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REVIEW

Pyroptosis: Induction and inhibition strategies for immunotherapy of diseases

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Abstract Cell death is a central process for organismal health. Pyroptosis, namely pyroptotic cell death, is recognized as a critical type that disrupts membrane and triggers pro-inflammatory cytokine secretion *via* gasdermins, providing a robust form of cytolysis. Meanwhile, along with the thorough research, a great deal of evidence has demonstrated the dual effects of pyroptosis in host defense and inflammatory diseases. More importantly, the recent identification of abundant gasdermin-like proteins in bacteria and fungi suggests an ancient origin of pyroptosis-based regulated cell death in the life evolution. In this review, we bring a general overview of pyroptosis pathways focusing on gasdermin structural biology, regulatory mechanisms, and recent progress in induction and inhibition strategies for disease treatment. We look forward to providing an insightful perspective for readers to comprehend the frame and challenges of the pyroptosis field, and to accelerating its clinical application.

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1. Introduction

As a critical step of life process, cell death is strictly regulated by multiple mechanisms to maintain physiological homeostasis[1]. Traditional cell death types can be divided into programmed cell death and nonprogrammed cell death (namely, necrosis). Different from necrosis caused by traumatic injury, programmed cell death is the result of a series of molecular events triggered by diverse endogenous and exogenous signals[2]. Pyroptosis is a newly identified programmed cell death pathway mediated by gasdermins, and has been gradually recognized as a key target against pathogen infection, cancer, inflammatory disease and so on[3].

Early in 1986, anthrax lethal toxin-induced cell death and cell contents leakage were first observed by Friedlander in primary mouse macrophages[4]. In 1992, ICE (interleukin-1β-converting enzyme) discovered in 1989, was identified as an inflammatory caspase with the function of converting precursor IL-1β to mature IL-1β[5,6]. In the same year, the phenomena of pyroptosis was found by Zychlinsky et al.[7] for the first time in *Shigella flexneri*-infected human macrophages, but was misclassified as apoptosis. In 1996, it was reported that invasion plasmid antigen B (ipaB) of *Shigella flexneri* activated ICE (caspase-1) through a direct interaction in infected macrophages, showing a different cell death mechanism from caspase-3/7/9-dependent apoptosis[8]. In 2001, the term of pyroptosis was first proposed by Cookson and Brennan to describe this proinflammatory programmed cell death with characteristics of pore-forming, membrane rupture and intracellular contents leakage, to distinguish from noninflammatory apoptosis[9]. In 2002, the multiprotein complex inflammasome was thought to activate pro-inflammatory caspases and the precursor IL-1β[10]. Notably, until 2015, gasdermin D (GSDMD) was identified to be a key pyroptosis executor, after cleaving by caspase-1 or caspase-11/4/5[11]. In addition, pyroptosis was further defined as gasdermin-mediated programmed cell death[12]. Since then, other gasdermins and related cleavage mechanisms have been dissected successively[13]. This progress rapidly promotes the translation of pyroptosis mechanisms to diseases treatment.

In this review, we bring a general overview of the pyroptosis pathway focusing on gasdermin structure biology, regulatory mechanisms, and recent progress in induction and inhibition strategies for disease treatment (Fig. 1). The part of induction strategies highlights the innovative means of gasdermin protein, DNA, RNA, reactive oxygen species (ROS), ion overload and novel small molecules as potent pyroptosis triggers for cancer immunotherapy. And in the part of inhibition strategies, the immune escape mechanisms of pathogens and artificial approaches including antibodies, small molecule inhibitors and ROS scavengers for pyroptosis-related inflammatory disease therapy are summarized in detail. Finally, we discuss potential challenges and promises in pyroptosis-targeting therapy. We look forward to providing an insightful perspective for readers to comprehend the frame and challenges of the pyroptosis field, and to accelerating the clinical application.

2. Pyroptosis pathways

Pyroptosis is recognized as a gasdermin-mediated lytic immunogenic form of programmed cell death. Clarifying the pathways involved in manipulating gasdermins has clearly been the core foundation for promoting therapeutic applications. Currently, multiple pathways including caspase and granzyme-mediated types have been discovered. Meanwhile, structural biology studies of gasdermins and related complexes have further illuminated the molecular mechanisms driving gasdermin activation and pore formation. In this section, pyroptosis-related structural biology and regulatory pathways are summarized to provide valuable insights into therapeutic strategies against inflammatory diseases, cancers and other conditions.

2.1. *Gasdermin protein family and structure*

The human gasdermin superfamily encoded by six paralogous genes includes gasdermin A (GSDMA), gasdermin B (GSDMB), gasdermin C (GSDMC), GSDMD, gasdermin E (GSDME, also named as DFNA5), and DFNB59 (also named as pejvakin, PJVK), which exhibit overall 23.9%−49.4% sequence similarity[14,15]. In addition to DFNB59, gasdermin family proteins share a conserved architecture containing a pore-forming N-terminal domain (NTD) and an inhibitory C-terminal domain (CTD) (Fig. 2). Recently, a series of fungal gasdermins and bacterial gasdermins (termed bGSDMs), which possess a quite similar architecture to that of mammal, were also discovered[16-18]. Gasdermins are autoinhibited in the resting stage[19]. In response to endogenous or exogenous stimuli, the interdomain linker region between the NTD and CTD can be proteolytically cleaved to liberate NTD fragments[20]. Through direct interactions with inner membrane lipids such phosphatidylinositide and phosphatidylserine, NTD fragments oligomerize to form pore structures and undergo remarkable conformational changes[20,21]. The pore structures (with inner diameters ranging from 15 to 21.5 nm[17]) in the cell membrane trigger pyroptosis, inducing osmotic cell swelling, membrane rupture, and the release of intracellular contents and cytokines (IL-1β and IL-18) to ultimately cause proinflammatory cell death[22].

In contrast to the conserved NTD and CTD, the interdomain linker regions are highly variable in sequence and length, indicating the cleavage of gasdermins by diverse proteases[20]. To date, the identified proteases involved in gasdermin cleavage mainly include caspases, granzymes, streptococcal pyrogenic exotoxin B (SpeB) and neutrophil elastase (Fig. 2). For GSDMA, only one cysteine protease SpeB, from virulence factors of group A *Streptococcus*, was recently identified to provoke pyroptosis in skin keratinocytes by cleaving human GSDMA (hGSDMA) at Gln246 and Leu244[23,24]. Three tandem alleles are expressed in mice, namely, mGSDMA1, mGSDMA2 and mGSDMA3. All of mGSDMA can be cleaved by SpeB at multiple sites (mGSDMA1 is identical to hGSDMA), suggesting a functional redundancy[23,24]. Notably, GSDMA in nonmammals including birds, amphibians, and reptiles, was recently found to be cleaved by host-encoded caspase-1 at a site similar to that of GSDMD[25]. Cyro-EM-based analysis revealed that the structural transition of mGSDMA3 NTD from the autoinhibition to the pore conformation is accompanied by the formation of two new membrane-inserted β-hairpins (Fig. 2)[26]. Additionally, the generation and stabilization of symmetry pore complexes (β-barrels) are associated with multiple factors: 1) The positively charged α1 helix to interact with acidic lipids, which is masked by CTD in full-length protein. 2) Extensive hydrophobic and charged interactions derived from neighboring inserted β-hairpins and globular domains. 3) In the inserted β-strands, residues facing the membrane are hydrophobic, while those facing the pore are mostly hydrophilic or charged[26,27]. The pore structure of mGSDMA3 might represent a relatively universal model for other gasdermin proteins.

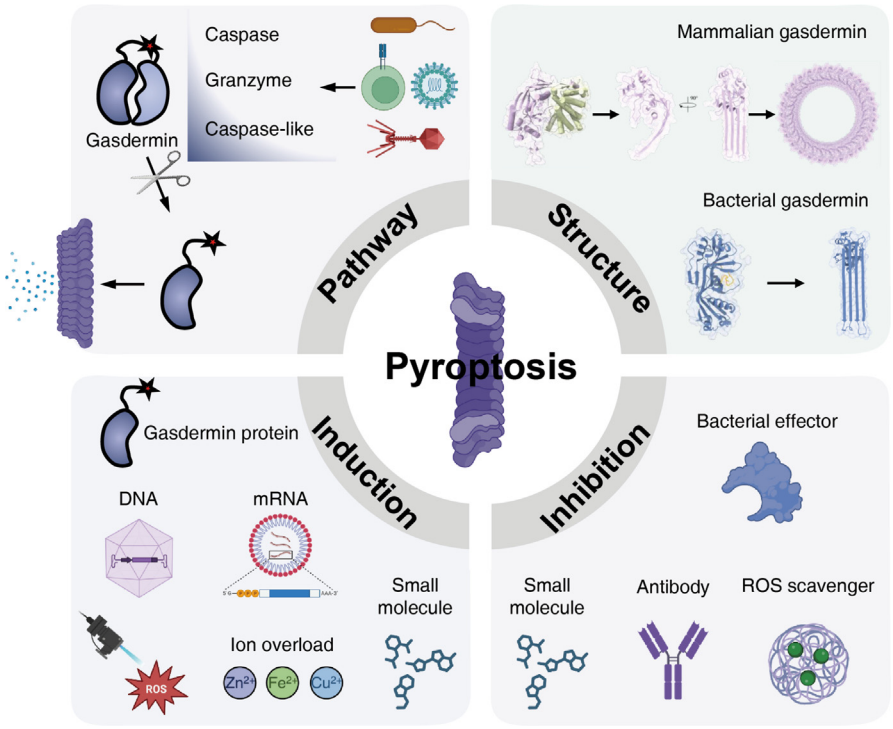


Figure 1 A schematic diagram of an overview of pyroptosis with a focus on gasdermin structure biology, regulating pathways, and induction and inhibition strategies. The image was created with BioRender. com.

Distinct from other gasdermin family members, human GSDMB (no mouse ortholog) contains five splicing isoforms (isoforms 1−5) with contrasting pyroptotic activities, due to the alternative splicing of exons 6 and 7[28-32]. The rule of isoform numbering refers to the work of Prof. Shao[29]. Isoforms 1−4 of GSDMB possess diverse interdomain linkers, identical CTDs and the core regions of the NTD. Isoform 5 lacks an interdomain linker and 28-residue fragment in the NTD and 58-residue fragment in the CTD[29]. Only isoforms 3 and 4 exhibit pyroptotic activity owing to the presence of an exon-6-derived sequence lying in the terminus of NTD and interdomain linker[28,29]. The fragment encoded by exon-6 forms β10 and the ordered tail of the GSDMB NTD is in an autoinhibited state. After cleavage by granzyme A (GzmA) at a conserved Lys site behind exon-6 in isoforms 3 and 4 (Fig. 2), oligomerization of the GSDMB NTD is promoted by the β9 (derived from exon-6) through hydrophobic interactions. In addition, alteration of key hydrophobic residues (such as Ile and Phe) in exon-6 abolishes the pyroptotic activities of isoforms 3 and 4[29,31,33].

For GSDMC, caspase-8 is identified to cleave human GSDMC at a conserved site of Asp365 across species (Fig. 2)[34]. However, when treated with α-ketoglutarate (α-KG), caspase-8 also cleaves human GSDMC and mouse GSDMC4 at site of Asp240 and Asp233, respectively[35], indicating a stimulus or cell type-dependent cleavage mechanism. As the most studied member, multiple proteases have been found to cleave GSDMD (Fig. 2)[22], including human caspases 1, 4 and 5 (mouse caspases 1 and 11)[11], caspase 8[36], cathepsin G[37], neutrophil elastase[38] and the Zika virus protease NS2B3[39]. Structural studies revealed that caspases 1, 4 and 11 possess a hydrophobic exosite to bind to GSDMD CTD, which is independent of the NTD and cleavage site in the interdomain region[40,41]. The CTD binding further promotes the dimerization of caspases to approximate the catalytic pocket to the tetrapeptide cleavage motif of the interdomain linker. The caspases mentioned above all cleave human GSDMD at the site of Asp275 (Asp276 of mouse), but trigger the release of different properties of cytokine (IL-1β, IL-18) due to differences in substrate specificity (Fig. 3)[11,22,40,42,43]. Except for caspases, GSDMD expressed in neutrophils can also be cleaved by the serine proteases cathepsin G and neutrophil elastase at several sites in the interdomain linker to release NTD, which is then inserting into neutrophil membranes of the nuclear, organelles and plasma[37,38].

Discriminating from other gasdermin family members, GSDME is found to be simultaneously involved in apoptosis and pyroptosis in a manner dependent on its expression level, owing to caspase-3[44-46]. Human GSDME with high expression is cleaved by caspase-3 at Asp270 to switch the form of cell death from noninflammatory apoptosis to pyroptosis[44]. In addition, granzyme B (GZMB) also directly cleaves GSDME at the same site (Asp270) as caspase-3, to function as an executor of cytotoxic lymphocyte-mediated death[47]. The last member, DFNB59, possesses a truncated CTD and its regulatory mechanism still remains unknown.

While, recent researches identified a kind of gasdermin homolog encoded in bacteria and fungi *via* bioinformatics, which might provide a new insight into DFNB59 (Fig. 2)[16-18]. Structural analysis revealed that some bacterial gasdermins (bGSDMs), such as *Vitiosangium* sp. and *Bradyrhizobium tropiciagri*, possess a lipophilic NTD with palmitoylation homologous to that of mammalian counterparts, and a much shorter CTD (approximately 20 residues) similar to that of DFNB59. In the autoinhibition state, the short peptide CTD wraps around the bGSDM NTD to restrict its interaction with the cytoplasmic membrane. Notably, the

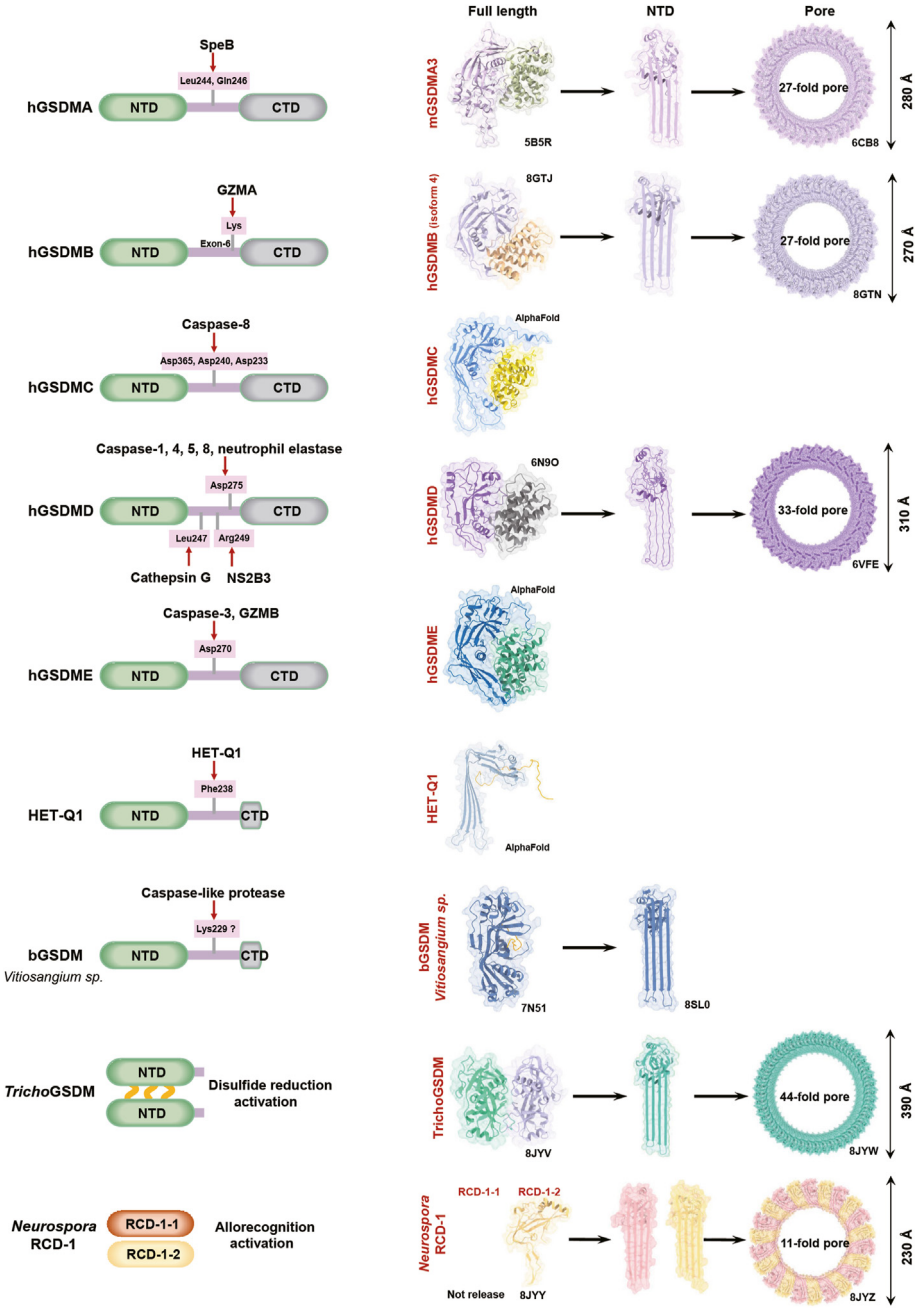


Figure 2 Activation mechanisms and structural biology of gasdermins and gasdermin-like proteins.

bGSDM NTD shows an identical conformation with that of mammalian NTD in the inactive state, despite the absence of the long α-helical CTD. After cleavage by associated proteases (mainly caspase-like peptidases), the bGSDM NTD forms membrane pores to elicit cell death to defend against phage infection, suggesting a bacterial origin for the mammalian gasdermin[16,17]. In addition to bGSDM above, a fungal gasdermin HET-Q1 was recently identified to induce the death of fungi and mammalian cells after the proteolytic cleavage by a subtilisin-like serine protease HET-Q2 at Phe238 (a key residue for HET-Q1 binding)[18]. Upon cleavage, the full-length HET-Q1 releases a ~5-kDa inhibitory C-terminal fragment to acquire the pore-forming activity. The characteristic of short C-terminal fragment is highly analogous to bGSDM, suggesting a conserved and ancient autoinhibition mechanism distinct from that of typical mammalian gasdermins. The structural biology of full length HET-Q1 and assembled HET-Q1 NTD still remains to be explored.

