



VIEWPOINT

Gasdermin-B (GSDMB) takes center stage in antibacterial defense, inflammatory diseases, and cancer

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One of the hottest topics in biomedical research is to decipher the functional implications of the Gasdermin (GSDM) protein family in human pathologies. These proteins are the key effectors of a lytic and pro-inflammatory cell death type termed pyroptosis (also known as “Gasdermin-mediated programmed cell death”). However, ever-growing evidence showed that GSDMs can play multiple and complex roles in a context-dependent manner. In this sense, Gasdermin-B (GSDMB; the only GSDM gene absent in mice and rats) has been implicated in antibacterial defense, numerous inflammatory pathologies (e.g., asthma, ulcerative colitis), and cancer, but both cell death-dependent and -independent functions have been reported in these diseases, fueling the debate on whether GSDMB has genuine pyroptotic capacity. Recently, a series of seminal papers cast light on the GSDMB multitasking capacity by showing that different GSDMB transcriptional isoforms have distinct biological activities. Nonetheless, there are still obscure areas to be clarified on the precise functional involvement of GSDMB translated variants in physiological and pathological conditions. In this viewpoint, we critically discuss the most recent and exciting data on this topic and propose a series of relevant challenges that need to be overcome before GSDMB-driven biomedical applications (as a biomarker of disease risk/progression/outcome or as specific therapeutic target) become a reality in clinical settings.

Introduction

Gasdermins (GSDMs) are ~50-kDa cytoplasmic proteins that, under specific circumstances, form pores in biological membranes leading to pyroptosis (“fiery death”) where dying cells release intracellular damage-associated molecules (DAMPs) and/or cytokines that provoke a strong inflammatory response [1,2]. The pyroptosis mechanism is complex but requires

the release of the GSDM N-terminal (NT) pore-forming domain [3,4] (Fig. 1A). Pyroptosis primigenial function, shared by all GSDMs (*GSDMA-E*) except *PJVK*, is the defense against pathogen infection (killing pathogens and/or infected cells) [5,6]. During evolution, the expansion of GSDM genes allowed the species to adapt their defensive arsenal against

Abbreviations

Aa, amino acids; CT, C-Terminal domain; DAMPs, danger-associated molecular patterns; ELANE, neutrophil elastase; GEMM, genetically engineered mouse models; GSDMs, Gasdermins; GVAS, genome-wide association studies; GZM, granzyme; IBD, inflammatory bowel diseases; INFs, interferons; IpaH7.8, invasion plasmid antigen gene H 7.8; LPS, bacterial lipopolysaccharide; NT, N-terminal domain; SNP, single nucleotide polymorphism.

