. Type II pneumocytes and alveolar macrophages in the lung express GrB. Increased perforin expression by CD8+ cells in the lung of smoking subjects with COPD
Asthma ^{17,122}	Intracellular/ extracellular	Increase in lymphocytes expressing GrB in the BAL fluid of patients suffering from allergic asthma after allergen challenge. Induction of GrB expression by basophils upon stimulation with IL-3 released by mast cells
Acute respiratory distress syndrome (ARDS) ¹⁴⁵	Intracellular/ extracellular	GrB and perforin mRNA levels are upregulated in the BAL of patients in the acute phase of ARDS
Pulmonary sarcoidosis ^{146,147}	Intracellular/ extracellular	GrB and perforin are expressed by CD8+ and some CD4+ T cells in the BAL fluid. Serum levels of GrB are decreased in patients with sarcoidosis
Hypersensitivity pneumonitis ¹²⁵	Extracellular	Granzyme B is increased in the BAL fluid of patients with hypersensitivity pneumonitis
<i>Oral</i>		
Chronic obstructive sialadenitis/sialolithiasis ¹⁴⁸	Intracellular(?)	GrB is expressed by periductal and periaccinar lymphocytes in patients with chronic sialadenitis
Papillon–Lefèvre syndrome (PLS) ^{149,150}		NK cells in patients with PLS fail to induce the caspase cascade in target cells because of an inactive form of GrB as a result of a mutation in cathepsin C Reduced active GrB in cytotoxic cells in patients with PLS compared with controls
<i>Blood disorders</i>		
Aplastic anemia ^{151,152}	Intracellular/ extracellular	No difference in GrB-expressing cytotoxic effector cells in disease patients compared with controls Increase in perforin but no increase in GrB in bone marrow clot sections of disease patients compared with controls
Idiopathic neutropenia ¹⁵³		No difference in GrB-expressing CD16+ cells in the blood between patients with chronic idiopathic neutropenia
Chronic idiopathic thrombocytopenic purpura (ITP) ^{154–156}	Intracellular(?)	Increased GrB-expressing T cells in the blood of patients with ITP compared with controls Increased GrB mRNA levels in CD8+ cells in patients with ITP compared with controls
<i>Skin diseases</i>		
Alopecia ^{157–159}	Intracellular	GrB-expressing CTLs are closely associated with hair follicles and may damage follicles. Substance P increases CD8+ T cells expressing GrB, which may cause hair follicle regression
UV photoaging ^{11,136,160}	Intracellular/ extracellular	GrB is expressed by keratinocytes in response to confluence, UVA, and UVB. UVB-treated keratinocytes have cytotoxic potential against co-cultured cells and GrB from UVA-treated keratinocytes can cleave fibronectin
Acne ¹⁶¹		GrB is upregulated in acne lesions
Atopic dermatitis/allergic contact dermatitis ^{162–165}	Intracellular	GrB-expressing CD4+ and CD8+ T cells are observed in the perivascular infiltrate and focally at spongiosis sites. In contact dermatitis, keratinocytes neighboring GrB-expressing cells are damaged

Table 2 Continued

Condition	Intracellular <i>versus</i> extracellular	Description
Vitiligo ^{166,167}	Intracellular	GrB-expressing CTLs cluster around disappearing melanocytes and may induce apoptosis in these cells
Lichen planus ^{165,168–172}	Intracellular	GrB-expressing cells are found in close proximity to apoptotic keratinocytes. DCs expressing GrB are found in lesions
Lichen sclerosus ^{173–175}		GrB is expressed in dermal infiltrate close to keratinocytes. Vasculitis associated with the disease contains GrB-positive cells in the perivascular infiltrate
Stevens–Johnson syndrome/ toxic epidermal necrolysis ^{176–180}	Intracellular/ extracellular	CTLs expressing GrB may induce apoptosis in keratinocytes. GrB-positive lymphocytes in blister fluid. GrB upregulation correlated to disease severity
Pityriasis rosea ¹⁶²		GrB is expressed by immune cells in pityriasis rosea lesions
Psoriasis ^{163,164}		GrB is expressed by some lymphocytes in psoriasis lesions
Bullous blistering skin lesions ¹⁸¹		GrB is expressed in bullous lesions by T cells
Discoid lupus erythematosus ^{165,182}		GrB is expressed on lesional lymphocytes expressing the skin-homing proteins CLA and MxA. GrB-positive cells are perivascular and located in the dermoepidermal junction
Pemphigus vulgaris (PV) ¹⁸³		Decreased <i>ex vivo</i> expression of GrB by circulating NK cells in patients with PV compared with controls
<i>Bones and joints</i>		
Rheumatoid arthritis (RA) ^{13,14,54,114,115,128,184–191}	Extracellular/ intracellular	GrB cleaves aggrecan and other cartilage components. Levels of GrB are markedly elevated in the synovial fluid and plasma of patients. All GrB-expressing cell types may also contribute to RA through GrB-mediated apoptosis, including chondrocytes that show the surface antigens of NK cells
Osteoarthritis ¹⁸⁷		mRNA and protein expression of GrB in the synovium of joints
Reactive arthritis ¹⁹²	Extracellular	GrB expressed in the synovial tissue
<i>Neurological disorders</i>		
Rasmussen's encephalitis (RE) ^{124,131,193–196}	Intracellular/ extracellular/ autoimmunity	GrB-expressing CTLs described in RE brains. GrB from CTLs is polarized toward neurons and astrocytes that express MHC I. Extracellular GrB levels in cerebrospinal fluid (CSF) are elevated. GrB cleaves the GLUR-3 receptor yielding an autoantigenic fragment
Multiple sclerosis (MS) ^{123,197–199}	Intracellular/ extracellular	CTLs are involved in neuronal toxicity and T _H 17 cells, which cross the blood–brain barrier, express GrB, and can kill neurons <i>in vitro</i> . Increase in extracellular GrB levels in the CSF in relapsing remitting MS
Guillain–Barré syndrome ²⁰⁰	Intracellular	GrB-expressing CTLs are increased and MHC I-expressing Schwann cells may be GrB targets. Implications in myelin sheath damage
Vasculitic neuropathy ²⁰¹		GrB is expressed in the peri-vascular infiltrate
Sensory perineuritis ²⁰²	Intracellular	GrB-expressing CTLs contribute to perineurial cell apoptosis
Ischemic stroke ²⁰³	Intracellular	GrB from CTLs and NK cells induce apoptosis of brain cells
Spinal cord injury ²⁰⁴	Intracellular	GrB levels are elevated and CTLs in close proximity to neurons in regions of damage
Myosina gravis ¹³³	Extracellular/ autoantigen	GrB cleaves the autoantigen AChR. GrB is present in myasthenia gravis thymus glands but absent in controls
<i>Autoimmune disease</i>		
Systemic lupus erythematosus (SLE) ^{205–209}	Intracellular/ autoimmunity	Frequency of GrB-expressing CTLs coincides with disease progression. GrB is involved in autoantigen processing of XRCC4 and other potential SLE autoantigens

