



# Channelling inflammation: gasdermins in physiology and disease

Xing Liu<sup>1,5</sup>, Shiyu Xia<sup>2,3,5</sup>, Zhibin Zhang<sup>2,4,5</sup>, Hao Wu<sup>2,3</sup> and Judy Lieberman<sup>2,4</sup>

**Abstract** | Gasdermins were recently identified as the mediators of pyroptosis — inflammatory cell death triggered by cytosolic sensing of invasive infection and danger signals. Upon activation, gasdermins form cell membrane pores, which release pro-inflammatory cytokines and alarmins and damage the integrity of the cell membrane. Roles for gasdermins in autoimmune and inflammatory diseases, infectious diseases, deafness and cancer are emerging, revealing potential novel therapeutic avenues. Here, we review current knowledge of the family of gasdermins, focusing on their mechanisms of action and roles in normal physiology and disease. Efforts to develop drugs to modulate gasdermin activity to reduce inflammation or activate more potent immune responses are highlighted.

## Gasdermins

A family of homologous pore-forming proteins whose N-terminal fragments permeabilize cell membranes when the protein is cleaved in the linker region connecting the active N-terminal and inhibitory C-terminal domains.

<sup>1</sup>The Center for Microbes, Development and Health, Key Laboratory of Molecular Virology and Immunology, Institut Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai, China.

<sup>2</sup>Program in Cellular and Molecular Medicine, Boston Children's Hospital, Boston, MA, USA.

<sup>3</sup>Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA, USA.

<sup>4</sup>Department of Pediatrics, Harvard Medical School, Boston, MA, USA.

<sup>5</sup>These authors contributed equally: Xing Liu, Shiyu Xia, Zhibin Zhang.

✉e-mail: xingliu@ips.ac.cn; wu@crystal.harvard.edu; judy.lieberman@childrens.harvard.edu  
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Gasdermins (GSDMs) are a recently characterized protein family encoded by six paralogous genes: *GSDMA*, *GSDMB*, *GSDMC*, *GSDMD*, *GSDME* (also known as *DFNA5*) and *DFNB59* (also known as *pejvakin*)<sup>1</sup>. Proteolytic cleavage of the GSDMs liberates an N-terminal (NT) fragment, which can assemble in membranes to form pores (verified for most, but not all, of the family). GSDM pores can disrupt cell membrane integrity to trigger an inflammatory cell death in which cellular contents, including inflammatory cytokines, are released into the extracellular space<sup>2–4</sup> (BOX 1; FIGS 1, 2). GSDM-mediated cell death is termed pyroptosis (fiery death).

The name ‘gasdermin’ comes from GSDM expression in the gastrointestinal tract and skin. Individual GSDMs are selectively expressed with varying abundance wherever the body encounters, detects and responds to infection<sup>3,5</sup>, being predominantly expressed in specific mucosal sites (TABLE 1). *GSDMA* is found in skin and the gastrointestinal tract, *GSDMB* is located in the lung, oesophagus, gastrointestinal tract and immune cells, *GSDMC* is found in keratinocytes and the gastrointestinal tract, and *GSDMD* is found in the gastrointestinal epithelia and in the sentinel cells of the immune system, macrophages and dendritic cells<sup>3</sup>. *GSDME* has a different pattern of expression in mesenchymal cells — muscle (both skeletal and cardiac), central nervous system (CNS) and placenta<sup>6</sup>. The physiological role of *GSDME* in these tissues, where inflammation might be harmful, is not entirely clear, although it may be involved in development<sup>7</sup>.

GSDMs A–E share highly conserved NT and C-terminal (CT) domains separated by a variable linker (FIG. 3a). The NT domain of GSDMs binds to acidic phospholipids in the inner leaflet of cell membranes

and forms pores<sup>8–11</sup>. In the full-length protein, the CT domain folds back on the NT domain to auto-inhibit pore formation<sup>9,12</sup>. GSDM cleavage in the linker region liberates the active, pore-forming NT domain. *GSDMD* is the most studied GSDM as it is the key executioner of inflammasome-induced pyroptosis and is probably the most important GSDM mediating disease-associated inflammation. *GSDMD* cleavage and pyroptosis are activated when cytosolic danger and infection sensors in macrophages and dendritic cells assemble into large supramolecular complexes called inflammasomes that recruit and activate inflammatory caspases<sup>13–15</sup>. The inflammatory caspases activate *GSDMD* by cleaving after Asp275 (mouse Asp276) in the linker<sup>13,14</sup>. By contrast, *GSDME* is activated during classical apoptosis (BOX 2), by caspase 3 cleavage after Asp270, which converts non-inflammatory apoptotic death into inflammatory pyroptotic death in cells that express *GSDME*<sup>16,17</sup>. Granzymes (Gzms) A and B, death-inducing proteases in natural killer (NK) cells and cytotoxic T lymphocytes (CTLs), also cleave *GSDMB* and *GSDME*, respectively, to activate pyroptosis during killer cell attack, which was previously thought of as non-inflammatory<sup>18,19</sup>. A recent study showed that *GSDMC* can be cleaved by caspase 8 after TNF-mediated death receptor signalling to trigger pyroptosis<sup>20</sup>. How *GSDMA* is activated is currently unknown.

GSDM pores in the plasma membrane act as channels through which low-molecular-weight cellular contents are released into the extracellular space to initiate inflammation (FIG. 2). Importantly, the pores mediate the ‘unconventional protein secretion’ of the pro-inflammatory cytokines (IL-1 $\beta$  and IL-18) that lack a signal peptide for secretion via the endoplasmic reticulum to Golgi secretory pathway<sup>21</sup>. Cellular alarmins, including ATP

**Pyroptosis**

A programmed inflammatory cell death, which occurs after the cleavage and activation of a gasdermin, leading to loss of cell membrane integrity and release of pro-inflammatory cytokines and cellular proteins that can act as danger signals.

**Inflammasome**

Intracellular complex of cytosolic sensors of invasive infection that recognize pathogen-associated molecular patterns (PAMPs) or of danger that recognize damage-associated molecular patterns (DAMPs) and then assemble into supramolecular complexes that activate inflammatory caspases; the canonical inflammasome activates caspase 1 and the noncanonical inflammasome activates caspase 4 or caspase 5 in humans or caspase 11 in mice.

**Inflammatory caspases**

Cysteine proteases that are activated by proximity-induced autocatalytic cleavage when they dimerize after recruitment to canonical (caspase 1) or noncanonical (caspase 4, 5 or 11) inflammasomes and are responsible for processing inflammatory cytokines and gasdermin D to induce pyroptosis or hyperactivation.

**Apoptosis**

A noninflammatory programmed cell death mediated by mitochondrial outer membrane permeabilization and activation of the effector caspases (3, 6 and 7).

and HMGB1, cleaved GSDMs and other cytokines and chemokines, are released at the same time to amplify inflammation in the tissue and recruit immune cells to respond to the infection or damage. Many small molecules and other proteins, which may differ between cell types, are probably released from pyroptotic cells. Pore formation triggers rapid pyroptotic cell death in which the damaged membrane forms large ballooning bubbles and dying cells appear to flatten as their cytoplasmic contents are released<sup>22</sup>. Both the rapidity of cell death and the morphological changes are hallmarks that distinguish pyroptotic from apoptotic cell death<sup>3</sup>. In some circumstances, GSDM-mediated cell membrane damage can be repaired by the ubiquitous damaged cell membrane repair response — the cell survives but inflammatory mediators are released through GSDM pores before the pores are removed from the membrane<sup>23</sup>. Macrophages that survive despite GSDMD cleavage have been termed ‘hyperactivated’, because they both stimulate inflammation and preserve their other antigen-presenting functions<sup>24</sup>.

The diverse physiological and pathological roles of GSDMs as executioners of pyroptosis are still being uncovered. However, associations of GSDMs with several diseases have emerged. GSDMD has been implicated in the pathogenesis of many inflammasome-associated diseases and tissue damage<sup>14,25,26</sup>. In addition, spontaneous mutations of GSDMs lead to disorders such as alopecia (GSDMA)<sup>27–31</sup>, asthma (GSDMB)<sup>32</sup> and hearing impairment (GSDME/DFNB59)<sup>6,33–39</sup> in mice and/or humans. Moreover, accumulating evidence suggests that GSDMs can inhibit or promote infection and cancers, implying complicated links between inflammation and pyroptosis and the development and progression of those diseases<sup>18–20,40–47</sup>.

The key roles of GSDMs in infection, inflammation and cancer have stimulated investigation of GSDM-targeting therapeutics. Strategies to therapeutically modulate GSDM activity are beginning to emerge, and several inhibitors of GSDMD have now been reported<sup>45,48,49</sup>.

**Box 1 | Discovery of pyroptosis**

Pyroptosis, a programmed, inflammatory cell death, is mediated by N-terminal (NT) gasdermin pores (FIG. 2). Pyroptotic cells lose cell membrane integrity, which leads to release of inflammatory cellular contents and formation of large ballooning bubbles, which are much larger than apoptotic blebs<sup>22,186</sup>. The dying cell appears to flatten as its cytoplasmic contents are released.

The first descriptions of pyroptosis were in the 1990s in macrophages that died after infection with invasive *Shigella flexneri* or *Salmonella enterica* subsp. Typhimurium. At that time, invasive bacteria-induced cell destruction was regarded as apoptosis, as markers of apoptosis (DNA fragmentation and membrane blebbing) were observed<sup>187–190</sup>. Further studies showed that macrophage death in response to bacterial infection was mediated by inflammatory caspase 1 rather than apoptotic caspases and was associated with pro-inflammatory cytokine secretion<sup>190–195</sup>. In 2001, Cookson and Brennan coined the term pyroptosis (fiery death) to distinguish inflammatory caspase-mediated cell death from apoptosis and unprogrammed necrosis caused by lack of membrane integrity<sup>196</sup>. Gasdermin D (GSDMD) was identified in 2015 as the inflammatory caspase substrate responsible for pyroptotic cell death in immune cells, and the following year the mechanism responsible for GSDMD-mediated death was identified as pore formation by the inflammatory caspase-generated GSDMD-NT<sup>8–11,13,14,22</sup>. Identifying the molecular basis of pyroptosis has prompted an explosion of research to understand the role of pyroptosis in disease.

This Review encapsulates our current understanding of the expression, activation and regulation of GSDMs and highlights their important physiological and pathological roles as perpetrators of cell death, cytokine secretion and inflammation. Emerging opportunities to develop GSDM-targeted drugs, particularly GSDMD inhibitors, and the associated challenges, are discussed.

**The gasdermin family**

Humans have six GSDM genes: *GSDMA*, *GSDMB*, *GSDMC*, *GSDMD*, *GSDME* and *DFNB59* (TABLE 1; FIG. 1). *GSDME* on human chromosome 7p15.3 is the most ancient gene with orthologues found in early metazoa and invertebrates<sup>17,50,51</sup>. The other GSDM genes likely arose as gene duplications — *GSDMA* and *GSDMB* on human chromosome 17q21 and *GSDMC* and *GSDMD* on human chromosome 8q2. Although in metazoa *DFNB59* is not truncated and is closely homologous to *GSDME*, mammalian *DFNB59* is a more distant and truncated gene, whose N terminus is homologous to the NT, pore-forming region of the other family members, but whether it possesses pore-forming activity is unknown. Mice lack a *GSDMB* orthologue, but have one *Gsdmd* on chromosome 15, one *Gsdme* on chromosome 6 and three *Gsdmas* (*Gsdma1–3*) and four *Gsdms* (*Gsdmc1–4*) clustered on chromosomes 11D and 15D1, respectively<sup>1</sup>.

Although published studies and web-based gene expression atlases are available for GSDM expression, these resources are inadequate for accurately predicting GSDM expression, which can be induced by cytokines and immune signalling in cells that are not ordinarily thought to express GSDMs, including lymphocytes. For example, *GSDMA* is upregulated by TGFβ<sup>52</sup>, *GSDMB* by interferon-α (IFNα), IFNβ, IFNγ and TNF<sup>19</sup>, *GSDMD* by IRF2 (REF.<sup>53</sup>) and *GSDME* by corticosteroids and forskolin<sup>54</sup>. Furthermore, expression of some of the GSDMs (especially of *GSDMA* and *GSDME* in cancer cell lines) is inferred to be epigenetically suppressed by DNA methylation, as DNA methyltransferase inhibitors can induce their expression<sup>55–60</sup>. Perhaps because of their expression in barrier cells, mutations in GSDM genes have been associated with skin defects, asthma and deafness in mice and humans (TABLE 2).

***GSDMA***. The first GSDM family member was identified by positional cloning of a gene causing abnormal skin and hair development in the mouse *Rim3* mutant<sup>5,61</sup>. Subsequent studies of mice with similar skin phenotypes<sup>27–31</sup> and bioinformatics<sup>62</sup> identified a *Gsdma1*, *Gsdma2* and *Gsdma3* gene cluster on mouse chromosome 11D and found that *Gsdma3* mutations caused alopecia (TABLE 2; FIG. 1). All these mutations localize to the CT domain of *Gsdma3* and behave as gain-of-function mutations that unmask the functional NT domain<sup>9</sup>. *Gsdma2* and *Gsdma3* are primarily expressed in stomach and skin, respectively<sup>27,52</sup>. The human *GSDMA* orthologue is also expressed in stomach and skin but is silenced in gastric cancer tissues and cell lines<sup>5</sup> (TABLE 1). The DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-aza-dC) upregulates *GSDMA* in these cancer lines, suggesting that *GSDMA* expression is suppressed by DNA methylation<sup>55</sup>. In pit cells of the gastric epithelium, TGFβ

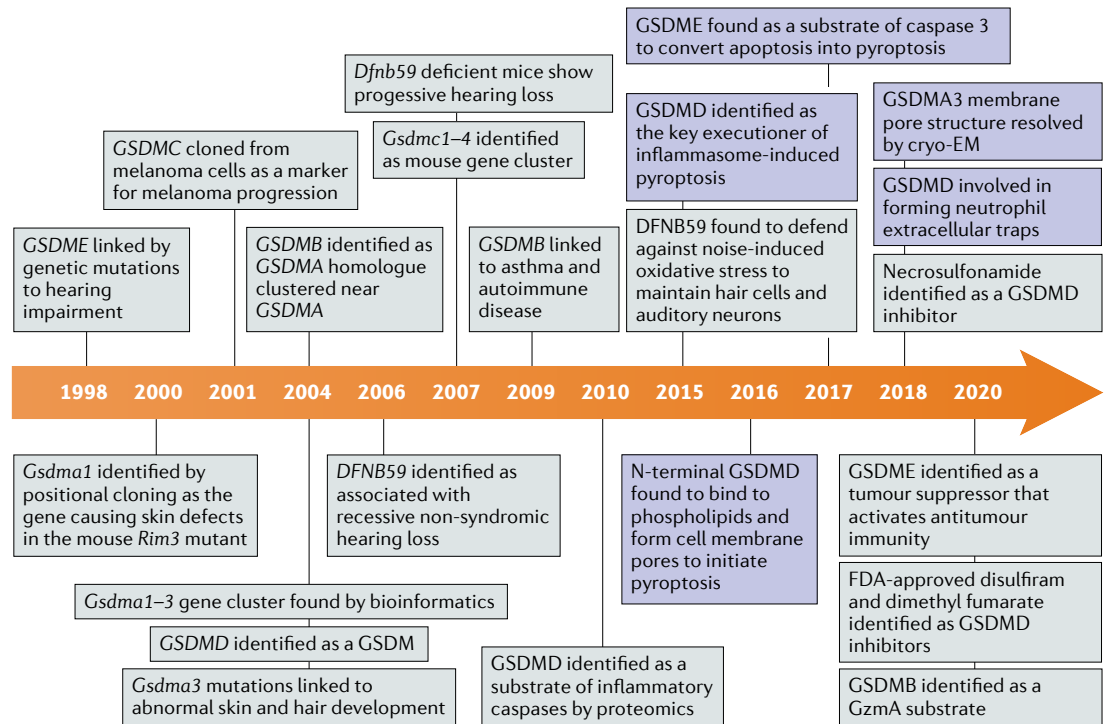


Fig. 1 | Key events in the history of gasdermins. The purple boxes indicate milestone discoveries of gasdermin (GSDM) research.

induces the LIM domain only 1 (LMO1) transcription factor to upregulate *GSDMA* and trigger pit cell death<sup>52</sup>. TNF similarly upregulates *Gsdma3* and causes death of mouse skin keratinocytes in vitro and in vivo<sup>63</sup>. It is worth noting that no obvious phenotype was observed in *Gsdma*<sup>-/-</sup> or *Gsdma3*<sup>-/-</sup> mice<sup>64,65</sup>, indicating that the *Gsdma* genes may be redundant in development and in the maintenance of gastric and skin barriers, or that their function is only apparent upon challenge by pathogens or other danger signals, which would be consistent with cleavage-dependent activation. *GSDMA* mutations have not been reported in human alopecia; however, *GSDMA* polymorphisms have been linked to systemic sclerosis (an autoimmune fibrotic disease of the skin and visceral organs), inflammatory bowel disease (IBD) and childhood asthma<sup>66–69</sup> (TABLE 2).