In addition to the canonical gasdermin structure of NTD + CTD, the gasdermin-like proteins *Tricho*GSDM and *Neurospora* RCD-1[48] with only a pore-forming domain have recently been identified (Fig. 2), showing a kind of activation independent of cleavage[49]. *Tricho*GSDM from the basal metazoan *Trichoplax adhaerens* only has an NTD and maintains the autoinhibitory state through disulfides-linked homodimers (three intermolecular disulfides: Cys46−Cys46, Cys47−Cys157, Cys157−Cys47)[49]. Similar to the structure of the autoinhibited

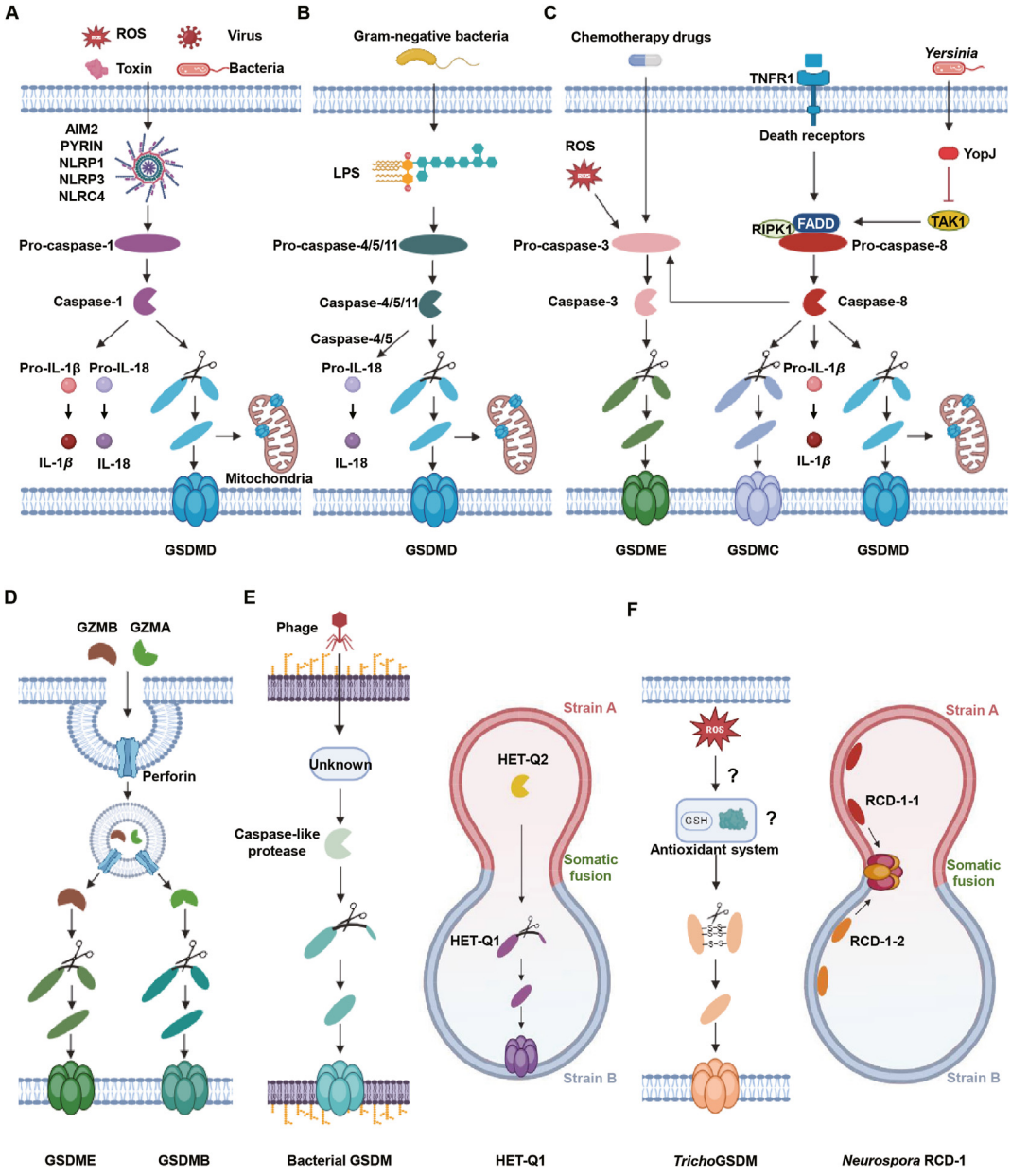


Figure 3 Schematic illustration of the different pyroptosis pathways[64]. (A) Caspase-1-mediated canonical inflammasome pathway. (B) Caspase-4/5/11-mediated noncanonical inflammasome pathway. (C) Apoptotic caspase-3/8-mediated pathway. (D) Granzyme-mediated cleavage. (E) Caspase-like protease-mediated cleavage of bacterial and fungal gasdermins. (F) Cleavage-independent gasdermin activation in the basal metazoan *Trichoplax adhaerens* (*Tricho*GSDM) and the filamentous fungus *Neurospora crassa* (*Neurospora* RCD-1). The image was created with BioRender. com.

gasdermin NTD in mammal, *Tricho*GSDM in homodimers exhibits a typical α/β fold with an extended twisted β sheet. When disulfides are reduced, *Tricho*GSDM switches from homodimers to monomers and further form s44-fold symmetric pores (currently the largest pore) on the liposomes containing cardiolipin. Distinct from *Tricho*GSDM, *Neurospora* RCD-1 adopts two homologous proteins RCD-1-1 and RCD-1-2 to regulate cell death during the allorecognition of filamentous fungus *Neurospora crassa*[48,49]. Both of individual RCD-1-1 and RCD-1-2 are inactive and display a similar structure with a twisted β sheet. Upon coexistence, the interaction of RCD-1-1 and RCD-1-2 generates substantial changes of $\beta2-\beta3$ and $\beta4-\beta5$ linking loops to generate β hairpins and form pores of 11 symmetric RCD-1-1/RCD-1-2 heterodimers (currently the smallest pore). These studies provide novel insight into the mechanistic diversities of gasdermins.

2.2. *Regulation of gasdermins for pyroptosis*

Gasdermins cleavage is precisely regulated by multiple pathways in response to various endogenous and exogenous stimuli, acting as a fundamental part of host immune defense to eliminate pathogens. Up to now, three types of caspases, including inflammatory and apoptotic-associated caspases, have been found to directly regulate gasdermin activity. Except for caspases, granzymes and caspase-like proteinase-mediated pathways are also recently

identified for gasdermins activation in mammals, fungi and bacteria, respectively. These different mechanisms indicate the wide involvement of gasdermins in the host defense system of the tree of life.

2.2.1. *Caspase-mediated cleavage*

2.2.1.1. Caspase-1-mediated canonical inflammasome pathway. Canonical pyroptosis pathway is dependent on the assembly of inflammasomes for the production of mature caspase-1[50]. Inflammasomes are important mediators of the innate immune response to infection and tissue damage. By recognizing molecules derived from invasive pathogens or damaged cells, inflammasomes contribute to coordinating the host defense against pathogens and initiating the inflammatory response that is essential for clearing the infection and promoting tissue repair[51]. Inflammasomes are multiprotein complexes typically composed of three parts: pattern-recognition receptor (PRR), apoptosis-associated speck-like protein (ASC) containing a caspase recruitment domain (CARD) and a pyrin domain (PYD), and inactive inflammatory pro-caspase-1[52]. The PRRs involved in canonical inflammasomes mainly include absent in melanoma 2 (AIM2), PYRIN, and nucleotide-binding oligomerization domain-like receptors (NLRP1, NLRP3 and NLRC4) [53]. When triggered by cytosolic stimuli such as bacteria, viruses and toxins, the PRRs are activated and assembled into hetero-oligomeric-complex inflammasomes to directly recruit pro-caspase-1 or indirectly recruit pro-caspase-1 with ASC (Fig. 3A)[10,54,55]. This assembly process switches pro-caspase-1 from monomers into tight interaction state, resulting in the self-cleavage of pro-caspase-1 into the active heterotetrameric caspase-1[56,57]. Mature caspase-1 proteolytically cleaves full-length GSDMD as well as processing proinflammatory pro-IL-1β and pro-IL-18, leading to pyroptosis and noncanonical secretion of IL-1β and IL-18 through gasdermin pores[11,21,58,59]. Notably, GSDMD cleavage and the processing of proinflammatory cytokines above by caspases are two independent processes (Fig. 3A)[11,60,61]. In addition to the plasma membrane, caspase-cleaved GSDMD by is also capable of rapidly damaging both the inner and outer membranes of mitochondria by binding cardiolipin, resulting in mitochondrial content release and ROS induction[62]. This in turn enhances the intensity of inflammasome activation to increase GSDMD cleavage. Moreover, mitochondrial pore formation occurs as soon as GSDMD is cleaved prior to plasma membrane damage, indicating a vital role of mitochondrial damage in pyroptosis by accelerating and amplifying related cell death signals[62].

2.2.1.2. Caspase-4/5/11-mediated noncanonical inflammasome pathway. Different from canonical NLR-ASC-caspase-1 paradigm, inflammasome assembly is absent in non-canonical inflammasome signaling which is directly mediated by caspase-4/5 (human), and caspase-11 (mouse ortholog) (Fig. 3B). Noncanonical inflammasome signaling plays a vital and unique role in the resistance to gram-negative bacteria such as *E. coli*, by virtue of detecting intracellular lipopolysaccharide (LPS, a component of cell walls). In the absence of classical PRRs, pro-caspase-4/5/11-self function as LPS sensors with their carrying CARDs, which further induces caspase-dimerization and self-cleavage activation to cleave GSDMD. Besides, extracellular LPS is mainly recognized by membrane-bound Toll-like receptor (TLR4) to stimulate type I interferon *via* the TRIF adaptor, which subsequently upregulates the expression of caspase-4/5/11[63,64]. This feedback loop represents a dual and interconnected LPS-sensing mechanism to defend against pathogens in different invasion stages. Another difference relative to caspase-1 is that caspase-4/5/11 specifically cleave GSDMD and display an extremely low efficiency in processing pro-IL-1β. With respect to IL-18, caspase-4/5, not caspase-11, display a comparable pro-IL-18-cleavage activity relative to caspase-1 (Fig. 3B)[42,43]. However, IL-1β is recognized as a gatekeeper cytokine for inflammation regulation and neutrophil recruitment against pathogens. To maximize the secretion amounts of IL-1β and IL-18, K^+ efflux and mitochondrial ROS elicited by GSDMD NTD pores in the membrane of cells and mitochondria, act as a compensatory stimuli to activate canonical inflammasomes such as NLRP3, and downstream caspase-1, enabling sufficient cytokine release[62,65,66].

2.2.1.3. Apoptotic caspase-3/8-mediated pathway. Caspase-3 is generally recognized as a hallmark of apoptosis[67]. In 2017, Shao and coworkers first revealed that pyroptotic death of cancer cells can be induced by chemotherapy through caspase-3-mediated GSDME cleavage[44]. This study provides new insight into cross-talk between pyroptosis and apoptosis bridged by apoptotic caspases. Chemotherapy, such as cycloheximide, only induce caspase-3-mediated pyroptosis in cells with a high GSDME expression level (Fig. 3C). Secondary necrosis develops after apoptosis in cells without sufficient GSDME, indicating that the loss of membrane integrity caused by secondary necrosis in apoptotic cells is not eliminated by efferocytosis[45,46]. ROS generated by endogenous or exogenous stimuli also act as caspase-3 activators for GSDME cleavage (Fig. 3C)[68]. Notably, normal tissues appear to express higher levels of GSDME than that of the majority of tumors due to immune evasion, implicating a potential intervention for alleviating chemotherapy-induced tissue damage[69]. In 2018, another apoptotic caspase, namely, caspase-8, was also shown to be involved in cleaving GSDMD for pyroptosis macrophages in the context of *Yersinia* infection (a causative agent of plague)[70,71]. Further study revealed that *Yersinia* produces an effector protein YopJ to block the activity of transforming growth factor-β-activated kinase 1 (TAK1), leading to the recruitment of a Fas-associated death domain (FADD)-receptor-interacting serine−threonine protein kinase 1 (RIPK1)−caspase-8 complex to lysosome-tethered Rag-Ragulator (Fig. 3C). Rag-Ragulator acts as a platform to activate caspase-8 for GSDMD NTD release and IL-1β processing, and itself does not influence caspase-8-triggered pyroptosis[70-72]. Except for GSDMD, caspase-8 can also cleave GSDMC to induce a noncanonical pyroptosis of cancer cells in response to TNF-α derived from macrophages, antibiotic chemotherapy drugs and α-KG treatment[34,35]. As Prof. Shao noted, these apoptotic caspase-mediated pathways demonstrate that the form of cell death is not entirely determined by the types of caspases (apoptotic or inflammatory), but by the substrates they recognize and hydrolyze[33].

2.2.2. *Granzyme-mediated cleavage*

Belonging to a family of serine proteases, granzymes are a critical executor of target cell lysis induced by cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells[73]. Upon recognizing the complex of MHC-I loaded with antigen peptides *via* T-cell receptors, CTLs primarily release cytotoxic granules containing granzymes and perforin to kill infected or transformed cells[74]. Granzymes are delivered into the cytosol of target cells through the membrane pores formed by perforin. Given that their activity is similar to that of apoptosis-associated caspases in cleaving substrate proteins, granzymes were previously considered to induce the silent

cell death of apoptosis without eliciting inflammation. The human granzyme family is encoded by five paralogous genes, namely, granzyme A (GZMA), GZMB, GZMH, GZMK and GZMM[75]. Among them, GZMA and GZMB have recently been found to mediate pyroptosis in target cells by directly cleaving GSDMB and GSDME, respectively[33,47]. As the most abundant serine protease in granzymes, GZMA from cytotoxic lymphocytes specifically cleaves GSDMB and eliminates tumor cells in a manner dependent on the GSDMB expression level (Fig. 3D), providing a guide for personalized immunotherapy. Notably, IFN-γ secreted from activated CTLs and NK cells can increase GSDMB expression in tumor cells through surface IFN-γ receptors, indicating a synergistic killing effect[33]. Similarly, GZMB also acts as a tumor suppressor by directly cleaving GSDME at a site identical to that of caspase-3 (Fig. 3D), to induce inflammatory lysis of tumor cells and facilitate immune cell infiltration[47]. Besides, GSDMB and GSDME expression are suppressed in some cancers and strongly positively correlated with patient survival[47,76].

2.2.3. *Caspase-like protease-mediated cleavage of bacterial and fungal gasdermins*

Mammalian, fungi and bacteria might share an ancient and conserved model for regulating pyroptotic cell death, namely caspase-like proteases and gasdermin-like executors, for host defense and other life processes (Fig. 3E). In 2022, Philip J. Kranzusch and coworkers identified a series of gasdermin homologs in bacterial and fungal genomes through a bioinformatic search of genes with similar NTD structures using Integrated Microbial Genomes (IMG) database[16]. Besides, associated proteases that can cleave corresponding bacterial gasdermins were also discovered based on phylogenetic analysis of upstream and downstream protease genes adjacent to bacterial or fungal gasdermin genes. The bGSDMs, such as those from *Bradyrhizobium, Runella* and *Vitiosangium*, exhibit a shared overall structure that is highly homology to the NTD of mammalian counterparts. Moreover, bGSDMs are also cleaved by caspase-like proteases such as *Runella* bGSDM-associated CHAT protease, to release activated NTD which further assembles into membrane pores with diverse pore sizes in response to bacteriophage infection[17]. Currently identified caspase-like proteases mainly include peptidase C14 and CHAT. Other proteases, such as peptidase C1-like, subtilase, peptidase U49 and trypsin-like, also display similar functions in corresponding bacteria. Similarly, in 2022, Daskalov et al. also characterized the gasdermin-like protein HET-Q1 in a filamentous fungus *Podospora anserine* (Fig. 3E)[18,77]. The pore-forming activity of HET-Q1 can be activated by a subtilisin-like serine protease HET-Q2 through proteolytic cleavage. The genes encoding HET-Q1 and HET-Q2 are idiomorphic to control the cell death during heterokaryon formation. These gasdermin-like cell death regulatory pathways reveal an evolutionary conservation in cell death signaling and execution. While, the mechanism involved in the pathway from exogenous or endogenous stimuli, such as phage infection, to caspase-like protease activation for gasdermin-like protein cleavage still remains to be explored.

2.2.4. *Cleavage-independent gasdermin activation*

In addition to the typical cleavage-mediated pathways described above, two atypical cleavage-independent mechanisms have been recently reported. In 2024, Li et al.[49] clarified the cleavage-independent activation mechanisms and assembly structures of two gasdermin-like proteins *Tricho*GSDM (metazoan) and *Neurospora* RCD-1 (fungus) (Fig. 3F). *Tricho*GSDM is a 250-residue protein only containing a pore-forming-domain with 24% sequence similarity to the hGSDME NTD, but shows no toxicity in the resting stage. They further found that *Tricho*GSDM adopts a covalent homodimer strategy (with three intermolecular disulfides) to block the pore-forming activity of monomers. When the disulfides are reduced with GSH or disulfide reductases, *Tricho*GSDM monomers efficiently assemble into pores (currently the largest gasdermin pores) and induce liposome-leakage. This study indicated that the reducing factors or the antioxidant system might be a caspase-like trigger to precisely regulate the *Tricho*GSDM-mediated cell death under certain conditions (Fig. 3F). *Neurospora* RCD-1 encoded by *rcd-1* was first identified to be a kind of gasdermin-like protein in the filamentous fungus *Neurospora crassa* by Daskalov and coworkers in 2020[48]. And *Neurospora* RCD-1 is involved in regulating the cell death during the conspecific nonself discrimination (namely, allorecognition). The *rcd-1* locus encodes agonistic RCD-1-1 and RCD-1-2, both of which show a strong binding affinity towards acidic phospholipids (such as cardiolipin). When somatic fusion occurs between incompatible *Neurospora crassa* strains (respectively expressing with RCD-1-1 and RCD-1-2), the fused cells undergo pyroptotic cell death (Fig. 3F). From the aspect of structural biology, Li et al.[49] revealed that heteropairing of RCD-1-1 and RCD-1-2 functioned as a trigger to induce the heterodimerization and pore formation. Notably, in the resting stage, both RCD-1-1 and RCD-1-2 exhibit membrane binding properties in *Neurospora crassa*. Therefore, the early interaction of RCD-1-1 and RCD-1-2 might directly occur on the membrane during the somatic fusion.

2.3. *Pyroptosis in different cells and diseases*

Pyroptosis has been observed in various cell types and is implicated in an increasing number of physiological (such as host defense) and pathological processes, making it a promising target for the intervention of diseases. Generally, human immune cells exhibit a wide expression profile of gasdermins, possibly due to the vital role of pyroptosis-related pathways in pathogen recognition and elimination[22,78]. Similar gasdermin expression patterns are also observed in respiratory tract, gastrointestinal tract and skin epithelium[22,78,79]. Specifically, GSDMA is mainly expressed in the skin and upper gastrointestinal tract[80]. GSDMB is expressed in immune cells and tissues of the liver, bladder, gut epithelium (such as the small intestine), esophageal epithelium and airway epithelium[22,33,80,81]. GSDMC is expressed in tissues of the spleen, tonsils, skin, gut epithelium (such as the small intestine and colon) and airway epithelium[22,78,80,82]. GSDMD is primarily expressed in immune cells and tissues of the gut epithelium (such as stomach), skin and liver[11,22,83-85]. Distinct from other gasdermins, GSDME and DFNB59 are widely expressed and identified in tissues such as the heart, brain, kidney, skin and gut[22,44,86].

Gasdermin expression patterns might be closely associated (but not predictive) with the pathogenic mechanism and therapeutic potential of pyroptosis. Indeed, pyroptosis has been observed to be involved in a variety of inflammatory diseases, due to excessive inflammation and tissue damage[87]. Pyroptosis-involved diseases mainly include cardiovascular diseases (such as myocardial ischemia/reperfusion (I/R) injury and atherosclerosis)[88], neurological diseases (such as Alzheimer's disease and Parkinson's syndrome)[89], kidney diseases (acute kidney injury, chronic kidney disease and diabetic kidney disease)[22], liver diseases (liver injury and liver fibrosis)[90], autoimmune diseases (systemic lupus erythematosus)[91], sepsis[92] and so on. On THE

other hand, the unique characteristics of pyroptosis in disrupting membrane and triggering pro-inflammatory cytokine secretion make it a potent choice for target cell lysis and immune activation. Pyroptosis induction has displays a preventive and therapeutic effects in multiple cancers including lung cancer (non-small cell lung cancer (NSCLC))[93], liver cancer[94], breast cancer (triple-negative breast cancer (TNBC))[95], melanoma[96], and leukemia[97], etc. Hence, modulating pyroptosis-related pathways with native or artificial strategies might provide novel and efficient interventions for diverse diseases.

3. Induction strategies

The excellent capability for direct membrane destruction and rapid release of proinflammatory cytokines and intracellular contents makes pyroptosis a robust weapon for disease treatment, especially for cancer immunotherapy. Besides, pyroptosis-induced immunogenic cell death is also capable of stimulating macrophages, dendritic cells and T lymphocytes to amplify the inflammatory responses and enhance immune phagocytosis. The current strategies for activating pyroptosis can be roughly divided into two categories: direct induction and indirect induction. The former one mainly focuses on directly adopting gasdermin protein as a trigger of pyroptosis in target cells for disease therapy, especially cancer therapy. Direct induction strategies can overcome the challenge of low or even no gasdermin expression and the need of upstream cleavage elements, which are generally discovered in cancer tissues as an immune escape mechanisms of T and NK cell-mediated cytotoxicity. Indirect activating strategies typically depend on the gasdermin expression levels and the maturity of caspases for gasdermin cleavage, which can be realized by diverse factors, such as ROS, LPS and the imbalance of cytosolic ion homeostasis.

3.1. *Gasdermin proteins as direct triggers*

Full-length gasdermins are inactive. Hence, NTD fragments are predominantly selected for various applications. However, the expression of gasdermin NTD alone is toxic to *E. coli.* In 2020, a bioorthogonal system was developed by Liu and coworkers for gold nanoparticle conjugate-mediated delivery of GSDMA3 NTD, initially demonstrating the therapeutic potential of pyroptosis-induced inflammation (Fig. 4A–C)[98]. To obtain the NTD, an engineered full-length GSDMA3 carrying a PPase cleavage site between the NTD and CTD was expressed and purified from *E. coli*. After digestion with PPase, the noncovalent complex GSDMA3 (NTD + CTD) was obtained and NTD (containing one Cys) was further conjugated to gold nanoparticles using a bioorthogonal responsive linker (Fig. 4A). Upon treatment with cell-penetrable Phe-BF_3, the linker silyl-phenolic ether was bioorthogonally split using a desilylation-induced [1,4] fragmentation process to release GSDMA3 NTD in living cells for generating pyroptosis (Fig. 4A–C). Besides, Phe-BF_3 also displayed a tumor selectivity and enrichment, which is highly compatible with the *in vivo* bioorthogonal application of the GSDMA3 NTD-nanoparticle conjugate. Intratumoral or intravenous treatment with the bioorthogonal conjugate caused the remarkable regression of 4T1 mammary tumors, which was dependent on $CD4^+$ helper and cytotoxic $CD8^+$ T cell-mediated killing and improved the response of checkpoint blockade. Moreover, this study also revealed that a small portion (<15%) of pyroptotic tumor cells are sufficient for tumor elimination, further indicating an efficient antitumor adaptive immunity initiated by gasdermin-mediated lytic immunogenic cell death[98]. Hence, in this context, the direct lysis of partial tumor cells by pyroptosis is just the beginning, and subsequent tumor regression is mainly driven by the lytic cell inflammation-mediated tumor-specific T cells.

