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# Knocking 'em Dead: Pore-Forming Proteins in Immune Defense

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## Keywords

complement, perforin, gasdermin, MLKL, granulysin, cytotoxic lymphocyte, necrosis, apoptosis, pyroptosis, necroptosis, microptosis

## Abstract

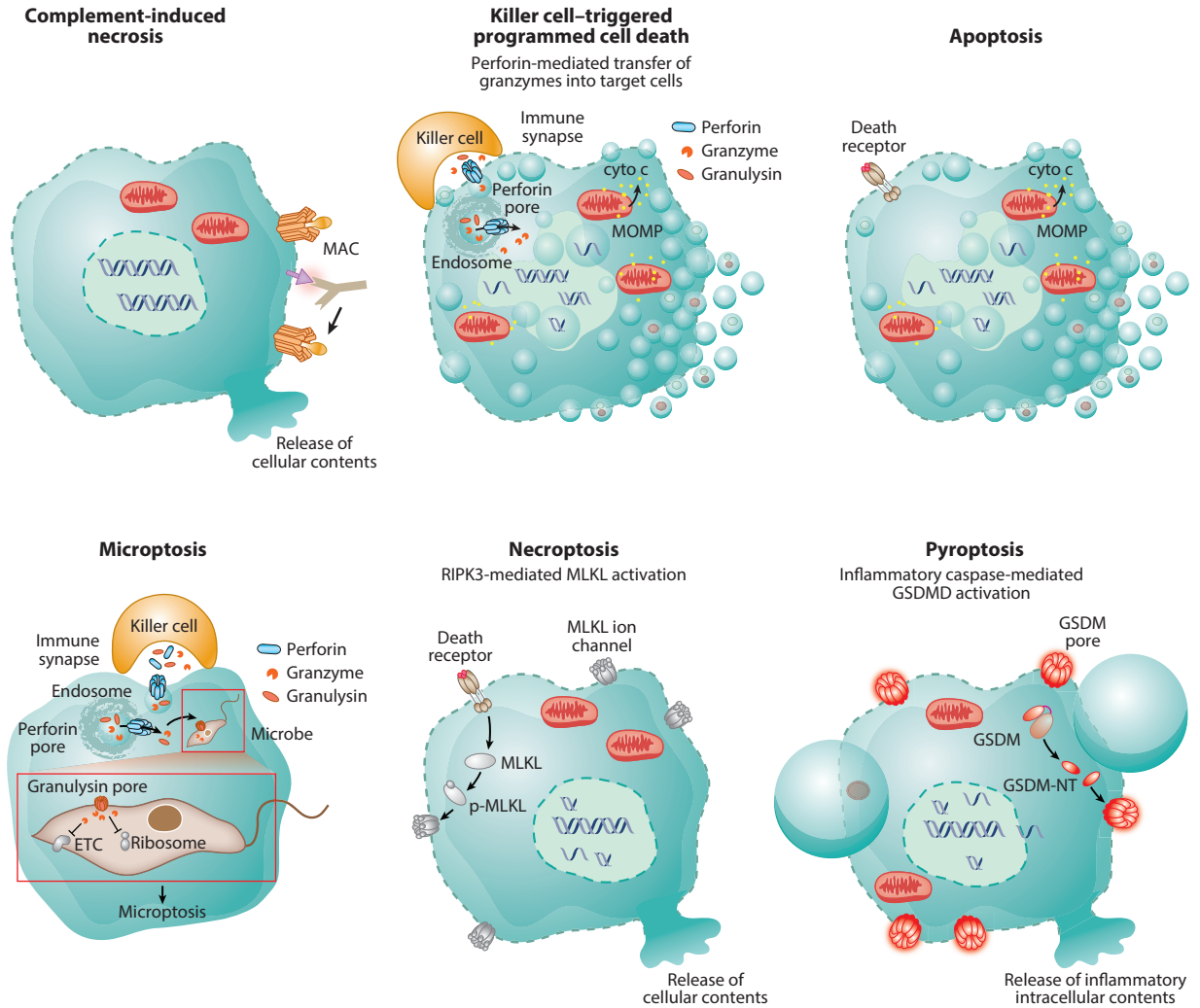
Immune cells use a variety of membrane-disrupting proteins [complement, perforin, perforin-2, granulysin, gasdermins, mixed lineage kinase domain-like pseudokinase (MLKL)] to induce different kinds of death of microbes and host cells, some of which cause inflammation. After activation by proteolytic cleavage or phosphorylation, these proteins oligomerize, bind to membrane lipids, and disrupt membrane integrity. These membrane disruptors play a critical role in both innate and adaptive immunity. Here we review our current knowledge of the functions, specificity, activation, and regulation of membrane-disrupting immune proteins and what is known about the mechanisms behind membrane damage, the structure of the pores they form, how the cells expressing these lethal proteins are protected, and how cells targeted for destruction can sometimes escape death by repairing membrane damage.

## 1. INTRODUCTION AND OVERVIEW

The immune system kills both microbial and host cells to control infection and tumors and sound alarms to recruit immune cells and activate a protective response to pathogens and other signs of danger. Until recently, cell death was divided into necrosis, in which the cell membrane is rapidly ruptured as the primary event, leading to inflammatory release of intracellular contents, and apoptosis, a noninflammatory programmed cell death in which the dying cell actively participates. Programmed cell death has characteristic morphological changes (cell membrane blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation) and is often mediated by disruption of the mitochondrial outer membrane and/or effector caspase activation, but it can be activated by other proteases, such as killer cell granzymes, independently of caspases. Cells undergoing these cell death programs are recognized and removed by phagocytic cells long before their cell membranes become permeabilized. Consequently, apoptosis and killer lymphocyte-mediated cell death have been thought of as noninflammatory, since secondary necrosis, if it occurs, happens inside phagosomes, and cellular contents are therefore not released into the extracellular milieu. The mechanistic basis for the eventual membrane disruption in secondary necrosis is not understood. In recent decades new forms of regulated cell death have emerged that include programmed inflammatory necrosis (necroptosis and pyroptosis) as well as other forms of caspase-independent programmed cell death.

The execution of these diverse cell death pathways relies on a handful of molecules that permeabilize membranes of cells targeted for immune elimination—complement, perforin, perforin-2, granulysin, gasdermins, and mixed lineage kinase domain–like pseudokinase (MLKL) (1–6) (**Figure 1**; **Table 1**). Each of these proteins primarily activates a distinct type of cell death: Complement punches holes in microbial or mammalian membranes to cause necrosis, perforin delivers granzymes to mammalian cells to induce caspase-independent and -dependent programmed cell death that resembles classical apoptosis, granulysin triggers both necrosis and programmed cell death in microbes (microptosis), gasdermins trigger pyroptosis induced after sensing invasive infection or cytosolic danger signals that activate inflammatory caspases, and MLKL mediates necroptosis induced by death receptor signaling when caspase-8 is inhibited (**Figure 2**; **Table 2**). Some of these proteins multimerize and insert into the target membrane to form large pores in the shape of  $\beta$  barrels (complement, perforin-1 and -2, gasdermins); others are smaller  $\alpha$ -helical proteins (granulysin, MLKL) that oligomerize and damage cell membranes through poorly understood mechanisms that may or may not involve transmembrane pores (7–14). Some of these proteins only efficiently disrupt microbial membranes (complement, granulysin), some only work on mammalian membranes (perforin), and some work on both (gasdermins) (2, 15–18). Some work primarily only by disrupting the targeted membrane (complement, gasdermins, MLKL), while others (perforin, granulysin) use their membrane-disrupting ability to deliver death-inducing enzymes that activate programmed cell death in target cells (2, 19). The strict separation of the death pathways in which these membrane-damaging molecules participate into inflammatory or noninflammatory cell death is no longer valid. For example, during classical apoptosis, caspase-3 activates gasdermin E (GSDME). GSDME processing converts relatively slow noninflammatory apoptosis to a more rapid inflammatory pyroptotic death in which the cell swells and forms bubbles, releases cytosolic molecules, and eventually bursts (20, 21). Another example of crossover between inflammatory and noninflammatory death pathways is caspase-8 activation by the inflammasome when caspase-1 is inhibited (22, 23). Cell membrane damage of any sort will activate secondary pyroptosis in any cell that expresses the NLRP3 NOD-like receptor, which senses ionic changes in membrane-damaged cells (24–26).

Most of these membrane-disrupting proteins first arose when vertebrates appeared, about the same time as the antigen receptors that are the basis of adaptive immunity. However, the strategies



**Figure 1**

Immune membrane damage-mediated cell death. Complement-mediated lysis is a classic example of necrotic cell death characterized by cell membrane rupture. Perforin delivers granzymes to induce programmed target-cell death that resembles apoptosis morphologically with cell shrinkage, membrane blebbing, chromatin condensation, and nuclear fragmentation. Granzyme B also causes apoptosis—it activates caspase-3 and also directly cleaves many caspase-3 substrates to cause mitochondrial outer membrane permeabilization and oligonucleosomal double-stranded DNA breaks, visualized on gels as DNA ladders. Perforin also delivers granulysin into the target cell. Granulysin permeabilizes the cell membrane of intracellular microbes to cause microbial necrosis and allows the granzymes to enter the microbe to proteolytically cause microptosis, programmed cell death of bacteria, fungi, and parasites. Death receptor ligation causes classic apoptosis if caspase-8 or -10 is activated or necroptosis if these caspases are inhibited. In necroptosis, MLKL is phosphorylated, binds to membranes, and perforates them probably by forming ion channels that cause swelling and hypotonic lysis. During pyroptosis, activated in response to invasive pathogens and sterile danger signals, gasdermins are cleaved and the N-terminal fragment forms large plasma membrane pores. Pyroptotic membranes form large ballooning structures, while apoptotic membranes cause small blebs. Because proteins are released through gasdermin pores, the pyroptotic cell likely maintains osmolarity and does not burst. Abbreviations: cyto c, cytochrome c; ETC, electron transport chain; GSDM, gasdermin; GSDM-NT, GSDM N-terminal fragment; GSDMD, gasdermin D; MAC, membrane attack complex; MLKL, mixed lineage kinase domain-like pseudokinase; MOMP, mitochondrial outer membrane permeabilization; p-MLKL, phospho-MLKL.