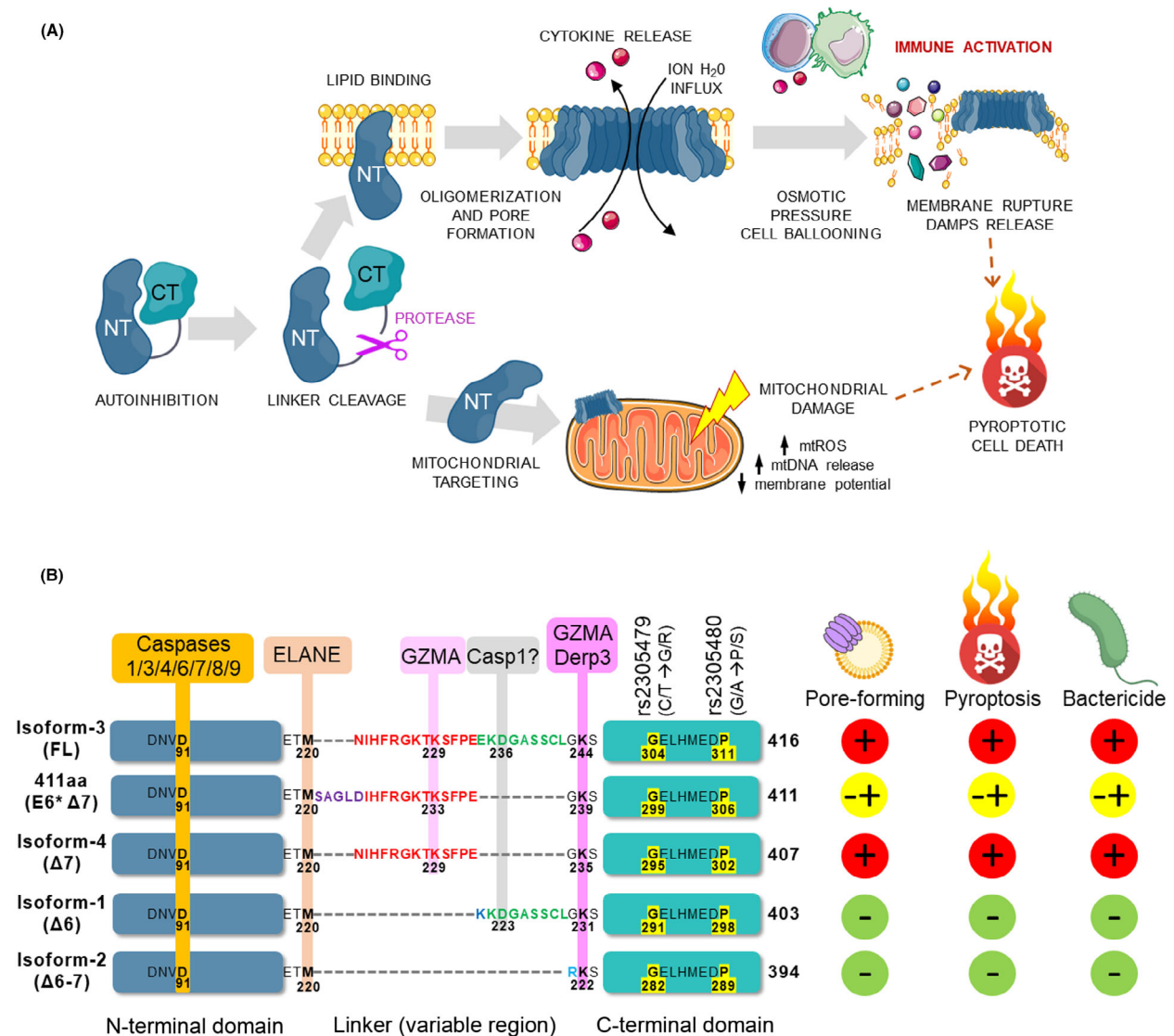


Fig. 1. General mechanism of Gasdermin (GSDM)-mediated pyroptosis and summary of the functional differences among translated Gasdermin-B (GSDMB) isoforms. (A) Schematic of the general steps of GSDM-mediated cell death. In basal conditions, GSDMs remain in the cytoplasm in a closed conformation since their N-terminal (NT) domains are inhibited by their C-terminal (CT) domains. In response to particular stimuli, specific proteases cleave the GSDM flexible interdomain region, releasing the NT domain. The released GSDM NTs bind to acidic lipids at the cell membrane and mitochondria, oligomerize (23–27 monomers) and form large transmembrane pores. In the plasma membrane, specific molecules (e.g., IL-1 β or IL-18) are released through these pores, leading to osmotic changes, and hence, osmotic shock. Osmotic shock provokes cell ballooning and membrane rupture, leading to the release of Danger Associated Molecular patterns (DAMPs). Altogether, cytokines, DAMPs, and other molecules activate the immune system. At the mitochondrial membrane, GSDM pores provoke organelle damage through oxidative stress (mtROS), the release of mitochondrial DNA (mt DNA), and the loss of membrane potential. Mitochondrial damage boosts pyroptotic cell death. (B) Details of the five translated GSDMB isoforms proven to have functional roles with protein size, important sequences, regions, and protease cleavage sites indicated. For every feature presented, the corresponding amino acid number (aa, below) is indicated for each isoform. The NT domain is shown in blue and the CT domain in green. Within the region that diverges among GSDMB isoforms (interdomain linker), the aa sequences encoded by the exon 6 are shown in red, those encoded by exon 7 are in green, while other divergent residues are identified by purple or blue letters. Missing residues are depicted as gray lines. The localization of each protease cleavage site is shown. The proteolytic events that can activate pyroptosis are shown in pink, those that hamper NT-pyroptosis in orange, and caspase-1 (debatable functional effect) in gray. In the CT, yellow letters highlight the localization and aa changes produced by two common single nucleotide polymorphisms (SNPs; nucleotide allele and the corresponding encoded aa is specified on top) that are differentially associated with asthma risk and IBD. The panel on the right shows the capacity of each isoform to induce functional pores on liposomes, pyroptosis in mammalian cells and the antibacterial effect ("bactericide") on gram-negative bacteria, based on the literature. "+" means strong effect, "-/+" = mild effect and "-" low or no effect. ELANE, neutrophil elastase; GZMA, Granzyme-A.

specialized pathogens [5,6]. Indeed, the *GSDMB* gene likely originated in mammals (from a late duplication and inversion of *GSDMA* [7]) to protect enterocytes from *Shigella* infection, but mice are naturally resistant to Shigellosis and thus, lack *GSDMB* [8,9].

Each GSDM is activated by specific stimuli (i.e., pathogens, cell damage, chemotherapy), proteases (e.g., GSDMB by Granzyme-A, GZMA) and biological contexts [1,2]. In fact, GSDMs can mediate not only additional cell death types (necrosis, apoptosis, NETosis, necroptosis, and/or autophagy) but also other cell-death-independent functions [10,11]. Thus, this protein family orchestrates multiple, sometimes opposing, tasks.

Importantly, the dysregulation of these GSDM functions can cause several diseases, and therefore the GSDMs are novel and promising therapeutic targets [1,2,10,12–14]. In this sense, GSDMB is involved in antibacterial defense [8,9], numerous inflammatory pathologies (asthma, inflammatory bowel disease – IBD, etc.) [15–22] and cancer [23–30]. However, both GSDMB cell death-dependent and -independent effects were reported in these diseases, fueling the controversy on whether GSDMB truly has pyroptotic capacity [14,31–33]. Recently, published seminal papers

demonstrate that GSDMB multifunctionality is controlled, in part, by the existence of distinct transcriptional variants (isoforms) with different biological activities [34–37]. The new data reinforce the potential of GSDMB isoforms as novel biomarkers and therapeutic targets in human diseases. However, there are still key issues to be resolved, before GSDMB-specific clinical applications become a reality. In this viewpoint, we present a critical review of the most compelling data and thoroughly discuss the following key questions.

Discussion

How many GSDMB isoforms are there? And why are they functionally different?

GSDMB mRNA is mostly expressed in the digestive and respiratory tract but also in lymphoid tissues and diverse cancer types [1,14,27,29,30,38]. Human *GSDMB* gene (a.k.a GSDML, PP4052, PRO2521), located at the 17q21.1 chromosome region, comprises 11 exons (but exon 1 is not translated). The usage of two complementary promoters [39] and alternative splicing events controls the expression of at least 15

Table 1. Translated GSDMB variants and cleavage products, features, and corresponding nomenclature from different sources. Variants are ordered by protein length (aa, amino acids), and their names in different databases or scientific literature (references cited) are shown.