Considering the inconvenience in obtaining gasdermin NTD, full-length protein represents another option and cleavage trigger is typically required to release NTD. In 2022, Li et al.[99] developed a bacteria-based full-length GSDMD (GSDMD FL) delivery system (Fig. 4D and E). To shuttle the protein into the cytosol, GSDMD FL was first cross-linked as nanocages (size of 130 nm) using GSH-responsive linkers, which were further conjugated to the bacterial surface using a facile amide reaction (Fig. 4D). To trigger the cleavage of GSDMD FL, an intracellular bacterium, namely, attenuated *Salmonella typhimurium*[100,101], was adopted as a delivery platform, which can efficiently enable the activation of caspase-1 using flagellum[102] (Fig. 4E). The endosomal sorting complexes required for transport (ESCRT) III acts as a potent membrane repair effector to inhibit the damage of membrane integrity induced by endogenous or exogenous factors including pyroptosis[103,104]. The assembly of ESCRT III is directly triggered by Ca^{2+} influx upon the membrane damage[105,106]. In this regard, the authors further designed the Ca^{2+} chelator BAPTA-AM-loaded dextran as an ESCRT inhibitor[107] to enhance the tumor pyroptosis by reducing the Ca^{2+} concentration outside cells (Fig. 4E). The combination of two nanoformulations into hydrogel or cell patches induced the remarkable pyroptosis in multiple tumors, including primary, metastatic and unresectable tumors, resulting in abundant necrotic tumor cells and tumor-specific T-cell responses for the subsequent tumor regression.

3.2. *DNA*

As noted in Central Dogma, DNA- and RNA-based strategies provide various options for developing functional protein drugs using cells as factories, with no need for expression *in vitro*. DNA and RNA vaccines have proved their great potential in fighting against virus infection, which has also led to increased research in other fields[108-110]. In 2020, Zhang et al.[47] revealed that the overexpression of mouse or human GSDME FL with plasmids in tumor cells greatly improved their recognition and killing by tumor-infiltrating lymphocytes which displayed increased GZMB, perforin, IFN-γ and TNF-α expression, relative to the inactive GSDME mutant and empty vector controls. In the same year, similar results were also observed by Zhou et al.[33] that the expression of GSDMB FL with plasmids in CT26 and B16F10 notably promoted antitumor immunity through GZMA-mediated cleavage. These studies together demonstrate the potent tumor suppressor role of intrinsic or extrinsic gasdermin-mediated pyroptosis for the first time, paving the way for the further biomedical applications. In 2021, a recombinant adeno-associated virus (rAAV) containing a GSDMD NTD DNA sequence was packaged using the Sf9/rBac system (rAAV-P1) and Cre/lox recombinase system (rAAV-P2)[111], respectively, to express GSDMD NTD in glioblastoma (C6) and breast cancer (4T1) tissues (Fig. 5A–D)[112]. The promoter mammal CBA (mCBA) was selected to initiate the expression, and did not induce the cytotoxicity associated with leaky expression towards Sf9 insect cells during rAAV-P1 package. To further avoid the toxicity during packaging, Cre/lox-based rAAV-P2 was developed which only initiates GSDMD NTD expression in presence of Cre recombinase expressed by rAAV-Cre. The rAAV-GSDMD NTD was able to induce the pyroptosis of multiple cancer cell lines *in vitro*

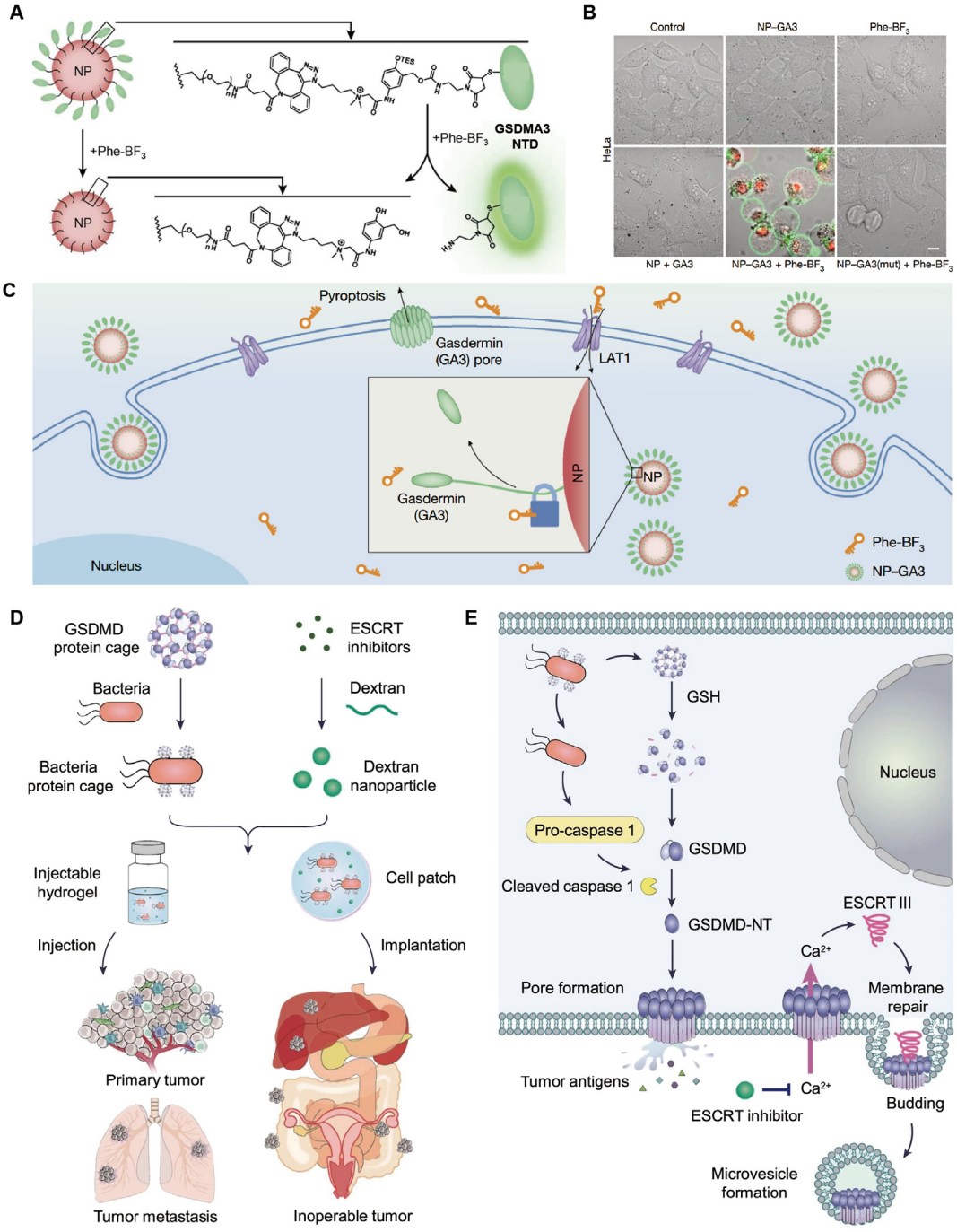


Figure 4 Using gasdermin proteins as pyroptosis triggers. (A) Schematic of Phe-BF_3-responsive release of GSDMA3 NTD from gold NPs using a silyl-ether-carbamate linker. (B) Confocal images of pyroptotic HeLa cells treated with the combination of Phe-BF_3 and GSDMA3 NTD-NP. (C) Schematic of GSDMA3 NTD-NP workflow from cellular uptake to BF_3-triggered GSDMA3 NTD release for pyroptosis[98]. Reproduced with permission. Copyright © 2020, Springer Nature. (D) Preparation and characterization of a pyroptosis-induced hydrogel composed of full-length GSDMD protein cage-anchored bacteria and dextran nanoparticles. (E) The cellular mechanism of synergistic tumor pyroptosis induced by GSDMD protein, bacteria and ESCRT inhibitor BAPTA-AM. Reproduced with permission[99]. Copyright © 2022, Springer Nature.

(Fig. 5C), and the regression of C6 glioblastoma (Fig. 5D) by virtue of entering the brain through temporarily opening the blood−brain barrier[113] and generating pyroptosis-mediated inflammation to efficiently recruit infiltrating lymphocytes. This was also the first validation of an rAAV carrier-based pyroptosis trigger.

Apart from rAVV, plasmid carrier nanocomplex have also directly been adopted as gasdermin expression sources[114-116]. In 2023, Zhong et al.[116] developed the GM@LR liposome containing GSDME FL plasmid and manganese carbonyl (Fig. 5E). The manganese carbonyl acted as a trigger for caspase-3 activation through the released CO. Besides, Mn^{2+} released from manganese

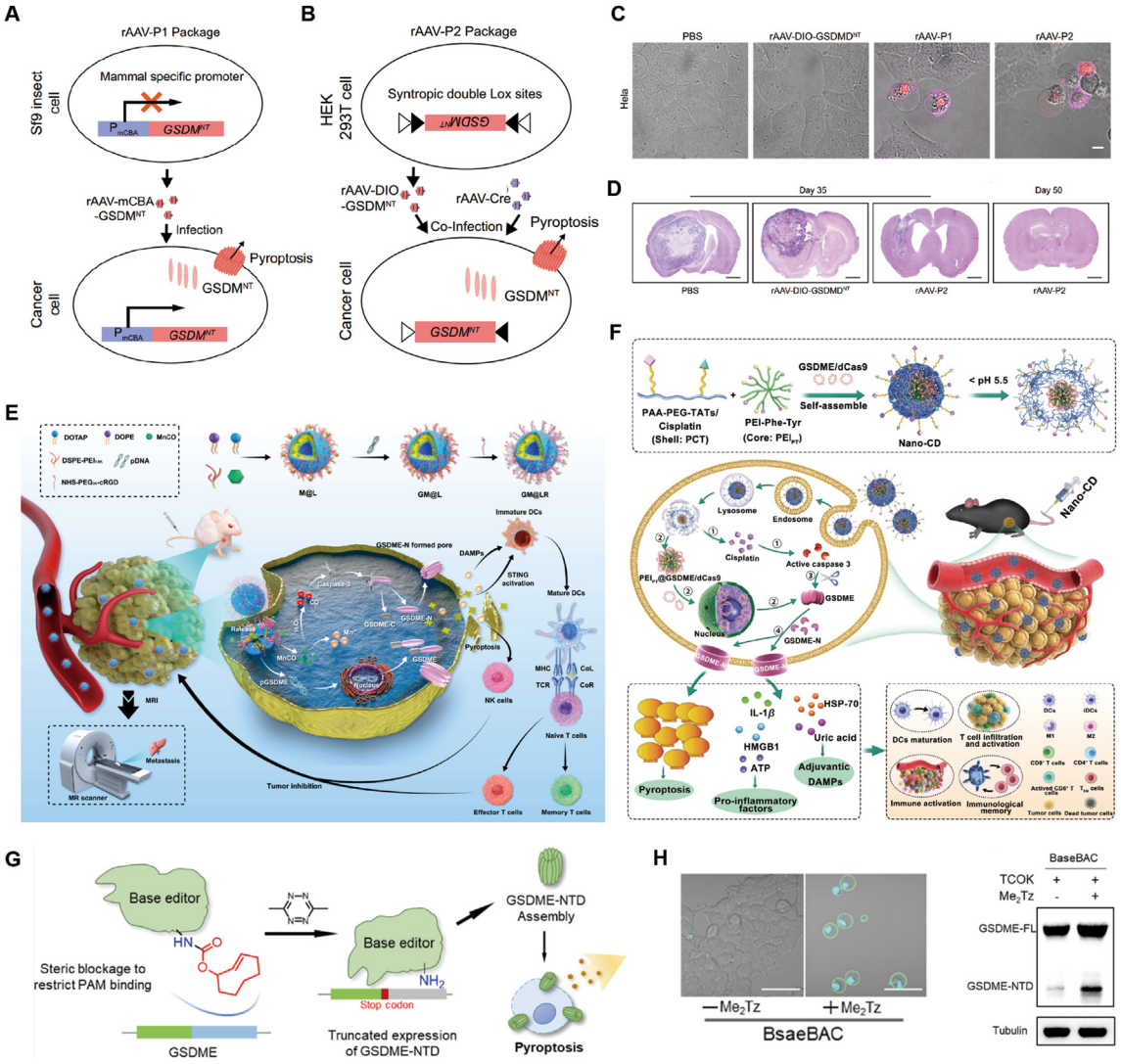


Figure 5 The rAAV, plasmid and CRISPR-based strategies for gasdermin protein expression and pyroptosis induction. (A) Design of the rAAV-GSDMD NTD (rAAV-P1) package using the mammal-specific promoter mCBA in the Sf9/rBac system. (B) Design of the rAAV-DIO-GSDMD NTD (rAAV-P2) package using Cre/lox system. rAAV-Cre reverts rAAV-P2 to initiate GSDMD NTD expression. (C) Confocal images of pyroptotic HeLa cells treated with rAAV-P1 and rAAV-P2. (D) H&E images of C6-luc tumor-bearing rat brain coronal sections after treatment with the indicated therapies. Reproduced with permission[112]. Copyright © 2021, Springer Nature. (E) Schematic diagram of MnCO and GSDME plasmid co-delivered nanodrug GM@LR for inducing pyroptosis[116]. Reproduced with permission. Copyright © 2023, American Chemical Society. (F) Preparation and working mechanism of nano-CRISPR scaffold (Nano-CD) containing chemotherapeutic cisplatin and the GSDME/dCas9 plasmid for endogenous GSDME-mediated pyroptosis. Reproduced with permission[115]. Copyright © 2023, Springer Nature. (G) Schematic diagram of endogenous GSDME NTD expression using the bioorthogonally activatable CRISPR base editor BaseBAC. (H) Confocal images and immunoblotting analysis of pyroptotic HEK293T cells treated with BaseBAC and Me_2Tz. Reproduced with permission[122]. Copyright © 2022, American Chemical Society.

carbonyl could also activate the cGAS-STING pathway[117-120] to cooperate with GSDME-mediated inflammation for the maturation of intratumoral DCs and infiltration of cytotoxic lymphocytes. Inspired by the immune escape of some tumors through down-regulation of gasdermin expression, some studies have sought to upregulate endogenous gasdermin expression through CRISPR/dCas9-based target gene transcription activation[121]. In 2023, Wang et al.[115] designed a Nano-CRISPR scaffold (Nano-CD) containing chemotherapeutic cisplatin and GSDME/dCas9 plasmid (Fig. 5F). Polyethyleneimine (PEI) was modified with amino acids Phe and Tyr to adsorb CRISPR/dCas9 plasmid through electrostatic interactions. The polyplex was further coated with PEGylated polyacrylic acid (PAA) modified with cisplatin and the cell-penetrating peptide TAT to improve the cellular uptake and cytosolic release. Compared with the control, Nano-CDs with an optimized small guide RNA (sgRNA) sequence increased GSDME expression by more than 7-fold. Besides, the protein level of GSDME NTD was also significantly increased *via* the cisplatin−caspase-3 activation−GSDME cleavage axis. The cooperation of self-supplying GSDME and cisplatin efficiently initiated the pyroptosis of B16F10 melanoma to reverse the immunosuppressive TME and boost T-cell responses, validating the potential of CRISPR/dCas9 for pyroptosis-related therapy. In addition, CRISPR-based base editing was also adopted to activate

pyroptosis through the editing of the cellular GSDME gene with a truncated expression of its NTD (from CAA to the stop codon TAA at Glu287) (Fig. 5G and H)[122].

3.3. RNA

Despite the prominent advantages of plasmid DNA vectors in terms of stability and expression efficiency, their application safety remains a concern regarding to the potential risk of exogenous gene insertion into host genomic DNA[123]. In comparison, RNA-based functional protein expression has gained increasing attention. The development of RNA structures[124] and delivery carrier designs[125,126] have greatly improved resistance to RNase. Moreover, the modification combination of 5′ Cap analog[127] and pseudouridine (Ψ) substitution[128] remarkably reduces the immunogenicity of mRNA to bypass cellular RNA sensors[129]. In 2023, Li et al.[130] reported a linear mRNA strategy for intracellularly expressing GSDMB NTD, using an ionizable lipid AA3-Dlin-based liposome formulation (Fig. 6A). The linear mRNA containing the 5′ UTR, GSDMB NTD coding sequence, 3′ UTR and polyA tail was obtained with an *in vitro* transcription (IVT) method and further modified with 5′ cap (a CleanCap analog) for initiating translation, improving expression efficiency and enhancing the stability. Compared with the Cap-0 of Anti-Reverse Cap Analog (ARCA), Cap-1 CleanCap-modified GSDMB NTD mRNA displayed a greater expression in orthotopic 4T1 breast tumors. The GSDMB NTD mRNA@liposome efficiently induced immunogenic pyroptosis in 4T1, HEK 293, HeLa, and B16F10 cells to initiate the leakage of cytosolic components such as HMGB1 and activate BMDCs *in vitro*[130]. When intratumorally injected into aggressive B16F10 and aPD-1-resistant 4T1 tumors, GSDMB NTD mRNA@liposome generated the GSDMB NTD expression *in vivo* to remodel the tumor microenvironment and induce the remarkable regression in combination with a checkpoint inhibitor.

In addition to liposome carriers, extracellular vehicles (EVs) have also been adopted to deliver gasdermin mRNA. As a natural ferry of cargos such as RNA for intercellular communications nearby and distant[131-133], EVs are promising carriers for gasdermin-based therapeutics. Besides, the 'homing' targeting capability[134] to the parent cells can further reduce the side effects of nonspecific organ toxicity induced by leakage expression. In 2023, an EV-based GSDMD NTD mRNA nanoformulation was designed by Xing et al.[135] for the first time to overcome the challenge of therapeutic delivery of GSDMD (Fig. 6B). Moreover, distinct from the IVT method, GSDMD NTD mRNA was generated in EV donor cells (HEK293) using transfection of GSDMD NTD plasmid. The translation of GSDMD NTD mRNA was repressed with puromycin[136] to avoid the pyroptosis-induced cytotoxicity and promote the encapsulation of mRNA into EVs.

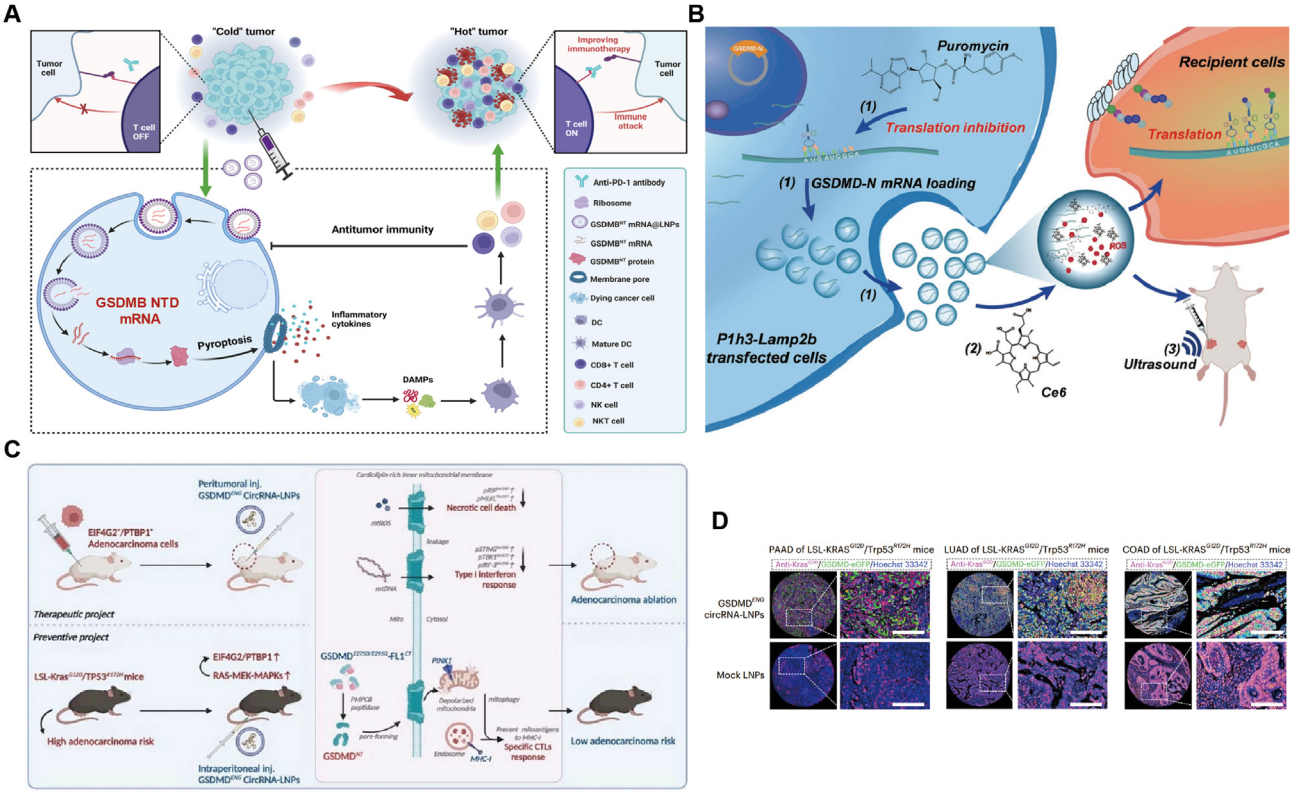


Figure 6 The mRNA-based gasdermin protein expression for pyroptosis induction. (A) Schematic of an intratumoral injection of mRNA liposomes encoding GSDMB NTD for pyroptosis-based immunotherapy combined with anti-PD-1[130]. Reproduced with permission. Copyright © 2023, Springer Nature. (B) Design of an extracellular vesicle-based GSDMD-NTD mRNA delivery system for cancer immunotherapy. Reproduced with permission[135]. Copyright © 2023, Wiley-VCH GmbH. (C) Schematic diagram of GSDMD^{ENG} circRNA-LNPs with dual features of tumor-specific expression and conditional activation through using HRV2-IRES for the treatment and prevention of KRAS mutation-related cancers. (D) Confocal images of immunofluorescent surgical samples after treatment with GSDMD^{ENG} circRNA-LNPs *in vivo*, which showed the colocalization of GSDMD with KRAS^{G12D} proteins in pancreatic adenocarcinoma (PAAD), lung adenocarcinoma (LUAD) and colon adenocarcinoma (COAD) from mice with KRAS^{G12D}/Trp53^{R172H}[138]. Reproduced with permission. Copyright © 2023, Springer Nature.