**GSDMB.** The human *GSDMB* gene clusters with *GSDMA*<sup>62</sup>, but no *Gsdmb* has been identified in rodents. *GSDMB* is expressed more broadly than *GSDMA*, especially in airway and gastrointestinal epithelia, liver, and neuroendocrine and immune cells<sup>32,70–72</sup> (TABLE 1). There are four *GSDMB* isoforms (1–4; Ensembl: ENSG0000073605), but whether these isoforms are expressed in a tissue or cell-specific manner is unknown. Genome-wide association studies (GWAS) have uncovered a strong association between *GSDMB* and susceptibility to chronic inflammatory diseases including IBD, asthma and type I diabetes<sup>32,73–76</sup>, and a single nucleotide polymorphism linked to increased *GSDMB* expression is associated with asthma<sup>32</sup> (TABLE 2). The link to childhood asthma and IBD is shared between *GSDMA* and *GSDMB*<sup>66,69,77</sup>, likely reflecting the close chromosomal

locations of these two genes and their potentially coupled expression.

*GSDMB* isoform 1 is the dominant isoform in human bronchial epithelial cells, and elevated isoform 1 expression is found in patients with asthma compared with healthy controls<sup>32</sup>. *GSDMB* isoform 1 overexpression increases the levels of genes involved in airway responsiveness and remodelling including the genes encoding TGFβ1, 5-lipoxygenase and MMP9, which could explain its role in asthma<sup>32</sup>. Surprisingly, unlike other GSDMs, *GSDMB* isoform 1 was found exclusively in the nucleus, consistent with a postulated role in amplification of transcription<sup>32</sup>, but making it uncertain whether *GSDMB*-mediated pyroptosis is involved in the pathogenesis of asthma or IBD. More in vitro and in vivo evidence is required to understand whether the effects of *GSDMB* depend on pore formation.

Unlike *GSDMA* and *GSDME*, *GSDMB* expression is markedly increased in various cancers, including cervical, breast, gastrointestinal and hepatic cancers, and its high expression is associated with poor prognosis<sup>71,78–80</sup> (TABLE 1). However, whether different isoforms of *GSDMB* are expressed in distinct cancers or how *GSDMB* affects tumour biology is not known. The novel nuclear localization and transcriptional effect of *GSDMB*<sup>32</sup> suggest that its function may be largely independent of pore-forming activity and raise the possibility that other GSDMs might also have unsuspected functions that are independent of their cleavage or ability to form pores.

**GSDMC.** *GSDMC* was first cloned from melanoma cells as a marker for melanoma progression<sup>81,82</sup>, and the mouse cluster (*Gsdmc1–4*) was identified by phylogenetic

**Granzymes**

Serine proteases stored in the cytotoxic granules of killer lymphocytes that are released during killer cell attack to cause programmed cell death in the target cell.

**Pro-inflammatory cytokines**

Small molecular weight proteins secreted by immune cells and some other cells that evoke inflammatory responses, activate immune cells and cause inflammation, fever and tissue damage.

**Alarmins**

Host molecules released from damaged or dying cells that act as danger signals to stimulate inflammation, also known as damage-associated molecular patterns (DAMPs).

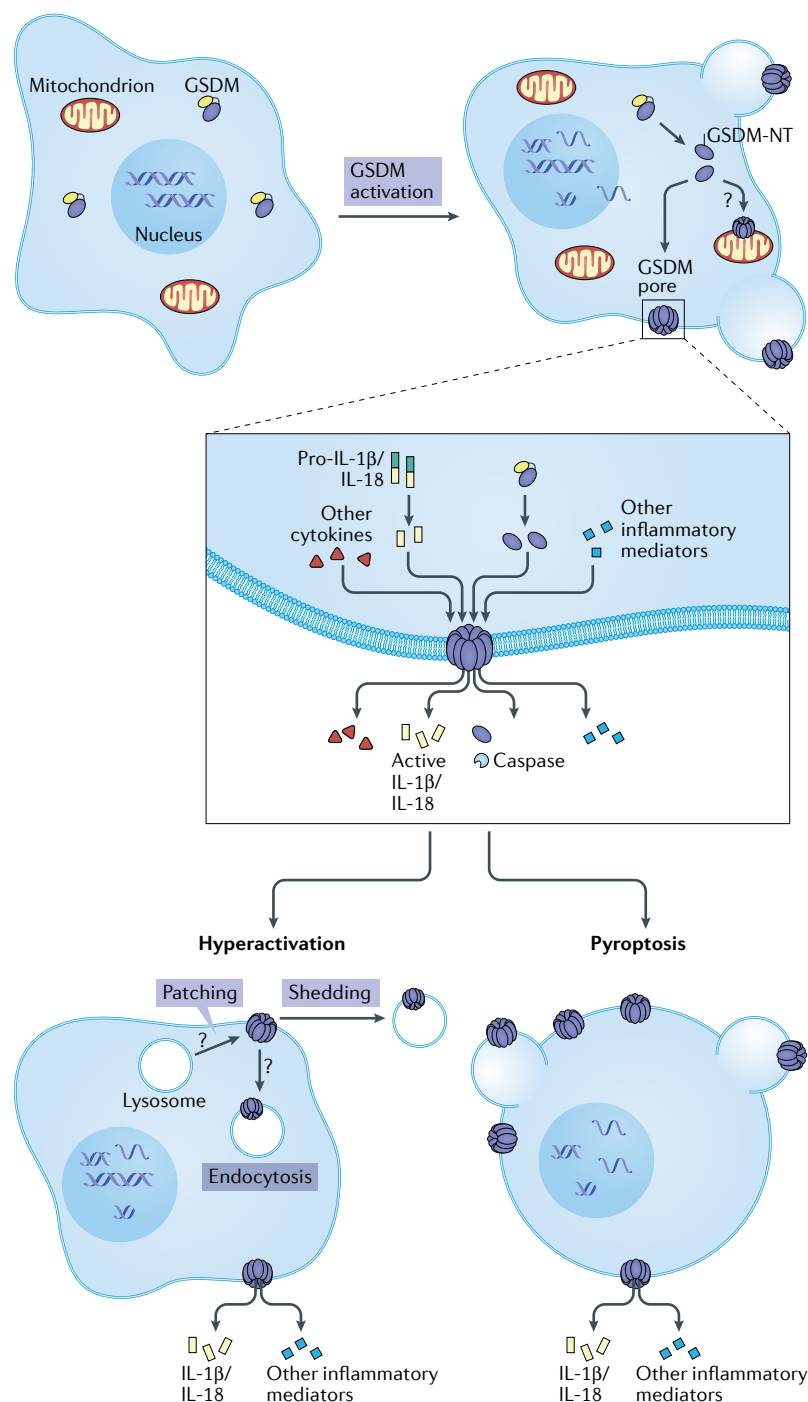
analysis<sup>1</sup>. *GSDMC* is expressed in trachea, spleen, oesophagus, small intestine, caecum, colon<sup>1,70,82</sup> and in skin keratinocytes, where expression is markedly increased by UV radiation via calcium-triggered NFATc1 signalling<sup>83,84</sup>. *GSDMC* expression can also be induced in tumour cells

by hypoxia-triggered STAT3 phosphorylation and its complexation with nuclear-translocated PDL1 (REF.<sup>20</sup>) (TABLE 1). *GSDMC* contributes to UV-induced MMP1 expression by activating ERK and JNK pathways<sup>84</sup>. *GSDMC* is cleaved and activated by caspase 8 in death receptor signalling, converting apoptosis into pyroptosis in cells that express *GSDMC*<sup>20</sup>. *GSDMC* is also upregulated in colorectal cancer and promotes cancer cell proliferation<sup>85</sup>, suggesting that *GSDMC* may function as an oncogene.

***GSDMD*.** *GSDMD* was originally identified by homology to other *GSDM* genes on chromosome 8 near *GSDMC*<sup>61</sup>. A 2010 proteomics study<sup>86</sup> identified *GSDMD* as the most efficient (and also selective) substrate of inflammatory caspases. However, the functional role of *GSDMD* and its cleavage were not explored further until the discovery of *GSDMD* as the key executor of inflammasome-induced pyroptosis reported by two groups in 2015, using a chemical mutagenesis screen in mice and a cell-based CRISPR screen<sup>13,14</sup>.

*GSDMD* is expressed in immune cells (especially macrophages and dendritic cells)<sup>3</sup>, placenta, gastrointestinal epithelium, various cancers including oesophageal and gastric cancers, pancreatic cancer, prostate cancer, melanoma and salivary gland tumour, and Jurkat T and Ramos B cancer cell lines<sup>70,81,87</sup> (TABLE 1). Transcription of *GSDMD* is upregulated by IRF2 (REF.<sup>53</sup>), which is a transcriptional suppressor of interferons. Although human *GSDMD* genetic polymorphisms have been identified, including a few that disrupt cleavage or pore formation<sup>88</sup>, most single nucleotide polymorphisms did not alter pore formation, and so far none has been linked to disease. The lack of known disease links does not mean that none exists, especially as the identification of *GSDMD* as the mediator of pyroptosis is so recent. Although *GSDMD* is widely expressed, no overt phenotype has so far been described in *Gsdmd*<sup>-/-</sup> mice, suggesting that *GSDMD* is largely dispensable for development and physiological homeostasis<sup>87</sup>. However, a recent study showed that the AIM2 cytosolic DNA-sensing inflammasome purges genetically compromised neuronal cells during neurodevelopment through *GSDMD* activation, preventing the accumulation of genetically aberrant cells and contributing to CNS homeostasis<sup>7</sup>. *Gsdmd*<sup>-/-</sup> mice, like *Aim2*<sup>-/-</sup>, *Asc*<sup>-/-</sup> or *Casp1*<sup>-/-</sup> mice, exhibited increased anxiety in behavioural tests<sup>7</sup>. In models of infection, *Gsdmd*<sup>-/-</sup> mice can be more or less susceptible to infection than wild-type mice, depending on the pathogen<sup>40,44,89,90</sup>. However, *Gsdmd*<sup>-/-</sup> mice are protected from death and morbidity in many models of genetic or induced inflammation, including sepsis, experimental autoimmune encephalomyelitis (EAE, a mouse model for multiple sclerosis (MS)), macular degeneration and neonatal onset multisystem inflammatory disease (NOMID, caused by gain-of-function mutation of the inflammasome gene *NLRP3*)<sup>14,25,26</sup>, highlighting the central role of *GSDMD* in inflammatory disease.

***GSDME*.** *GSDME* was first described as a mutated gene (*DFNA5*) that results in an autosomal dominant form of hearing impairment, caused by cochlear hair



**Fig. 2 | Gasdermins function as gatekeepers of pyroptosis.** In response to invasive pathogens, sterile danger signals or cytotoxic T cell attack, gasdermins (GSDMs) are activated by proteolytic cleavage, which releases the N-terminal (NT) fragment, which forms large cell membrane pores. The GSDM pore behaves as a gatekeeper for initiating downstream inflammatory cascades and pyroptotic cell death. Pyroptotic cells form large balloon-like membrane structures. Small intracellular molecules, including cytokines and cellular alarmins, are released through GSDM pores, causing inflammation. Some cells, termed 'hyperactivated', repair GSDM pores by shedding the damaged membrane and survive, but still induce inflammation by releasing IL-1 family cytokines.



Table 1 | Human and mouse gasdermin genes

| Human gene (chromosomal location)            | Mouse gene (chromosomal location)           | Expression in normal tissues   | Expression in tumours   | Activation pathway   | Biological function   | Disease links  | Refs                        |
|--|---|--|---|--|-----------------------|--|-----------------------------|
| <i>GSDMA</i> (17q21.1)                       | <i>Gsdma1–3</i> (11D)                       | Gastric and skin epithelia   | Silenced in gastric cancer tissues and cell lines   | Not known  | Not known             | Systemic sclerosis in humans, alopecia in mice   | 1,27,28,30,31,61            |
| <i>GSDMB</i> (17q21.1)                       | None  | Airway, oesophagus, gastrointestinal tract, liver and colon epithelium, neuroendocrine cells, immune cells | Expressed in colon, rectal, pancreatic and cervical cancers, and barely expressed in breast, lung and liver cancers                 | Granzyme A   | Not known             | Inflammatory bowel disease, asthma, type I diabetes  | 19,32,72,74–80              |
| <i>GSDMC</i> (8q24.21)                       | <i>Gsdmc1–4</i> (15D1)                      | Keratinocytes, trachea, spleen, oesophagus, small intestine, caecum, and colon                             | Upregulated in colorectal cancer and melanoma   | TNFR–caspase 8   | Not known             | Not known  | 20,82–85                    |
| <i>GSDMD</i> (8q24.3)                        | <i>Gsdmd</i> (15D3-E1)                      | Immune cells, placenta, oesophagus and gastrointestinal tract epithelium                                   | Expressed in oesophageal and gastric, pancreatic, prostate cancers, melanoma, salivary gland tumours, Jurkat T cells, Ramos B cells | Inflammasome caspases; neutrophil elastase; cathepsin G; RIPK1–caspase 8 | Pyroptosis; NETosis   | Sepsis, experimental autoimmune encephalomyelitis, macular degeneration, neonatal onset multisystem inflammatory disease | 13,14,25,43,105,106,131,144 |
| <i>GSDME</i> (7p15.3) (a.k.a. <i>DFNA5</i> ) | <i>Gsdme</i> (6B2.3) (a.k.a. <i>Dfna5</i> ) | Cochlea, placenta, heart, brain, kidney  | Epigenetically inactivated by DNA methylation in gastric, colorectal and breast cancer, and most human cancer cell lines            | Granzyme B, caspase 3  | Pyroptosis            | Autosomal dominant nonsyndromic hearing loss   | 16–18,33–38                 |
| <i>DFNB59</i> (2q31.2) (a.k.a. <i>PJVK</i> ) | <i>Dfnb59</i> (2C3) (a.k.a. <i>Pjvk</i> )   | Brain, eye, inner ear, heart, lung, kidney, liver, intestine, testis                                       | Not known   | Not known  | Hair cell maintenance | Recessive nonsyndromic hearing impairment  | 39                          |

a.k.a., also known as.

cell loss<sup>6,33–38,91</sup>. These mutations in *DFNA5* lead to skipping of exon 8, premature termination and activation of *GSDME* to cause cell death. *GSDME* mRNA is detected in cochlea, placenta, heart, brain and kidney<sup>6,92</sup> (TABLE 1). *GSDME* is cleaved and activated by caspase 3 (and possibly other apoptotic effector caspases, caspases 6 and 7) when cells expressing *GSDME* are triggered to undergo apoptosis. *GSDME* cleavage converts noninflammatory apoptosis into inflammatory pyroptosis. Why *GSDME*-activating mutations specifically cause auditory damage, with no apparent effect on other organs in which it is highly expressed, is unclear. One possibility is that because the autoactive *GSDME* protein is unstable, pyroptosis could be limited to especially vulnerable cochlear hair cells<sup>17</sup>. The poor stability of autoactive *GSDMs* could also explain why there are no reports of disease-associated autoactive mutant *GSDMs* except for human *GSDME* and mouse *GSDMA*.

