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Review Article

The multifaceted roles of gasdermins in cancer biology and oncologic therapies

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ABSTRACT

The involvement of the Gasdermin (GSDM) protein family in cancer and other pathologies is one of the hottest topics in biomedical research. There are six GSDMs in humans (*GSDMA, B, C, D, GSDME/DFNA5* and *PJVK/DFNB59*) and, except PJVK, they can trigger cell death mostly by pyroptosis (a form of lytic and pro-inflammatory cell death) but also other mechanisms. The exact role of GSDMs in cancer is intricate, since depending on the biological context, these proteins have diverse cell-death dependent and independent functions, exhibit either pro-tumor or anti-tumor functions, and promote either sensitization or resistance to oncologic treatments. In this review we provide a comprehensive overview on the multifaceted roles of the GSDMs in cancer, and we critically discuss the possibilities of exploiting GSDM functions as determinants of anti-cancer treatment and as novel therapeutic targets, with special emphasis on innovative GSDM-directed nano-therapies. Finally, we discuss the issues to be resolved before GSDM-mediated oncologic therapies became a reality at the clinical level.

1. The gasdermin gene family

The gasdermin family (*GSDM*) comprises six genes in humans (*GSDMA, B, C, D, GSDME/DFNA5*, and *PJVK/DFNB59*) and ten in mice (*Gsdma1–3, Gsdmc1–4, Gsdmd, Gsdme*, and *Dfnb59*) [1–3]. *GSDME* (A.K.A *DFNA5*, deafness autosomal dominant 5) is the most ancient *GSDM* gene, being present in some invertebrates like corals [2,4], while *GSDMB* appears only in specific mammalian species, being the only *GSDM* member not present in the mouse and rat genomes [2]. *GSDM* usually cluster in specific chromosomal regions (17q21 contains *GSDMA* and *GSDMB*; 8q24 *GSDMC* and *GSDMD*; Table 1) indicating that gene duplications occurred during vertebrate evolution [2,4]. In fact, *GSDMB* likely originated from a local duplication and DNA-strand inversion of *GSDMA* in some mammals [2,4]. The name Gasdermin originates from the "Gastric and dermal" expression of *GSDMA*, the first identified *GSDM* gene [5]. *GSDMs* are generally expressed in the digestive tract, where they show specific expression patterns: in the gastro-esophageal epithelium *GSDMA* is mostly detected in differentiated cells, *GSDMB* in the basal layers, *GSDMC* in the suprabasal and differentiated regions and *GSDMD* in the differentiating cells [1,3]. In addition, *GSDMs* show specific expression patterns in multiple tissues/organs. For example, *GSDMA* is expressed in skin, lung, mammary glands; *GSDMB* in liver, lung, colon and immune cells; *GSDMC* in skin and spleen; *GSDMD* in the intestine and leukocytes; *GSDME* in reproductive organs and nervous system and *PJVK* in testes and the auditory nervous system, among others [1–4,6,7].

2. GSDM at the crossroads between pyroptosis and other cell death mechanisms

GSDMs are cytoplasmic proteins (around 50 KDa) with nine

Abbreviations: CT, C-terminal domain; DAMPs, damaged-associated molecular patterns; DCT, Decitabine; GZM, Granzyme; KO, Knock-out; LDH, Lactate dehydrogenase; LPS, lipopolysaccharide; NLRP3, NOD-like receptor protein 3; NC, Nanocapsules; NT, N-terminal domain; NP, Nanoparticles; PLK1, Polo like kinase 1; ROS, reactive oxygen species; SCC, Squamous Cell Carcinoma; TGF-β, transforming growth factor-β; TNF, Tumor necrosis factor; TSG, Tumor suppressor gene; WB, Western blot; WT, Wild-type.

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conserved motifs (containing leucine-rich regions) of still unknown function, distributed throughout the protein [3]. GSDMs, with the exception of PJVK, share a moderately conserved N-terminal (NT) and C-terminal (CT) domains that are interconnected by a central hinge region, which is specific for each family member [2]. For many years, GSDMs functions have been unclear until the discovery, in 2015, that GSDMD trigger inflammasome-dependent lytic cell death [8–10] by forming membrane pores [11–15]. Further studies revealed that other GSDMs exhibit a similar protein 3D structure [15–18] and share the pro-cell death function that is activated by a common mechanism [15,19,20]. The common model shows that cytosolic GSDMs have an inactive conformation in which the NT pore-forming domain is auto-inhibited by binding to specific residues in the hydrophobic core of the CT [11–15,19,20]. This folded conformation is maintained by the linker interdomain region, which is cleaved by specific proteases activated after particular stimuli (Fig. 1A). The released GSDM NT binds to specific acid lipids (*e.g.*, phosphoinositides and phosphatidylserine) and inserts into the cell membrane internal layer, oligomerizing and forming large transmembrane pores [11–15,19,20] (Fig. 1B). The upstream pathways leading to GSDM NT release are specific of each GSDM [7,19,20]. Regarding GSDMD, multiple pathogen signals (*e.g.*, LPS, flagellin) or cell damage molecular patterns "DAMPs" (*e.g.*, reactive oxygen species "ROS", dsDNA) activate the multiprotein complex inflammasome, which then provokes the release of GSDMD NT domain by the pro-inflammatory caspases1/4/5/11 [7,19,20]. GSDMD NT pores facilitate the secretion of mature inflammatory cytokines (IL1β, IL18) and other intracellular molecules (*i.e.*, Lactate dehydrogenase; LDH), while the influx of water and ions might be responsible for cell swelling [7,19,20]. Later, the activation and oligomerization of Ninjurin-1 (NINJ1) produces the extensive membrane rupture and cell lysis (Fig. 1B), which results in the release of diverse intracellular content, including the pro-inflammatory DAMP HMGB1 [21]). This inflammasome/GSDMD-mediated mechanism is termed pyroptosis ("fiery death") and can be divided into "canonical" (caspase 1) or "non-canonical" (caspase 4/5/11) depending on the stimuli and caspases involved [7,19,20]. Pyroptosis occurs mostly in leukocytes and epithelial cells and provokes an inflammatory reaction of the surrounding cells [7,19,20]. Pyroptosis is mechanistically and biologically different to other programmed cell death types, such as apoptosis, necroptosis or ferroptosis, and dying cells exhibit distinct morphologic features: extensive cell swelling and membrane ballooning in absence of cell detachment, intact nucleus with chromatic condensation, among others [22]. Except for PJVK, the NT of other GSDMs can produce lytic cell death [15,19,20] with the release of LDH and other molecules. Therefore, pyroptosis was renamed as "Gasdermin-mediated programmed cell death" [23], but accumulating evidences proved that GSDMs can orchestrate other cell death processes and additional death-independent functions.

Indeed, each GSDM is activated by specific proteases in a cell context-dependent way (Fig. 1A) that could lead to different biological consequences: GSDMD NT is released by inflammatory caspases during canonical and non-canonical pyroptosis [9,10], whereas *Yersinia* infection [24,25] or RIPK3 signaling [26] provokes caspase-8-mediated GSDMD activation. Cathepsin-G activates GSDMD during NOD-like receptor protein 3 (NLRP3)-stimulated pyroptosis in macrophages and neutrophils [27], but neutrophil elastase (NE) cleavage in neutrophils could result in pyroptosis [28] or NETosis [29]. Of note, GSDMD pores can release cytokines in leukocytes in absence of cell death [30], and GSDMD-cell death can be inhibited by the Endosomal sorting complexes required for transport (ESCRT-III) mechanism [31]. GSDME is activated by apoptotic caspase-3, in response to multiple stimuli [32,33], leading to cell lysis considered by some authors as "necrosis secondary to apoptosis" [32,34], while GSDME processing *via* killer cell-derived Granzyme-B (GZMB) induces pyroptotic and immunogenic cell death in tumor cells [35]. Surprisingly, even caspase-8 (after *Yersinia* infection) and caspases 4/11 can initiate signaling pathways leading to GSDME cleavage and cell death [36]. Immunocyte-released Granzyme-A (GZMA) cleavage of GSDMB can produce either lytic cell death in cancer cells [37] or selective killing of *Shigella* intracellular bacteria but not the infected cells [38]. Moreover, unprocessed GSDMB enhances caspase-4/GSDMD non-canonical pyroptosis [39]. Finally, both TNF-α + hypoxia [40] and α-Ketoglutarate [41] induce Caspase-8 cleavage of GSDMC but in different residues (Fig. 1A). GSDMC NT provokes pyroptosis in cancer cells but it could result in chronic tumor necrosis [40]. The proteases activating human and mouse GSDMA and PJVK proteins are still unknown. Notably, cell-death function can be inhibited by protease processing within the GSDM NT domain, like GSDMD cleavage by caspases-3/7 [42] or enterovirus 3C protease [43]. Likewise, diverse caspases (1/3/6/7) can cleave and inactivate the GSDMB NT [16,39], contradicting the work by Panganiban and collaborators, which suggested that caspase-1 cleavage of GSDMB linker induced pyroptosis during asthma [44].

Of note, GSDM-mediated cell death is more complex than merely forming cell membrane pores, since some GSDM NT can also target intracellular organelle (mitochondria, neutrophil granules and possibly the nucleus) [4,7] (Fig. 1C). In particular, activated GSDMs (GSDMA/A3/D/E) trigger mitochondrial damage [45–48], and conversely, altered mitochondrial function induces GSDMD/E cleavage [49]. In fact, time lapse microscopy reveals that during pyroptosis mitochondrial damage precedes cell lysis [45,46]. Mitochondria dysfunction can occur in diverse ways. After caspase-3/GSDME cleavage, GSDME pores permeabilize the mitochondrial membrane leading to downstream apoptosome activation [49]. Thus, GSDME activation cause a positive feedback loop enhancing mitochondrial apoptosis and/or pyroptosis. By contrast, constitutively active mutant GSDMA3 proteins could cause mitochondrial damage by two mechanisms: binding to the mitochondrial chaperone Trap1, which promotes oxidative stress and loss of the mitochondria membrane potential triggering apoptosis-independent cell death [47], or through stimulation of pro cell-death autophagy [48]. Consistent with the GSDM mitochondrial targeting, GSDM NTs exhibit strong binding affinity for cardiolipin, a lipid enriched in the internal mitochondrial membranes [15,50].

Taken together, these evidences proved that GSDMs coordinate an extensive and complex cross-talk between diverse cell death pathways [22,34], but the precise functions of each GSDM in physiology and disease has only started to emerge.

The molecular mechanisms regulating inflammasome-dependent and independent pyroptosis and the implication of GSDMs in physiological processes (*e.g.*, response to infectious agents) and pathologies has been extensively revised elsewhere [7,19,20]. As examples, GSDM pro-cell death functions are involved in multiple inflammatory pathologies such as sepsis, autoimmune encephalomyelitis (GSDMD), asthma and inflammatory bowel diseases (GSDMB), among others. Additionally, rare *GSDM* mutations (Fig. 1) provoke pathogenic phenotypes in mice (*Gsdma3* mutations that impede CT protein inhibitory function trigger skin inflammation and hair loss, among other defects) and humans (*GSDME* and *PJVK* truncating mutations produce hereditary deafness disorders) [7,19,20].

In this review we provide a comprehensive overview on the multifaceted roles of the GSDM family in cancer, covering not only their pro-cell death activities in particular tumor contexts, but also other functions that eventually could lead to tumor progression. Moreover, we will focus on the implication of GSDMs in mediating cancer response to treatment and the relevance of GSDMs as novel therapeutic targets for clinical oncology management, with special emphasis on novel GSDM-directed nano-therapies.

3. Anti-tumor and pro-tumor effects of GSDMs

The pro-cell death activity of GSDMs could lead to the idea that these proteins play mainly an anticancer function. Yet, GSDM genes are often located in genomic regions frequently amplified in cancers (Table 1),

Table 1
Expression and functional roles of GSDMs in human cancers.

