CHAPTER 37

Cell-Mediated Cytotoxicity

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INTRODUCTION

The most effective way the immune system can control the threats of intracellular infection and cellular transformation is by destroying infected and cancerous cells.[1–7] When killer lymphocytes recognize harmful cells, they can target them for elimination by triggering programmed cell death. The main killer cells are natural killer (NK) cells of the innate immune sytem and cluster of differentiation (CD)8+ T-lymphocytes af adaptive immunity, although some CD4+ T lymphocytes, particularly T_H1 and regulatory T (T_{reg}) cells, also express and deploy the specialized cell death machinery. All killer lymphocytes contain specialized secretory lysosomes, called cytotoxic granules, that are filled with death-inducing enzymes, called granzymes ("granule enzyme"). When the killer cell is activated, the cytotoxic granules move to the immune synapse formed with the target and fuse their membranes with the killer cell membrane, dumping their contents into the immune synapse in a process termed granule exocytosis. Perforin, a pore-forming protein in the granules, delivers the death-inducing granyzmes into the cytoplasm of the target cell to initiate its death. In this encounter, the killer cell remains unharmed.[8] It is a serial killer that can detach from one target to seek and destroy others.[9] Killer cells can also activate programmed cell death by using cell surface receptors to ligate cellular death receptors, such as Fas, on target cells. Granule-mediated cell death is key to control viral and intracellular bacterial infection and cancer because perforin-deficient mice and humans homozygous for perforin mutations or deficent in molecules needed for granule exocytosis are highly vulnerable to infection with intracellular pathogens and prone to develop spontaneous lymphomas.[10] The death receptor pathway regulates lymphocyte homeostasis. Patients genetically deficient in the death receptor Fas or its ligand FasL develop autoimmunity.[11] Target cells destroyed by cytotoxic granules or death receptor ligation die a highly regulated death (programmed cell death or apoptosis) rather than by necrosis. Programmed cell death minimizes inflammation and damage to nearby tissue as target cells undergoing programmed cell death are rapidly recognized and cleared by immune phagocytes, especially macrophages.[12] The topic of this chapter was reviewed in more depth in a recent issue of *Immunological Reviews*.[13]

In this chapter we first describe the killer cells: which immune cells are able to kill and how they develop this capacity and are regulated. Because of its destructive potential, cytotoxicity needs to be carefully regulated. We next focus on the death machinery used for granule- and death receptor–mediated cytotoxicity and how it is mobilized and used to destroy the target cell. We also discuss what is known about how killer cells are protected against their own weapons of destruction. Some granzymes are expressed without perforin in nonkiller cells. We also discuss the increasing evidence for noncytotoxic proinflammatory roles of killer molecules.

THE KILLER CELLS

The major killer cells are NK cells in innate immunity and CD8 T cells in adaptive immunity. Naïve T cells that have not previously seen antigen do not express either granule effector molecules or death receptors, and are incapable of cell-mediated cytotoxicity.[14] Within about 5 days of activation, naïve CD8 T cells differentiate into effector cytotoxic T lymphocytes (CTLs) that express both types of cytotoxic molecules. At the same time, these cells downregulate adhesive and chemokine receptor molecules that retain them in lymph nodes and acquire receptors that allow them to traffic to tissue sites of infection and tumor invasion. Activation to cytotoxic effector cells is tightly regulated. It requires not only antigen-receptor activation, but also costimulation, and is greatly enhanced when antigen-presenting cells are stimulated by danger and pathogen-associated pattern motif receptors or when naïve T cells are stimulated by exogenous inflammatory and antiviral cytokines, including type I interferons (IFNs), interleukin (IL)-1, and IFNγ. Upon activation, effector CD8 T cells also begin to express the Fcγ receptor CD16, also present on NK cells, which enables them to recognize and lyse target cells that have been coated with IgG antibodies in a process called antibody-dependent cell-mediated cytotoxicity.[15] In situations of persistent and extensive antigen, however, such as occur in tumors and chronic viral infection, many of the CD8 T cells that have the surface protein expression of CD8 effector cells no longer express perforin and are not cytotoxic.[16–18] Effector CD8 T cells that lack cytotoxicity have been termed "exhausted." Most effector cells in an immediate immune response die within a few weeks, but some survive and develop into memory cells. Memory cells downregulate expression of cytotoxic effector proteins, but the kinetics of downregulation varies with the molecule and with the particularities of the immunostimulatory environment.[14,19] In particular, activation of CD8 T cells without CD4 T-cell help leads to an unimpaired primary cytotoxic response, but greatly impairs the development of antigen-specific memory cells.[20]

The immunosuppressive drug rapamycin directs antigen-stimulated CD8 T cells to differentiate preferentially into memory cells rather than to effector CTLs.[21] Memory CD8 T cells rapidly reacquire cytotoxic capability within hours of restimulation. The molecular basis for this rapid response is not well understood, although recent studies suggest that in memory CD8 T cells, the chromatin of cytolytic effector gene promoters and of eomesodermin, the master transcription factor that regulates CD8 effector genes, bears epigenetic marks that poise them for transcription compared to naïve T cells.[22–26] These cells may also store messenger ribonucleic acids (mRNAs) for perforin and granzyme that can be rapidly translated upon activation. Some types of activated CD4 T cells, especially T_H1, NKT, and T_{reg} cells, also express granzymes and perforin and have cytotoxic activity. Murine T_{reg}s express granzyme B, but probably not granzyme A.[27,28] Although immunosuppression by T_{reg}s is mediated by soluble factors, there is also a poorly understoood component that requires cell-to-cell contact. Direct lysis of cognate T cells and potentially other immune cells by granule-mediated and death receptor pathways by T_{reg}s is likely an important mechanism for suppressing immune activation.[29–31]

Because it takes a week to 10 days for naïve CD8 T cells to proliferate and differentiate into a large population of antigen-specific CTLs, the immediate response to intracellular infection in individuals that have not been vaccinated or previously exposed is mediated by NK cells. Although freshly minted NK cells were previously thought to immediately express granzymes and perforin, it now seems clear that—at least in mice—resting NK cells have minimal cytotoxic activity.[32] They constitutively express mRNAs for granzymes A and B and perforin, but only have granzyme A protein. Because they lack substantial perforin protein, cytotoxicity is limited. However, perforin and granzyme proteins and cytotoxicity are upregulated rapidly when NK cell–activating receptors are stimulated. Less differentiated NK cells that highly express the neural cell adhesion molecule or CD56 are poorly cytotoxic, while more differentiated $CD56^{dim}$ NK cells are potent killer cells.[33] In the circulation, $CD56^{dim}$ NK cells have about a log more perforin than $CD56^{bright}$ NK cells. NK-activating receptors recognize cell surface changes in tumors, stressed cells, and infected cells, such as downregulation of major histocompatibility complex/human leukocyte antigen molecules or cell surface expression of nonclassical major histocompatibility complex molecules, such as MICA and MICB, that are induced by stress. A longstanding dogma of innate immunity is that innate immune responses are not altered by antigen exposure. However, it is now clear that NK cytotoxicity to infection and other stimuli can be greatly increased by previous antigen exposure.[34] NK cell memory of prior exposure probably results from the expansion of NK cells bearing activating receptors specific for different important pathogens. These receptors, many of which are poorly conserved during mammalian evolution, may have coevolved with important pathogens. The link between individual NK receptors and pathogen recognition remains to be defined.

CYTOTOXIC GENE EXPRESSION

There are 5 human granzymes and 10 mouse granzymes expressed from three gene clusters that arose by gene duplication. In humans, the genes encoding granzymes A and K, tryptases that cleave after basic amino acids, are clustered on chromosome 5; the genes for granzyme B, which cleaves after aspartic acid residues like the caspases, and granzyme H (or C in mice), which cleaves after hydrophobic residues, are clustered with myeloid cell proteases like mast cell chymase on chromosome 14; and the gene for granzyme M, which is highly expressed in NK cells and cleaves after Met or Leu, is found on chromosome 19 (Fig. 37.1). The mouse granzyme B cluster is uniquely expanded by multiple gene duplications to encode, in addition, granzymes D, E, F, G, L, and N. Nothing is known about these mouse-specific enzymes, but they may have evolved to defend against specific common mouse pathogens.[1] Granzyme A and granzyme B are the most abundant granzymes and the most studied. Killer cells, including NK cells, cytotoxic CD4 and CD8 T cells, and even some T_{reg} cells, express highly individualized and tightly regulated patterns of granzymes that depend on both cell type and mode of activation.[35,36]

Expression in Noncytolytic Cells

Perforin is only expressed by cytotoxic cells. Although granzymes were previously also thought to have similarly restricted expression, noncytotoxic cells can express granzymes without perforin.[37] Granzyme transcripts can be amplified from prothymocytes in fetal liver and double negative thymocytes.[38] Although granzyme A transcripts are detected in thymocytes with the potential to develop into CD8+ cells, granzyme A activity is detected only in the most mature CD4–CD8+ thymocytes. These results suggest posttranscriptional regulation of granzyme translation (see the following for additional examples). Granzyme B, but not granzyme A, is expressed in T_{reg} cells and plays an important perforin-dependent role in T_{reg} function in mice. Benign and transformed B cells can be induced to express granzyme B by IL-21 alone or when combined with anti–B-cell-receptor antibody.[39] Granzyme B is also expressed without perforin in many different types of myeloid cells. Within the immune system, granzyme B is expressed in human plasmacytoid dendritic cells (pDCs).[40] There are comparable levels of granzyme B transcripts in resting and activated pDCs, but significantly higher amounts of granzyme B protein in activated cells, suggesting posttranscriptional regulation of expression. Granzyme B is also expressed in both normal and neoplastic human mast cells in vitro and in vivo.[41] It localizes to mast cell granules and is secreted when they are activated. In mice, skin-associated mast cells and bone marrow–derived in vitro differentiated mast cells express granzyme B but lung mast cells do not.[42] Neither granzyme A nor perforin are detected in mouse mast cells. The granzyme B gene is encoded within a few hundred kilobases of mast cell proteases. Thus, the granzyme B/mast cell chymase and tryptase genomic region is likely open and active in mast cells. In human basophils, IL-3 induces granzyme B, but not granzyme A or perforin,

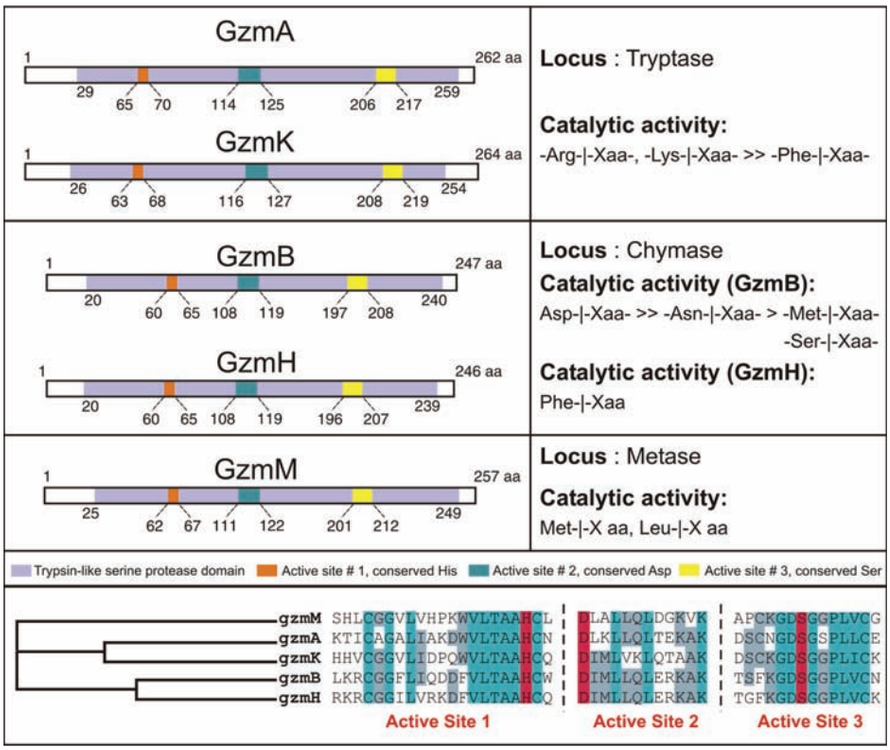

FIG. 37.1. The Human Granzymes are Encoded in Three Clusters. (Figure reprinted with permission from Chowdhury D, Lieberman J. Death by a thousand cuts: granzyme pathways of programmed cell death. *Annu Rev Immunol.* 2008;26: 389–420.)

expression.[43] Expression of granzyme B in mast cells and basophils suggests a role of granzyme B in mediating allergic disease. In fact, granzyme B has been found in bronchoalveolar lavage fluid after allergen exposure. Several studies have suggested that granzyme B and perforin are expressed in human neutrophils, but this is controversial.[44–47]

Granzyme B is also expressed in the absence of perforin in the human reproductive system in developing spermatocytes and in placental trophoblasts,[48] and by granulosa cells of the human ovary in response to follicle stimulating hormone.[49] In addition, granzyme B has been detected in a subset of primary human breast carcinomas and in chondrocytes of articular cartilage.[50] The granzyme M transcript is expressed at low levels in the photoreceptor cells of the retina in the mouse.[51] An alternatively spliced form (aGM) is exclusively expressed in these cells at much higher levels. Like granzyme M, granzyme K has an alternatively spliced form exclusively expressed in the brain.[52] The physiologic significance of the alternative transcripts of granzymes M and K is unclear.