Size (aa)	NCBI kDa ^a	NCBI name	Differences from full-length variant (aas)	NCBI Prot ref seq	UniProt	ENSEMBL transcript ID	Alternative names [references]
416	47.3	Isoform 3	Full-length (CANONICAL)	NP_001159430.1	Q8TAX9-4	ENST00000418519.6	GSDMB ^{iso3} [36]
411	46.8	–	221N → SAGLD Lacks exon 7 (234–242)	NP_001375349.1	Q8TAX9-1	ENST00000360317.7	1-416 [34] GSDMB ^{isoU} [36] isoformU [36]
407	46.5	Isoform 4	Lacks exon 7 (234–242)	NP_001375350.1	Q8TAX9-6	ENST00000520542.5	GSDMB ^{iso4} [36] 1-416Δ7 [34]
403	45.8	Isoform 1	Lacks exon 6 (221–234)	NP_001035936.1 NP_001356331.1 NP_001375351.1	Q8TAX9-3	ENST00000309481.11 ENST00000394179.5	GSDMB-1 [23] GSDMB ^{iso1} [36] 1-416Δ6 [34]
394	45.0	Isoform 2	Lacks exons 6 & 7 (221–243)	NP_001375352.1 NP_061000.2	Q8TAX9-2	ENST00000394175.6	GSDMB-2 [23] GSDMB ^{iso2} [36] 1-416Δ6,7 [34]
312	35.4	Isoform 5	Lacks exons 5–9 (193–296)	NP_001375353.1	–	–	–
237	27.7	–	Short exon 6; aberrant ORF → 237-STOP	–	–	–	Isoform 6 [37] F ^a [22]
163	18.1	–	Missing 1–253	–	Q8TAX9-5	–	–
244 ^b	28.2	–	NT portion after cleavage by GZMA	–	PRO0000451672	–	p30 [36]
172 ^b	19.1	–	CT portion after cleavage by GZMA	–	PRO0000451673	–	p16 [36]

^aMolecular weight calculated with ProtParam tool (<https://web.expasy.org/protparam/>); ^bThese are not transcriptional variants but cleavage products of GZMA.

different transcripts, but only a few are protein-coding (Table 1). Unfortunately, there are important divergences in the number and nomenclature of translated variants among NCBI, ENSEMBL, and Uniprot databases, as well as in the scientific literature, thus provoking confusion on their functional roles (Table 1 shows the equivalence in diverse databases and publications; and Fig. 1B shows the sequence and functional features of relevant variants). In this article, we will use the NCBI coding, translating other nomenclatures into this format, and the variants not listed in NCBI will be referred based on their amino acid (aa) size. Accordingly, isoforms 1–4 differ in the usage of alternative exons 6 (13aa) and 7 (9aa), being isoform-3 the full-length (416aa) protein (hereafter used as reference for residue numbering). Despite being widely used in the literature, the 411aa variant is scarcely expressed in tissues/cancer cells [36,37] and does not originate from canonical transcripts (it contains unusual exon 6 sequence; 221SAGLD₂₂₅, Fig. 1B) but maybe from aberrant splicing of unknown origin. Similarly, the rs11078928 SNP (in the intron 5 acceptor splice site) can provoke either full exon-6 skipping (isoform-1), shorter exon-6 sequences (237aa variant) and/or several truncated transcripts between exons 5 and 9 (isoform-5) [21,22] (Table 1).

Recently, four independent reports demonstrated that only the NT from GSDMB variants containing full exon-6 (isoforms-3/4) can generate entirely functional pores on biological membranes/liposomes and produce effective pyroptosis to kill HEK293T cells [34–37], cancer cells [34,36,37] or Gram-negative bacteria [35–37] (Fig. 1B). These results not only have deep implications for understanding the GSDMB roles in pathologies (see sections below) but also explain why previous studies using isoforms 1, 2, or 411aa found no strong evidence of GSDMB-driven pyroptosis [8,19,23,40,41]. It should be noted that exon 6-encoded residues (N221-E233) span the NT domain and the flexible linker interdomain [9,35–37,40]. Structural and mutational analyses prove that the exon 6-encoded basic patch (R225/K227/K229) is essential for efficient pyroptosis, but its precise function is debatable, with conflicting results showing a role in either NT lipid binding [35], oligomerization [34,36] or proper membrane insertion of oligomers [37]. Moreover, supporting the fundamental role of exon-6 in pyroptosis, several exon-6-containing GSDMB fragments are cytotoxic [3,18,34] being the 1–232 construct [18], the shortest peptide identified to date with this capacity. Importantly, inconsistent data regarding the minimum cytotoxic region [3,18,20,34,42] can be attributable to experimental

divergences among studies, like adding tags (i.e., GFP) to protein constructs, which indeed could abolish pyroptosis of some NT fragments [34].

Additionally, diverse studies on GSDMB 3D structure (mostly in complex with bacterial IpHa7.8; see ‘How does GSDMB mediate antibacterial defense?’) not only revealed interesting divergences among isoforms 1, 4, and 411aa but also suggested that GSDMB exhibits unconventional mechanisms of autoinhibition, lipid binding affinities, and pore formation compared with other GSDMs (GSDMD, GSDMA3) [9,35–37,40,43–45]. Unfortunately, neither the full-length isoform-3 nor isoform-2 (without exons 6–7) has been crystalized yet; thus, essential information is lacking about the functionally structural divergences among GSDMB variants and other GSDMs.