Relative to normal tissue, *GSDME* expression is epigenetically suppressed by methylation in gastric, colorectal and breast cancers, and most human cancer cell lines. By contrast, p53, which is inactivated in many cancers, reportedly activates *GSDME* transcription in DNA-damaged cells<sup>93</sup>, which could promote the removal

of cancer cells by inflammatory recruitment and activation of phagocytic macrophages. *GSDME* is also mutated in a variety of other cancers, consistent with its postulated role as a tumour suppressor gene<sup>56–60,94,95</sup>. When expression of *GSDME* is induced by DNA methyltransferase inhibitors in vitro, colony formation in gastric and colorectal cancers and invasiveness in breast cancer are reduced<sup>56–59</sup>. Notably, cancer-associated *GSDME* mutations in the NT domain are primarily loss-of-function (LOF) mutations, which lead to impaired pore-forming ability<sup>18</sup> (TABLE 2).

***DFNB59***. *DFNB59* is a more distantly related *GSDM* family member with a truncated non-homologous CT domain. *DFNB59* transcripts are detected in brain, eye, inner ear, heart, lung, kidney, liver, intestine and testis<sup>39,96</sup> (TABLE 1). Like *GSDME*, *DFNB59* is linked by familial genetic mutations to hearing loss in humans, but these mutations are recessive, rather than dominant<sup>39</sup>. Patients with *DFNB59* mutations show either dysfunctional cochlear outer hair cells<sup>97</sup> or auditory neuropathy<sup>39</sup>, which impairs neural transmission of the auditory signal (TABLE 2). Mutations in mouse *Dfnb59* were also found to be associated with deafness by forward genetic

**AIM2**

(Absent in melanoma 2). An inflammasome that senses microbial or mislocalized host DNA in the cytosol to form a canonical inflammasome.

**Experimental autoimmune encephalomyelitis**

(EAE). Animal models of autoinflammatory disease in the central nervous system that resemble human multiple sclerosis, which are triggered by injecting immunogenic self peptides that sensitize CD4<sup>+</sup> T cells.

### Neonatal onset multisystem inflammatory disease

(NOMID). A rare severe autoinflammatory disease, caused by constitutively active mutations of the *NLRP3* inflammasome gene, that occurs in infancy with symptoms of fever, chronic meningitis, skin rash and arthropathy, also known as chronic infantile neurological cutaneous articular (CINCA) syndrome.

### IL-1 family

A group of 11 pro-inflammatory cytokines and their natural antagonists (IL-1 $\alpha$ , IL-1 $\beta$ , IL-1RA, IL-18, IL-33, IL-36 $\alpha$ , IL-36 $\beta$ , IL-36 $\gamma$ , IL-36RA, IL-37 and IL-38) that share a conserved  $\beta$ -trefoil structure and have important roles in promoting or regulating inflammatory responses to infection or sterile insults.

### NLRP3

A member of the NOD-like receptor subfamily of pattern recognition receptors, which recruits adaptor protein ASC and caspase 1 to assemble a large protein complex named the inflammasome in response to danger signals. Also known as NALP3 and cryopyrin.

### Pyrin

A cytosolic inflammasome sensor encoded by the *MEFV* gene, predominantly expressed in phagocytes, that is triggered by bacterial toxins or effectors.

screening<sup>97</sup>. However, *Dfnb59*<sup>-/-</sup> mice, but not *Gsdme*<sup>-/-</sup> mice, show hearing impairment<sup>39,97–99</sup>, suggesting that they cause deafness through different mechanisms.

DFNB59 is a peroxisome-associated protein required for oxidative stress-induced peroxisome proliferation in both hair cells and auditory neurons<sup>96</sup>, which protects against sound-induced oxidative damage. DFNB59 senses sound-induced reactive oxygen species (ROS) and recruits the autophagy machinery to degrade damaged peroxisomes<sup>100</sup>. It is unclear whether DFNB59 is a pore-forming protein or whether it is constitutively active, as its extremely short CT domain might not inhibit pore formation. It is worth exploring whether DFNB59 forms pores in peroxisome membranes and whether other interacting proteins inhibit or activate it. In support of the latter possibility, DFNB59-CT interacts with the ROCK2 Rho-associated protein kinase and the IQGAP1 scaffold protein<sup>99</sup>.

### Role of gasdermins in pyroptosis

The best-characterized pyroptosis pathway is that induced by GSDMD downstream of inflammasome activation in immune sentinel cells and some epithelial cells at mucosal and skin barriers (FIG. 3). Depending on the upstream inflammasome signals, the nature of the danger molecules released from dying cells may be different. Most canonical inflammasomes first require a transcriptional priming step (for example by lipopolysaccharide (LPS)-mediated Toll-like receptor signalling) to upregulate the expression of inflammasome sensors and pro-forms of IL-1 family cytokines<sup>101</sup>. When these canonical inflammasomes are activated, such as through the AIM2 sensor by cytosolic double-stranded DNA (dsDNA), the NLRP3 sensor by a large array of stimuli that disturb cell membrane integrity (for example bacterial toxin nigericin, extracellular ATP, uric acid crystals, cholesterol crystals and amyloid aggregates) or the pyrin sensor by the *Clostridioides difficile* toxin TcdB, IL-1 family cytokines IL-1 $\beta$  and IL-18 are processed to maturity<sup>102,103</sup> and GSDMD is cleaved at Asp275 (mouse Asp276) in the interdomain linker<sup>13,14</sup>. By contrast, noncanonical inflammasomes that activate caspases 4, 5 and 11 proteolyse only GSDMD, but not IL-1 family cytokines even with priming; however, secondary GSDMD pore-induced membrane damage and NLRP3 activation result in cytokine maturation in addition to GSDMD processing<sup>14,104</sup>.

GSDMD pores in the plasma membrane trigger rapid pyroptotic cell death in which the damaged membrane forms large ballooning bubbles and dying cells appear to flatten as their cytoplasmic contents are released<sup>22</sup> (FIG. 2). How or whether GSDMD pore-induced cell death is regulated is not clear, but cells with activated GSDMD pores do not always die. Both the rapidity of cell death and the morphological changes are hallmarks that easily distinguish pyroptotic from apoptotic cell death<sup>3</sup>. The cellular contents that are released from pyroptosis can include IL-1 family cytokines IL-1 $\beta$  and IL-18, as well as IL-1 $\alpha$  that does not require processing for its biological activity. Of note, neither full-length nor processed IL-1 family cytokines have a signal peptide for secretion via the endoplasmic reticulum to Golgi secretory pathway, but pyroptosis provides a mechanism for

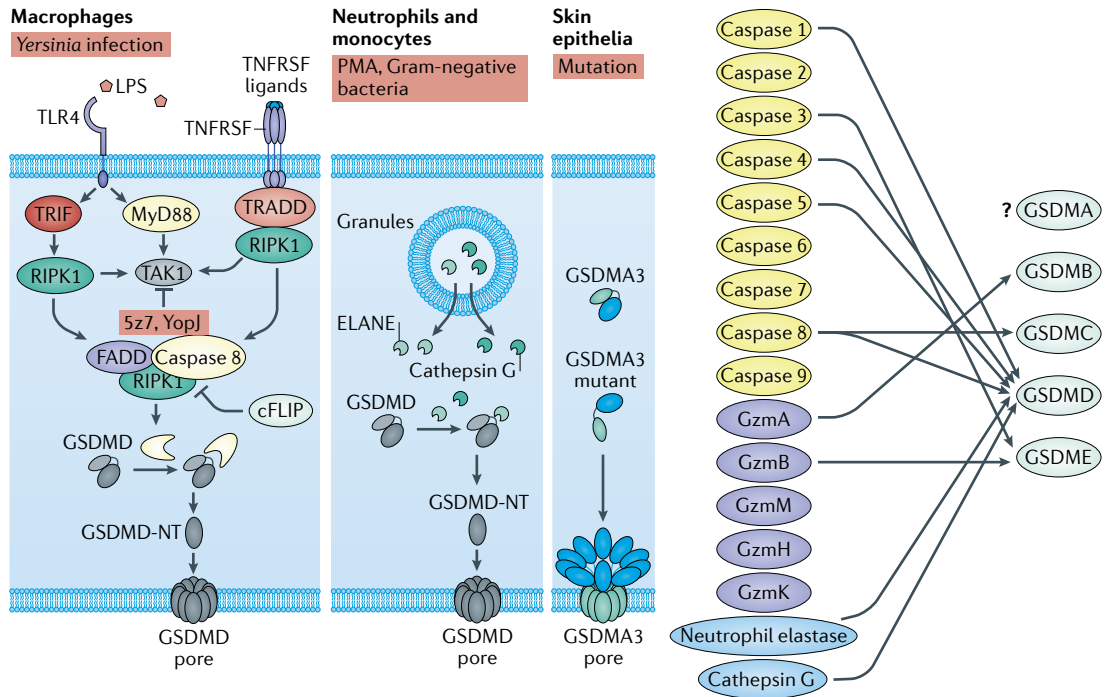
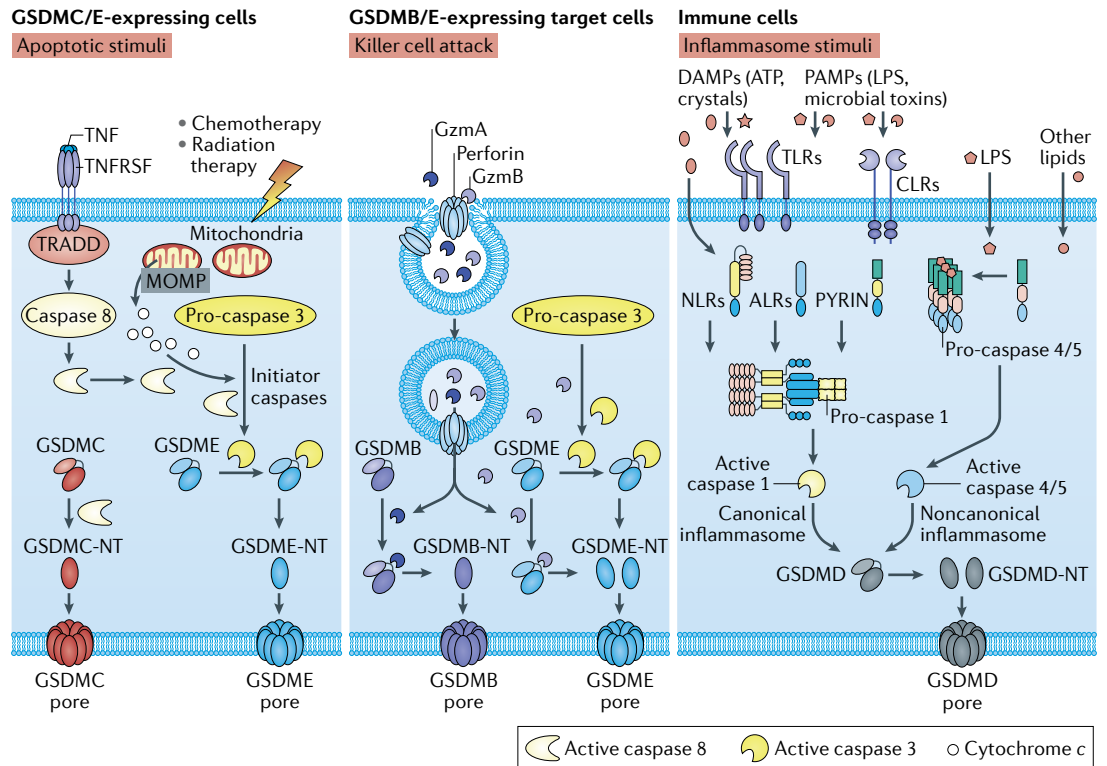
their release by ‘unconventional protein secretion’. Other released contents include cellular alarmins, such as ATP and HMGB1, cleaved GSDMD, inflammatory caspases, chemokines and other cytokines. These signals amplify inflammation in the tissue and recruit immune cells to respond to infection or danger. Many small molecules and other proteins, which may differ from cell type to cell type and from stimulus to stimulus, are probably released from pyroptotic cells. Detailed unbiased studies are required to elucidate what molecules are released under what condition and which released molecules are particularly important for amplifying inflammation.

In addition to inflammatory caspases, caspase 8, which is activated by cell surface death receptor ligation and oligomerization, can trigger GSDMD-dependent pyroptotic cell death (FIG. 3). During pathogenic *Yersinia* infection, TAK1 or I $\kappa$ B kinase inhibition by the *Yersinia* effector protein YopJ unleashes RIPK1–caspase 8-dependent GSDMD cleavage, which contributes to host defence against *Yersinia* infection<sup>105–107</sup>. Meanwhile, GSDME also gets cleaved and activated by an unknown mechanism and functions in concert with active GSDMD to trigger pyroptosis of infected cells. Similarly, in intestinal epithelial cells that lack FADD, RIPK1 and RIPK3-mediated caspase 8 activation leads to GSDMD-dependent pyroptosis and causes ileitis<sup>108</sup>. Like FADD, cellular FLICE-like inhibitory protein (cFLIP) negatively regulates pyroptotic cell death and inflammation. Deficiency of cFLIP relieves the inhibition of LPS-induced formation of a death receptor complex, which contains RIPK1 and caspase 8, leading to caspase 8-dependent cleavage of GSDMD<sup>109</sup>.

Pyroptosis can be induced by other GSDMs that are not activated by inflammasomes and without involving the IL-1 family cytokines (FIG. 3). During classical apoptosis, caspase 3 activates GSDME by cleaving after Asp270 (mouse Asp271), which converts relatively slow non-inflammatory apoptotic death into a more rapid inflammatory pyroptotic death in cells that express GSDME<sup>16,17</sup>. By contrast, activated caspase 3 cleaves GSDMD within the N terminus and destroys its ability to form pores<sup>110</sup>. GzmB, which is a serine protease that, like caspases, cleaves after Asp residues, is released from killer lymphocytes into target cells and can also directly cleave cytosolic GSDME to trigger pyroptosis<sup>18</sup>. GzmB also cleaves and activates caspase 3, which amplifies its effectiveness at killing target cells<sup>111–113</sup>. GzmA, another death-inducing protease in killer lymphocytes, cleaves GSDMB that is expressed in digestive tract epithelial tumours and causes pyroptotic cell death in target tumour cells<sup>19</sup>. Collectively, although immune-mediated elimination of infected or cancerous target cells has always been thought of as non-inflammatory, killer cells cause inflammatory pyroptosis in target cells expressing GSDME or GSDMB. Furthermore, a recent study showed that GSDMC can also be cleaved by caspase 8 after TNF-mediated death receptor signalling to trigger pyroptosis<sup>20</sup>.