Extracellular Signals Regulating Granzyme Expression

The kinetics and expression of the individual granzymes and perforin vary in different clonal populations in vitro and in vivo and depend on how they are activated.[53–55] Most circulating CD8+ T-lymphocytes that express any granzyme, express both granzyme A and granzyme B, but some cells are positive for only one granzyme. Single-cell expression profiles of granzymes, perforin, and IFNγ have been investigated in in vitro or in vivo activated CD8+ T cells using reverse transcription-polymerase chain reaction in mice[35] and intracellular staining and flow cytometry in humans.[56] Individual T cells show diverse expression of these genes. Although some pairs of genes (perforin and IFNγ) are coexpressed more frequently than others, no specific combination of genes is consistently coexpressed. During in vitro activation of mouse naïve lymphocytes with antibodies to CD3, CD8, and CD11a and IL-2, the expression of granzyme A and granzyme C is delayed compared with cytolytic activity and expression of perforin and granzyme B.[35] When mouse CTLs are activated in vivo by influenza virus infection, most antigen-specific CD8 T cells found in the lung 1 week after infection express both granzymes A and B, and about a third of them also express perforin. Moreover, there is no in vivo difference in the kinetics of induction of granzyme A, granzyme B, or perforin. Granzyme C is not induced by influenza infection in vivo. The diversity of expression of individual granzyme and perforin genes suggests that each gene is regulated independently, although it is likely that these genes will share some common transcription factor recognition sites and epigenetic changes. Differences in TcR avidity, costimulatory and inhibitory receptor engagement, danger and innate immune receptor activation, cytokine milieu, type and state of activation of the antigen-presenting cell, and presence of helper or regulatory CD4 T cells will likely influence the induction of the granzyme and perforin genes. Moreover, the cell's prior history of activation will affect cytolytic gene expression during subsequent encounters with antigen. Surprisingly little is known about this subject.

The perforin and granzyme genes are induced during T-cell activation. However, the only signal shown consistently to upregulate granzyme A and B and perforin is IL-2.[57] IL-2 regulates perforin and granzyme expression directly and independently of its effect on CD8+ T-cell survival and proliferation.[58] Mice genetically deficient in IL-2 retain the ability to elicit a CTL response against many viruses, tumors, and allografts,[59,60] although there are deficiencies in cytotoxicity under certain conditions.[61] The other γ_c-dependent cytokines (IL-4, IL-7, IL-9, IL-15, and IL-21) likely substitute for IL-2 in its absence. IL-15 is particularly important because it also shares the γ-chain with the IL-2 receptor. IL-15 induces the expression of perforin, granzymes A and B, IFNγ, and Fas ligand in primary mouse lymphocytes.[62] IL-21 works synergistically with IL-15 to upregulate granzyme A and B expression in mouse CD8 T cells.[63] In vivo in mice, IL-21 exhibits potent antitumor function by enhancing NK and CD8 T-cell cytotoxicity.[64] Similarly in human peripheral blood CD8 T cells, IL-15 and IL-21 both activate granzyme B and perforin expression, but IL-21 does so without inducing CD8 T-cell proliferation.[65] Members of the IL-6/IL-12/IL-27 family also can upregulate granzyme and perforin expression.[66,67]

Transcriptional Regulation of Perforin and Granzymes

Two key transcription factors, T-bet (*TBX21*) and eomesodermin (*EOMES*), that belong to the T-box family are the key master regulators of cytotoxic gene expression and survival of committed CD8 memory cells.[68–71] After naïve CD8 T-cell activation, T-bet is induced before eomesodermin.[72] Notch signaling and the Runx3 transcription factor upregulate eomesodermin, but also directly upregulate expression of perforin and granzyme B genes.[72,73] Mice deficient in both T-bet and eomesodermin genes are unable to control tumors and intracellular infection.[74–76] They develop a wasting syndrome caused by anomalous differentiation to IL-17–secreting cells, suggesting that these two genes not only positively regulate cytotoxic gene expression and other genes required for CTL survival and function, but also suppress differentiation to alternate lineages.

Chromosome transfer experiments have shown that expression of the perforin gene (*PRF1/prf1*) is regulated by cis-regulatory regions extending about 150 kb around the gene.[72,77,78] These include a core promoter located 120 bp upstream of the transcription start site and two enhancer regions and a locus control region (LCR) that are altered during T-cell differentiation and activation. The LCR is open for transcription specifically in cytotoxic cells. The region around the presumed LCR is more accessible to DNase I digestion (and therefore its chromatin is open) in murine CD8 CTLs than in CD4 T_H1 cells, likely explaining their approximately 20-fold increase in *prf1* mRNA. Increased IL-2 does not enhance the accessibility of the LCR. The enhancers are both activated by IL-2R signaling mediated by signal transducer and activator of transcription (STAT)5 binding to two sites in each enhancer. Other STAT family members activated by alternate cytokines can also activate them. Activation of the more proximal enhancer also depends on IL-2–activated NF-κB binding. Both enhancers also contain binding sites for AP-1 and Ets transcription factors, while the distal enhancer has an E-box and NFAT binding site and the proximal enhancer contains eomesodermin, Ikaros, and CREB binding sites. Recruitment of ribonucleic acid (RNA) pol II to the transcription start site and activation of transcription increase with IL-2 stimulation. The key factors involved in activating transcription at the *prf1* promoter are Runx3 and eomesodermin. T-bet does not appear to play a direct role in activating prf1 transcription, but likely acts indirectly by increasing IL2-Rβ expression and enhancing IL-2 signaling. The current model suggests that Runx3 is needed to open the extended *prf1* locus during T-cell differentiation, while eomesodermin plays a more direct role in activating transcription near the promoter. Other transcription factors also likely participate in transactivating the perforin promoter, including an ets transcription factor, probably MEF.

Much less is known about the details of gene regulation of the granzymes. Enhancers or other long-range regulatory region of granzyme genes remain to be defined. Granzyme B is the only granzyme whose expression has been studied. A distal DNase hypersensitivity site 3.9 kb upstream of the granzyme B transcription start site is accessible only in activated, but not resting, CD8 T cells.[79] Inclusion of this region in a GFP reporter in transgenic mice enhances CTL-specific expression, suggesting that this region may have enhancer activity. Induction of the expression of granzyme transcripts requires at least two independent stimuli: activation of the TcR and costimulation by cytokines of the γ_c family. The signals from several distinct signal transduction pathways are integrated in the nucleus in the form of transcription factors that bind to granzyme gene regulatory elements and activate transcription. Early studies identified a 243-bp fragment upstream of the mouse granzyme B transcription start site that potentially regulates granzyme B transcription.[80] This region contains binding sites for two ubiquitous transcription factors, activating transcription factor/cyclic AMP-responsive element binding protein and activator protein-1, and two lymphoid specific factors, Ikaros and core-binding factor (PEBP2).[81] Several of these transcription factor binding sites are evolutionarily conserved between the human and mouse granzyme B promoters.[82,83] Analysis of reporter assays using promoters that had been systematically mutated at these sites in primary cells and cell lines revealed subtle differences in the importance of some transcription factors in primary cells versus cell lines. For example, activator protein-1, cyclic AMP-responsive element binding protein, and core-binding factor were not as important for transcription in primary cells as they appeared to be in cell lines.[82,84] These studies suggested that combinations of transcription factors (particularly, activator protein-1 and core-binding factor) activate granzyme B expression in primary cells. The most compelling difference between the mouse and human granzyme B gene promoter is the importance of the Ikaros site only in human granzyme B expression.[82,84] Studies in *Stat1*-deficient mice indicate that STAT1 mediates

granzyme B induction by IFNα or IL-27.[67,85] IL-27–induced augmentation of granzyme B expression also depends on T-bet.[67] eomesodermin also drives granzyme B expression.[71] Direct binding of T-bet and eomesodermin to the granzyme B promoter has not been examined.

Posttranscriptional Regulation

Several examples of cells expressing perforin and/or granzyme transcripts, but not protein, were described previously, including resting NK cells, thymocytes, and unactivated pDCs and mast cells. Murine memory CTLs also express abundant granzyme B mRNA but no protein.[86] All these results point toward a general mechanism of "prearming" cytotoxic lymphocytes with effector mRNAs, allowing these cells to rapidly respond to external stimuli. This type of gene regulation is well known to regulate cytokine expression, presumably for the same purpose. Two recent studies provide evidence for negative regulation of granzyme B and perforin expression by microRNAs miR-27* and miR-223 in NK cells.[87,88] It will be interesting to see if expression or processing of these microRNAs declines rapidly after NK cell activation.

GRANULE-MEDIATED CELL DEATH

Killer Cell Granules

Killer cells contain cytotoxic granules that are acidic, electron-dense, specialized secretory lysosomes[89] (Fig. 37.2). These granules are mobilized like secretory vesicles in other secretory cells, such as neurotransmitter-containing vesicles near the synapses of neurons and melanin-containing vesicles of melanocytes. Cytotoxic granules contain the granzymes, trypsin-like serine proteases, whose major job is to initiate programmed cell death in cells marked for immune elimination. Cytotoxic granule proteins also regulate the survival of activated lymphocytes and may also cause inflammation by acting on extracellular substrates. The granzymes are trypsin-like serine proteases that use a classic histidine, serine, aspartic acid catalytic triad to cleave their substrates. Human granzymes A, B, C, and M, rat granzyme B, and human progranzyme K have all now been crystalized with high resolution.[90–95] The active granzymes are produced by cleavage of a dipeptide from the N-terminus of the proenzyme. Activation is accompanied by a radical conformational change. Progranzyme K has a more rigid structure lacking an open active site than the active

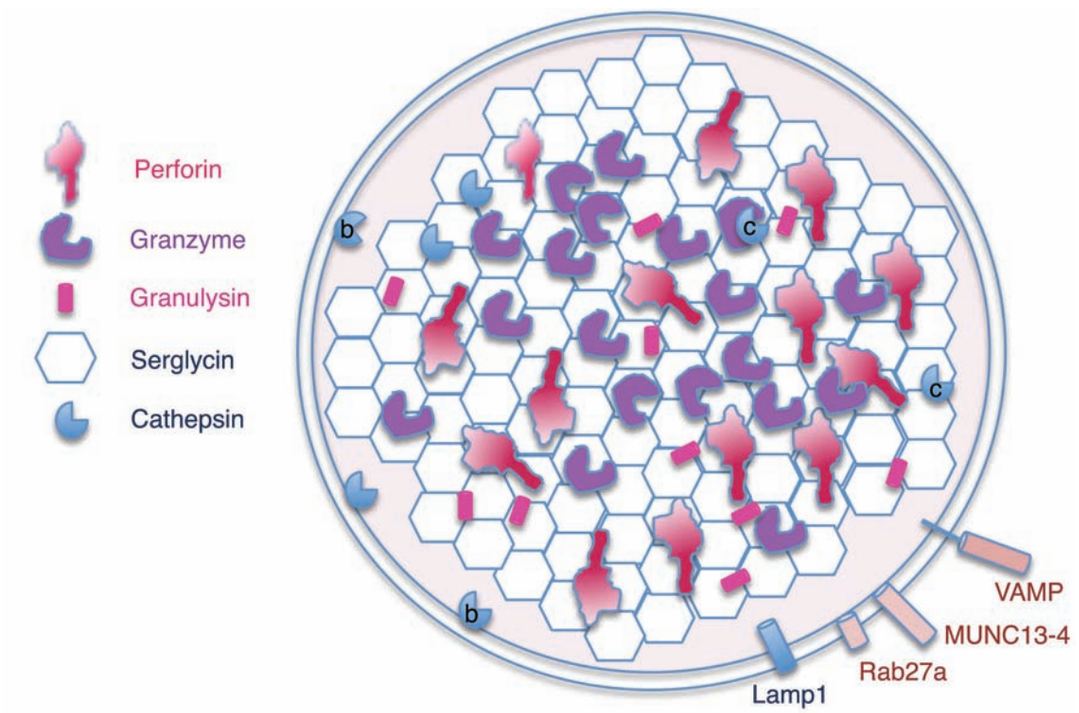

FIG. 37.2. Key Components of Cytotoxic Granules. The cytolytic effector molecules, perforin, granzymes, and granulysin, are bound to the serglycin proteoglycan. Cytotoxic granules also contain molecules found in all lysosomes, such as Lamp1 (CD107a) and cathepsins, as well as membrane-associated proteins specific to secretory lysosomes, such as vesicle-associated soluble N-ethylmaleimide-sensitive factor accessory complex component (VAMP)7 or VAMP8, Munc13-4, and Rab27a, which are essential for granule exocytosis. Cathepsins B and C play a special role in cytotoxic granules: cathepsin C processes the progranzymes to the active enzyme and membrane-associated cathepsin B helps protect the killer cell from membrane damage in the immune synapse by perforin. Other cathepsins may substitute for these cathepsins when they are absent or mutated.

granzymes. Detailed information about the conformation surrounding the active sites of granzyme A and granzyme B has provided the structural basis for understanding how subtle differences in the active site conformation lead to substantial differences in substrate specificity. As a consequence, mouse granzyme B is preferentially able to cleave mouse procaspase-3, while human granzyme B is better able to cleave the human orthologue. Granzyme A differs from the other granzymes in forming a covalent homodimer; the other granzymes are monomeric. Dimerization creates an extended site for substrate binding that is believed to confer a high degree of specificity to granzyme A for its substrates.[90,96] In particular, because of the extended exosite for substrate binding, granzyme A substrates do not share a common short peptide sequence around the cleavage site.