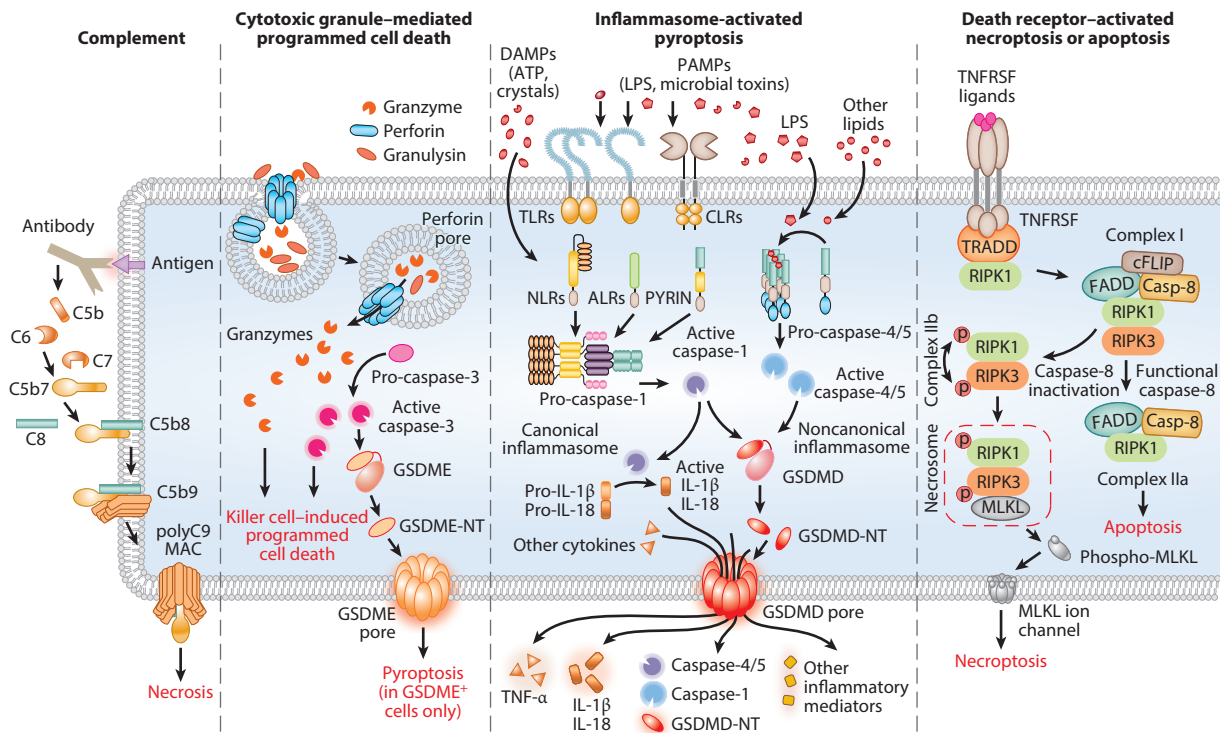
**Table 1 Immune membrane-disrupting proteins**

Name	Type of membrane damage	Activation pathway	Inflammatory?	Type of cell death	Biological function	References
Complement	$\beta$ -Barrel pore	Proteolytic complement cascade	Yes	Necrosis	Lyse bacteria and opsonized cells	17, 32, 37, 45, 172, 173
Perforin	$\beta$ -Barrel pore	Killer cell attack and degranulation $\text{Ca}^{2+}$ -dependent	No	Killer cell programmed cell death	Eliminate infected and cancerous cells	13, 47, 59, 64, 69, 70
Perforin-2	Presumed $\beta$ -barrel pore	Not known	Not known	Not known	Enhance clearance of invading bacteria	31, 75, 77, 78, 79
Gasdermins	$\beta$ -Barrel pore	Inflammasome-inflammatory caspase-GSDM cleavage Apoptosis-caspase-3-GSDME	Yes	Pyroptosis	Recruit immune cells and activate response to infection and sterile signs of danger	7, 14, 18, 19, 23, 24
MLKL	Ion channel?	TNFRSF-RIPK1/RIPK3 DAI-RIPK3 TLR-TRIF/RIPK3	Yes	Necroptosis	Cell death in setting of caspase inactivation Involved in infection, inflammation, sepsis, ischemia injury, neurodegeneration	10, 16, 112, 113, 117, 118
Granulysin	Not known	Killer cell degranulation	Yes	Microptosis	Kill intracellular and possibly cell-free bacteria, fungi, and parasites	9, 11, 137, 150, 151

Abbreviations: GSDM, gasdermin; GSDME, gasdermin E; MLKL, mixed lineage kinase domain-like pseudokinase; TLR, Toll-like receptor; TNFRSF, tumor necrosis factor receptor superfamily.

used to disrupt membranes are similar to mechanisms used throughout evolution even in prokaryotes. None of these membrane-permeabilizing proteins, except some complement components, binds to a specific receptor. This means that microbes and cancer cells cannot readily evade immune elimination by mutating or downregulating a receptor, but it also means that systems need to be in place to avoid membrane damage to the cell that expresses these dangerous molecules and to enable selective targeting without damaging bystander cells. Each cell death-inducing protein needs a strategy to optimize disruption of membranes of cells to be killed and avoid harming membranes of cells that need to be left unharmed.

This review describes the membrane-disrupting proteins used in immune responses to infection, cancer, and danger, focusing on the newly identified members of this group (gasdermins, MLKL, perforin-2) and recent research that identifies unappreciated biological functions of



**Figure 2**

Molecular mechanisms that activate membrane damage. The complement system is activated by the classic, lectin, or alternative pathway, all of which share a common terminal pathway in which C5 is cleaved into the chemoattractant C5a and the pore-initiating protein C5b. C5b then sequentially binds C6, C7, and C8, which assemble on the target membrane and recruit C9, which oligomerizes to form the MAC pore. Killer cells release cytotoxic granules' contents (perforin, granzymes, granulysin) into the immune synapse formed with the target cell. Perforin oligomerizes to form short-lived pores in the target cell membrane that trigger membrane repair, which activates endocytosis of the damaged membrane with bound perforin, granzymes, and granulysin. Perforin then forms pores in the endosome, releasing granzymes and granulysin into the target cell cytosol. Cytosolic granzymes induce both caspase-independent and -dependent programmed cell death by cleaving and activating downstream effector proteins. In target cells expressing GSDME, granzyme B-activated caspase-3 can cleave and activate GSDME to convert noninflammatory cell death to inflammatory pyroptosis. In response to cytosolic sensing of microbial infection or danger signals, canonical or noncanonical inflammasomes assemble to activate inflammatory caspases (caspase-1 and caspases-4/-5/-11, respectively). Activated inflammatory caspases cleave GSDMD, releasing its N-terminal fragment, GSDMD-NT, which forms pores in the plasma membrane to cause pyroptosis. Ligand-bound death receptors direct the recruitment and formation of distinct complexes that activate necroptosis or apoptosis. Complex I contains TRADD, RIPK1 and E3 ubiquitin ligases, which inhibit apoptosis by poly-ubiquitinating RIPK1. If RIPK1 is not ubiquitinated, complex IIa activates caspase-8 and initiates apoptosis, whereas when caspase-8 activation is blocked, complex IIb assembles when RIPK1 recruits and phosphorylates RIPK3, which initiates necroptosis by phosphorylating MLKL. Abbreviations: ALR, AIM2-like receptor; CLR, C-type lectin receptor; DAMP, danger-associated molecular pattern; GSDMD, gasdermin D; GSDMD-NT, GSDMD N-terminal fragment; GSDME, gasdermin E; GSDME-NT, GSDME N-terminal fragment; LPS, lipopolysaccharide; MAC, membrane attack complex; MLKL, mixed lineage kinase domain-like pseudokinase; NLR, nucleotide-binding oligomerization domain-like receptor; PAMP, pathogen-associated molecular pattern; TLR, Toll-like receptor; TNFRSF, tumor necrosis factor receptor superfamily; TRADD, TNF-receptor-associated death domain protein.

granulysin. A homolog of perforin, perforin-2 (PFN-2 or MPEG1), that is constitutively expressed in myeloid cells and that damages the cell membranes of intracellular or phagocytosed bacteria, has recently been described (27, 28). Here we do not review the small antimicrobial peptides, like the defensins, produced by mucosal epithelia to control infection. We describe

**Table 2** Types of cell death

Type	Immune effectors	Morphological features	Molecular features	Characteristic
Complement-induced necrosis	Complement	Cell swelling, plasma membrane rupture	Cell membrane pores	Inflammatory
Killer cell-induced programmed cell death	Perforin/granzymes	Cell shrinkage, membrane blebbing, chromatin condensation, nuclear fragmentation	Caspase activation (granzyme B), mitochondrial ROS, loss of mito $\Delta\psi$ , proteolytic cascade, global mRNA decay, MOMP, cytochrome c release, genomic DNA fragmentation	Noninflammatory
Apoptosis	Death receptors/ effector caspases, granzyme B	Cell shrinkage, membrane blebbing, chromatin condensation, nuclear fragmentation	Caspase activation, mitochondrial ROS, loss of mito $\Delta\psi$ , proteolytic cascade, global mRNA decay, MOMP, cytochrome c release, genomic DNA fragmentation	Noninflammatory
Microptosis	Perforin/granulysin/granzymes	Parasites undergo cell death that morphologically resembles mammalian cell apoptosis	Generation of superoxide anion, disruption of antioxidant defenses, disruption of protein synthesis and central metabolism	Inflammatory
Pyroptosis	Inflammasome/ inflammatory caspases/ gasdermins	Membrane bubbles, cell flattening	Inflammatory caspase activation, gasdermin cleavage, cell membrane pore, release of inflammatory intracellular contents	Inflammatory
Necroptosis	Death receptors/ MLKL	Cell swelling, cell membrane rupture	RIPK1/3, MLKL phosphorylation, membrane ion channel, release of cellular contents	Inflammatory

Abbreviations: MLKL, mixed lineage kinase domain-like pseudokinase; MOMP, mitochondrial outer membrane permeabilization; ROS, reactive oxygen species.

recent advances in understanding how these proteins damage membranes, made possible by new structural biology methods to study large membrane-associated complexes. Throughout we highlight the important unsolved questions in the field. We begin with an introductory description of each of the immune membrane-disrupting proteins and then describe how each of them addresses the biological challenges of selective disruption of target membranes and the target cell response to membrane damage.