### Regulation of gasdermin activity

Structural studies over the past few years have helped to reveal the elegant mechanisms that control and activate GSDMs, from auto-inhibition to processing, and



finally to formation of large  $\beta$ -barrel pores (FIG. 4a). The molecular basis of GSDM auto-inhibition has been illustrated by crystal structures of full-length, inactive mouse GSDMA3 and human and mouse GSDMD<sup>9,12</sup>. Whereas GSDMA3 was readily crystallized<sup>9</sup>, extensive loop truncations were required to crystallize either human or mouse GSDMD<sup>12</sup>. In both GSDMA3 and GSDMD full-length structures, the pore-forming NT

segments of the proteins comprise a twisted  $\beta$ -sheet surrounded by  $\alpha$ -helices, and a large part of the polypeptide chain that inserts as extended  $\beta$ -strands into the membrane is disordered. The corresponding GSDM-CTs, the repressive segment of the proteins, are compact and almost exclusively  $\alpha$ -helical and interact extensively with the GSDM-NTs to inhibit pore formation.

◀ Fig. 3 | **Molecular mechanisms that activate gasdermins.** In response to apoptotic signalling, gasdermin C (GSDMC) and GSDME are processed by caspase 8 and caspase 3, respectively, converting apoptosis into pyroptosis. Granzymes (Gzms) secreted by killer cells are delivered by perforin into target cells, where GzmA and GzmB can directly cleave and activate GSDMB and GSDME, respectively, to trigger pyroptotic cell death. In immune sentinel cells, both cytosolic canonical inflammasomes that respond to microbial infection or danger signals and the noncanonical inflammasome that responds directly to lipopolysaccharide (LPS) or endogenous oxidized phospholipids activate the inflammatory caspases (caspases 1, 4, 5 and 11), which cleave GSDMD and generate pore-forming N-terminal GSDMD (GSDMD-NT). TAK1 inhibition by the *Yersinia* effector protein YopJ or the small molecule 5z7 triggers caspase 8-dependent GSDMD cleavage and activation. GSDMD can also be directly processed and activated by neutrophil elastase (ELANE) and cathepsin G. In addition to protease-mediated release of active GSDMD-NT, mutations in *Gsdma3* lead to abolition of C-terminal GSDM inhibition and trigger GSDMA3 pore-forming activity. Diagram at bottom right indicates the proteases known to cleave and activate each of the gasdermins (yellow, caspases; purple, lymphocyte granzymes; blue, myeloid cell granule proteases). ALR, AIM-2 like receptor; CLR, C type lectin receptor; DAMP, damage-associated molecular pattern; NLR, NOD-like receptor; MOMP, mitochondrial outer membrane permeabilization; PAMP, pathogen-associated molecular pattern; TLR, Toll-like receptor.

Some mutations, especially in the C terminus of GSDMs, that disrupt the inhibitory interaction between the CT and NT fragments activate GSDMs spontaneously (TABLE 2). Both spontaneous and mutagenesis-induced *Gsdma3* mutants acquire pore-forming activity and induce inflammatory cell death, which leads to mouse skin abnormalities<sup>27–31,61</sup>. *GSDME* mutations that cause familial hearing loss result in a truncated GSDME with spontaneous pore-forming activity<sup>6,33–38</sup>. The pores formed by these mutants are believed to be constructed with the dissociated C terminus still attached, although this has not been formally verified.

Based on the GSDMA3 structure, there are two auto-inhibitory GSDM NT–CT interfaces. Interface I is located near the centre of the protein and interface II at the periphery (FIG. 4b). The biophysical determinants of these two interfaces differ remarkably. Interface I, formed between the positively charged  $\alpha 1$  helix and  $\beta 1$ – $\beta 2$  loop of GSDM-NT and an acidic groove of GSDM-CT, is primarily mediated by charge–charge interactions. By contrast, interface II is established through mainly hydrophobic interactions between the  $\alpha 4$  helix of GSDM-NT and GSDM-CT. Gain-of-function mutations in GSDM-associated diseases disrupt either of the auto-inhibitory contacts (TABLE 2), bypassing the requirement of proteolysis in GSDM activation and leading to membrane penetration by full-length GSDMs<sup>9,12</sup>.

Enzymatic processing of GSDMD for its activation (FIG. 4a) is more complex than classical substrate engagement by inflammatory caspases. The crystal structure of active caspase 1 in complex with a GSDMD interdomain linker fragment suggested that the catalytic pocket of inflammatory caspases recognizes the FLTD tetrapeptide motif (residues 272–275 in human GSDMD)<sup>114</sup>. However, the crystal structures of active caspases 1/4/11 in complex with GSDMD-CT and further functional characterization revealed a conserved mechanism whereby these inflammatory caspases interact with GSDMD-CT, instead of the tetrapeptide motif in the linker region, to drive the recognition and processing of GSDMD<sup>115</sup> (FIG. 4b). The GSDMD mutant with FLTD

mutated to AAAAD was equally processed in vitro and in cells, and rescued GSDMD deficiency in LPS-induced pyroptosis<sup>115</sup>. In the structures of the complex between caspase 1/4/11 and GSDMD-CT, the autoprocessed inflammatory caspase forms a two- $\beta$ -strand protrusion that acts as a substrate-binding exosite to recruit GSDMD by hydrophobic and hydrogen bonding interactions with the CT. This recruitment likely places the interdomain linker near the caspase catalytic site for cleavage, a hypothesis now supported by the crystal structure of full-length GSDMD in complex with caspase 1 (REF.<sup>116</sup>) (FIG. 4b). Thus, GSDMD-CT not only auto-inhibits GSDMD-NT, it also provides the platform for inflammatory caspase recruitment and GSDMD activation. It remains to be seen whether similar mechanisms regulate activation of other GSDMs.

### Mechanism of gasdermin pore formation

The mechanism of GSDM pore formation was revealed from the structure of the GSDMA3-NT pore<sup>117</sup>. Because GSDM-NT pores are cytotoxic, preparing the GSDMA3-NT protein for structural studies involved expressing an engineered recombinant full-length GSDMA3 in which a human rhinovirus 3C protease site was inserted in the interdomain linker. The recombinant purified protein was then cleaved by the 3C protease in the presence of liposomes containing the acidic lipid cardiolipin. GSDMA3-NT pores formed on the liposomes were then solubilized in detergent and imaged by cryo-electron microscopy (cryo-EM). The high-resolution structure is a strikingly large, predominantly 27-subunit transmembrane  $\beta$ -barrel with 108 extended  $\beta$ -strands and an inner diameter of 18 nm (FIG. 4b). This observed dimension for GSDMA3-NT pores is on a par with the 20 nm ring dimension for GSDMD-NT pores measured by atomic force microscopy<sup>10,118</sup>. GSDM pores are sufficiently large to allow the passage of small or medium-sized proteins, including the IL-1 family cytokines (FIG. 4b).

The membrane-inserted GSDMA3-NT subunit resembles a left hand with its soluble globular domain as the palm and two membrane-inserted  $\beta$ -hairpins as fingers (FIG. 4c). Comparison of auto-inhibited with membrane-inserted GSDMA3-NT structures indicated drastic conformational changes that result in the extension of the fingers as if a closed fist unfurls to an open hand, including melting of the  $\alpha 4$  helix at the tip of a  $\beta$ -hairpin<sup>9,117</sup> (FIG. 4c). In close juxtaposition to the inserted  $\beta$ -strands is the positively charged  $\alpha 1$  helix, which interacts with cardiolipin in the cryo-EM structure and serves as a major acidic lipid-binding element. This  $\alpha 1$  helix looks like a thumb folded over the palm. The  $\alpha 1$  and  $\alpha 4$  (FIG. 4c) helices of GSDM-NT, which are important in lipid binding and membrane insertion, respectively, are masked in the full-length protein by interactions with GSDM-CT (FIG. 4b), which explains how GSDM-CT represses GSDM activity. Oligomerization in the GSDMA3-NT pore is mediated by the sides of the hand-shaped subunit, from the membrane-inserted fingers, folded thumb to the palm<sup>117</sup>. In particular, the  $\alpha 1$  thumb helices in the oligomerized pore align head to tail on the membrane to form a stabilizing helical belt.

#### Exosite

Secondary binding sites of an enzyme that are remote from the active site but modulate substrate binding and enzymatic activity.



Box 2 | Overview of apoptosis

Classical apoptosis, a physiological noninflammatory programme of cell suicide, is triggered by a variety of stimuli including death receptor signalling, cytotoxic drugs, radiation and a variety of cellular stresses (including growth factor deprivation and hypoxia) that lead to mitochondrial outer membrane permeabilization (MOMP) and the activation of apoptotic effector caspases (caspases 3, 6 and 7)<sup>197,198</sup>. During apoptosis, mitochondria generate reactive oxygen species (ROS), lose their transmembrane potential and release cytochrome c into the cytosol, cell membrane lipids become scrambled and externalize phosphatidyl serine, chromatin condenses, genomic DNA is fragmented, mRNA is globally degraded and new protein synthesis is blocked<sup>197,199–201</sup>. Phagocytic cells, which recognize cell surface phosphatidylserine on apoptotic cells, rapidly engulf and remove apoptotic cells in vivo before their plasma membrane loses its integrity<sup>202,203</sup>. Thus, apoptotic cells do not release their cellular contents into the extracellular milieu, and apoptotic death is usually noninflammatory and immunologically silent. Although pyroptosis and apoptosis have historically been considered separate pathways, recent studies discussed in this Review show that apoptotic stimuli can trigger pyroptosis under certain circumstances. For example, activated caspase 3 can cleave gasdermin E (GSDME) to cause pyroptosis instead of apoptosis<sup>16,17</sup>. Similarly, in cells infected with pathogenic *Yersinia*, TNF receptor activation activates caspase 8 to trigger GSDMD-dependent pyroptosis<sup>105,106</sup>.

Interestingly, phosphorylation of Thr6 of  $\alpha 1$  in GSDME (equivalent to Thr8 in GSDMA3) inhibits oligomer formation and pyroptosis<sup>119</sup>, presumably by altering the  $\alpha 1$  helix conformation to interfere with oligomerization. The kinases responsible for phosphorylating GSDME or other GSDMs and how phosphorylation is regulated are unknown.

**Gasdermin D-mediated cell hyperactivation**

In certain situations, macrophages, dendritic cells and neutrophils survive inflammasome-activated GSDMD cleavage without membrane rupture and pyroptosis, and are called hyperactivated cells because they both stimulate inflammation and preserve their other functions<sup>24,120</sup> (FIG. 2). Based on the cryo-EM structure of the GSDMA3-NT pore with an ~18 nm inner diameter<sup>17</sup>, GSDMD-NT pores are also big enough for chemicals (such as ATP) and small proteins to pass through<sup>9,117</sup>. Hyperactivation and pyroptosis can be distinguished by measuring cell culture supernatants for lactate dehydrogenase (LDH), which is too large to exit through GSDMD-NT pores and relies on cell lysis for its release, and for mature IL-1 family cytokines and possibly HMGB1, which are small enough to be readily released through these pores. While pyroptotic cells release both LDH and cytokines, hyperactivated cells do not release LDH, but do release cytokines<sup>31</sup>. Because hyperactivated cells survive, they may even process and pump out more inflammatory mediators than cells that die. An unbiased proteomics analysis of molecules released from hyperactivated cells and an analysis of their roles in infection and inflammation will be informative.

A number of processes that activate live cell cytokine secretion or hyperactivation have been described. In dendritic cells, a mixture of oxidized phosphorylcholine derivatives (oxPAPC) or an isolated component 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine (PGPC) produced in dying mammalian cells induces noncanonical inflammasome-dependent release of IL-1 $\beta$  without causing cell death<sup>120</sup>. Multiple stimuli, including an *N*-acetylglucosamine fragment of bacterial

peptidoglycan and oxPAPC, can also hyperactivate macrophages and induce inflammasome-mediated IL-1 $\beta$  release from living macrophages in a GSDMD pore-dependent process<sup>21,24,121,122</sup>. Human, but not murine, circulating monocytes can respond to extracellular LPS to activate an alternative NLRP3-ASC-caspase 1 inflammasome independently of K<sup>+</sup> efflux and without ASC speck formation, leading to IL-1 $\beta$  secretion without cell death<sup>123</sup>. A TLR4-TRIF-RIPK1-FADD-caspase 8 signalling axis was dissected genetically as upstream of alternative inflammasome activation<sup>123</sup>, but the exact mechanism remains unknown. A hyperactive state can also be brought about by deleting the sterile- and armadillo-motif-containing protein 1 (SARM1), which also contains a Toll-IL-1R (TIR) domain<sup>124</sup>. *Sarm1*<sup>-/-</sup> macrophages essentially recapitulate NLRP3-dependent hyperactivation after priming and nigericin treatment, with release of IL-1 $\beta$  but without cell death<sup>124</sup>. Potentiation of cell death by SARM1 involves SARM1 clustering at the mitochondria and SARM1-dependent mitochondrial depolarization<sup>124</sup>.

Interestingly, exposure to *Salmonella enterica* subsp. Typhimurium, ATP or nigericin, which cause inflammasome activation and pyroptosis in macrophages, induces GSDMD-dependent IL-1 $\beta$  release in neutrophils, without killing them<sup>122,125,126</sup>. With some stimuli, GSDMD-NT in neutrophils predominantly targets primary granules and LC3<sup>+</sup> autophagosomes, instead of the plasma membrane, and IL-1 $\beta$  appears to be secreted via an autophagy-dependent mechanism<sup>127</sup>. The resistance of neutrophils to pyroptotic death is unique among inflammasome-signalling cells so far described. It allows neutrophils to secrete IL-1 $\beta$  at sites of infection without compromising the crucial inflammasome-independent neutrophil antimicrobial functions that would be impaired if neutrophils died. The inflammasome pathway must somehow be modified in neutrophils to optimize host pro-inflammatory and antimicrobial responses during pathogen challenge.

What determines whether GSDMD cleavage triggers pyroptosis or hyperactivation is not well understood. However, a likely mechanism responsible for cell survival in the face of membrane damage by GSDM pores is activation of the ubiquitous plasma membrane damage repair response in eukaryotic cells, which rapidly restores membrane integrity, allowing the cell to survive. All cells sense damage to the plasma membrane by an immediate increase in intracellular Ca<sup>2+</sup>, which triggers membrane repair by recruiting the endosomal sorting complexes required for transport (ESCRT) machinery to damaged membrane areas and removes them in ectosomes<sup>23</sup>. The plasma membrane repair response to other types of membrane damage also involves increased endocytosis of damaged membrane and patching of damaged membrane by donated vesicular membranes (mostly from lysosomes)<sup>128</sup>. Whether these other membrane repair phenomena occur in cells with GSDMD-damaged plasma membranes has not been examined. The ability of cells to survive and repair the damage likely reflects a contest between how much and how rapidly membrane damage is induced and whether it is spatially localized, versus the robustness of the

**Hyperactivation**

A response to gasdermin activation and pore formation in which cells release IL-1 and other inflammatory mediators, but do not die.