And puromycin in EVs might be inactivated by ROS generated from coloaded Chlorin e6 (Ce6) to realize the mRNA translation in recipient cells. To improve the targeting ability, a humanized HER2 single-chain antibody P1h3 was fused with Lamp2b (an exosomal membrane protein[137]) to display on the surface of EVs, through an overexpression plasmid. Antibody-anchored significantly enhanced the specific tumor accumulation of EVs, reducing the leaky cytotoxicity. The engineered GSDMD NTD-mRNA EVs effectively suppressed Her2-positive breast cancer *via* pyroptosis to directly induce immunogenic tumor cell killing and generate robust tumor-specific cytotoxic T lymphocytes.

To further avoid the side effects of leaky cytotoxicity in nontarget cells and tissues, Feng et al.[138] constructed a selective mRNA translation strategy using internal ribosomal entry sites (IRESs) from human rhinovirus type 2 (HRV2) (Fig. 6C). The IRES is a highly structured region (approximately 500 nt) in mRNA for triggering the internal initiation of translation, independent of normal cap-dependent processes[139]. IRES-based mRNA translation generally requires IRES-transacting factor (ITAF, such as PTBP1[140]) and the interaction of initiation factors (IF, such as eIF4G2[141]) to recruit the 40S ribosomal subunit and start the translation[142,143]. Based on this requirement, the study adopted an IRES-containing mRNA reporter platform to screen for IRES sequences with conditional expression capabilities towards eIF4G2 and PTBP1 which are highly expressed in tumor tissues. An IRES from HRV2 was shown to specifically trigger the GSDMD NTD expression in cancer cells. However, the HRV2 IRES-GSDMD NTD mRNA encapsulated in liposome did not induce more prominent tumor cytotoxicity than that in normal cells, possibly due to ESCRT complex-mediated membrane repair. To bypass the ESCRT process, a short mitochondrial targeting sequence was added into mRNA to direct the GSDMD NTD into mitochondria and promote cell death through pore-forming-induced mitochondrial damage. To improve the half-life of mRNA, the authors further constructed a circRNA containing the HRV2 IRES-GSDMD NTD using a permuted intron-exon (PIE) splicing strategy[144,145]. The engineered circRNA liposomes, namely, $GSDMD^{ENG}$ circRNA-LNPs, efficiently ablated various adenocarcinomas, gliomas and hematological tumors through mitochondrial content leakage and tumor-specific T-cell response generation. More importantly, the $GSDMD^{ENG}$ circRNA-LNPs could also represents a promising preventive therapy against $KRAS^{G12D}$ mutation-driven adenocarcinogenesis (Fig. 6D), providing a novel application for pyroptosis.

3.4. *Photo-induced ROS*

ROS, namely, reactive oxygen species, generally refer to an array of oxygen derivatives such as H_2O_2 derived from reduction−oxidation and electronic excitation in aerobic life[146]. Under normal condition, the ROS concentration is tightly controlled and maintained at a low level. However, the elevated ROS concentrations can result in molecular damage and even cell death through diverse pathways (oxidative distress). In recent years, an increasing number of studies have revealed a complicated relationship between ROS and cell death such as pyroptosis, ferroptosis[147] and apoptosis[148], providing a novel intervention strategy for pyroptosis. As a central element in regulating cell life, caspases, especially caspase-3, have also been identified as major targets for ROS-induced pyroptosis[149]. Briefly, the generation of excessive ROS can lead to the cleavage of caspases, which further cleave gasdermins to initiate pyroptosis. In this section, we review photo-induced ROS, namely, photodynamic therapy (PDT), for pyroptosis with a focus on membrane-targeting strategies.

The typical photo-induced ROS usually requires photoactive agents, namely photosensitizers (PSs), to absorb photon energy and reach the excited singlet state[150]. After an internal conversion process, the excited triplet state of PSs is formed and acts as the main type for ROS induction *via* electron transfer and energy transfer pathway[151]. The generated ROS such as singlet oxygen ($^1O_2$) and hydroxyl radical ($\cdot OH$) can react with various cellular components and damage cellular membrane-related structures (mitochondria, endosomes, etc.)[152], to activate inflammasome-dependent or inflammasome-independent caspase for gasdermin cleavage. Currently, caspases 1 and 3 have been discovered to be involved in ROS-induced pyroptosis. Subcellular organelle-anchored PSs display unique advantages in precisely and efficiently inducing ROS and have been a rising direction for PDT application. Currently studied types mainly include cell membrane, mitochondrion, endoplasmic reticulum (ER), and lysosome/endosome.

3.4.1. *Cell membrane-anchored photosensitizers*

In 2021, Wu et al.[153] designed a kind of cationic lipid-modified cell membrane anchored PS TBD-R as a pyroptosis inducer (Fig. 7A). The decoration of cationic quaternary ammonium lipid chain promoted the membrane localization of TBD-R at a chain amount (1−3) dependent manner. Besides, TBD-R exhibited the characteristics of aggregation-induced emission on cell membrane, leading to more efficient ROS generation *in situ*. Further experiments revealed that TBD-R with laser irradiation activated caspase-1 and GSDMD cleavage for pore-formation. The coordination of direct membrane destruction by ROS and pyroptosis facilitated the release of LDH, IL-1β and IL-18, representing a potential therapy for tumor ablation. Actually, this study was also the first validation of ROS-mediated pyroptosis *in vitro*.

In the following work in 2022, Wang et al.[154] further performed the *in vivo* antitumor experiments and certificated the therapeutic potential of TBD-3C with the capabilities of precise control, pyroptosis induction and tumor environment remodeling (Fig. 7B). Similarly, in 2023, Tang et al.[155] synthesized a dimeric PS D1 with the capability of aggregation-induced emission for cell membrane-targeting pyroptosis (Fig. 7C). The membrane anchoring of D1 might be attributed to the hydrophobic octyl linker between dimer and positively charged pyridinium (Fig. 7C). The aggregation-induced emission of D1 effectively promoted the generation of type I ROS $^1O_2$, $OH\cdot$ and $O_2^{-\cdot}$, resulting in direct membrane damage and caspase-1-GSDMD-meidated pyroptosis for eliminating primary tumors and boosting adaptive responses against metastasis and recurrence.

Recently, Lu et al.[156] also designed a membrane-tethered and enzyme-responsive PS aPYCI as a pyroptosis inducer, using zwitterionic lipid-based anchors (Fig. 7D). The aPYCI4 with two lipid chains showed an apparent membrane localization characteristic which was not observed in PYCI3 (with one lipid) and PYCI1-2 (with one or two cholesterol-based anchors). Upon aminopeptidase N cleavage (decaging) and near-infrared (NIR) irradiation, aPYCI efficiently induced ROS (mainly singlet oxygen) production for caspase-1-GSDMD axis-mediated tumor cell-targeting lytic death. This further led to the conversion of “cold” TME into “hot” one through CTLs infiltration for local and abscopal tumors regression.

In addition to lipid chains, the ligands of transmembrane proteins have also been adopted as cell membrane anchors of PSs.

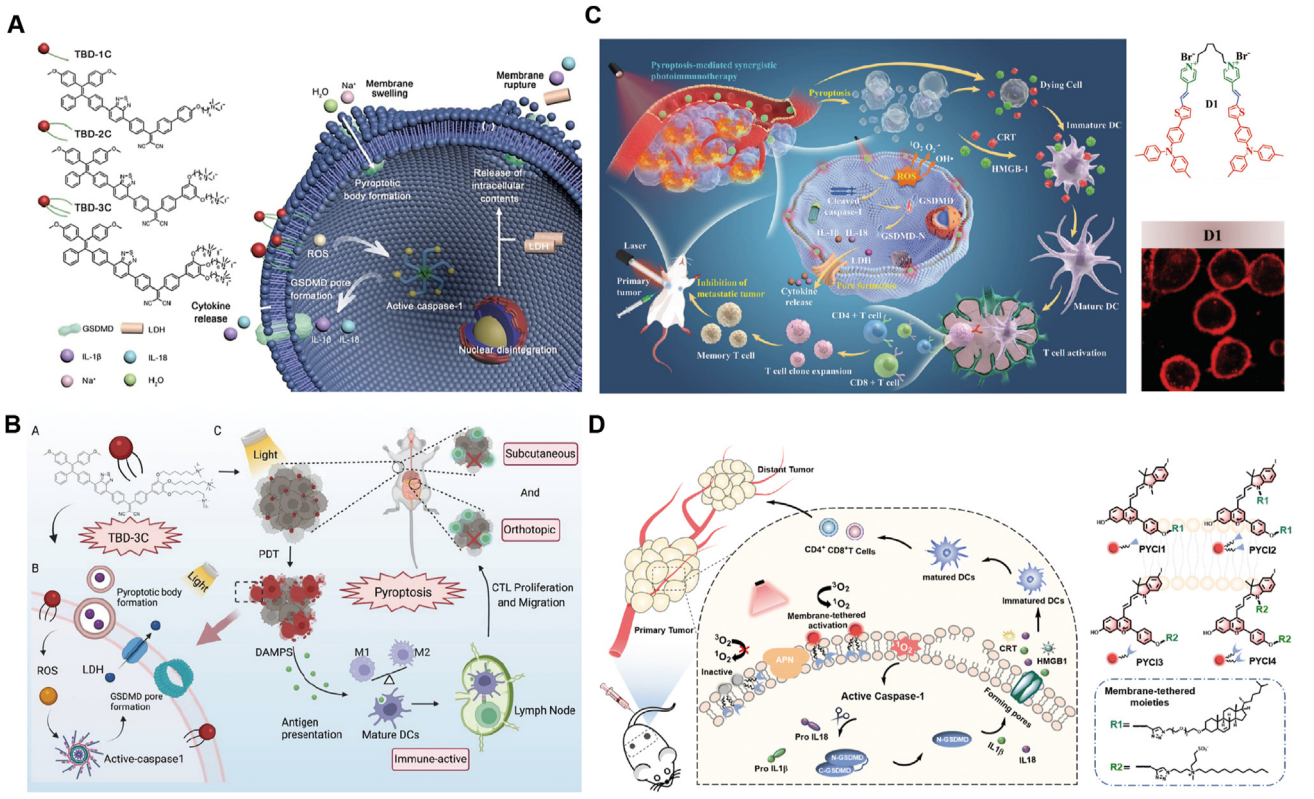


Figure 7 Cell membrane-anchored photosensitizers for inducing ROS-mediated pyroptosis. (A) Structures and pyroptosis-induced mechanism of membrane-anchored photosensitizers TBD-1C, 2C, and 3C[153]. Reproduced with permission. Copyright © 2021, Wiley-VCH GmbH. (B) Schematic diagram of TBD-3C photosensitizer-induced photodynamic pyroptosis for cancer immunotherapy *via* a pathway of caspase-1/GSDMD[154]. Reproduced with permission. Copyright © 2022, Wiley-VCH GmbH. (C) Structure of the dimeric photosensitizer D1 and the schematic illustration of D1-mediated photothermal/photodynamic pyroptosis *via* a pathway of caspase-1/GSDMD[155]. Reproduced with permission. Copyright © 2023, Wiley-VCH GmbH. (D) Structures of membrane-tethered photosensitizers PYCI1, PYCI2, PYCI3 and PYCI4 and the schematic of aPYCI4-induced photodynamic pyroptosis *via* a pathway of caspase-1/GSDMD[156]. Reproduced with permission. Copyright © 2023, The Royal Society of Chemistry.

In 2022, Su et al.[157] developed a rhenium(I)-based PS CA-Re for initiating GSDMD-mediated pyroptosis using benzene sulfonamide, a binding moiety towards carbonic anhydrase IX (CAIX), as a membrane anchor. CAIX is specifically overexpressed in tumor cells as a transmembrane protein localized on the cell membrane. CA-Re was indeed found to be distributed on cell membrane and efficiently generated ROS including $\cdot O_2^-$ and lipid peroxidation upon light radiation, resulting in caspase-1-mediated GSDMD cleavage for pyroptosis. This promoted the remarkable cell death of MDA-MB-231 cells with an IC_{50} of 20 nmol/L *in vitro*, and induced potent antitumor T-cell responses for bilateral 4T1 tumors regression.

3.4.2. *Mitochondria-anchored photosensitizers*

Apart from cell membrane, other subcellular organelle-targeting PSs also display unique characteristics in promoting photo-induced ROS for pyroptosis. In 2022, Zeng et al.[158] constructed a series of PSs modified with different functional moieties to target subcellular organelles including mitochondrion (Mito-ZS), ER (ER-ZS) and lysosome (Lyso-ZS), respectively (Fig. 8A and B). The three PSs successfully generated ROS of $^1O_2$ to cause oxidative damage towards the corresponding organelles, resulting in pyroptotic cell death *via* the pore-forming pathway of caspase-1-GSDMD. The mechanism was soundly validated by the plasma membrane rupture, Western-blot and RNA-Seq analyses. Besides, PSs targeting mitochondrion and ER were observed to induce a higher degree of pyroptosis compared with the one localized in lysosome, which was evidenced by *in vitro* LDH release percentage (Fig. 8B) and the *in vivo* capacity of pyroptotic inflammation-mediated distant tumor eradication. This might be attributed to the central role of mitochondrion (ATP production) and ER (protein and lipid synthesis) in maintaining the tumor proliferation.

In 2022, Wang et al.[159] developed a supramolecular PS LDH@ZnPc with a property of mitochondria anchored (Fig. 8C). LDH@ZnPc was obtained through electrostatic adsorption of zinc phthalocyanines (ZnPc) on the positively-charged surface of layered double hydroxides (LDH). The LDH@ZnPc displayed a remarkable mitochondrial localization to produce ROS and depolarize mitochondrial membrane potential, leading to the TC-1 tumor regression through PDT and pyroptosis of caspase-1-mediated GSDMD cleavage. In 2023, Zhang et al.[160] developed a mitochondria-targeting pyroptosis inducer termed ZPHM, using a FDA-approved PS hexyl 5-aminolevulinate hydrochloride (HAL) encapsulated in ZIF-8 MOF (Fig. 8D). HAL is a PS precursor with the capability of mitochondrial orientation, which could be transformed into active PpIX in cancer cells[161]. Upon irradiation, PpIX accumulated in mitochondria effectively induced ROS to activate NLRP3 inflammasomes for caspase-1-GSDMD-mediated pyroptosis. Meanwhile, an autophagy inhibitor

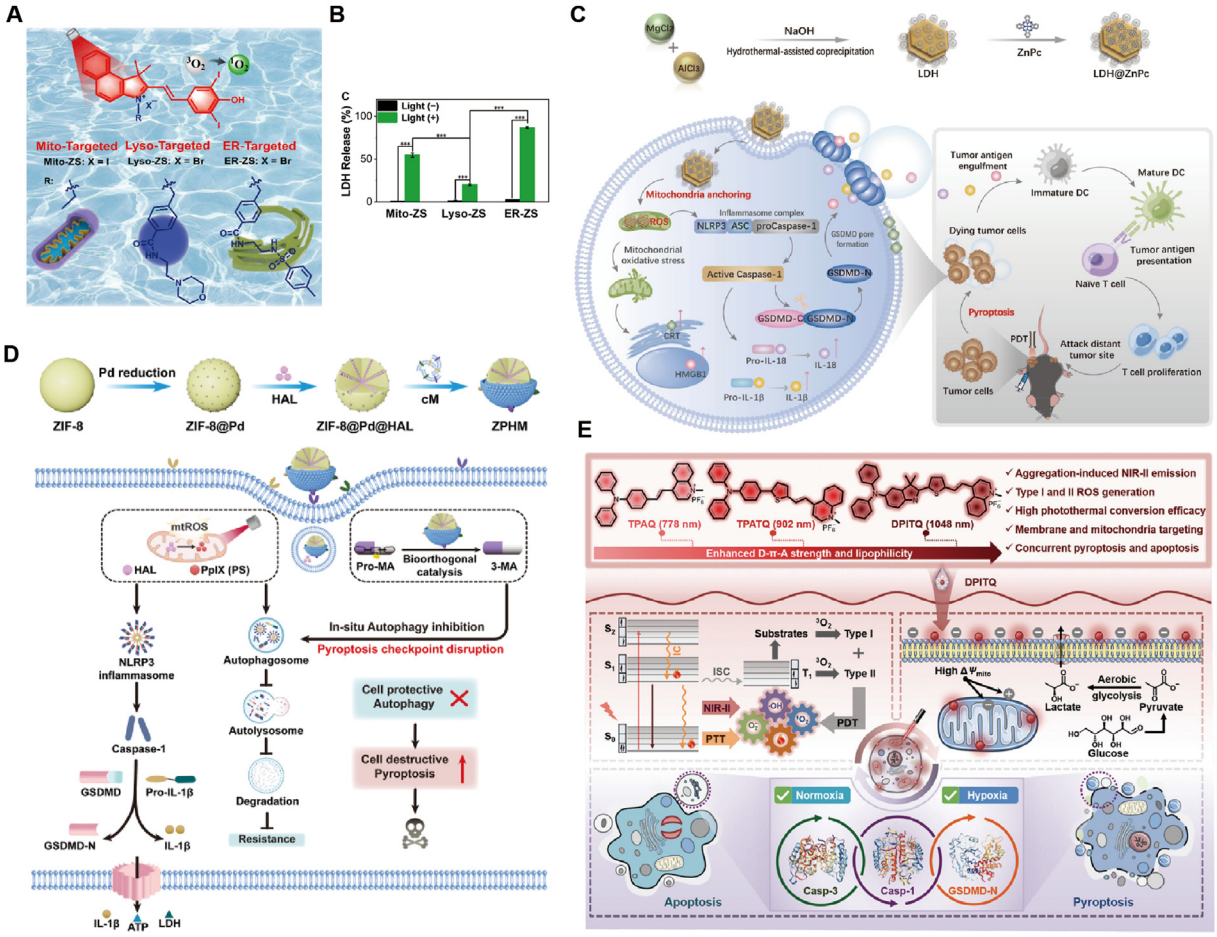


Figure 8 Mitochondria-anchored photosensitizers for inducing ROS-mediated pyroptosis. (A) Structures and subcellular organelle targeting of photosensitizers Mito-ZS, Lyso-ZS, and ER-ZS. (B) LDH release levels in cells incubated with Mito-ZS, Lyso-ZS, and ER-ZS, respectively[158]. Reproduced with permission. Copyright © 2022, Elsevier Ltd. (C) Structure and preparation of LDH@ZnPc and its mechanism in mediating photodynamic pyroptosis *via* mitochondria-anchored and a pathway of caspase-1/GSDMD[159]. Reproduced with permission. Copyright © 2022, Springer Nature. (D) Fabrication of ZPHM nanoregulator containing hexyl 5-aminolevulinate hydrochloride (HAL) and 3-methyladenine (3-MA), and the diagram of ZPHM in bioorthogonally boosting pyroptosis *via* a pathway of caspase-1/GSDMD. Reproduced with permission[160]. Copyright © 2023, American Chemical Society. (E) Structure of NIR-II photosensitizer DPITQ and its application mechanism of photodynamic caspase-1/GSDMD-mediated pyroptosis *via* dual targeting of membrane and mitochondria[162]. Reproduced with permission. Copyright © 2023, Wiley-VCH GmbH.

3-methyladenine (3-MA) was coloaded to inhibit the degradation of proteins improve the pyroptosis efficiency. Compared to targeting mitochondria alone, the combination anchoring strategy might induce a better pyroptosis efficiency. In 2023, Zhuang et al.[162] constructed an aggregation-induced NIR-II emissive PS DPITQ as the pyroptosis inducer with a capability of plasma membrane and mitochondria anchored (Fig. 8E). The targeting ability might be owed to the suitable lipophilicity. Upon 635 nm irradiation, anchored DPITQ generated type I and II ROS to specifically cause the plasma membrane and mitochondrial dysfunction, ultimately promoting caspase-1-GSDMD-mediated cell death.