## 2. THE PLAYERS

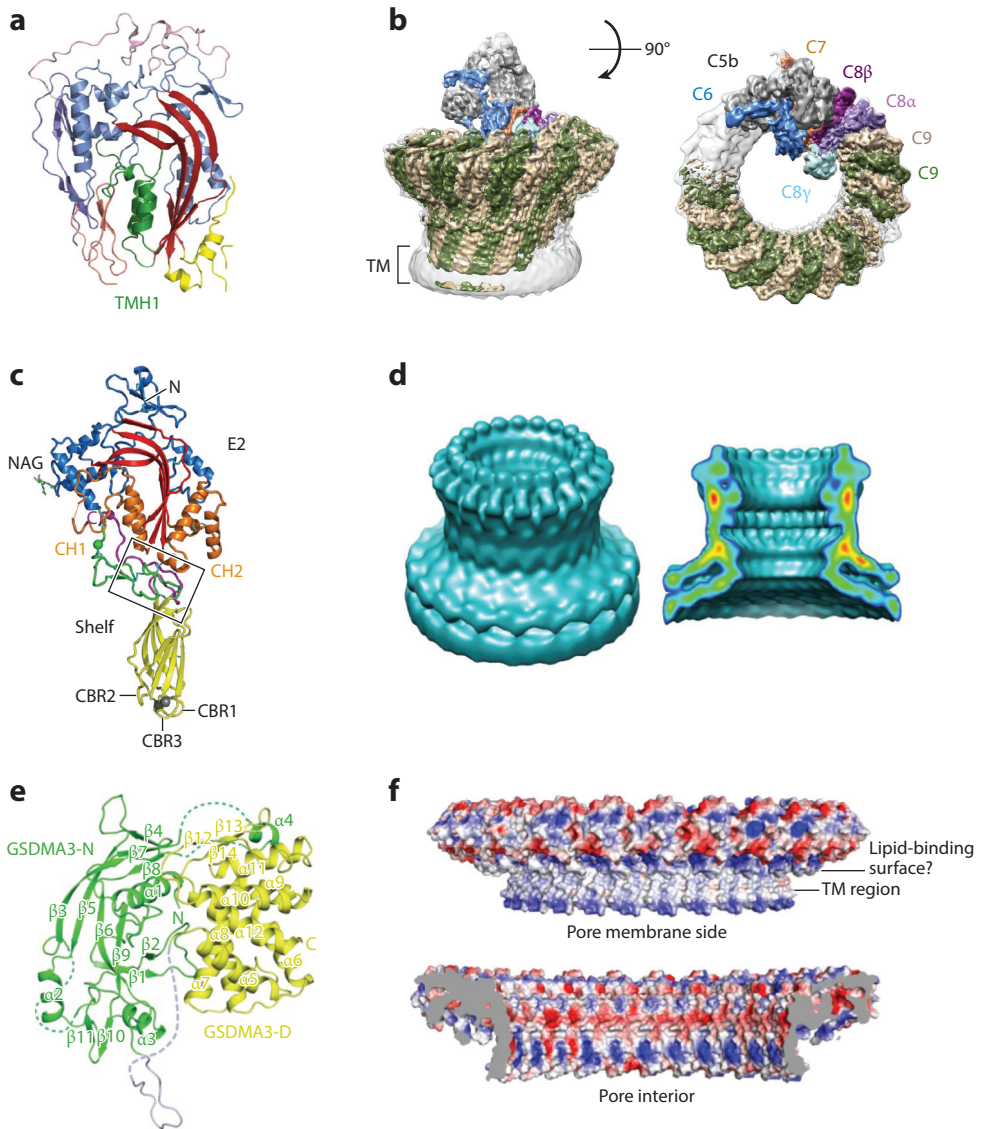
### 2.1. The Complement System

Complement was the first described immune pore-forming system, first identified by Paul Ehrlich in 1899 as a hemolytic activity in blood that complemented the activity of antibodies (29). It is a

collection of mostly circulating precursor proteins that work together in a proteolytic cascade to form large membrane pores. Its main function is to destroy enveloped viruses and microbes, especially when they are opsonized by antibodies, although complement also removes damaged mammalian cells and immune complexes. The complement system is composed of more than 30 soluble and membrane-bound proteins that are activated by binding to microbial carbohydrates and immunoglobulins and by multiple proteolytic cascades that converge on cleaving soluble C5 complement into C5a, a powerful chemoattractant, and C5b, which triggers pore formation by nucleating a pore, the membrane attack complex (MAC) (30, 31) (**Figures 3a,b** and **4a**). C5b, C6, and C7 first form a lipophilic complex that binds to and inserts in membranes when it recruits C8 (**Figure 2**). This complex recruits C9, which multimerizes with the membrane-inserted complex to form a 110-Å-diameter membrane pore, composed of C5b, C6, C7, heterotrimeric C8 ( $\alpha$ ,  $\beta$ ,  $\gamma$ ), and  $\sim$ 18 copies of C9. C6, C7, C8 $\alpha$ , C8 $\beta$ , and C9 are members of the MAC/perforin (MACPF)/cholesterol-dependent cytolytic (CDC) protein superfamily, which includes complement, perforin, and many gram-positive bacterial toxins (1, 32, 33). More than 1,000 proteins from all kingdoms of life contain a MACPF domain that might form pores (34). The MAC is the only heteromeric immune pore-forming protein. MAC pores cause necrosis. Assembly of the complement MAC on bacteria is triggered when mannose-binding lectin (MBL) binds to bacterial carbohydrates or when antibody-opsonized bacteria are recognized by C1q in the C1 complex. The MAC kills bacteria by perforating bacterial cell membranes (35). Patients with complement deficiency are vulnerable to encapsulated bacteria, such as *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Salmonella enterica* serovar Typhimurium. Some organisms hijack the complement system to increase their virulence, for example, by using complement receptors to enter cells (36), while some viruses and intracellular bacteria bind complement regulatory proteins and receptors to escape complement-mediated death (37).

## 2.2. Perforin

Perforin, a pore-forming protein encoded by the *PRF1* gene, has an N-terminal MACPF domain (5, 38, 39) that is similar to the pore-forming domains of the C6–C9 components of the complement MAC (especially C9) and bacterial CDC (40) (**Figure 3c,d**). Unlike the soluble complement components, which are expressed mostly by hepatocytes and secreted into the blood, perforin is expressed only in killer lymphocytes, which store it in cytotoxic granules, specialized secretory lysosomes (41). When a killer cell recognizes a target cell, its cytotoxic granules migrate along microtubules to the immune synapse, where they dock and fuse with the killer cell plasma membrane, releasing perforin and other cytotoxic effector proteins (granzymes and granulysin) into the immune synapse (42). Perforin then forms pores in the target cell membrane, which lead to cytosolic delivery of the other effector proteins. However, delivery does not occur directly through plasma membrane pores (43–45). Although like complement, perforin pokes holes in target cell membranes that would ordinarily cause necrosis, the membrane damage by killer cells is rapidly repaired by the ubiquitous cell membrane repair pathway, perhaps because damage is localized to the immune synapse. Membrane repair triggers endocytosis of perforin together with the death-inducing granzymes, which bind to the target cell membrane by charge interactions, which allows them to be coendocytosed with perforin (46, 47). Perforin then forms pores in the endosomes of target cells, which deliver the granzymes into the target cell cytosol, where they cause programmed cell death. Although most of the granzymes do not activate the caspases, granzyme B cleaves and activates caspase-3, which amplifies killer cell-mediated death (48). The perforin MACPF domain is followed by an EGF domain that contributes to the pore structure and a Ca<sup>2+</sup>-binding C2 domain, responsible for perforin's Ca<sup>2+</sup>-dependent binding to target cell membranes (9, 49)

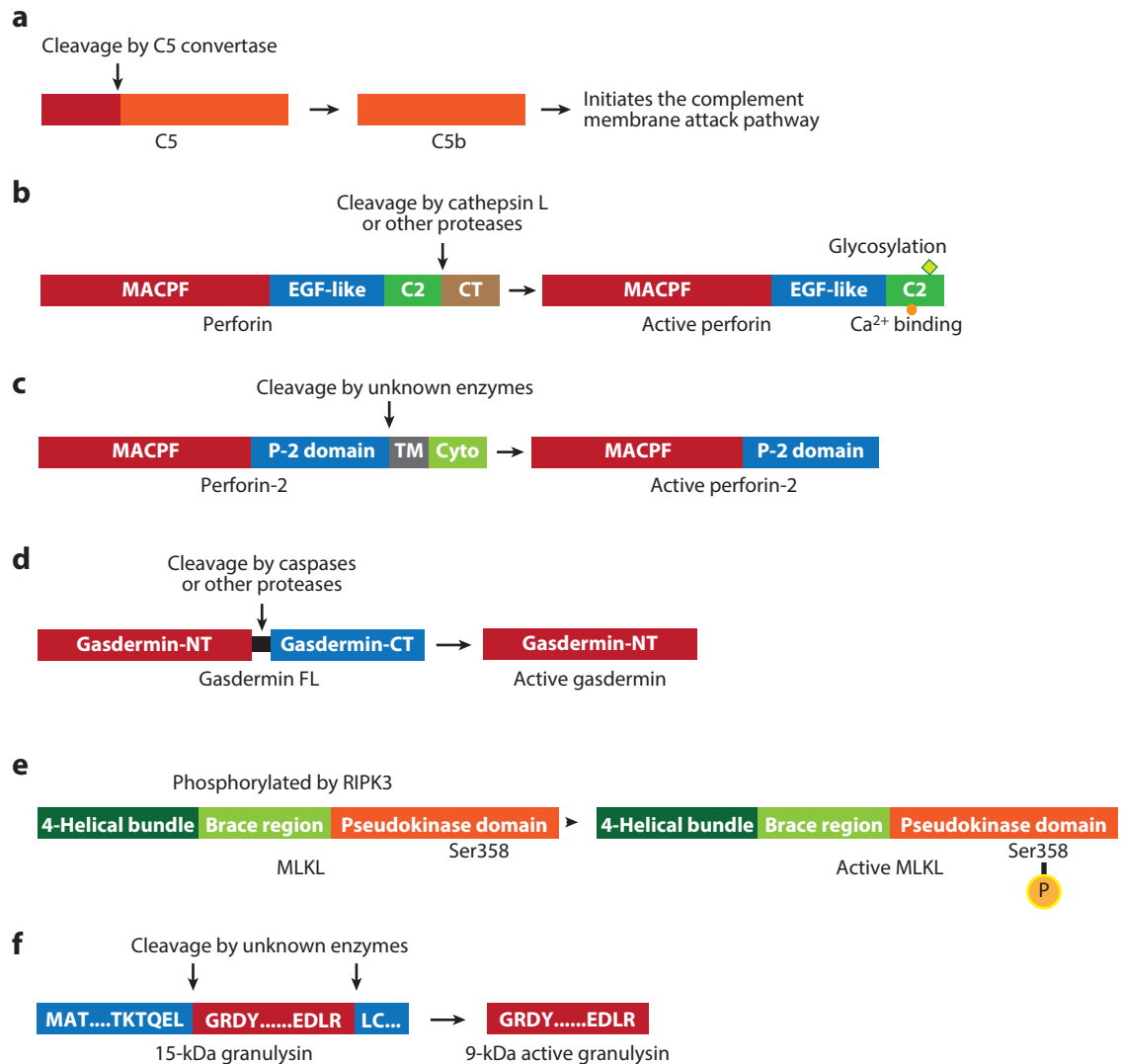


**Figure 3**

Structures of immune pore-forming proteins. (*a,c,e*) Crystal structures of the monomers of (*a*) complement C9, (*c*) perforin, and (*e*) GSDMA3. (*b,d,f*) Cryo-electron microscopy reconstruction of the membrane pores formed by (*b*) the complement membrane attack complex, (*d*) perforin, and (*f*) GSDMA3. Abbreviations: GSDMA3, gasdermin A3; TM, transmembrane. Panels *a*, *b*, *e*, and *f* adapted from References 10, 13, 15, and 163, respectively; panels *c* and *d* adapted from Reference 9.