The cytotoxic granules also contain perforin, a pore-forming molecule that delivers the granzymes into the target cell. Another pore-forming molecule, granulysin, that is homologous to the saposins, is cationic and selectively active at disrupting negatively charged bacterial and possibly fungal and parasite cell membranes. Granulysin is expressed in humans and nonhuman primates and orthologues are found in some other species (pigs, cows, and horses), but not in mice. The positively charged cytotoxic effector molecules are bound in the granule to an acidic proteoglycan, called serglycin, after its many Ser-Gly repeats.[97,98] In addition to these specialized molecules, the cytotoxic granules also contain lysosomal enzymes, the cathepsins, and internal lysosomal membrane proteins, such as CD107 (Lamp1). The outside of the granule membrane binds soluble N-ethylmaleimide-sensitive factor accessory protein receptor (SNARE) proteins, synaptotagmins and Rab GTPases, that regulate vesicular trafficking and cytotoxic granule release. Some of these molecules, including Rab27a and Munc13-4, which are important for granule exocytosis, are only incorporated into cytotoxic granules as they mature by fusion of cytotoxic granules with specialized exocytic vesicles, formed in secretory cells by fusion of late endosomes and recycling endosomes. Some of the granule-associated molecules associate with lysosomes in all cells, while some have a specialized function in killer cells.

Steps in Granule Exocytosis

When CTLs and NK cells form an immune synapse with a target cell, engagement of activating receptors, including the T-cell receptor, NK cell–activating receptors, and Fc receptors, stimulates the killer cell to destroy the target cell[7] (Figs. 37.3 and 37.4). Activation for cytolysis is enhanced by binding of CD8 or CD4, costimulatory receptors, and adhesion molecules like LFA-1, which cluster in well-defined concentric rings within the immune synapse. Killer cell activation causes a calcium flux that induces lytic granules to cluster around the microtubule organizing center and then align along the immunologic synapse.[99–103] Granules move to the immune synapse via both the microtubule network and actin cytoskeleton. The latter interaction is via myosin IIA in NK cells.[104] The actin meshwork thins around the site of the synapse to make room for granules to move through it.[105–107] Cytotoxic granules then dock to the killer cell plasma membrane in the central region of the immune synapse (c-SMAC). In T cells, granule docking and fusion may localize to a distinct (secretory) region of the central cluster (c-SMAC) of the immune synapse that is separate from the signaling domain containing the T-cell receptor and associated kinases.[108] Recent studies did not observe a separation of signaling and secretory domains in the c-SMAC of NK cells. Cytotoxic granule docking is orchestrated by bind-

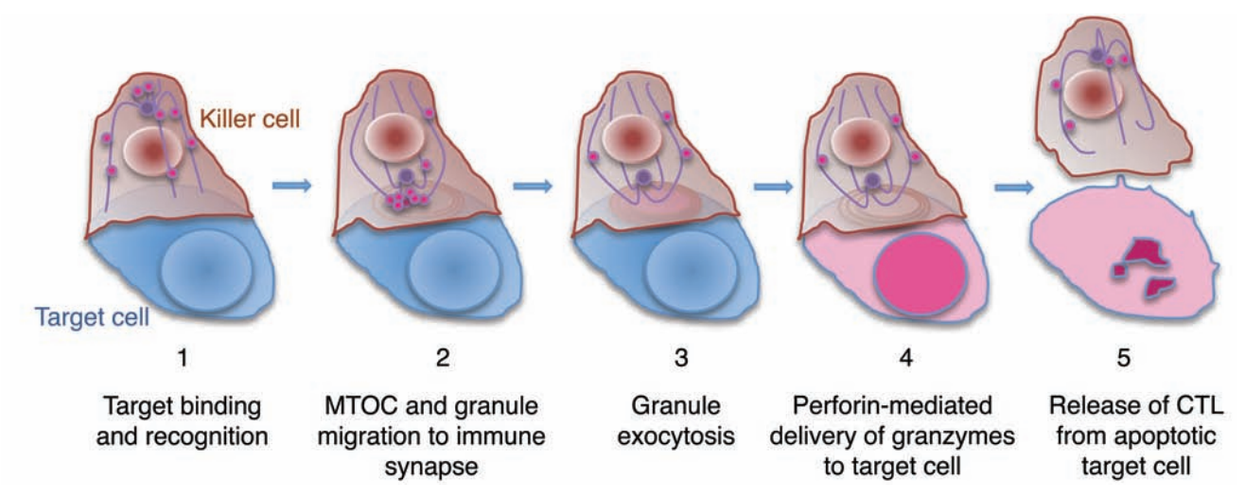

FIG. 37.3. Steps in Granule-Mediated Cytotoxicity. After the killer cell recognizes a target cell (1), an immune synapse is formed at the interface and the microtubule organizing center moves to the synapse, reorganizing the microtubule network (2). Cytotoxic granules move along microtubules to dock at the killer cell membrane in the c-SMAC of the immune synapse. Granule membranes fuse with the killer cell plasma membrane, releasing their contents (*magenta*) into the immune synapse (3). Perforin delivers the granzymes into the cytosol of target cells (4) where they initiate apoptotic death (5). The granzymes concentrate in the nucleus of target cells. The killer cell then detaches from the dying cell and is free to seek out additional targets.

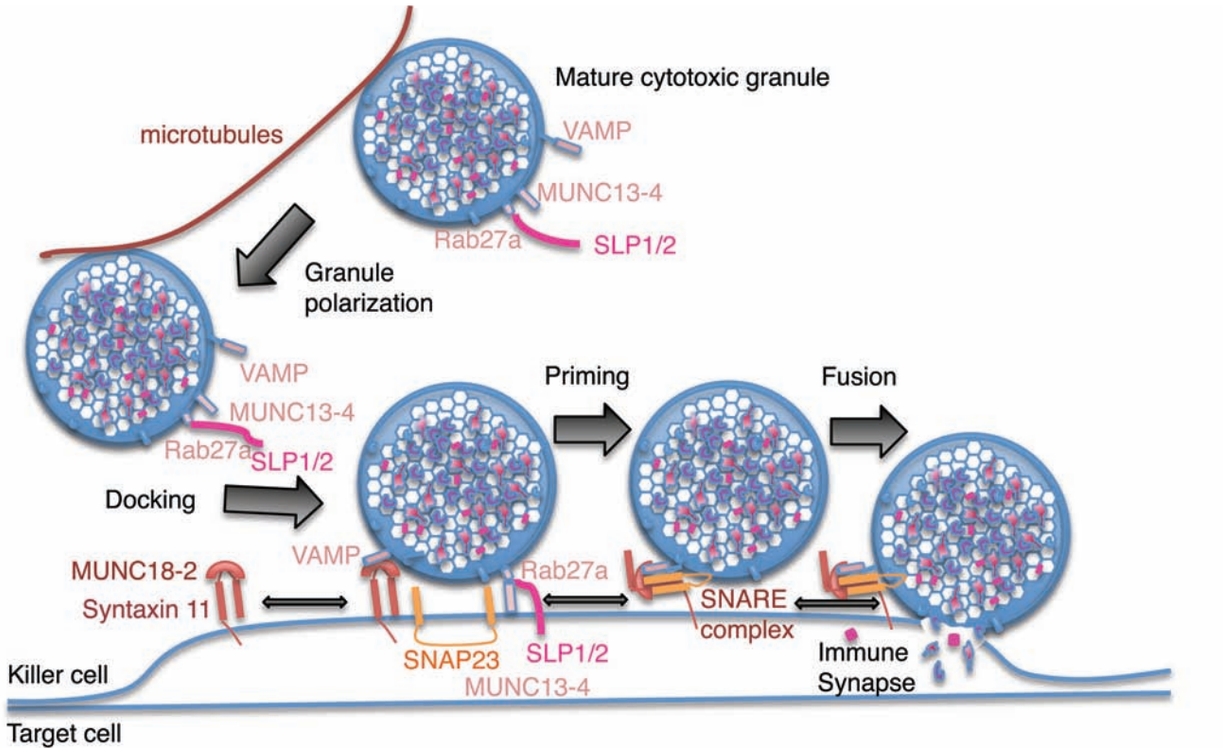

FIG. 37.4. Model of Granule Exocytosis. In response to antigen recognition, the mature cytotoxic granule moves along microtubules, probably with assistance from the actin-myosin cytoskeleton (*not shown*), to dock at the cell membrane at the immune synapse. A cytotoxic granule vesicle-associated soluble N-ethylmaleimide-sensitive factor accessory (SNARE) complex component (VAMP) protein binds to MUNC18-2, which is associated with plasma membrane syntaxin 11. Cytotoxic granule proteins Rab27a and Munc-13-4, in association with a synaptotagmin SLP1 or SLP2, help anchor the granule to the membrane. A SNARE complex forms between plasma membrane SNAP23 and Syntaxin 11 and granule membrane VAMP to initiate fusion of the granule membrane to the plasma membrane. Following membrane fusion, the cytotoxic granule contents are released into the immune synapse. After fusion, granule membrane-associated cathepsin B (*not shown*) is displayed on the killer cell membrane and protects it from perforin membrane damage. Figure adapted from de Saint Basile et al.[7]

ing of Rab27a on the cytosolic side of the mature granule membrane with synaptotagmin-like proteins, SLP1 or SLP2, which are anchored in the cell membrane. Docked granules are then primed for fusion by the interaction of Munc13-4 on their surface with syntaxin 11 on the killer cell membrane. This triggers the formation of a SNARE complex, the molecular machine for granule membrane fusion, between a cytotoxic granule vesicle-associated SNARE complex component (VAMP) protein with syntaxin 11 and SNAP23 on the cell membrane. Of the seven human VAMP proteins, studies in cytotoxic T cells have suggested that VAMP8 is required, while in NK cells both VAMP4 and VAMP7 are needed for different steps leading to granule exocytosis.[109,110] Granule membrane fusion also requires participation of Munc18-2 to trigger the conformational activation of the SNARE complex. Although the general mechanism of granule exocytosis described previously is used by all killer cells, some of the details of granule trafficking and fusion at the synapse may differ between killer T cells and NK cells (although apparent differences may disappear when the same high resolution techniques are applied to both types of killer cells). Cytotoxicity and granule fusion may occur even in the absence of a stable synapse.[111]

Genetic Diseases Caused by Defects in Perforin or Granule Exocytosis

Inherited deficiencies in perforin or the genes encoding syntaxin 11, Munc13-4, and Munc18-2 that orchestrate cytotoxic granule trafficking and release are linked to defective cytotoxicity and profound immunodeficiency.[112–118] Patients with mutations in these genes develop the familial hemophagocytic lymphohistocytosis (FHL) syndrome. These patients are handicapped in controling viral infections and develop a severe immune activation syndrome that is often fatal in childhood unless treated with bone marrow transplantation. Some patients with milder perforin mutations that do not completely eliminate cytotoxic function are not diagnosed until adulthood. These adult patients with FHL not only have impaired antiviral immunity, but are also more prone to develop lymphoma (like perforin-deficient mice). The most prominent and sometimes fatal clinical manifestation of FHL is an inflammatory syndrome caused by uncontrolled activation and expansion of CD8 T cells, often in response to poorly controlled herpesvirus infections, that leads to systemic activation of macrophages, which infiltrate tissues and overproduce proinflammatory cytokines. Macrophage activation is driven by excessive production of IFNγ by activated CD8 T

cells.[10,119] Sequencing of perforin mutations in patients with FHL has identified nonsense, frameshift, and missense mutations that disrupt perforin synthesis, folding, or activity.[5,120] The importance of some of these have been validated by testing cytolytic function of rat basophilic leukemia cells engineered to express mutant perforin and granzyme B. Defects in genes encoding the AP3 adaptor, needed to shuttle cargo from the Golgi to secretory lysosomes, the lysosomal trafficking regulator LYST, or Rab27 lead to human syndromes (Hermansky-Pudlak syndrome type 2, Chediak-Higashi syndrome, and Griscelli syndrome type 2, respectively) and corresponding mouse models (*pearl*, *beige*, and *ashen* mice, respectively) in which cytotoxicity as well as other processes involving secretory lysosomes are defective. In fact, mice and humans with defects in these genes have defects in pigmentation due to defective melanosome transport.

Lessons from Knockout Mice

Mice genetically deficient for granzymes A, B (and the granzyme B cluster), and M, and perforin provide important tools for probing the importance of these effector molecules in immune defense.[1] Perforin-deficient mice[112] closely recapitulate the symptoms of humans with genetic perforin deficiency. They are severely immunodeficient and compromised in their ability to defend against viruses and tumors, and develop the inflammatory syndrome of FHL when infected with mouse cytomegalovirus.[121] Mice deficient in any 1 of the 10 granzymes, or even of the granzyme B cluster, only have subtle differences compared to wild-type animals. These experiments highlight the functional redundancy of the granzymes. While only one molecule (perforin) effectively delivers the granzymes into target cells, each of the granzymes can trigger cell death. However, target cells may be selectively resistant to one or another of the granzymes (ie, by bcl-2 overexpression or by expression of viral serpins). Requirements for a single granzyme have been shown in some cases by specific immune challenges. For example, granzyme A–deficient mice are more susceptible to the poxvirus ectromelia[122] and granzyme B–deficient mice have less GvHD.[123] In constructing genetically deficient mice, genetic alterations of one gene can affect the expression of nearby granzyme genes. In the original granzyme B knockout mice, the PGK-neo cassette remaining in the granzyme B locus impedes the expression of other granzyme cluster genes (granzymes C, D, and F). The granzyme B gene has also been deleted keeping the expression of granzymes C, D, and F intact.[124] Cytotoxic T cells from the granzyme B–specific deletion mouse are significantly more effective at inducing apoptosis than those from the granzyme B–cluster knockout animal, underlining the importance of the other granzyme B cluster granzymes, especially when granzyme B is absent.

