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REVIEW

Structural and functional insights into GSDMB isoforms complex roles in pathogenesis

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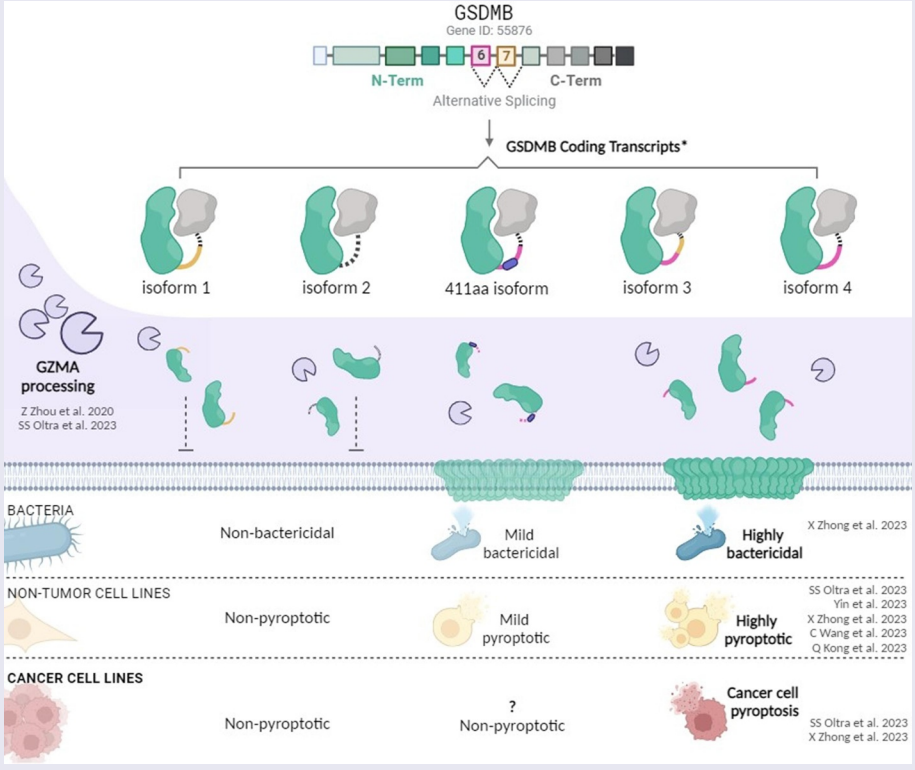
ABSTRACT SHADS

Gasdermins (GSDMs) have garnered significant scientific interest due to their protective and detrimental roles in innate immunity, host defense, inflammation, and cancer alongside with other pathologies. While GSDMs are mostly recognized as key effectors of a lytic type of pro-inflammatory cell death known as pyroptosis, they do also take part in other cell death processes (NETosis, secondary necrosis, or apoptosis) and exhibit cell-death independent functions depending on the cellular context. Among GSDMs, Gasdermin B (GSDMB) pyroptotic capacity has been a subject of conflicting findings in scientific literature even when its processing, and subsequent activation, by Granzyme A (GZMA) was decoded. Nevertheless, recent groundbreaking publications have shed light on the crucial role of alternative splicing in determining the pyroptotic capacity of GSDMB isoforms, which depends on the presence of exon 6-derived elements. This comprehensive review pays attention to the relevant structural differences among recently crystalized GSDMB isoforms. As a novelty, the structural aspects governing GSDMB isoform susceptibility to GZMA-mediated activation have been investigated. By elucidating the complex roles of GSDMB isoforms, this review aims to deepen the understanding of this multifunctional player and its potential implications in disease pathogenesis and therapeutic interventions.



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Introduction

Gasdermin protein Family; pyroptosis executors

The GSDM family comprises six members in humans (GSDMA-E, and pejvakin -PVJK-), each playing crucial roles in the etiopathology of various human diseases. Among them, GSDMB is the most recent evolutive member, being present in certain mammal species but absent in the mouse and rat genomes. *GSDMB*, along with *GSDMC* and *GSDMD*, originated from a duplication event of the ancestral *GSDMA* gene [1]. Nevertheless, the sequence identity between them is less than 30% [1]. Despite the significant variability in their amino acid sequences, all GSDMs, except for PVJK, share a common three-dimensional (3D) structure consisting of N-terminal (NTD) and C-terminal (CTD) domains connected by a linker region, unique to each specific GSDM member.

Although identified in the early 2000s, the GSDM family has experienced a surge of research interest in recent years. A pivotal discovery in 2015 unveiled GSDMD role as the final effector of pyroptosis, an inflammatory form of lytic cell death [2–4] triggered by the formation of biological membrane pores [5,6] (Figure 1a). Hastily, ensuing research confirmed that this lytic function is shared among all GSDM members, except PJVK [7]. This common feature can be attributed to their typical structural motif, which is critical for pyroptosis execution [7,8]. In resting cells, GSDMs adopt an inactive conformation in the cytoplasm, with the NT pore-forming domain auto-inhibited through interactions with specific residues within the inhibitory CTD [9]. Certain stimuli, such as pathogens (e.g. bacteria, viruses), damage associated molecular patterns, or antitumor drugs can trigger a pyroptotic response by activating specific proteases that cleave the linker interdomain region (i.e. Granzyme-A; GZMA cleaves GSDMB at the K244 residue [10]). Upon cleavage, the liberated NTD experiences conformational changes and binds to specific phospholipids (including sulfatides, cardiolipin, and others [11–15]) from the internal plasmatic membrane where it oligomerizes and forms pores that facilitate the secretion of intracellular molecules (i.e. cytokines) and the influx of water and ions, leading to cell swelling and membrane ballooning (Figure 1a). Lastly, the compromised cell membrane collapses, the intracellular content is released, and cell undergoes pyroptosis [5–7,9,16].