How are GSDMB cell death functions regulated by proteases?

Recent data point out that GSDMB-mediated cell death is controlled by distinct proteases (Fig. 1B) in a complex way.

Granzyme-A (GZMA)

GSDMB interdomain linker is specifically cleaved by GZMA (also mouse GZMA but with lower efficiency [8,27]) not the other GZMs [27,37]. GZMA is delivered through porins by activated NK and T cells into either infected cells, to kill intracellular bacteria [8] (Fig. 2A), or GSDMB-expressing epithelial cells (Fig. 2B,C) and cancer cells (Fig. 2D), provoking pyroptosis. The GSDMB K244 residue (common to all isoforms; Fig. 1B) is the main and physiological cleavage site for GZMA, producing the p30 NT fragment [27,34]. Although there is a minor cleavage site at K229 (Fig. 1B) that produces p28 NT, this cleavage only occurs *in vitro* with GZMA incubation and not during immunocyte killing in coculture experiments [27]. Accordingly, K244A mutation but not K229A greatly abrogates GZMA-mediated GSDMB pyroptosis [27].

Significantly, while all isoforms are cleaved by GZMA at K244 with equal efficacy *in vitro* [27], only the NT fragments from exon-6-containing variants are cytotoxic [27,34,37]. However, upon immunocyte challenge (coculture of HEK293 or cancer cells with NKs) GZMA seems to cleave more efficiently exon-7-containing isoforms-1/3 [34,37], suggesting that exon-7 residues might facilitate GZMA cleavage. Resolving the crystal structure of each GSDMB isoform in complex with GZMA would permit testing this hypothesis.

Caspases

The functional effect of caspases on GSDMB cell death is extremely controversial (Fig. 1B). Originally, Chao *et al.* [40] reported that apoptotic caspases 3/6/7 could cleave *in vitro* GSDMB at the ₈₈DNVD₉₁ site producing 10 KDa NT (residues 1–91) and 37Kda C-terminal (92–416 aas) fragments. Later, two reports [8,20] demonstrated that most caspases (1/3/4/6/7/8 and 9) cleaved GSDMB at this site (common to all isoforms; Fig. 1B). Interestingly, this cleavage equals the processing of GSDMD at D92 by apoptotic caspase-3 that produces a pyroptotic-deficient NT domain [46]. Indeed, the overexpression of neither 1–91 nor 92–416 GSDMB fragments induces cell death [20,34], indicating that caspase activation may act as a regulatory mechanism to dampen GSDMB-mediated pyroptosis [34]. In striking contrast, Panganiban *et al.* [18] reported that caspase-1 cleaved GSDMB D236 residue (Fig. 1B) and triggered GSDMB-NT pyroptosis, but only in isoform-3 (contains exon 6) and not the isoform-1 (lacks exon 6). Moreover, based on the evidence that the minor allele (C) of rs11078928 SNP associates with decreased asthma risk, and provokes exon-6 skipping during transcription [21], the authors propose that this genetic variant would reduce the levels of caspase-1 activatable pyroptotic GSDMB (exon-6-containing) variants, and this would be functionally linked to asthma biology (see ‘Do GSDMB pyroptotic or non-pyroptotic functions cause inflammatory diseases?’ and Fig. 2B). However, this hypothesis requires further verification since the effect of caspase-1 on GSDMB pyroptosis was validated neither by other groups nor under physiological asthma conditions or in clinical samples.