Table 2 Continued

Condition	Intracellular <i>versus</i> extracellular	Description
Neonatal lupus erythematosus ²¹⁰		GrB expression in the left ventricle of hearts from fetuses/infants with complete atrioventricular block
Scleroderma (SSc) ^{130,205,211,212}	Autoimmunity/ extracellular	GrB cleaves the autoantigens topoisomerase I, NOR-90, fibrillarin, B23, and others. SSc patients with ischemic digital loss have autoantibodies for CENP-C, which may be useful as biomarkers for IDL. The GrB cleavage product angiotatin inhibits angiogenesis and may be responsible for the poor circulation in SSc
Sjögren syndrome (SS) ^{205,213–220}	Intracellular/ autoimmunity	GrB cleaves the autoantigens SS-B (La) autoantigen, α -fodrin, β -fodrin, type 3 muscarinic acetylcholine receptor, and others. CD4+ and CD8+ T cells induce apoptosis of epithelial cells through the granule pathway and these cells are only present in SS glands
Myositis ^{205,221–224}	Intracellular/ autoimmunity	GrB cleaves autoantigens such as PMS-1 and HisRS. GrB-expressing cells are found in the endomysial sites of polymyositis and are proposed to cause muscle cell damage
Type 1 diabetes ²²⁵	Intracellular	Human and mouse β -cells undergo apoptosis in the presence of GrB, which correlates with a loss in islet insulin secretion capacity
<i>Bile/liver/intestinal diseases</i>		
Lymphocytic gastritis (LG) ^{226–228}	Intracellular?	Intraepithelial CD8+ cells from LG children with celiac disease lack GrB Increase in GrB expressing intraepithelial lymphocytes in patients with acute gastric mucosal lesions compared with controls Increased GrB expressing intraepithelial lymphocytes in patients with non-celiac disease associated LG compared with patients with celiac disease associated LG
Autoimmune cholangitis (AC) and primary biliary cirrhosis (PBC) ²²⁹		GrB expressing T cells found in the bile duct epithelium. No difference in the number of GrB-expressing lymphocytes between patients with AC versus PBC
Nodular regenerative hyperplasia (NRH) of the liver ²³⁰		Increased CD8+ lymphocytes expressing GrB in liver biopsy samples from patients with NRH compared with controls.
Inflammatory bowel disease ²³¹		Increased GrB-expressing intraepithelial lymphocytes in patients with Crohn's disease and ulcerative colitis
<i>Vascular diseases</i>		
Atherosclerosis ^{10,135,189,232–236}	Extracellular/ intracellular	GrB levels increase with increased disease severity. Present in high levels in advanced atherosclerotic and TVD plaques. Elevated in lipid-rich regions Granzyme B in the blood is significantly higher in patients with unstable atherosclerotic plaques. The study also showed that raised plasma levels of GrB in unstable carotid plaques were associated with an increased frequency of cerebrovascular events (ie, strokes), suggesting that GrB may be a marker of plaque instability GrB expressed in macrophages in atherosclerotic plaques Perforin deficiency in LDLR-KO mice does not affect atherosclerosis. Supports role for extracellular GrB or its role in late-stage/advanced atherosclerosis GrB in the absence of perforin can induce smooth muscle cell apoptosis through the cleavage of extracellular matrix proteins. Fibronectin identified as a substrate GrB production from PBMCs of unstable angina pectoris (UAP) patients was significantly higher than those with stable angina (SAP). GrB production from PBMCs increased with the increasing TIMI risk score in UAP patients. The percentage of GrB-positive lymphocytes to CD3-positive lymphocytes in UAP patients was significantly higher than in SAP

Table 2 Continued

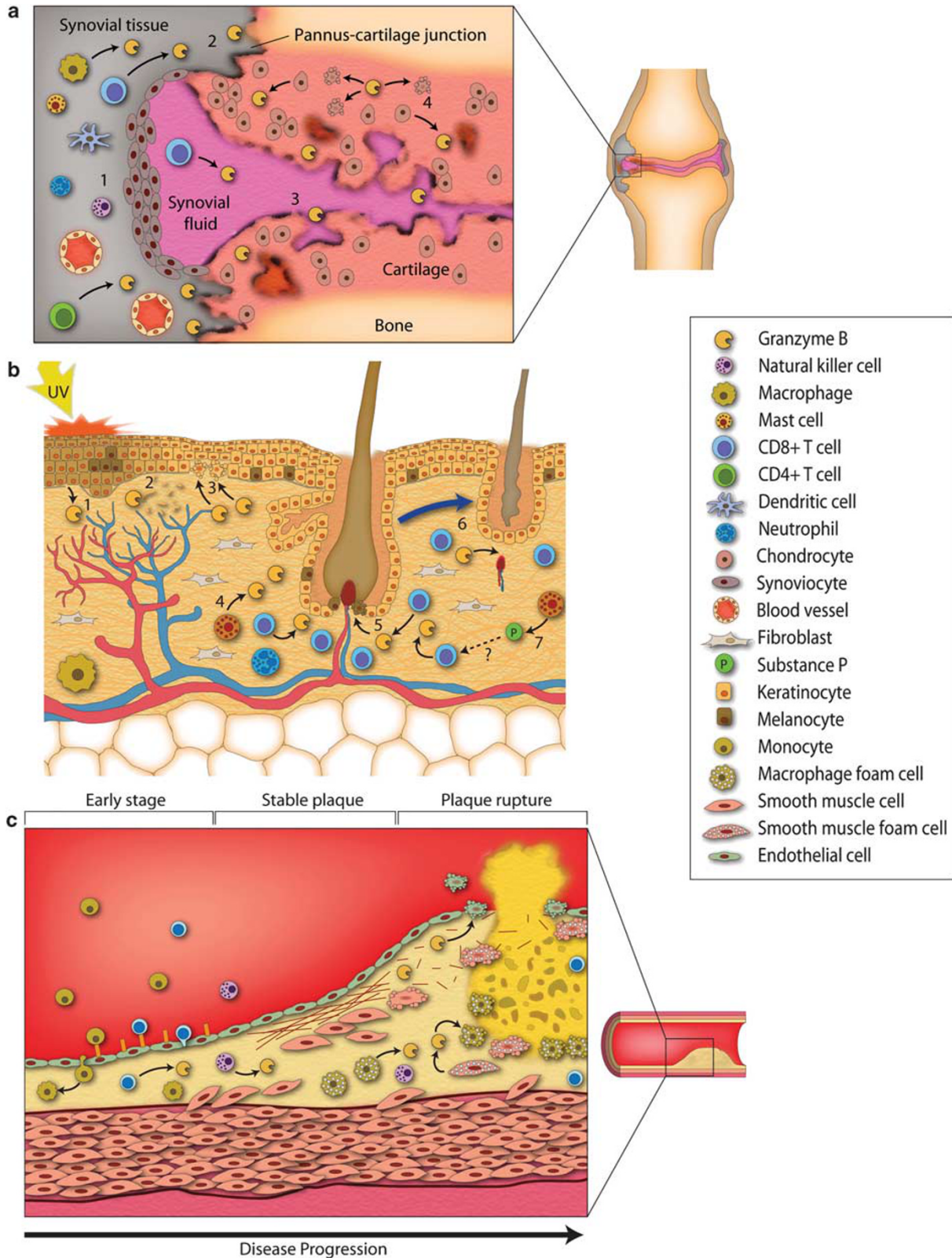
Condition	Intracellular versus extracellular	Description
Acute transplant rejection ^{237–245}	Intracellular	GrB-mediated apoptosis that occurs during the recruitment of inflammatory cells after the nonspecific injury to graft vessels. GrB can contribute to lesion formation through processes that include GrB-mediated apoptosis, and the promotion of EC activation and SMC migration
Allograft vasculopathy ^{10,246,247}	Intracellular	GrB/perforin pathway involved in endothelial and smooth muscle cell apoptosis
Kawasaki disease ^{248–250}	Extracellular	Children with Kawasaki's show elevated vascular inflammation and often die from fatal aortic dissections or aneurysms. Elevated levels observed in lesions, aneurysms, and plasma of Kawasaki's patients; however, its involvement of GrB disease pathogenesis requires further elucidation
<i>Kidney diseases</i>		
Crescentic glomerulonephritis (CG) ²⁵¹	Intracellular	Perforin-neutralizing antibody protects against the progression of CG in rats
Goodpasture's disease (GD) ²⁵²		Glomerular GrB expression and GD pathogenesis is reduced upon administration of anti-CD8+ antibody
<i>Esophagus</i>		
Achalasia ²⁵³		The inflammatory infiltrate found in the myenteric plexus contains cytotoxic T cells, some of which express GrB
Esophagitis ²⁵⁴		Significant increase in GrB-expressing intraepithelial lymphocytes in biopsy specimens from patients with esophagitis compared with controls
Crohn's disease ²⁵⁵		Increase in GrB-expressing cells in esophagus biopsy specimens taken from patients with CD compared with controls
<i>Other</i>		
Eosinophilic fasciitis ²⁵⁶		The inflammatory infiltrate in eosinophilic fasciitis contains some CD8+ cells expressing GrB, suggesting a cytotoxic immune response
Cryptorchidism ²⁵⁷		Lymphocytes expressing GrB are decreased in the testis of patients with cryptorchidism
Histiocytic-necrotizing lymphadenitis (HNL)/Kikuchi disease ^{258,259}	Intracellular/ apoptotic necrosis	GrB-expressing cells found in necrotizing lesions of patients with Kikuchi disease
Chediak-Higashi syndrome ²⁶⁰		CTL granules are unable to release their contents upon recognition of a T-cell receptor, express normal levels of GrB
Duchenne muscular dystrophy (DMD)/facioscapulohumeral dystrophy (FSHD) ²⁶¹	Intracellular	GrB expression detected in muscle biopsy specimens from patients with DMD and FSHD but absent in control samples