Because granzyme A and granzyme B are the most abundant granzymes in T cells, granzyme A/B doubly deficient mice are more immunodeficient than the single knockouts.[125–127] CTLs from granzyme A/B–deficient mice, although somewhat impaired in cytotoxicity relative to wild-type cells, nonetheless largely retain the ability to kill target cells.[128–130] However, the timing of key molecular events during apoptosis, such as externalization of phosphatidylserine (annexin V staining), is delayed during cell death induced by granzyme A/B–deficient CTLs versus wild-type CTLs.[131] Cytotoxic T cells lacking granzyme A and granzyme B induce a modified form of cell death that seems morphologically distinct from either perforin-mediated necrosis or wild-type CTL-mediated apoptosis.[131] Granzyme A/B–deficient animals do not develop spontaneous tumors and clear many viruses normally. The likely explanation of these results is that the other "orphan" granzymes (particularly H/C, K, and M),[132–134] substitute for granzyme A and granzyme B.

Although granzyme M is highly expressed in innate immune killer cells, including NK cells, NKT cells, and γδ T cells, granzyme M–deficient mice have normal NK and T cell numbers and NK activity against tumors.[135] Defense against the mouse poxvirus ectromelia and implanted NK-sensitive tumors is unimpaired in granzyme M–deficient mice compared to wild-type mice. Deficient mice are somewhat impaired in responding to mouse cytomegalovirus infection as they have higher viral levels, but they are eventually able to clear the infection. Thus granzyme M does not appear to be essential for NK cell–mediated cytotoxicity.

Perforin Delivery of Cytotoxic Molecules into Target Cells

When the granule membrane fuses with the killer cell membrane, the granule contents are released into the synapse. Granzymes and perforin probably dissociate from serglycin in the immune synapse before they enter target cells.[136] Granzymes bind to the target cell membrane by electrostatic interactions (granzymes are very positively charged and the cell surface is negatively charged)[137–139] and also by specific receptors, such as the cation-independent mannose-6-phosphate receptor.[140] However, specific receptors are not required for binding, internalization, or cytotoxicity.[137,139,141,142] The lack of a receptor enables all types of cells to be eliminated and limits escape from immune surveillance. The granzymes are delivered into the target cell (but not the killer cell) by perforin, where they initiate at least three distinct pathways of programmed cell death. While perforin is essential for granule-mediated cytotoxicity to deliver the granzymes into cells, the granzymes are redundant, as each granzyme can independently activate cell death. Although genetic deficiency of one or a few granzymes does not lead to severe immunodeficiency, mice lacking one or another granzyme display subtle differences in their ability to control specific viral infections. Why are there so many granzymes? The immune system needs to contend with a wide variety of tumors and infections, some of which have elaborated strategies to evade apoptosis and immune destruction. Some of the granzymes may have evolved to disarm specific intracellular pathogens. The interplay between granzyme B and granzyme H and adenovirus illustrates how multiple granzymes may have evolved to eliminate important pathogens.[143–145] Although both enzymes can cleave and inactivate at least two adenoviral proteins, the virus has also developed a way of inactivating granzyme B. Granzyme H potentiates the effect of granzyme B by destroying an adenoviral granzyme B inhibitor.

Perforin delivers granzymes and other effector molecules into the target cell cytosol[146,147] (Fig. 37.5). At high concentra-

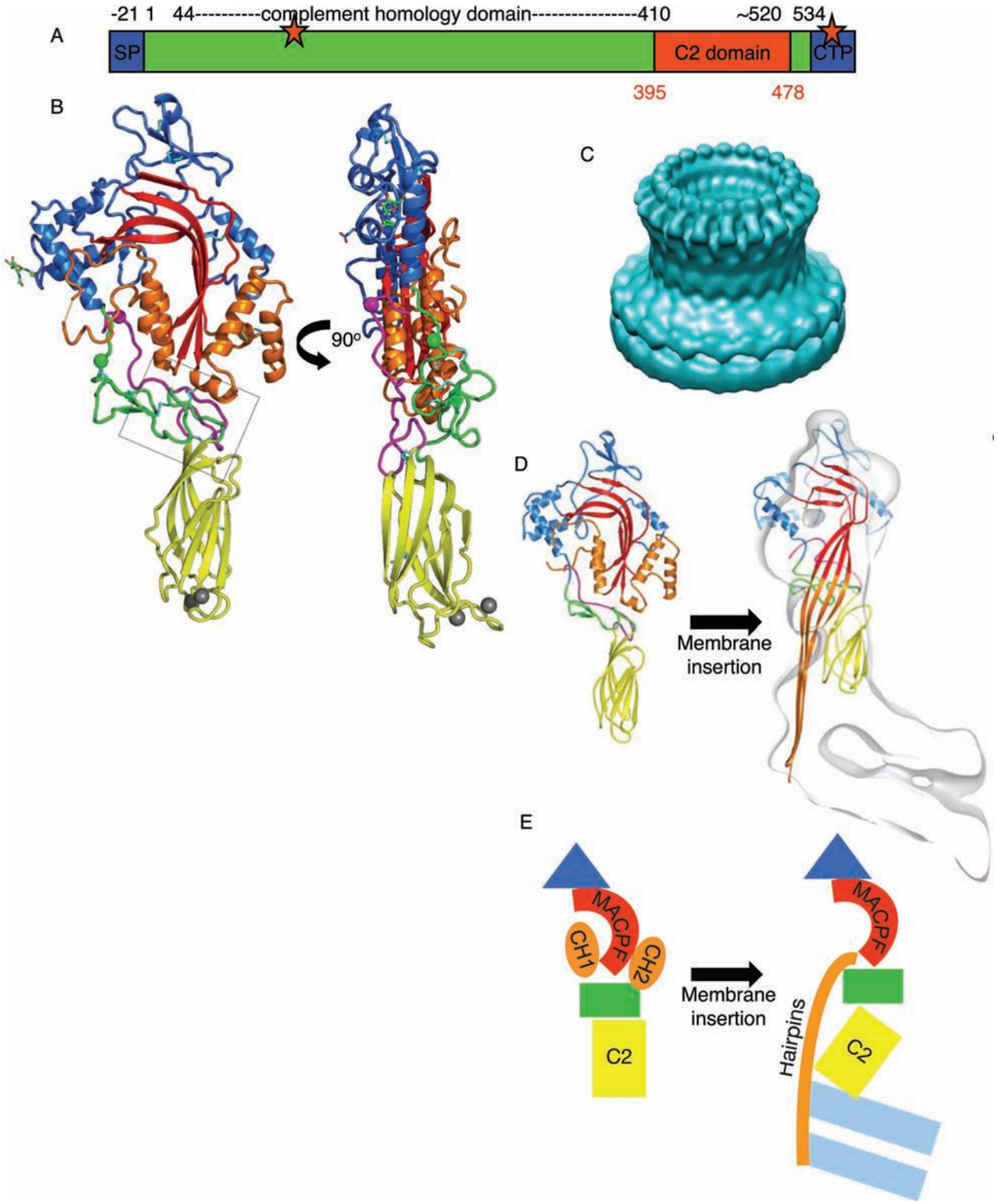

FIG. 37.5. Perforin. **A:** Perforin is synthesized with a leader peptide, complement homology domain (composed of a complement membrane attack complex/perforin [MACPF] joined to two α-helical domains [CH1, CH2] and an epidermal growth factor [EGF] domain), linked to a C2 membrane binding domain, followed by a C-terminal peptide. Glycosylation sites are indicated by *stars*. **B:** Crystal structure of the perforin monomer, color coded with the MACPF domain in *red*, the CH1 and CH2 helices in *orange*, the EGF domain in *green*, and the C2 domain in *yellow*. The *grey balls* indicate calcium binding to the C2 domain in the crystal structure. **C:** Cryoelectron microscopy reconstruction of the large perforin pore. **D,E:** Model of perforin conformational change induced by membrane binding of the C2 domain to form a multimerized pore based on the structure of the monomer and the reconstruction of perforin pore densities. Domains in **(E)** are color coded as in **(B)**. *Blue* indicates the membrane. This figure is adapted with permission from Law RH, Lukoyanova N, Voskoboinik I, et al. The structural basis for membrane binding and pore formation by lymphocyte perforin. *Nature*. 2010;468:447–451.

tions, perforin multimerizes in a cholesterol- and calcium-dependent manner in the plasma membrane of cells to form 5- to 30-nm pores.[148–152] Recent crosslinking and biophysical studies suggest that perforin may form at least two types of pores in membranes: small pores composed of about seven monomers that are not stable and much larger stable pores.[153,154] Cryoelectron microscopy reconstructions suggest that the large pores are composed of approximately 19 to 24 subunits and have a lumen large enough for granzyme monomers or granzyme A dimers to readily pass through. The precursor of human perforin is a 555 amino acid protein synthesized with a 21 amino acid leader sequence. The N terminal region of the mature 67 kDa protein (residues 44-410 of the human protein) is homologous to domains in complement proteins C6, C7, C8a, C8b, and C9 that form the complement membrane attack complex (MAC). The crystal structure of monomeric mouse perforin was recently solved.[152] The complement homology domain, termed the MAC/perforin domain (MACPF), is similar in structure to that of bacterial pore-forming cholesterol-dependent cytolysins, although they insert into membranes in opposite orientations. The MACPF domain of perforin is followed by an epidermal growth factor (EGF)-like domain; a C2 domain, a domain present in synaptotagmins and other calcium-dependent proteins, which becomes able to bind to lipid membranes after a conformational change in response to calcium; and a short 12 amino acid C-terminal peptide. The docking of the calcium-bound C2 domain is the first step in pore formation. Docking likely triggers both multimerization and a major conformational change in which two clusters of α-helices in the MACPF domain jackknife into the membrane. It is unclear whether multimerization to form a pore occurs before or after this conformational change. Perforin is glycosylated at two sites: one in the MACPF domain and one in the C-terminal peptide. Glycosylation of at least one site is needed for targeting perforin to cytotoxic granules, probably via binding of the glycan to the mannose-6-phosphate receptor.[155] En route to or in the granule, the glycosylated C-terminal peptide is removed from human (but not mouse) perforin by an undefined cysteine protease to produce the mature active protein.[156]

The original model for how perforin delivers granzymes into cells was that granzymes entered cells through perforin pores in the target cell plasma membrane. This model predicts that granzymes directly pass and disperse into the target cell cytosol. However, granzymes do not directly enter the cytosol, but instead are first endocytosed into clathrin-coated vesicles and transported to endosomes.[154,157,158] Thus the original model is not correct. Current data suggest that perforin indeed forms target cell plasma membrane pores, but these pores are small and transient (Fig. 37.6). However, calcium flows into the target cell through these pores and remains elevated for a few minutes. Because intracellular calcium is low in cells with an intact cell membrane, the cell senses a calcium influx as a sign of disruption of the plasma membrane. The elevated calcium triggers a cellular membrane damage response (also known as cellular wound healing) in which intracellular vesicles move to the plasma membrane and fuse their membranes to patch holes and any damaged membrane is rapidly removed and internalized into endosomes.[158–161] At the same time, membrane-bound granzymes, granulysin, and perforin are endocytosed. Elevated cytosolic calcium activates endosomal fusion, and granzyme- and perforin-containing endosomes fuse to form giant endosomes approximately 10 times larger than normal endosomes that have been termed gigantosomes. In the endosomal membrane, perforin forms larger and more stable pores through which granzymes begin to leak out into the cytosol. About 15 minutes after cell death has been triggered, the gigantosomes rupture, releasing any remaining cargo to the cytosol where they begin to activate programmed cell death. When the membrane repair response is inhibited, because the cell remains leaky, target cells die by necrosis, instead of by slower, regulated and energy-dependent programmed cell death.

Although perforin is the major molecule responsible for granzyme delivery, under some circumstances other molecules might serve that function. For example, bacterial and viral endosomolysins can substitute for perforin in vitro (and are widely used as laboratory reagents for intracellular delivery[162]) and potentially might play a similar role in vivo. The heat shock protein hsp70, which chaperones some peptides across cell membranes, can also carry granzyme B (and presumably other granyzmes) into cells.[163] Hsp70 is on the surface of some stressed cells or tumor cells and might help to remove these cells from the body.