Cancer type (suggested function*)	Expression in tumors (method) & effects in untreated cancer cells.	Ref
Human *GSDMA* (*GSDM, GSDM*1) [17q21.1] **Mouse *Gsdma1/2/3* [11D]**		
Breast, gastric & ovarian (anti-tumor)	No expression was detected in any of the 24 cancer cell lines (Northern blot), even though *GSDMA* gene was amplified in 4/4 (100%) breast and 2/8 (25%) gastric HER2 cell lines.	[52]
Esophageal & Gastric (anti-tumor)	Undetectable expression in 58/60 (97%) tumors and 17/21 (81%) cell lines (RT-PCR). Overexpression decreases colony formation in MKN28 cells.	[1]
Gastric (anti-tumor)	Undetectable expression in 11/18 (61%) tumors and 8/11 (73%) cell lines (RT-PCR). *GSDMA* is restored by inhibition of promoter hypermethylation in 6/8 (75%) lines. Overexpression in cell lines decreases cell growth and induces cell death.	[51]
Ovarian (pro-tumor)	Greater mean expression in tumors ($n = 379$) compared to normal tissue ($n = 88$) (mRNA DBs). Higher *GSDMA* cancer expression associates with poor survival.	[53]
Human *GSDMB* (*GSDML*, *PRO2521*) [17q21.1] **Not present in mouse**		
Bladder (pro-tumor)	Larger mean expression in tumors compared to normal tissue ($n = 19$) (mRNA DBs). GSDMB and USP24 protein expression are positively correlated in bladder cancers ($n = 80$; IHC). GSDMB silencing in T24 and 5637 cells dampens proliferation, migration and invasion and reduces *in vivo* tumor growth of T24 xenografts. GSDMB controls glycolysis and *in vivo* cancer growth *via* STAT3 signaling.	[59]
Breast (pro-tumor)	Greater mean expression in tumors compared to normal tissue (RT-PCR, $n = 18$). Higher mRNA tumor expression associates with poor prognosis ($n = 1628$; mRNA DBs). Its over-expression in MCF7 cells induces cell motility, invasion, gelatin degradation *in vitro* & *in vivo* tumor growth and metastasis (mostly isoform 2). GSDMB silencing reduces migration and invasion of HCC1954 cells.	[56]
HER2 Breast (pro-tumor)	Overexpressed (80/212; 66%) (IHC) and gene-amplified (FISH) (73/123; 59%) in HER2-positive tumors. Higher levels ($n = 212$; IHC & $n = 2096$; mRNA DBs) associate with metastasis, poor prognosis and treatment relapse.	[55]
HER2 Breast (pro-tumor)	GSDMB expression promotes migration in HCC1954, SK-BR-3, and BT474 cells. Enhances lung metastasis in MDA-MB-231 cells.	[61]
Cervical (pro-tumor)	More frequently expressed in tumors (16/21; 76%) than corresponding non-neoplastic areas (8/21; 38%) (IHC). Its silencing reduces cell growth in Hela cells.	[57]
Cervical (uncertain)	Equal expression frequency in tumors (23/27; 85%) than corresponding non-neoplastic areas (24/27; 89%) (IHC).	[37]
Colorectal (uncertain)	Similar expression frequency in tumors (166/230; 72%) than corresponding non-neoplastic areas (154/230; 67%) (IHC).	[37]
Esophageal (anti-tumor)	Less frequent expression in tumors (44/80; 55%) than corresponding non-neoplastic areas (72/80; 90%) (IHC).	[37]
Esophageal & Gastric (pro-tumor)	Expressed in 47/60 (78%) tumors and 21/21 (100%) cell lines (RT-PCR). Co-amplified with HER2 in 2/8 (25%) gastric cancers. Its overexpression in MKN28 cells does not affect colony formation.	[1]
Gastric (anti-tumor)	Less frequent expression in tumors (34/75; 45%) than corresponding non-neoplastic areas (66/75; 88%) (IHC).	[37]
Gastric (pro-tumor)	More frequently expressed in tumors (44/52; 85%) than normal tissues (29/82; 35%) (RT-PCR). Stronger expression in tumor cells compared to normal cells (ISH, $n = 5$).	[58]
Gastric, CRC & HCC (uncertain)	Similar mean expression between tumors (gastric n = 21; HCC $n = 15$; colon $n = 9$) than corresponding non-neoplastic areas (RT-PCR).	[6]
HCC (uncertain)	Similar expression frequency in tumors (17/21; 81%) than corresponding non-neoplastic areas (21/21; 100%) (IHC). Its silencing does not affect cell growth in HepG2 cells.	[57]
Lung (pro-tumor)	Higher mean expression in lung adenocarcinomas than normal tissue ($n = 515$; mRNA DBs).	[54]
Multiple cell line types	GSDMB protein endogenous expression detected only in 18/54 (33%) cell lines (WB). Its expression could be induced by IFN-ɣ in 11 cell lines (WB). GZMA induces pyroptosis killing of GSDMB-expressing cell lines.	[37]
OSCC (pro-tumor)	Higher mean expression in lymph-node metastatic cancers *vs* non-metastatic ($n = 53$; microarrays and RT-PCR)	[60]
Pancreatic (uncertain)	Similar expression frequency in tumors (41/77; 53%) than corresponding non-neoplastic areas (45/77; 45%) (IHC).	[37]
Human *GSDMC* (*MLZE*) [8q24.21] **Mouse *Gsdmc1–4* [15D1]**		
Breast (pro-tumor)	Higher levels associate with worse overall survival ($n = 626$; IHC).	[40]
Colorectal (pro-tumor)	Higher mean expression in tumors *vs* adjacent normal tissue ($n = 44$; RT-PCR). GSDMC protein detected only in tumors, not in normal tissue (n = 44; IHC). GSDMC was silenced (DLD-1 and LoVo cells) or over-expressed (SW480 and WiDr cells). GSDMC upregulation promoted *in vitro* proliferation, anchorage-independent growth and *in vivo* tumorigenesis.	[68]
Esophageal & Gastric (anti-tumor)	Expressed in 34/60 (57%) of tumors and 16/21 (76%) cell lines (RT-PCR). Its overexpression reduces slightly colony formation in MKN28 cells.	[1]

(*continued on next page*)

Table 1 (*continued*)

Cancer type (suggested function*)	Expression in tumors (method) & effects in untreated cancer cells.	Ref
Lung (pro-tumor)	Greater mean expression in lung adenocarcinomas *vs* normal tissue (n = 515; mRNA DBs). Higher *GSDMC* expression associates with poor prognosis, metastasis and radio-resistance (mRNA DBs). Hypermethylation associates with gene silencing.	[54]
Melanoma (pro-tumor)	Expressed in 8/26 (30%) melanomas but not in nevi (n = 5). More frequently expressed in metastatic (6/11; 55%) than non-metastatic (2/15; 13%) melanomas (IHC).	[67]
Human *GSDMD* (*GSDMDC1*, *DFNA5L*, *DF5L*, *FKSG10*) [8q24.3] Mouse *Gsdmd* [15D3-E1]		
ESCC (uncertain)	Higher mean expression in tumors than in normal tissues ($n = 148$; mRNA DBs and $n = 30$; IHC).	[133]
Esophageal & Gastric (anti-tumor)	Expressed in 38/60 (63%) of tumors and 21/21 (100%) cell lines (RT-PCR). Its overexpression strongly reduces colony formation in MKN28 cells.	[1]
Gastric (anti-tumor)	Strong expression more frequent in normal tissue (41/61; 67%) than in tumors (27/102; 27%) (IHC). mRNA levels higher in normal *vs* matched tumor tissue ($n = 39$; RT-PCR). GSDMD expression in BGC823 cells reduces *in vitro* proliferation and *in vivo* cancer growth.	[69]
Lung (pro-tumor)	Higher expression (IHC score; $n = 168$) in adenocarcinomas and SCCs than normal tissue. Overexpression associates with increased tumor size, stage and lower survival in adenocarcinomas. GSDMD silencing in PC9, H1703 and H1975 cells reduces proliferation, promotes apoptosis and attenuates *in vivo* tumor growth.	[70]
Osteo-sarcoma (pro-tumor)	Expressed in 20/41 (49%) tumors and none of non-neoplastic areas (IHC). Greater mean protein expression in tumors than matched normal tissues ($n = 61$; WB). Higher levels associate with metastasis, resistance to chemotherapy, and poor survival.	[134]
Salivary ACC (pro-tumor)	More frequently expressed in ACC (33/33; 100%), than adenomas (23/29; 79%) & normal tissues (24/33; 73%) (IHC). Strong IHC expression only seen in ACC. Over-expression in ACC-LM and ACC-83 cells enhances *in vitro* invasion.	[71]
Human *GSDME* (*DFNA5*) [7p15.3] Mouse *Dfna5* [6B2.3]		
Breast (anti-tumor)	Lower mean expression in tumors *vs* normal samples ($n = 1142$; mRNA DBs). Reduced *GSDME* in tumors does not associate with survival. Greater *GSDME* levels in ER-negative and lobular carcinomas. Higher promoter hypermethylation in tumors. *GSDME* gene body methylation associates with reduced overall survival.	[79]
Breast (anti-tumor)	Reduced mean expression in cancer *vs* normal samples ($n = 2509$; mRNA DBs). Higher tumor *GSDME* does not associate with survival but correlate with immune-related genes. No association between promoter methylation and gene expression.	[96]
Breast (anti-tumor)	Decreased mean expression in cancers *vs* normal samples ($n = 10$; RT-PCR). Higher methylation in tumors. GSDME expression can be activated with demethylating agents. GSDME silencing increases colony formation, proliferation and invasion of MDA-MB-231 cells.	[75]
Breast and colon (anti-tumor)	Lower mean expression in breast ($n = 1097$) and colon ($n = 286$) cancers *vs* normal samples (mRNA DBs). GSDME suppresses *in vivo* tumor growth and promotes anti-tumor immunity of murine cells (EMT6, CT26, 4 T1 & B16–10) in immunocompetent mice.	[35]
Colorectal (anti-tumor)	Lower expression in cancers *vs* normal tissue ($n = 5$) (RT-PCR). Higher hypermethylation in tumors (65%) than normal tissue (3%). GSDME in HCT116 cells reduces cell growth and colony formation.	[73]
Colorectal (uncertain)	Using *Gsdme* KO mice, no clear effects were seen in two experimental models of intestinal cancer: the chemical induction by azoxymethane "AOM" or crossing with the Apc1638N/+ strain.	[82]
ESCC (anti-tumor)	Greater expression (IHC score) in cancers compared to normal tissue (IHC). Increased GSDME in SCCs ($n = 104$; IHC) associates with enhanced therapy response and better prognosis.	[116]
Gastric (anti-tumor)	Frequent promoter hypermethylation in tumors (53%). GSDME expression upregulated by demethylating agents in gastric cell lines. GSDME over-expression reduces colony formation and augments apoptosis in NUGC3 cells.	[74]
GBM (uncertain)	Higher mean expression in tumors than in normal brain (mRNA DBs), but GSDME levels do not associate with patients' survival.	[89]
Lung (uncertain)	Expressed in 59% of cancers, similar levels than normal tissues (IHC). Expressed in 95% of cell lines (WB). GSDME does not affect tumor growth of human NCI-H3122 and HCC827 xenografts.	[78]
Multiple cell lines (anti-tumor)	Protein detected in 30/57 (53%) cell lines, strong expression in 11/57 (19%) (WB). *Gsdme* KO mice do not exhibit developmental defects.	[33]
Melanoma (anti-tumor)	GSDME silencing in B16-Ova cells increases *in vivo* tumor growth.	[49]
Human *PJVK* (*Pejvakin*, *DFNB59*) [2q31.2] Mouse *Dfnb59* [2C3]		
Ovarian (anti-tumor)	Lower mean expression in tumors ($n = 379$) compared to normal tissue (mRNA DBs). Lower PJVK cancer expression associated with poor survival.	[53]

Table rows are ordered alphabetically by *GSDM* gene name (aliases indicated in parentheses and gene locus in square brackets) and then by tumor types. * The overall effect (proven or inferred) on tumor biology (excluding effects on cancer treatment) according to study results. Abbreviations: ACC: Adenoid Cystic Carcinoma; CRC: Colorectal; ESCC: Esophageal Squamous Cell Carcinoma; GBM: Glioblastoma Multiforme; IHC: Immunohistochemistry; ISH: *in situ* hybridization; mRNA DBs: Data Bases of mRNA expression (microarrays or RNAseq); SCC: Squamous Cell Carcinoma; WB: Western Blot.

such as the 17q12–21 region (*GSDMA* and *GSDMB*), that contains the *HER2/ERBB2* oncogene, or the 8q24 region (*GSDMC* and *GSDMD*), close to *MYC* oncogene. Moreover, GSDMs are either upregulated or downregulated (mRNA and/or protein) in a wide range of cancer types compared to their respective normal tissues (Table 1). In fact, accumulating evidences indicate that GSDMs play complex roles in cancer biology, possibly having either pro-tumor or anti-tumor functions depending on the cellular context (summarized in Table 1). The involvement of GSDMs in oncologic treatment response will be presented in section 4.