(Figure 4b). Nineteen to twenty-four perforin monomers assemble (at least in lipid monolayers) into a pore with a lumen diameter of  $\sim 160$  Å, large enough to deliver the granzymes (9). Perforin pore formation depends on membrane cholesterol; hence, perforin does not damage microbial membranes that lack cholesterol (2, 50). Why perforin forms pores only in cholesterol-containing membranes is not understood. At the immune synapse, perforin binding to the killer cell





**Figure 4**

Activation of immune membrane-disrupting proteins. (a) The complement cascade is initiated when C5 is processed to generate C5b. (b) Perforin consists of a MACPF domain, an EGF-like domain, and a calcium-binding C2 domain followed by a C-terminal peptide. It is synthesized as an inactive precursor that is glycosylated in its C2 domain and processed in cytotoxic granules by cathepsin L or other unknown proteases. (c) Perforin-2, a membrane-bound protein, consists of a MACPF domain, conserved perforin-2 domain, transmembrane domain and cytoplasmic domain. Its pore-forming activity may be enhanced by proteolytic activation by unknown enzymes to remove the transmembrane domain and release it from vesicular membranes. (d) Gasdermins have N-terminal and C-terminal domains separated by a linker; the C-terminal domain folds back on the N-terminal domain to autoinhibit membrane pore formation. Cleavage in the linker generates the N-terminal pore-forming domain. (e) MLKL, composed of an N-terminal four-helical bundle domain followed by the brace region and a C-terminal pseudokinase inhibitory domain, is activated by phosphorylation of Ser358 by RIPK3. (f) The inactive 15-kDa granulyisin propeptide is cleaved after Leu62 and Arg136 by unknown enzymes to produce active granulyisin (9 kDa). Abbreviations: CT, C-terminal; Cyto, cytoplasmic; EGF, epidermal growth factor; FL, full length; MACPF, membrane attack complex/perforin; NT, N-terminal; MLKL, mixed lineage kinase domain-like pseudokinase; P-2, perforin-2; TM, transmembrane.

membrane does not harm the killer cell, for reasons that are not entirely clear. Following cytotoxic granule fusion with the killer cell plasma membrane, cytotoxic granule cathepsin B is exposed on the killer cell membrane at the synapse and proteolytically inactivates any perforin that binds to the killer cell (51). However, cathepsin B genetic deficiency does not lead to killer cell death during target cell attack, suggesting other uncharacterized protective mechanisms (52).

*Prf1*<sup>-/-</sup> mice are profoundly immunodeficient and are unable to protect themselves from viruses and prone to develop lymphoma (53, 54). Humans bearing hypomorphic *PRF1* are impaired in handling intracellular infection and can develop an often-fatal inflammatory syndrome, familial hemophagocytic lymphohistiocytosis, due to unresolved infection, high levels of IFN- $\gamma$ , and macrophage activation that can be treated by bone marrow transplantation or the recently approved anti-IFN- $\gamma$  antibody emapalumab (55, 56). Individuals bearing less severe *PRF1* mutations can be asymptomatic until adulthood and may develop lymphoma.

### 2.3. Perforin-2

Recently a weakly paralogous protein PFN-2 that contains a MACPF domain and is expressed from the *MPEG1* gene mainly in macrophages and other myeloid cells has also been identified and is hypothesized to also form membrane pores (27, 28, 57) (**Figure 4c**). *MPEG1* was the first MACPF domain-containing gene to appear in eukaryotes during evolution (in sponges, where it functions in antibacterial defense), and *PRF1* may have arisen as a gene duplication of *MPEG1*. *MPEG1* is constitutively expressed in professional phagocytic cells (macrophages, dendritic cells, neutrophils, microglia), in some innate or innate-like lymphocytes [ $\gamma\delta$  T cells, natural killer (NK) cells], and in some epithelia, including keratinocytes. It is broadly induced by interferons in epithelia, endothelia, fibroblasts, and lymphocytes, suggesting it may function in many cell types (58). Unlike other mammalian MACPF proteins, PFN-2 contains a transmembrane domain and may be bound to endosome and phagosome membranes oriented with its MACPF domain in the vesicular lumen (59). It has been proposed to disrupt membranes of phagocytosed bacteria to control bacterial infection (60), and *MPEG1* knockdown reduces in vitro macrophage clearance of diverse bacterial species (61). A recent study linked heterozygotic mutations of *MPEG1* to persistent atypical mycobacterial infection, providing support for an immune protective role in humans, especially for bacteria that infect macrophages (62). The MACPF domain of the protein might be proteolytically released into phagosomes to access bacterial cell membranes. Indeed, a cleaved PFN-2 was visualized by immunoblot after bacterial infection, although the responsible protease has not been identified (63). Overexpression of PFN-2 in HEK-293T cells or interferon-induced expression in bacteria-infected mouse embryo fibroblasts leads to pores in both bacterial and host cell membranes, visible by transmission electron microscopy (EM) (63). Pores are only formed if the membrane preparations are trypsinized, consistent with the hypothesis that an active soluble pore-forming protein is liberated by proteolytic digestion of the membrane-bound protein. The inner diameters of PFN-2 pores are  $\sim 100$  Å, similar to the complement and PFN-1 MAC pores. A recent cryo-EM study of soluble PFN-2 lacking the C-terminal transmembrane and cytosolic domains visualized large oligomeric membrane-bound prepores (hypothesized to take shape before assembly into a membrane-spanning pore) formed by 16 monomers that associated with lipids by their juxtamembrane P-2 domain (14). Soluble PFN-2 permeabilized red blood cells, but only in acidic media, consistent with its need to function in acidic phagolysosomes. In this study rare images (not enough to solve the structure of the pore) captured a pore in one liposome connected to another liposome, suggesting that PFN-2 may not need to be cleaved from the membrane to form pores in phagocytosed bacteria. In one study *Mpeg1*<sup>-/-</sup> mice were more susceptible than littermate control mice to bacterial challenges with *Staphylococcus aureus* and *S. Typhimurium* (63).

These studies need to be considered as provisional, since the knockout mice were not backcrossed to the parental strain, but they suggest that PFN-2 is important in eliminating phagocytosed bacteria.

However, many questions remain. Does PFN-2 pore-forming activity depend on proteolytic activation, and what is the protease? On what membranes does PFN-2 localize, and what bacterial and eukaryotic membranes does the activated protein permeabilize? Does it permeabilize (phagolysosomal) membranes to release lysosomal proteases to kill host cells? If not, how is bacterial specificity regulated? Is PFN-2 active against other microbes, such as fungi and protozoan parasites, or enveloped viruses? Do any antimicrobial or cytolytic effector proteins enter through PFN-2 pores to contribute to killing? How important is PFN-2 in overall microbial defense in vivo?

## 2.4. Gasdermins

The gasdermins (GSDMs) are a family of homologous pore-forming proteins that cause pyroptosis, an inflammatory death associated with loss of cell membrane integrity and release of inflammatory mediators. In humans, this gene family consists of 6 genes, *GSDMA–E* and *DFNB59*. *GSDME*, also known as *DFNA5*, and *DFNB59*, also known as *pejvakin*, have both been linked by genetic mutation to hearing loss. Mice have three *GSDMA* counterparts (*Gsdma1–3*), four *GSDMC* counterparts (*Gsdmc1–4*), and no *Gsdmb* (64). As the name implies, the family members are constitutively expressed in the skin and mucosal epithelial cells. Although their expression overlaps to varying degrees, the GSDMs are differentially abundant in different sites: GSDMA in the skin, GSDMB in the lung, GSDMC in the gut. GSDMD is also highly expressed in immune antigen-presenting cells, macrophages, and dendritic cells. GSDMs can also be induced in many cell types, but how the expression of these genes is regulated is not known.

The GSDMs share highly conserved N- and C-terminal domains, connected by highly variable flexible linker regions (15) (**Figures 3e** and **4d**). They are mostly cytosolic and are proteolytically activated to bind to membranes and form pores when the linker region is cut to separate the N-terminal pore-forming domain from the autoinhibitory C-terminal domain (15). The exceptions are GSDMB, which appears to be mostly nuclear, and DFNB59, which has a truncated C terminus. GSDMD membrane damage is triggered by cytosolic sensing of invasive infection or signs of danger that cause the formation of large amyloid-like complexes called inflammasomes, which activates inflammatory caspases (caspase-1 in mouse and human, -11 in mouse, -4 and -5 in human) to cleave GSDMD and activate it to form pores. The inflammatory caspases cut GSDMD in the linker. Some myeloid cell proteases (neutrophil elastase and cathepsin G) also cleave GSDMD directly (65, 66). Two independent groups reported that TAK1 inhibition by the *Yersinia* effector protein YopJ triggers caspase-8-dependent GSDMD cleavage and activation, leading to pyroptosis (67, 68). However, it is unclear whether caspase-8 or another protease directly cleaves GSDMD.