NOR-90, fibrillar, and others.²⁰⁵ Some SSc patients also develop complications from the disorder, such as ischemic digital loss (IDL).²¹¹ Serum samples from SSc patients with IDL contain autoantibodies for the specific GrB cleavage

fragments of CENP-C, pointing toward GrB as a mediator of IDL.²¹¹ Moreover, as there is a need for predictive biomarkers for digital loss in SSc, specific IDL autoantigens cleaved by GrB maybe a useful tool for diagnosis.

In addition to autoimmunity, extracellular cleavage fragments created by GrB also have other functional roles in SSc. The reduction of blood flow characteristic of SSc may be due

to an imbalance between proangiogenic and antiangiogenic factors that control new vessel growth. Plasma from SSc patients shows reduced endothelial cell migration and tube



formation *in vitro*. The serum sample was found to contain higher levels of the antiangiogenic protein angiostatin, which Mulligan-Kehoe *et al*¹³⁰ found to be synthesized by GrB through the cleavage of plasminogen (in the presence of another granule protein) as well as from plasmin. As plasmin is proangiogenic in nature, GrB may also be responsible for decreasing levels of plasmin in SS serum, thereby decreasing angiogenesis through the synthesis of angiostatin and the degradation of plasmin.¹³⁰

Interestingly, autoantigen release may be dependent on cell type in SS. B23 is resistant to GrB-mediated cleavage in many cell types but appears to be exclusively cleaved in vascular SMCs.²¹² Whether this cell-type exclusiveness in SMCs is due to a cell-specific abnormality of this protein (ie, post-translational modification, etc.) is unknown. Regardless, the selective production of this autoantigen and potentially others in vascular cells may contribute to the extensive vascular pathogenesis apparent in SS.

SS

SS is a systemic autoimmune disease characterized by chronic inflammation in the salivary and lacrimal glands with focal infiltrate around ductal and acinar epithelial cells. This infiltrate interacts with epithelial cells and induces apoptosis. CD4+ and CD8+ T cells induce apoptosis in epithelial cells through the Fas/FasL and perforin/GrB pathways.²¹³ In support of this notion, perforin/GrB-expressing infiltrate was found only in SS salivary glands and not in controls, suggesting that GrB is a culprit in epithelial cell death.²¹⁴ The majority of cells surrounding apoptotic acinar epithelial cells are CD8+ T cells expressing the integrin $\alpha_E\beta_7$, suggesting that GrB from these integrin-expressing T cells may be involved in the pathogenesis of SS.²¹⁵

During epithelial cell apoptosis, GrB may cleave several autoantigens in SS, namely the SS-B (La) autoantigen, α -fodrin, β -fodrin, and type 3 muscarinic acetylcholine receptor.^{216–218} GrB-mediated cleavage of the La protein resulted in its translocation from the nucleus into the cyto-

plasm, where the autoantigen is easily accessible to antigen-presenting cells and can initiate an immune response.²¹⁹ As such, GrB may not only synthesize autoantigens in SS but may also render them more readily accessible for immunorecognition.

Myositis

Myositis is an autoimmune inflammatory skeletal muscle disease resulting in muscle weakness. Areas of muscle damage show a characteristic mononuclear cell infiltrate consisting of T lymphocytes and NK cells, which express major histocompatibility complex II (MHC-II) molecules. These cells invade and destroy muscle fibers, leading to muscle fiber necrosis, potentially through muscle cell MHC-I. GrB- and perforin-expressing cells were found in the endomysial sites of polymyositis (PM) and may cause muscle cell damage, apoptosis, and necrosis in PM.²²¹ However, although there is a significant amount of inflammation and cytotoxic molecules in PM, muscle cells are generally resistant to apoptosis, which may be linked to their expression of human IAP-like protein, a cell death repressor.²²²

In addition to its potential role in muscle cell integrity, GrB may be a source of autoantigen production in myositis through the processing of the DNA mismatch repair enzyme PMS-1 protein fragment, as 7.5% of myositis patient serum tests positive were for the autoantibody.²⁶⁸ The sera from myositis patients also recognized other unidentified protein fragments, suggesting there may be several more unidentified autoantigens derived from GrB cleavage in myositis.