Nevertheless, this common mechanism is an oversimplification of the multifaceted functions of GSDMs, since, in addition to plasma membrane pyroptosis, recent studies have proved the capacity of specific GSDMs to interact with diverse cellular organelles (e.g. nucleus, endosomes, autophagosomes or mitochondria) and to orchestrate alternative cell-death pathways (i.e. apoptosis, necroptosis, NETosis) as well as cell-death-independent mechanisms (like "hyperinflammatory cytokine release" or autophagy) governing cellular homeostasis (reviewed in [17]). The precise mechanisms controlling these sublytic GSDM functions in a biological context-dependent manner have started to be elucidated [18] while GSDM pyroptosis has been substantially investigated in different pathological situations (infection, cancer, and inflammation) (reviewed in [19]). For instance, GSDMD-mediated pyroptosis plays a prominent role in multiple infectious or inflammatory diseases whereas GSDME has emerged as a compelling subject of study, due to its anti-tumor properties through its capacity for cancer cell lysis [19]. Meanwhile, the pyroptotic capacity of GSDMB, and its relevance in pathogenesis, has remained uncertain until recently. The discovery that GZMA processing can unleash GSDMB pyroptotic potential under specific conditions [10] marked a turning point in understanding its functional significance. Subsequent investigations have delved into the structural properties of GSDMB, shedding light on the context-dependent nature of its cell death activity.

Accordingly, in this review, we discuss these recent advances in GSDMB research with a focus on the structural mechanisms that control its activation, pore formation, and the biological consequences of GSDMB-induced membrane permeabilization.

GSDMB, from DNA to protein structural properties

Human *GSDMB* gene localizes at the 17q21.1 locus and consists of 11 exons (being exon 1 non-translated). Its expression is regulated by

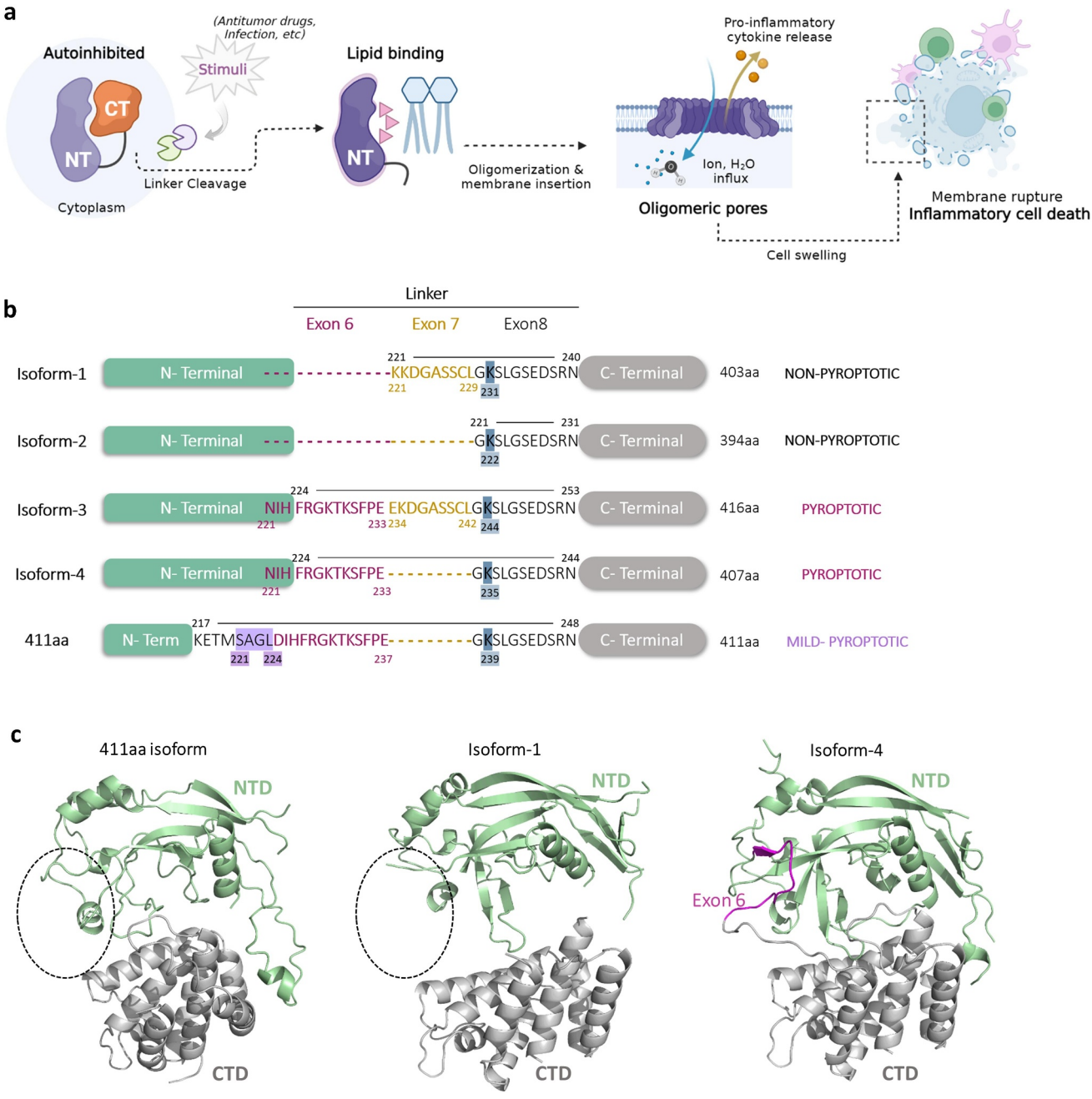


Figure 1. Common model for GSDM-mediated pyroptosis and comparison of GSDMB isoforms. a) main steps in the general mechanism of pyroptotic cell-death mediated by Gasdermins (GSDMs). In basal conditions, GSDMs remain in the cytoplasm in a closed conformation: NTD inhibited by CTD (NTD: amino terminal and CTD: carboxy terminal domains). In response to different stimuli, specific proteases cleave the GSDM interdomain region, releasing the NTD. After structural reorganization, NTDs, now active, form large transmembrane pores, typically consisting of 23–27 monomers, releasing pro-inflammatory molecules (IL-1β/IL-18) and allowing H_20 and ion influx. Osmotic shock causes cellular ballooning and membrane rupture, secreting DAMPs that together with cytokines activate the immune system. b) scheme of aligned amino acid (aa) sequences from GSDMB functional isoforms highlighting the NTD (green), CTD (gray) and the unique linker regions. The differences in residues from exon 6 (magenta) and 7 (ochre) are indicated. Likewise, both the aa insertion from the non-canonical 411aa isoform and the Grazyme A (GZMA) cleavage site (at K244 within exon 8) are highlighted in purple and blue respectively. Each feature in the scheme is accompanied by its corresponding aa number, which varies for each represented isoform. On the right it has been indicated the pyroptotic capacity. c) crystal structures of full-length GSDMB isoforms – 1 (PDB-ID: 8GTK), –4 (PDBid:8GTJ) and 411aa (PDBid:8EFP). NTD (green), CTD (gray) and exon 6 residues (magenta).