3.4.3. Lysosome/endosome-anchored photosensitizers

Distinct from other subcellular organelles, the unique pH reduction characteristic of endosome and lysosome enables the precise control of photo-induced ROS-mediated pyroptosis. As mentioned, in 2022, Chen et al.[163] prepared a series of acid-responsive amphiphilic copolymers ANPS conjugated with PS for realizing endosomal maturation stage-dependent pyroptosis in tumor tissues (Fig. 9A). The copolymers were composed of a mPEG-based hydrophilic block and a tertiary amine-based ionizable hydrophobic block. The PS Ce6 and fluorescence quencher QSY21 were respectively conjugated with copolymers which were further assembled into nanoparticle ANPS with an equal amount of Ce6 and QSY21. ANPS library exhibited a wide pH transition ranging from 6.9 to 5.3, allowing for the restoration of the PS activity in different maturation stages of endosomes, and using a spatiotemporally controllable approach (Fig. 9A). Upon irradiation, the disassociated ANPS in early endosomes produced a high level of $^1O_2$ to induce lipid peroxidation of early endosome membrane, which further activated phospholipase C (PLC) to elicit caspase-3-GSDME-mediated pyroptosis. The ANPS achieved apparent tumor suppression in multiple cancer models expressing GSDME, along with minimal side effects towards normal tissues with low level or no GSDME expression. Similarly, in 2022, Wu and coworkers designed a ruthenium-based PS modified on the surface of TiO_2 nanoparticles (TiO_2@Ru-

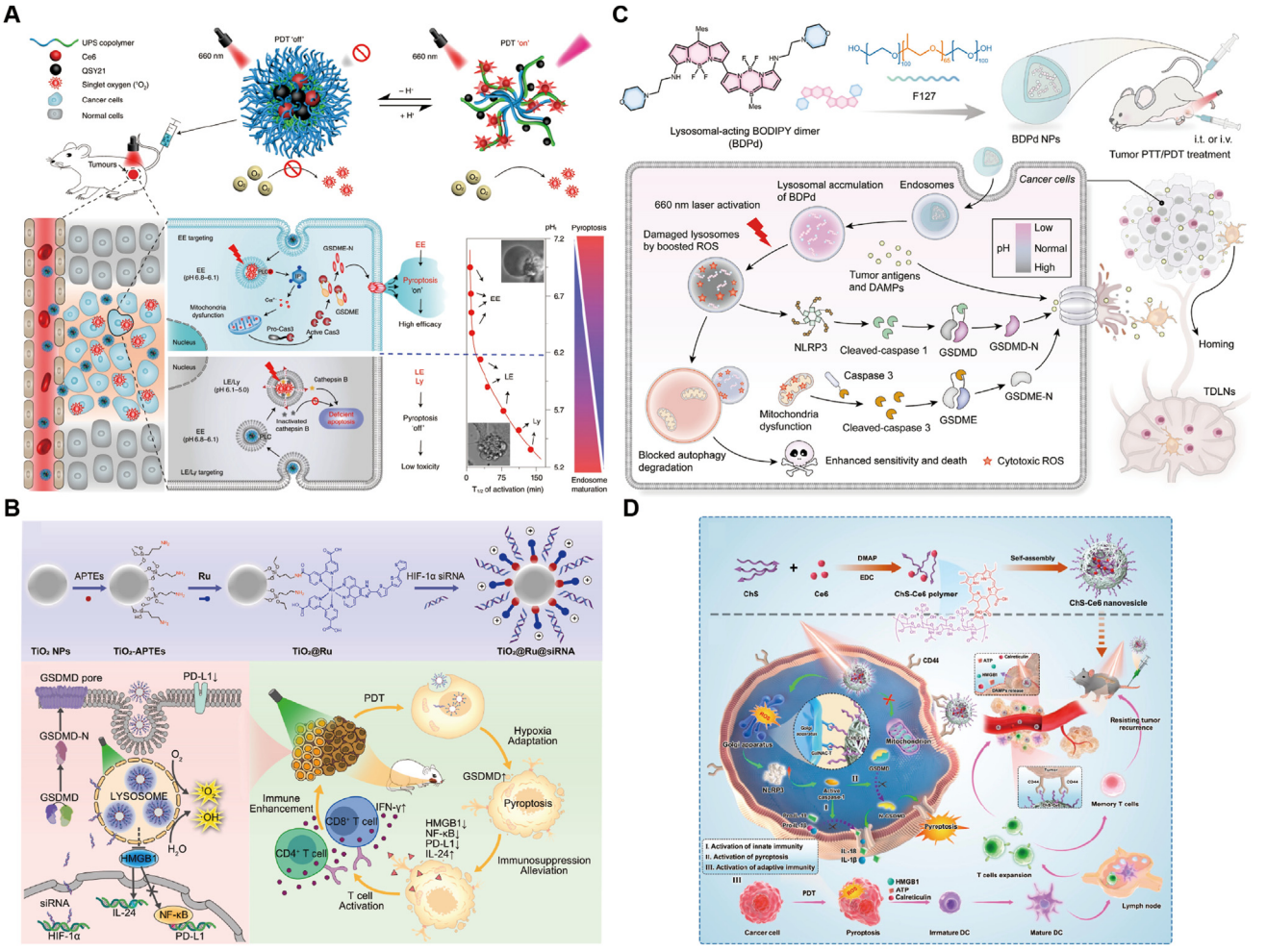


Figure 9 Lysosome/endosome and Golgi apparatus-anchored photosensitizers for inducing ROS-mediated pyroptosis. (A) Schematic diagrams of ANPS and its property of tunable photodynamic pyroptosis dependent on temporal differentiation of endosome maturation[163]. Reproduced with permission. Copyright © 2022, Springer Nature. (B) Fabrication of TiO_2@Ru@siRNA and its action mechanism in generating lysosomal ROS for GSDMD-based pyroptosis[164]. Reproduced with permission. Copyright © 2022, Elsevier Ltd. (C) Construction of BDPd nanoparticles and the schematic of photodynamic ROS in lysosomes for lysosome damage-mediated caspase-1/GSDMD activation[165]. Reproduced with permission. Copyright © 2024, American Chemical Society. (D) Schematic of construction and workflow of ChS-Ce6 nanovesicles for Golgi apparatus-targeting-based ROS induction and subsequent caspase-1/GSDMD activation[167]. Reproduced with permission. Copyright © 2023, American Chemical Society.

@siRNA) to generate ROS in lysosomes and further cause lysosomal membrane damage, resulting in caspase-1-GSDMD-mediated pyroptosis (Fig. 9B)[164]. In 2023, Sun et al.[165] also developed a lysosomal-targeting boron-dipyrromethene dimer BDPd as a pyroptosis inducer (Fig. 9C). With the help of morpholine group, BDPd nanoparticles specifically accumulate in lysosomes and generate ROS upon 660 nm activation to disrupt the lysosomal membrane and simultaneously induce the activation of GSDMD and GSDME.

3.4.4. *Golgi apparatus-anchored photosensitizers*

NLRP3 acts as a critical sensor of stimulants for caspase activation. Some studies reveal that the recruitment, self-assembly, and activation of NLRP3 depend on the Golgi apparatus, rather than the mitochondria, indicating a potential intervention for inducing pyroptosis[166]. In 2023, Hu et al.[167] designed a Golgi apparatus-targeting pyroptosis inducer using self-assembled chondroitin sulfate conjugated with a classical PS Ce6 (Fig. 9D). Owing to the Golgi apparatus-targeting property of chondroitin sulfate, Ce6 was accumulated and generated ROS for NLRP3 activation to induce GSDMD cleavage upon irradiation. Besides, the NLRP3 expression was also observed to be upregulated after irradiation. These factors synergistically triggered a robust pyroptosis to reprogram the tumor microenvironment for distant tumor inhibition.

3.5. *Imbalance of ion homeostasis*

As the critical component of cell homeostasis, the intra/extracellular ion concentrations are tightly regulated. Ions are widely involved in various cellular processes such as signaling cascades, osmotic pressure, and redox potential. Drastic changes in ion concentrations in the cytosol and subcellular organelles will generally result in programmed cell death such as apoptosis, ferroptosis, cuproptosis as well as pyroptosis. Notably, ferroptosis and cuproptosis are the typical representatives of ion homeostasis imbalance (Fe^{2+} and Cu^{2+} overload). Hence, disrupting the

cellular balance of certain ions provides a new induction strategy for pyroptosis. The currently involved ions include iron, zinc, calcium, copper, and some other ions. The pyroptosis mechanism of ion overload can be roughly summarized as the following route: extensive ion-ROS generation-caspase-1/3 activation-GSDMD/GSDME cleavage.

3.5.1. *Iron ions*

In 2020, Ploetz et al.[168] designed an iron-based metal−organic framework Lip-MOF coated with DOPC lipids to substantially improve the cytosolic iron concentration in tumor cells (Fig. 10A). The MOF MIL-100(Fe) was synthesized using Fe^{3+} and trimesic acid. After efficient cellular internalization with the help of

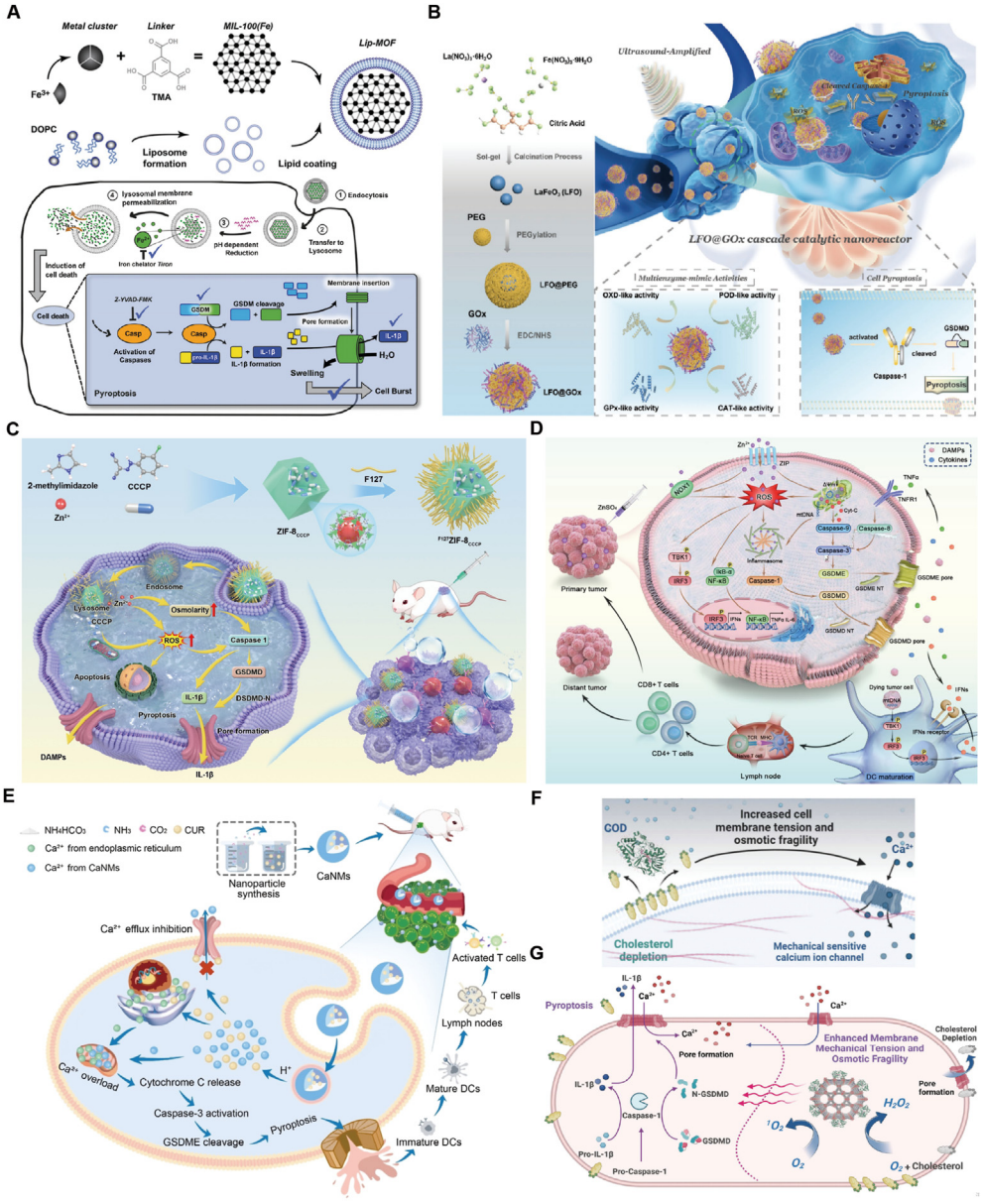


Figure 10 Disruption of iron, zinc and calcium homeostasis to induce pyroptosis. (A) Schematic diagrams of Lip-MOF preparation and its mechanism of low pH-responsive extensive iron ion release into lysosomes and the cytosol for triggering GSDMD-mediated pyroptosis[168]. Reproduced with permission. Copyright © 2020, Wiley-VCH GmbH. (B) Schematic illustration of LFO@GOx fabrication and the scheme of cascade catalysis-mediated Fe^{2+} overload in activating ROS-TXNIP-NLRP3-GSDMD pathway[169]. Reproduced with permission. Copyright © 2023, Wiley-VCH GmbH. (C) Construction of $^{F127}ZIF\text{-}8_{CCCP}$ nanoparticles and the schematic of Zn^{2+} overload for ROS/caspase-1/GSDMD activation[170]. Reproduced with permission. Copyright © 2023, Wiley-VCH GmbH. (D) Schematic of ROS generation mediated by Zn^{2+} through mitochondrial aerobic respiration electron leakage and the NADPH oxidation pathways for activating caspase-1/GSDMD and caspase-3/GSDME pyroptosis[171]. Reproduced with permission. Copyright © 2024, Chinese Chemical Society. (E) Schematic of biodegradable CaNMs as a pyroptosis inducer through mitochondrial Ca^{2+} overload[172]. Reproduced with permission. Copyright © 2022, Wiley-VCH GmbH. (F) Schematic of membrane tension and susceptibility to oxidative stress increased by COD-mediated cholesterol depletion. (G) Schematic of Hf-TBP/COD in membrane mechanical tension increase and ROS generation for Ca^{2+} overload and caspase-1/GSDMD activation[173]. Reproduced with permission. Copyright © 2023, Wiley-VCH GmbH.

DOPC, Lip-MOF was degraded at low pH in lysosomes and released cysteine-reduced Fe^{2+} into the cytosol, resulting in lytic cell death. Distinct from that iron overload usually induces ferroptosis, Lip-MOF induced caspase-GSDMD-mediated pyroptosis. While, the mechanism involved in iron overdose-induced caspase activation and the *in vivo* application potential of Lip-MOF remain to be explored. Similarly, in 2023, Chen and coworkers adopted the perovskite nanocrystal $LaFeO_3$, termed LFO@GOx, as a pyroptosis inducer *via* Fe^{2+}-mediated ROS production (Fig. 10B)[169]. Along with ultrasound stimulation, Fe^{3+} in LFO@GOx was transformed into Fe^{2+} to boost ROS induction, ultimately leading to thioredoxin-interacting protein(TXNIP)-NLRP3-GSDMD pathway activation.

3.5.2. *Zinc ions*

Notably, zinc is the second most abundant metal element in organisms and has emerged as a critical factor in immunotherapy of diseases. In 2023, Ding et al.[170] further discovered that the typical ZIF-8 MOF assembled from Zn^{2+} and 2-methylimidazole act as a pyroptosis inducer (Fig. 10C). ZIF-8 was modified with pluronic F127 (a surfactant polyol) to obtain ^{F127}ZIF-8_{CCCP} to enhance the stability and biocompatibility. After uptake by cancer cells, ^{F127}ZIF-8_{CCCP} was pH-responsively degraded to release extensive Zn^{2+} into the cytosol, causing fluctuations in osmolarity and ion homeostasis. This ionic overload eventually led to caspase-1-GSDMD-dependent pyroptosis. Besides, loading with carbonyl cyanide m-chlorophenyl hydrazone (CCCP), an ROS generator, further improved the pyroptosis intensity *in vitro* and *in vivo* (Fig. 10C). Moreover, in 2024, Yang et al.[171] presented a comprehensive mechanistic research of zinc ions in regulating immune responses for immunotherapy (Fig. 10D). Just as mentioned earlier, excessive Zn^{2+} leads to ROS production. They revealed that the ROS generation was achieved through two pathways, namely, mitochondrial aerobic respiration electron leakage and the NADPH oxidation. The Zn^{2+} imbalance and ROS generation further trigger the pyroptosis *via* the pathways of caspase-1/GSDMD and caspase-3/GSDME. These studies provide a cornerstone for Zn^{2+}-mediated metalloimmunotherapy[171].

3.5.3. *Calcium ions*

In 2022, Lin and coworkers constructed a new ion overload-based pyroptosis inducer CaNMs through targeting mitochondrial Ca^{2+} homeostasis (Fig. 10E)[172]. Similar to the mechanism of ZIF-8, the CaNMs synthesized from Ca^{2+}, NH_4HCO_3 and curcumin were degraded in endosomes to increase the cellular Ca^{2+} concentration. Curcumin acted as a Ca^{2+} modulator to promote Ca^{2+} accumulation in mitochondria, as evidenced by a mitochondrial Ca^{2+} probe (Rhod-2 AM), and inhibit the Ca^{2+} efflux. These effects resulted in Ca^{2+} overload-mediated increase in ROS to activate caspase-3 for GSDME cleavage-based pyroptosis. In addition to direct ion surges mediated by cytosolic delivery, cell membrane tension has also been found to affect ion homeostasis. In 2023, Zhen et al.[173] reported a cholesterol oxidase-absorbed MOF Hf-TBP/COD as a pyroptosis inducer *via* increasing membrane tension and osmotic fragility (Fig. 10F and G). The cholesterol oxidase in Hf-TBP/COD was used for the depletion of cholesterol in cancer cell membrane, which increased membrane tension and susceptibility to oxidative stress (Fig. 10F). The combination of increased membrane tension and ROS induced by PS in MOF promoted Ca^{2+} influx (imbalance) and the rupturing propensity, eventually leading to caspase-1/GSDMD mediated pyroptosis (Fig. 10G). Similarly, Qiu et al.[174] recently constructed a mesoporous polydopamine nanoparticle DMP@P as a pyroptosis inducer. DMP@P was coloaded with the chemotherapeutic agent mitoxantrone and the DNA methyltransferase inhibitor decitabine. Upon NIR laser irradiation, the polydopamine core acted as a PS to prompt the sharp influx of Ca^{2+}, which synergized with decitabine to significantly boost the mitoxantrone-mediated caspase-3 activation and GSDME cleavage.

3.5.4. *Copper ions*

Similar to ferroptosis, a copper accumulation-induced programmed cell death is recently identified and termed as cuproptosis, which is caused by Fe−S cluster protein destabilization and mitochondrial lipoylated proteins aggregation[175,176]. In addition to cuproptosis, copper accumulation or overload was also found to result in pyroptosis. In 2023, Zhao et al.[177] designed a kind of copper hydroxyphosphate nanoparticles $Cu_2(PO_4)$ (OH) NPs as a copper-overload inducer for cuproptosis and pyroptosis-based cancer therapy (Fig. 11A). In response to high levels of H_2S in colon cancer tissue, $Cu_2(PO_4)$ (OH) NPs dissociated and transformed into ultrasmall Cu_9S_8 NPs through sulfidation, which were efficiently taken up by cancer cells. The disruption of Cu ion homeostasis further generated ROS for NLRP3 activation and GSDMD-mediated pyroptosis. Besides, the copper overload also triggered the cuproptosis, which collaborated with pyroptosis to achieve the remarkable HCT116 tumor regression. In 2023, Zhang et al.[178] developed a Cu-TBB nanosheet assembled from Cu^{2+} and bacteriochlorin (a PS) as a GSH and photo-responsive pyroptosis inducer (Fig. 11B). In the presence of high GSH levels, the disassembly of Cu-TBB occurred to release Cu^{2+} and TBB into the cytosol. The overload of Cu^{+} derived from Cu^{2+} reduction led to the generation of $O_2^{-\cdot}$ and $^1O_2$-based ROS storms *via* a metal reducing reaction, which further activated GSDMD-based pyroptosis. The pyroposis process was also enhanced by irradiated TBB-induced ROS. The combination of Cu^{2+} overload and PS irradiation promoted the pyroptosis-mediated inflammatory cytokines release for activating DC and T-cell priming.

3.5.5. *Other ions*

Elements such as Bi, W, Zr, and Mn etc. have also been used to disrupt ion homeostasis[179-181]. In 2021, Jia et al.[179] synthesized a novel 10-nuclear heteroatom cluster polyoxometalate {BiW8} (Fig. 11C), which could induce pyroptotic cell death of multiple cancer cell lines with IC_{50} values ranging from 200 to 900 μmol/L. The cell death IC_{50} values were positively correlated with intracellular ROS levels induced by {BiW8}. The increased ROS levels might attributed to the glutathione metabolism inhibition mediated by the extensive metallic ions released from $\{BiW_8\}$, which further induced caspase-mediated pyroptosis (the involved gasedermin is unknown). Similarly, Ding et al.[180] designed a biodegradable upconversion nanoparticles ZrNPs (K_3ZrF_7:Yb/Er) to induce pyroptosis for cancer treatment (Fig. 11D and E). ZrNPs were found to destroy the balance of intracellular osmolarity and ionic homeostasis by quickly releasing large amounts of$[ZrF7]^{3-}$, F^- and K^+, which led to the generation of ROS, and caspase-1 activation-mediated GSDMD cleavage (Fig. 11E). The ionic homeostasis imbalance supposition was also confirmed using a chemically stable control $NaYF_4$:Yb/Er which was almost inert in promoting cell death and GSDMD cleavage. This study revealed for the first time a pyroptosis-targeting application mechanism of upconversion nanoparticles[180].