Myositis patients can also develop interstitial lung disease and Levine *et al*²²³ showed that this lung pathogenesis may be due to a difference in the protein structure of the autoantigen histidyl-transfer RNA synthetase (HisRS). The conformation of the lung-specific HisRS makes it more susceptible to GrB cleavage compared with the normal HisRS conformation present in the rest of the body. This lung-specific conformation exposes a cryptic GrB cleavage site, resulting in HisRS fragmentation and GrB-dependent synthesis of an

Figure 3 Granzyme B in the pathogenesis of rheumatoid arthritis, skin disease, and atherosclerosis. **(a)** Infiltrating immune cells in RA (CTLs, macrophages, NK cells, and T-helper cells) express and release GrB in joints and induce apoptosis in resident cells (1). GrB may contribute to proteoglycan degradation as GrB-positive cells are found at the pannus–cartilage junction, an area of cartilage destruction (2). Extracellular GrB levels are elevated in the synovial fluid of RA joints and are believed to further degrade matrix (3). Chondrocytes express GrB in RA and are capable of inducing apoptosis in neighboring cells and secreting GrB into the extracellular milieu, which causes further extracellular damage (4). **(b)** GrB may contribute to skin aging, alopecia, and disease through various intracellular and extracellular pathways. UVA light, which is believed to be responsible for visible aging that occurs in the skin, induces reactive oxygen species production, which leads to GrB expression in keratinocytes (1). GrB from keratinocytes cleaves the ECM protein fibronectin (2), inhibits cell migration, and can also induce apoptosis in neighboring cells (3). Mast cells from the skin are another cell type that express GrB (4). GrB from CTLs has been proposed to induce melanocyte apoptosis in vitiligo (5). Invading CTLs express GrB in alopecia areata and influence hair follicle regression (6). Substance P secreted by skin mast cells increases GrB-positive CTLs in the skin, further promoting AA (7). Whether substance P induces mast cell degranulation leading to the release of GrB is unknown. **(c)** At the early stages of atherosclerosis, after endothelial dysfunction and intimal lipid retention, CTLs, and monocytes infiltrate the vessel wall and migrate into the intima. SMCs and macrophages engulf oxidized lipoproteins and become lipid-laden foam cells, leading to GrB expression in these cells. The latter may promote foam cell apoptosis in developing plaques. GrB can also cleave various extracellular matrix proteins that maintain fibrotic cap stability. In addition to a loss in matrix integrity, ECM cleavage will also result in a loss of SMC–ECM interactions, which may promote apoptosis. SMC expression of PI-9 is decreased in unstable plaques, rendering SMCs susceptible to GrB-dependent apoptosis and further promoting plaque instability and rupture.

autoantigenic peptide, specific to the lung.²²³ It is interesting to speculate that GrB-specific autoantigens are cleaved when protein conformation is altered, thereby uncovering otherwise hidden GrB cleavage sites. Protein conformational changes due to post-translational modifications, pH changes, mechanical damage, dissociation from binding partners, changes in subcellular localization, and other factors may have a role in the susceptibility to GrB cleavage of not just autoantigens but also to intracellular and extracellular substrates involved in a vast array of activities, having endless potential implications in signaling, apoptosis, and extracellular activities *in vivo*.

RA

RA is a chronic autoimmune inflammatory disease involving tissue destruction in joints and tendons. GrB has been implicated intracellularly and extracellularly in this disease through apoptosis and cartilage matrix destruction (Figure 3a). GrB is present in several cell types in areas of inflammation as well as in regions of matrix disruption, making it a potentially important mediator of RA pathogenesis.

The expression of GrB by activated CTLs in the SF from RA patients was first described by Young *et al*,¹⁸⁴ with ~15% of SF lymphocytes expressing GrB. Since then, soluble extracellular GrB has been reported as increased in the SF and plasma of patients with RA, with levels averaging as high as 3 and 1 ng/ml, respectively.^{54,114} The presence of extracellular GrB in the SF of RA complements the study by Froelich *et al*¹²⁸ showing that GrB degrades the ECM synthesized by chondrocytes *in vitro*. One of these substrates was identified as aggrecan, a major constituent of cartilage; however, no other substrates have been identified to date. Fibronectin, as well as bioactive fibronectin fragments, has also been reported in inflammatory arthritis SF.^{137,140,269,270} Fibronectin fragments are chemotactic for macrophages and neutrophils, and these fragments can release proteoglycans and cause chondrolysis by binding to cartilage.^{271,272} However, despite the fact that fibronectin is a well-established substrate of GrB, no one has reported whether extracellular GrB activity contributes to the synthesis of these fragments.

Froelich *et al*¹²⁸ proposed that CTLs secrete GrB extracellularly in the joint, which degrades and remodels the interstitial ECM. Complementary to this study is that of Roday *et al*¹⁸⁵ showing that GrB cleaves proteoglycans from bovine cartilage explants, resulting in glycosaminoglycan release. GrB-positive cells were identified at the invasive front of the inflamed synovial tissue (pannus) and in the cartilage-pannus junction, suggesting that GrB may be responsible for the cartilage invasion and destruction that occurs in this junction.^{185,186}

In addition to CTLs, other cell types may act as sources of GrB in RA. GrB-expressing NK cells are present in the synovial tissue of RA patients and these cells are the main GrB-expressing cell type in the RA joint.^{187,188} Macrophages

express GrB in the lining and perivascular areas of the synovial tissues of RA joints. Macrophage numbers are increased in RA compared with normal joints, suggesting that macrophages are also a significant source of GrB, and may contribute to cartilage destruction.¹⁸⁹ Although CD4 + T cells are believed to act largely as T-helper cells in RA, CD4 + CD28– cells have been suggested to show cytotoxicity. Unlike their CD28 + counterparts, which express GrB but not perforin, CD4 + CD28– T cells express both perforin and GrB and show cytotoxic activity *in vitro*. CD4 + CD28– perforin-expressing T cells account for up to 50% of CD4 + T cells in synovial tissue, suggesting they may be a significant source of GrB in RA.¹⁴ Mast cells, DCs, and neutrophils are other cell types upregulated in RA, which express GrB under specific circumstances.^{15,20,28} However, it is unknown whether these cell types express GrB in RA.

GrB and perforin are also expressed by articular chondrocytes as GrB and perforin transcript/protein levels are increased in diseased RA cartilage, as detected by semi-quantitative RT-PCR and immunostaining.^{13,115} Increased GrB and perforin levels in these cells correspond to chondrocyte apoptosis, particularly in the pannus region invading the cartilage, suggesting that self-synthesized GrB may induce self-regulated apoptosis. Cultured chondrocytes also express the surface antigens of NK cells and show cytotoxicity against co-cultured cells, suggesting that in addition to having a self-inducing-apoptosis capacity, chondrocytes may also act as resident cytotoxic cells in the cartilage of RA patients.¹¹⁵ Finally, secreted GrB from chondrocytes may have cartilage degradation potential as well; suggesting that GrB from this cell source has both intracellular and extracellular implications.¹³

Despite the fact that RA is an autoimmune disease and that increased levels of GrB are associated with rheumatoid factor autoantibody-positive patients, little is known regarding the GrB generation of RA-specific autoantigens.¹⁹⁰ A rat chondrocyte cDNA library was immunoscreened with serum from an RA patient and the DNA-binding protein AHNAK was detected as a potential autoantigen. This protein is cleaved *in vitro* by GrB, suggesting that it may be involved in the autoantigen processing of this protein.²⁰⁹ GrB-mediated autoantigen processing could be another mechanism by which GrB may contribute to the pathogenesis of RA, as little study has been done thus far in this area.