two alternative promoters and can generate multiple transcript variants through alternative splicing. The ENSEMBL genomic database reports up to 15 such variants (www.ensembl.org/Homo_sapiens/Gene/Summary?g=ENSG00000073605;r=17:39904595–39919854), some of them derive from out-of-frame splicing events and thus are susceptible to downregulation by nonsense-mediated mRNA decay [20]. Meanwhile, the in-frame splicing variants translate into biologically active isoforms with varying lengths. Significantly, conflicting information regarding the number and nomenclature of GSDMB isoforms can be found through various databases and among the literature. For ease of reference, nomenclature from NCBI will be used in this review and the distinct aliases used within literature [11–13,21] have been compiled in Table 1. According to NCBI, there are five translated variants (isoforms 1–5), being isoform-5 a largely truncated protein and likely non-functional (Table 1). Indeed, most functional studies have focused on isoforms-1 to −4, which share the canonical CTD and the core regions of the NTD sequences although differ in the alternative presence of exons 6 and 7 within the linker region (Figure 1b). Specifically, isoform-1 lacks exon 6, isoform-2 lacks exons 6 and 7, isoform-3 comprises the full-length sequence and exon 7 is missing in the isoform-4 (Table 1, Figure 1b). It is worth noting that UniProt database includes six isoforms (Q8TAX9–1 to −6), from which four correspond to NCBI isoforms −1 to −4 but with different numbering (Table 1). The extra UniProt isoform Q8TAX9–5 lacks the NTD, while Q8TAX9–1, hereinafter referred to as "411aa isoform", closely resembles isoform-4 as it likewise lacks exon 7, but uniquely features a four-residue insertion followed by an asparagine-to-aspartate substitution at the beginning of exon 6 (Table 1, Figure 1b). Remarkably, this largely studied 411aa isoform is not derived from canonical transcripts but maybe from aberrant splicing events of unknown origin. The five functional translated GSDMB variants are cytosolic proteins of around 45-48 kDa.

Structurally, GSDM proteins are highly similar, featuring the cited two-domain (NTD and CTD) architecture with a linker region. This 3D configuration was first demonstrated for GSDMA3 [7], the earliest member to be crystallized, and afterward for human and murine GSDMD [23]. Meanwhile, initial efforts to crystallize GSDMB resulted in a partial representation of the linker and CTD (residues 220 to 411) from the non-canonical 411aa isoform linked to maltose binding protein [15]. Due to the highly cytotoxic nature of GSDMB, more complete protein structures could not be resolved until recently, when the bacterial leucine-rich repeat (LRR) domain of IpaH7.8 ubiquitin ligase was used to stabilize and permit crystallization of diverse GSDMB isoforms. Thus, the 411aa variant/IpaH7.8 complex was resolved in two recent studies by Yin et al., (2023) and Wang et al., (2023) [12,24], using cryo-electron microscopy (Table 2). Almost simultaneously, another study led by Zhong et al., (2023) successfully crystallized GSDMB isoform-1 and −4 again [11] (Table 2). Unfortunately, no crystallized structures have been reported for the full-length isoform-3 nor for the shortest isoform-2 so far.

Aside confirming that GSDMB exhibits the two-domain arrangement similar to GSDMA3 and GSDMD, these studies yield significant novel insights into the structural features of GSDMB (Figure 1c). First, the NTD of GSDMB is characterized by a central ten-stranded antiparallel β-sheet (nine in 411aa isoform, see section 3) tightly packed against five α-helices, while the globular CTD, the smallest among human GSDMs, consists of eight tandem α-helices. Second, the major structural singularity of GSDMB lies in its stronger autoinhibition forces. Besides the β1-β2 hydrophobic interactions, which are the primarily responsible for tethering the NT-domain to the CT-domain in GSDMA3 and GSDMD, GSDMB auto-inhibition is reinforced by polar interactions [11,12,24].

Moreover, the comparison among the crystal structures of GSDMB isoforms also provided some hints on the importance of the sequence variations over the protein structure. Thus, key structural differences occur between isoforms −1 and −4 due to the absence of the exon 6: **i)** the elimination of a minor interdomain interface and the unfolding of α6 due to a re-orientation of the CTD relative to the NTD, **ii)** the lack of relevant structures for pore formation including β10 sheet, and **iii)** a structural disorder of the NTD and the linker interdomain including exon 7. Additional

Table 1. NCBI nomenclature-based organization of reported GSDMB isoforms. the accession numbers for the corresponding mRNA and proteins from different indicated sources are provided. The protein length (aa; amino acids), exon skipping or key differences at sequence level when compared to the canonical isoform-3, the corresponding pyroptotic capacity (if determined) as well as their alternative names among literature (references cited) are indicated.