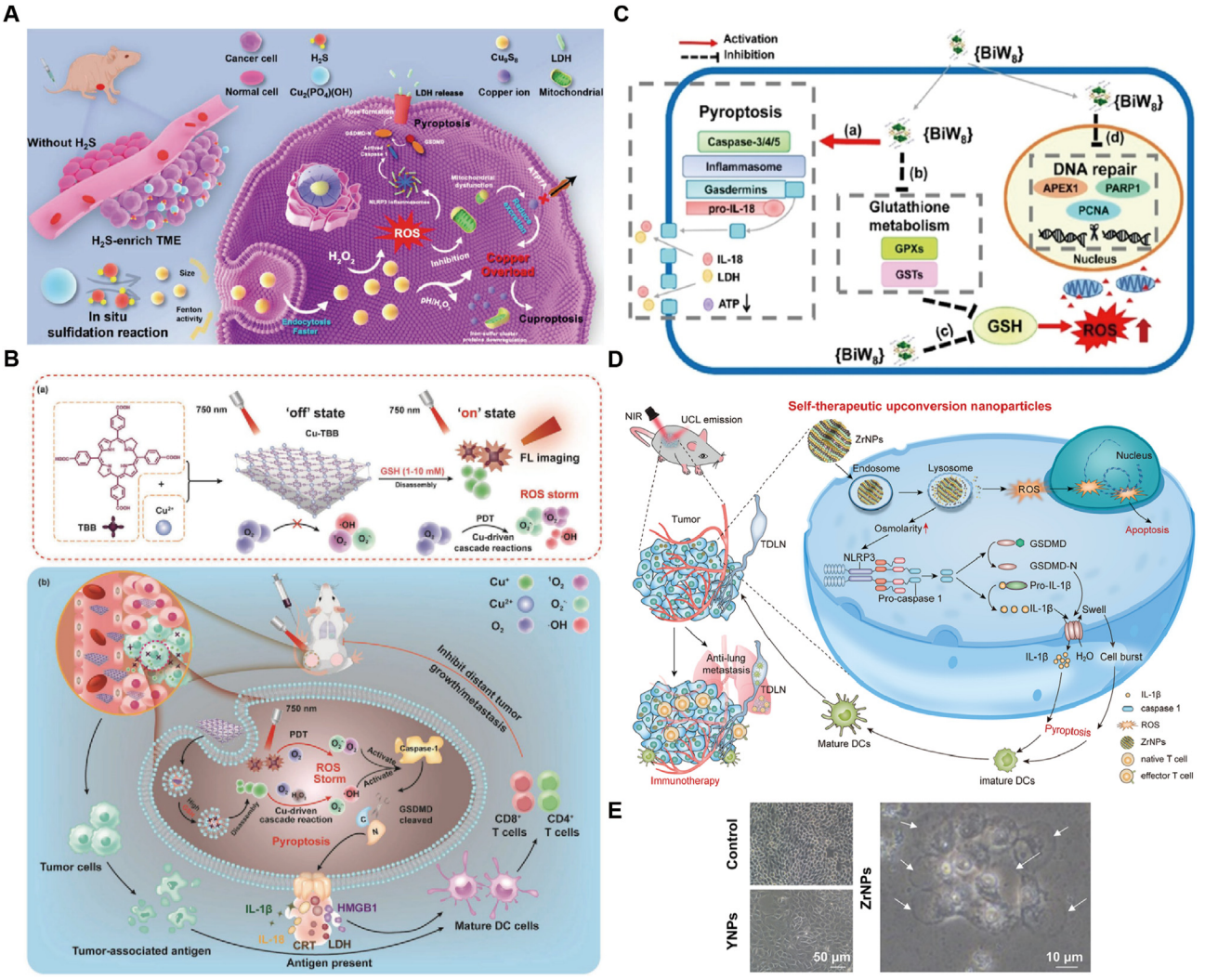


Figure 11 Disrupting copper and other ion homeostasis for inducing pyroptosis. (A) Schematic diagrams of $Cu_2(PO_4)$ (OH) NPs and its mechanism of copper homeostasis disruption for triggering caspase-1/GSDMD-mediated pyroptosis[177]. Reproduced with permission. Copyright © 2023, Wiley-VCH GmbH. (B) Schematic illustration of Cu-TBB fabrication and the scheme of extensive Cu^{2+} release from Cu-TBB triggered by high GSH levels for ROS/caspase-1/GSDMD activation[178]. Reproduced with permission. Copyright © 2023, Wiley-VCH GmbH. (C) Schematic illustration of $\{BiW_8\}$-induced pyroptosis and ROS generation[179]. Reproduced with permission. Copyright © 2021, Wiley-VCH GmbH. (D) Schematic of biodegradable ZrNPs in activating the caspase-1/GSDMD pyroptosis pathway through destroying the balance of intracellular osmolarity and ionic homeostasis. (E) Morphology images of cells treated with ZrNPs[180]. Reproduced with permission. Copyright © 2021, American Chemical Society.

Table 1 Small-molecule pyroptosis inducer.

Classification	Drug	Mechanism	Gasdermin	Disease
Chemotherapy drug	Cisplatin + Decitabine	Caspase-3 activation and GSDME expression increase	GSDME	Breast cancer (4T-1)[182]
	Doxorubicin + JQ1	Caspase-3 activation and PD-L1 level reduction	GSDME	Breast cancer (4T-1)[183]
	Paclitaxel + P18	ROS and caspase-3 activation	GSDME	Colon cancer (CT26)[184]
	Doxorubicin	Caspase-3 activation	GSDME	Colon cancer (CT26)[185]
	Methotrexate	Caspase-3 activation	GSDME	Obstructive extrahepatic cholangiocarcinoma[186]
PROTACs	DeFer-2	Ferritin degradation-induced iron stress, caspase-3 activation	GSDME	Melanoma (B16F10)[187]
	C-02	HK2 degradation-induced mitochondrial homeostasis damage, caspase-3 activation	GSDME	Breast cancer (4T-1)[188]

Table 1 (*continued*)

Classification	Drug	Mechanism	Gasdermin	Disease
	Oleanolic acid	Proteasome activation-induced UBE4B and PLIN2 degradation, caspase-1, 3 activation	GSDMD, GSDME	Osteosarcoma (K7M2)[189]
Inflammasome activators	Val-boroPro	Human CARD8 and mouse NLRP1b activation	GSDMD	Acute myeloid leukemia[190]
	CQ31	Human CARD8 activation	GSDMD	Acute myeloid leukemia (*in vitro*)[191]
	DNF@LIPO	AIM2 and caspase-1 activation	GSDMD	Breast cancer (4T-1)[192]
	Monosodium urate	NLRP3 and caspase-1 activation	GSDMD	Gout[193]
	Nigericin and decitabine	NLRP3 activation and GSDMD expression increase	GSDMD	Breast cancer (4T-1), bladder cancer (MB49)[194]
Mitochondrial dysfunction	Oligomycin A	BAX/BAK-mediated mitochondrial outer membrane permeabilization and caspase-3 activation	GSDME	Melanoma (B16)[195]
	$CyNH_2$	Destruction of mitochondrial membrane and caspase-3 activation	GSDME	Breast cancer (4T-1)[196]
	Dichloroacetate	Disturbing the energy supply homeostasis through PDHK1 inhibition	GSDMD	Osteosarcoma (K7M2)[197]
	TPA-2TIN	Aggregating in mitochondria to cause dysfunction and caspase-3 activation	GSDME	Breast cancer (4T-1)[198]

3.6. *Small-molecule inducers*

In this section, we present a summary of small molecules that trigger pyroptosis, including chemotherapy drugs, PROTACs, inflammasome activators and molecules that cause mitochondrial dysfunction (Table 1). These small molecules display different mechanisms of pyroptosis induction compared with the strategies summarized above.

3.6.1. *Chemotherapy drugs*

In 2017, Wang et al.[44] for the first time identified chemotherapy drugs as a pyroptosis inducer *via* caspase-3 activation. In the following years, numerous studies have adopted the characteristic to develop new single and combination therapies for cancer and other diseases (Table 1). In 2019, Fan et al.[182] designed a combination of chemotherapy nanodrugs and epigenetic inhibitors for cancers with low GSDME expression (Fig. 12A). The hypermethylation of promoters in cancer cells represses the functional protein-encoding gene transcription[199]. Decitabine, a DNA methyltransferase (DNMT) inhibitor[200], was used to relieve GSDME silencing in cancer cells (an immune escape mechanism) (Fig. 12A). The coordination of decitabine and liposomal cisplatin facilitated the pyroptosis occurrence to suppress the tumor growth and recurrence. In 2020, Zhao et al.[183] also developed a nanoarray using a combination of doxorubicin (DOX) and JQ1 (an epigenetic modulator) as an efficient pyroptosis inducer (Fig. 12B). DOX and JQ1 were coloaded into hyaluronic acid-modified polydopamine which was cross-linked using a borate ester-based ROS-responsive liner to obtain DOX/JQ1-IBRN. JQ1 acts as an inhibitor of bromodomain and extraterminal protein BRD4 to selectively reduce the expression of downstream effector PD-L1[201]. The combination of DOX-induced pyroptosis and JQ1 reprogrammed the tumor environment and generated antitumor immunity to efficiently inhibit 4T1 recurrence and metastasis. In 2021, Xiao et al.[184] developed a paclitaxel-based nanoprodrug MCPP as a pyroptosis inducer (Fig. 12C). Two paclitaxel molecules were covalently linked with a disulfate linker to consume the GSH in the tumor microenvironment and release free paclitaxel. Through the coassembly of P18 photosensitizer, MCPP efficiently induced durable pyroptotic tumor cell death upon irradiation, to promote the maturation and tumor antigen presentation of DCs for conferring tumor inhibition and immunological memory. Similarly, in 2022, Liang et al.[185] also constructed a cascaded pH-activated DOX nanoprodrug PDNP as a pyroptosis inducer (Fig. 12D). The cascaded pH from the tumor environment to the endosome triggered the release of DOX to activate caspase-3/GSDME for inducing antitumor immunity.

In addition to cancer models in mice, chemotherapy drug-based pyroptosis has also been successfully applied in patients with end-stage cholangiocarcinoma (CCA). In 2020, Gao et al.[186] reported a kind of apoptotic human tumor-cell-derived microvesicles loaded with methotrexate (MTX) to alleviate obstructive extrahepatic CCA through pyroptosis. After perfusion into patients' bile-duct lumen, MTX-microvesicles was found to degrade the stromal barrier of the CCA and induce the CCA-cell pyroptosis through a GSDME-dependent mechanism. This further attracted neutrophils infiltration through GSDME-mediated intracellular content release and proinflammatory cytokine secretion. This process was closely related to the high GSDME expression in CAA cells and microvesicle encapsulation. This study provided a potent validation of pyroptosis-based treatment for clinical obstructive extrahepatic CAA and for other potential indications[186].

3.6.2. *PROTACs*

PROTACs, namely proteolysis targeting chimeras, have been recognized as a promising strategy to degrade proteins of interest through the ubiquitin-proteasome pathway[202]. Recently, PROTACs have also displayed the potential in pyroptosis inducers by degrading vital proteins in maintaining cellular homeostasis (Table 1). For example, the ferritin acts as a depository for storing iron to regulate iron ion homeostasis, and can be a target for

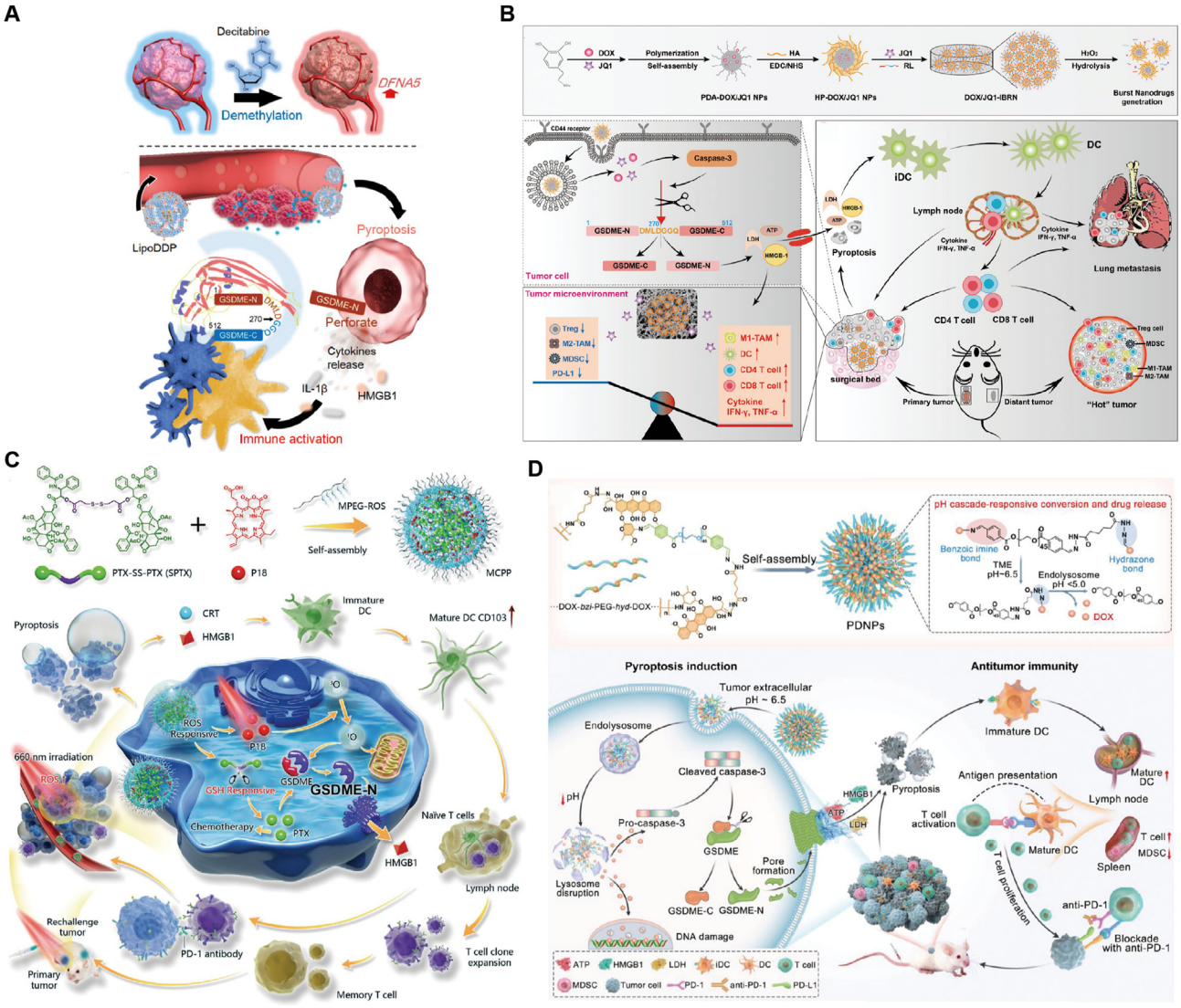


Figure 12 Chemotherapy drugs as pyroptosis inducers. (A) Schematic diagrams of the coordination of the epigenetic inhibitor decitabine and liposomal *cis*-platinum for triggering GSDMD-mediated pyroptosis[182]. Reproduced with permission. Copyright © 2019, American Chemical Society. (B) Illustration of DOX/JQ1-IBRN fabrication and the scheme of the combination of DOX and JQ1 (an epigenetic modulator) for generating pyroptosis-mediated adaptive immunity against tumors[183]. Reproduced with permission. Copyright © 2020, Wiley-VCH GmbH. (C) Schematic illustration of MCPP preparation and the workflow of paclitaxel in MCCP-mediated GSDME cleavage for cancer immunotherapy[184]. Reproduced with permission. Copyright © 2021, Wiley-VCH GmbH. (D) Schematic of PDNP construction and its mechanism of cascaded pH-activated DOX release for caspase-3/GSDME-mediated pyroptosis[185]. Reproduced with permission. Copyright © 2022, Wiley-VCH GmbH.

pyroptosis. In 2023, Hu and coworkers[187] synthesized a series of conjugates of oleic acid (a ferritin dimer binder) and the ligand VH032 of Von Hippel-Lindau (VHL) E3 ligase (Fig. 13A and B). After screening, the obtained DeFer-2 efficiently degraded ferritin, rapidly resulting in iron ion accumulation and ROS generation, namely, iron stress. This further triggered the activation of caspase-3 to consequently cleave GSDME for pyroptotic cell death (Fig. 13B). Moreover, the nanoformulation of albumin-binding DeFer-2 (aDeFer-2) displayed a robust B16F10 melanoma inhibition effect *in vivo* and improved mice survival. In the same year, Sang et al.[188] also developed a Hexokinase 2 (HK2)-targeting PROTACs to disturb aerobic glycolysis and induce mitochondrial damage (Fig. 13C). HK2 functions as a principal rate-limiting enzyme in glycolysis process, which also binds to voltage-dependent anion channels (VDACs) in the mitochondrial membrane to maintain normal metabolite exchange. The optimized HK2 degradation candidate C-02 substantially degraded HK2 proteins in breast cancer cells to block the glycolysis and disrupt mitochondrial homeostasis, which consequently led to GSDME-mediated immunogenic cell death for effective 4T1 suppression (Fig. 13C). Except for typical PROTACs, nonspecific activation of the proteasome for crucial protein degradation can also be a trigger for homeostasis imbalance-induced pyroptosis. In 2023, Luo et al.[189] reported that oleanolic acid (OA) nanomicelles directly provoke the activation of cellular proteasome system (Fig. 13D). Subsequent experiments revealed that OA nanomicelles directly interacted with 20S proteasome subunit alpha 6 (PSMA6) to change its NTD conformation which affected the recruitment of proteins into the 20S proteasome. This enhanced the hydrolysis of proteins including UBE4B and PLIN2, resulting

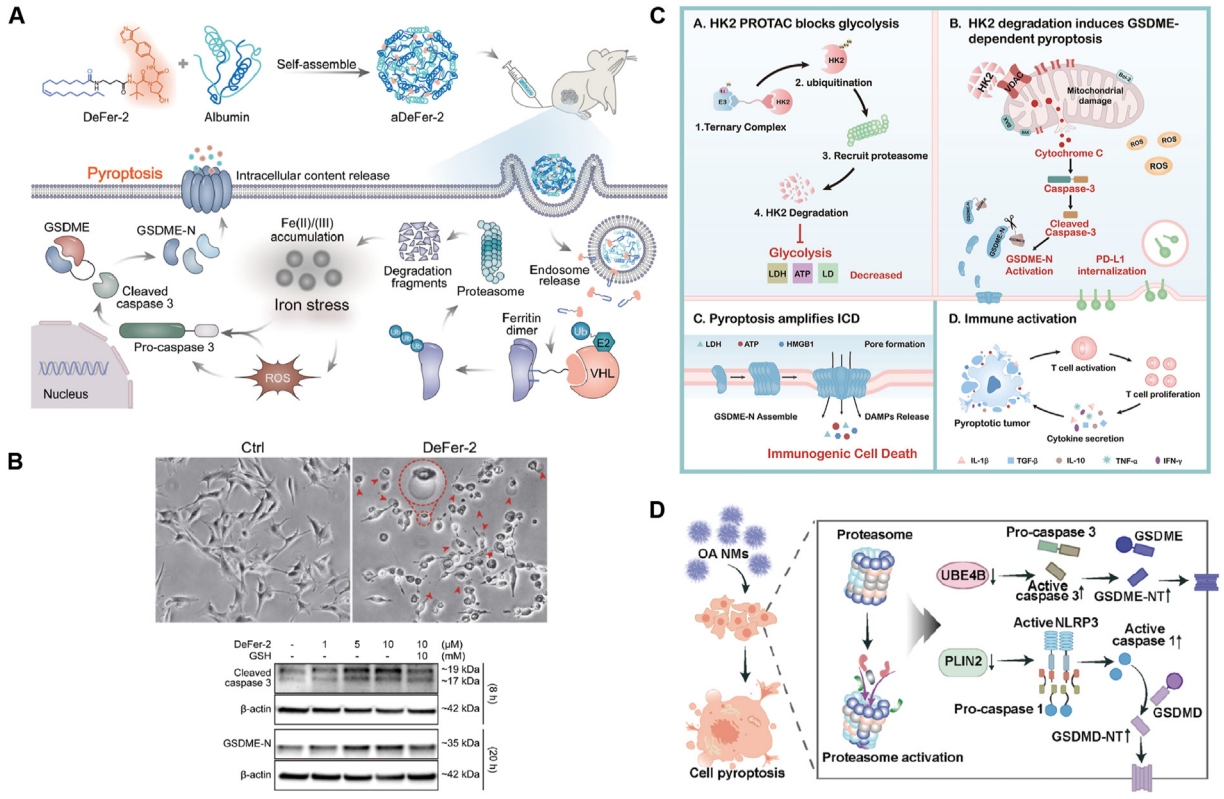


Figure 13 PROTACs as pyroptosis inducers. (A) Schematic diagram of aDeFer-2 preparation and its activity of ferritin degradation for inducing iron stress and further activate the caspase-3/GSDME pathway. (B) Morphology images and Western blot analysis of B16F10 cells incubated with DeFer-2[187]. Reproduced with permission. Copyright © 2023, American Chemical Society. (C) Illustration of HK2 PROTAC-mediated HK2 degradation to inhibit glycolysis to further disrupt mitochondria and induce following GSDME-related pyroptosis[188]. Reproduced with permission. Copyright © 2023, American Chemical Society. (D) Schematic of OA nanomicelles-mediated PLIN2 and UBE4B degradation by activating proteasome, resulting in caspase-3/GSDME and caspase-1/GSDMD activation[189]. Reproduced with permission. Copyright © 2022, Wiley-VCH GmbH.

in the activation of caspase-3 and caspase-1 to cleave GSDME and GSDMD, respectively. Besides, OA nanomicelles displayed a potent dose-dependent antitumor activity *in vivo*.