GSDMA was initially described as frequently lost in gastro-esophageal cancers [1,51] and breast cancer cells [52], even in HER2-positive cell lines with GSDMA gene co-amplification [52]. GSDMA expression could be restored by methylation inhibitors [51]. In gastric cancer cells, GSDMA upregulation, mediated by TGF-β (transforming growth factor-β) and the transcription factor LMO1 (LIM domain only 1), promoted an apoptotic signal leading to reduced cell growth [51]. These data point to a potential tumor-suppressor gene (TSG) role for GSDMA. Contrarily, in expression databases *GSDMA* mRNA was upregulated in ovarian carcinomas *versus* normal tissue, and higher *GSDMA* levels associated with worse survival [53]. Likewise, *GSDMA* mRNA is upregulated in some lung cancer datasets [54] and breast tumors [55,56], but the relevance of these observations is uncertain. In fact, despite being the first GSDM gene identified [5], the mechanism of cleavage/activation and the precise functional effects of GSDMA in cancer remains unclear.

Unlike other GSDMs, GSDMB does not consistently reduce cell growth of gastric cancer cells or other cell models [1,56,57]. Moreover, contrary to its neighbor gene *GSDMA*, *GSDMB* is frequently expressed (mRNA and/or protein) in several human tumor types and cancer cell lines, including gastric, hepatic, and breast, among others (Table 1). Nonetheless, compared to normal tissues, GSDMB levels are either upregulated [1,54–59], downregulated [37] or unchanged [6,37,57] depending on the tumor type and the methods used to measure its expression (Table 1). In terms of mRNA, *GSDMB* over-expression associates with advanced/metastatic disease in oral SCC [60] and gastric tumors [58], shorter disease free and metastasis free survival in breast cancer [56], and overall survival in only 3 of 33 cancer types tested by Zhou and colleagues [37]. However, since *GSDMB* mRNA is detected in various normal cell types [7] the true GSDMB expression in tumors should be assessed by immunohistochemistry. Using a validated GSDMB antibody and a specific FISH probe, Hergueta-Redondo and colleagues [55] demonstrated that GSDMB is over-expressed/gene-amplified in >60% of HER2 breast cancers. Similarly, in gastro-esophageal cancers *GSDMB* and *HER2* co-amplification is a common finding [1]. Importantly, in HER2 breast carcinomas, GSDMB protein upregulation associated significantly with various adverse clinical parameters: disease progression, relapse and response after neoadjuvant therapy, as well as distant metastases and lymph node positivity in the adjuvant setting, independently of hormonal receptors status [55]. Moreover, GSDMB expression mediates multiple pro-tumor functions in breast neoplasias: increases *in vitro* cell migration, possibly by modulating the Rac GTPases, invasion and gelatin degradation [56], stimulates *in vivo* metastatic dissemination [56,61], and reduces sensitivity to anti-HER2 therapy. Actually, all these pro-tumor activities could be decreased with a novel anti-GSDMB nanotherapy [61], thus demonstrating that GSDMB plays a key role in the biology and clinical behavior of HER2 breast carcinomas. Similarly, in bladder cancer cells GSDMB promotes proliferation,

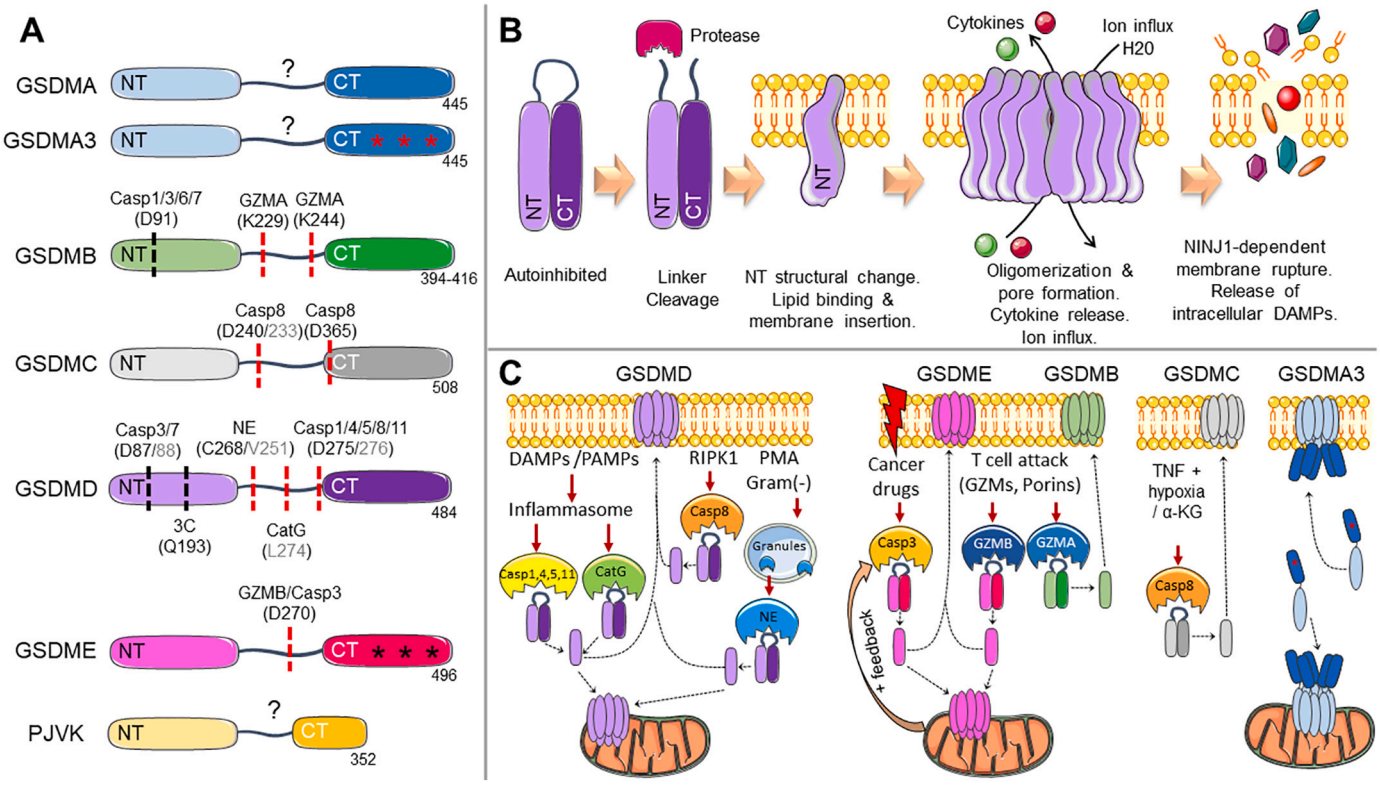

Fig. 1. GSDM pore-forming functions. A: Regulation of GSDM function by cleavage or mutation. Schematic representation of GSDM regions (NT, N-terminal domain; CT, C-terminal; and linker interdomain) showing the known proteases and respective cleavage sites (in parentheses, the cleavage sites of murine proteins are indicated in grey letters). The cleavage events inducing pore-forming activity are shown in red lines, and inhibitory processing in black. Gain-of–function mutations in the CT of GSDMA3 and GSDME are indicated by asterisks. **B:** Main steps in the formation of GSDM membrane pores. There are differences in the structure and size of GSDM pores. GSDMA3 form pores containing around 27 monomers and with an internal pore diameter of 16–18 nm; GSDMD, 33 monomers and up to 21 nm internal diameter. **C:** Summary of mechanisms of GSDM activation and biological effects. Casp, Caspase; CatG Cathepsin G; GZM, Granzyme; NE, Neutrophil Elastase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

migration and invasion [59]. These evidences and the previous observations in gastric carcinomas [1,58] indicate that GSDMB could act like an oncogene. However, the *in vivo* effect of GSDMB on tumorigenesis and progression might depend on the cancer models, since it promotes tumor growth in MCF7 breast [56] and T24 bladder cancer xenografts [59] but no other human [61] and murine [37] cancer models (Table 1). At this point, it is important to highlight the existence of multiple *GSDMB* transcriptional variants [6,57] and at least four distinct translated protein isoforms (ENSEMBL:ENSG00000073605). Though, for unknown reasons the 411 aa protein sequence -Q8TAX9-, which was partially crystalized [16], and is regarded as "canonical" in all databases, does not match any of the transcriptional variants. The four translated isoforms, which differ on the alternative usage of exons 6–7, can play different functions in cancer [56] and inflammatory diseases [44,62]. The isoform differential expression was reported in normal and cancer tissues [6,57,58,63–65] and could be regulated by specific genomic elements [58,64,65] and SNPs [44,63,66]. However, there is scarce information on the precise functions of each translated variant in normal and pathological contexts. In this regard, Hergueta-Redondo et al. reported that in MCF7 breast cancer cells both GSDMB isoform 1 (GSDMB-1; lacks exon 6) and 2 (GSDMB-2; the shortest isoform, lacking exons 6–7) enhanced migration and invasion *in vitro*, while only GSDMB2 promoted *in vivo* tumor growth and metastatic dissemination in immunodeficient mice [56].

Opposing its pro-tumor functions, GSDMB can, under specific stimuli, exhibit pyroptotic activity in cancer cells [37,61], and thus an antitumor effect. Specifically, in a context of an activated antitumor immune response, the lymphocyte-derived Granzyme A (GZMA) can cleave GSDMB within cancer cells (Fig. 1A,C), activating caspase-independent pyroptosis and promoting tumor clearance [37]. Contrasting with other studies (Table 1), Zhou and cols [37] reported a lower GSDMB immunohistochemical staining in gastro-esophageal tumors compared to normal tissues, but not other cancer types (Table 1), suggesting that GSDMB could act as tumor suppressor in these neoplasias. Unfortunately, this study did not evaluate the association of GSDMB staining with clinicopathological variables or disease survival, thus the clinical relevance of these observations remains unclear.