Programmed Cell Death Pathways Activated by Granzymes

Once in the cytosol, the granzymes independently activate several parallel pathways of programmed cell death[6] (Table 37.1). Granzyme B cleaves and activates the caspases and also directly cleaves many important caspase substrates. Granzyme B can activate cell death that mimics caspase activation, even when the caspases are inhibited or in cells in which the caspase mitochondrial pathway is deficient. Granzyme A activates a distinct programmed cell death pathway that does not involve the caspases or disrupt the mitochondrial outer membrane. The substrates of the two major granzymes are largely nonoverlapping. The exceptions, lamin B and PARP-1, may indicate common features needed for cells undergoing all forms of programmed cell death, such as disruption of the nuclear membrane, inhibition of deoxyribonucleic acid (DNA) repair, or maintaining cellular adenosine triphosphate levels. What is known about cell death executed by the other (so-called orphan) granzymes is briefly described in the following. The orphan granzymes may be more highly expressed under conditions of prolonged immune activation.[164] The orphan granzymes are functionally important as mice genetically deficient in the whole granzyme B cluster are less efficient at clearing allogeneic tumors than mice deficient in just granzyme B.[124] Although some key granzyme proteolytic substrates are in the cytosol (ie, Bid, caspase-3, and ICAD for granzyme B), other important targets are in other membrane-bound cellular compartments, especially the nucleus and mitochondrion. In the cytosol, granzymes B, H, and possibly K also directly cleave the proapoptotic BH3-only BCL2

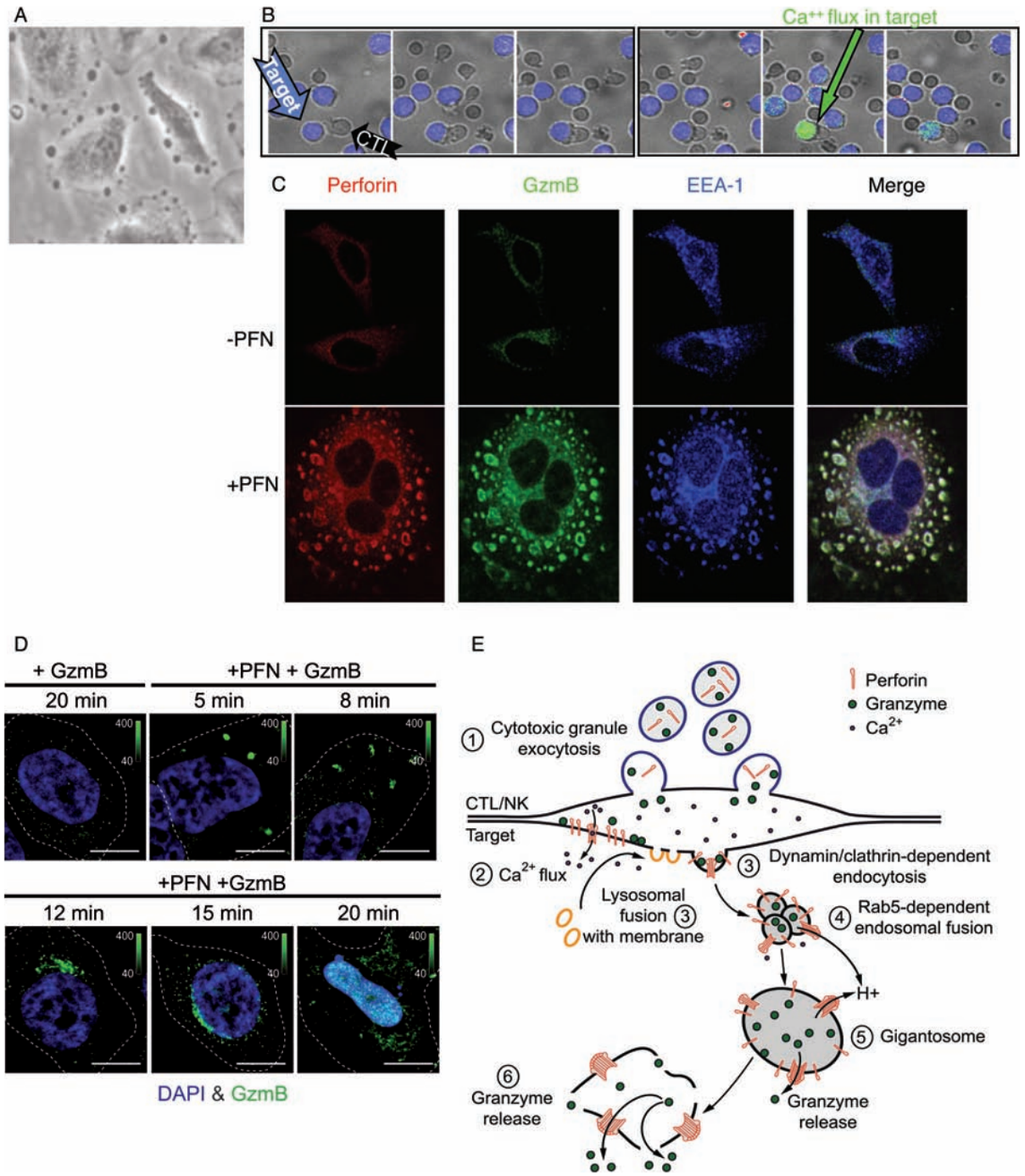

FIG 37.6. Current Model of Perforin Delivery of Granzymes into the Target Cell. A: Perforin treatment of HeLa cells causes dramatic membrane perturbation and blebbing. **B:** Killer cell degranulation causes a transient calcium influx in target cells that persists for a few minutes. In this experiment from Keefe et al.,[157] PHA-activated human cytotoxic T-lymphocytes were incubated with Fura-2-loaded, anti–cluster of differentiation 3-coated U937 cells and images were obtained every 30 seconds. The Fura-2 indicator dye is *blue* when calcium is low and *green* when it is elevated. **C:** Perforin and granzyme B are endocytosed into giant EEA-1–staining endosomes (image courtesy of Jerome Thiery). **D:** When HeLa cells are treated with perforin and granzyme B, within 5 minutes, granzyme B (*green*) concentrates in gigantosomes and is released beginning after about 12 minutes. The released granzyme concentrates in the target cell nucleus. **E:** Model for perforin delivery. After cytotoxic granule exocytosis into the immunological synapse (*1*), perforin multimerizes in the target-cell membrane to form small transient pores through which calcium enters (*2*), triggering a plasma membrane repair response (*3*) in which lysosomes fuse with the damaged plasma membrane and perforin and granzymes are rapidly internalized by endocytosis. Perforin and granzyme-containing endosomes then fuse in response to the transient calcium flux (*4*) to form gigantosomes. Within gigantosomes, perforin continues to multimerize to form larger pores, preventing acidification and causing some granzyme release (*5*), before inducing endosomal rupture and complete granzyme release into the target-cell cytoplasm (*6*). **(D)** and **(E)** are reprinted from Thiery J, Keefe D, Boulant S, et al. Perforin pores in the endosomal membrane trigger the release of endocytosed granzyme B into the cytosol of target cells. *Nat Immunol.* 2011;12:770–777.

TABLE 37.1 Features of the Distinct Cell Death Pathways Induced by the Granzymes

Granzyme	A	B	C/H	K	M
Expression					
Cytolytic CD8 T cells	++	++	+	+	+/–
Cytolytic CD4 T cells	+	+			
CD4 T_{reg}s	–	+			
NK cells	+	+/–			++
Myeloid cells	–	+			
Common features					
Rapid loss of membrane integrity	+	+	+	+	?
Annexin V staining	+	+	+	+	?
Chromatin condensation	+	+	+	+	?
DNA damage	+	+	+	+	?
Mitochondrial depolarization	+	+	+	+	?
Caspase activation	–	+	–	–	?
Type of DNA damage					
Oligonucleosomal DNA fragmentation	–	+	–	–	?
Single-stranded DNA nicks	+	–	+	+	–
TdT labeling	+	+	+	+	?
Klenow labeling	+	+	+	+	–?
Type of mitochondrial damage					
Inhibition by bcl-2 overexpression	–	+	?	?	?
Cytochrome c release	–	+	+?	?	?
Mitochondrial swelling	+	+	++	+	+
Autophagy	–	–	–	–	+?

CD, cluster of differentiation; DNA, deoxyribonucleic acid; NK, natural killer; TdT, terminal deoxynucleotidyl transferase; T_{reg}, regulatory T.
Table modified from Chowdury and Lieberman.[6]

family member Bid to initiate the classical mitochondrial apoptotic pathway that leads to mitochondrial outer membrane permeabilization (MOMP) and release of cytochrome c and other proapoptotic proteins from the intermembrane space.[165–171] Granzymes A and B (and possibly other granzymes) enter mitochondria through an unknown mechanism to cleave important substrates including electron transport proteins.[172–174] Granzyme C (in mice) and granzyme H (in humans) activate caspase-independent cell death with a pronounced mitochondrial phenotype. All of these events cause mitochondrial depolarization and production of superoxide anions and other reactive oxygen species, which is a key first step in killer lymphocyte–mediated death, as superoxide scavengers block granzyme-mediated cell death.[173] Granzyme A and granzyme B rapidly translocate to and concentrate in the nucleus,[175,176] where proteolytic cleavage of key substrates is important to induce programmed cell death by both granzyme A (SET, Ape1, lamins, histones, Ku70, PARP1) and granzyme B (lamin B, PARP1, NuMa, DNA-PK_{cs}). Nuclear translocation of the granzymes may be mediated by importin-α.[177]

Granzyme A

Granzyme A induces caspase-independent cell death, which is morphologically indistinguishable from apoptosis[178–180] (Fig. 37.7). Granzyme A is the most ancient of the granzymes; tryptases homologous to granzyme A are found in cytotoxic cells in bony fish.[181] Granzyme A was the first granzyme described and is the most widely expressed. Cells treated with granzyme A and perforin die rapidly: within minutes they undergo membrane blebbing and have evidence of mitochondrial dysfunction (increased reactive oxygen species, loss of mitochondrial transmembrane potential [$\Delta\Psi_m$], disruption of mitochondrial morphology).[172,173] Within half an hour, externalization of phosphatidyl serine (measured by annexin V staining) occurs, and DNA damage, chromatin condensation, and nuclear fragmentation become apparent within 1 to 2 hours. DNA is damaged by single-stranded cuts into megabase fragments that are much larger than the oligonucleosomal fragments generated during caspase or granzyme B–activated cell death.[182] Because the DNA fragments are too large to be released from the nucleus, assays that measure DNA release into culture supernatants are typically negative. Mitochondria are damaged without MOMP or release of proapoptotic mediators, such as cytochrome c, from the mitochondrial intermembrane space.[173] In mitochondria, granzyme A cleaves Ndufs3 in electron transport chain complex I to interfere with mitochondrial redox function, adenosine triphosphate generation and maintenance of $\Delta\Psi_m$ and to generate superoxide anion.[129,172,173] The superoxide generated

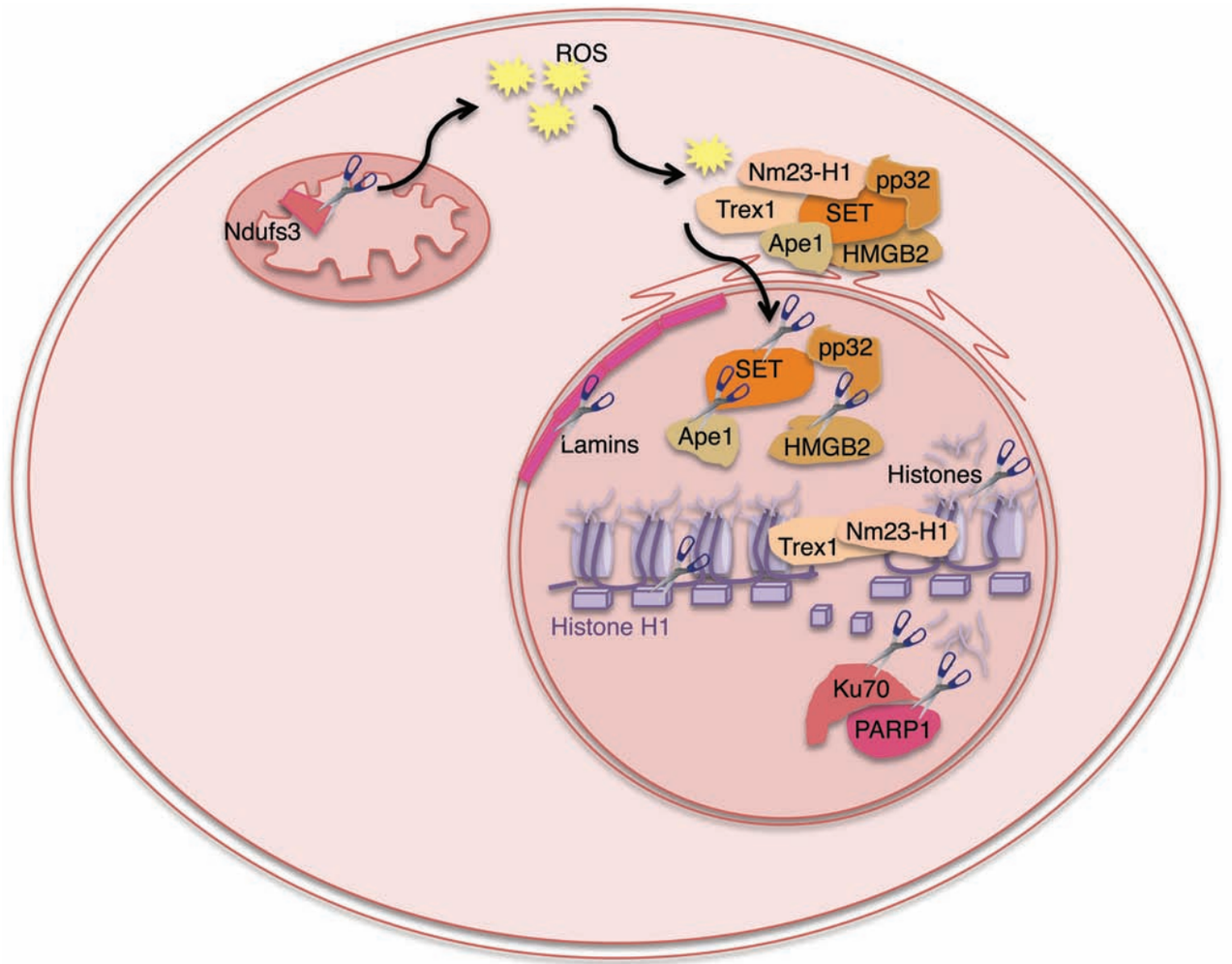

FIG 37.7. The Granzyme A Pathway of Cell Death. Reactive oxygen species generated by granzyme A (represented by *scissors*) cleavage of Ndufs3 in electron transport complex I in mitochondria drives the endoplasmic reticulum–associated SET complex into the nucleus. Granzyme A enters the nucleus by an unknown pathway. In the nucleus, Granzyme A cleaves three components of the SET complex (SET, HMGB2, and Ape1) to activate two nucleases in the complex to make single-stranded deoxyribonucleic acid (DNA) lesions; NM23-H1 makes a nick, which is extended by the exonuclease TREX1. Granzyme A also degrades the lamins and the linker histone H1 and removes the tails from the core histones, opening up chromatin and making it more accessible to these nucleases. DNA repair proteins Ku70 and PARP1 are also targets.