3.6.3. *Inflammasome activators*

As shown in canonical inflammasome pathways, cytosolic stimuli can trigger the assembly of inflammasomes to activate caspase-1 for pyroptosis induction. Therefore, in recent years, biomimetic strategies targeting inflammasome activation have been developed to induce pyroptotic cell death (Table 1). Early in 2018, Bachovchin and coworkers found that serine dipeptidases DPP8/9 inhibitor Val-boroPro could activate inflammasome sensors of human CARD-containing protein CARD8 and mouse NLRP1b in myeloid cells to induce caspase-1-GSDMD-mediated pyroptosis (Fig. 14A)[190], which efficiently inhibited the progression of acute myeloid leukemia *in vivo*. Moreover, in 2022, they further reported a small molecule CQ31 with specific activity towards CARD8 nor NLRP1 to induce GSDMD-based pyroptosis (Fig. 14B)[191]. CQ31 was discovered to inhibit M24B aminopeptidases and Xaa-Pro aminopeptidase 1, which further resulted in DPP8/9 inhibition due to proline-containing peptides accumulation. In 2023, Xu et al.[192] presented a virus-like DNA particle DNF@LIPO through the self-assembly of long DNA building blocks synthesized by rolling-circle amplification (Fig. 14C). With the help of cationic lipofectamine 3000 coating, the DNA particles were efficiently internalized by tumor cells to trigger the activation of AIM2 inflammasome and cGAS−STING pathways[203-206]. The AIM2 oligomerization promoted the caspase-1 activation to induce tumor cell pyroptosis. Besides, this study also demonstrated that the products of rolling-circle amplification could act as a virus-like stimuli to activate cytosolic PRRs for cancer immunotherapy. Recently, Chen et al.[193] revealed a new pathogenesis of gout caused by monosodium urate through inducing pyroptosis (Fig. 14D). Monosodium urate crystals were observed to induce a size-dependent pyroptosis to release cytokines and recruit neutrophils and macrophages into joints, through triggering NLRP3/caspase-1/GSDMD activation. Besides, this finding was confirmed using a GSDMD inhibitor dimethyl fumarate which effectively alleviated the acute inflammatory response of gout in mice model. Except for canonical inflammasome pathway, LPS-mediated noncanonical pathway has also shown a potential for pyroptosis application. In 2023, Kumari et al.[207] revealed that circulating host-derived EVs were capable of binding blood-borne LPS to promote the intracellular transfer of LPS and trigger GSDMD-based pyroptosis. Moreover, LPS-loaded EVs were also observed to enhance the expression of NLRP3 and pro-caspase-1*via* Toll-like receptor 4 (TLR4). This study provides an LPS-EV platform for pyroptosis-based immunotherapy. Nigericin, an antibiotic and NLRP3 agonist, is also widely used for inducing caspase-1/GSDMD-mediated pyroptosis[194].

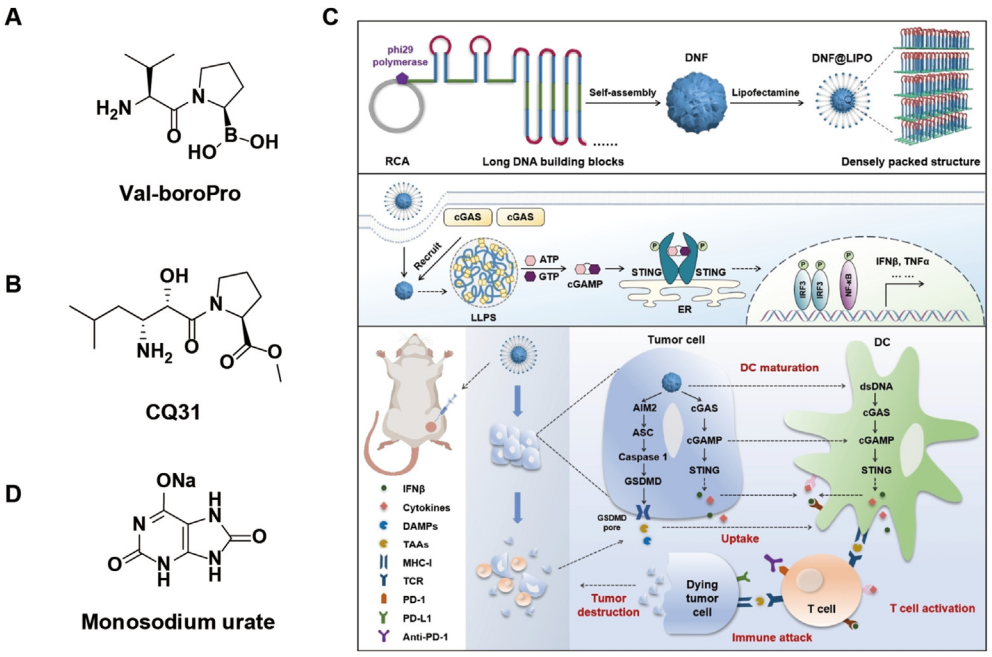


Figure 14 Inflammasome activators as pyroptosis inducers. (A) Molecular structure of Val-boroPro[190]. (B) Molecular structure of CQ31[191]. (C) Illustration of DNF@LIPO preparation and its activity in AIM2 activation for inducing caspase-1/GSDMD pyroptosis[192]. Reproduced with permission. Copyright © 2023, Wiley-VCH GmbH. (D) Molecular structure of monosodium urate[193].

3.6.4. Molecules for mitochondrial dysfunction

Similar to mitochondrion-targeting ROS induction for pyroptosis, other molecules affecting mitochondrial functionalities are also able to induce caspase-mediated pyroptosis under some conditions (Table 1)[208]. In 2021, Wang et al.[195] reported that oligomycin A, an inhibitor of ATP synthase in mitochondria, was found to trigger the BAX/BAK-mediated mitochondrial outer membrane permeabilization (Fig. 15A). This process led to the intracellular oxidative stresses and cytochrome *c* leakage to activate caspase-3, thereby cleaving GSDME for melanoma pyroptosis. By encapsulating oligomycin A with MOF of MIL101-NH_2-Fe, the obtained MOF FeOA NPs exhibited a robust activity towards B16 melanoma suppression. In the same year, Wang et al.[196] revealed for the first time that the amino-modified hemicyanine $CyNH_2$, a common NIR dye, was capable of activating pyroptosis through selectively accumulation in mitochondria (Fig. 15B). This resulted in the destruction of mitochondrial membrane to release cytochrome *c*, subsequently activating caspase-3-GSDME-based pyroptosis. Moreover, the prodrug $NCyNH_2$ was further developed to enhance the tumor-specific killing through responsive cleavage by overexpressed NAD(P)H: quinone oxidoreductase isozyme 1 (NQO1).

In 2022, Jin et al.[197] developed a dichloroacetate (DCA)-conjugated polymer micelle OPDEA-PDCA to induce pyroptosis-based osteosarcoma immunotherapy (Fig. 15C). DCA is a specific inhibitor of mitochondrial pyruvate dehydrogenase kinase 1 (PDHK1) which plays a vital role in regulating the inflow of tricarboxylic acid cycle. Besides, PDHK1 expression is associated with osteosarcoma invasion. DCA treatment disturbs the energy supply homeostasis, resulting in mitochondrial oxidative stress. Due to the ability of OPDEA to target mitochondria *via* the tertiary amine-oxide group, OPDEA-PDCA micelles were observed to be accumulated in mitochondrial of K7M2 osteosarcoma cells to induce PDHK1 inhibition-mediated mitochondrial oxidative stress. This ultimately led to pyroptotic cell swelling and remarkable K7M2 tumor regression in combination with anti-PD-L1 therapy. Similarly, in 2023, He and coworkers also synthesized a mitochondrion-localized aggregation-induced emission luminogen (AIEgen) TPA-2TIN to promote the pyroptosis of 4T1 tumor cells without the need for laser irradiation (Fig. 15D)[198]. The AIEgen TPA-2TIN was found to selectively aggregates in mitochondria to cause dysfunction, consequently triggering caspase-3 activation and GSDME cleavage.

4. Inhibition strategies

Just as a coin has two sides, pyroptosis is also a double-edged sword in pathogenicity and therapeutics. Overall, pyroptosis has emerged as a potential target for treating inflammatory diseases, due to its potent activities in rapid cell damage and cytokine bursts[87,209-211]. From stimulation to membrane rupture, multiple proteins are involved in pyroptosis pathways, providing diverse options for intervening pyroptosis-related diseases. Inhibitors of caspases and inflammasomes have been reviewed in detail elsewhere[3,212]. In this section, natural and artificial inhibition strategies towards gasdermin, ninjurin-1 (NINJ1) and ROS were summarized.

4.1. Natural strategies

Shigella fexneri is a gram-negative bacterium which lives freely in the cytosol. In 2021, Shao et al. reported that OspC3, a type III secretion system (T3SS) effector in *Shigella fexneri*, could block caspase-11/4-mediated noncanonical pyroptotic cell death to promote the intracellular proliferation and cause lethal shigellosis[213,214]. Further biochemical dissections revealed that OspC3, not OspC1 or OspC2, was able to recognize and covalently modify caspase-11/4 at Arg314 (caspase-4) and Arg310 (caspase-11) *via*

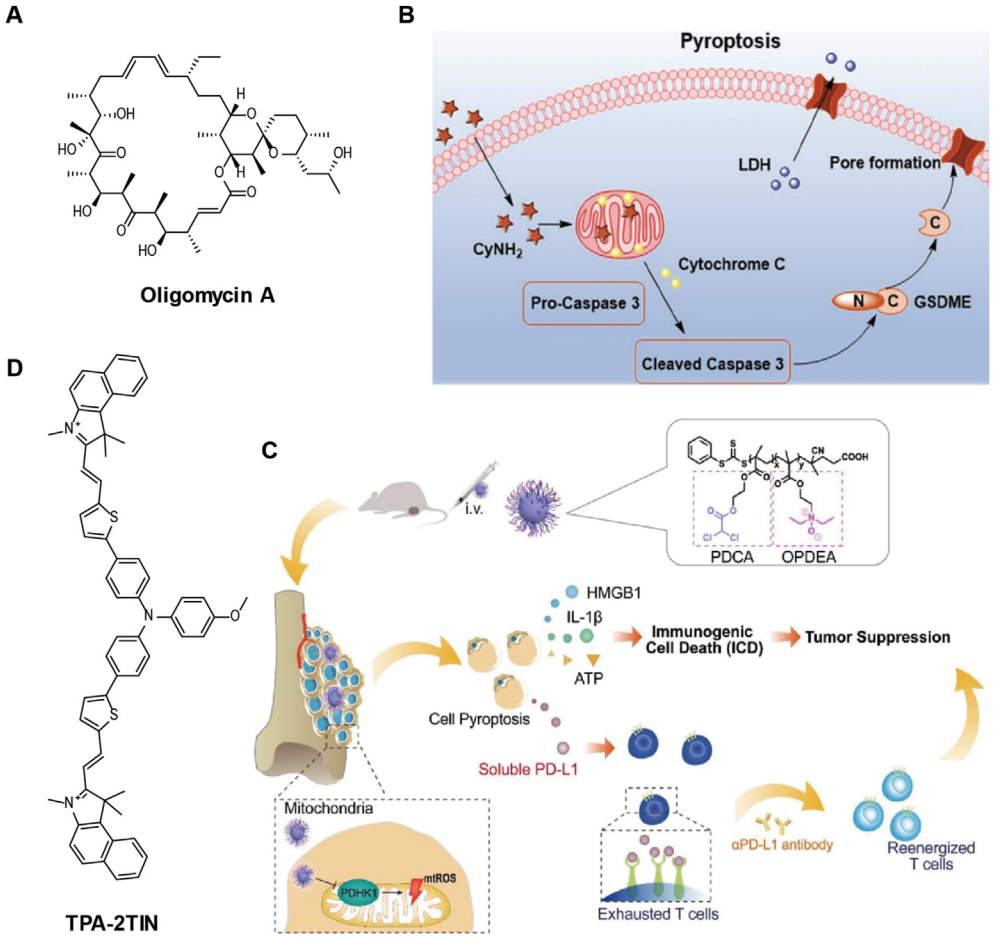


Figure 15 Mitochondrial dysfunction molecules as pyroptosis inducers. (A) Molecular structure of oligomycin A[195]. (B) Schematic diagram of $CyNH_2$-induced pyroptosis through selective accumulation in mitochondria and disruption of the mitochondrial membrane to activate caspase-3/GSDME[196]. Reproduced with permission. Copyright © 2021, Wiley-VCH GmbH. (C) Schematic of pyroptosis-induced by OPDEA-PDCA through inhibiting PDHK1 to cause mitochondrial oxidative stress[197]. Reproduced with permission. Copyright © 2022, American Chemical Society. (D) Molecular structure of TPA-2TIN[198].

an ADPriboxanation with NAD^+ as a donor (Fig. 16A and B). The ADPriboxanation modification abolished the functions and activities of caspase-11/4 in autoprocessing and GSDMD cleavage triggered by LPS. Considering the critical role of OspC3 in paralyzing pyroptosis-based host defense, the *Shigella fexneri* with mutated OspC3 might act as a live attenuated vaccine to induce anti-*Shigella fexneri* protective immunity. In the following work of 2023, they further dissected the structural mechanism about ADPriboxanation modification of caspase-11/4 by Ca^{2+}-free calmodulin-binding OspC3 (Fig. 16C)[215].

In addition to OspC3, IpaH7.8, an ubiquitin ligase effector from *Shigella fexneri*, was also identified as a pyroptosis inhibitor through the proteasomal degradation of hGSDMD (not mGSDMD) (Fig. 16B). Besides, as a *Shigella fexneri* species-specific molecular determinant, IpaH7.8 was observed to modify hGSDMD pore-forming domain with ubiquitin for degradation using host proteasome. Notably, ubiquitinated hGSDMD still possessed the capability of pore formation, indicating the key role of proteasomal degradation. Accordingly, *Shigella flexneri* without IpaH7.8 led to an increased percentage of GSDMD-mediated pyroptotic cell death.

Mycobacterium tuberculosis causes tuberculosis, which led to a total of 1.3 million people died in the year of 2022. In the same year, Liu and coworkers identified a secreted effector PtpB from *Mycobacterium tuberculosis*, with a function of suppressing canonical inflammasome-mediated host pyroptosis (Fig. 16D)[216]. Subsequent experiments revealed that PtpB acted as a phospholipid phosphatase to dephosphorylate the phosphoinositides PI4P and $PI(4,5)P_2$ in the plasma membrane. The phosphates in PI4P and $PI(4,5)P_2$ are the essential group for the membrane localization of cleaved GSDMD. This process greatly inhibited the host cell pyroptosis induced by *Mycobacterium tuberculosis* infection-mediated NLPR3 inflammasome activation. In addition, the host ubiquitin was discovered to be an activator for the phosphatase activity of PtpB by binding to UIM-like domain (Fig. 16D).

4.2. *Artificial strategies*

4.2.1. *Antibody*

Directly targeting gasdermin is recognized as an attractive method to dampen inflammation. In 2023, Geyer and coworkers prepared six human GSDMD-targeting nanobodies, through phage display identification of nanobodies obtained from the recombinant human GSDMD protein immunization in the alpaca[15]. Surface plasmon resonance determination identified nanobodies $VHH_{GSDMD-1}$, $VHH_{GSDMD-2}$, $VHH_{GSDMD-3}$, and $VHH_{GSDMD-5}$

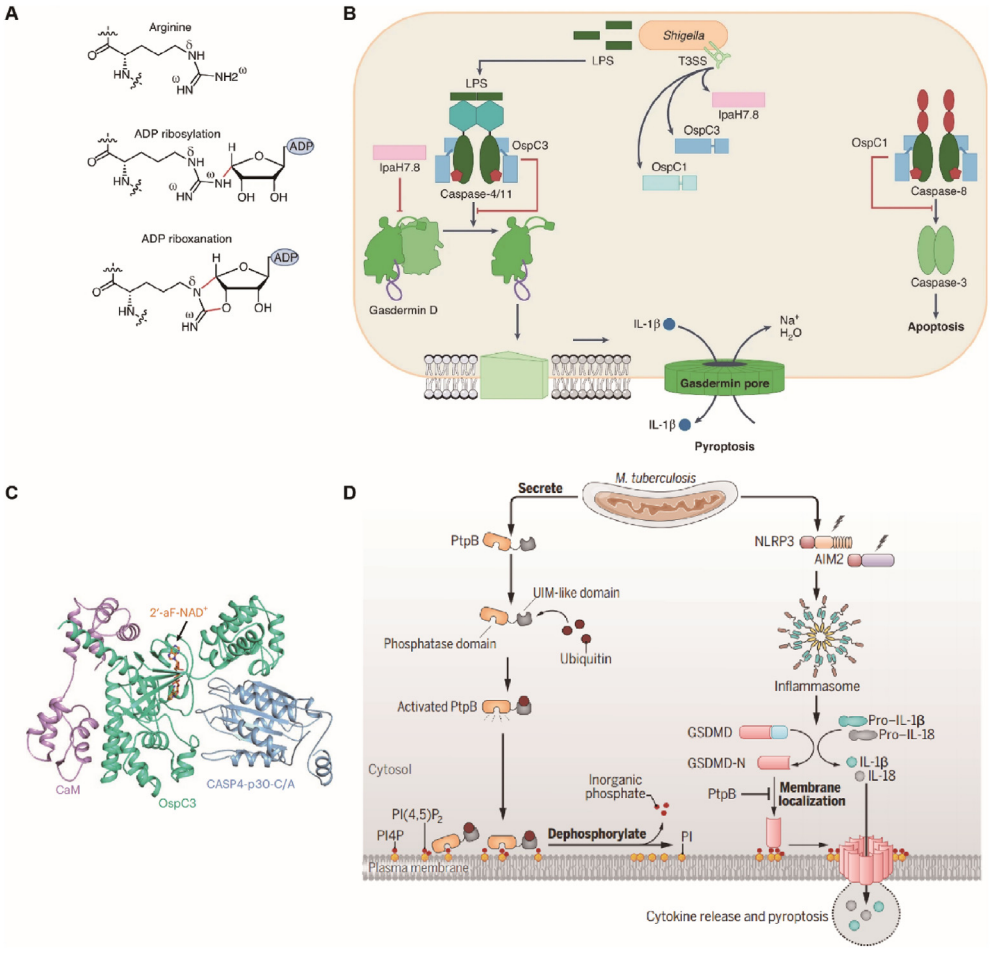


Figure 16 Bacterial effectors for pyroptosis inhibition. (A) Molecular structures of native Arg, ADP-ribosylated Arg and ADP-riboxanated Arg. (B) Schematic diagram of the pyroptosis inhibition activities of OspC3 and IpaH7.8 secreted from *Shigella fexneri* through ADP-riboxanation-based caspase-4/11 inactivation and GSDMD degradation, respectively[213,214]. Reproduced with permission. Copyright © 2021, Springer Nature. (C) Crystal structure of CaM−OspC3−2′-aF-NAD$^+$−caspase-4 quaternary complex[215]. Reproduced with permission. Copyright © 2023, Springer Nature. (D) Schematic diagram of pyroptosis inhibition activities of PtpB secreted from *Mycobacterium tuberculosis* through host ubiquitin hijack-mediated activation to alter the host membrane phospholipid composition[216]. Reproduced with permission. Copyright © 2022, American Association for the Advancement of Science.

with nanomolar level binding affinities. To evaluate the activity towards inhibiting GSDMD NTD assembly, a liposome leakage assay was constructed by adding a mixture of nanobody, caspase-4 and full-length GSDMD protein into calcein-packed liposomes. Based on that, they further found that only $VHH_{GSDMD\text{-}1}$ and $VHH_{GSDMD\text{-}2}$ displayed a remarkable inhibitory effects (IC_{50} = 0.22 and 0.65 μmol/L, respectively), similar to that of the caspase inhibitor VX-765. Using X-ray diffraction, the authors investigated the binding mechanism of human GSDMD and nanobody complex, in which nanobody recognized the oligomerization interface of GSDMD NTD to block the pore forming process (Fig. 17A and B)[15].