GSDME is cut by caspase-3 after Asp270 and activated during apoptosis, which switches noninflammatory apoptotic death to pyroptosis in GSDME-expressing cells (20, 21). Although GSDME is widely expressed in epithelial cells, many cancer cell lines from the same tissues suppress its expression by promoter methylation, perhaps because pyroptosis of the cancer cell would recruit and activate antitumor immune cells to the tumor. How the other GSDMs are activated is not known. The GSDMs can permeabilize both bacterial and mammalian cell membranes (15, 16). A high-resolution structure of the pore formed by N-terminal GSDMA3 has been solved by cryo-EM (10) (**Figure 3f**; see below). Although the amino acid sequences of the GSDMs and the MACPF domains of complement and PFN are not at all homologous, the GSDMA3 pore

assembles from approximately 27 monomers into a 180-Å pore in the form of a  $\beta$  barrel that closely resembles the structure of the complement and PFN pores. GSDM pores are examples of convergent evolution to a 3-D structure that resembles MACPF/CDC pores.

Up to now, little has been known about the physiological functions of gasdermins except for GSDMD. Polymorphisms or mutations in *GSDM* genes are associated with skin defects, asthma, and hearing loss (69–73). Gasdermin a3 (*Gsdma3*) was identified as a gene whose mutation causes developmental defects in hair follicle and sebaceous gland development. Postnatal activation of *Gsdma3* causes alopecia. Three spontaneous (*Dfl*, *Re-den*, *Rim3*) and six ENU mutagenesis-induced *Gsdma3* (*Fgn*, *Bsk*, *Rco2*, *Ae*, *Gsdma3*<sup>M1Btlr</sup>, *Gsdma3*<sup>I359N</sup>) mutant mouse lines are associated with skin abnormalities. These mice bear either mutations or loss of the C-terminal domain of *Gsdma3* (71, 74–78). However, no skin defects are observed in *Gsdma3*<sup>-/-</sup> mice. Both the *Gsdma3* mutant (*Ae*) and the N terminus of *Gsdma3* similarly induce cell death. These findings suggest these *Gsdma3* mutations are gain-of-function mutations in which the mutant C-terminal domain no longer fully inhibits the N-terminal domain (79). This idea is supported by the *Gsdma3* structure, which showed that these mutations abolish autoinhibitory interactions between the domains to activate *Gsdma3* constitutively (15).

GSDMB is highly expressed in bronchial epithelia, and its polymorphisms are associated with asthma susceptibility (80). However, the pathogenesis may not be related to inflammation. In one study, which needs to be confirmed, GSDMB was nuclear (unlike the other GSDMs) and GSDMB induced airway hyperresponsiveness and airway remodeling by activating the expression of TGF- $\beta$ 1 and 5-lipoxygenase (81).

*DFNA5* and *DFNB59* mutations are responsible for autosomal dominant and recessive non-syndromic hearing impairment, respectively (72, 82). Unlike the other GSDM genes expressed primarily in immune and epithelial cells, *DFNA5/GSDME* is mainly expressed in heart, brain, and kidney, while the *DFNB59* transcript is detected in brain, eye, inner ear, heart, lung, kidney, liver, intestine, and testis (72, 82). *DFNA5*-related hearing loss is attributed to gain-of-function mutation via aberrant splicing of the transcript. *DFNB59* was found to modulate peroxisome function to defend against noise-induced oxidative stress in cochlear hair cells to maintain hair cell and auditory neuron homeostasis (83).

## 2.5. MLKL

Death receptor signaling, mediated by TNF- $\alpha$ , Fas ligand, or TRAIL binding, can trigger diverse outcomes: NF- $\kappa$ B activation of inflammation and cell proliferation, apoptosis, or programmed necrosis (necroptosis), depending on the posttranslational modifications (phosphorylation, ubiquitylation) and availability of receptor-associated proteins and formation of distinct receptor-associated complexes (4, 6). The death receptor-associated kinase RIPK1 integrates these signals. When caspase-8 activation is inhibited, RIPK1 is ubiquitylated and binds to RIPK3 via a homotypic interaction of their RIM homotypic interaction motif (RHIM) domains, which leads to the formation of the necrosome, the execution complex of necroptosis. Necroptosis is a form of programmed necrotic cell death, morphologically characterized by organelle and cell swelling and plasma membrane rupture (84). The necrosome, which appears to assemble into a large amyloid-like supramolecular organizing center (SMOC), phosphorylates and activates MLKL to bind to phosphoinositides on the inner leaflet of the cell membrane and permeabilize it (85–90). MLKL is composed of an N-terminal four  $\alpha$ -helical bundle domain and a C-terminal pseudokinase inhibitory domain (Figure 4e). When RIPK3 phosphorylates the pseudokinase domain, the helical domain is exposed, binds to the cell membrane, and assembles into disulfide-bonded tetramers and probably higher-order oligomers that permeabilize it. How MLKL disrupts the cell

membrane is unclear. There are many  $\alpha$ -helical bundle small peptides expressed in prokaryotes and eukaryotes that oligomerize and disrupt microbial or cell membranes, including the antimicrobial cytotoxic granule peptide granulysin and the mitochondrial outer membrane-disrupting bax, but understanding how they cause membrane permeabilization will probably need to await structural information about the oligomers they form and how they assemble in membranes. RIPK3 can also phosphorylate and activate MLKL and necroptosis independently of death receptor signaling and RIPK1, such as by binding to TRIF or other mediators of interferon signaling or by binding to the intracellular DNA sensor ZBP1 (also known as DAI) (91, 92).

The biological roles of necroptosis are still not completely clear. *Ripk3* and *Mkl1* are found in some vertebrates and mammals, but marsupials lack both genes and carnivores lack *Mkl1*, suggesting that necroptosis is not needed for normal development or effective immunity. In fact, mice deficient in *Mkl1*, the final executioner of necroptosis, develop normally and have no known phenotype or immunological deficit under homeostatic conditions (85, 93). Moreover, *Mkl1* deficiency has more modest effects than *Ripk1* or *Ripk3* deficiency in some of the challenge models that were used to show the importance of necroptosis, suggesting that some of the biological functions attributed to necroptosis are in fact not caused by necroptosis. Necroptosis has been implicated in a broad range of pathological conditions including infection, inflammation, sepsis, ischemia injury, and neurodegeneration (6, 94–98). The in vitro and in vivo models to probe the function of necroptosis generally are somewhat artificial, since they involve genetic deficiencies in key death receptor and apoptosis pathway molecules or other methods to inhibit caspase activation. Many of the studies assessing the importance of necroptosis have relied on inhibition of RIPK1, using inhibitors like necrostatin (Nec)-1 or the more specific inhibitor Nec-1s, or more recently developed inhibitors of RIPK3 (99–101). However, RIPK1 can trigger both apoptosis and necroptosis, and RIPK3 likely also has inflammatory functions that are independent of necroptosis (102–106). The MLKL inhibitor necrosulfonamide, which inhibits MLKL disulfide bonding required for pore formation, is a Cys-reactive drug, which belongs to a class of drugs that are not specific because of their ability to modify cysteine residues on many proteins (107). Perhaps the most convincing (if indirect) evidence for the importance of necroptosis in disease pathogenesis comes from models of infection. Many microbes and cancer cells have elaborated mechanisms to block classical apoptosis, which could consequently favor necroptosis. The identification of viral and bacterial virulence factors that suppress necroptosis and in vivo data showing that *Ripk3* deficiency alters infection in some models suggest that necroptosis contributes to host immunity, although this needs to be explored in *Mkl1*<sup>-/-</sup> mice to determine whether protective effects are in fact mediated by necroptosis or other downstream effects of the RIP kinases (108–111).

## 2.6. Granulysin

Granulysin (GNLY) is a saposin-like, five  $\alpha$ -helical domain lipid-binding protein in killer lymphocyte cytotoxic granules that at high concentrations acts as a human antimicrobial peptide with broad activity against bacteria, fungi, and parasites (2, 112, 113) (**Figure 4f**). Granulysin is expressed in many mammalian species, but not in rodents, which indicates it is not essential for immune defense. Saposins assemble into  $\alpha$ -helical proteins with a common fold that is held together by disulfide bonds. Granulysin is expressed exclusively in some innate (NK), innate-like ( $\gamma\delta$  T, NKT), or adaptive (CD4 and CD8 T) lymphocytes when they become cytotoxic. However, in healthy human donors, granulysin is only expressed in a minority of the cytotoxic lymphocytes that express granzymes or perforin. Granulysin is highly expressed in killer cells in the skin and in decidual NK cells in the placenta during the first trimester of pregnancy, again suggesting a role in protecting the barrier epithelia from infection (114, 115). Exposure of T cells to killed bacteria

or IL-2 family cytokines that use the IL-2 receptor common  $\gamma$  chain (CD132) upregulates granulysin expression in vitro. A small N-terminal lipopeptide from a *Mycobacterium leprae* lipoprotein stimulated dendritic cells to secrete IL-12 and induce granulysin expression in cocultured T cells (116), suggesting that recognition of microbial pathogen-associated molecular patterns (PAMPs) may trigger GNL $\gamma$  expression. However, the microbial molecules (PAMPs), signaling pathways, and transcription factors that upregulate gene expression, other than Stat5, are not known.

Granulysin is processed from a 15-kDa inactive precursor molecule by cleavage at both ends to a 9-kDa active membrane-disrupting protein, probably by cathepsins in cytotoxic granules, where it is stored with perforin and granzymes (112, 113). The mechanism for membrane permeabilization is not known. The homologous, saposin-like amoebapore A protein from *Entamoeba histolytica*, which kills phagocytosed bacteria, dimerizes to expose a His residue near its C terminus that is believed to be important for membrane intercalation (117, 118). Gel filtration experiments suggest that higher-order oligomers (hexamers?) might oligomerize in the membrane. Granulysin has a very positively charged surface and binds to acidic phospholipids and cardiolipin, like gasdermins and MLKL. A patch of four basic Arg residues between helix 2 and helix 3 is important for lipid binding (119). Granulysin is about a thousand times more active at disrupting microbial membranes of bacteria, fungi, and parasites than mammalian membranes and is inhibited by cholesterol in mammalian membranes by an unknown mechanism (2, 119).