Type I Diabetes

Type I diabetes results from the selective destruction of the insulin-producing β -cells in pancreatic islets, and is primarily a T-cell-mediated autoimmune disease directed against one or more β -cell autoantigens.²⁷³ Lymphocyte infiltration (termed insulinitis) occurs early in models of type I diabetes and is necessary but not sufficient to cause β -cell destruction, insulin deficiency, and hyperglycemia.²⁷⁴ It is now accepted that β -islet destruction is caused by islet antigen-specific autoreactive T cells and that both CD4 + and

CD8+ T cells are required for the disease to occur (reviewed in refs. 273,275,276) Although still an area of active investigation, β -cells are believed to be destroyed primarily by granule-mediated apoptosis.^{274,276–281}

Mouse models of diabetes have provided support for T-cell-induced cell death with β -cell apoptosis observed in both spontaneous diabetes in non-obese diabetic mice^{277,279} and in more accelerated diabetes models.^{277,278} Furthermore, cell death mediators, including perforin, cytokines, and death ligands, have been identified in the insulinitis lesions of NOD mice.²⁸² Both CD4+ and CD8+ T-cell subsets are required for the development of diabetes in the NOD mouse; however, CD8+ T effector cells have a fundamental role in the destruction of pancreatic β -cells and contribute to sustaining islet inflammation.^{283,284} Pancreatic β -cells express MHC-I molecules that present antigenic peptides to CD8+ T cells,²⁸² and NOD mice lacking MHC-I expression and CD8+ T cells are resistant to type I diabetes.^{285,286} Perforin has a key role in β -cell apoptosis in the NOD model, as perforin-deficient NOD mice are reported to develop insulinitis but have a markedly reduced incidence of diabetes.^{75,287} This implies that CTLs destroy β -cells, at least in part, by granule-mediated apoptosis. Support for GrB in β -cell damage is provided by *in vitro* studies indicating that both human and mouse β -cells undergo apoptosis in the presence of GrB, which correlates with a loss in the islet insulin secretion capacity.²²⁵

Lastly, there is evidence that GrB has a predominant role in the destruction of β -cells after pancreatic islet transplantation, with several recent studies showing elevated GrB levels in plasma preceding islet graft rejection.^{288,289} This suggests that GrB may be a reliable indicator of ongoing graft loss after β -islet transplantation. Evidence from both human and mouse models has shown that β -cell death observed in diabetes occurs through apoptosis and that granule-mediated cytotoxicity is involved in β -cell loss. In summary, although studies to date would suggest that the GrB/perforin pathway contributes to β -cell apoptosis, it remains to be determined whether there is an extracellular role for GrB in diabetes. Whether GrB has a role in type 2 diabetes remains unclear.

Neurological Disorders

The central nervous system is regarded as an immune-privileged site because of the blood–brain barrier and an immunosuppressive environment. However, in many neurological conditions, there is a loss of immune privilege and, as a result, GrB has been implicated in the pathogenesis of a number of neurological disorders through mechanisms of immune cell-mediated apoptosis, receptor cleavage, autoantigen synthesis, and potential extracellular activities, as described below.

There are several neurological diseases where the role of GrB is just emerging, largely in an immune cell-mediated, pro-apoptotic role. In vasculitic neuropathy, the characteristic perivascular infiltrate expresses GrB and GrB-positive

cells are significantly upregulated.²⁰¹ In sensory perineuritis, GrB-expressing infiltrates were detected and T cells are believed to contribute to perineural cell apoptosis.²⁰² During cerebral ischemia, in a rat model of ischemic stroke, GrB secreted from CTLs and NK cells results in the breakdown of Hsp-70 and AIF translocation from the mitochondria to the nucleus, ultimately resulting in apoptosis of brain cells.²⁰³ Finally, in a rat model of spinal cord injury, GrB levels were elevated and CTLs were found in close proximity to neurons in damaged regions of the spinal cord. Many of the neurons undergoing death were positive for GrB, suggesting that GrB is responsible for neuron cell death in spinal cord injury.²⁰⁴

In addition to inducing apoptosis intracellularly, GrB may contribute to neurological disease through other mechanisms, such as through receptor signaling or through direct cleavage of receptors. In myasthenia gravis (MG), a decrease in acetylcholine receptors (AChR) at neuromuscular junctions is observed as a result of autoimmune attack. Furthermore, GrB and the autoantigen fragments of AChR are observed in the thymus of MG patients but are not present in healthy controls. Casciola-Rosen *et al*¹³³ showed that GrB cleaves AChR, suggesting that GrB-mediated cleavage of this protein exposes cryptic antigens that facilitate an autoimmune response in the disease and results in a loss of functional AChR. G-protein-coupled receptors are also linked to GrB and neurotoxicity. The addition of recombinant GrB to neuronal cell cultures results in neurotoxicity, even in the absence of perforin through the G α -coupled receptor.²⁹⁰ Whether GrB facilitates cytotoxicity by cellular interaction or uptake, by cleaving the receptor itself, or by creating an ECM fragment that may act as a ligand is not known.

Rasmussen's encephalitis

Rasmussen's encephalitis (RE) is an autoimmune chronic inflammatory disease occurring mainly in childhood, characterized by seizures and a loss of motor skills and speech. Immune infiltrates consisting mainly of GrB-positive CTLs are found in the brains of these patients. Using confocal microscopy, Bien *et al*¹⁹³ found that GrB was polarized toward neurons, some of which expressed MHC-I. This suggests that MHC-I-initiated GrB-mediated cytotoxicity is responsible for the loss of neurons in this disease. In similar studies, Bauer *et al* described GrB-positive lymphocytes in close proximity to astrocytes on the border of astrocyte-deficient lesions. Some of these cytotoxic cells showed GrB polarized toward astrocytic membranes. Similarly to neurons, astrocytes express MHC-I, suggesting that GrB is contributing to astrocyte loss in RE.¹⁹⁴ In another study, V β T-cells expressing GrB were found in close apposition to neurons and astrocytes in patients, further supporting the aforementioned studies.¹⁹⁵

Extracellular GrB activity has also been linked to RE as extracellular GrB levels in CSF are elevated in patients with RE, with the original cellular source believed to be CTLs.¹²⁴

It is possible that an elevation in extracellular GrB could be used as a biomarker for RE, as it is present at earlier stages of the disease and remains slightly elevated as the disease progresses. Extracellular GrB is capable of cleaving the neuronal glutamate receptor subunit 3 (GLUR3) and producing an autoantigenic fragment. Antibodies against GLUR3 are present in the serum of children with this disease. However, GrB can only cleave the non-glycosylated form of the receptor, suggesting that normal tolerance is escaped when neurons produce the unglycosylated form of the protein. The change in glycosylation of this protein makes it more susceptible to GrB cleavage, possibly by exposing a cryptic cleavage site not present in the glycosylated form of the receptor.¹³¹ This suggests that GrB cleavage of ECM and receptors may also be regulated by post-translational modifications, such as glycosylation, an area that has been scarcely described and may be significant in disease pathogenesis in general.