GSDMC was initially identified as a marker of melanoma progression, being particularly over-expressed in metastatic melanoma cells [67]. Further studies reported opposing results regarding its expression and functional effects in tumors. On one hand, Saeki and cols found GSDMC expression in 50% of gastro-esophageal tumors and cell lines but was mostly lost in diffuse-type gastric carcinomas. In gastric cancer cells, GSDMC expression reduced cell proliferation, suggesting a possible tumor suppressor function [1]. On the other hand, GSDMC upregulation in lung [54] and breast [40] tumors associates with adverse clinical parameters (Table 1), and increases proliferation along with *in vivo* tumor growth in colorectal cancer cells [68], suggesting a pro-tumor role. Activation of GSDMC cleavage by drugs or metabolites (α-KG) [41] induces pyroptosis in cancer cells (Fig. 1; Table 2), but can also provoke chronic necrosis [40]. In the latter case, under hypoxia nuclear PD-L1 activates GSDMC transcription in breast cancer cells. In parallel, activated macrophages secrete TNF-α resulting in caspase-8 activation within cancer cells. The coordinated upregulation of GSDMC and caspase-8 induces GSMDC cleavage, causes pyroptosis and subsequent necrosis in breast cancer cells [40]. Paradoxically, chronic tumor necrosis fuels cancer progression, and therefore patients with GSDMC-overexpressing breast cancers show poor survival [40]. This study challenges the idea that GSDM-mediated cancer cell death can have beneficial effects on cancer progression.

Whereas GSDMD role in leukocyte pyroptosis is well known, its implication in cancer is debatable. Depending on the study, both GSDMD upregulation and downregulation (mRNA and/or protein) have been described in tumors, where GSDMD overexpression associates with either good or unfavorable prognosis, as well as antitumor or pro-tumor effects (Table 1). For instance, GSDMD expression diminishes proliferation in gastric cancer cells, either *via* cell death induction [1] or by regulating cell cycle-related proteins [69], but it increases lung cancer growth *in vivo* [70] and invasion of salivary gland adenocarcinomas [71].

Among the GSDMs, GSDME exhibits the most consistent anti-tumor functions in the literature and is commonly considered as a potential TSG for several reasons. First, GSDME expression in cancer models (untreated cells) inhibits cell growth and/or provokes cell death [72,73]. Second, diverse anti-oncologic treatments induce GSDME cleavage and enhance cancer death (Table 2; discussed in section 4). Third, GSDME is usually expressed in normal tissues but is frequently downregulated in diverse cancer types, being this alteration sometimes associated with increased malignancy or poor prognosis (Table 1). Fourth, promoter hypermethylation (a common mechanism of silencing TSGs) of *GSDME* is commonly observed in cancers [72–77]. Besides, *GSDME* loss-of-function mutations occur in a small proportion of tumors, as an alternative mechanism to methylation for inactivating GSDME-mediated cancer cell death [35]. Finally, the tumor suppressor activity of GSDME is mediated by its cleavage by killer-cell GZMB, resulting in caspase-independent pyroptosis of cancer cells and enhancement of antitumor immunity [35].

Despite these evidences, and the consistent effect of GSDME on tumor sensitization to cancer therapies (Table 2), it is still questionable if GSDME *per se* generally acts as a *bona fide* TSG, and whether the evaluation of tumor GSDME protein levels have a reliable prognostic utility (Table 1). For instance, GSDME protein upregulation was observed in the majority in lung tumors and cell lines, and GSDME cleavage (pyroptotically active) was detected even in untreated specimens [78]. Besides, GSDME promoter hypermethylation does not universally correlate with gene silencing in diverse cancer datasets (Table 1) [77]. Interestingly, the gene body of *GSDME* is frequently hypomethylated in cancer compared to normal tissues, and in fact, the particular patterns of methylation in the *GSDME* gene promoter and body regions, rather than *GSDME* expression, can be used as a powerful biomarker for pan-cancer detection [79–81]. Apart from human samples, studies with *in vivo* animal models reported seemingly contradictory results regarding GSDME role in tumor development and progression. In xenograft cancer models, GSDME does not affect tumor growth of human lung cancer cells [78], but reduces tumor growth of murine melanoma [49], breast, and colon cell lines [35]. Moreover, comparing *Gsdme* Knock-out (KO) mice with wildtype (WT) animals in two experimental models of intestinal cancer, no clear effects on carcinogenesis, tumor differentiation and progression were evidenced, though an increased tumoral inflammation was observed in WT mice [82]. By contrast, *Gsdme* KO mice exhibit reduced tumorigenesis in inflammation–mediated colitis-associated colorectal cancer models [83], suggesting that GSDME-mediated pyroptosis and inflammation play a role in cancer initiation. Nonetheless, these conflicting data could be partly explained considering new findings demonstrating that GSDME tumor suppressive effect depends mostly on the subsequent tumor inflammation and immunogenic activation (see section 6).

Finally, hitherto, the implication of PVJK is largely unknown. Recently, using ovarian carcinoma expression databases, *PJVK* was found downregulated in tumors compared with normal samples, and lower *PJVK* mRNA levels in cancers associated with poor survival rates [53].

4. GSDMs modulate tumor response to anti-cancer treatments

Evading apoptosis is a cancer hallmark that can provide resistance to anti-cancer therapies. In this scenario, activating pyroptosis or other cell death mechanisms could lead to tumor regression. GSDM-mediated cell death, which can proceed in caspase-dependent and independent ways (Fig. 1), occurs in response to multiple cell-damaging stimuli and anti-cancer treatments (chemotherapy, targeted drugs and immunotherapy) (Table 2). These data would bring to the idea that GSDM-

Table 2
Effect of GSDMs on anti-cancer therapy response.

Tumors /cell lines	Treatment	Effect on cancer cells	Ref
GSDMB			
HER2 Breast tumors, PDXs & cell lines	Trastuzumab	Higher levels associate with poor treatment response (adjuvant or neoadjuvant contexts) in human tumors. Trastuzumab resistance associates with GSDMB upregulation in human PDXs. GSDMB expression promotes drug survival in SK-BR-3 and HCC1954 cells.	[55]
HER2 Breast cell lines	Trastuzumab	GSDMB upregulation increases trastuzumab survival in HCC1954, SK-BR-3, and BT474 cells. This effect can be partially blocked with anti-GSDMB nanotherapy.	[61]
Colon & Melanoma cell lines	Anti-PD1	Exogenous GSDMB over-expression in murine CT26 and B16-F10 cell line xenografts sensitizes to anti-PD1 treatment *in vivo*.	[37]
GSDMC			
Breast cell line	Multiple chemo drugs	Most drugs upregulate GSDMC but only the antibiotic-type induce caspase-8/GSDMC pyroptosis in MDA-MB-231 cells.	[40]
Multiple cell lines	DM-α-KG	Induces ROS and activation of DR6/caspase-8 axis leading to GSDMC pyroptosis in multiple human and mouse cancer cell lines *in vitro*. DM-α-KG reduces *in vivo* tumor growth (Hela and B16 xenografts) and metastasis (B16) and this effect depends on the presence of GSDMC and DR6.	[41]
GSDMD			
AML primary cultures and lines	Val-boroPro (DPP8/9 inhibitor)	Activates CARD8/caspase-1/GSDMD-mediated pyroptosis and efficiently kills AML primary cells and 12/17 AML cell lines *in vitro*. Halts tumor progression of one PDX and MV4;11 cell line xenografts *in vivo*.	[86]
NPC cell lines	Taxol	Caspase-1 cleaves GSDMD and activates pyroptosis *in vitro* in HNE-2, 5-8F cell lines. GSDMD silencing does not affect tumor growth *in vivo* in untreated 5-8F xenografts. GSDMD sensitizes 5-8F xenografts to taxol treatment.	[135]
Esophageal SCC cell lines	Metformin	Metformin upregulates miR-497, which in turn downregulates PELP1. PELP1 reduction increases GSDMD pyroptosis in KYSE510 and KYSE140 cells.	[133]
Ovarian cell lines	Alpha-NETA	Induces caspase-4 and GSDMD upregulation and cell death in Ho8910PM cells. GSDMD silencing partly increases cell survival to the compound.	[136]
GSDME			
(TN) Breast cancer cell lines	Tetra-arsenic hexoxide	Induces mitochondrial ROS-mediated caspase-3/GSDME cleavage and pyroptosis in mouse EO771, 4T1 and human Hs578T, MDA-MB-231 cancer cells but not in non-tumorigenic cell lines (MCF10A, NMuMG). GSDME silencing reduces drug-induced cell death.	[125]
(TN) Breast cell lines	Cetuximab	miR-155-5p antagomir upregulates GSDME and switches apoptosis to pyroptosis after cetuximab treatment in EGF-overexpressing MDA-MB-231 and MDA-MB-468 cells.	[112]
Colon cell lines	Lobaplatin	After lobaplatin, caspase-3 cleaves GSDME and promotes pyroptosis *via* ROS/JNK/bax mitochondrial signaling pathway in HT-29 and HCT116 cells. GSDME silencing switched from pyroptosis to apoptosis but did not affect *in vivo* tumor growth upon lobaplatin treatment.	[90]
Colon cell lines	TNF + CHX or navitoclax	These drugs induce BAK/BAX/Caspase-3 activation and GSDME cleavage and pyroptosis in HCT116 cells. GSDME silencing reduces cell death to treatment.	[121]
Colon cell lines	Ionizing radiation (IR)	IR induces GSDME-pyroptosis *via* the LncRNA NEAT1 and miR488 in HCT116 cells.	[113]
Gastric cell lines	5-FU	Stimulates Caspase-3 and GSDME cleavage and pyroptosis in SGC-7901 and MKN-45 cells. GSDME knock out switches pyroptosis to apoptosis.	[137]
GBM cell lines	Galangin (natural flavonoid)	Simultaneously induces autophagy plus caspase-3/GSDME cleavage and pyroptosis in U87MG and U251 cells. GSDME silencing switches pyroptosis to apoptosis. Inhibition of autophagy plus Galangin increases cell death *in vivo* in U87MG xenografts.	[89]
Esophageal SCC cell lines	Cisplatin and BI2536	The Plk1 inhibitor BI2536 combined with cisplatin provokes caspase-3/GSDME cleavage and pyroptosis in nine SCC cell lines *in vitro*. The drug combination severely reduces *in vivo* tumor growth of KYSE150 tumor xenografts.	[116]
Head & Neck cell lines	Triptolide	Triptolide activates BAD/BAX-caspase 3-GSDME pyroptosis by repressing mitochondrial associated hexokinase-II and provoking ROS in HK1 and FaDu cells. Combining Triptolide with erastin (SLC7A11 inhibitor) halts *in vivo* tumor growth in HK1 xenografts.	[88]
Liver cell lines	Miltirone	Induces ROS and mitochondrial damage leading to caspase-3/GSDME cleavage and pyroptosis in HepG2 and Hepa1–6 cells *in vitro* and cell death *in vivo* using Hepa1–6 tumor xenografts. GSDME silencing switches pyroptosis to apoptosis.	[126]
Lung cell lines and primary human tumors	Trametinib, Erlotinib or Ceritinib	GSDME sensitizes to Trametinib, erlotinib and ceritinib targeted therapies *in vitro* and *in vivo* using multiple cell lines. GSDME knock out switches pyroptosis to apoptosis but does not affect tumor growth when untreated. GSDME pyroptosis was observed in patients treated primary tumors.	[78]
Lung cell line	Cisplatin or paclitaxel	Cisplatin provokes a stronger activation of caspase-3/GSDME cleavage and pyroptosis than paclitaxel in A549 cells. GSDME knockdown inhibits cisplatin- but not paclitaxel-induced pyoptosis *in vitro*.	[138]
Lung & neuroblastoma cell lines	Dasatinib	Dasatinib upregulates GSDMD and GSDME protein levels and provokes caspase-3/GSDME cleavage and pyroptosis in A549 and SH-SY5Y cells *in vitro*.	[139]
Melanoma Cell lines	Doxorubicin	Stimulates autophagy plus caspase-3/GSDME cleavage and pyroptosis in SK-MEL-5, SK-MEL-28, and A-375 cells. Inhibition of eEF-2K decreases autophagy and upregulates sensitivity to doxorubicin and pyroptosis *in vitro*.	[140]
Melanoma cell line	Iron plus ROS activators	Iron upregulates ROS which in turn activates Tom20/bax/caspase-3 and GSDME pyroptosis in A375 cells. GSDME silencing but not GSDMD prevents Iron/CCCP pyroptosis *in vitro*. Iron supplementation plus sulfasalazine inhibits xenograft tumor growth and metastasis through GSDME-pyroptosis induction.	[124]
Melanoma cell lines	BRAF and MEK inhibitors	BRAFi+MEKi activate caspase-3 / GSDME cleavage and pyroptosis *in vitro* and *in vivo* in diverse melanoma models. GSDME-pyroptosis stimulates immune anti-tumor response.	[85]
Multiple cell line types	Multiple chemo drugs	Chemotherapy drugs provoke caspase-3/GSDME cleavage and pyroptosis in SH-SY5Y, Mewo, Hela, NCI-H522 and EMT6 cancer cells but also in normal keratinocytes, placental epithelial cells and smooth muscle cells. *Gsdme* KO mice display reduced *in vivo* toxicity of normal tissues to Cisplatin, 5-FU or bleomycin treatment.	[33]

AML: Acute myeloid leukemia; DM-α-KG: Dimethyl-α-Ketoglutarate; GBM: Glioblastoma; NPC: nasopharyngeal carcinoma; SCC: Squamous Cell Carcinoma; TN: Triple Negative Breast Cancer.

expressing cancer cells may be generally more sensitive to oncologic therapeutic challenge, but the reality is much more complex.