by damaged mitochondria drives an endoplasmic reticulum (ER)-associated oxidative stress response complex, called the SET complex, into the nucleus where it plays a critical role in granzyme A–induced nuclear damage.[173,182] The SET complex contains three nucleases (the base excision repair endonuclease Ape1, an endonuclease NM23-H1, and a 5′-3′ exonuclease Trex1), the chromatin modifying proteins SET and pp32, which are also inhibitors of the tumor suppressor protein phosphatase 2A, and a DNA binding protein that recognizes distorted DNA, HMGB2.[183–187] One of the normal functions of the complex is to repair abasic sites in DNA generated by oxidative damage. Recent studies also implicate the cytosolic SET complex as binding to the human immunodeficiency virus preintegration complex and facilitating human immunodeficiency virus infection.[188] The SET complex exonuclease Trex1 digests cytosolic DNA produced by endogenous retroviruses and infectious viruses to inhibit the innate immune response to cytosolic DNA.[189–192] Mutations in *TREX1* that inactivate its nuclease activity or cause its mislocalization are linked to human inflammatory and autoimmune diseases, including Aicardi-Goutiere syndrome and systemic lupus erythematosis.[193–197] Granzyme A, which traffics to the nucleus by an unknown mechanism, converts this DNA repair complex into an engine for DNA destruction by cleaving SET, an inhibitor of the endonuclease NM23-H1.[185] This allows NM23-H1 to nick DNA; the exonuclease Trex1 then extends the break.[184] At the same time, granzyme A cleaves and inactivates HMGB2 and Ape1 to interfere with base excision repair.[186,187] In addition to disabling base excision repair, granzyme A also interferes with DNA repair more generally by interfering with the recognition of damaged DNA by cleaving and inactivating Ku70[198] and PARP-1.[199] Within the

nucleus, granzyme A also opens up chromatin by cleaving the linker histone H1 and removing the tails from the core histones, making DNA more accessible to any nuclease, and disrupts the nuclear envelope by cleaving lamins.[200,201]

Granzyme B

Granzyme B is unique among serine proteases because it cleaves after aspartic acid residues, like the caspases[202,203] (Fig. 37.8). It induces target cell apoptosis by activating the caspases, particularly the key executioner caspase, caspase-3.[204,205] Human granzyme B, but not the mouse enzyme, also activates cell death by directly cleaving the key caspase substrates, Bid and ICAD, to activate the same mitochondrial and DNA damage pathways, respectively, as the caspases.[92,166–168,206–208] As a consequence, caspase inhibitors have little effect on human granzyme B–mediated cell death and DNA fragmentation, while the same inhibitors significantly block the action of the mouse enzyme. Thus, human CTLs and NK cells may be more effective than mouse killer cells at eradicating virus-infected cells or tumors that have developed methods for evading the caspases. Both human and mouse enzymes cleave many of the same substrates as the caspases (including PARP-1, lamin B, NuMa, DNA-PK_{cs}, tubulin) and have substrate specificity close to that of caspases-6, -8, and -9.[209] However, human granzyme B cleaves optimally after the tetrapeptide IEPD, while mouse granzyme B has somewhat different peptide specificity, preferring to cleave after IEFD.[92,206] Moreover, other regions including the P' region (C-terminal to the cleavage site) and more distal regions contribute to substrate specificity. Because of subtle

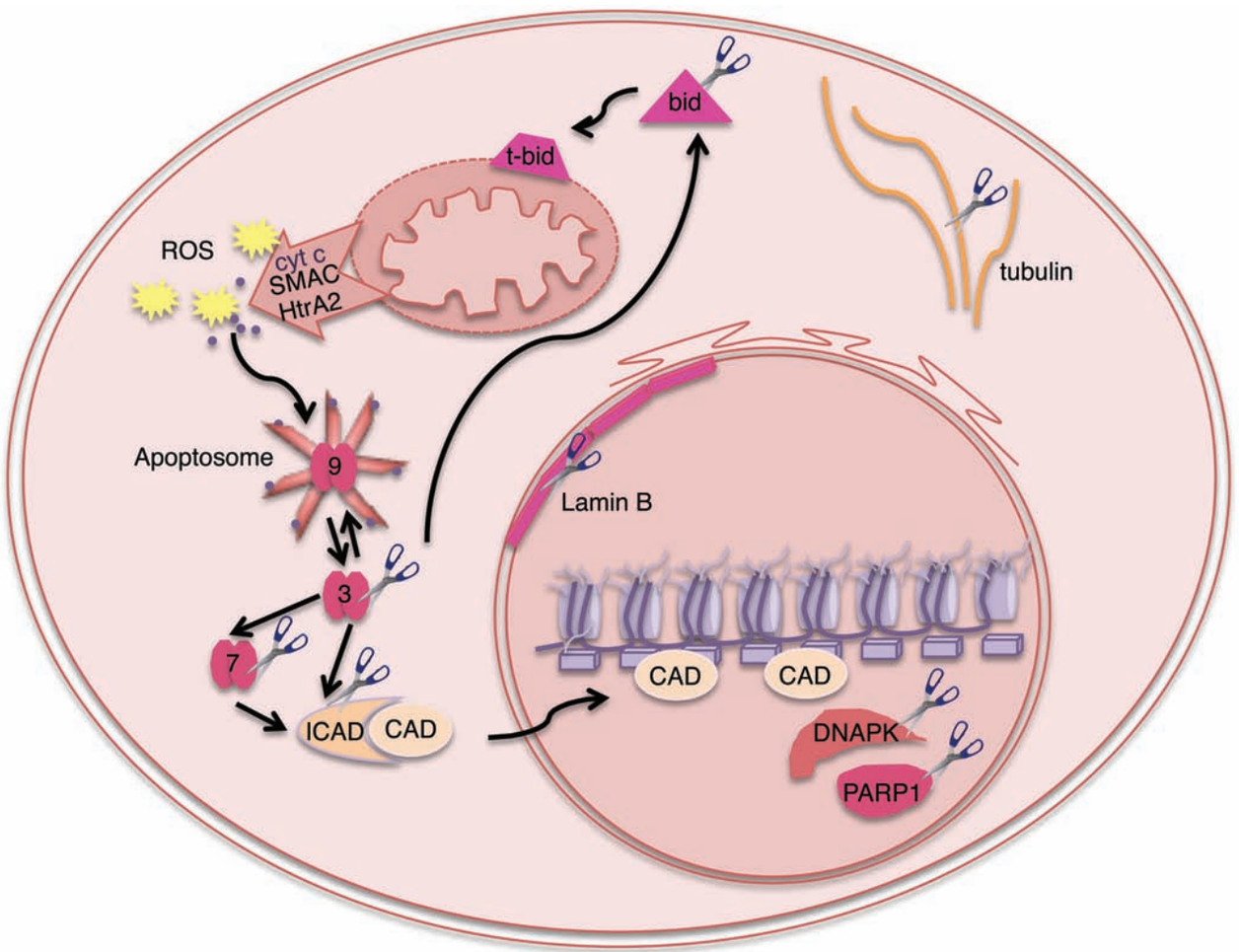

FIG 37.8. The Granzyme B Pathway of Cell Death. Human granzyme B (represented by *scissors*) cleaves bid to produce truncated bid (t-bid), which initiates the classical mitochondrial apoptotic pathway characterized by reactive oxygen species generation, loss of the mitochondrial transmembrane potential, and disruption of the mitochondrial outer membrane to release proapoptotic mediators in the intermembrane space, including cytochrome c (*blue balls*, cyt c), SMAC/Diablo, and HtrA2/Omi. Cytochrome c binds to Apaf-1 to form the apoptosome that activates caspase-9. Caspase-9 activates caspase-3 and the apoptotic cascade that includes cleavage of ICAD, the inhibitor of the caspase-activated DNase (CAD), allowing CAD to enter the nucleus and make oligosomal DNA double strand breaks. Human granzyme B on its own can directly activate caspase-3 and some key downstream caspase targets, including ICAD, lamin B, tubulin, and the DNA repair proteins DNA-PK_{cs} and PARP1. Mouse granzyme B lacks direct proteolytic activity on some important substrates (see text).

differences in sequence, the human and mouse granzyme B can differ in important ways with respect to their substrates and the efficiency with which they are cleaved.

The granzyme B (and caspase) mitochondrial pathway leads to reactive oxygen species (ROS) generation, dissipation of $\Delta\Psi_m$, and MOMP, with release of cytochrome c and other proapoptotic molecules from the mitochondrial intermembrane space. Human granzyme B activates this pathway directly by cleaving Bid, while mouse granzyme B activates it indirectly. However, granzyme B targets mitochondria in other ways, including by cleaving antiapoptotic Mcl-1 and Hax-1, a protein that helps maintain the mitochondrial transmembrane potential.[174,210] Loss of $\Delta\Psi_m$, but not cytochrome c release, occurs in the presence of pancaspase-inhibitors (even using mouse granzyme B) and in mice genetically deficient for Bid, Bax, and Bak (the latter two Bcl-2 family members are required for Bid-induced mitochondrial damage).[132,169,211,212] Granzyme B can also activate ROS by activating extramitochondrial nicotinamide adenine dinucleotide phosphate-oxidase.[213] DNA damage by granzyme B is mediated primarily by the activation of the caspase-activated DNase (CAD) following proteolytic cleavage of its inhibitor ICAD either directly by human granzyme B or indirectly by executioner caspases, such as caspase-3.

In humans, there is a common polymorphism of granzyme B in which three amino acids (Q^{48}, P^{88}, Y^{245}) are mutated to $R^{48}A^{88}H^{24}$.[214] This polymorphism does not seem to affect cytotoxicity and does not have any known clinical significance.[215]

Granzymes C and H

Mouse granzyme C and human granzyme H, homologous granzymes encoded downstream from granzyme B, are predicted to cleave after aromatic residues.[133,216] Granzyme H arose during primate evolution, independently of granzyme C, in an intergenic recombination event between granzyme B and a mast cell chymase.[217] Both induce caspase-independent death with hallmarks of programmed cell death: ROS generation, dissipation of $\Delta\Psi_m$, chromatin condensation, and nuclear fragmentation.[133,216] DNA destruction by granzyme C (and probably granzyme H as well) is via single-stranded nicks and does not involve CAD. Rapid mitochondrial swelling and disruption of mitochondrial ultrastructure are particularly striking in cells treated with granzyme C. The mitochondrial pathways activated by granzyme C and granzyme H may be different; granzyme C triggers cytochrome c release, a sign of MOMP, while granzyme H does not.[133,216]

Granzyme H cleaves two adenoviral proteins: a DNA binding protein (also a granzyme B substrate) and the adenovirus 100K assembly protein, a previously described inhibitor of granzyme B.[143,145] Cleavage of DNA binding protein interferes with viral DNA replication, while cleavage of 100K restores granzyme B function in adenovirus-infected cells. Granzyme H also cleaves the cellular La protein, an RNA binding protein that participates in the posttranscriptional processing of mRNAs transcribed by RNA polymerase III and some t-RNAs and viral RNAs.[218] Cleavage mislocalizes La from the nucleus and decreases translation of hepatitis C virus proteins. Therefore, granzyme H may play a special role in immune defense against certain viruses. Because granzyme H is expressed in NK cells, it may help eliminate these viruses early in infection, before adaptive immunity has had a chance to develop.

Granzyme K

Granzyme K is another tryptase in mice, rats, and humans that is encoded downstream near granzyme A on human 5q11-12 (or the syntenic region of mouse chromosome 13). It is much less expressed than granzyme A, and unlike granzyme A, is a monomer, not a dimer. Mice genetically deficient in granzyme A express granzyme K, which may explain the lack of a significant phenotype of granzyme A–/– mice, except when challenged with some viruses.[219,220] Purified rat and recombinant human granzyme K have been available for some time,[221,222] but little was known about its cell death activation until recently. Like granzyme A, purified rat granzyme K efficiently induces caspase-independent cell death, characterized by mitochondrial dysfunction without MOMP (ROS and loss of $\Delta\Psi_m$, but without cytochrome c release).[132] However, unlike granzyme A, rat granzyme K–induced cell death was originally reported to be inhibited in cells overexpressing Bcl-2.[132] This finding was surprising, as Bcl-2 inhibits MOMP, which leads to cytochrome c release, which was not detected in granzyme K–treated cells. In fact, a more recent study found that cell death by recombinant human granzyme K did not activate caspase-3 and was unaffected by caspase inhibitors or Bcl-x_L overexpression.[171] Granzyme K mimics granzyme A DNA damage[171]: it causes caspase-independent nuclear fragmentation and nuclear condensation and single-stranded DNA breaks by targeting the SET complex. Like granzyme A, granzyme K causes SET complex nuclear translocation and hydrolyzes and inactivates SET, Ape1, and HMGB2 in the SET complex.[171] Presumably, cleavage of SET, the inhibitor of NM23-H1, triggers DNA damage by the granzyme A–activated DNases, NM23-H1, and Trex1 in the SET complex.[184,185] The same group recently reported that granzyme K causes mitochondrial damage that includes not only ROS generation and dissipation of $\Delta\Psi_m$, but also Bid cleavage (to a fragment that appears to be the same size as is generated by granzyme B) and MOMP with release of cytochrome c and endoG.[171] This needs to be verified because rat granzyme K does not cause cytochrome c release,[132] and this same group showed that caspases are not activated by granzyme K and overexpression of Bcl-x_L does not interfere with human granzyme K–induced cell death,[171] as would be expected if MOMP is triggered. Although granzyme K appears to duplicate the nuclear damage pathway of granzyme A, further studies are needed to determine whether the mitochondrial granzyme K pathway resembles that activated by granzyme A (no MOMP) or granzyme B (Bid cleavage, MOMP), or is a hybrid of both. A proteomics analysis that compared granzyme A and granzyme K suggested that although the two enzymes share many substrates, some may be unique to granzyme K.[223] In fact, recent studies suggest that granzyme K cleaves and inactivates p53, which should interfere with cellular repair pathways, and interferes with the ER unfolded protein response by cleaving multiple components of the ER degradation complex.[224,225]