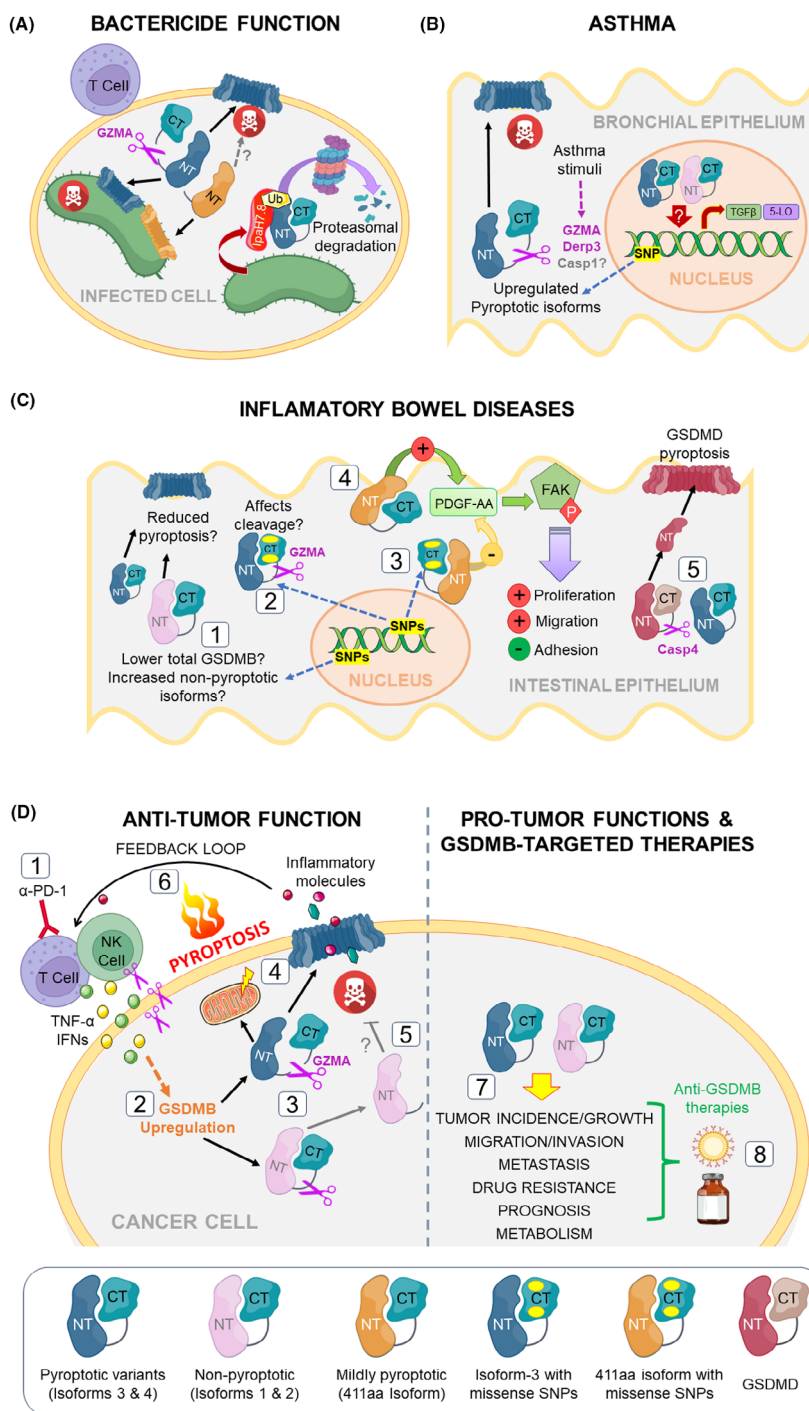
Furthermore, caspases certainly activate cell death in other GSDMs, specifically GSDMD, where caspase-1 triggers canonical pyroptosis (stimulated by nigericin plus bacterial Lipopolysaccharide, LPS) and caspases 4/5 non-canonical pyroptosis (directly activated by intracellular LPS) [47–49]. Therefore, different studies have evaluated if GSDMB isoforms expression might interfere with GSDMD-mediated pyroptosis, but contradictory results were reported. Thus, while in bronchial epithelial cells isoform-3 (but not isoform-1) exogenous overexpression increased nigericin-stimulated cell death [18], in THP1 cells treated with LPS + Nigericin neither GSDMB isoform 3 nor 2 (which were subsequently cleaved at D91; Fig. 1B) affected the levels of GSDMD-pyroptosis [34]. In contrast, Chen *et al.* [20] reported that GSDMB expression in THP1 cells increased cell death by enhancing caspase-4 cleavage of GSDMD via non-canonical pyroptosis (Fig. 2C).

Other proteases

Neutrophil elastase (ELANE) cleaves GSDMB at the M220 residue (common to all isoforms; Fig. 1B) producing an NT fragment with no pyroptotic activity [34]. Moreover, ELANE also cleaves and decreases the pyroptotic effect of highly cytotoxic GSDMB-NT fragments transfected into cancer cells. Despite the experiments were performed *in vitro* in the absence of neutrophils, the authors speculate that ELANE activation might downregulate GSDMB pyroptosis or control the way cancer cells die during antitumor immune response [34]. Therefore, NK-derived GZMA could trigger pyroptosis, while ELANE might produce a switch from lytic cell death (perhaps to control excessive inflammatory response) to noninflammatory cell death mechanisms (apoptosis). Finally, Derp3, a serine protease commonly found in asthma-inducing allergens, also cleaves GSDMB at K244 (Fig. 1) in human bronchial epithelial cells [50], suggesting that direct pyroptotic activation of GSDMB by asthma-sensitizing proteases may cause this pathology (Fig. 2B).

Is mitochondrial damage required for GSDMB-mediated cell death?

Accumulating evidence indicates that GSDM-mediated cell death involves not only cell membrane lysis but also mitochondrial dysfunction [10,51] (Fig. 1A). Interestingly, in other GSDM members, GSDM-NT mitochondrial localization and subsequent damage can either fuel pyroptosis (GSDMD) or trigger complementary/secondary cell death mechanisms, such as apoptosis (GSDMA, GSDME), autophagic cell death (GSDMA3) or necroptosis (GSDMD) in specific biological contexts [42,52–55]. Regarding GSDMB, several GSDMB-NT constructs largely accumulate in mitochondria [34,42] but only those with pyroptotic activity (exon-6 containing) provoke diverse signs of mitochondria impairment (Fig. 1A). At least in HEK293 cells (that lack other endogenous GSDMs [27]), GSDMB-NT mitochondria damage and lytic cell death seems to be independent of other secondary cell death mechanisms, like apoptosis or necroptosis [34]. However, it is unknown if GSDMB mitochondrial damage requires pore formation and if this process is necessary for efficient pyroptosis. Confocal timelapse microscopy showed that diverse GSDMB-NT constructs initially localize in mitochondria and cytoplasm, being focal membrane accumulation just observed prior to cell lysis [34]. This suggests that GSDMB-NT might (alike GSDMA [56]) show initial



preference for mitochondria but later cell membrane engagement results in cell rupture. Therefore, like other GSDMs [56–58], GSDMB-NT cell membrane pore formation might be sufficient for cell lysis, being mitochondrial dysfunction a cooperative mechanism to accelerate/ensure cell death.

How does GSDMB mediate antibacterial defense?