GSDMB isoforms NCBI	NM accession number (mRNA record)	ENSEMBL Transcript ID	GSDMB isoforms UniProt	aa Length	Skipping Exons/ differences from canonical	Lytic response	Alternative names among literature
Isoform 1	NM_001042471.2/ NM_001369402.2/ NM_001388422.1	ENST00000309481.11	Q8TAX9–3	403	221–234 (Exon 6): missing	NO	GSDMB-1 [22], GSDMB^{iso1} [11], 1–416Δ6 [21], isoform 3 [12], GSDMB1 [13]
Isoform 2	NM_001388423.1/ NM_018530.3	ENST00000394175.6	Q8TAX9–2	394	221–243 (Exon 6&7): missing	NO	GSDMB-2 [22], GSDMB^{iso2} [11], 1–416Δ6,7 [21], GSDMB2 [13]
Isoform 3	NM_001165958.2/ NM_001388420.1	ENST00000418519.6	Q8TAX9–4	416	Full- length	HIGH	GSDMB^{iso3} [11], 1–416 [21] Isoform 4 [12], GSDMB3 [13]
Isoform 4	NM_001165959.2/ NM_001388421.1	ENST00000520542.5	Q8TAX9–6	407	234–242 (Exon 7): missing	HIGH	GSDMB^{iso4} [11], 1–416Δ7 [21], Isoform 6 [12], GSDMB4 [13]
Isoform 5	NM_001388424.1	-	-	312	313–416: missing	?	
-	-	-	Q8TAX9–5	163	1–253: missing	?	
-	-	.	Q8TAX9–1	411	221–221: N SAGLD 234–242 (Exon 7): Missing	SUBLYTIC*	GSDMB^{isoU}/isofomrU [11] Isoform 1 [12], GSDMB5 [13]
-		-	-	237		?	GSDMB6 [13] F [20]

*The pyroptotic potential of the "411aa-isoform" is relatively low in both bacteria and HEK293T cells.

Table 2. Structure of GSDMB isoforms. The crystallization status, the accession number from protein data Bank (PDB), from cited references have been indicated. NC: non-crystalized.

Isoform	3D structure	PBDid	Structural Determination
Isoform-1	Crystalized	8GTK	X-ray crystallography [11]
Isoform-2	Predicted		NC
Isoform-3	Predicted		NC
Isoform-4	Crystallized	8GTJ	X-ray crystallography [11]
411aa isoform	Crystallized, Cryo-EM	7WJQ, 8EFP (CT domain: 5TIB, 5TJ2, 5TJ4)	X-ray crystallography for CT domain (92–411 aa) [15] X-ray crystallography [24] Cryo-electron microscopy [12]

structural studies on the non-canonical 411aa isoform [12,24] have revealed heterogeneity in the linker region between the NTD and the CTD (Figure 1c). This variability can be attributed to the different structure determination techniques employed (X-ray crystallography versus Cryo-EM respectively, Table 2), as well as to the impact of the extra sequence present in the 411aa isoform. Specifically, the four-amino-acid insertion replaces certain residues involved in the formation of β10, which may introduce local disorder disrupting the correct folding of this β-sheet and preventing the stability of the NT domain beyond K217 (thus not appearing in the crystal structure, Figure 1c).

As above mentioned, the crystal structures of both isoform-2 and, importantly, the full-length isoform-3 are yet to be elucidated. Therefore, further investigations are needed to fully clarify the structural implications of these sequence differences and their functional consequences in GSDMB biology.

Alternatively spliced exons determine GSDMB-NTD pyroptotic capacity

The emergence of alternative splicing-derived isoforms of a gene is a highly regulated process that endows the cell with distinct functional capabilities. Still, previous studies had primarily focused

on one single GSDMB isoform to determine its pore-forming function, neglecting to adequately consider the potential impact of distinct splice variants, frequently even the full-length isoform. This has sparked controversy regarding the role of GSDMB in cell death and its association with human pathophysiology [16,25]. Thereby, whereas certain studies have supported a GSDMB-proficient pyroptotic activity [10] or a partial functionality restricted to disrupting bacterial-derived membranes as a host defense mechanism [14]; others have denied GSDMB-NTD pyroptotic capacity [14,26] or instead assigned GSDMB pyroptotic-independent roles such as promoting proliferation and migration during epithelial restitution [27], transcriptional regulation [27], cancer invasion, metastasis, or tumor growth/incidence [22,28–31] or inducing pro-survival autophagy in response to specific antitumor drugs [32].

The answer to whether GSDMB is a pore-forming protein has come to fruition through seminal investigations published in 2023, finally clarifying the functional diversity among the distinct GSDMB isoforms. Notably, the study conducted by Oltra SS et al. 2023 [21] revealed for the first time the differential role of GSDMB isoforms in cancer cell death and clinical behavior. This report and almost coeval studies demonstrate that only the released NTDs of isoforms-3/-4, which contain exon 6, exhibit pore-forming activity in liposomes [11,12], gram-negative bacterial membranes [11], nonmalignant HEK293T cells [11–13,21] and cancer cell lines [11,21]. Furthermore, the GSDMB cytotoxic NTDs were shown to cause a dual impact by targeting both cellular and mitochondrial membranes [21] akin to the behavior of NTDs from other GSDMs [2,33–35].

Several studies have consistently implied that residues from exon 6, along with exon 7, are structural contributors of the linker region [11–13,21] but still, the precise residues contributing to the interdomain linker were not steadily defined. However, it has been precisely thanks to the recent structural discoveries from these studies that we can now finally clarify this matter. Here we reveal that the first three residues within exon 6, along with M220 from exon 5 ($_{220}$MNIH$_{223}$) display a beta sheet secondary structure (β9 region in the NTD, β10 in the autoinhibited structure); and therefore, must be considered part of the *bona fide* NTD (Figure 1b). The rest of the exon 6 amino acid residues, from F224 to E233, although essential in the cytotoxic NTD, do exhibit structural disorder thus forming part of the flexible linker region. This observation points out that the structural NTD, with secondary structure, is indeed shorter than the NT cytotoxic region.

Functionally, the GSDMB-pore requires the interaction of NT adjacent monomers through key residues. In this sense, the hydrophobic residues I222 and F224 from this β9 were predicted to create a concave pocket that enables the insertion of F144 within the cytosolic globular domain of the adjacent subunit in the process of GSDMB-NTD pore assembly [11]. Indeed, *in vitro* analysis demonstrated that the disruption of I222-mediated interactions significantly reduces the pore-forming activity in liposomes and, consequently, the pyroptotic capacity of isoform-4 NTD [11]. Similarly, mutation on the first four residues from exon 6 (N221G/I222G/H223G/F224G) has been associated with a decreased pore-forming activity of isoform-3 NTD [12]. These results confirm the essential role of exon 6 for the GSDMB-NTD mediated cytotoxicity and suggest a conserved oligomerization mechanism among the GSDM family members, evidenced by the analogue localization of the first few residues of the interdomain linker in human and mouse GSDMD (V229–F232 and I230-V233, respectively) in the center of the oligomerization interface I [23].