Granzyme M

Granzyme M is the most distinctive of the granzymes. It likely arose from a gene duplication of a neutrophil protease, as it is encoded near a cluster of other neutrophil proteases in human chromosome 19p13.3 (or a synteic region of mouse chromosome 10) and is slightly more homologous to one of them (complement factor D) than to the other granzymes.[226] Unlike the other granzymes, granzyme M cuts after Met or Leu.[227,228] None of the serine protease inhibitors that block the other granzymes, including the pangranzyme inhibitor 3,4-dichloroisocoumarin, effectively inhibit granzyme M.[229] Moreover, granzyme M appears to function primarily in innate immunity, as it is expressed mostly in NK cells and γδ T cells and only in the subset of CD56+ T cells.[230,231] Until recently, it was not clear whether granzyme M induces cell death.[134] Granzyme M−/− mice have unimpaired NK- and T-cell development and NK cell–mediated cytotoxicity, but are less able to defend against mouse cytomegalovirus infection.[135]

The literature does not agree about the type of cell death activated by granzyme M. Kelly et al., using recombinant human granzyme M expressed from baculovirus in insect cells, found that granzyme M induced rapid, caspase-independent cell death that looked like autophagic death and did not find evidence for DNA fragmentation, mitochondrial depolarization, phosphatidyl serine externalization, or caspase activation.[134] On the other hand, using human granzyme M expressed in yeast, the Fan laboratory argued that granzyme M activated caspase-dependent cell death, in part by cleaving and inactivating both the apoptosis inhibitor survivin and ICAD, with phosphatidyl serine externalization, caspase activation, CAD activation with oligonucleosomal DNA laddering, PARP cleavage, and mitochondrial disruption with MOMP (mitochondrial swelling, dissipation of $\Delta\Psi_m$, ROS generation, cytochrome c release).[232–234] This group also suggested that another granzyme M substrate may be TRAP75, a heat shock protein that inhibits granzyme M–induced ROS generation.[233] However, one aspect of this study that may not be completely consistent with what is known about granzyme M is that the Fan paper[232,233] claims that granzyme M cleaves ICAD after a Ser residue, while peptides containing Ser at the P1 site are not substrates of granzyme M expressed in yeast. Therefore, further work will be needed to determine whether granzyme M activates granzyme B–like caspase-dependent cell death or a novel pathway distinct from that activated by the other granzymes. The mouse and human isoforms may also have diffeent substrates.[235] Examining cell death induced by native purified granzyme M may be necessary to determine what type of cell death is induced by these enzymes. One intriguing other activity of granzyme M might be to cleave and inactivate the granzyme B serpin inhibitor Serpin B9 (PI-9), which it has been shown to do in vitro.[228] If this proves to be a physiologically relevant substrate in cells, then one function of granzyme M might be to potentiate the activity of granzyme B. Mice genetically deficient in granzyme M are more susceptible to cytomegalovirus infection, and granzyme M cleaves a cytomegalovirus structural protein and inhibits its replication.[236] Thus an important function of granzyme M may be to help protect us from this important human pathogen.

Granulysin

Human cytotoxic granules of cytotoxic T cells and NK cells also contain another effector molecule: the membrane perturbing saposin-like molecule granulysin.[237] The granulysin gene (*GNLY*) was first identified as a late activation gene expressed 3 to 5 days after T-cell activation, which coincides with the expression of the other cytotoxic effector molecule genes in naïve T cells.[238] Granulysin is synthesized as a 15 kDa protein that is cleaved at both ends to produce a 9 kDa peptide. Both forms can form membrane pores in membranes. The larger form is secreted by NK cells and cytotoxic T cells, while the 9 kDa form is stored and released from cytotoxic granules during NK-cell or cytotoxic T–cell attack. Granulysin preferentially disrupts bacterial membranes and has been postulated to play a role in immune elimination of bacteria, fungi, and parasites.[239–242] It may also have some antitumor activity, but this requires very high granulysin concentrations in vitro that may not be physiologically relevant. There is also some evidence that secreted granulysin can act as a chemoattractant for dendritic cells and other immune cells and can induce them to express proinflammatory cytokines.[243,244] Purified granulysin is only active as a cytotoxic agent against bacteria and other pathogens when experiments are performed under hypotonic or acidic conditions that are not found extracellularly. Thus, granulysin's membrane perturbing activity likely only operates within cells, perhaps to target intracellular bacteria and other pathogens located in acidic intracellular vesicles, such as phagolysosomes. Perforin is needed to deliver granulysins into target cells.[239,245,246] Understanding the importance of granulysin in antibacterial defense and immunopathology (it is overexpressed at sites of immune activation and in blistering skin diseases) will be facilitated by the recent generation of transgenic mice that express granulysin.[247]

How is the Killer Cell Protected from its Cytotoxic Molecules?

The killer cell is not injured by its own granules. It delivers the "kiss of death" and escapes the encounter with the cell targeted for elimination unharmed and then can find and destroy other targets. How the killer cell determines that it has killed its target and is ready to detach is unknown. Several mechanisms ensure that the killer molecules are inactive during protein synthesis, processing, and storage within the granule. Within the killer cell, the cytotoxic molecules are synthesized as proenzymes that are only processed to their active form within the granule. The granzymes and perforin are expressed with a signal sequence that directs them to the ER. The high concentration of calreticulin in the ER likely serves as a sink for free calcium, which prevents perforin activation.[248–251] Cleavage of the signal peptide of the granzymes produces an inactive proenzyme that contains an N-terminal dipeptide that needs to be removed to produce an active protease. During synthesis, perforin is also rapidly transported from the ER to the Golgi. This is facilitated by a conserved C-terminal tryptophan residue by an unknown mechanism.[155] Mutation of the terminal tryptophan leads to enhanced death of the killer cell. In the Golgi, mannose-6-phosphate–containing glycans are added

to progranzymes and perforin, which serve as sorting signals for transport to lysosomes.

Within the cytotoxic granule, the N-terminal dipeptide on all progranzymes is removed by cathepsin C (dipeptidyl peptidase I) to generate the active enzyme.[252] However, mice and humans genetically deficient in cathepsin C have only partially reduced granzyme activity and cytolytic function and modestly reduced immune defense against viral infection.[253,254] This suggests that alternate ways can activate progranzymes. In fact, IL-2 treatment stimulates cathepsin C–independent dipeptide cleavage in NK cells from patients with Papillon-Lefevre syndrome, who have loss of function of cathepsin C.[255] Cathepsin H and probably other cathepsins can process progranzyme B.[256] Granzymes, which are highly basic, are bound, as are perforin and presumably granulysin, to the acidic serglycin proteoglycan within the granule, which helps keep them inactive. Serglycin is responsible for the electron dense core and may also enhance effector protein storage in the granules.[257] Granzyme proteolytic activity and perforin pore formation is also negligible at the acidic pH (pH 5.1 to 5.4) of the granule.

Although granzyme and perforin trafficking within cytotoxic cells minimizes leakage of active death effector molecules out of granules, any stray molecules in the cytoplasm could cause cell death.[258] During granule exocytosis, some granzymes might inadvertently reenter effector cells. Because CTLs typically kill several targets in succession without harming themselves, an important question is how CTLs protect themselves from their own cytotoxic molecules. An important protective mechanism against killer cell suicide is serpin (serine proteinase inhibitor) expression in the killer cell cytoplasm.[259] Serpins are members of a superfamily of protease inhibitors with over 1500 family members.[260,261] Serpins inactivate their target proteases either by covalently and irreversibly binding to the active site of the enzyme or by forming noncovalent complexes that are so strong they resist the denaturing conditions of SDS-PAGE gel electrophoresis.[261,262] Serpins that inactivate granzyme B (SerpinB9, also known as PI-9, in human cells[263] or its ortholog Spi6 in mouse cytotoxic cells[264]) are expressed in killer cells. Mice genetically deficient in *Spi6* have reduced numbers of memory CD8 T cells, suggesting that CTL survival is compromised by their own granzyme B.[265] In fact, cytotoxic T cells from these mice show granule membrane instability and have increased cytosolic granzyme B and apoptosis.[266] However, no killer cell serpins are known that inactivate the other granzymes. A recent report suggests that SerpinB4 is a potent granzyme M inhibitor that may render some tumors resistant to granzyme M.[267] However, NK cells or activated killer lymphocytes are not known to express this serpin.

When perforin and granzymes are released into the immune synapse, why is killing unidirectional? How is the killer cell membrane protected from perforin damage? During granule exocytosis, the cytotoxic granule membrane fuses with the killer cell plasma membrane, exposing internal granule membrane–associated proteins. These include cathepsin B, which inactivates by proteolysis perforin redirected toward the killer cell.[8] However, killer cells genetically deficient in cathepsin B survive unscathed when they kill targets.[268] This suggests that other membrane-bound granule cathepsins (or perhaps other CTL surface proteases or other perforin inhibitors) might also inactivate perforin redirected at the killer cell.

Cellular Resistance to Granule-Mediated Death

The human granzyme B serpinB9 is not only expressed by lymphocytes[259,269] but also by dendritic cells,[270] cells at immune privileged sites (testis and placenta),[48,271,272] endothelial and mesothelial cells,[273] and mast cells.[274] Similar results hold for Spi-6 in mice.[264,265,275] Modulators of inflammation like LPS, IFNγ and IL-1β[276,277] induce *SERPINB9* AQ1 expression. SerpinB9 expression is enhanced by estrogen and hypoxia because of estrogen responsive elements and hypoxia inducible factor 2 binding sites, respectively, in its promoter.[278,279] In particular, serpinB9 is induced by hypoxia in neuroblastomas.[280] This expression pattern suggests that serpinB9 not only protects killer cells and myeloid cells that express granzyme B from autodestruction, but also may protect antigen-presenting cells, bystander cells at sites of inflammation, and cells in immune privileged sanctuaries from granzyme B–mediated killing. It may also be a mechanism for tumor evasion of immune surveillance.

No intracellular inhibitors of granzyme A have yet been identified. However, some trypsin inhibitors also inhibit granzyme A. Granzyme A is bound and irreversibly inhibited in the circulation by two trypsin inhibitors, α-2 macroglobulin and antithrombin III.[281] Extracellular granzyme A complexed to proteoglycans is resistant to these two protease inhibitors.[282] A recent study identified another granzyme A inhibitor, pancreatic secretory trypsin inhibitor, from pancreatic secretions.[283] Pancreatic secretory trypsin inhibitor is found in the blood, particularly in patients with severe inflammation and tissue destruction.[284,285] Unlike the other two granzyme A inhibitors, pancreatic secretory trypsin inhibitor inhibits granzyme A complexed to proteoglycans.[283] It is unclear whether any of these granzyme A inhibitors are expressed in cytotoxic lymphocytes.

Viral Granzyme Inhibitors

A number of viruses produce inhibitors of apoptosis or Bcl-2–like proteins that inhibit caspase-mediated apoptosis, which consequently also inhibit granzyme B–mediated cell death. The pox virus–encoded cytokine response modifier A gene (CrmA) inhibits granzyme B.[286] CrmA directly binds and inhibits granzyme B both in vitro and in vivo. Overexpression of CrmA in target cells inhibits CTL-mediated cell death. CrmA also strongly binds and inhibits caspases-1 and -8 and weakly inhibits other caspases like caspase-3.[287] Parainfluenza virus type 3 specifically inhibits granzyme B by degrading granzyme B mRNA in infected T cells.[288] Importantly, granzyme A transcripts are not affected by this virus. The mechanism of virus-mediated granzyme B mRNA decay is not known.

Human granzyme B is inhibited by the adenoviral assembly protein (Ad5-100K) by a unique "un-serpin"-like mechanism.[143] Ad5-100K rapidly complexes with granzyme B and gets cleaved very slowly at specific sites. Granzyme B that enters the infected target cell during killer cell attack is

saturated by the abundant Ad5-100K protein. Importantly, the slow kinetics of the cleavage reaction ensures that there is always a molar excess of Ad5-100K protein relative to granzyme B. Ad5-100K, which is also needed for virus assembly,[143] does not inhibit caspases or other apoptotic pathways.[144] In fact, the inhibitory activity of Ad5-100K is specific for human granzyme B and not its mouse or rat ortholog.