At micromolar concentrations granulysin permeabilizes microbial membranes. Such a high granulysin concentration is probably never reached in bodily fluids, except in the skin blisters of patients undergoing severe, often fatal, drug reactions (Stevens Johnson syndrome or toxic epidermal necrolysis) where disease pathology is linked to granulysin-dependent killing of keratinocytes (114, 120). Lower nanomolar concentrations of granulysin permeabilize bacterial membranes to deliver granzymes intracellularly and enable them to kill cell-free bacteria. However, it is unclear whether secreted granulysin, with or without secreted granzymes, efficiently kills cell-free bacteria in vivo. Yet, when killer lymphocytes recognize and kill cells harboring intracellular bacteria and parasites, they not only destroy the host cell but also kill the microbes, in either phagosomes or the cytosol, in a granulysin-, perforin-, and granzyme-dependent manner (121, 122). Granulysin binds to microbial cell membranes and delivers the death-inducing granzymes into the microbes, where they proteolytically disrupt essential pathways needed for viability (electron transport, antioxidant defenses, protein synthesis, and central metabolism) to cause microbial programmed cell death (microptosis) (123). Because of their complementary specificities for microbial and host cell membranes, granulysin and perforin work together to deliver the death-inducing granzymes into both the host cell and microbial cytosol. Some T and NK cells also bind directly to cell-free fungi, form an immune synapse with them, release their cytotoxic granules, and kill fungi in a granulysin-dependent manner (124, 125).

The importance of granulysin in immune defense has been examined by comparing wild-type mice that lack granulysin with GNL $\gamma$ -transgenic mice that express GNL $\gamma$  only in killer cells at levels similar to those in humans (121, 122, 126). In the three reported intracellular infection models (*Listeria monocytogenes*, *Toxoplasma gondii*, and *Trypanosoma cruzi*), GNL $\gamma$ -transgenic mice handled these infectious challenges much better than wild-type mice. In a nonfatal *L. monocytogenes* infection model, bacterial loads were about 2 logs lower in the livers and spleens of transgenic mice. In lethal challenges with the protozoan parasites, most GNL $\gamma$ -transgenic mice survived and cleared the infection. In two tumor models, GNL $\gamma$  protection from lymphoma was modest—in one lymphoma the GNL $\gamma$  transgene did not significantly change survival, while in another lymphoma, 20% of transgenic mice survived a tumor xenograft challenge that was lethal to all wild-type mice. The modest protection of GNL $\gamma$  in tumor models is consistent with its low activity in disrupting mammalian cell membranes.

**Table 3 Assays that distinguish membrane-disrupting cell death pathways**

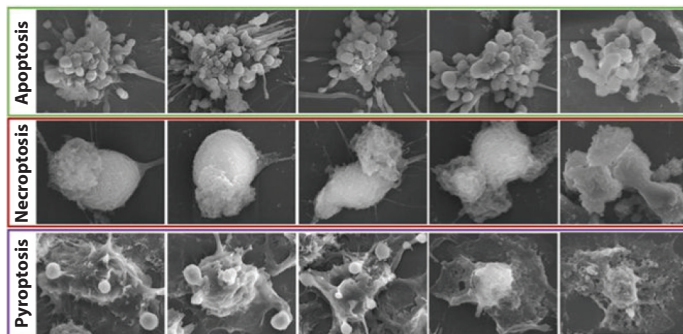
Assay	Apoptosis	Pyroptosis	Necroptosis
Count viable surviving colonies	↓	↓	↓
Measure intracellular ATP	↓ (late)	↓ (early)	↓ (early)
Observe cell morphology	Cell shrinkage, membrane blebbing	Membrane bubbles, cell flattening	Cell swelling, cell membrane rupture
Detect the activation of mediators	Effector caspase cleavage	Inflammatory caspase/ GSDM cleavage	MLKL phosphorylation
Uptake of cell-impermeable small-molecule dyes (SYTOX Green or propidium iodide)	Late	Early	Early
LDH release	Late	Yes	Yes
HMGB1 release	No	Yes	Yes
Annexin V staining	Positive	Positive	Positive
DNA ladder assay, TUNEL assay	++	+/-	+/-

Abbreviations: GSDM, gasdermin; MLKL, mixed lineage kinase domain-like pseudokinase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

### 3. ASSAYS THAT DISTINGUISH MEMBRANE-DISRUPTING CELL DEATH PATHWAYS

Studying membrane-disrupting pathways depends on having reliable ways to monitor and distinguish whether they are activated and cause cell death (**Table 3**). Overall cell death of any type can be quantified by counting the reduction in viable surviving colonies or by measuring the amount of intracellular ATP, which is proportional to the number of viable cells. Death is inflammatory or noninflammatory depending on how rapidly cell membrane integrity is disrupted compared to when the dying cell is recognized in vivo and phagocytosed and on whether membrane damage leads to protein release.

Necroptosis, pyroptosis, and apoptosis can be distinguished morphologically by phase contrast light microscopy with time-lapse imaging or using fixed cells (127, 128) (**Figure 5**). Both apoptotic and pyroptotic adherent cells detach rapidly with similar kinetics as soon as the caspases or gasdermins are activated. In necroptosis, activated MLKL forms ion channels that cause



**Figure 5**

Morphological features of different types of cell death. Adapted from Reference 128.

osmotic swelling. The cell body swells, and the cell membrane suddenly bursts (129). In contrast during pyroptosis, large gasdermin cell membrane pores allow the cell to maintain its osmolarity (127). The damaged membrane forms large ballooning bubbles, which are much larger than apoptotic blebs and easy to distinguish, but the dying cell does not rupture and appears to flatten as its cytoplasmic contents are released and the cytoskeleton and intermediate filaments are proteolytically disrupted (127, 130). Small membrane blebs, cell shrinkage, nuclear contraction, and chromatin condensation are characteristics of apoptosis and killer cell-mediated cell death (5). The induction of each cell death program can be timed by measuring the specific activation of its mediators (cleavage of effector or inflammatory caspases and gasdermins, MLKL phosphorylation) by immunoblot or fluorescent inhibitor of caspase-3 or caspase-1 (FLICA) flow cytometry assays. Necroptosis or pyroptosis can also be confirmed or timed by assessing the translocation of GSDMD or MLKL, respectively, to the plasma membrane using fluorescence microscopy or cell fractionation (15, 16). In addition, downstream processing of important selective caspase-3, granzyme, or inflammatory caspase substrates, such as bid, PARP1, or pro-IL-1 $\beta$ , indicates which cell death pathways have been activated. The specific forms of cell death can also be verified by showing the effect of specific inhibitors, such as DEVD-fmk for classical apoptosis, EGTA for perforin-mediated killer cell death, Ac-YVAD-fmk for caspase-1, disulfiram for GSDMD pore formation, or necrosulfonamide or necrostatin-1 for necroptosis (107, 131, 132).

DNA fragmentation is a hallmark of apoptosis that distinguishes apoptotic from necrotic death. DNA fragmentation is induced by the caspase-activated DNase (CAD) in classical apoptosis (133), which is also activated by granzyme B (134), and by NM23-H1 and Trex1, which are activated by granzyme A (135, 136). CAD activation can be detected by visualizing a DNA ladder assay on agarose gels. CAD- and granzyme-mediated DNA damage can also be visualized by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. An alternative method for examining DNA fragmentation is to measure DNA content by propidium iodide (PI) staining and flow cytometry. Apoptotic cells have sub-G<sub>0</sub>/G<sub>1</sub> DNA content because DNA fragments are released. Although there are no known programs of DNA damage in necroptosis or pyroptosis, DNA breaks are sometimes observed, so detecting DNA damage does not necessarily indicate noninflammatory death.

Loss of plasma membrane integrity is easily assessed by detecting uptake of cell-impermeable small-molecule dyes, such as SYTOX Green or PI, by flow cytometry, plate-reader or fluorescence microscopy. Because these dyes bind nucleic acids, once taken up into cells they bind DNA and stably stain the cells. The release of LDH, a large ~140-kDa tetrameric enzyme that is too large to pass through smaller pores, detected by a colorimetric plate reader assay that measures enzymatic activity, may occur after small-dye uptake, for example, when a necrotic cell bursts. Inflammatory disruption of cell membrane integrity can also be assessed by measuring HMGB1 in culture supernatants by immunoblot or ELISA. Release of processed inflammatory cytokines from immune cells is an indicator of pyroptosis.

Phagocytic cells recognize early signs of membrane disruption by detecting externalized phosphatidylserine (PS) on the dying cell, which is experimentally assessed by annexin V binding. During apoptosis a scramblase transfers PS from the inner to outer leaflet of the plasma membrane; during necroptosis or pyroptosis the calcium influx from the extracellular milieu when the cell membrane is damaged activates the scramblase transmembrane protein 16F to flip PS to the outer leaflet of the cell membrane (136a, 136b). Annexin V staining occurs early in programmed cell death at about the same time the effector caspases or MLKL is activated. Phagocytosis of apoptotic cells or of cells targeted by killer lymphocytes is a key mechanism for avoiding inflammation since cell lysis (i.e., secondary necrosis) of these dying cells is a late event that mostly occurs within phagosomes of phagocytic cells *in vivo* (137–140). By contrast, proteins that cause inflammatory



death (complement, MLKL, and gasdermins) cause cytolysis more rapidly and presumably before or coincident with phagocytosis, which is inflammatory because endogenous danger signals or danger-associated molecular patterns (DAMPs) are released extracellularly instead of being contained within phagosomes (141). In the absence of phagocytic cells in *in vitro* cultures, all dying cells eventually lyse, so distinguishing the different forms of death requires careful attention to the timing of membrane disruption relative to caspase, gasdermin, or MLKL activation.

#### 4. MOLECULAR MECHANISMS FOR REGULATING MEMBRANE DISRUPTION

Each immune pore-forming protein needs to control damage to the cells that express it. Three principal mechanisms are used: (a) expression of a prepore protein that needs to be proteolytically cleaved before it can oligomerize (complement, perforin, perforin-2, granulysin, gasdermins); (b) storage in membrane-delimited compartments under conditions at which it is inactive (perforin, perforin-2, granulysin); and (c) requirement of posttranslational modifications for oligomerization [MLKL (phosphorylation) and possibly perforin (glycosylation)] (Figure 4).

Complement proteins are only activated outside the cell, so the cells that express them are not vulnerable to membrane damage. Extracellular complement activation occurs in three phases—recognition, activation of convertase enzymes that cleave the key complement components C3 and/or C5, and assembly of the MAC leading to cytolysis (142). A large variety of microorganisms, including bacteria, viruses, fungi, and protozoa, become complement targets when the mannan-binding lectin (MBL) recognizes surface glycans (143).