Additionally, recent structural investigations suggest that the linker region coded by exon 6 regulates the membrane-permeabilizing activity of GSDMB-NTD [11–13,21]. Atomic modeling of NTD pores from isoform-3 and −4, but neither from isoform-1 nor probably isoform-2, has revealed a cluster of basic residues within the liker disordered region of exon 6 (R225, K227, and K229); which are likely oriented with their positively charged side chains toward the membrane [12,13]. This cluster could form a fourth lipid-binding site, accompanying the three conserved ones that GSDMB shares with other GSDMs [12]. Remarkably, these interactions, based on salt bridges in a lipid environment, provide high stability to the subunit interaction surface, which joins the continuity of the β-sheets to

form the inner flat surface of the pore [21]. Contrary to these results, investigations by Kong et al. (2023) propose that this linker region may be uninvolved in lipid binding or homo-oligomerization but rather be participating in the pore insertion step during pore formation [13]. Although their precise function awaits further clarification, it is evident that this cluster of residues accounts for the essential role of exon 6, as their substitution by alanine or glutamic acid – but not the doble mutant K225A/K227A – significantly compromises the ability of GSDMB-NTD to permeabilize the membrane [11,12,21].

The crucial role of exon 6 in governing GSDMB-NTD membrane insertion/oligomerization/pore insertion processes supports the pyroptotic-null function of exon 6-skipping variants of the protein. Notably, NTD from isoform-1/-2, characterized by the lack of important structures for pore formation including the β9 sheet and a key lipid-binding surface, completely lose their ability to permeabilize the membrane [11–13,21]. Additionally, in the 411aa variant, the substitution of the N220 acidic residue by a four-amino-acid insertion $_{222}$AGLD$_{225}$ results in the replacement of the canonical basic residue K225 by a negatively charged D225 and the loss of secondary structure beyond L217 (Figure 1b), potentially weakening membrane attachment. This diminished membrane permeabilization potential may be insufficient to pass membrane repair mechanisms while still being effective in killing bacteria lacking membrane repair machinery according to Wang et al. (2023) [12].

GZMA, activator of GSDMB cell death functions

The physiological activation of GSDM pore-forming activity is a highly regulated process that necessarily implies the proteolytic cleavage and subsequent release of the cytotoxic NTD. Current literature refers to diverse cysteine and serine proteases, like inflammatory caspases, apoptotic caspases, cathepsins, neutrophil elastase (ELANE), and granzymes as specific regulators of exclusive GSDM members [36].

Likewise, the cleavage of GSDMB by specific peptidases could differentially regulate its cell death activity. In this case, while the functional effect of caspases is controversial, many studies conclude that caspases 1/3/4/6/7/8 and 9 cleave GSDMB at the $_{88}$DNVD$_{91}$ motif, common to all GSDMB isoforms [14,15,26]. Interestingly, caspase cleavage produces a pyroptotic-deficient GSDMB-NTD [21,26] equal to the one resulting from the processing of GSDMD at D92 by apoptotic caspase-3 [37]. Thus, activation of most caspases may act as an inhibitory control mechanism for GSDMB pyroptosis [21]. Besides caspases, ELANE also exhibits proteolytic activity toward both GSDMB and GSDMD, albeit yielding distinct outcomes [21]. While ELANE processing produces a GSDMD-NTD that actively forms pores in neutrophils [38], in the case of GSDMB the cleavage occurs at the last exon 5 residue (M220), common to all isoforms, and subsequently results in the formation of an exon 6-excluding NTD that lacks pyroptotic capacity [21].

To date, Granzyme A (GZMA) and Der p3 allergen are the only known proteases identified as capable of generating pyroptotic GSDMB-NTDs [10,13,14,21,39], being the K244 residue the primary physiological cleavage site for both proteolytic enzymes [10,21]. GZMA cleavage has been demonstrated through *in vitro* approaches (incubation of purified GZMA and GSDMB proteins [10]; or electroporation of GZMA into GSDMB-overexpressing HEK293T cells [10]) and cellular co-culture experiments that model immunocyte attack on cancer cells [10,13,21]. In these experiments, natural killer (NK) cells and cytotoxic T lymphocytes effectively killed GSDMB-expressing cells (either HEK293T or cancer cell lines) through perforin-GZMA-mediated cleavage of GSDMB at K244 [10,13,21]. Additionally, GZMA can also process GSDMB at K229 (a residue exclusively present on isoforms-3/-4) but only under *in vitro* reactions with purified proteins. This cleavage does not occur during NK cell killing experiments, and therefore, K299 is considered a minor and non-physiological cleavage site [10]. Notably, since the K244 residue locates within GSDMB exon 8, GZMA can cleave the four GSDMB splicing isoforms [10,13,21]. However, only the NTDs released from exon 6-including variants (−3/-4) can exert pyroptotic activity upon GZMA activation [13,21]. Furthermore, there appears to be a higher efficiency in GZMA cleavage of isoform-1/-

3, which share the presence of exon 7 [13,21], suggesting a potential role of exon 7 residues in facilitating GZMA cleavage.

Novel structural insights into GZMA-GSDMB interaction

Despite the key functional role of GZMA in activating GSDMB, the absence of a crystallized structure of the full-length isoform-3 adds further complexity to the study of the mechanistic details of GSDMB/GZMA functional interaction. Addressing this challenge for the first time, we have used computer simulation techniques at the atomic level. A model of isoform-3 has been constructed based on the crystal structure of isoform-1 [11], with a focus on the spatial positioning of K244 (Figure 2a, B). Classical homology modeling and molecular dynamics (MD) simulations (100ns) were utilized to generate and stabilize the structure, following established protocols [21,40]. The initial interaction between GSDMB and GZMA was generated using the HADDOCK method [41,42] employing the crystallized structure of GZMA (PDBid: 1ORF) [43]. The resulting model after MD trajectory highlights the interface between both proteins, with exons 6 and 7 playing a crucial role, (Figure 2c) and it localizes the positively charged K244 within the negatively charged active center of GZMA, specifically interacting with D206 GZMA residue (Figure 2d).