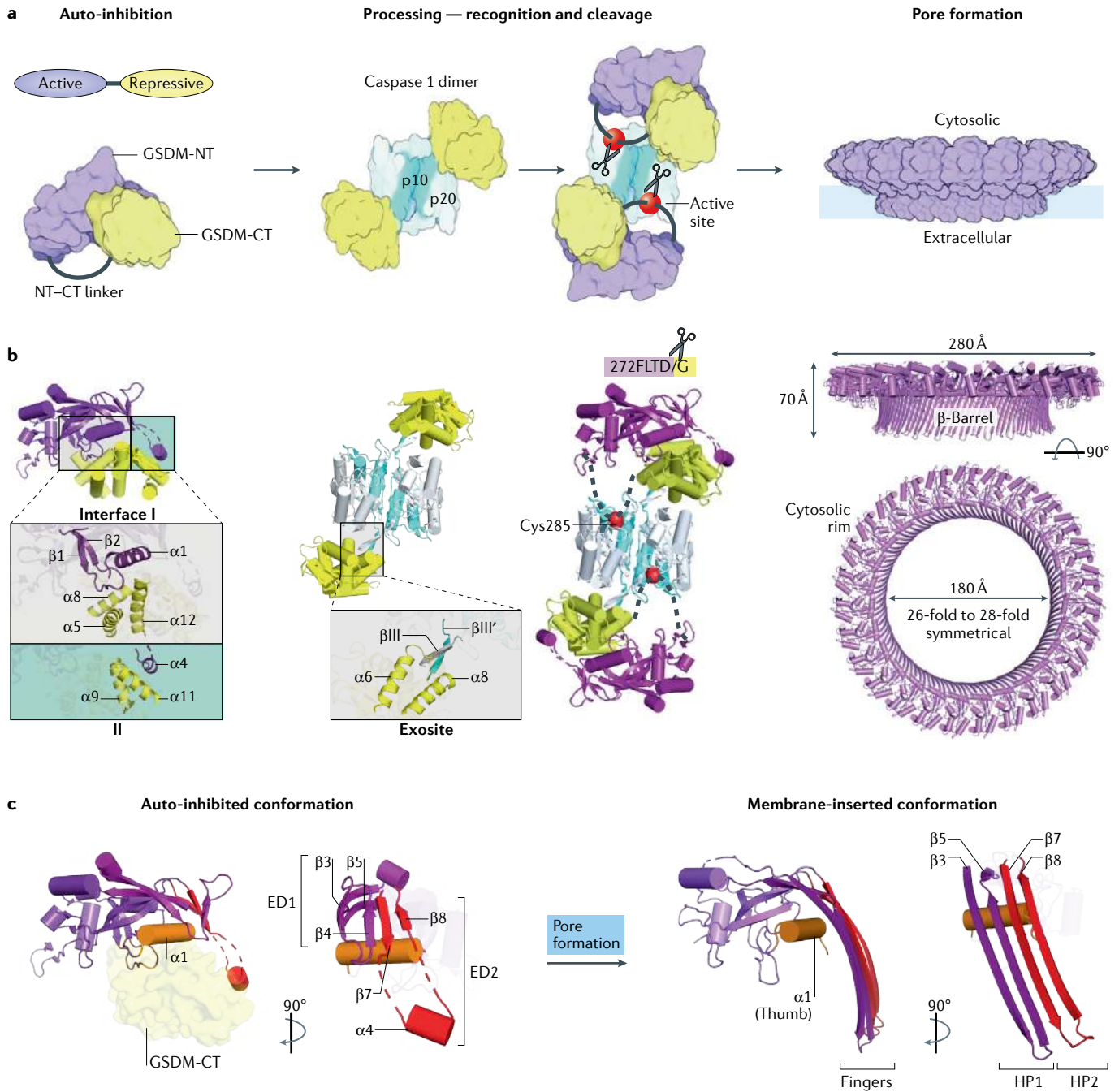
**oxPAPC**

A mixture of oxidized phospholipids generated by the oxidation of 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (PAPC), some of which can bind to and activate or antagonize innate immune sensors of lipopolysaccharide.

Table 2 | Disease-linked gasdermin mutations

| Gene <sup>a</sup> | Nucleotide change  | Amino acid change  | Consequence   | Disease link                     | Refs          |                             |  |                                  |           |
|-------------------|--|--|---|----------------------------------|---------------|-----------------------------|--|----------------------------------|-----------|
| <b>GSDMA</b>      | 53G>A  | R18Q   | Unknown   | Asthma/systemic sclerosis        | 67            |                             |  |                                  |           |
|                   | 382G>A   | V128M  | Elevated IgE and bronchial hyperresponsiveness  | Asthma                           | 69            |                             |  |                                  |           |
| <b>Gsdma3</b>     | –  | Lacks auto-inhibition interfaces I and II:   | Disrupts auto-inhibition, and <i>Gsdma3</i> becomes constitutively active to induce pyroptosis in hair follicle cells | Alopecia                         | 9,27–31       |                             |  |                                  |           |
|                   | B2 insertion   | 1–259 + RDW  |   |                                  |               |                             |  |                                  |           |
|                   | –  | Disrupts auto-inhibition interface I:  |   |                                  |               |                             |  |                                  |           |
|                   | 832A>C   | T278P  |   |                                  |               |                             |  |                                  |           |
|                   | 1028T>C  | L343P  |   |                                  |               |                             |  |                                  |           |
|                   | 1030T>C  | Y344H  |   |                                  |               |                             |  |                                  |           |
|                   | 1031A>G  | Y344C  |   |                                  |               |                             |  |                                  |           |
|                   | 1042G>A  | A348T  |   |                                  |               |                             |  |                                  |           |
|                   | –  | Lacks auto-inhibition interface II:  |   |                                  |               |                             |  |                                  |           |
|                   | 1096A>T  | K366* (stop codon)   |   |                                  |               |                             |  |                                  |           |
|                   | –  | Disrupts auto-inhibition interface II:   |   |                                  |               |                             |  |                                  |           |
| 1231_1236dup      | Duplication of E411A412  |  |   |                                  |               |                             |  |                                  |           |
| <b>GSDMB</b>      | 871G>A; 892C>T   | G291R; P298S<br>Reduce conformational flexibility and increase positive surface charge   | Unknown   | Asthma                           | 133           |                             |  |                                  |           |
|                   | 236–1199G>A  | Intron variant   | Elevated IgE and bronchial hyperresponsiveness  |                                  | 69            |                             |  |                                  |           |
|                   | 661+742T>C   | Exon 6 (13 aa) deletion  | Unknown   |                                  | 77            |                             |  |                                  |           |
| <b>GSDME</b>      | 990+503_990+1691delins132<br>991-15_991-13del<br>991-6C>G<br>1183+4A>G<br>991-15_991-13del | Exon 8 skipping and truncated GSDME containing aa 1–330 and an aberrant 41-residue C-terminal sequence due to frameshift (lacks auto-inhibition interfaces I and II) | Truncated GSDME is constitutively active to induce pyroptosis in cochlear hair cells                                  | Autosomal dominant hearing loss  | 6,17,33–38,92 |                             |  |                                  |           |
|                   | 410T>C   | I137T  | Most GSDME mutants have reduced pore-forming activity and may have reduced tumour inhibition                          | Cancer                           | 18            |                             |  |                                  |           |
|                   | 650T>A   | I217N  |   |                                  |               |                             |  |                                  |           |
|                   | –  | Disrupts oligomerization interface:  |   |                                  |               |                             |  |                                  |           |
|                   | 53A>T  | D18V   |   |                                  |               |                             |  |                                  |           |
|                   | 70A>G  | N24D   |   |                                  |               |                             |  |                                  |           |
|                   | 635C>T   | P212L  |   |                                  |               |                             |  |                                  |           |
|                   | –  | Lacks extension domains I and II:  |   |                                  |               |                             |  |                                  |           |
|                   | 138G>A   | W46*   |   |                                  |               |                             |  |                                  |           |
|                   | 628G>T   | E210*  |   |                                  |               |                             |  |                                  |           |
|                   | <b>DFNB59</b>  | 113dupT  |   |                                  |               | K41Efs*8 (48 aa truncation) | T54I and R183W mutants result in auditory neuropathy while other truncations lead to defect in cochlear hair cell function | Autosomal recessive hearing loss | 39,97–100 |
|                   |  | 122delA  |   |                                  |               | K41Sfs*18                   |  |                                  |           |
|                   |  | 161C>T   |   |                                  |               | T54I                        |  |                                  |           |
| 406C>T            |  | R136*  |   |                                  |               |                             |  |                                  |           |
| 499C>T            |  | T167*  |   |                                  |               |                             |  |                                  |           |
| 547C>T            |  | R183W  |   |                                  |               |                             |  |                                  |           |
| 726delT           |  | F242Lfs*7  |   |                                  |               |                             |  |                                  |           |
| 988delG           |  | V330Lfs*7  |   |                                  |               |                             |  |                                  |           |
| <b>Dfnb59</b>     | 868A>T   | K290*  | Defect in cochlear hair cell function   | Autosomal recessive hearing loss | 98            |                             |  |                                  |           |

aa, amino acid; GSDM, gasdermin. <sup>a</sup>Upper case gene names refer to human genes, and lower case refers to mouse genes.



repair processes, which likely depends on how much GSDMD is in the cell and how quickly and efficiently it is cleaved. The latter will in turn depend on the intensity of inflammatory stimuli and degree of inflammasome and inflammatory caspase expression and activation, as well as the effectiveness of mechanisms for regulating and clearing inflammasomes, activated caspases and GSDMs. Given the importance of IL-1 $\beta$  secretion and pyroptosis in human inflammatory diseases, a better understanding of unconventional cytokine secretion and the regulation of pyroptosis is important.

Processing of IL-1 family cytokines removes an NT acidic region that converts pro-IL-1 $\beta$  from an acidic precursor into a more basic mature cytokine that binds to PIP2-rich acidic regions on the plasma membrane. Plasma

membrane association was proposed to be a necessary step for efficient release<sup>129</sup>. It is worth noting that *Gsdmd*<sup>-/-</sup> macrophages can still release caspase 1-processed mature IL-1 $\beta$  but the GSDMD-independent release, which may also rely on PIP2-mediated plasma membrane binding, is much slower than GSDMD-dependent release<sup>129</sup>.

**Gasdermin-NTs and mitochondrial damage**

The activated NT domain has also been shown to bind to internal cellular membranes<sup>119,127,130,131</sup>. GSDMD-NT binds with apparently higher affinity to the mitochondrial and bacterial lipid, cardiolipin, than to plasma membrane lipids<sup>8,9</sup>. GSDMD-NT and GSDME-NT can translocate to mitochondria, permeabilize the outer membrane and disrupt mitochondrial function, leading

◀ Fig. 4 | **Mechanisms of gasdermin auto-inhibition, processing and pore formation.** **a** | Cartoon representation of gasdermin (GSDM) pore formation. Interaction between the N-terminal and C-terminal fragments of a GSDM (GSDM-NT, in purple and GSDM-CT, in yellow) auto-inhibits protein activation. Active caspase 1, a dimer of the p10 (in pale blue)–p20 (in silver) complex, recognizes two copies of GSDM through the GSDM-CT. This exosite recognition mechanism places the long interdomain linker (in grey) between GSDM-NT and GSDM-CT near the active site of caspase 1 (red balls). Active caspase 4 and caspase 11 use a similar mechanism to process GSDMD. Linker cleavage liberates GSDM-NT for pore formation at the membrane. The fully formed GSDM pore features a large cytosolic rim in addition to a transmembrane  $\beta$ -barrel. **b** | Structural illustration of GSDM at each stage in pore formation depicted in part **a**. In auto-inhibited GSDM (PDB: 5B5R for GSDMA3), GSDM-NT contacts GSDM-CT at two interfaces. Interface I is formed primarily by charged interactions of the  $\alpha$ 1 helix and the  $\beta$ 1– $\beta$ 2 loop of GSDM-NT with the  $\alpha$ 5– $\alpha$ 8– $\alpha$ 12 helix cluster of GSDM-CT. Interface II is formed mainly by hydrophobic interactions between  $\alpha$ 4 of GSDM-NT and  $\alpha$ 9 and  $\alpha$ 11 of GSDM-CT. GSDMD recognition (PDB: 6KN0 and 6VIE) by caspase 1 is mediated by a substrate-binding exosite, comprised of two anti-parallel  $\beta$ -strands —  $\beta$ III of caspase 1-p20 and  $\beta$ III' of caspase 1-p10. The exosite binds to  $\alpha$ 6 and  $\alpha$ 8 of GSDMD-CT through primarily hydrophobic interactions. The GSDMD NT–CT linker is positioned near the active site Cys285 of caspase 1 for cleavage. The GSDM pore contains roughly 27 subunits according to the structure of the GSDMA3 pore (PDB: 6CB8). With each subunit contributing four transmembrane  $\beta$ -strands, the membrane-inserted  $\beta$ -barrel contains 108  $\beta$ -strands. The inner and outer diameters of the pore are approximately 180 Å and 280 Å, respectively. A soluble rim, rich in  $\alpha$ -helices and loops, decorates the  $\beta$ -barrel on the cytosolic side. **c** | GSDM-NT conformations before (left) and after (right) pore formation. The positively charged lipid-binding helix  $\alpha$ 1 (in orange, like a thumb) is masked by GSDM-CT in the auto-inhibited conformation, and in the membrane-inserted conformation it interacts with lipids with negatively charged head groups found on the inner leaflet of the plasma membrane. Drastic conformational changes of GSDM-NT occur at extension domains 1 and 2 (ED1, in magenta and ED2, in red), which are located at the  $\beta$ 3– $\beta$ 4– $\beta$ 5 and  $\beta$ 7– $\alpha$ 4– $\beta$ 8 regions in auto-inhibited GSDM-NT, respectively. Through pore formation, ED1 and ED2 become the two transmembrane  $\beta$ -hairpins (HP1, in magenta and HP2, in red, like four fingers), respectively.

to ROS generation, loss of transmembrane potential and cytochrome *c* release, which also activates caspase 3 and enhances overall cell death<sup>119</sup>. As cardiolipin resides in the mitochondrial inner membrane, which is not accessible to the cytosol<sup>132</sup>, further study of mitochondrial targeting by GSDMs and its consequences is needed. NT fragments of GSDMA3, GSDMA and GSDME bind to the same phospholipids as GSDMD-NT<sup>9,17</sup>. Intriguingly, an *in vitro* lipid-binding assay showed that both full-length GSDMB and its NT domain bind to sulfatide and phosphoinositides, but not to cardiolipin<sup>133</sup>. The somewhat distinct phospholipid-binding properties of GSDMB suggest that it might bind to different membranes from the other GSDMs, but this has not been shown in cells.

### Role of gasdermin D in NETosis

GSDMD plays a key role in NETosis, a regulated form of neutrophil cell death triggered by microbial infection, activation and danger signals, during which the nuclear envelope and plasma membrane are perforated to release large web-like structures, called neutrophil extracellular traps (NETs), composed of genomic DNA, histone and antimicrobial proteins<sup>43,131,134</sup> (FIG. 3). In phorbol 12-myristate 13-acetate (PMA)-induced NET formation, serine proteases such as neutrophil elastase (ELANE) are released from granules, which cleave and activate GSDMD. GSDMD in turn permeabilizes primary azurophilic granules to further release ELANE in a feed-forward loop, allowing its nuclear translocation, histone processing, chromatin expansion and neutrophil

lysis<sup>43</sup>. Similarly, GSDMD is required for Gram-negative bacterial infection-triggered NETosis whereby LPS activates the noncanonical inflammasome, which activates GSDMD, which permeabilizes primary granules and the neutrophil plasma membrane to facilitate protease release, nuclear decondensation and NET extrusion<sup>131</sup>. Although chromatin is extruded through the nuclear envelope during NETosis, there is no evidence that activated GSDMD localizes to the nuclear envelope or is directly involved in its permeabilization.