First, the precise effect of GSDMs on cell death depends on the balance of different stimuli, signaling pathways, molecular alterations, and cellular contexts. In fact, the co-activation of specific signaling pathways might be required for GSDM pyroptosis. For instance, in HepG2 cells GSDME enhances etoposide cell death only in the presence of WT p53 [84] and GSDMC is upregulated by diverse chemotherapy drugs in MDA-MB-231 cells, but only the antibiotic-type (doxorubicin/epirubicin) induce caspase-8/GSDMC-dependent pyroptosis [40]. Second, the GSDMs mediate an extensive crosstalk among diverse cell death mechanisms, and these could cooperate or compete in a biological context-dependent way. For example, inflammasome-triggered caspases (1/4/5/11) activate GSDMD pyroptosis but the apoptotic caspases 3/7 inhibits pyroptosis in monocytes [42]. Third, tumor cells could exhibit intrinsic or acquired resistance to pyroptosis. While loss of function mutations in human GSDM genes have been reported only for GSDME so far [35], alterations in upstream signaling pathways or caspases could also occur. Accordingly, GSDME-positive $BRAF^{V600E/K}$ melanomas resistant to BRAF+MEK inhibitors exhibit weak induction of the pro-apoptotic proteins BIM-EL and BMF, and thus reduced caspase-3 activity [85]. Similarly, 5/17 AML lines were intrinsically resistant to ValboroPro agent due to the lack of pro-caspase-1, which was required for GSDMD-mediated pyroptosis [86].

Apart from these considerations, the general picture shows that the GSDMs are mainly involved in switching apoptosis to lytic and inflammatory cell death processes (pyroptosis and necrosis, but not necroptosis or ferroptosis) [87] after cancer treatment (Table 2). GSDME is the main determinant for this apoptosis-to-pyroptosis switch in multiple *in vitro* and *in vivo* models treated not only with a myriad of anti-cancer agents (Table 2) but also with specific natural antitumor products, such as the epoxide triptolide [88] or the flavonol galangin [89]. In clinical specimens, GSDME-pyroptosis was also evidenced (measuring serum LDH concentrations) in lung cancer patients after chemo- or EGFR inhibitor-based treatments [78]. Nonetheless, in many studies it is unclear if this apoptosis-pyroptosis switch translates into a bigger cancer killing. For example, in lobaplatin-treated colorectal cancer cells GSDME silencing did not affect tumor response (growth rate) *in vivo* and *in vitro* [90], and TRAIL treatment resulted in similar Hela cell death quantities, irrespective of GSDME levels [35].

Like GSDME, GSDMD acts as a therapy sensitizer or mediates apoptosis-pyroptosis switch in particular cancer cell types and oncologic treatments (Table 2), thus, these GSDMs could be important modulators of cancer therapy response. Unfortunately, many of the studies listed in Table 2 were not performed in clinical human specimens, so the impact of GSDM-mediated pyroptosis on cancer patient survival is still to be verified.

Contrary to other GSDMs, GSDMB upregulation has been associated with therapy resistance. Specifically, in HER2 breast carcinomas GSDMB renders cancer cells more resistant to the anti-HER2 agents trastuzumab and lapatinib, but no to taxol chemotherapy [55,61], likely in a pyroptosis independent mechanism. For this reason, GSDMB significantly associates with worse breast cancer patients' prognosis in both adjuvant and neoadjuvant anti-HER2 treatments [55].

Summarizing, the final effect of GSDMs on tumor treatment response depends on multiple factors, including the co-activation of specific signaling pathways, the drug used, cellular context and, as discussed later, the effect on the tumor microenvironment.

5. Targeted activation of GSDM cytotoxicity in tumor cells as novel therapeutic options

Diverse oncologic treatments induce GSDM-mediated cell death, thus suggesting that targeted approaches specifically aimed at activating of GSDM cytotoxicity in tumors can be exploited as novel therapeutic options (Fig. 2). To this end, promising therapeutic effects has been recently obtained using two general types of approaches: a) Target activation of the intrinsic GSDM pro-cell death function in cancer cells through nanomedicines; b) Delivering GSDM cytotoxic peptides/expression constructs into tumors.

Within the first type of approaches, three studies targeted GSDME-pyroptosis with nanocarriers that combined agents to induce *GSDME* transcription (such as the DNA methylation inhibitor decitabine, DCT) with anti-cancer drugs (Fig. 2A). Fan and colleagues [91] used tumor-targeting nanoliposomes loaded with cisplatin (LipoDDP) and DCT to trigger GSDME-pyroptosis in 4T1 breast xenografts. The intravenous administration of LipoDDP-DCT upregulated GSDME expression (by inhibiting its gene promoter hypermethylation), induced caspase-3/GSDME cleavage and the subsequent pyroptosis led to a reduced tumor growth and metastatic spread *in vivo*. Similarly, Zhao and collaborators [92] designed biomimetic nanoparticles (BNP), by fusing breast cancer membranes onto a poly-lactic-*co*-glycolic acid (PLGA) core, that were loaded with the photosensitizer indocyanine green (ICG) and DCT. In 4T1 tumor xenografts the "BNP" nanomedicine was activated by low-dose photo-activation (local hyperthermia) and induced GSDME-dependent pyroptosis by the combination of two effects: On one hand, ICG-mediated puncture of cancer cell membranes prompted a sharp increase in cytoplasmic Ca^{2+}, provoking cytochrome c release and caspase-3 activation. On the other hand, DCT release up-regulated *GSDME* transcription. Importantly, this "activatable" therapeutic agent reduced significantly the size of primary tumor and metastasis by activating a strong systemic antitumor immune response. Finally, Hu et al. [93] designed a nano-drug delivery system based on mPEG-PLGA-PLL copolymer that was loaded with arsenic trioxide (As_2O_3) as therapeutic agent for hepatocellular carcinoma (HCC) treatment. Intratumor administration of these nanoparticles (As_2O_3-NPs) resulted in caspase-3/GSDME cleavage and pyroptosis of Huh7 xenografted tumor cells. Moreover, As_2O_3 reduced the expression of DNA methyltransferases (Dnmt1, 3a and 3b) which modulate the transcriptional levels of *GSDMD* or *GSDME* in a cell line-dependent way. In terms of pre-clinical efficacy, compared to free As_2O_3, As_2O_3-NPs greatly inhibited tumor growth and showed no obvious systemic toxicity.

Instead of using drugs/compounds affecting GSDM expression and/or activation, Molina-Crespo and collaborators [61] created the first nanotherapy targeting directly a GSDM protein with a therapeutic antibody (Fig. 2B). Specifically, the nanomedicine (termed AbGB-NC) combined a therapeutic anti-GSDMB monoclonal antibody (AbGB) with biocompatible nanocapsules (NCs) functionalized with hyaluronic acid (HA). Since GSDMB upregulation promotes multiple pro-tumor effects in HER2 breast carcinomas [55,56], the authors next validated the therapeutic effect of this nanomedicine in different HER2/GSDMB+ breast cancer models. The results demonstrated that the intracellular released AbGB, but not an irrelevant antibody, was able to impact significantly on tumor behavior in diverse ways: a) decreasing *in vitro* cell migration; b) increasing the sensitivity to trastuzumab anti-HER2 therapy; c) reducing tumor growth *in vivo* of orthotopic breast cancer xenografts; and d) limiting lung metastasis colonization *in vivo,* with no obvious unwanted cytotoxic effects. At the mechanistic level, *in vitro* tests indicated that the binding of the AbGB to GSDMB (within the CT region and close to the hinge region) could affect the protein configuration thus enhancing the interaction of GSDMB protein with sulfatides. This interaction would subsequently decrease cell migration, and importantly may release the intrinsic pro-cell death activity of GSDMB. In agreement with this, the AbGB-NCs, but no the control treatments, increased the cell death rate (TUNEL assay, not specific test for pyroptosis) of cancer cells *in vivo*, and specifically of GSDMB-overexpressing tumors (Fig. 2B). Overall, this study proved that GSDMB is a novel therapeutic target for aggressive HER2/GSDMB+ breast cancers, and indicated, for the first time, that GSDMB cytotoxic effect and other functions in cancer can be modulated directly with intracellular antibodies.

Whereas the above nanotherapies rely on the endogenous expression

of the GSDMs by the tumor cells, other therapeutic approaches have been designed for extrinsically delivering GSDM cytotoxic constructs into cancer cells. As a potential treatment for schwannoma tumors Ahmed and collaborators [94] generated an adeno-associated serotype-1 virus (AAV1)-based vector that, under the control of a Schwann cell-specific promoter (P0), expresses the pyroptotic NT region of GSDMD (residues 1–276). The intratumoral injection into intra-sciatic nerve of the AAV1-P0-GSDMD-NT in human and syngeneic mouse cells xenograft schwannoma models reduced tumor burden by increasing cell death and reducing cell proliferation. Moreover, this treatment alleviated tumor-associated pain while causing no evident neurologic toxicity.

Recently, Wang et al. [95] devised a very clever approach by which the release of the pyroptotic GSDMA3 protein occurs specifically within tumor cells (Fig. 2C). Specifically, the authors designed a complex nano-bioorthogonal system based on two interacting components. As first component, the mouse GSDMA3 cleaved protein (NT plus CT domain) was conjugated *via* a triethylsilyl (TES) ether linker to gold nanoparticles (NP) to generate the NP-GSDMA3, a biocompatible nanosystem that accumulated mostly into tumor tissue of 4T1 cells xenografts. As second component, the authors used the tumor-imaging probe phenylalanine trifuoroborate (Phe-BF_3), which was taken up specifically cancer cells. Phe-BF3 desilyated the silyl ether bond of NP-GSDMA3 resulting in the intracellular release of a pytoptotic active GSDMA3 protein and the killing of 4T1 tumor cells *in vivo*. After three rounds of injection with Phe-BF3 plus NP-GSDMA3 the tumor burden reduced enormously, while the administration of Phe-BF_3 or NP-GSDMA3 alone, or a loss-of-function GSDMA3-NP did not have any effect. No obvious cytotoxic effects were observed in other organs in mice treated Phe-BF_3 plus NP-GSDMA3 [95].

Remarkably, some of these GSDM-targeted nanotherapies achieved tumor regression through the activation of a potent anticancer inflammatory reaction (Fig. 2) (discussed below), thus indicating that pyroptosis-inducing nanotherapies are promising approaches that could halt tumor progression simultaneously by attacking tumor cells and enhancing immune response.