GZMA-released GSDMB-NT can form pores and kill specific gram-negative bacteria species (Fig. 2A). To counterattack this bactericidal function in human gut,

Fig. 2. Model of the complex functional implications of Gasdermin-B (GSDMB) isoforms in diverse pathologies. (A) Antibacterial effect of GSDMB on Gram-negative bacteria. Granzyme A (GZMA) cleaves cytosolic GSDMB in response to immunocytic (T-cell) attacks on bacterial-infected cells. Highly pyroptotic GSDMB isoforms can kill both bacterial and host cells by forming membrane pores, but the 411aa isoform can preferentially kill bacteria, since it has lower cytotoxic effect on mammalian cells [8]. *Shigella flexneri* can secrete the IpaH7.8 ubiquitin ligase that marks GSDMB for proteasomal degradation, thus allowing bacteria survival. (B) Implications of GSDMB in asthma. Left: Most intragenic or extragenic SNP alleles associated with asthma-risk upregulate total GSDMB levels in leukocytes and/or bronchial cells. Furthermore, the rs11078928 SNP asthma-risk allele allows transcription of pyroptotic isoforms. Asthmatic stimuli can activate GSDMB-mediated pyroptosis via immunocyte-GZMA, caspase-1 ("?"; conflicting results) or the dust mite-originated Derp3 protease [18,27,50]. Alternatively, both pyroptotic (isoform-3) and nonpyroptotic (isoform-1) GSDMB variants can enter the nucleus of bronchial cells, where they may indirectly ("?"; mechanism unknown) regulate the transcription of genes involved in airway remodeling [17]. (C) Implication in Inflammatory Bowel Diseases (IBD; Crohn's disease and ulcerative colitis). (1) Contrary to asthma, most single nucleotide polymorphisms (SNP) associated with IBD-risk downregulate total GSDMB levels in leukocytes and/or intestinal cells. Moreover, the rs11078928 SNP impedes transcription of pyroptotic isoforms. (2) The rs2305479 and rs2305480 SNPs produce missense mutations in the C-terminal (CT) of GSDMB that can interfere with proper GZMA cleavage of the pyroptotic isoform-3 [60]. (3) Alternatively, the same missense mutations (at least reported for the 411aa) hamper the intestinal epithelial repair function of GSDMB [19]. (4) In this context, full-length GSDMB localizes to the plasma membrane and activates proliferation and migration while decreasing adhesion via PDGF-AA signaling and FAK-phosphorylation. (5) GSDMB can bind and activate caspase-4 mediated cleavage of GSDMD, leading to GSDMD-dependent noncanonical pyroptosis [20]. D: Dual role of GSDMB isoforms in cancer. Left: antitumor (pyroptotic) function. (1) Immunotherapy (anti-PD1) activates immunocytes that release GZMA and cytokines (TNF- α , INFs, represented by yellow and green circles) (2) which upregulate GSDMB expression [27]. (3) Activated cytotoxic isoforms of GSDMB provoke mitochondrial damage and cause cell lysis. (4) the released molecules fuel (6) antitumor inflammatory response (positive feedback loop) that can synergize with anti-PD-1 immunotherapy. (5) Cleaved noncytotoxic isoforms might reduce the cell death capacity of pyroptotic isoforms [37]. Right: pro-tumor functions. (7) GSDMB overexpression favors diverse pro-tumor activities and affect clinical behavior and disease prognosis. (8) These activities could be tackled with GSDMB-targeted nanotherapies [25] and other approaches. Note: The figure legend for each subpanel specifies the precise isoforms used in the cited studies. However, since the effect of every isoform has not been evaluated in each disease, for simplicity, the pyroptotic isoforms 3 and 4 are represented together (blue NT) and the non-pyroptotic isoforms 1 and 2 (pink NT) are also grouped. Moreover, the isoform 411aa (orange) is represented independently to highlight its intermediate pyroptotic capacity.

Shigella flexneri has developed a virulence mechanism to disseminate infection [8,9]. Specifically, the ubiquitin-ligase IpaH7.8 labels the NT of all GSDMB isoforms and drives their proteasomal degradation [8,9]. Additionally, IpaH7.8 might dampen GSDMB pore-forming activity independently of degradation [35]. Interestingly, one study proposed that GSDMB-NT preferentially binds bacterial over mammalian cell membranes, thus killing intracellular pathogens but not the host cells [8] (Fig. 2A). It should be noted that this study used the 411aa variant, which has reduced pore-forming capacity in liposomes and overall bactericidal activity (compared to full length isoform-3 [35,36]). Indeed, no clear microbicidal effect was observed *in vivo* using a Genetically Engineered Mouse Model (GEMM) expressing 411aa variant upon infection with the murine tropic pathogen STm [8]. Some authors suggest that GSDMB-NT might preferentially bind to lipids enriched in the internal membrane of bacteria (and this might be the reason why they also target the evolutionary-related mitochondria; see "How does GSDMB mediate antibacterial defense?"). Hence, different membrane composition among gram-negative species and mammalian cells could determine GSDMB-NT targeting efficiency. Despite this is a very attractive hypothesis, divergent results on lipid affinity

and liposome leakage were obtained varying lipid composition, like cardiolipin and sulfatides [8,35–37,40], and the GSDMB isoform used. Moreover, GSDMB-NT can kill bacteria from either inside (when expressed in *Escherichia coli*; [36,37]) or outside (incubation with cytotoxic peptides [8,9,35]); thus, further studies are required to define how differential composition between external and internal membranes affects GSDMB isoforms pore-forming efficacy.