In another study, the granule-associated protease cathepsin G (CatG) present in both macrophages and neutrophils cleaves GSDMD directly at Leu274, only two residues upstream of the caspase cleavage site at Asp276, to activate its pore-forming activity<sup>135</sup> (FIG. 3). The catalytic activity of CatG on GSDMD appears to be stronger than that of other myeloid granule enzymes, including ELANE and proteinase 3, and even exceeds that of caspase 1 and caspase 11, based on measured  $k_{\text{cat}}/K_{\text{M}}$  values. Sublethal LPS-induced systemic inflammation in mice depends on CatG and GSDMD and is suppressed by CatG-inhibiting serpins. Canonical NLRP3 inflammasome activation-dependent IL-1 $\beta$  release in bone marrow-derived macrophages (BMDMs) by nigericin or ATP also depends on CatG and GSDMD. Thus, GSDMD activation in both neutrophils and macrophages permeabilizes their granules to release CatG, which in turn further processes GSDMD in a feed-forward loop to enhance secretion of processed pro-inflammatory cytokines<sup>135</sup>.

### Gasdermins and disease

**Gasdermin D in infection.** The affinity of GSDMD for bacterial cell membrane lipid cardiolipin allows it to bind, permeabilize and kill bacteria in the cytosol or phagosomes, as well as cell-free bacteria when it is released from pyroptotic cells<sup>8,9,89</sup>. Indeed, GSDMD was shown to mediate caspase 11-dependent defence against *S. Typhimurium*  $\Delta$ *sifA*<sup>41</sup> and *Brucella abortus*<sup>42</sup>. Similarly, *Gsdmd* deficiency compromises AIM2-mediated host defence and makes mice susceptible to *Francisella novicida* infection<sup>40</sup>. GSDMD protects against *Burkholderia pseudomallei* using both canonical and noncanonical pathways<sup>89</sup>. For *Legionella pneumophila* infection, *Gsdmd*<sup>-/-</sup> mice showed only mildly increased susceptibility compared with wild-type mice and failed to recapitulate the greater susceptibility in *Nlr4*<sup>-/-</sup> mice<sup>46</sup>. The NAIP5/NLRC4 inflammasome also activates caspase 8, which cleaves and activates caspase 7, and GSDMD and caspase 7 together are responsible for restricting *L. pneumophila* infection<sup>46</sup>.

However, even though GSDMD-NT can permeabilize and kill bacteria, GSDMD is not always protective in infection and even exacerbates some bacterial infections by diverse mechanisms. In one study, *Gsdmd*<sup>-/-</sup> mice were better able to survive pathogenic *Escherichia coli* infection because neutrophils, which are the main cellular defenders against bacteria<sup>43–45</sup>, survived longer without *Gsdmd*<sup>44</sup>. In another study, *Mycobacterium tuberculosis* infection in a human macrophage cell line induced plasma membrane damage and secondary activation of NLRP3 and GSDMD-dependent pyroptosis and inflammatory cytokine release, which increased

#### NETosis

A unique form of neutrophil cell death that is characterized by release of decondensed chromatin to form neutrophil extracellular traps (NETs) that trap microbes but can also capture and activate platelets to trigger clotting.



bacterial cell-to-cell spread *in vitro*<sup>136</sup>. However, the overall effect of GSDMD and pyroptosis on mycobacterial infection would need to be studied *in vivo*.

In the case of parasites, *Toxoplasma gondii* has been reported in one study to induce AIM2, ASC and caspase 8-dependent, but GSDMD-independent, death in human macrophages<sup>137</sup>. However, another study suggests that *T. gondii* infection in human monocytes activates a Syk–CARD9–NF- $\kappa$ B signalling pathway and the NRLP3 inflammasome to release IL-1 $\beta$  in a GSDMD-independent manner<sup>138</sup>. IL-1 $\alpha$ , but not IL-1 $\beta$ , has been shown to be crucial for parasite control. IL-1 $\alpha$  release from microglia depends on GSDMD, and *Gsdmd* deficiency in mice reduces IL-1 $\alpha$  secretion and compromises immune protection in response to *T. gondii* infection in the brain<sup>139</sup>. GSDMD is activated in macrophages *in vitro* when another parasite, *Entamoeba histolytica*, binds and activates the noncanonical caspase 4 inflammasome by sensing an unknown ligand<sup>140</sup>. *In vitro*, *E. histolytica* causes caspase 4 and GSDMD-dependent macrophage hyperactivation with IL-1 $\beta$  secretion but without pyroptosis. Determining whether the net physiological impact of GSDMD is to improve immune control or exacerbate tissue damage in *E. histolytica* infection will require *in vivo* experiments.

The role of inflammasome activation and GSDMD in viral infection is not well studied. Viruses activate Toll-like receptors that sense viral nucleic acids on the cell surface or in endosomes to prime expression of innate immune genes. Cytoplasmic DNA and RNA sensors can trigger an interferon response (that is, cGAS, RIGI and MDA5) and/or an inflammatory response (IFI16, AIM2, NLRP6 and NLRP9) to viruses. Many viral proteins inhibit the antiviral interferon response, but so far viral mechanisms to inhibit inflammasome activation and pyroptosis have not been identified. In one study, the NLRP9 inflammasome, which recognizes stretches of viral dsRNA, protected intestinal epithelial cells from rotavirus infection in a GSDMD-dependent manner<sup>141</sup>. By contrast, inflammasome and GSDMD activation might contribute to viral pathogenesis for viruses whose disease course has a strong inflammatory component (such as HIV, influenza A or SARS coronaviruses). In rhinovirus and norovirus infections, NLRP3- and GSDMD-dependent pyroptosis increases pathological inflammation, contributing to viral pathogenesis<sup>90,142</sup>.

#### **Gasdermin D in autoinflammatory genetic diseases.**

Autoactivation of inflammasome pathways causes or contributes to multiple sterile inflammatory diseases. Familial Mediterranean fever (FMF), the most prevalent monogenic autoinflammatory disease worldwide, is caused by missense mutations in *MEFV*. *MEFV* encodes the pyrin inflammasome, which senses bacterial toxin inactivation of RhoA GTPases<sup>143</sup>. *MEFV* mutation reduces the threshold for activating the pyrin inflammasome, causing recurrent bouts of fever and pain. GSDMD is crucial for FMF autoinflammatory pathology. In a mouse FMF model that expresses a hypersensitive pyrin, *Gsdmd* deficiency abolishes auto-inflammatory symptoms<sup>144</sup>. Similarly, autoactive NLRP3 mutants result in autoinflammatory disorders, including NOMID, which

features severe systemic inflammation that is not adequately treated with current cytokine inhibitor drugs. Ablation of *Gsdmd* in NOMID mice prevents disease symptoms<sup>26</sup> (FIG. 5).

#### **Gasdermin D in complex diseases exacerbated by inflammation.**

GSDMD is probably the most important GSDM in mediating the inflammation that is associated with many diseases. It is the convergent target of canonical and noncanonical inflammasome signalling in immune sentinel cells, and excessive activation of GSDMD-mediated pyroptosis and IL-1 family cytokine secretion can lead to inflammatory diseases and pathological consequences (FIGS 4,5). An unrestrained inflammatory response to infection is responsible for cytokine release syndrome (CRS), sepsis, disseminated intravascular coagulation (DIC) and acute organ damage, including acute respiratory distress syndrome (ARDS) and acute kidney injury (AKI). Release of IL-1 family cytokines is crucially dependent on GSDMD activation<sup>21,122</sup>. Binding of these cytokines to the IL-1 receptor triggers the activation and release of other pro-inflammatory cytokines, including IL-6 and TNF<sup>145</sup>. GSDMD-mediated pyroptosis in macrophages also triggers release of tissue factor, which activates coagulation, leading to DIC in sepsis<sup>146,147</sup>. Similarly, GSDMD-dependent neutrophil NETs capture platelets and serve as a nidus for thrombus formation, which can also contribute to DIC<sup>148</sup>. The noncanonical inflammasome plays a key role in sepsis in mouse models<sup>14,104,149</sup>. In LPS and caecal ligation and puncture (CLP)-induced sepsis, *Gsdmd* deficiency protects mice from lung, intestine, liver and kidney injury and DIC<sup>14,150</sup>. CRS and ARDS, triggered in patients with clinical deterioration during pathogenic influenza and SARS respiratory infections, could also be mediated or exacerbated by pyroptosis in tissue-resident macrophages, but this has not been shown.

GSDMD has been clearly linked to noninfectious diseases that are not caused by a single genetic mutation, in which inflammation plays an important pathogenic role. The NLRP3 inflammasome is implicated in EAE in mice. *Gsdmd* deficiency suppresses EAE neuroinflammation, and GSDMD inhibitors disulfiram (DSF) and dimethyl fumarate (DMF) attenuate EAE<sup>25,48</sup>. In EAE, GSDMD-mediated pyroptosis is activated in peripheral myeloid cells as well as in microglia and oligodendrocytes in the CNS<sup>25,151</sup>, but which cell types are most crucial for neuroinflammation is unclear. In a mouse bone marrow transplantation model, a recent study indicated that radiation causes severe damage to bones and spleen through NLRP3- and AIM2-mediated GSDMD-dependent pyroptosis, which is prevented only when GSDMD is deficient in both donor and host cells<sup>152</sup>. Activation of the noncanonical inflammasome pathway in mice worsens graft-versus-host disease, and *Gsdmd* deficiency reduces intestinal inflammation, tissue damage, donor T cell expansion and mortality in allogeneic haematopoietic stem cell transplantation<sup>153</sup>. Genetic deficiency or knockdown studies have also implicated GSDMD in the pathogenesis of alcoholic hepatitis, nonalcoholic steatohepatitis, noninfectious liver injury and ischaemia–reperfusion injury in humans and mice<sup>154–158</sup>. Both caspase 8 and GSDMD have also been

#### **Familial Mediterranean fever**

(FMF). A rare spontaneous autosomal recessive genetic disease, characterized by recurrent fever and peritonitis, caused by mutations in the pyrin inflammasome gene (*MEFV*).

#### **Autoinflammatory disorders**

A diverse group of diseases characterized by recurrent episodes of inflammation, which result from poorly controlled activation of innate immunity.

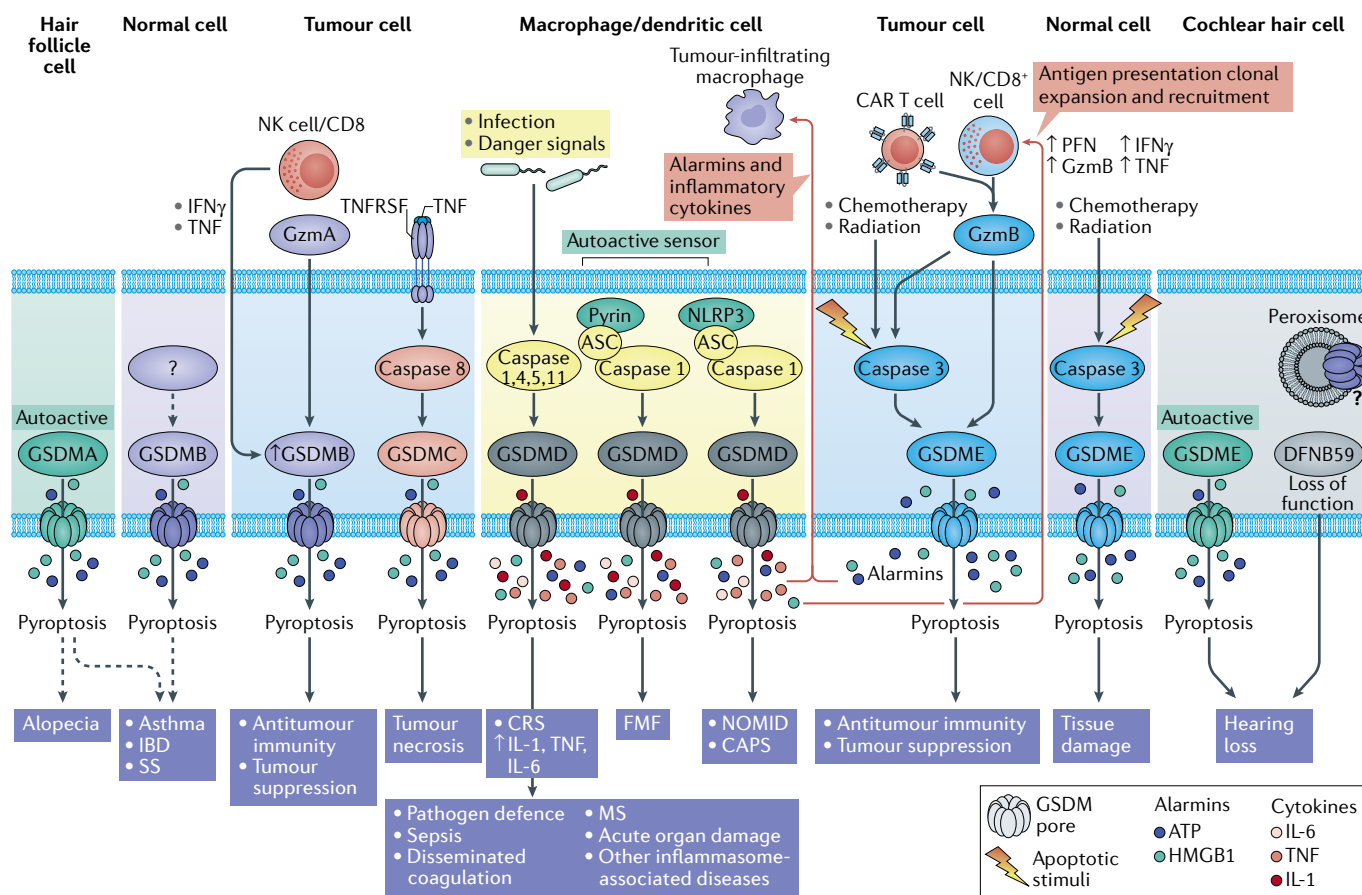
#### **Cytokine release syndrome**

(CRS). A systemic life-threatening inflammatory response syndrome caused by massive inflammatory cytokine release.

implicated in mixed-lineage kinase domain-like protein (MLKL)-independent ileitis in mice with intestinal epithelial FADD deficiency<sup>108</sup>. The serum of children with acute vasculitis in Kawasaki disease contains elevated levels of GSDMD and IL-1 $\beta$  and induces endothelial cell pyroptosis in vitro, suggesting that GSDMD-mediated pyroptosis plays an important role in endothelial damage in Kawasaki disease<sup>159</sup>. GSDMD-mediated pyroptosis has also been implicated in diabetic kidney disease<sup>160</sup>, diabetic cardiomyopathy<sup>161</sup>, contrast-induced kidney injury<sup>162</sup>, early brain injury after subarachnoid haemorrhage<sup>163</sup> and traumatic brain injury<sup>164</sup>. Although activation of canonical and noncanonical inflammasomes has been implicated in the inflammation and pathogenesis of many noninfectious human diseases, the

role of GSDMD in all these conditions has not yet been verified by studies in *Gsdmd*-deficient mice.