6. GSDM-mediated cell death: effects on the tumor microenvironment and the cancer response to immunotherapy

GSDM pyroptosis and the immune response are engaged in a complex bidirectional crosstalk. On one hand, cancer cell lysis and the subsequent release of DAMPs and cytokines promotes an immunostimulatory tumor microenvironment that could lead to cancer rejection [87]. On the other hand, cytotoxic T cells and NK cells can directly trigger pyroptosis by cleaving GSDME or GSDMB in cancer cells *via* perforin-mediated release of GZMB and GZMA, respectively [35,37]. Therefore, pyroptosis and activated immunocytes could generate a positive feedback loop in anticancer immunity (Fig. 2).

GSDME-mediated pyroptosis generally associates with a strong immune antitumor response [87]. GSDME upregulation in human cancers correlate positively with immune infiltration [96], and its overexpression in murine breast cancer and melanoma xenografts in immunocompetent mice significantly augmented the number of tumor infiltrating lymphocytes (TILs), mostly CD8+ T and NK cells. Activated TIL cytotoxicity and increased phagocytosis by tumor-associated macrophages provoked tumor eradication [35]. Moreover, after therapeutic challenge by BRAF–MEK inhibitors GSDME-mediated pyroptosis in murine melanoma models increased T cell infiltration and improved treatment responses *in vivo* [85]. Consistent with the beneficial effect of GSDME pyroptosis in cancer treatment, as commented before, the GSDME targeted nanotherapies LipoDDP-DCT and BNP induced a strong immune reaction, including the release of cytokines and pro-inflammatory molecules, recruitment and activation of cytotoxic T cells, and dendritic cell maturation, that altogether provoked tumor eradication [91,92], Alike GSDME nanotherapies, the efficacy of the NP-GSDMA3 + Phe-BF3 nanomedicine depends on the immune anti-tumor reaction. In fact, the pyroptotic killing of a small fraction of tumor cells (20%) was sufficient to activate, *via* IL1-β secretion, a potent anti-tumor effect (T cells, NK and macrophages) resulting in the elimination of the bulk tumor [95] (Fig. 2C).

Remarkably, the GSDME tumor suppressor function in both untreated and therapy-challenged tumors seem to rely on the presence of activated cytotoxic cells. Thus, depletion of NK and CD8+ T cells or knocking-out perforin reverses GSDME effects on tumor growth of untreated cancers [35] and depletion of CD4+/CD8+ T-cells significantly dampens the response of xenografted melanoma cells to BRAF–MEK inhibitors. Consequently, immunocompetent (*e.g.*, Balb/c) and not immunodeficient (nu/nu) mouse strains should be used to unveil the full effects of GSDME on cancer. In fact, differences in mouse strains may explain in part the conflicting results obtained with GSDME-positive cancer xenografts (discussed in section 2).

Of note, while *in vitro* GSDME activation in cancer cells occurs *via* caspase-3 (resulting in necrosis secondary to apoptosis), *in vivo* cleavage by killer-cell GZMA provokes immunogenic cell death (Fig. 2), a form of cell death that is sufficient to activate an adaptive immune response [35,87]. Actually, the "vaccine" inoculation of immunocompetent mice with GSDME-overexpressing cancer cells produces the efficient killing of the secondary tumor implantation. Supporting this idea, GSDME-mediated pyroptosis triggers an immune response against residual cancer, since *Gsdme*-silenced murine melanomas recurred more frequently than *Gsdme*-expressing ones after the interruption of BRAF-MEK inhibitors therapy [85].

In opposition to the intrinsic tumor suppression mediated by GSDME, GSDMB promotes diverse pro-tumor effects in breast cancer [55,56], and its exogenous expression in murine colon and melanoma cells does not affect tumor growth in immunocompetent mice [37]. In fact, to expose the potential GSDMB antitumor effect, the stimulation of the antitumor immune response by PD-1 immune checkpoint inhibitors is required for efficient GZMA/GSDMB-mediated tumor pyroptosis and regression [37] (Fig. 2B). This shows that triggering GSDMB cytotoxicity within tumors may require additional signals from the tumor microenvironment in order to enhance immune recognition and cancer killing. In this sense, GSDMB expression in cancer cells is upregulated by cytokines (IFNs and TNF-α) produced by TILs [37], and this may contribute to maintaining a positive feedback loop of pyroptosis (Fig. 2B). Moreover, this work indicates that pyroptosis and immunotherapy could synergize to produce a protective immune reaction. Supporting this idea, Wang et al. 2020 proved that the NP-GSDMA3 + Phe-BF_3 therapy sensitized 4T1 tumors to *in vivo* anti-PD-1 treatment [95] (Fig. 2C). Therefore, pyroptosis-induced inflammation, *via* GSDM agonists, can cooperate with immune checkpoint inhibitors, thus leading to an improved effectiveness of cancer immunotherapy. Based on these data, the current hypothesis is that GSDM-mediated pyroptosis could convert immunologically "cold" to "hot" tumors, and likely respond better to immune check-point inhibitors.

7. Fine tunning of GSDM-mediated cell death for oncologic treatment: remaining questions

Although the data described above reveal a crucial role of GSDMs as novel therapeutic targets and as key determinants of chemotherapeutic and immunotherapy treatments there are still a number of questions to be resolved before GSDM-mediated oncologic therapies became a reality at the clinical level.

7.1. Controlling the specificity, intensity and timing of GSDM-mediated cancer cell death

Currently, predicting the final effect of pyroptosis in therapy outcome is intricate. In order to exploit successfully GSDM-pyroptosis in cancer therapy, there are three key parameters that need to be finely controlled (specificity, timing and intensity of cancer lysis and

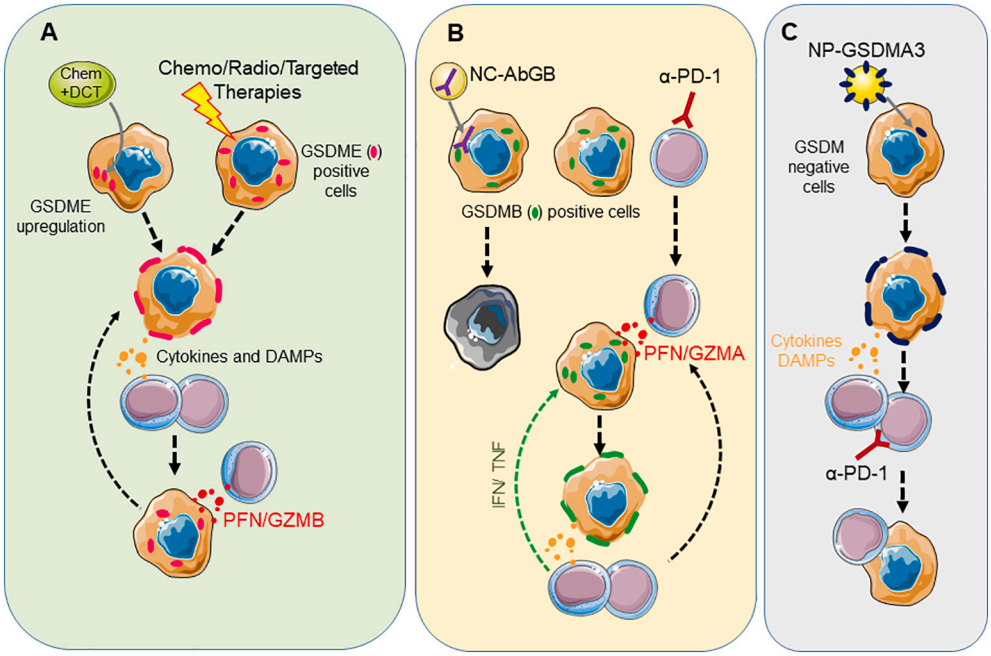

Fig. 2. Nanotherapies and standard therapies inducing GSDM-mediated cell death. A: GSDME-mediated therapies. **Left:** Nanomedicines combining Decitabine (DCT) with other chemotherapy agents can upregulate *GDSME* transcription and then trigger GSDME-mediated pyroptosis [91,92]. **Right:** Multiple chemotherapy agents or targeted therapies can initiate caspase-3 mediated GSDME pyroptotic activation. GSDME pyroptotic cells could release cytokines (IL1β) or DAMPs that enhance anti-tumor immune response through killer T-cells. T cells secrete perforins (PFN) and GZMB, which in turn activates GSDME-mediated pyroptosis in other tumor cells (positive feed-back loop). **B:** GSDMB-based therapies. **Left:** intracellular delivery of an anti-GSDMB antibody (AbGB) *via* Nanocapsules (NC) activates GSDMB intrinsic cell death activity in cancer cells [61]. The exact type of cell death was not determined. **Right:** Immune stimulation with anti-PD-1 treatment enhances immune recognition and targeting of GSDMB-positive cancer cells through NK and cytotoxic T cells attack. Killer T cells introduce perforins (PFN) and GZMA, which in turn activates GSDMB-mediated pyroptosis and the subsequent enhancement of anti-tumor immune response. T cells also produce interferons and TNFs that upregulates *GSDMB* transcription in cancer cells (positive feed-back loop). **C:** Delivery of GSDMA3 cytotoxic peptides *via* gold nanoparticles (NP) results in pyroptotic cell death, which activates the immune system. Combined treatment with anti-PD-1 immunotherapy boots further immune activation and cytotoxic T cell attack of tumor cells [95]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

inflammation) (Fig. 3). Regarding specificity, the pyroptosis of GSDM-expressing normal cells is partly responsible for the side effects of chemotherapy. Indeed, GSDME-expressing mice exhibit enhanced normal tissue damage and body weight loss after cisplatin treatment [33]. To limit normal cell pyroptosis, two studies revealed that the activation of vitamin-D/VDR signaling could reduce the side effects of platinum. Huang et al. [97] showed that in OSCC cells vitamin-D administration reduced the caspase-3/GSDME activation in normal tissues, thus lessening chemotherapeutic side effects, while Jiang et al. [98] reported that paricalcitol treatment (VDR agonist) inhibited NF-κB-mediated NLRP3/Caspase-1/GSDMD pyroptosis in a mouse model of cisplatin-induced acute kidney injury. Instead of activating endogenous GSDMs, like chemotherapy does, introducing GSDM-specific expressing vectors or peptides specifically into cancer cells (*i.e.* GSDMA3-NP [95])

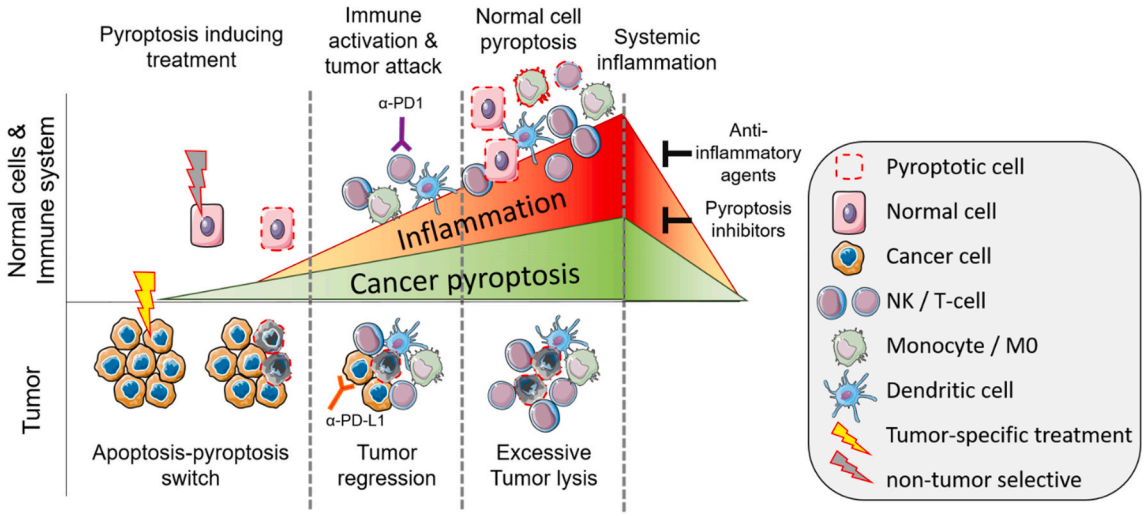

Fig. 3. Beneficial and negative effects of therapy-induced GSDM-mediated cell death depend on the balance between immune activation and the level of tumor specific pyroptosis. Therapies activating GSDM-mediated pyroptosis in both normal and tumor cells result in cytotoxic side effects. Tumor-selective agents can initially switch apoptosis to pyroptosis cell death. Depending on the amount of cancer pyroptosis, the inflammatory response can halt tumor progression. Immunotherapy agents (like anti-PD-1 or PD-L1) can synergize with pyroptosis-inducing drugs for efficient tumor eradication. However, excessive tumor pyroptosis could provoke an exaggerated inflammatory response that could lead to secondary pyroptosis of GSDM-expressing immune cells or normal cells and finally deleterious systemic inflammation. To limit inflammation and the amount of pyroptosis in normal cells GSDM-specific inhibitors may be useful.

could be a way to circumvent normal cell pyroptosis. However, even if these approaches were completely specific of tumor cells, they would require further refinement to increase their safety. It would be desirable the development of systems based on "activatable" GSDM peptides, rather than fully cytotoxic ones, in which the activation could be controlled (either by cleavage or other methods) in a time and dosage dependent way.