To examine how the distinct GSDMB isoforms interact with GZMA and the dynamics of proteolytic cleavage, a model for each isoform was subjected to docking simulations with GZMA, followed by MD (100ns) to inspect the stability of the assembly. Analysis of the root-mean-square-deviation (rmsd) values from the trajectories revealed that the absence of exon 6 in isoform-1, exon 7 in isoform-4, or both exons in isoform-2 led to decreased stability in the interaction with GZMA compared to the trajectory of full-length isoform-3 (Figure 2e). Furthermore, the interaction energy values of GZMA coupled with each of the five GSDMB isoforms (isoform-1 to −4 and the non-canonical 411aa isoform) models after MD simulations (calculated using the PRODIGY server [44,45]), showed a marked difference in ΔG and Kd values for isoform-3 compared to the other four isoforms, indicating its greater stability (Figure 2f) and perhaps, therefore, improved cleavage kinetics.

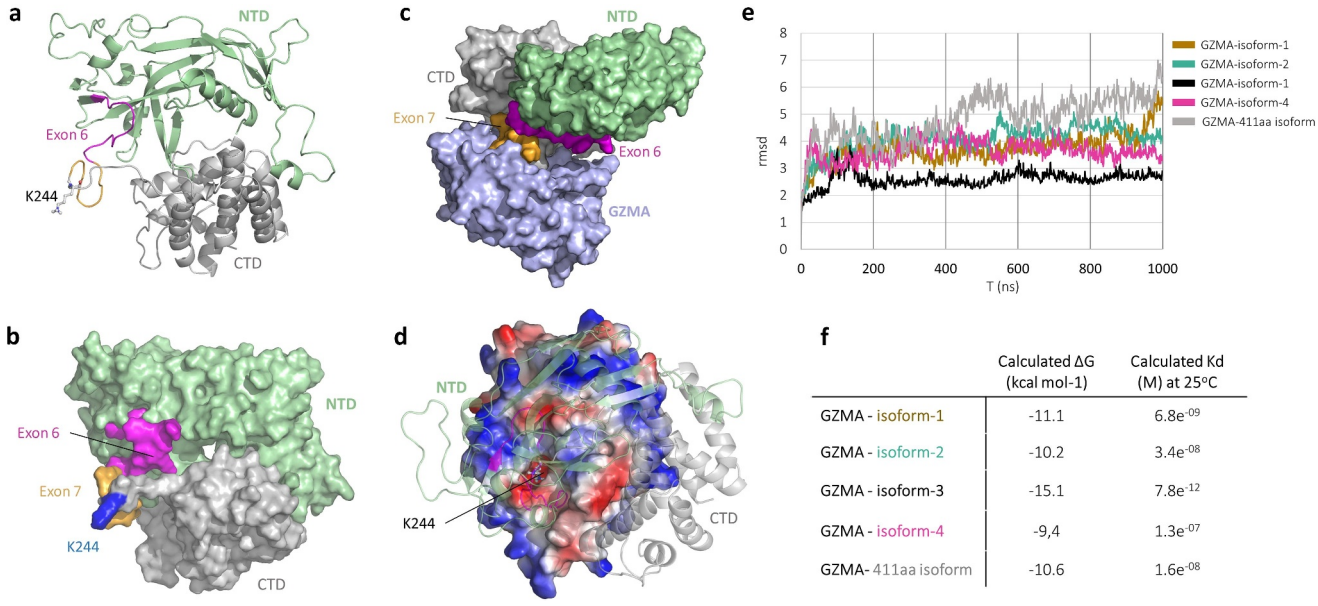
Overall, these novel structural findings, together with previous data from Kong et al. (2023) investigations [13], point out that despite both isoforms −3/-4 being capable of mediating lymphocyte-mediated pyroptosis, isoform-4 exhibits a greater resistance to GZMA cleavage. Collectively, these observations suggest a potential molecular mechanism underlaying the distinct susceptibility of these isoforms to GZMA-mediated proteolysis.

Multifaceted roles of GSDMB in pathology

It is believed that the GSDMB primigenial function is the pore-forming bactericidal activity against species-specific intracellular pathogens, like *Shigella*. Since mice are naturally resistant to Shigellosis, they lack *GSDMB* gene, unlike other mammals [14,24]. Nonetheless, in the knock-in (KI) mouse models expressing human GSDMB, this protein still retains similar biological functions than those observed in human pathologies, implying that GEMMs are relevant to study human diseases (see comments below).

In humans, GSDMB displays a widespread expression pattern in various tissues, prominently in the digestive tract epithelia, liver, lung, colon, and immune cells [46], alongside its presence in diverse cancer types [16]. Functionally, GSDMB exerts both cell death-dependent and independent effects, and its multifunctionality may be governed by different mechanisms including the expression of distinct transcriptional variants with varied biological activities. Perturbations in the regulation of these isoform-specific functions may lead to the development of pathological conditions.