Demyelination diseases: MS and Guillain–Barré syndrome

MS is a complex autoimmune disease of the central nervous system resulting in demyelination of nerves and GrB has been implicated in MS pathogenesis. In patients with relapsing remitting MS, GrB levels in peripheral T cells decrease, whereas plasma GrB levels do not change. However, there is an increase in extracellular GrB in the CSF as quantified by ELISA, suggesting that extracellular granzyme may contribute to the disease. There was no correlation between the CSF cell count and levels, suggesting that this is not due to an increase in cell number and that there is localized GrB release in the CSF/central nervous system of MS patients.¹²³ As there are low numbers of NK cells in the CSF of MS patients, it is believed that the cellular source of GrB is CTLs. Although it has yet to be shown, the extracellular localization of GrB in CSF implies that the enzyme has access to the ECM in the spinal cord and brain, suggesting that it may be actively cleaving this matrix, perhaps similar to what has been described in arthritis.¹⁸⁵

There have been several studies reporting an intracellular pro-apoptotic role for GrB in MS. As oligodendrocytes and neurons express MHC-I, GrB-expressing CTLs may be responsible for the induction of cytotoxicity resulting in axonal damage and demyelination.¹⁹⁷ In addition, neurons expressing MHC-1 resist Fas-mediated killing but are susceptible to granule-mediated apoptosis.¹⁹⁸ $\gamma\delta$ T cells have also been suggested to direct cytotoxicity to the myelin-oligodendrocyte unit; however, inhibition of GrB in these cells only partially reduced cytotoxicity.²⁹¹ T_H17 lymphocytes crossing the blood–brain barrier express GrB and have the capacity to kill human fetal neuron cultures *in vitro*, suggesting that this cell type is also a source of cytotoxic GrB in MS.¹⁹⁹

In Guillain–Barré syndrome, an inflammatory demyelinating disease of the peripheral nervous system, GrB-expressing CD8+ T cells were increased and localized close to neurons of the dorsal root ganglion. MHC-I molecules were also detected on Schwann cells and myelin sheaths,

suggesting that these T cells may be responsible for myelin damage in both the peripheral nervous system and the central nervous system.²⁰⁰ This could be intracellularly and/or extracellularly mediated and more studies are required to determine the role of GrB in demyelination.

Skin Disorders

GrB expression in the skin

The role of GrB in dermatological conditions is emerging and the contribution of GrB to skin aging, alopecia, and disease is illustrated in Figure 3b. There are several potential sources of GrB in the skin, namely CLs, DCs, macrophages, mast cells, and keratinocytes.^{117,160} Keratinocytes express GrB *in vitro* after treatment with UVA, UVB, and at high confluence.^{11,136,160} Interestingly, UVB and high confluence induce both GrB and perforin expression, whereas GrB is expressed without perforin in response to UVA. UVA induced GrB expression in human skin *in vivo*.¹³⁶ In the latter study, UVA treatment of cultured keratinocytes caused a reactive oxygen species (ROS)-dependent release of macrophage migration inhibitor factor (MIF), and MIF induced GrB expression by keratinocytes. This GrB expression was p38 mitogen-activated protein kinase (MAPK)-dependent and resulted in the phosphorylation of its subsequent substrate MAPKAPK2. During UVB treatment, ROS production results in signaling through EGFR, MAPK activation, and subsequent MAPKAPK-2 phosphorylation.¹¹ It should be noted that it is unclear as to whether these pathways are involved in the induction of GrB expression in other cell types.

Perforin was expressed in parallel with GrB in response to UVB and increased confluence, and UVB-irradiated Ha-CaT keratinocytes showed cytotoxic activity against various skin cell types *in vitro*, suggesting that GrB in this cell type shows cytotoxic potential.¹¹ GrB from keratinocytes decreased *Staphylococcus epidermidis* growth, suggesting that keratinocyte GrB may be protective *in vivo* against skin bacterial infection.¹⁶⁰ This is only the second report of GrB-mediated cytotoxicity by a nonimmune cell type (the first was described in chondrocytes), as most cell types do not express perforin simultaneously.¹¹⁵ Despite the evidence of UV induction of GrB, it remains unclear whether GrB from keratinocytes contributes to UV-dependent photoaging or wrinkling, as GrB should have ECM cleavage potential if released extracellularly.

GrB is also expressed by mast cells in mouse skin *in vivo*, as well as in cultured skin mast cells after IgE treatment.^{28,117} Mast cells do not express perforin and GrB that is released extracellularly from these cells induces fibroblast anoikis, suggesting that it has a role in ECM degradation in the skin. No follow-up studies have been published describing GrB from mast cells or keratinocytes contributing to skin diseases *in vivo*, and the majority of dermatological studies to date exclusively examine CTLs as the sole source of GrB in the skin. As mast cells do not express perforin and keratinocytes

express GrB (but not perforin) in response to stimuli such as UVA, there is clearly a potential extracellular, perforin-independent capacity for GrB in the skin. As the skin contains a high proportion of ECM such as collagen, elastin, and ground substance, future extracellular studies will be useful in further examining the extracellular activity of GrB and its role in disease.

Alopecia areata

Alopecia areata (AA) is an inflammatory condition that results in non-scarring patchy hair loss. Perifollicular and intrafollicular CTLs have been implicated in AA, suggesting that there is an immune cell attack on the normally immune privileged hair follicle during the growth phase (anagen) of the hair cycle.^{292,293} GrB-expressing cytotoxic cells were found closely associated with human hair follicles in chronic AA patients, suggesting that GrB may contribute to CTL-mediated follicular damage.¹⁵⁷ However, in a contradictory study, Sato-kawamura *et al*¹⁵⁸ did not see GrB-positive cells around follicles in lesions of AA. In a C3H/HeJ mouse model of AA, GrB expression was described in immune cells in the intrafollicular dermis, although few of these cells expressed CD8.¹⁵⁹ Interestingly, supplying the neuropeptide substance P to the skin of AA-affected mice led to an increase in CD8 + cells expressing GrB, possibly leading to an increase in cytotoxicity in the skin. Substance P also resulted in regression of hair follicles out of the growth phase into catagen, suggesting this could be related to a substance P-dependent increase in CD8 + GrB-expressing cells.¹⁵⁹ Although more studies are required to determine causation and to define the mechanism by which GrB contributes to AA, there is an association between the disease and GrB expression.

Other skin disorders

GrB has been linked to other skin conditions such as acne, vitiligo, atopic dermatitis, allergic contact dermatitis, psoriasis, lichen planus (LP), lichen sclerosus, Stevens–Johnson syndrome, and toxic epidermal necrolysis.^{161–164,166,168,173,176} GrB expression in most of these diseases was found to be localized to CTLs and GrB-specific disease implications were almost exclusively believed to be through intracellular CTL-mediated apoptosis of keratinocytes and other resident cell types. As more cellular sources of GrB have been identified as of late and the emerging role of extracellular GrB activity gains traction, determining other GrB sources and examining the importance of extracellular GrB activity will be a necessity in dermatological research involving GrB.