Indeed, the amount of tumor pyroptosis and the subsequent anti-tumor inflammatory response is a key factor to be controlled. Excessive cell lysis and release of pro-inflammatory signals could lead to over-activation of the immune system, secondary pyroptosis of immune or other normal cells and finally to systemic inflammation (Fig. 3). For example, chimeric antigen receptor (CAR) T cells [99] treatment strongly activating GSDME-mediated pyroptosis in tumors then induced GSDMD-regulated cell death in macrophages, leading to a cytokine release syndrome that could threaten patients' lives.

Finally, cancer progression and treatment response also depend on the duration of cancer pyroptosis and inflammation, as exemplified by GSDMC-mediated tumor necrosis, which favors breast cancer progression [40]. To explain this paradoxical effect of GSDMC-pyroptosis, Hou and collaborators [40] suggested that chronic pyroptosis leads to tumor necrosis, and this overpowers anti-tumor immunity by increasing tumor growth, while acute pyroptotic-mediated inflammation would augment the immune anticancer response.

Summarizing, only the right balance between cancer pyroptosis and inflammatory response may halt tumor progression without unwanted side-effects. The use of immunotherapy agents (*e.g.*, anti-PD-1/PD-L1) could synergize with pyroptosis-inducing drugs for efficient tumor eradication, and to limit excessive inflammation or of pyroptosis in normal cells, anti-inflammatory agents or GSDM-specific inhibitors may be required (Fig. 3).

7.2. Deciphering the mechanisms that regulate directly GSDM expression and protein function

Currently there is still limited knowledge on the precise mechanisms that control, in physiological and pathological contexts, GSDM expression and functions at diverse biological levels (transcriptional, post-translational, intracellular localization, *etcetera*) [100,101]. Understanding in detail these processes will help for the rational design of therapeutic agents and boost the possibilities of exploiting GSDM-mediated cell death in clinical oncology and other diseases [19,100,101]. The known regulatory mechanisms of GSDM expression and posttranslational modifications are schematized in Fig. 4 and discussed below.

Concerning transcription, *GSDM* gene silencing by promoter hypermethylation was reported in tumors/cancer cells (*GSDMA*, *GSDMC* and *GSDME* [51,54,77,102]), but also in leukocytes or other normal cells (*GSDMA*, *GSDMB, GSDMD* and *GSDME* [77,102–104]). Accordingly, demethylating agents can upregulate *GSDMA* [102], *GSDME* [33,92] or *GSDMD* [93,103] in cancer and normal cells. Since demethylating drugs are not tumor-specific, in order to induce *GSDM* upregulation and GSDM-mediated cytotoxicity selectively into cancer cells, these agents should be delivered using tumor-targeted nanomedicines, as described before [92].

The information about the transcription factors and regulatory elements modulating GSDM expression is currently limited and depends on the cell type (Fig. 4). In this sense, it has been described that TGFβ recruits LMO1 to the *GSDMA* promoter and activates its expression in gastric cells [51]. Moreover, *GSDMA*, *GSDMB* and other neighbor genes within 17q12–21 region are coordinately regulated by complex interactions of *cis* and *trans* elements [104]. Actually, *GSDMB* transcription is particularly complex, since it contains two alternative promoters, the cellular and the viral origin HERV-H LTR element (long terminal repeats of human endogenous retroviruses), which is integrated antisense-oriented into the *GSDMB* 5′-region [64,65]. Both promoters have specific activity in healthy tissues and cancer cells [64,65], being the activation of the LTR-derived promoter associated with the malignant transformation of precancerous tissues and tumor progression [58,64,65,105]. Regarding transcription factors, the Alu element of *GSDMB* cellular promoter contains Ikaros Family Zinc Finger 1 (IKFZ1) binding sites controlling gene expression in gastric epithelium [58]. Additionally, TNF-α (also IFN α/β/ɣ) upregulates *GSDMB* in cancer cell lines [37] and NFκB-signaling increases *GSDMB* during non-canonical pyroptosis in THP1 cells [39].

GSDMC transcription, but not other GSDMs, is upregulated by nuclear PD-L1. Thus, after hypoxia or antibiotic-type chemotherapy, p-Stat3 interacts with PD-L1 and facilitates its nuclear translocation, enhancing *GSDMC* expression in MDA-MB-231 cancer cells [40]. Besides, UV exposure upregulates *GSDMC* in skin keratinocytes [106]. In mice *Gsdmc2* and *Gsdmc4* rise after blocking TGFβ signaling in a colon cancer model [68], while all four *Gsdmc* genes are upregulated by IL-14 and IL-13 in worm-infected gut [107].

GSDMD promoter is directly activated by the interferon regulatory factor 2 (IRF2), and to a lesser extent by IRF1, during canonical or non-canonical pyroptosis in monocytes and endothelial cells [108]. In mouse adipocytes NFκB upregulates *GSDMD*, by binding its promoter, whereas melatonin treatment counteracts this effect [109]. Finally, TFAP2A directly drives *GSDMD* expression in hyperoxia-induced A549 lung cancer cells [110].

GSDME is upregulated by glucocorticoids and forskolin in leukemia cell lines [111], and downregulated by the miR-155-5p in breast cancer cells [112]. Moreover, upregulation of the LncRNA NEAT1 in response to irradiation enhances *GSDME* by inhibiting miR-448 levels in colorectal cancer cells [113]. In HepG2 cells, p53 directly induces *GSDME* transcription after different types of genotoxic stress [84], and in MCF7 cells both p53 and p63γ upregulate *GSDME* by binding to its p53 response element [114]. Finally, noise exposure upregulates *Pjvk* in mouse organs of Corti [115] but its transcriptional regulation in other normal or cancer cells is unknown.

At the protein level, the phosphorylation, likely by Polo like kinase 1 (Plk1), of specific Threonines in GSDMA (T8) and GSDME (T6) inhibits their oligomerization and pore formation [4,49]. This means that despite endogenous GSDM expression in cancer cells, phosphorylated GSDMs may not perform its cell death function. Consistent with this, cisplatin treatment effectiveness in esophageal cancer cells with high endogenous *GSDME* improved when a Plk1 inhibitor was co-administered [116], thus GSDME phosphorylation inhibition might strengthen chemotherapy response. The functional effect of potential phosphoresidues in other GSDMs (Fig. 4) is still untested.

Numerous studies highlight the importance of cysteines in controlling GSDM pyroptotic activity and demonstrate that small molecules acting on these residues have therapeutic utility in sepsis and other inflammatory diseases (reviewed in [19,20,100,101]) (Fig. 4). Succination by endogenous fumarate or Dimethyl-fumarate (DMF) on multiple cysteines block GSDMD cleavage, and likely GSDME too [117]. Necrosulfonamide, Bay 11–7082 and disulfiram covalently modify the GSDMD C191 (C192 in mouse) and inhibit GSDMD oligomerization/pore formation [118,119], while itaconate binds to GSDMD C77 (mouse) and might reduce GSDMD cleavage [100]. On the other hand, specific modification of Cys could activate GSDM pyroptosis. Inflammasome-induced mitochondrial ROS produces oxidation of GSDMD cysteines and favors pyroptosis [120]. Additionally, GSDME palmitoylation on C407/C408 is required for chemotherapy-induced pyroptosis in HCT116 cells, and palmitoylation inhibition reduced cell death [121]. Mechanistically, ZDHHC palmitoyltransferases palmitoylate GSDME CT and decrease the inhibitory interaction between NT and CT domains [121]. Moreover, cysteine residues can also play key roles in regulating non-pyroptotic functions of GSDMs, such the case of PJVK, where the C328 and C343 are required for ROS-induced LC3B-binding and pexophagy (see section 8).