Based on several Genome-Wide Association Studies (GWAS), multiple single nucleotide polymorphisms (SNPs) within or near the GSDMB gene have been associated with predisposition to numerous inflammatory disorders (e.g. allergy, asthma, inflammatory bowel diseases, type-1 diabetes, multiple sclerosis, or rheumatoid arthritis; reviewed in [47,48]; and Table 3). Some of these SNPs are relatively frequent (>34%), and consistently



	Calculated ΔG (kcal mol-1)	Calculated Kd (M) at 25°C
GZMA - isoform-1	-11.1	$6.8e^{-09}$
GZMA - isoform-2	-10.2	$3.4e^{-08}$
GZMA - isoform-3	-15.1	$7.8e^{-12}$
GZMA - isoform-4	-9,4	$1.3e^{-07}$
GZMA- 411aa isoform	-10.6	$1.6e^{-08}$

Figure 2. GSDMB isoform-3 structure in complex with GZMA. Model of the general structure of GSDMB isoform-3 based on 8GKT crystal (isoform-1) [11], depicted in two forms: a) ribbon diagram showing structural domains and b) protein surface representation. NTD in green, CTD in gray, with exons 6 and 7 dyed in magenta and ochre, respectively. Position of GZMA cleavage residue K244 is indicated. c) model for the interaction between GSDMB and GZMA refined through 100ns of molecular dynamics (MD) simulations and a d) detailed view of the interface between modeled GSDMB isoform-3 in complex with GZMA. GZMA surface is colored according to electrostatic potentials, with the negatively charged pocket (red) surrounding the GSDMB residue K244. e) stability analysis in terms of root-mean-square deviation (rmsd in angstroms -Å-) of the interaction between GZMA and the distinct GSDMB variants during 100ns of MD simulation. f) Contact-based prediction of binding affinity in GSDMB-GZMA complexes for the diverse GSDMB isoforms. In protein complex formation, lower calculated free energy (ΔG, kcal mol-1) and dissociation constant (kd, (M) at 25°C) correspond to higher protein affinity.

associated with disease risk among different studied populations (Table 3). Despite the strong genetic association, the functional impact of most SNPs on GSDMB activity are still unknown, being their effect on total GSDMB levels sometimes different among distinct tissues (leukocytes vs epithelial cells) [47,48]. Most studied SNPs are those provoking missense changes within GSDMB proteins (Table 3): rs2305479 that causes a G304R change (isoform-3 sequence), and rs2305480 changing P311 residue to Serine. Moreover, the rs11078928 (within the intron 5 acceptor splice site) could be very important for GSDMB functions, as it significantly modifies the balance among exon-6-containing (pyroptotic) and exon-6-null (non-pyroptotic) GSDMB transcripts in human disease samples and cellular models [49,50]. Among inflammatory disorders, most functional data come from studies on asthma and IBDs, and thus will be briefly commented here.

GSDMB upregulation (correlated with several SNPs) in airway epithelial cells or leukocytes appears as a risk factor for childhood-onset of asthma as well as for the severity and exacerbations of the disease [50–52]. Moreover, the rs11078928-G allele, that favors the transcription of non-pyroptotic GSDMB isoforms −1/-2, decreases asthma risk while the T-allele (that enables the transcription of pyroptotic variants −3/-4) has the opposite effect on disease susceptibility. This data suggest that increased proinflammatory cell death may be responsible for asthma pathogenesis [46,49], but, strikingly, other evidence points to cell-death-independent GSDMB functions in this disease. Indeed, the KI mice ubiquitously expressing GSDMB isoform-3 presented an asthmatic phenotype [52], that was mediated by GSDMB nuclear localization and transcriptional regulation of genes related to airway remodeling and hyperresponsiveness. Interestingly, the same functional mechanism was observed in human bronchial cells expressing the pyroptosis-deficient isoform-1 [52].

Interestingly, several GSDMB SNP alleles that predispose to asthma in turn associate with decreased risk of IBDs, implying opposite

Table 3. Selection of common GSDMB intragenic SNPs and their associations with human diseases.

SNP (LOCALIZATION)#	ALLELES (VAF*)	EFFECT ON GSDMB EXPRESSION/FUNCTION	DISEASE ASSOCIATION
rs11078927 (17:39908152; intronic)	C > T (0.28)	C allele associates with higher GSDMB mRNA expression.	C allele increases asthma risk.
rs11078928 (Chr 17:39908216; acceptor splice site of intron 5)	A > G (0.29)	G allele associates with lower overall expression. Moreover, it reduces transcripts with exon 6 (isoforms GSDMB3/4) but increases variants with truncated exon 6 (13-nucleotides-shorter).	G allele increases risk of IBD, MS, T1D & COPD. A allele increases asthma risk.
rs12450091 (17:39912368; exon 3)	*T* > C (0.029)	C allele: Protein missense variation E122G	C allele raises asthma risk
rs2290400 (17:39909987; intronic)	*T* > C (0.42)	T allele associates with higher GSDMB mRNA expression.	T allele raises asthma and ALL risk. C Allele increases T1D risk.
rs2305479 (17:39905964; exon 10)	C > T (0.31)	T allele associates with lower GSDMB mRNA expression. Protein missense variation G304R	T allele increases IBD risk but decreases asthma susceptibility. G299R change (GSDMB 411aa isoform) dampens the intestinal epithelial repair function of GSDMB**
rs2305480 (17:39905943; Exon 10)	G > A (0.29)	A allele associates with lower GSDMB mRNA expression. Protein missense variation P311S	A allele increases IBD & RA risk but decreases asthma susceptibility. P306S change (GSDMB 411aa isoform) dampens the intestinal epithelial repair function of GSDMB**
rs4795399 (17:39905186; intronic)	T>A>C (0.29)	T allele associates with higher GSDMB mRNA expression.	T allele raises asthma susceptibility.
rs4795400 (17:39910767; intronic)	C > T (0.30)	C allele associates with higher GSDMB mRNA expression.	C allele increases allergy (asthma, hay fever and/or eczema) risk.
rs7216389 (17:39913696; intronic)	*T* > C (0.33)	T allele associates with higher GSDMB mRNA expression.	T allele raises asthma susceptibility.
rs921650 (17:39912823; intronic)	A > G (0.33)	A allele associates with higher GSDMB mRNA expression.	A allele raises allergy susceptibility.