**Gasdermins in cancer.** The role of GSDMs in cancer is complicated (reviewed elsewhere<sup>47</sup>). The 2000 paper<sup>5</sup> that named GSDMA based on its restricted expression in the gastrointestinal tract and skin hinted at a possible role of GSDMs in cancer by noting that *GSDMA* expression is suppressed in human gastric cancers and that it might be a tumour suppressor gene<sup>5</sup>. In fact, GSDMA, GSDMC and GSDMD all reportedly suppress gastric cancer cell proliferation<sup>70,165</sup>. However, reduced GSDMD expression attenuates tumour proliferation and predicts a favourable prognosis in non-small-cell lung cancer<sup>166</sup>. *GSDMB* is amplified and highly expressed in cervical,



**Fig. 5 | Role and function of gasdermins in human disease.** Autoactive gasdermin A (GSDMA) triggers pyroptosis in hair follicle cells and causes alopecia in mice but so far no human link to alopecia has been reported. GSDMB is implicated in autoimmune diseases such as childhood asthma and inflammatory bowel disease (IBD), but the underlying mechanism remains unclear. Granzyme A (GzmA) in killer lymphocytes activates GSDMB in target cells to cause pyroptosis. TNF can activate caspase 8 to trigger GSDMC-mediated pyroptosis in tumours. Upon sensing infection or danger, assembly of inflammasomes activates inflammatory caspases to cleave GSDMD and trigger pyroptosis, initiating immune responses. However, excessive pyroptosis leads to lethal sepsis, cytokine release syndrome (CRS) and severe inflammation and tissue damage. Autoactive pyrin or NLRP3 constitutively activate GSDMD and cause pyroptosis leading to familial Mediterranean fever (FMF) and neonatal-onset multisystem inflammatory disease (NOMID), respectively. Natural killer (NK) and CD8<sup>+</sup> killer lymphocytes and chimeric antigen receptor (CAR) T cells activate pyroptosis

in GSDME-expressing tumours when GzmB cleaves and activates GSDME and caspase 3. Alarmins released from pyroptotic tumour cells can also activate GSDMD-mediated pyroptosis in tumour-infiltrating macrophages and dendritic cells to enhance antigen presentation and functional activation of tumour-infiltrating lymphocytes (TILs). Secondary activation of macrophages can also mediate CRS during CAR T cell therapy. Chemotherapy drugs and radiotherapy activate caspase 3 in GSDME-expressing cells to convert apoptosis into pyroptosis, increasing their effectiveness at suppressing tumour growth by activating antitumour immunity; at the same time normal tissue cell damage and cytotoxicity is increased by activating GSDME-dependent pyroptosis especially in gut epithelial cells and haematopoietic precursor cells. Autoactive GSDME induces cochlear hair cell pyroptosis, leading to nonsyndromic hearing impairment. Loss-of-function mutation of *DFNB59* also causes recessive nonsyndromic hearing loss. CAPS, cryopyrin-associated periodic syndromes; MS, multiple sclerosis; SS, systemic sclerosis.

hepatic, colon and HER2<sup>+</sup> breast cancer and may function as an oncogene in those cancers<sup>71,79</sup>. GSDMB expression in HER2<sup>+</sup> breast cancer correlates with increased metastasis and poor prognosis<sup>78,80</sup>. Overexpression of *GSDMB* in HER2<sup>+</sup> MCF7 cells promotes cell mobility and invasion, probably by activating *Rac1* and *Cdc42* (REF.<sup>78</sup>). Moreover, introducing anti-GSDMB into HER2<sup>+</sup> tumour cells using a hyaluronan-coated nanoparticle reduces xenograft growth and metastasis *in vivo*<sup>167</sup>. By contrast, *GSDMB* expression is suppressed in primary oesophageal and gastric tumours<sup>19</sup>, and its expression correlates with better outcome in patients with bladder carcinoma or melanoma. GSDMC is upregulated in melanoma, colorectal cancer and lung adenocarcinoma, promotes tumour growth and metastasis, and might function as an oncogene in those cancers<sup>82,85,168</sup>. These studies suggest that the roles of GSDMs in cancer are context, GSDM and cancer type specific, reflecting the complex role of inflammation in tumorigenesis, cancer proliferation and antitumour immunity<sup>169</sup>. Because of this complexity, these studies need to be interpreted with caution. *In vitro* experiments and studies in immunodeficient mice do not take into account the interaction between the tumour and the immune system, where GSDMs may play their most important *in vivo* role.

GSDME, which is activated by caspase 3 during apoptosis, is more strongly implicated in tumour suppression. *GSDME* is epigenetically suppressed by promoter DNA methylation in gastric, colorectal and breast cancers and most human cancer cell lines; and most tumour-associated *GSDME* mutations studied compromise its ability to induce pyroptosis<sup>17,18,56–60</sup> (TABLE 2). Moreover, *GSDME* expression, induced by 5-aza-dC *in vitro*, suppresses colony formation and tumour cell proliferation in gastric cancer, melanoma and colorectal cancer, and reduces invasivity of breast cancer<sup>56,57,60,119</sup>. Worse 5-year survival and an increase in lymph node metastases are associated with reduced *GSDME* in breast cancer<sup>57</sup>. Moreover, lack of *GSDME* promotes melanoma cell line resistance to etoposide, which is rescued by *GSDME* overexpression<sup>170</sup>. In addition, treatment of lung cancer cells with KRAS, EGFR or ALK inhibitors leads to caspase 3-mediated *GSDME* activation, which increases the antitumour effectiveness of these drugs<sup>171</sup>.

The tumour-suppressive effect of *GSDME* is dependent on immune surveillance *in vivo* by converting NK and CD8<sup>+</sup> T killer cell-mediated noninflammatory apoptosis into inflammatory pyroptosis in the tumour, which augments antitumour immunity<sup>18</sup> (FIGS 3,5). This effect is abrogated in tumours that express noncleavable *GSDME* and in mice that lack all lymphocytes, only killer lymphocytes or perforin that is needed for cytotoxic granule-mediated killing<sup>18</sup>. *Gsdme* expression in mouse tumours suppresses tumour growth and enhances the number and functions of tumour-infiltrating NK and CD8<sup>+</sup> T lymphocytes<sup>18</sup>. When killer cells recognize a tumour target, they deliver the cytotoxic protease GzmB into the tumour, which induces *GSDME*-mediated pyroptosis, both directly because GzmB cleaves and activates *GSDME* and indirectly because GzmB also cleaves and activates caspase 3 (REF.<sup>18</sup>). Similar findings were reported

using chimeric antigen receptor (CAR) T cells, which induce pyroptosis in *GSDME*-expressing target cells, but how much *GSDME* pyroptosis contributes to the effectiveness of CAR T cell therapy has not been evaluated<sup>172</sup>. Caspase 3 activation by GzmB generates a positive feedback mechanism as caspase 3 also cleaves and activates *GSDME*. As *GSDME* can be cleaved and activated by both caspase 3 and GzmB, *GSDME* may be a more potent tumour suppressor than the other GSDMs. Caspase 3 activation is common in cancers, triggered by an inadequate supply of oxygen or nutrients, especially in poorly vascularized regions, by treatment with chemotherapy or radiation and by killer cell attack. Because *GSDME* is suppressed by promoter hypermethylation in many cancers<sup>53–57</sup>, epigenetic modifying drugs could promote antitumour immunity. Indeed, combining 5-aza-dC with chemotherapy in one study enhanced the immunogenic effect of chemotherapy<sup>173</sup>. However, it is unclear how much pyroptosis contributes to the 5-aza-dC-induced antitumour effect as 5-aza-dC should derepress many genes, including tumour suppressor genes.

The potential therapeutic role of inducing pyroptosis in tumours has been demonstrated in a number of examples. Like GzmB-induced *GSDME* activation, the killer lymphocyte cytotoxic protease GzmA cleaves *GSDMB* and induces pyroptosis in tumour cells targeted for immune elimination<sup>19</sup> (FIGS 3,5). When combined with anti-PD1, ectopic expression of human *GSDMB* in murine colorectal cancer CT26 and melanoma B16 promotes tumour clearance in mice. The effectiveness of BRAF and MEK inhibitors against *GSDME*-expressing *BRAF*<sup>V600E/K</sup> mutant melanoma was shown to depend on the induction of *GSDME*-mediated pyroptosis to activate CD8<sup>+</sup> T cell-dependent antitumour immunity<sup>174</sup>. In another study, the serine dipeptidase DPP8/9 inhibitor Val-boroPro, which activates the CARD8 inflammasome in human myeloid cells, induced *GSDMD*-mediated pyroptosis in most human acute myeloid leukaemia (AML) cell lines and primary patient samples, but not cell lines from other lineages<sup>175</sup>.

These data suggest that *GSDM*-mediated pyroptosis plays a crucial role in antitumour immunity for some tumours, and harnessing this process could be an effective way to treat cancer. However, activating *GSDMs* in *GSDM*-expressing normal tissues and immune cells can also cause severe tissue damage, such as in chemotherapy-induced caspase 3-mediated *GSDME* activation in normal tissues<sup>17</sup> (FIGS 3,5). Although a DPP8/9 inhibitor induces pyroptosis in AML cells, it may also activate *GSDMD*-mediated pyroptosis in B cells and other CD34<sup>+</sup> cells<sup>175</sup> and cause treatment-associated toxicity. Activation of *GSDME*-mediated pyroptosis in normal mouse tissues also causes extensive tissue damage after chemotherapy<sup>17</sup>. During CAR T cell anticancer therapy of B cell leukaemia, GzmB activates both *GSDME* and caspase 3, causing pyroptosis. Moreover, culture supernatants from B-ALL human cell lines or primary leukaemias cocultured with CAR T cells activate caspase 1–*GSDMD*-mediated pyroptosis in macrophages. Activation of pyroptosis by CAR T cells is responsible for CRS, one of the serious side effects of this therapy<sup>172</sup> (FIGS 3,5). Thus, selective activation of *GSDMs* in tumours

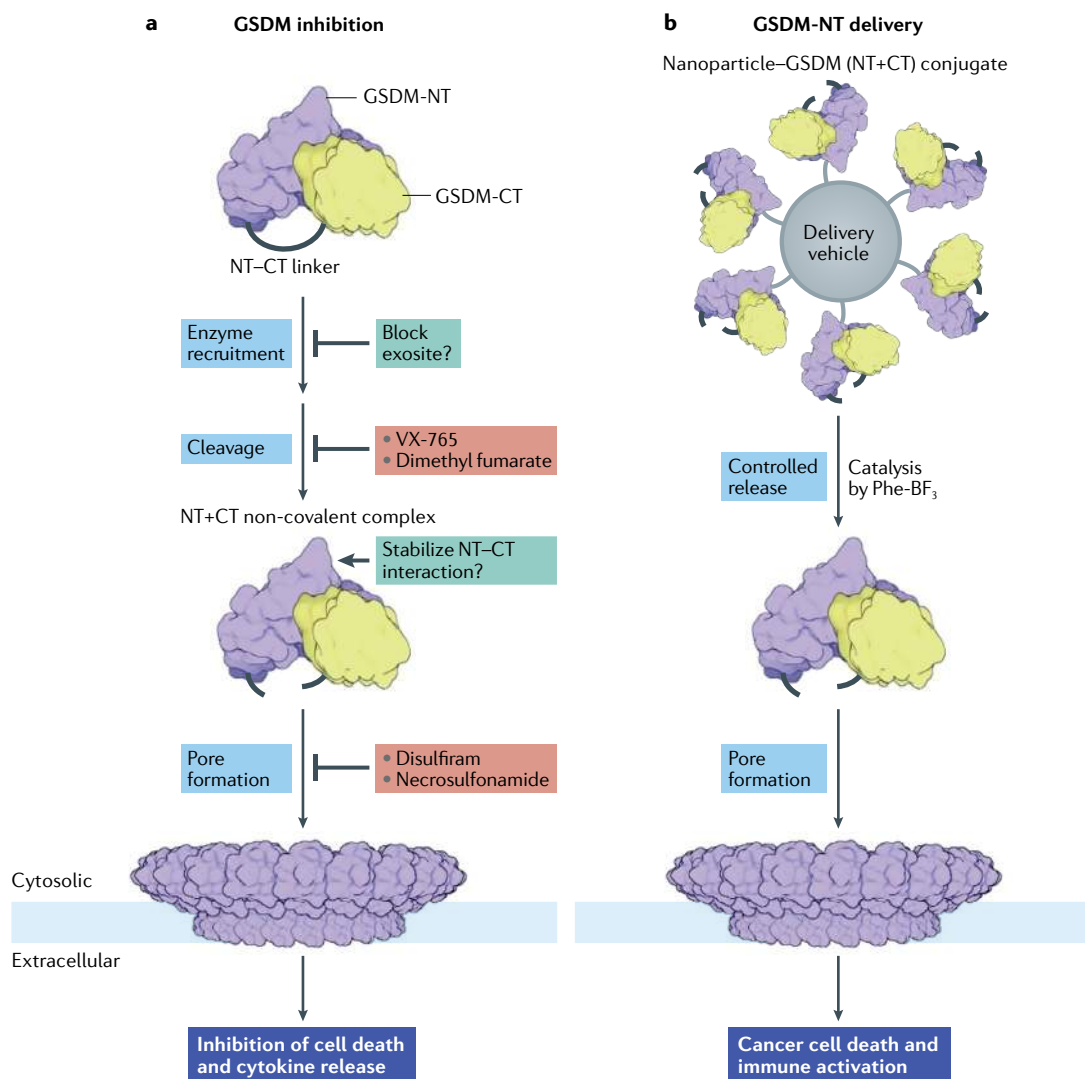


Fig. 6 | **Strategies to treat gasdermin-associated diseases.** **a** | Steps in gasdermin (GSDM) activation that can be targeted, and the mechanisms used by existing GSDMD inhibitors<sup>45,48</sup>. Other yet unused potential mechanisms are shown with a question mark. **b** | Cellular delivery of GSDM for cancer cell killing and activation of antitumour immunity<sup>180</sup>. See text for details. CT, C-terminal; NT, N-terminal.

rather than normal cells could be crucial for developing pyroptosis-inducing cancer therapy.

### Therapeutically targeting gasdermins

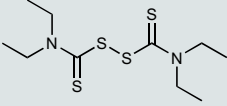
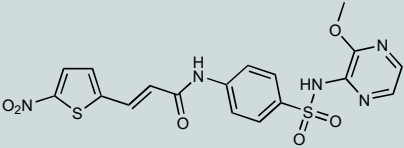
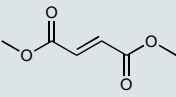
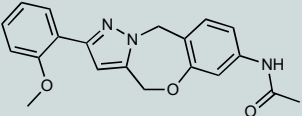
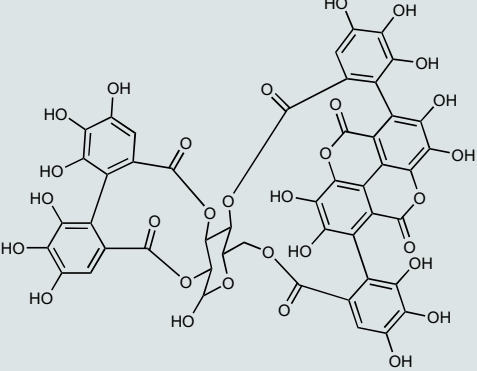
The key roles of GSDMs in infection, inflammation and tumour suppression have prompted investigation of GSDM-targeting therapeutics (FIG. 6). Inflammasome pathways have already been extensively targeted indirectly for disease modulation by inhibiting IL-1 $\beta$ , the signature cytokine released in a GSDMD activation-dependent manner, or its receptor, using the FDA-approved drugs anakinra, a recombinant natural human IL-1 receptor antagonist, and canakinumab, an anti-IL-1 $\beta$  monoclonal antibody<sup>176</sup>. These drugs are used to treat severe manifestations of autoinflammatory diseases, including RHEM, NOMID, severe systemic autoimmune diseases (rheumatoid arthritis, systemic juvenile idiopathic arthritis, adult-onset Still's disease) and bronchiolitis obliterans that do not respond to other treatments. Small-molecule inhibitors of caspase 1, such as VX-765, have also been

developed<sup>151</sup>, although they have not proved useful for treating human disease. Inhibitors of inflammasome sensors are under clinical development<sup>177</sup>. However, GSDMD is an especially attractive drug target because it is the bottleneck for all inflammasome effects, acting upstream of IL-1 and alarmin secretion and downstream of all inflammasome sensors (FIGS 2,3,5). Inhibition of GSDMD and possibly other GSDMs may therefore be the most efficacious strategy for treating diseases that are primarily inflammatory and/or autoimmune and may also be useful for treating diseases exacerbated by pyroptosis, such as cardiovascular disease and CRS in severe COVID-19 infection. On the other hand, induction of GSDM-mediated cell death may be useful for amplifying immune responses, such as in vaccination or cancer.