Perforin and granulysin are synthesized as proproteins that need to be cleaved to become active (112, 144). Both are only expressed once a lymphocyte gains cytotoxic capability. They are likely only processed into their active form by cathepsins within killer lymphocyte cytotoxic granules, where they are stored. Cytotoxic granules are very acidic (pH ~ 5); at this pH, perforin is inactive. Moreover, perforin insertion in membranes requires that the C2 domain bind to free Ca<sup>2+</sup>, which is reduced in cytotoxic granules, because they contain calreticulin, which chelates free calcium (144, 145), helping to safeguard the killer cell. Under some circumstances, such as in activated regulatory T cells (Tregs), which express granzymes and perforin, granzymes leak out of cytotoxic granules and kill the activated T cell (146). Self-killing reduces the half-life of activated Tregs, which may explain the transience of adoptively transferred Treg cell lines being developed as a therapy to block transplant rejection. Granzyme leakage might be caused by perforin pore formation in cytotoxic granules in the activated effector cell, but this has not been shown. GNLY is also stored in cytotoxic granules. However, because granulysin does not damage mammalian membranes except at extremely high and probably supraphysiological concentrations, granulysin leakage may not damage the killer cell. Secreted granulysin on its own (at high concentrations) or with granzymes (at lower concentrations) kills cell-free bacteria *in vitro*, but it is inactive against intracellular bacteria because it does not efficiently get into mammalian cells without perforin (2).

Gasdermins and MLKL are both cytosolic with ready access to cell and organelle membranes, but membrane damage is regulated because their membrane-damaging activity needs to be activated. The gasdermins are activated by proteolytic cleavage by caspases. Cleavage in the linker region triggers a radical conformational change of the N-terminal fragment that exposes membrane binding residues and induces oligomerization (15, 16, 127, 147, 148). It is not clear which happens first—membrane binding of a monomer followed by insertion and oligomerization in the membrane or oligomerization to form a prepore that then inserts in the membrane. The cryo-EM structure of GSDMA3 visualized both a membrane-penetrating pore and a 27-mer prepore

structure in which the N-terminal domains had not yet much changed conformation (10). Similar prepores have been seen for the bacterial CDC, pneumolysin (149, 150), and perforin-2 (14). In prepore models, a prepore ring assembles at the membrane before membrane insertion. MLKL is activated by phosphorylation at T357 and S358 (90, 107) by activated RIPK3, which causes a conformational change that triggers formation of MLKL tetramers and possibly higher-order oligomers that organize into amyloid-like fibers and bind to cell membranes (12, 86–90, 151). For both gasdermins and MLKL, activation relocalizes these cytosolic proteins to the cell membrane. Recent evidence suggests that activated GSDMs and MLKL also disrupt mitochondria and other organelle membranes, including possibly the nuclear envelope (152–154). Disruption of these intracellular organelles, especially mitochondria, may promote cell death (153).

Membrane permeabilization by immune membrane-disrupting proteins other than MLKL is not known to be regulated by posttranslational modifications. However, a recent study indicated that GSDME Thr6, which is located in a region important for membrane binding, may be phosphorylated, which inhibits oligomerization, membrane binding, and pyroptosis (153). This Thr is conserved in GSDME, and phosphomimetic mutation of an analogous Thr (Thr8) in GSDMA also inhibits pore formation, suggesting that phosphorylation in this region may inhibit pyroptosis by multiple gasdermins. The kinase(s) that phosphorylate GSDME or pathways that trigger its phosphorylation are not yet known. Other posttranslational modifications of gasdermins, such as ubiquitylation, sumoylation or cysteine oxidation, which regulate other innate immune processes (155, 156), might also be important. GSDMD pore formation depends on a redox-sensitive Cys (Cys191 in humans, Cys192 in mice), whose oxidation will likely regulate pore formation (16, 132, 157).

## 5. LIPID BINDING AND MEMBRANE RECOGNITION

The binding of membrane-disrupting proteins to lipids and cellular proteins influences which membranes they permeabilize. Complement is the only system whose activity is regulated by binding to cell surface proteins, which helps provide target cell specificity. Since complement pore-forming constituents are soluble blood proteins that have access to many cells and tissues, a way to identify microbes or antibody-coated cell targets is needed. C3b complexes serve as both the assembly platform and protease to initiate pore formation (36). In the classical pathway, C3 is activated and targeted to target membranes by antibody opsonization; in the alternative pathway, other features of microbial membranes serve as recognition sites. The complement cascade is initiated when C5 binds to a cell surface enzyme complex that contains activated C3 (C3b), which cleaves C5 to generate a labile fragment C5b. C5b is stabilized by binding to C6 and then binds C7. The C5bC6C7 complex is responsible for irreversible binding to the membrane and then initiates MAC pore formation. The C5bC6C7 complex binds to lipid vesicles composed of any of the abundant phospholipids—phosphatidylcholine (PC), phosphatidylinositol, phosphatidic acid (PA), PS, and phosphatidylglycerol—found in all membranes of prokaryotes and eukaryotes. Therefore, complement can form pores in any membrane, including mammalian cell membranes. Specificity for bacterial versus mammalian membranes is achieved by the presence on mammalian membranes of CD55 (also called decay-accelerating factor) or CD46 (also called membrane cofactor protein), which degrade the C3 convertase or the activated subunit of C3, which initiates the complement cascade on cells; and CD59, which inhibits binding of C8 and C9 to the growing pore (158, 159). CD55 and CD59 are both GPI-anchored proteins. Individuals with genetic deficiency of *PIGA*, an X-linked gene whose product is needed to synthesize GPI anchors, or *CD59* develop a hemolytic disease (paroxysmal nocturnal hemoglobinuria) caused by MAC pores in red blood cell membranes (160).

Perforin does not use accessory proteins for its  $\text{Ca}^{2+}$ -dependent membrane binding. Perforin binds to PC in a  $\text{Ca}^{2+}$ -dependent manner and also binds to immobilized phosphatidylethanolamine (PE) and the phosphatidylinositol phosphates (PIPs), but not phosphatidylserine (PS) (16). In principle, perforin could permeabilize mammalian cell membranes from either the inside (if it leaks from cytotoxic granules in the killer cell) or the outside (when it is released from cytotoxic granules). Although perforin can bind to microbial membranes, it does not form pores in them since pore formation by perforin, like the bacterial CDCs, depends on cholesterol, which is missing from microbial membranes. The other cytotoxic granule membrane-disrupting protein, granulysin, binds to the same phospholipids as perforin, but it also binds to cardiolipin, present on mitochondrial and bacterial inner membranes, and could be especially important for damaging microbial membranes (16). Although cardiolipin is initially transported to inner membranes, it also translocates to mitochondrial and gram-negative bacterial outer membranes, making them vulnerable to granulysin. In contrast to perforin, GNLV membrane damage is inhibited by cholesterol, making it 1,000-fold more active in microbial than mammalian membranes (119). It is not known how cholesterol inhibits granulysin membrane damage. Since granulysin binds to cardiolipin, it might also bind to and damage target cell mitochondrial membranes, as mitochondrial membranes are cholesterol poor and rich in cardiolipin. The distinct lipid preferences of perforin and granulysin mean that cytotoxic granule components could permeabilize and deliver granzymes into any cellular compartment to kill host cells and intracellular microbes, whether they are replicating in membrane-delimited phagosomes or the cytoplasm (2). Whether perforin or granulysin damages mitochondrial, nuclear, or organellar membranes during killer cell granule-mediated death has not been studied. However, the granzymes concentrate in target cell nuclei and mitochondria through not well appreciated mechanisms that do not require perforin (161, 162).

Full-length gasdermins and their C-terminal fragments do not bind to membranes, but their N-terminal fragments bind to cardiolipin and other acidic phospholipids (PIPs, PA, PS) found exclusively on the inner leaflet of the plasma membrane of live cells; however, they do not bind to outer leaflet phospholipids (PE, PC) (15, 16). Thus, N-terminal gasdermins rupture the cell membrane of the inflammatory cell only from the inside and do not damage membranes of bystander cells when they are released during pyroptosis. They may also bind to and damage some organelle membranes, including those of mitochondria (153). The affinity for cardiolipin is higher than for other phospholipids (15, 16). As a consequence, the GSDMD N-terminal domain kills both intracellular and extracellular bacteria, potentially helping control the infections that trigger pyroptosis.

MLKL preferentially binds to PIPs (restricted to the inner leaflet of the cell membrane) via a positively charged patch in the four-helix bundle domain of MLKL that becomes accessible after RIPK3 phosphorylation and oligomerization (88).

## 6. STRUCTURAL BASIS OF PORE FORMATION

The structure of pore-forming proteins in both monomer and pore forms illuminates how these pore-forming proteins function, including what conformational changes are required for membrane insertion, what residues mediate oligomerization and lipid binding to form the pore, and how these interactions may be targeted to disrupt pore formation by genetic mutations, posttranslational modifications, or inhibitors (**Figure 3**). Although solving the structures of monomeric proteins or soluble oligomers is usually not too difficult, solving the structures of large complexes, especially when they are membrane bound, is challenging. Indeed, structures of most of the immune membrane-disrupting monomers have been solved or can be modeled based on homologous

proteins (7, 9, 14, 85, 163). However, solving the structure of the perforin monomer required mutating an amino acid required for multimerization to maintain the protein as monomers at high concentration (9). Until recently the membrane-bound oligomeric structures remained unknown. No structural information is available for oligomeric, membrane-bound granulysin or MLKL. The monomeric structure of both these molecules, like many antimicrobial peptides, consists of a handful of  $\alpha$  helices, and membrane binding is localized to a small patch of positively charged amino acids. It is still not completely clear how many molecules assemble to disrupt the membrane or whether these assemble to span the membrane as a pore. A few biochemical studies suggest that phosphorylated MLKL assembles into octamers that span the membrane to create a small ion channel, but a structural model of membrane-associated oligomerized MLKL would provide a clearer picture (164, 165). Moreover, we need to understand whether formation of an MLKL ion channel causes osmotic swelling and cell membrane rupture.