Except for antibody directly binding to gasdermin proteins, other proteins involved in the membrane lysis process can also be antibody-binding targets for blocking pyroptosis effects. In 2023, Kayagaki et al.[217] described a monoclonal antibody D1 against NINJ1 protein to inhibit NINJ1 oligomerization and subsequent plasma membrane rupture. NINJ1 is a transmembrane protein located on the cell surface, which actively mediates the rupture of the plasma membrane following cellular osmotic pressure increase induced by pyroptosis, necroptosis etc.[218]. As the end point of pyroptosis-mediated cell death, the randomly distributed NINJ1 monomers undergo large oligomerization to lyse the membrane and release proinflammatory cytoplasmic molecules (Fig. 17C)[219]. Treatment with the D1 antibody remarkably inhibited NINJ1 oligomerization (Fig. 17D) and NINJ1-dependent plasma membrane rupture induced by nigericin-mediated pyroptosis and intracellular content leakage[217], providing a potential therapy for the inflammation and tissue injury induced by pyroptosis as well as other types of cell death.

4.2.2. *Small molecules targeting gasdermin and NINJ1*

Compared with small molecule inhibitors targeting upstream proteins such as caspases and inflammasomes, directly inhibiting gasdermins might provide the better therapeutic efficacy and specificity with minimal side effects. The previously identified gasdermin inhibitors mainly include necrosulfonamide[220], disulfiram and dimethyl fumarate, which all block the pore forming activity of GSDMD *via* covalent modification at the Cys191 (human)/Cys192 (mouse) site (Table 2). Notably, Cys191/192

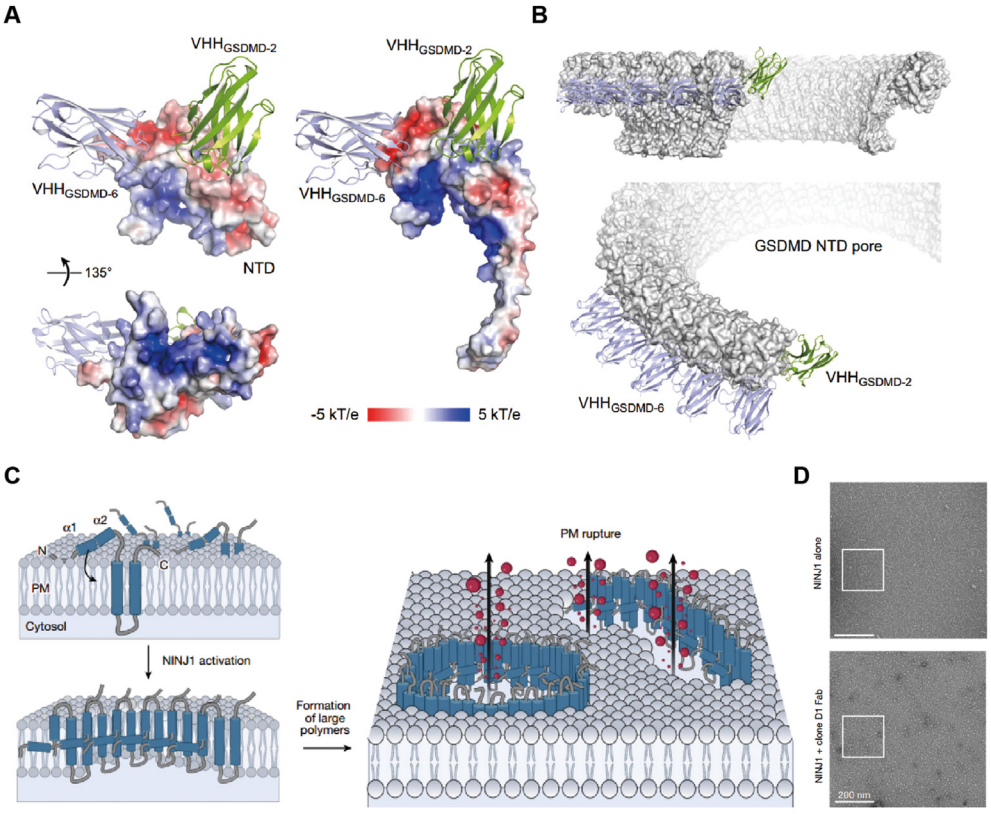
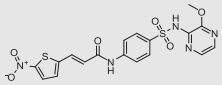
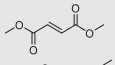
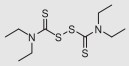
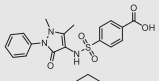
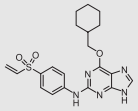
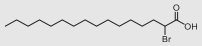


Figure 17 Antibodies for pyroptosis inhibition. (A) Interaction interfaces between GSDMD and nanobodies of $VHH_{GSDMD-2}$ and $VHH_{GSDMD-6}$. (B) Overlay of $VHH_{GSDMD-2}$ and $VHH_{GSDMD-6}$ binding to GSDMD pores[15]. Reproduced with permission. Copyright © 2023, Springer Nature. (C) Schematic diagram of NINJ1-mediated plasma membrane rupture[219]. Reproduced with permission. Copyright © 2023, Springer Nature. (D) Negative-stain electron microscopy of NINJ1 with or without D1 antibody[217]. Reproduced with permission. Copyright © 2023, Springer Nature.

Table 2 Small-molecule pyroptosis inhibitors targeting gasdermin and NINJ1.

Molecule	Structure	Target	IC_{50}	Off-target	Disease model
Necrosulfonamide		Cys191 in GSDMD	≈10 μmol/L	MLKL	LPS-induced sepsis and pulmonary fibrosis[220]
Disulfiram		Cys191 in GSDMD	<10 μmol/L	Multiple targets such as caspase-1, caspase-3	LPS-induced sepsis, autoimmune encephalitis[221]
Dimethyl fumarate		Cys191 in GSDMD	≈10 μmol/L	Multiple targets such as NF-κB, NLRP3, Nrf2	LPS-induced sepsis, pneumonia[222]
GI-Y1		Arg7 in GSDMD	N/A	N/A	Cardiac disorder[224]
NU6300		Cys191 in GSDMD	≈0.9 μmol/L	hERG potassium channel	Colitis and LPS-induced sepsis[225]
2-Bromopalmitate		GSDME palmitoylation	N/A	Palmitoyltransferase DHHC	TNF-α+CHX-induced pyroptosis[223]
Muscimol		NINJ1	N/A	$GABA_A$ receptors	LPS-induced sepsis[227]

uniquely existed in GSDMD, providing a specific target for distinguishing GSDMD from other gasdermin family members. Necrosulfonamide was previously reported as an inhibitor of the mixed lineage kinase domain-like protein (MLKL), a necroptosis effector, through selectively inhibiting the function of MLKL−RIP1−RIP3 complex. In 2018, Rathkey et al.[220] revealed that necrosulfonamide directly binds to Cys191 of human and mouse GSDMD *via* a Michael addition reaction, which further

disrupts GSDMD NTD oligomerization by preventing the cysteine disulfide formation ($IC_{50} \approx 10$ μmol/L). Moreover, necrosulfonamide-based inhibition of pyroptosis displays a therapeutic effect in a sepsis model.

Disulfiram is the first FDA-approved medication for the chronic alcohol dependence. In 2020, the old drug disulfiram was identified as a potent pyroptosis inhibitor by Hu et al.[221] using a fluorogenic liposome leakage-based high-throughput screen. By covalently binding to Cys191 (human)/Cys192 (mouse) of GSDMD, disulfiram abrogates the oligomerization of GSDMD NTD to inhibit the pyroptotic cell death ($IC_{50} \sim 10$ μmol/L) and proinflammatory cytokine (IL-1β) release, without disturbing GSDMD cleavage. In an LPS-induced sepsis model, the intraperitoneal injection of disulfiram significantly reduced the levels of IL-1β, TNF-α, and prolonged the survival of mice. Similarly, in 2020, dimethyl fumarate and endogenous fumarate were observed to block the activity of GSDMD through covalently modifying Cys191 to form *S*-(2-succinyl)-cysteine[222]. Distinct from necrosulfonamide and disulfiram, dimethyl fumarate-induced modifications disturb the interaction between GSDMD and caspases to inhibit the processes of cleavage, oligomerization and cell death ($IC_{50} < 10$ μmol/L). Besides, dimethyl fumarate treatment shows a protection effect against LPS shock and ameliorates chronic inflammatory diseases (such as experimental autoimmune encephalitis). In the same year, Hu et al.[223] revealed that the commonly used palmitoylation inhibitor 2-bromopalmitate (2-BP) was capable of inhibiting TNF-α+CHX-induced pyroptosis by blocking GSDME-C palmitoylation.

Recently, the GSDMD-specific inhibitors GI-Y1[224] and NU6300[225] were identified to block pyroptosis by binding Arg7 and Cys19, respectively (Table 2), which were evaluated in the models of ischemia/reperfusion injury, dextran sodium sulfate−induced colitis and LPS-induced sepsis. In addition to small molecules, Mg^{2+} has also recently been found to impede GSDMD NTD oligomerization and membrane localization by blocking Ca^{2+} influx, resulting in pyroptosis inhibition and remarkable survival of mice with LPS-induced sepsis[226]. Regardless of the potent activities in blocking the pore-forming of gasdermins, all of the above inhibitors exhibited the off-target effects, severely limiting their applications in pyroptosis-related inflammatory diseases. This also triggers an urgent need for novel inhibitors with high activity and specificity.

In addition to directly disturbing the pore-forming process, small molecules inhibiting NINJ1-mediated membrane rupture following gasdermin oligomerization also exhibit therapeutic potential in LPS-induced sepsis (Table 2). In 2023, Fink and coworkers identified a small molecule, muscimol, with the novel activity of blocking NINJ1 oligomerization to reduce the release of proinflammatory HMGB1 after *Salmonella* infection[227]. Subcutaneous administration of muscimol efficiently suppressed the renal damage and the lethality of LPS-induced sepsis. The off-target effect also exists in muscimol which is a well-characterized neuronal $GABA_A$ activator. Muscimol and NINJ1 antibodies demonstrated that blocking NINJ1 oligomerization might provide another promising target for pathological conditions related to pyroptosis[217,227].

4.2.3. *ROS scavengers*

ROS have gradually been recognized as a critical source for triggering caspase-mediated pyroptosis. Thus, scavenging ROS might represent a therapeutic strategy for pyroptosis-associated diseases. In 2022, Ma et al.[228] constructed an artificial prussian blue-based nanozyme (termed PBzyme) to scavenge ROS for alleviating the progression of neurodegenerative diseases by inhibiting pyroptosis (Fig. 18A). PBzyme was obtained using hydrothermal method and raw materials of potassium ferricyanide, poly(vinylpyrrolidone). Due to its remarkable capability in ROS scavenging, PBzyme significantly reduced the activation of NLRP3 and caspase-1, and the subsequent generation of GSDMD-mediated inflammatory factors in microglia. Moreover, intracerebroventricular injection of PBzyme alleviated dopaminergic degeneration and neuroinflammation generation in a Parkinson's disease mouse model (induced with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, MPTP). Similarly, in 2022, Chen et al.[229] developed a tea polyphenol EGCG-based nanoparticles as the radical species (O, N) scavengers (Fig. 18B), efficiently inhibiting pyroptosis during LPS-induced sepsis to reduce organ damage and prolong mouse survival. Notably, the polymeric EGCG nanoparticles might directly disturb the oligomerization of GSDMD NTD to block the LPS-induced pyroptosis, and the detailed mechanism remains to be investigated. In 2023, EGCG was also adopted by Zhou et al.[230] to synthesize the metallopolyphenol nanoparticle PG@Cu-FP as an ROS scavenger for pyroptosis inhibition (Fig. 18C and D). Besides, with the help of a mitochondrial targeting peptide, PG@Cu-FP effectively reduced mitochondria-derived ROS to alleviate the oxidative stress-induced damage towards mitochondria in nucleus pulposus cells (NPCs). This further weakened the NLPR3 inflammasome activation to inhibit GSDMD-mediated pyroptosis, thereby efficiently delaying excessive inflammation-associated intervertebral disc degeneration in mouse model (Fig. 18D). Other designs, such as using NAD^+[231] and antioxidases[232] as ROS scavengers, have also been reported to act as pyroptosis inhibitors for the treatment of pyroptosis-related hepatitis and sepsis.

5. Conclusions and perspectives

The past years have witnessed the rapid development of pyroptosis in molecular mechanisms and immunotherapy of diseases. Nonetheless, many challenges remain to be addressed for facilitating the clinical application, particularly considering the double-edged sword effects of pyroptosis. The open questions are described as follows.

5.1. *The triggering mechanism*

As a critical type of regulated cell death, pyroptosis is recognized as a potent host defense system against infection either in metazoans or in fungi and bacteria. In metazoans, multiple proteins including PRRs, TNF receptors, and caspases are currently identified to act as sensors and adaptors for gasdermin cleavage. The interaction networks of pyroptosis, is complex and still unclear, such as the ROS-inflammasome-caspase network. There might be other underlying pathways for triggering metazoan pyroptosis, which can provide new intervention targets for diseases. In fungi and bacteria, the pyroptosis activation is hypothesized to adopt a model similar to that of metazoans, namely, stimulants, PRRs, caspase-like proteins and gasdermin-like proteins. An increasing number of studies have indicated that redox regulation, such as ROS induction and the antioxidant system, represents a vital factor to influence pyroptosis through multiple mechanisms, including inflammasome activation, palmitoylation and disulfide bond reduction[49,62,233]. Hence, ROS might be relatively universal effectors or triggers of pyroptosis in metazoans, fungi and

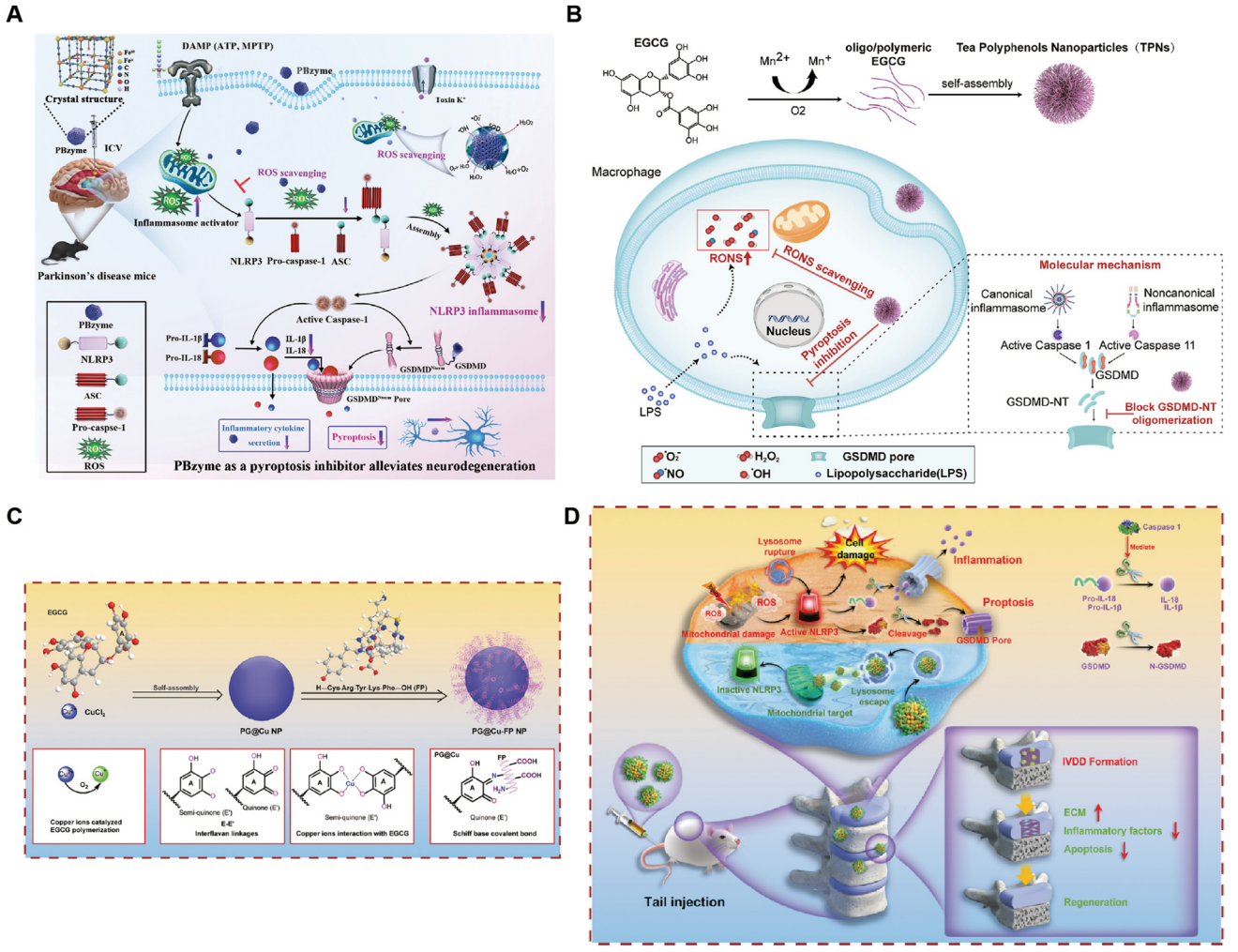


Figure 18 ROS scavengers for inhibiting pyroptosis. (A) Schematic diagram of the PBzyme structure and its activity of mitochondrial ROS-scavenging to inhibit NLRP3 and subsequent microglial pyroptosis[228]. Reproduced with permission. Copyright © 2022, Wiley-VCH GmbH. (B) Schematic illustration of TPN preparation and the pathway of TPN in pyroptosis inhibition through RONS scavenging[229]. Reproduced with permission. Copyright © 2022, American Chemical Society. (C) Schematic of PG@Cu-FP assembly. (D) The mechanism of PG@Cu-FP for intervertebral disc degeneration therapy through mitochondrial ROS-scavenging to inhibit NLRP3-GSDMD-mediated pyroptosis[230]. Reproduced with permission. Copyright © 2023, Wiley-VCH GmbH.

bacteria, remaining to be explored. To date, numerous questions remain from infection to pore formation in fungi and bacteria, particularly regarding the molecular mechanisms of caspase-like protein activation triggered by stimulant components derived from the infection process. Besides, the mechanisms revealed in fungi and bacteria might also provide inspiration for metazoan pyroptosis research.

5.2. *Gasdermin cleavage, organelle location and new functions*

In addition to caspases, granzymes, other effectors mediating gasdermin cleavage might also exist. Recently, ROS-dependent palmitoylation is found to be required for GSDMD pore forming[233,234], suggesting cofactors involved. In addition to the plasma membrane, gasdermins also translocate to mitochondria and lysosomes to assemble into transmembrane pores. In particular, the pore-formation in mitochondria is essential for amplifying caspase activation signals and efficient gasdermin cleavage. Thus, whether other membranous organelles are damaged by gasdermins and the underlying functions require further research. Notably, GSDMD-mediated transient pore formation in the plasma membrane is recently observed to regulate the release of proinflammatory S100A8/S100A9 from neutrophils, revealing a novel function in cell communication[207]. Other remaining questions include the functions of the released gasdermin CTD, and effects of pore formation in fungi and bacteria except for cell death.

5.3. *Pyroptosis-based pathogenesis*

Pyroptosis has been found to be involved in the pathogenesis of multiple diseases such as cancer, cardiovascular diseases, neurodegenerative diseases, infectious diseases, renal inflammation/fibrosis, retinal vascular inflammatory diseases, inflammatory bowel diseases, and autoimmune diseases[235]. These findings firmly demonstrate the potential role of pyroptosis in therapeutic intervention, and also provide a novel entry point for understanding the pathogenesis of other diseases. However, the understanding of pyroptosis-involved pathological mechanisms is still

limited: what cell types exhibit abnormal gasdermin expression, well defined pyroptotic characteristic in diseased states; the necessity of activated gasdermin-mediated pathway of identified cell type in disease phenotypes *in vivo*; and the cross-talk between pyroptosis and other cell death pathways in diseased states. The future direction involves the systematic evaluation of pyroptosis-targeting therapeutic value, and the dissection of pyroptosis-mediated pathogenesis in more disease models.

5.4. *Therapeutic intervention*

For induction intervention, how to avoid the side effects of pyroptotic cell death towards normal tissues and uncontrollable cytokine bursts is the main question. The majority of current induction strategies are too complex to be used in the clinic. Thus, there is an urgent need for efficient, simple and precise pyroptosis-induction therapeutics. Given the heterogeneity of gasdermin expression, epigenetic regulation can be combined with the strategies involving the cleavage of endogenous gasdermins. Relying on the conspicuous capability in target cells elimination, pyroptosis induction therapeutics can be expanded to other diseases such as preventive clearance of diseased cells (infected, early cancerous and mutant one etc.), rather than limited to tumors. If this is achieved, it will greatly promote the related clinical applications. As to inhibition intervention, developing small molecule inhibitors with high activity and specificity towards gasdermins for *in vivo* therapy is still a challenge remaining to be addressed. Additionally, the use of antibodies to block pore formation represents another efficient option. Currently, there is no antibody identified to directly bind gasdermin and block oligomerization outside the cell membrane. If this is achieved, this will avoid the use of vectors or mRNAs for the cytosolic delivery of antibodies. As a native negative regulatory mechanism of pyroptosis, ESCRT-related pathways might represent alternative targets for blocking pyroptosis-induced tissue damage[104].

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Author contributions

Junjun Wu: Writing − review & editing, Writing − original draft, Supervision, Funding acquisition, Conceptualization. Hong Wang: Writing − review & editing, Resources. Pu Gao: Writing − review & editing. Songying Ouyang: Writing − review & editing, Supervision, Funding acquisition, Conceptualization.

Conflicts of interest

The authors declare no conflict of interest.

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