Vitiligo is characterized by a loss of pigmentation in the skin due to a progressive loss of melanocytes, the cell type that produces the pigment melanin. In the perilesional area of vitiligo biopsies, the border between the unpigmented lesion and normal pigmented skin, CD8 + CTLs were shown to cluster around disappearing melanocytes with ~60% of these T cells expressing perforin and GrB, respectively. Furthermore, GrB-expressing T cells were not found elsewhere in

vitiligo or in non-diseased control skin.¹⁶⁶ As there have been several accounts that melanocytes are insensitive to FasL-mediated apoptosis, GrB from surrounding CTLs is believed to mediate the death of melanocytes in vitiligo and may be a major contributor to the vitiligo phenotype.¹⁶⁷

GrB-expressing T cells have also been linked to atopic dermatitis and allergic contact dermatitis. In atopic dermatitis, a chronic inflammatory skin disease with eczematous lesions, an increase in GrB expression in CD4 + and CD8 + lymphocytes was described, particularly in the perivascular infiltrate and focally at spongiosis sites (areas of intracellular fluid accumulation in the dermis).¹⁶⁴ In contact dermatitis, a skin disease caused by hapten exposure in sensitized individuals, a very similar GrB expression pattern was found, within CD4 + and CD8 + cells in perivascular infiltrate and spongiosis sites.¹⁶³ Interestingly, keratinocytes in close proximity to GrB-positive lymphocytes showed signs of cell damage at sites of spongiosis. Weak GrB and perforin staining was described in these keratinocytes, suggesting they may be exposed to GrB from neighboring lymphocytes or may express the proteins themselves.

Lesions of the chronic inflammatory skin diseases, lichen sclerosis and LP, also showed abundant T cell expression of GrB. In lichen sclerosis, GrB mRNA expression was found in dermal infiltrate close to keratinocytes in the intra-epidermal area, was highly expressed at early stages of the disease, and levels were over 100 times greater than that of normal skin.^{173,169} GrB-positive cells were found in close vicinity to apoptotic keratinocytes and through electron microscopy GrB was found to be transferred from a lymphocyte into an apoptotic keratinocyte, suggesting that GrB from lymphocytes is contributing to keratinocyte cell death in this disease.¹⁶⁸ GrB-expressing plasmacytoid DCs usually found in blood were seen in oral LP lesions and may contribute to GrB-associated damage in this disorder.¹⁷⁰

Stevens–Johnson syndrome and toxic epidermal necrolysis are rare and potentially life-threatening diseases involving keratinocyte apoptosis and epidermal necrosis, which are largely believed to be drug induced.¹⁷⁶ GrB-positive lymphocytes were found in blister fluid and GrB upregulation correlated to increased disease severity. T cells in lesions express perforin and GrB, and it has been suggested that T cells may trigger keratinocyte cell death in these skin conditions.^{176–180}

Vascular Diseases

Atherosclerosis

Atherosclerosis a lipid-driven inflammatory disease responsible for the majority of heart attacks, strokes, and lower limb loss, and, as such, is now the leading cause of death world-wide.^{294–297} The most abundant inflammatory and immune cells in atherosclerotic lesions are T lymphocytes and macrophages, and their role in atherosclerosis is well documented.^{189,298–300} Small populations of neutrophils, mast cells, DCs, and NK cells also are present.^{301–303} The role

of GrB in atherosclerotic plaque progression is illustrated in Figure 3c. Until recently, GrB expression was believed to be limited to CTLs and NK cells. However, CD4⁺/CD8⁺ macrophages infiltrating inflammation sites and lipid-laden foam cells within atherosclerotic plaques also express GrB.^{10,189,304} In atherosclerotic lesions, GrB expression increases with disease severity and is observed in both lipid-rich areas and cellular regions surrounding the core of developing plaques, as well as in the shoulder regions of advanced plaques.¹⁰ Within the intima and media, GrB is found within SMCs, macrophages, and T cells, but can also be detected extracellularly. Furthermore, GrB is found to localize to TUNEL-positive foam cells, suggesting that it could be contributing to cell death in lipid-rich regions of developing plaques.¹⁰

The presence of GrB in advanced atherosclerotic lesions and its association with increased disease severity suggests that GrB may affect plaque stability. Indeed, elevated levels of GrB are detected in the plasma of patients with atherosclerosis, with the highest levels detected in patients with unstable plaques, lending support to the hypothesis that GrB influences plaque instability. In a study by Skjelland *et al*,²³² increased GrB levels in the plasma corresponded to carotid plaque instability and increased incidence of cerebrovascular events in humans. Elevated GrB production was also observed in peripheral blood mononuclear cells isolated from patients with unstable angina pectoris compared with cells from patients with stable angina pectoris.²³³ Thus, evidence to date indicates that GrB might be involved in the initiation of plaque instability and rupture. In addition, the repercussions of elevated GrB levels may extend beyond plaque rupture and into post-infarct ventricular remodeling as Kondo *et al* recently reported elevated GrB levels in the plasma of patients with myocardial infarction (MI) in both the acute and subacute phases. They also found that sustained levels of GrB was an independent factor for late-stage left ventricular remodeling.²³⁴

The mechanisms by which GrB may contribute to plaque stability include ECM fragmentation, release of ECM-sequestered cytokines, and/or induction of macrophage or SMC apoptosis in the fibrous cap. To the latter point, Choy *et al*¹³⁵ showed, using a cell culture model, that GrB could induce SMC apoptosis in the absence of perforin through the disruption of SMC-ECM interactions and induction of anoikis. Conversely, levels of the endogenous GrB inhibitor PI-9 in SMC markedly reduced in unstable plaques *versus* stable plaques, implying a role for GrB-induced SMC apoptosis. It should be noted, however, that although the GrB-inhibitory role of PI-9 is well-established, its effects on GrB as a potential mechanism were not considered nor discussed in the latter study. Rather, reduced PI-9 was associated with increased SMC caspase-1 activity and IL-1 β production.³⁵ With regard to its extracellular function in atherosclerosis, GrB is capable of cleaving multiple ECM proteins, including fibronectin, vitronectin, and laminin.^{134,135} These

data suggest that GrB may contribute to plaque instability through both intracellular and extracellular activities, leading to the formation of a necrotic core and thinning of the fibrous cap, and ultimately plaque rupture.¹⁰ Although ongoing, preliminary studies have been reported using apolipoprotein E \times GrB double knockout mice in which GrB deficiency was found to be associated with reduced atherosclerosis, suggesting that this protease may be important in this disease.³⁰⁵

Although GrB appears to be involved in the pathogenesis of atherosclerosis, it should be noted that the precise effects of elevated GrB levels may depend on the stage of lesion development. During the early stages of lesion development, GrB-induced SMC apoptosis may be a beneficial way to reduce intimal hyperplasia,³⁰⁶ whereas in more advanced lesions, increased SMC apoptosis may decrease plaque stability through decreased cellularity and increased ECM proteolysis.