Do GSDMB pyroptotic or non-pyroptotic functions cause inflammatory diseases?

Several GWAS studies proved that several Single Nucleotide Polymorphisms (SNPs) regulating GSDMB expression are strongly associated with asthma, allergy, and inflammatory and autoimmune pathologies, but, surprisingly, the risk alleles are constantly opposite for autoimmune diseases and asthma, suggesting contrary immunopathogenic mechanisms for these disorders (revised in [15,16]). Most SNP alleles that upregulate *GSDMB* mRNA in leukocytes cells, and to a lesser extent in bronchial cells, associate with asthma susceptibility, severity, and exacerbations [15,16]. Moreover, rs11078928 asthma-risk allele (T) allows transcription of pyroptotic variants (isoforms-3/4) [18,21], suggesting

that excessive pyroptosis in asthma is pathogenic (Fig. 2B). Accordingly, activation of specific GSDMB isoforms by proteases (see “[How are GSDMB cell death functions regulated by proteases?](#)”) might trigger pyroptosis in bronchial cells in response to asthma stimuli [18,50]. Contrarywise, GSDMB can play non-pyroptotic roles in this disease. Indeed, the knock-in mice ubiquitously expressing isoform-3 (pyroptosis-proficient variant) exhibit an asthmatic phenotype, but instead of increased cell death, GSDMB nuclear localization triggered the upregulation of *TGF- β 1* and *5-lipoxygenase* genes and the subsequent airway remodeling [17] (Fig. 2B). Interestingly, identical genes were transcriptionally regulated by nuclear GSDMB isoform-1 in human bronchial cells [17]. GEMM with nuclear/cytoplasmic isoform-2 expression evidenced other infrequent lung pathologies (atelectasis and emphysema) [41], but asthma was not experimentally induced in this and the other GEMM available (411aa variant [8]). Therefore, it is yet untested if several GSDMB variants share the transcriptional regulation function in asthma and other biological contexts.

Opposing asthma, the SNP risk alleles for IBD generally downregulate total *GSDMB* mRNA in gut/immune cells [15,59] while the rs11078928 risk allele (C) abolishes transcription of exon-6 pyroptotic GSDMB variants thus favoring noncytotoxic isoforms [21]. These data suggest that inefficient pyroptosis may trigger IBD (Fig. 2C). Conversely, increased GSDMB protein (cleaved and uncleaved) was reported in IBD-inflamed tissues compared to nonaffected tissues and healthy individual controls [19,20]. Moreover, there are two common IBD-risk SNPs (rs2305479 and rs2305480) provoking missense variations within GSDMB CT (Fig. 1B) that significantly alter protein conformation and perhaps NT-CT autoinhibition [40] and/or other GSDMB functions. Interestingly, Pizarro's group proved that these SNP-mediated aa changes hamper gut epithelial restitution in IBDs, through pyroptosis-independent mechanisms [19]. Specifically, while wild-type GSDMB mediates effective wound healing (increased proliferation, migration, and reduced adhesion) via PDGF-AA/FAK signaling, these mechanisms are impeded by IBD-associated GSDMB variations (Fig. 2C). They also showed that the immunosuppressor methotrexate upregulates GSDMB expression and cell membrane localization of uncleaved GSDMB in gut epithelium [19]. However, this work used the 411aa, questioning whether this cell death-independent functions also apply for fully pyroptotic variants. Indeed, other authors indicate that the corresponding mutations in the pyroptotic-proficient isoform-3 (Fig. 1B) affects GZMA cleavage efficacy [60] (Fig. 2C), potentially

regulating cell death activity in IBD. Finally, as unexpected twist, Chen *et al.* [20] propose that GSDMB does not itself provoke pyroptosis in IBD but enhances non-canonical GSDMD-pyroptosis through caspase-4 activation (see “[How are GSDMB cell death functions regulated by proteases?](#)” and Fig. 2C).

It should be noted that it is still unclear how the differing cleavage products produced by distinct protease could affect the diverse GSDMB functions in inflammatory diseases.

Can we exploit GSDMB pro-tumor and antitumor (cell death) activities for developing novel anticancer therapies?

GSDMB displays its greatest multitasking capacity in cancer, where it can promote diverse pro-tumor or antitumor functions [14,33] (Fig. 2D). GSDMB mRNA/protein overexpression occurs in different cancer types (stomach, bladder, liver, cervix, and breast) but, unfortunately, in most studies, the precise GSDMB isoform expression was not defined [14,23,24,28–30]. GSDMB upregulation is particularly common (> 60%; frequently due to *GSDMB* gene co-amplification with *ErbB2/HER2/NEU* oncogene) in HER2-positive breast and gastroesophageal tumors and cell lines [24,26], where the endogenous GSDMB (undefined isoforms) and/or the exogenous isoform-3 induces resistance to anti-HER2 therapies via prosurvival autophagy [26]. Moreover, in breast cancer, the overexpression of GSDMB isoforms 1, 2, and 3 boost motility and invasion [23,25], and the isoform 2, which is preferentially upregulated in breast carcinomas [23,34], also enhances tumor growth and metastasis *in vivo* (xenografts [23]) and doubles mammary carcinoma incidence in the HER2/NEU GEMMs [41]. Interestingly, many of the GSDMB pro-tumor functions in HER2 breast carcinomas can be reduced *in vitro/in vivo* with a novel nanotherapy loaded with a specific GSDMB antibody (that recognizes all isoforms) [25], probing the feasibility of therapeutically targeting GSDMB in cancer (Fig. 2D). Furthermore, GSDMB upregulation promotes bladder cancer growth and regulates glycolytic metabolism [28], but in this study the GSDMB isoforms were not clearly specified. Together, these data suggest that GSDMB isoforms could distinctively control multiple pro-tumor functions, but further research is required to prove if these activities are unique of specific tumor types and whether they are controlled by proteases.