#According to ENSEMBL database and EMBL-EBI GWAS catalog.
*VAF: Variant allelic frequency.
**Effect of the combination of G299R and P306S.
IBD, Inflammatory Bowel Diseases, MS, Multiple Sclerosis; T1D, Type-1 Diabetes; COPD, chronic obstructive pulmonary disease; ALL, acute lymphoblastic leukemia; RA, rheumatoid arthritis.

immunopathogenic processes between these disorders [47–49]. Sadly, the precise GSDMB activities underlying IBD etiology remain unclear. In this sense, the consequences of the GSDMB missense variations provoked by IBD risk-associated SNPs rs2305479 and rs2305480 is debatable, as one study suggested an effect on GSDMB structure flexibility and likely lipid binding affinity [15] and a recent report proved a more complex and pyroptotic-independent function [27]. In the latter study, GSDMB translocation to the plasma membrane resulted in the upregulation of intestinal epithelial cell proliferation and migration, and these effects were dampened by SNP-mediated GSDMB missense mutations [27]. Unfortunately, in these two studies the non-canonical 411aa isoform (mildly pyroptotic) was used, and thus the role of cytotoxic isoforms in Crohn's disease and ulcerative colitis is yet to be clarified.

Pyroptosis plays an important role in cancer progression by inducing cancer cell death and anti-tumor immune responses. However, in contrast to other members of the family – specifically GSDMA or E – with clearer antitumoral roles [16], GSDMB exhibits multifaceted effects on cancer progression. It is frequently overexpressed in various tumor types, including gastric, hepatic, lung, bladder, uterine-cervix, and breast cancers [16]; and notably its overexpression has been detected in >60% of HER2+ breast and gastric carcinomas, correlating with poor clinical outcomes [31,32]. The biological effects on cancer of individual

GSDMB isoforms has been evaluated in very few papers. Specifically, isoform-2 upregulation, but not isoform-1, triggers *in vivo* breast cancer tumor growth and metastasis [22] and it is the only isoform associated with unfavorable clinicopathological and prognostic features in human breast cancer cohorts [21]. Furthermore, in HER2 breast cancer GEMMs, the expression of human GSDMB isoform-2 doubled mammary carcinoma incidence [29]. Importantly, none of these experimental models showed cell death induction by isoform-2 (nor isoform −1). Similarly, the pyroptotic proficient isoform-3 boosts cell migration and invasion *in vitro* as well as tumor growth and metastatic dissemination *in vivo* [28], along with reduced sensitivity to anti-HER2 therapy through pro-survival autophagy induction [28,32]. Altogether, these data suggest that GSDMB could promote multiple pro-tumor activities under certain biological contexts [22,28,29,31,32]. Nonetheless, on the flip side, the cleavage of GSDMB pyroptotic isoform-3 by lymphocyte-derived GZMA unleashes its pore-forming activity promoting tumor clearance and antitumor immunity [10,13,21]. Despite these findings hold promise for targeted immunotherapy, it is important to note that tumor cells often co-express both pyroptosis-competent and incompetent isoforms [11]. In fact, the presence of non-pyroptotic variants is frequently observed in cancer cell lines [11,13] and tumors [21], potentially interfering with cytotoxic GSDMBs [13]. Summarizing, the multiple GSDMB isoform expression in different tumoral contexts adds complexity to the therapeutic implications and warrant further investigation.

Concluding remarks

Recent groundbreaking studies have shed light on the molecular mechanisms that underlie GSDMB pyroptotic capacity. Notably, exon 6 skipping by alternative splicing has been identified as a critical factor in shaping the lytic efficacy of proteolyzed GSDMB. In this study, we have integrated freshly available crystallographic data – detailing the NTD pores across diverse GSDMB isoforms [11–13] – alongside novel results from advanced MD simulations [21] that highlight the key role of exon 6-derived structures in the pore-forming steps, including lipid binding, oligomerization, and pore insertion. In summary, the cogency of these findings not only elucidates the mechanisms behind GSDMB pyroptotic capacity but also validates earlier studies that questioned its cytotoxicity by overlooking GSDMB lytic potential due to their focus on the non-canonical spliced 411aa isoform [14,27].

Beyond their role in dictating the lytic function, alternative splicing events may be also orchestrating GSDMB susceptibility to proteolytic cleavage by GZMA [13]. Remarkably, MD simulations performed in this study have revealed that the interaction of GZMA with the full-length GSDMB protein (isoform-3) is more stable than shorter GSDMB isoforms. This heightened stability suggests that not only exon 6 but also exon 7 within GSDMB linker region may be decisive in the interaction interface of the GZMA-GSDMB complex preceding the proteolytic cleavage. This interplay between alternative splicing and proteolytic processing underscores the multifaceted nature of GSDMB functional diversity, shedding light on a previously uncharted layer of its regulation.

Aside GZMA regulation, deciphering in detail other mechanisms that control distinct (pyroptotic and non-pyroptotic) functions in each GSDMB variant, is fundamental to understand the complex GSDMB roles in the genetic predisposition, initiation, progression, and therapeutic response of inflammatory diseases and cancer. To this aim, since mice genome lack *GSDMB* gene, there is an urgent need for developing novel GSDMB isoform-specific knock-in mouse models that precisely mimic human diseases and could be used for the translational evaluation of novel GSDMB-targeted therapies.

In tumors, the potential of GSDMB-mediated pyroptosis to turn “cold” into “hot” tumors arises as a promising opportunity for cancer therapy. In fact, recent advancements using GSDMB-NTD mRNA-containing nanoparticles have shown encouraging antitumor immunity, sensitizing tumors in an anti-PD-1-resistant 4T1 breast cancer mouse model [53]. However, triggering pyroptosis via immunotherapy might be beneficial only in tumors over-expressing cytotoxic variants. Indeed, tumors are prone to up-regulate

noncytotoxic variants while down-regulate pro-death GSDMB isoforms-3 and −4 to avoid pyroptosis, and the potential co-expression of both lytic and sub-lytic isoforms has been suggested to dampen pyroptotic-proficient cytotoxic effect [11,13,21]. Moreover, under specific tumoral contexts, GSDMB isoforms promote cell-death independent activities with pro-tumorigenic outcomes [16,22,28,29,31,32]. In summary, unraveling the precise mechanisms regulating GSDMB isoform-specific expression and accurately quantifying the cytotoxic/non-cytotoxic isoform ratio in tumors is essential for near-future therapeutic perspectives.

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Authors' contributions

All authors wrote and revised the manuscript. DRP and PGP have carried out the structural and molecular dynamics techniques. SC and PGP made the figures.

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