The development and improvement of cryo-EM methods to tackle membrane-bound complexes have enabled high-resolution structures of three large ( $>100$  Å inner diameter) immune pores: complement, perforin, and gasdermin (8–11) (**Figure 3**). These monomers all undergo radical conformational changes to form similar large  $\beta$ -barrel structures that are hydrophobic on the outside and hydrophilic on the inside and that insert into and span the membrane to puncture it. Each monomer contributes two  $\beta$  hairpins (four  $\beta$  strands) to form the  $\beta$  barrel. These large channels are not ion selective and allow soluble mediators and small- and medium-sized proteins to exit the cell (without any known charge selectivity). Which molecules are released through these pores and which are retained in the dying cell are not known. However, these large pores can release proteins lacking secretion signals, including the processed proinflammatory cytokines IL-1 $\beta$  and IL-18, and other inflammatory mediators, such as HMGB1, and presumably many nonprotein components. Consequently, death by these large pores is highly inflammatory. The exception is killer cell death that generates perforin pores at immune synapses. In this setting, the target cell avoids necrosis by using its membrane repair capability (see below) to rapidly remove disrupted membrane and restore membrane integrity. Because all ions and many proteins can diffuse through these pores, the dying cell maintains its osmolarity and cells dying with these pores are unlikely to burst by hypotonic lysis, as was proposed for pyroptosis before the mechanism of cell lysis was identified.

The recent cryo-EM structure of the MAC (8, 13), solved to 4.9 Å near-atomic resolution, consists of three parts: an asymmetric region (C5b, C6, C7, and C8), a hinge (C7, C8, and two C9 molecules), and a C9 oligomer (**Figure 3b**). The C5bC6C7 complex anchors to the membrane outer leaflet and lowers the activation energy for bending the membrane. C8 binding bends the membrane and C8 traverses the bilayer and recruits C9, which inserts into the membrane to oligomerize to complete the MAC pore, which contains about 18 C9 monomers and has an  $\sim 110$ -Å inner diameter. In the C9 transition from the soluble monomer to the pore form, two helical regions, TMH1 (transmembrane hairpin 1) and TMH2, unravel and insert into the membrane as amphipathic  $\beta$  hairpins (166). There is no evidence that complement MAC forms a prepore intermediate before membrane insertion.

Perforin multimerizes to form a homomeric cylindrical pore, whose structure was solved by cryo-EM at  $\sim 28$  Å resolution a decade ago (9) (**Figure 3d**). There is as yet no atomic resolution structure of the pore form of perforin. Most perforin pores in the early landmark study were composed of 19–24 subunits and had an inner diameter of 130–200 Å. The perforin MACPF domain at the N terminus of the molecule is followed by an epidermal growth factor (EGF) domain and a type II C2 Ca<sup>2+</sup>-binding domain that mediates membrane binding (9). The MACPF domain of perforin is formed from a bent and twisted four-stranded  $\beta$  sheet flanked by

two clusters of amphipathic  $\alpha$  helices named CH1 and CH2, which extend into amphipathic  $\beta$  hairpins that traverse the membrane to oligomerize and form pores.

Structures of monomeric full-length GSDMA3 and murine and human GSDMD revealed common features (15, 167) (**Figure 3e**). The globular pore-forming N-terminal domains and globular inhibitory C-terminal domains are connected by a disordered linker, which is the site of proteolytic cleavage, which frees the functional N-terminal gasdermin to form pores. Mutations that disrupt the interface between the two regions cause inflammatory syndromes. It is unclear whether because of weaker binding of the two halves of the protein, the full-length mutated protein assembles into pores or becomes more susceptible to cleavage to the active N-terminal fragment. The high-resolution ( $\sim 3.8$  Å) cryo-EM structure of the GSDMA3 pore in a cardiolipin-containing bilayer is the only resolved gasdermin pore structure (10). Most GSDMA3 pores showed 27-fold symmetry to form a large  $\beta$  barrel composed of 108  $\beta$  strands with an  $\sim 180$ -Å inner and 280-Å outer diameter cylindrical structure. As with other pore-forming proteins, the monomer undergoes a radical conformational change from the globular N-terminal domain to a hand-shaped structure whose fingers are the  $\beta$  strands that penetrate the membrane in the pore form. Mutational studies indicated that a basic patch of three Arg residues in the N-terminal  $\alpha 1$  helix binds to membrane lipids. Although the pores formed by GSDMA3 and MACPF domain proteins assume a similar final  $\beta$ -barrel structure, a comparison of the order of  $\alpha$  helices and  $\beta$  sheets and the mechanism of membrane insertion of GSDMA3 and pneumolysin [a CDC whose pore structure has been solved (150)] suggests that GSDMs probably evolved independently of MACPF proteins to produce a protein with a similar final pore structure.

## 7. MEMBRANE REPAIR RESPONSES TO MEMBRANE DAMAGE

Not all cells in which pore-forming proteins are activated die. Eukaryotic cells respond to plasma membrane damage with a stereotypic membrane repair response, sometimes called cellular wound-healing, that can rapidly restore membrane integrity (168, 169). Many of the molecules that regulate vesicular trafficking in cells have  $\text{Ca}^{2+}$ -sensing domains that react rapidly to the sudden rise in intracellular  $\text{Ca}^{2+}$  that occurs when membrane integrity is breached. If the membrane's damage is not severe, its integrity can be restored in less than a minute by a combination of three processes: (a) patching of damaged membrane using membranes donated by intracellular organelles, especially lysosomes, that move to and fuse with the damaged membrane; (b) endocytosis of damaged membrane mediated by flotillin (caveolae)- and/or clathrin-dependent pathways; and (c) exocytosis of damaged membrane in a process that depends on the endosomal sorting complexes required for transport (ESCRT).  $\text{Ca}^{2+}$ -dependent membrane repair is known to be mobilized in response to pores formed by bacterial CDCs, complement, perforin, gasdermins, and MLKL (43, 170–174). Without membrane repair, membrane permeabilization, especially by large pores like those formed by complement, perforin, and gasdermins, leads to necrosis and inflammation, because of the release of cytokines, chemokines, and other danger signals. However, in some circumstances, cell death can be averted or converted to noninflammatory apoptosis and the dying cell can continue to live and perform its functions or even become activated. After some inflammasome-activating stimuli in macrophages and dendritic cells, GSDMD is cleaved to form pores through which IL-1 $\beta$  is secreted, but the cells survive because of ESCRT-dependent membrane repair and become activated for phagocytosis and antigen presentation (172, 175–178). Because of their preserved functions and secretion of inflammatory cytokines and chemokines, these surviving immune sentinel cells have been termed hyperactivated. Similarly, cells triggered to undergo necroptosis that survive because of ESCRT-dependent membrane repair release more cytokines and chemokines than cells that die (173). As a consequence,

adaptive immune responses are enhanced compared to those generated when membrane repair is inhibited. Complement- and perforin-mediated necrotic death are very concentration dependent (43). At lower concentrations, damaged cells survive because of membrane repair. In the case of perforin-dependent killer lymphocyte-mediated death, the membrane repair process is required for delivering granzymes into the target cell—perforin pores in the plasma membrane lead to accelerated clathrin-dependent coendocytosis of granzymes and perforin from the damaged target cell membrane at the immune synapse (43–45). However, the target cell saved from necrosis still dies. Although the cell membrane barrier is restored, endocytosed perforin forms pores in endosomes, releasing granzymes into the target cell cytosol, where they activate programmed cell death that morphologically resembles apoptosis.

What determines whether the cell is resuscitated and avoids necrotic death is not understood. These outcomes presumably depend on the extent of membrane damage, perhaps on whether it is localized and the effectiveness of membrane repair. With perforin, plasma membrane repair seems to succeed in most target cells under physiological conditions and is critical for granzyme-mediated programmed cell death. In contrast, the outcome in cells attacked by complement seems to be mostly cell lysis without much of a role of plasma membrane repair. The outcome might be different because soluble complement components typically form pores scattered throughout the membrane, while perforin causes damage localized to a small area within the immune synapse. Localized damage may be easier to repair. Cell resuscitation by membrane repair presumably also depends on how robust membrane repair pathways are. However, whether these repair pathways are regulated has not been studied.

## 8. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Membrane-disrupting proteins play crucial roles in immune responses to infection, cancer, and signs of cellular damage. Recent studies identified novel membrane-disrupting proteins that act, either alone or in conjunction with other immune effector enzymes, especially granzymes, to cause a multiplicity of different forms of cell death. Some of these proteins (complement, perforin, perforin-2, and gasdermins) form large  $\beta$ -barrel pores, whose structures have just begun to be elucidated by cryo-EM. These pores release all sorts of cytosolic (and possibly noncytosolic) molecules that can cause inflammation and recruit other immune cells to sites of danger. The membrane-associated structure of others (MLKL, granulysin) is unknown; they may form smaller ion channels or perturb the membrane in other ways. Small channels can cause osmotic lysis, which can be highly inflammatory as cells burst, releasing their contents. In all cases of membrane damage to host cells, the targeted cell has the chance to survive if the membrane damage is repaired by the ubiquitous membrane repair response. As far as is known, bacterial cells have no comparable membrane defense. Some membrane-disrupting proteins are specific for bacterial versus host cell membranes, and some damage proteins from the outside and others from the inside. Cell target specificity is mostly (except for complement) not mediated by recognition of protein receptors, which means that target cells cannot escape by downmodulating expression of a receptor but have specificity determined by binding to specific membrane lipids or by the cholesterol content of the membrane, which may alter membrane stiffness.

There is still so much we do not know about these powerful proteins. Structural studies are needed to really understand how granulysin and MLKL damage membranes. Very little is known about how the expression of these membrane-damaging proteins is regulated and what cells express them. Additional mechanisms for protecting the expressing cell and regulating membrane damage likely exist and need to be investigated. Little is known about what happens to intracellular membranes of nuclei, mitochondria, endosomes, phagosomes, lysosomes, and other organelles

when these molecules are activated. Are these membranes also damaged, causing organelle dysfunction, and does their damage contribute to cell death or inflammation? Which molecules are released from cells attacked by these membrane-disrupting proteins? Do they differ according to the mediator of membrane damage, and which released molecules are most inflammatory? Importantly, little is known about how much the newly described membrane disruptors contribute to immune defense and pathology in vivo. For example, because in vivo models of necroptosis rely heavily on gene knockouts, the normal physiological role of necroptosis in normal immune defense and pathology is not well understood. Almost nothing is known about the functions of perforin-2. As we understand better the mechanisms behind membrane damage and the roles of these membrane-perturbing proteins in disease, can we develop small-molecule inhibitors or antibodies or harness their clever strategies for killing bacterial or mammalian cells or delivering other molecules inside cells for therapeutic purposes? The next few years should see a lot of research to answer these questions.

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