EXTRACELLULAR ROLES OF GRANZYMES

Although most research has focused on the cell death–inducing properties of granzymes, there is increasing evidence of extracellular functions of granzymes in promoting inflammation and coagulation and degrading extracellular matrix. Low amounts of granzymes A, B, and K are detected in the serum of healthy donors.[289] During inflammation and infection, elevated levels of granzymes are found in serum and other bodily fluids, including the serum of patients undergoing acute cytomegalovirus infection and chronic human immunodeficiency virus infection, the joints of patients with rheumatoid arthritis, and the bronchoalveolar lavage fluid of allergen-challenged patients with asthma and patients with chronic obstructive pulmonary disease.[43,282,290–294] Elevated granzyme levels have also been found in the serum of patients with endotoxemia and bacteremia, reflecting the fact that granzymes (but not perforin) are expressed and secreted by activated myeloid cells and a few other cell types, not just by lymphocytes.[41,44,45,295–298] In fact, in patients with sepsis, not only is serum granzyme K elevated, but its natural inhibitor (inter-α protein) is depleted, so the free active form of the enzyme is circulating and might cause damage.[299] Granzyme B has also been detected in macrophages of atheromatous lesions and rheumatoid joints.[296] Proteolysis by extracellular granzymes will be inhibited by serum and extracellular protease inhibitors, such as the trypsin inhibitors, antithrombin III and alpha-2-macroglobulin.[282] Some conditions that induce extracellular granzymes may also increase the release of intracellular serpins.[300]

Extracellular granzymes might arise from direct secretion —bypassing granule exocytosis— or by leakage from the immune synapse or by release from necrotic cells. Most directly secreted granzymes are secreted as proenzymes, which are inactive.[301] However, some of the proenzymes might be activated extracellularly by serum proteases. It is not known whether the immune synapse forms a perfectly tight gasket that completely prevents granzymes from leaking into the extracellular space during degranulation. Asymmetric synapses termed kinapses, which are less stable and less tight, and are formed by cytolytic CD4 T cells and probably under circumstances where the integrated activating signal from the target cell is weaker, may be leakier than the canonical stable synapse.[302] Although extracellular granzymes are not likely to get into the cytoplasm of cells to induce cell death without a high local concentration of perforin, they can proteolyze cell surface receptors or extracellular proteins. Recent studies suggest extracellular granzymes A and K activate macrophages to produce and secrete inflammatory cytokines, although the mechanism for this is not known.[303–305] These experiments performed with recombinant and purified granzymes need to be confirmed using cytotoxic cells because macrophages are exquisitely sensitive to immune activation by endotoxin, and it is not possible to verify the absence of endotoxin in preparations of these trypsin-like enzymes, because the endotoxin assay measures trypsin activity.[180] Some of the reported extracellular functions/substrates of the granzymes are summarized subsequently, but it is likely that these proteases, despite their high degree of substrate specificity, could have multiple, as yet unappreciated, destructive effects, particularly if present at high concentrations at inflamed sites in the absence of natural inhibitors. The physiologic significance of these extracellular activities is still unclear. One provocative study found a dramatic increase in granzyme B–sufficient versus granzyme B–deficient mice in the rate of rupture of aortic aneurysms in atherogenic mice (deficient in ApoE) that were also perforin deficient.[306] This result suggests that extracellular granzyme B contributes to the pathogenesis of atherosclerosis.

The known extracellular activities of granzymes suggest a proinflammatory effect. Granzyme A can activate the proinflammatory cytokine IL-1β directly,[307] and granzyme B can convert pro–IL-18 to its active form.[308] Granzyme K can activate proinflammatory cytokine production from lung fibroblasts, probably by cleaving their surface protease-activated receptor-1.[309] Granzymes also degrade extracellular matrix. Granzyme A may be able to degrade heparin sulfate proteoglycans, collagen type IV, and fibronectin.[310–312] Granzyme B can remodel the extracellular matrix by cleaving vitronectin, fibronectin, and laminin.[313] Proteolysis of the extracellular matrix might facilitate lymphocyte migration to sites of infection or inflammation or cause tissue destruction at sites of inflammation.[313,314] Granzyme A may also inhibit clotting by cleaving the thrombin receptor and von Willibrand factor[315–317] or by activating prourokinase to activate plasminogen.[318] In the central nervous system, granzyme B cleaves a glutamate receptor (GluR3), potentially contributing to immunoneurotoxicity, excitation, and autoimmunity in the brain.[319,320] Granzyme B on its own causes death of neurons in a pertussis toxin–sensitive manner, suggesting possible cleavage or involvement of G protein–coupled receptors.[321] Other potential granzyme B receptor targets are Notch1 and FGFR1, which might inhibit growth signals to developing or malignant cells.[322]

DEATH RECEPTOR PATHWAYS

NK cells and cytotoxic T cells can also trigger apoptosis by ligating and activating cell surface tumor necrosis factor (TNF) receptor family members that contain a cytoplasmic approximately 80 amino acid long death domain on target cells[323,324] (Fig. 37.9). Death by death receptor ligation can be distinguished from granule-mediated cell death because it is calcium independent and is not inhibited by calcium chelation. The death receptors on target cells form trimers when they are activated. In humans, six members of the larger TNF receptor family contain death domains: FAS (CD95, activated by FAS ligand [FasL, CD95L]), TNFR1 (activated by TNF), DR3 (activated by TNF ligand–related molecule 1 [TL1 or TNFS15]), DR4 and DR5 (activated by TNF-related apoptosis-inducing ligand [TRAIL]), and DR6 (unknown ligand). There

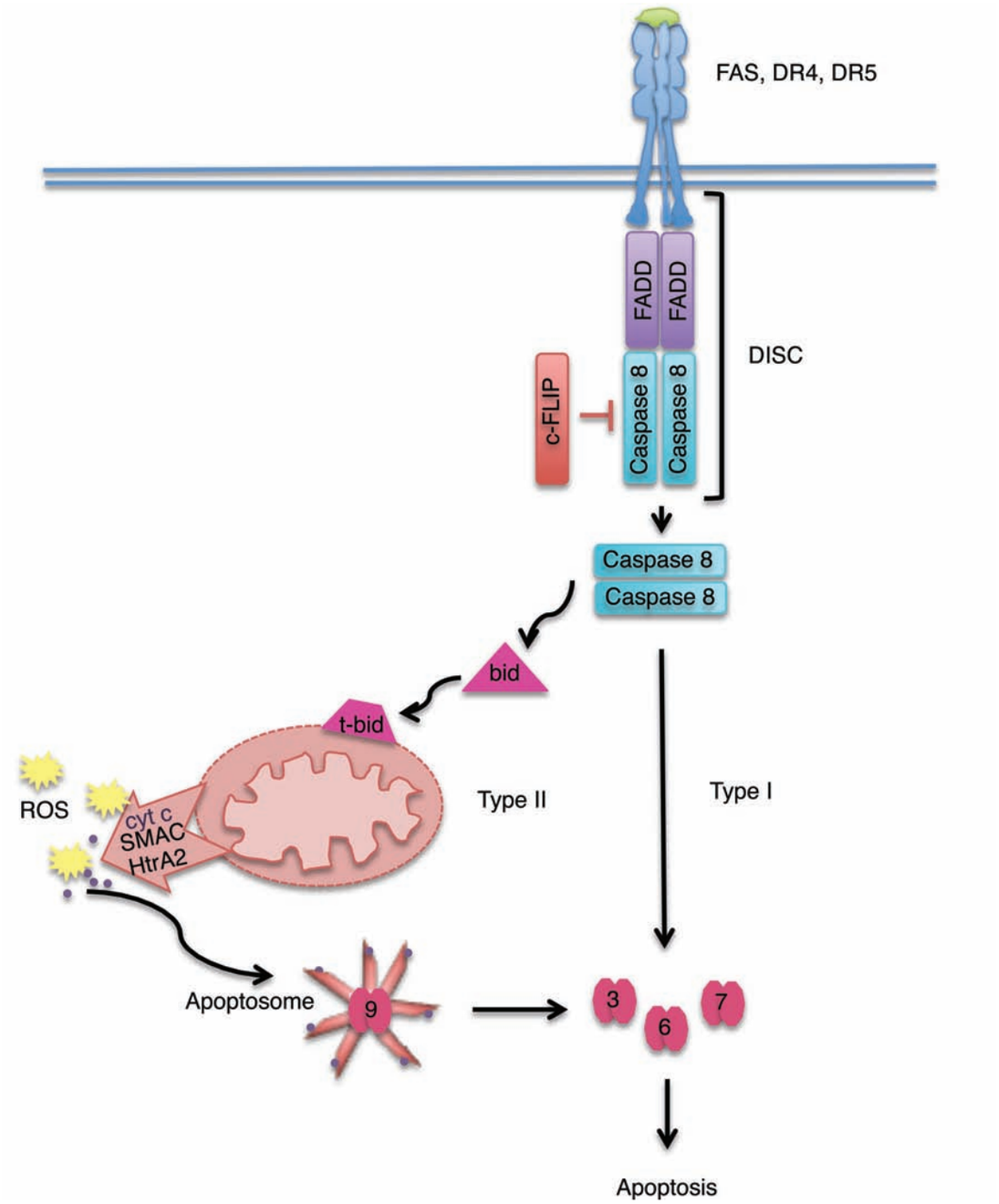

FIG. 37.9. Death Receptor Pathways of Apoptosis. Ligation of a death receptor trimer on target cells recruits the death-induced signaling complex, which activates caspase-8, releasing it to the cytoplasm where it can cleave bid to activate mitochondrial apoptotic pathways and cleave and activate the effector caspases-3, -6, and -7. In type I cells, apoptosis does not require mitochondrial amplification, whereas type II cells die only if mitochondrial mediators of apoptosis are released. The caspases activated downstream of caspase-8 are represented by numbered dimers. Cytochrome c required for caspase-9 activation in the apoptosome is represented by a *blue ball*. Caspase-8 activated by death receptor signaling and granzyme B–mediated death are very similar, although the granzyme B–mediated death is much more rapid. Fas-associated death domain can also recruit an alternate signaling complex that leads to cell activation rather than apoptosis (*not shown*).

are mouse orthologs for all of these, except DR5. The death domains recuit one of two adapter molecules, Fas-associated death domain (FADD) or TNF receptor–associated death domain (TRADD), that serve as a platform for recruiting signaling complexes. Depending on the cellular context, signaling by the death receptors can either trigger caspase-mediated apoptosis or proliferative and proinflammatory responses. In general, the receptors that predominantly recruit FADD (FAS, DR4, and DR5) are more likely to trigger apoptosis, while signaling from the TRADD-associated receptors (TNR1, DR3, and possibly DR6) is more likely to activate cell survival and proliferation pathways via activation of the NF-κB transcription factor and the JNK and p38 MAP kinase pathways. When cell death is triggered in cells in which the caspase pathway is inhibited, targeted cells undergo an alternate programmed cell death pathway termed necroptosis, mediated by a kinase (RIP1) that is recruited by TRADD.[325,326]

After death receptor activation and recruitment of FADD, FADD recruits the apical caspases 8 and/or 10 (the latter has no mouse ortholog) to form the death-induced signaling complex (DISC) at the cell membrane. Within the DISC, these caspases are autoproteolyzed and activated. The activated caspases are then released to the cytoplasm where they can cleave the Bcl-2 family member Bid to activate mitochondrial damage and also cleave and activate the executioner caspases (3, 6, and 7). The mitochondrial pathway amplifies caspase activation by activating caspase-9. Some cells (called type I [eg, thymocytes]) undergo apoptosis without requiring activation of the mitochondrial pathway, while others (type II cells [eg, B-lymphocytes]) are resistant to cell death if the mitochondrial pathway is blocked.

Humans and mice that are genetically deficient in either FAS or its ligand are able to defend against intracellular pathogens, but develop an autoimmune syndrome called autoimmune lymphoproliferative syndrome.[11,327] FAS-mediated death is required to eliminate chronically activated T cells and contributes to elimination of self-reactive immune cells. Mice with genetic deficiencies in these genes develop similar symptoms. Although caspase-8 is considered the main initiator caspase that associates with and is activated by death receptor signaling, humans bearing caspase-8 mutations have defects in T-cell activation and immunodeficiency rather than autoimmunity, which highlights the importance of the nonapoptotic signaling that results from death receptor engagement.[328] Of note, humans with caspase-10 mutations develop autoimmune lymphoproliferative syndrome, suggesting that under certain circumstances caspase-10 substitutes for caspase-8 in initiating death receptor–mediated apoptosis.[329] Nonapoptotic death receptor signaling, mediated by activation of NF-κB, JNK, and MAP kinase pathways, not only promotes cell proliferation but has a proinflammatory effect, which involves activating chemokine and cytokine production by macrophages and dendritic cells. The relative strength of pro-apoptotic and nonapoptotic signaling is determined in part by cellular expression of c-FLIP, an inhibitor of caspase-8, that is recruited to the DISC and promotes recruitment of RIP1 and TRAF2 to activate nonapoptotic pathways. c-FLIP mRNA can be spliced into alternate isoforms: c-FLIP$_L$ (long) and c-FLIP$_S$ (short). DISC recruitment of c-FLIP$_S$, which is homologous to caspase-8 but is enzymatically inactive, inhibits apoptosis, while the concentration of c-FLIP$_L$ determines whether it is proapoptotic (at low concentrations) or antiapoptotic (at high concentrations). Some tumor cells as well as some activated T cells and NK cells overexpress c-FLIP$_L$, which renders them insensitive to death receptor-mediating apoptosis and promotes their survival.

Mouse studies suggest that DR5 (the ortholog of human DR4 and DR5) and its ligand TRAIL may play an important role in innate immune tumor surveillance.[330] TRAIL may also participate in eliminating activated CD8 CTLs that were primed in the absence of effective CD4 help.[20] Immature NK cells express TRAIL, and DR5-deficient mice are prone to develop tumors and metastases in several endogenous mouse tumor models. As a consequence, soluble TRAIL and agonistic antibodies to DR4 or DR5 are currently being developed for potential tumor immunotherapy.[331,332]

CONCLUSION

Killer lymphocytes in the innate and adaptive immune responses protect us from infection and cellular transformation by releasing cytotoxic granules and help control immune cell proliferation and autoimmunity by both cytotoxic granule release and death receptor–activated cell death. Killer cells trigger multiple programs of cell death, which ensures that the immune system can control pathogens that have devised strategies to resist individual cell death pathways. Lymphocyte-targeted cells are recognized by scavenger cells like macrophages that rapidly engulf them and remove them to limit inflammation that occurs when cells die by encrosis. Research in the next few years should provide a better understanding of how cytotoxic gene expression is regulated, how killer cells are protected from their own molecules of destruction, the alternate cell death pathways activated by the multiple granzymes, and how the granzymes overcome the strategies by which viruses and tumors try to evade elimination. Further research will clarify the mechanisms and physiological importance of inflammatory noncytotoxic effects of killer cell enzymes.

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