Contrasting pro-tumor effects, triggering GSDMB pyroptosis in cancer cells (only those expressing the pyroptotic-activatable variants [27,34,37]) can be an

exciting therapeutic strategy for GSDMB-positive tumors. *In vivo* studies probed that exogenously over-expression of isoform-3 in murine cancer cell xenografts did not affect tumor growth, but the immune stimulation with immunotherapy (anti PD-1) resulted in T-cell/GZMA-dependent tumor suppression [27] (Fig. 2D). Moreover, inflammation further upregulated GSDMB expression in tumors, provoking a positive feedback loop for tumor death (Fig. 2D). These results prompted excitement on the possibility to use GSDMB as a determinant of immunotherapy efficacy. Unfortunately, in these experiments, GSDMB cleavage was not shown *in vivo*; thus, it is uncertain the efficacy of endogenous mouse GZMA and the percentage of pyroptotic cancer cells required for efficient tumor eradication. Likewise, the importance of other cleavage processes, which could counterattack pyroptosis (see “How are GSDMB cell death functions regulated by proteases?”), in tumor progression is uncertain.

Importantly, tumor cells usually express diverse cytotoxic and noncytotoxic variants at the same time [36,37], and noncytotoxic isoforms could interfere with the antitumor effect of cytotoxic ones [37]. Therefore, the balance among isoforms, and their distinct pro-/antitumor functions, may govern the clinical tumor behavior. Accordingly, higher GSDMB isoform-2 mRNA expression associated with poor prognosis in breast [34] and renal clear cell carcinomas [37], while isoform-3 upregulation correlated with favorable outcome in bladder urothelial carcinoma [37]. The dominant GSDMB variant might determine the way to attack GSDMB therapeutically.

Concluding remarks and future challenges

In several human diseases, GSDMB orchestrates critical biological (cell death-dependent and independent) functions that could be differentially controlled by distinct protein isoforms. Consequently, GSDMB variants are promising novel biomarkers of disease risk, aggressiveness, and/or outcome as well as potential therapeutic targets. However, the rational design of clinically effective GSDMB-targeted biomedical applications still faces some important challenges:

First, is necessary a consensus standardization of the nomenclature and a precise functional, biochemical, and structural characterization of all GSDMB isoforms.

It is crucial to unveil the regulation of GSDMB isoforms by proteases (both activators and inhibitors) and the functional and biological effects of the cleaved products in normal and pathological conditions. For this, it would be convenient to resolve the crystal

structure of each GSDMB isoform/protease complex, and to generate *in vitro/in vivo* systems that allow the accurate control of GSDMB isoform expression and protease activation at physiological levels.

Moreover, the precise quantification of the expression and status (cleaved or uncleaved) of GSDMB protein isoforms in healthy and disease tissues/cell types is imperative. Unfortunately, to date, there is no antibody that can distinguish GSDMB variants (for its use in immunohistochemistry or western blot) in biological samples. The mRNA methods used in the literature detect several translated and untranslated transcripts.

Discovering the exact mechanisms controlling GSDMB isoforms expression (at multiple levels: genetic, epigenetic, translational) would allow to experimentally/therapeutically upregulate specific functions in a context-dependent way (e.g., enhance cytotoxic isoforms in tumor cells). Methotrexate and cytokines (TNF α , INFs) are known to enhance GSDMB transcription in multiple cell types, but there are inconclusive results on their specific effect on isoforms.

Since mice lack *GSDMB* gene, there is an urgent need for developing novel GSDMB GEMMs that recapitulate the isoform-specific tissue/cell expression in each disease. If shared among the scientific community, these models could boost translational research to test GSDMB-isoforms-targeted therapies *in vivo*. So far, three GEMMs have been generated (isoforms-2, 3, 411aa) but they were not studied in the same diseases.

Defining the exact intracellular events during GSDMB cell death. For example, to uncover the kinetics of GSDMB-mediated mitochondrial impairment and cell lysis, additional technical approaches are required in which GSDMB-NT release could be controlled and visualized accurately.

To exploit GSDMB antibiotic activity for gram-negative bacteria infection, it is essential first to characterize how GSDMB variants might differentially target internal and external bacteria membranes and host plasmatic or mitochondria membranes.

For asthma and IBD risk assessment and treatment, it is fundamental to clarify the contribution of pyroptotic and nonpyroptotic GSDMB-isoform activities, and their potential modulation by genetic traits (SNPs). If excessive pyroptosis is the cause of disease, developing inhibitors of GSDMB activation would be beneficial.

Finally, to design GSDMB-targeted anticancer treatments, it is mandatory to quantify the ratio of cytotoxic/noncytotoxic isoforms in tumor cells and microenvironment (immune and other normal cells

may express GSDMB). Triggering pyroptosis via immunotherapy might be beneficial in tumors over-expressing cytotoxic variants, but further research is required to define the adequate balance among cancer pyroptosis and local or systemic inflammation in clinical settings to avoid side effects. In tumors over-expressing nonpyroptotic isoforms, blocking GSDMB pro-tumor functions or enhancing the expression of cytotoxic variants would be desirable.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

All authors wrote and revised the manuscript. The main figures were created by DS and the graphical abstract figure by SC.

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