Besides, very little is known about the mechanisms that control

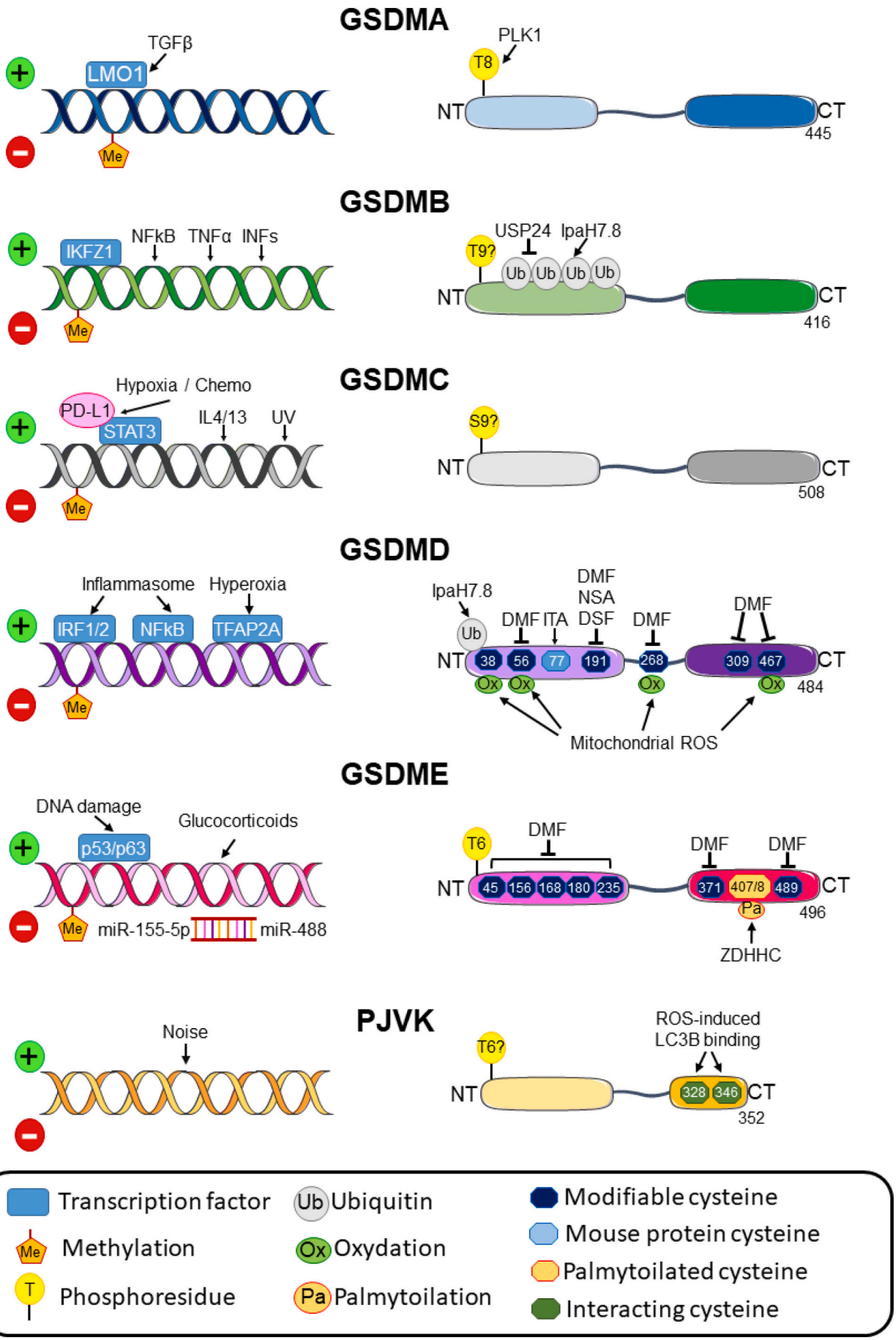

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Fig. 4. Transcriptional and posttranslational regulation of the Gasdermins. Left: Positive (+) and negative (−) transcriptional regulatory mechanisms. Transcription factors known to bind to each GSDM promoter are depicted on the DNA strand, and their specific regulatory stimuli are indicated on top (arrows). Other factors or stimuli with still undefined binding site are indicated by arrows. See text for detailed explanation and references. **Right:** Posttranslational modifications modulating GSDM functions. The activating (arrow) or inhibitory (bar-headed arrow) signals/drugs are shown on top of the residues. Phosphorylation: The phosphorylation of the first Threonine in GSDMA and GSDME blocks its pyroptotic function. Similar potential phosphoresidues (question mark) exists in GSDMB/C/PJVK but their effect are still unproven. Ubiquitination: bacterial IpaH7.8 polyubiquitinates GSDMB/D, and prompts their proteosomal degradation. Ubiquitin-specific peptidase 24 (USP24) may deubiquitinate GSDMB and increase its levels. Cysteine modification: important Cysteine residues, unless specified in the legend, are numbered according to the human GSDM proteins. Intrinsic fumarate and Dimethyl-fumarate (DMF) succinates diverse cysteines in GSDMD/E and block pyroptosis. Disulfiram (DSF) and Necrosulfonamide (NSA) bind covalently and block GSDMD oligomerization. Itaconate (ITA) might decrease cleavage of mouse GSDMD. Cysteine oxidation enhances GSDMD pyroptosis. Zinc finger DHHC domain-containing (ZDHHC) enzymes palmitoylate GSDME and increase pyroptosis. Two cysteines in PJVK C-terminus are required for LC3B binding. See text for further explanation and references.

GSDM protein synthesis, folding, stability and function. So far, ubiquitination control of GSDM levels has only been proven for GSDMB and GSDMD. In fact, human GSDMB and GSDMD, but not other GSDMs or mouse GSDMD, are polyubiquitinated by the *Shigella flexneri* IpaH7.8 ubiquitin ligase [122]. In *Shigella*-infected cells, GSDMB or GSDMD NT ubiquitination drives GSDM proteasomal degradation, therefore preventing GSDMB-pore-mediated bacterial killing [38] or GSDMD cell pyroptosis [122], respectively. In bladder cancer cells, the ubiquitin-specific peptidase 24 (USP24) binds and likely deubiquitinates GSDMB, thus increasing GSDMB protein levels [59]. Additionally, the interaction between GSDMB and the chaperone Hsp90β seems to regulate GSDMB protein stability, in particular the GSDMB-2 isoform, in breast cancer cells [56]. Surprisingly, GSDMB-1 overexpression in primary human bronchial epithelium cells activates the transcription of Hsp60/Hsp70 [62]. Interestingly, the activated GSDMA3 NT interacts with diverse chaperones (Hsp90α/β, Hsp70, Hop and Trap1), while Hsp90 inhibition reduces GSDMA3 NT mitochondrial targeting and import through Tom70, and the subsequent mitochondria damage and cell death, without affecting total GSDMA3 protein levels. Although preliminary, these data point to Hsp chaperones as potential regulators of GSDM protein stability and function.

7.3. Modulating the upstream/downstream regulators of GSDM pyroptosis

Apart from these direct regulatory mechanisms of GSDM genes and proteins, the GSDM pyroptotic function could be controlled biologically or pharmacologically by modulating upstream/downstream regulators at multiple levels (reviewed in [19,20,100,101]): a) Inflammasome activation. Both inhibitors of NLRPs, like MCC950, and activators, such as Talabostat, have been reported [19,100,101]; b) GSDM cleavage. Protease inhibitors (i.e VX-740) and molecules interfering with GSDM-protease interaction (e.g Ac-DMLD-CMK) can diminish pyroptosis [100,101]; c) GSDM oligomerization and pore formation. The Ragulator-Rag-mTORC1 pathway does not affect GSDMD-NT cell membrane targeting but is necessary for oligomerization and pore formation in macrophages. Thus, inhibiting Ragulator-Rag function blocks the subsequent events, including mitochondrial dysfunction and cell ballooning [123]; d) Ion influx and membrane repair. GSDM pores allow the influx/efflux of ions. Thus, Ca^{2+} influx can either activate the ESCRT-II complex, which encapsulate GSDMD pores into vesicles, leading to cell membrane repair [31], or enhance inflammasome activation, intracellular organella damage and cell death. Extracellular Mg^{2+} seems to partially block these effects [100,101]; e) Mitochondrial dysfunction and cell death. Mitochondrial and lysosome damage lead to cell death, prior to cell lysis [45,46]. Thus, increasing mitochondrial ROS fuels pyroptosis in cancer cells, while protecting mitochondrial damage dampens cell death [88,90,124–127]. f) Cell lysis. The final membrane rupture in pyroptosis and other cell death types is controlled by the NINJ1 proteins. While NINJ1 does not bind to GSDM pores, controlling NINJ1 function could block membrane burst and the secondary release of DAMPs [21]; e) Effects of DAMPs and cytokines on cell microenvironment. Indeed, agents that block interleukins (e.g anakinra) or HMGB1 have been shown to modulate pyroptosis-mediated inflammation in cancer [19,83,95,101].

In summary, drugs that target either directly GSDM proteins or their upstream/downstream effectors could be used in the near future to modulate pyroptosis and inflammation during cancer treatment (Fig. 3).

8. Other functions of GSDMs in normal and cancer cells

Apart from pyroptosis, GSDMs can mediate other diverse functions in physiological and pathological contexts, including cancer.

Originally, GSDMs were proposed to be involved in differentiation and tissue regeneration [2] and recently in the acquisition of the epidermal cornification program during species evolution [128]. GSDM function in skin differentiation and homeostasis seems to be complex and may involve cell death-dependent and independent mechanisms. Accordingly, UV (an inductor of differentiation and tumorigenesis) upregulates *GSDMC* in skin keratinocytes and this results in MMP1 induction *via* the ERK/JNK pathways [106], but higher doses of UVB irradiation provoke GSDME or GSDMD cleavage and pyroptosis [129,130].

Apart from this, GSDMs functions and autophagy are intermingled, and this crosstalk could result in cell death or other biological consequences. Precisely in NLRP3-activated neutrophils, GSDMD NT localizes to azurophilic granules and LC3+ autophagosomes, but not the plasma membrane, leading to pyroptosis-independent autophagy-mediated IL-1β secretion [131]. In contrast, GSDMA3 mutant proteins lacking autoinhibition or the GSDMA3 NT shuttle to mitochondria, and in parallel, upregulate LC3-II autophagy, finally leading to cell death [48]. Furthermore, after sound-induced oxidative stress, PJVK is upregulated and recruits LC3B to the damaged peroxisomes prompting pexophagy (autophagy of peroxisomes) and peroxisome proliferation leading to protection of auditory cells [132], but it is still unknown if PJVK forms pores in the peroxisomes. As autophagy is a key biological process in cancer development, the potential involvement of GSDMs in autophagy-associated mechanisms should be studied further.

Among GSDMs, particularly GSDMB can execute diverse cell death-independent functions, including bactericidal activity [38]. First, GSDMB in breast cancer cells promotes motility, potentially *via* Rac1 and Cdc42 GTPases, and cell invasion, which is associated with enhanced gelatin degradation activity and a particular expression pattern of metalloproteases [56,61]. Second, unlike other GSDMs, the full length GSDMB protein can bind *in vitro* to specific lipids, including sulfatides, suggesting that GSDMB might be involved in regulating intracellular lipid transport in cancer and inflammatory diseases [16]. This GSDMB-lipid interaction could also regulate cancer cell motility [61]. Third, GSDMB possesses a putative nuclear localization signal and mutation/deletion of this sequence excludes the protein from the nucleus [57,62]. In fact, GSDMB is commonly observed in the cell nucleus of normal and cancer tissues [6,57]. The biological function of nuclear GSDMB is uncertain but evidences suggest that GSDMB somehow regulates transcription of particular genes. Specifically, in human bronchial epithelial cells, nuclear accumulation of GSDMB was required for transcriptionally inducing TGF-β1 and 5-lipoxygenase [62]. In fact, the increased asthma exacerbation and airway remodeling observed in the mouse hGSDMBZp3-Cre model (ubiquitously expressing human

GSDMB) was functionally linked with the upregulation of the same set of genes [62], indicating that transcriptional control, rather than pyroptosis was the key pathological effect of GSDMB in asthma. Unfortunately, the function of nuclear GSDMB in cancer cells has not been identified yet.

Summarizing, GSDM pyroptosis-independent functions warrant further study to stablish the precise implication of these proteins in cancer.

9. Conclusions and future perspectives

- The GSDMs not only control "pyroptosis" in normal and cancer cells but also other cell death mechanisms (apoptosis, necrosis, autophagic cell death) in a biological context-dependent way. Whereas triggering GSDM-mediated cancer cell death could have positive impact in cancer treatment, resulting in tumor rejection and enhanced anticancer immunity, it can also have detrimental effects on normal cells (i.e chemotherapy side-effects) and the immune system (*e.g.*, systemic inflammation). Therefore, to exploit successfully GSDM-pyroptosis in cancer therapy, further research is needed to identify tumor-specific treatments and to define the right balance of intensity and timing between cancer pyroptosis and inflammation in clinical settings (Fig. 3). In these sense, the development of both inducers and inhibitors/"attenuators" of GSDM-triggered pyroptosis might be useful to control therapy response and disease progression.
- Altered expression of GSDMs have been documented in multiple tumor types, but the inconsistencies in the methods performed and data analyses have prevented so far defining the potential utility of GSDMs as predictive/prognostic biomarkers of cancer progression or clinical behavior. To this aim, it is required the standardization of immunohistochemical methods with fully validated antibodies in large series of human tumors with available clinical data.
- GSDMs have multiple functions (including cell-death independent) and their effects on tumor biology (pro-tumor or anti-tumor activities) may depend on the cellular context, in particular the interaction of cancer cells with the tumor microenvironment. Therefore, in order to unveil the precise roles of these proteins in cancer and to prove the potential of exploiting GSDM-mediated cell death in clinical oncology, it is essential more investigation on the specific mechanisms governing GSDM expression/functions in normal and neoplastic cells (i.e transcriptional regulation, protein cleavage and posttranslational modifications), as well as the development of *in vivo* GSDM GEMM models that could recapitulate the complex tumor-microenvironment interactions.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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