**Small-molecule gasdermin inhibitors.** A few studies have identified small-molecule GSDMD inhibitors (TABLE 3). DSE, an FDA-approved drug for treating chronic alcoholism (Antabuse), was identified in a



Table 3 | Gasdermin D inhibitors

| GSDMD inhibitor  | Description   | In vitro IC <sub>50</sub>   | Cellular IC <sub>50</sub>   | Mechanism of action   | In vivo effect  | Refs  |
|--|---|---|---|---|---|-------|
| <p>Disulfiram</p>         | Commonly known as Antabuse, an FDA-approved alcoholism drug | ~300 nM alone in GSDMD-induced liposome leakage; ~200 nM in combination with Cu(II) | ~7.7 μM in canonical inflammasome-dependent pyroptosis in THP-1; ~10.3 μM in noncanonical inflammasome-dependent pyroptosis in murine iBMDMs; ~400 nM in combination with Cu(II) in THP-1 | Binds GSDMD (K <sub>d</sub> = ~12.8 μM), covalently modifies Cys191 in GSDMD, and inhibits GSDMD-NT oligomerization/insertion                             | Increased survival and suppression of inflammatory responses in murine models of sepsis and EAE   | 25,48 |
| <p>Necrosulfonamide</p>   | An inhibitor of MLKL in necroptosis                         | ~9.5 μM in GSDMD-induced liposome leakage   | ~10–15 μM in canonical inflammasome-dependent pyroptosis in iBMDMs  | Binds GSDMD (K <sub>d</sub> = ~32 μM), potentially modifies Cys191 in GSDMD, and inhibits GSDMD-NT oligomerization/insertion                              | Increased survival and suppression of inflammatory responses in a murine model of sepsis  | 45,48 |
| <p>Dimethyl fumarate</p>  | An FDA-approved drug (Tecfidera) for the treatment of MS    | NA  | NA  | Succinates GSDMD at Cys191 and other reactive cysteines, prevents interaction between GSDMD and caspases, and inhibits GSDMD-NT oligomerization/insertion | Increased survival and suppression of inflammatory responses in murine models of sepsis, EAE and FMF; decreased levels of GSDMD-NT and IL-1β in patients with MS treated with Tecfidera | 48,49 |
| <p>LDC7559</p>          | A compound based on the pyrazolo-oxazepine scaffold         | NA  | ~5.6 μM in PMA-induced NETosis in murine neutrophils; ~300 nM in cholesterol crystal-induced NETosis in murine neutrophils  | Binds GSDMD in pulldown experiments using cell lysates; mechanism of action unclear   | NA  | 43,48 |
| <p>Punicalagin</p>      | A complex polyphenol from pomegranate extract               | NA  | ~3.9, 3.7 and 7.5 μM in IL-1β secretion, LDH release and membrane permeabilization, respectively, in ATP-treated murine BMDMs   | Potentially interferes with membrane fluidity and inhibits GSDMD-NT insertion   | NA  | 179   |

The half-maximal inhibitory concentration (IC<sub>50</sub>) and equilibrium dissociation constant (K<sub>d</sub>) values were taken from the referenced studies. EAE, experimental autoimmune encephalomyelitis; FMF, Familial Mediterranean fever; GSDM, gasdermin; iBMDMs, immortalized murine bone marrow-derived macrophages; LDH, lactate dehydrogenase; MLKL, mixed-lineage kinase domain-like protein; NA, not available; NT, N-terminal; MS, multiple sclerosis; PMA, phorbol myristate acetate; THP-1, a human monocytic cell line.

high-throughput liposome leakage-based screen as an inhibitor of GSDMD pore formation on liposomes with a half-maximal inhibitory concentration ( $IC_{50}$ ) in the nanomolar range<sup>48</sup>. In human THP-1 cells and mouse immortalized BMDMs (iBMDMs), stimulated by the canonical NLRP3 and AIM2 inflammasomes and the noncanonical inflammasome, respectively, DSF blocked pyroptosis and cytokine release without affecting inflammasome speck formation, caspase activation or GSDMD cleavage, suggesting that it was acting by blocking pore formation. In a mouse model of LPS-induced septic shock, DSF protected wild-type and caspase 1-knockout mice from death and greatly reduced serum inflammatory cytokine levels. The potency of DSF was enhanced when it was combined with copper gluconate, which reportedly stabilizes diethylthiocarbamate (DTC), the cellular metabolite of DSF<sup>178</sup>. The efficacy of DSF in protecting against GSDMD-mediated inflammatory disorders was also demonstrated in EAE<sup>25</sup>. Necrosulfonamide (NSA), a drug previously known to inhibit MLKL in necroptotic cell death, was also shown to inhibit GSDMD pore formation, with low micromolar cellular  $IC_{50}$  in blocking cytokine release and pyroptosis downstream of NLRP3 and NLRC4 inflammasomes<sup>45</sup>. Like DSF, NSA extended survival of wild-type mice in a model of LPS-induced sepsis.

DMF, FDA-approved Tecfidera for the treatment of MS, was reported to prevent GSDMD from interacting with caspases, limit GSDMD processing and oligomerization, and block pyroptosis; endogenous fumarate similarly inhibited GSDMD<sup>49</sup>. In murine models of sepsis, EAE and FMF, DMF extended survival and alleviated inflammation. Patients with MS treated with DMF exhibited decreased plasma GSDMD-NT and IL-1 $\beta$  compared with untreated controls, consistent with a model in which GSDMD exacerbates MS, as was shown in mouse EAE. These strong inhibitory effects of DMF contrast with the lack of inhibition of GSDMD-induced liposome leakage by DMF reported in an earlier study<sup>48</sup>. These two studies suggest that DMF may inhibit GSDMD cleavage, while DSF and NSA work downstream in the pathway to inhibit pore formation. Further characterization of DMF is necessary to evaluate its potency as a GSDMD inhibitor.

DSF, NSA and DMF are all Cys-reactive drugs that inhibit GSDMD by modifying Cys residues (TABLE 3). DSF and NSA covalently modify Cys191, but not other cysteines, in GSDMD to inhibit pore formation<sup>45,48</sup>, while DMF and endogenous fumarate modify multiple Cys residues, including Cys191, leading to GSDMD succination<sup>49</sup>. Consistently, GSDMD with mutated Cys191 and other GSDMs that lack this reactive cysteine are not inhibited by DSF or NSA<sup>45,48</sup>. GSDMD Cys191 is an exposed cysteine on the  $\alpha 4$  helix that plays an important role in membrane penetration (FIG. 4b). Modification of Cys191 may interfere with membrane insertion and therefore abrogate formation of transmembrane  $\beta$ -hairpins (FIGS 4c, 6a). Although the exposed position of Cys191 may facilitate its modification, a downside of Cys-reactive compounds is that they are less selective and likely also target molecules other than GSDMD. Therefore, the mechanisms of action of DSF, NSA and DMF *in vivo* should be interpreted with caution.

Two other molecules, LDC7559, a pyrazolo-oxazepine-based compound, and punicalagin, an antioxidant polyphenol from pomegranates, have been reported to inhibit PMA-induced NETosis and NLRP3/AIM2 inflammasome-dependent pyroptosis, respectively, with activity in the low micromolar range<sup>43,179</sup>. In pulldown experiments using cell lysates, LDC7559 specifically enriched GSDMD detection by mass spectrometry. However, the interaction between LDC7559 and GSDMD was not directly verified, and LDC7559 does not inhibit GSDMD pore formation on liposomes<sup>48</sup>, hence leaving its mechanism of action unexplained. Likewise, considering that punicalagin also protects macrophages from detergents and may indirectly affect GSDMD pore formation by rigidifying the plasma membrane, further studies are needed to elucidate whether punicalagin directly and specifically binds to GSDMD.

On the basis of a structural understanding of GSDM auto-inhibition, molecules that stabilize the GSDM NT-CT interface may inhibit the full-length protein by locking it in its inactive conformation (FIG. 6a). The recent identification of an exosite for inflammatory caspase recognition of GSDMD suggests that another possible way of inhibiting GSDMD would be to identify a compound that blocks the exosite (FIG. 6a). It is likely that other post-translational modifications and mechanisms to regulate pore formation will be uncovered that might be attractive drug targets.

**Manipulating gasdermin expression in cells.** In an effort to induce GSDM-mediated cancer cell pyroptosis to prime adaptive immunity, intratumoural delivery of nanoparticle-conjugated, pre-cleaved GSDMA3 (NT + CT) into cells was combined with the cancer-imaging compound phenylalanine trifluoroborate (Phe-BF<sub>3</sub>), which catalyses the release of GSDMA3 from nanoparticles, causing selective tumour cell pyroptosis<sup>180</sup> (FIG. 6b). Pyroptosis of <15% of tumour cells was sufficient to suppress the entire tumour graft in mice, because the treatment activated the adaptive immune response to the tumour. Immunodeficiency or T cell depletion abrogated the tumour-suppressive response. Further, a partially effective intravenous treatment with the nanoparticles synergized with checkpoint blockade and sensitized the tumours to anti-PD1, suggesting that pyroptosis could be exploited to trigger or enhance anti-tumour immunity. This study provides a proof of concept for targeting GSDMs in cancer; however, care needs to be taken to avoid excessive pyroptosis, which could be toxic to normal cells or cause cytokine release with systemic consequences. Delivery of GSDM-NT or identification of GSDM agonists could also in principle be used as vaccine adjuvants or to enhance the immune response to infection.

### Considerations for therapeutic GSDMD inhibition

The wide variety of diseases in which GSDMD likely mediates inflammation and its crucial role as a final bottleneck for pyroptosis suggest that GSDMD inhibition could be an extremely effective therapeutic strategy. However, interfering with the antimicrobial roles of GSDMD could lead to increased susceptibility to

#### Cys-reactive drugs

Drugs that covalently bind to the sulfhydryl (SH) group of Cys in cysteine-containing proteins.

infection. In addition, innate immune responses are complex, and each potential therapeutic indication will need to be examined carefully in preclinical models. In a study using the monosodium urate (MSU)-induced gout model, although GSDMD was activated through the NLRP3 inflammasome, it was not needed for cell death or IL-1 $\beta$  release, and the GSDMD inhibitor NSA did not ameliorate disease<sup>181</sup>. Suppressing pyroptosis can activate other innate immune responses. For instance, GSDMD inhibition in a cell can activate the interferon response<sup>182</sup>. In a mouse study that modelled systemic lupus erythematosus (SLE) using injected imiquimod, the severity of SLE was unexpectedly exacerbated in *Gsdmd*<sup>-/-</sup> mice, leading to more severe kidney and pulmonary pathology, more autoantibodies and reduced mortality<sup>183</sup>. Type I interferons play an important driving role in many SLE models and in patients with SLE. GSDMD may have suppressed interferon induction in these mice, although a change in interferon signalling in this SLE model in *Gsdmd*<sup>-/-</sup> mice was not examined.

Mouse models may not always accurately mirror human disease pathogenesis, and mouse models of inflammatory diseases can be affected by small differences in experimental protocol or differences in the microbiome of mouse colonies or other poorly defined environmental or genetic differences. For example, two recent studies of dextran sodium sulfate-induced colitis found opposite results: in one study *Gsdmd* deficiency exacerbated colitis and enhanced cGAS-STING-mediated interferon induction<sup>184</sup>, while in another study lack of *Gsdmd* reduced disease severity<sup>185</sup>.

The role of GSDMD in various inflammatory diseases and potential interactions of GSDMD activation with other innate immune responses will need to be examined carefully to select disease indications and anticipate possible toxicity of GSDMD-modulating compounds as they become available.

### Concluding remarks and future perspectives

Since the initial discovery of GSDMD as the substrate of inflammatory caspases, much progress has been made in understanding the activation mechanisms of the GSDM family. Two ways to activate GSDMs have so far been identified: first, protease-mediated cleavage in the linker region to liberate the NT active domain, and second, CT domain mutations including many germline-encoded disease mutations that disrupt the interaction of the NT and CT domains and diminish the ability of the CT domain to inhibit NT domain pore formation (FIGS 2–5). Although the physiological stimuli that activate GSDMs are still being uncovered, a theme of GSDMs as sensors of cytosolic protease activation has emerged. Currently

these proteases include caspases, granule-associated proteases, such as neutrophil elastase and cathepsin G, and granzymes delivered to target cells by killer T cells. The flexibility and accessibility of the GSDM linker region increases its vulnerability to protease attack. Direct protease activation of a GSDM could induce inflammatory death in diverse types of cells beyond immune and barrier cells. It will not be surprising if GSDMs are found to be cleaved and activated by additional cellular proteases as well as by pathogen-encoded proteases.

As inflammation causes or contributes to many poorly controlled and sometimes lethal diseases and strongly augments antigen-specific immunity, there is a need to further elucidate the regulation of GSDMs at the root of inflammation. For example, inflammasome activation also exacerbates many mouse models of disease, including atherosclerosis, type II diabetes, gout, nonalcoholic steatohepatitis and several neurodegenerative diseases, including Alzheimer disease. GSDMs not only cause cell death, but are also required for unconventional protein secretion of the IL-1 family cytokines and other intracellular danger molecules such as HMGB1 that lack a secretion signal. What regulates whether GSDM activation causes lytic cell death or hyperactivation without cell death remains unclear. A related question is whether pyroptosis or hyperactivation generates a stronger innate immune signal. Factors released by GSDM activation must be quite different in pyroptotic versus hyperactive cells. There is no doubt that future studies will link the GSDM family to more physiological and pathological processes and uncover how these processes are controlled.

As the key perpetrators of inflammation, GSDMs are strategically situated for therapeutical manipulation of inflammation. Developing GSDM agonists or antagonists could transform the treatment of inflammatory disease and potentially lead to better vaccine adjuvants or immune therapy. So far, identifying specific compounds that inhibit GSDMs has been challenging. The currently available GSDMD inhibitors, NSA, DSF and DMF, all covalently modify reactive cysteines and are thus not completely specific. Further mechanistic understanding of GSDM pore formation and the structural intermediates that form in the transition from the inactive to the active state will undoubtedly inform drug identification and optimization. Similarly, understanding the molecular pathways that regulate pore formation could provide additional therapeutic targets. Given the crucial function of GSDMs in inflammation, the search for GSDM-modulating drugs is likely to be very active in the coming years.

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#### Competing interests

H.W. and J.L. are co-founders of Ventus Therapeutics. The other authors declare no competing interests.

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