Cardiac allograft vasculopathy

Transplantation is the recommended therapy for end-stage organ disease. Although the prognosis for acute rejection has improved considerably with the introduction of immunosuppressive agents, long-term allograft survival has not improved appreciably since the early 1980s. Numerous studies have reported elevated GrB levels in the plasma and inflammatory infiltrate of biopsies from patients experiencing acute rejection, suggesting GrB may be a useful biomarker for the prediction of acute rejection.²³⁷⁻²⁴⁵ Although acute rejection constitutes a major challenge in the early postoperative period, the major impediment to long-term survival for solid organ transplant recipients is allograft vasculopathy (AV), which is an accelerated, immune-mediated form of occlusive arteriosclerotic vascular disease that affects vessels of solid organ allografts.^{307,308} The pathogenesis of AV is complex and characterized by intimal accumulation of SMCs, T lymphocytes, and macrophages, as well as extensive medial thinning that is associated with increased SMC apoptosis.^{10,308,309}

GrB is abundant in the intima, media, and adventitia of cardiac AV (CAV) vessels, and increased GrB corresponds to increased CAV disease severity.¹⁰ In the intima, GrB localizes to macrophage foam cells, whereas in the media and adventitia, GrB was found extracellularly, as well as in SMC and leukocyte infiltrates.¹⁰ Similar to developing atherosclerotic lesions, GrB within CAV lesions localizes to TUNEL-positive foam cells within the deep intima, suggesting that it contributes to cell death within the lesions.¹⁰ Roles for both GrB and perforin in CAV pathogenesis have been assessed using a mouse heterotopic cardiac transplant model of CAV.^{246,247} In these studies, hearts transplanted into GrB-KO mice or perforin-KO mice showed smaller lesions, reduced luminal narrowing, and a marked reduction in endothelial cell apoptosis when compared with wild-type recipients.^{246,247} Similar to its predicted role in atherosclerosis,

GrB may contribute to CAV pathogenesis through apoptosis of SMC and endothelial cells, and through extracellular activities such as SMC anoikis and cleavage of ECM proteins. Both mechanisms are likely to contribute to the overall integrity of blood vessels and the development of CAV.

Other cardiovascular diseases

Inflammation, activated T cells, and SMC death are associated with other inflammatory vascular diseases such as Kawasaki disease, Takayasu myocarditis, and giant cell arteritis.^{248,249,310–313} Kawasaki disease is a childhood form of vasculitis that affects the coronary arteries and is characterized histologically by inflammation and ECM destruction, resulting in the formation of coronary artery aneurysms.³¹⁴ Elevated levels of CTLs, macrophages, and B cells have been noted in the lesions, aneurysms, and the plasma of Kawasaki's patients; however, the involvement of GrB in Kawasaki disease pathogenesis requires further elucidation.^{248–250} Increased plasma levels of GrB, in addition to GrB-positive CTL and macrophages, have been reported in Kawasaki disease; however, a more recent study showed elevated CTLs in acute Kawasaki disease in which neither GrB nor perforin could be detected.^{249,250} Whether the variations in GrB and perforin expression reflect alterations in differentiated T-cell populations, genetic differences in GrB/perforin production, or variation in preparation are unknown, but will prove to be an interesting area of future research.

Inflammatory Lung Diseases

COPD

COPD is an inflammatory lung disorder characterized by features of emphysema, chronic bronchitis, and small airways disease and is associated with chronic pulmonary and systemic inflammation.³¹⁵ COPD patients who smoke have shown increased levels of CD8+ cells expressing perforin in the BAL fluid.¹⁴³ An increase in the number of T cells and NK cells expressing GrB in the blood and BAL fluid of patients with COPD has also been reported.¹⁴⁴ A plausible apoptotic role for GrB in COPD was suggested by Hodge *et al*,¹⁴⁴ who showed a positive correlation between T cell-derived GrB in the BAL fluid and apoptosis of bronchial epithelial cells. CD8+ CTLs and NK cells infiltrating the lung in COPD patients are active and express GrB while GrB protein and mRNA were also detected in type II pneumocytes from patients with COPD.¹⁸ Although these findings raise the possibility of an interesting new source of GrB delivery into the parenchymal spaces of the diseased lung, more studies are needed to determine whether GrB release by these cells do in fact contribute significantly to COPD pathogenesis. Given the apoptotic and degenerative nature of COPD along with the pro-apoptotic and matrix-degrading ability of GrB, speculation of a major role for GrB in COPD pathogenesis continues to gain momentum.

Asthma

Asthma is a T_H2-dependent inflammatory disease of the lung characterized by reversible airway obstruction, bronchial hyper-reactivity, and chronic inflammation leading to tissue remodeling. Little is known regarding the specific role of GrB in allergic asthma; however, Bratke *et al*¹²² were able to show an increase in GrB-expressing T cells and NK cells, as well as extracellular GrB in the BAL fluid of patients suffering from allergic asthma after allergen challenge, leading to speculation that GrB contributes to pathogenesis through matrix destruction and remodeling. These findings are supported by Tschopp *et al*,¹⁷ who reported an alteration in the granule content of basophils and the induction of GrB expression upon stimulation with IL-3 released by mast cells. Despite a lack of perforin expression, human basophils showed NK-like cytotoxicity, which was abolished after GrB inhibition further supporting a possible perforin-independent extracellular role of GrB in allergic asthma.

Other inflammatory lung diseases

Several other lung diseases are affected by GrB activity as well. It has been reported that GrB and perforin mRNA levels are increased in the BAL fluid of patients in the acute phase of acute respiratory distress syndrome (ARDS).¹⁴⁵ A functioning and unbroken alveolar epithelium is critical for recovery from ARDS and increased granzyme-mediated apoptosis of alveolar epithelial cells could compromise the integrity of this barrier leading to disease progression. Furthermore, hypersensitivity pneumonitis has been shown to be associated with an increase in GrB protein levels in the BAL fluid supporting the notion that GrB contributes to alveolar destruction and matrix remodeling observed in this disease.¹²⁵ GrB-expressing CD4+ and CD8+ T cells have also been detected in the BAL fluid of patients with pulmonary sarcoidosis, a disease characterized by a T_H1-dominant immune response and the formation of granulomas often found in the lung.¹⁴⁶ Reduced levels of GrB have also been detected in the serum of sarcoidosis patients, leading to speculation that the protease is increased in tissues elsewhere in the body where it participates in the cytotoxicity observed in pulmonary sarcoidosis.¹⁴⁷ However, as with all inflammatory lung diseases, much work remains to be done to confirm a specific role for GrB in disease pathogenesis and to determine the intracellular and/or extracellular mechanisms that might be involved.

CONCLUSION

GrB is emerging as a multifunctional protease that may have important roles in a number of inflammatory and/or degenerative pathologies. The realization that this protease can be expressed and secreted by both immune and non-immune cells, in addition to the fact that the function of this protease extends beyond the induction of CTL/NK-induced apoptosis suggests that there is much to be learned about this protease in disease. Nonetheless, it is becoming apparent that

GrB may have a larger role in chronic inflammatory, autoimmune, and/or degenerative diseases than previously believed. Future studies may show GrB as a novel therapeutic target for such diseases.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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