Lighting a Fire: Can We Harness Pyroptosis to Ignite Antitumor Immunity?

Zhibin Zhang, Ying Zhang, and Judy Lieberman

ABSTRACT

The impressive success of current cancer immunotherapy in some patients but lack of effectiveness in most patients suggests that additional strategies to promote antitumor immunity are needed. How cancer cells die, whether spontaneously or in response to therapeutic intervention, has a profound effect on the type of immune response mobilized. Here, we review research that highlights a previously unappreciated role of gasdermin-mediated inflammatory death (pyroptosis) to promote antitumor immunity and identifies gasdermin E as a tumor suppressor. Immune elimination of tumor cells by natural killer cells and cytotoxic T lymphocytes, which is the final key event in antitumor immunity, was previously thought to be noninflammatory. The research shows that gasdermin expression in tumor cells converts immune cell–mediated killing to inflammatory pyroptosis when cell death–inducing granzymes directly cleave and activate gasdermins. Granzyme B cleaves gasdermin E, and granzyme A cleaves gasdermin B. The data suggest the potential to harness pyroptosis in the tumor to ignite an effective immune response to immunologically cold tumors. Gasdermin expression also augments toxicity of cancer therapy—gasdermin E expression by B-cell leukemias and lymphomas is a root cause of chimeric antigen receptor (CAR) T–cell cytokine storm, and its expression in normal tissues promotes the toxicity of chemotherapeutic drugs. Even though our knowledge about the role of pyroptosis in cancer is growing, there is still a lot to learn—what activates it, how it is regulated, when it is beneficial, and how it can be harnessed therapeutically to improve cancer immunotherapy or reduce therapy-related toxicity.

Introduction to Gasdermins and Pyroptosis

To better harness the potential power of antitumor immunity, novel strategies are needed to recruit and activate tumor-infiltrating lymphocytes (TIL) to eliminate cancer cells and to overcome cancer cell editing, which enables tumors to be ignored or resist antitumor immunity. How cancer cells die, whether spontaneously in response to stresses in the tumor microenvironment, such as hypoxia or unmet metabolic needs, or by attack by killer lymphocytes or cancer therapy, has a profound effect on immune tumor control and the effectiveness of therapeutic interventions. Cells can die by apoptosis, a noninflammatory death that leads to rapid phagocytic clearing of the dying cell, which is generally not immunogenic, or by programmed necrosis (either necroptosis or pyroptosis) that rapidly disrupts the cell membrane, releasing intracellular inflammatory mediators, including inflammatory cytokines (IL1β, IL18, and IL6) and alarmins, such as ATP and HMGB1 (1, 2). Necroptosis and pyroptosis are inflammatory and immunogenic, and recruit and activate immune cells in the tumor (3–5). However, inflammation is a double-edged sword that can promote both tumorigenesis and antitumor immunity at all stages of tumor development (6). Whether tumor-promoting or -inhibitory effects dominate, the effect of inflammation likely depends on the particular genetic and epigenetic features of the tumor as well as differences in host inflammatory status and immunity. Which dominates also likely varies during tumor development. The tumor-promoting effects of inflammation may have their strongest effect in the early stages of tumorigenesis but become much less important once a tumor is well established.

The gasdermins (GSDM), a family of cytosolic proteins expressed under basal conditions mostly in macrophages and dendritic cells, the skin, and mucosal epithelia, are the final mediators of pyroptosis (7, 8). In fact, pyroptosis is now defined as GSDM-mediated inflammatory programmed cell death (9). Humans express five GSDMs (A–E). A sixth, more distantly related protein, DFNB59 (also known as PJVK), is not known to cause cell death. GSDMs A–E are composed of an active N-terminal membrane-disrupting domain connected by a flexible linker to a C-terminal domain that binds to the N-terminal domain to suppress its activation. Cleavage of the linker liberates the N-terminal domain to bind to cell membranes and assemble into pores that kill the cell and serve as conduits to release inflammatory mediators (10–14). The flexible linker is especially vulnerable to attack by proteases and may serve as a simple alarm system for detecting and responding to mislocalized cytosolic proteases, which are normally confined to membrane-delimited organelles (4). GSDMD, which is most highly expressed in hematopoietic cells, is the most well-studied GSDM, because it is largely responsible for inflammation triggered when immune sentinel cells sense pathogen- or host danger–associated molecular patterns (PAMPs or DAMPs, respectively). GSDMD is cleaved by the inflammatory caspases [caspase-1 (in mice and humans), caspase-4/-5 (in humans), and caspase-11 (in mice); refs. 7, 8), which are recruited to large supramolecular complexes called inflammasomes. After cytosolic sensing of PAMPs or DAMPs, inflammasomes assemble and activate the inflammatory caspases by proximity-induced autoproteolysis. Activated caspase-1 processes key proinflammatory IL1 family cytokines into their active forms that cause fever, but GSDM-mediated pyroptosis is needed to release them, because they lack a signaling peptide for secretion. IL1 amplifies inflammation by triggering cascades that activate NFκB and lead to secretion of other inflammatory cytokines, such as IL6 and TNFα, and chemokines that recruit immune cells to sites of inflammation.

Program in Cellular and Molecular Medicine, Boston Children's Hospital and Department of Pediatrics, Harvard Medical School, Boston, Massachusetts.

Corresponding Author: Judy Lieberman, Boston Children's Hospital and Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115. Phone: 617-713-8600; Fax: 617-713-8620; E-mail: judy.lieberman@childrens.harvard.edu

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Although IL1 release depends on GSDMD pores, after some stimuli, macrophages and other cells can repair the damage to the cell membrane by the pores and survive (15, 16). These surviving macrophages have been termed "hyperactivated" because they seem to have increased antigen-presenting and other immune functions compared with macrophages in which the inflammasome pathway is not activated.

GSDME: A Tumor Suppressor?

Unlike GSDMD, GSDME is activated by caspase-3 after cells expressing GSDME receive classic apoptotic signals (17, 18). Although GSDME expression in cells triggered to undergo apoptosis does not change how many cells die, it converts slow noninflammatory apoptosis into more rapid and inflammatory pyroptotic death that can stimulate an effective antitumor immune response (4). How GSDMs A–C are activated and their physiologic role are not as well understood (9). How GSDM expression is regulated also has not been well studied, although these genes can be upregulated by cytokines and immune signaling in cells that do not ordinarily express them, including lymphocytes. For example, GSDMA is upregulated by TGFβ (19), GSDMB expression can be induced by IFNα, IFNβ, IFNγ, and TNFα (5), GSDMD by IRF2 (20), and GSDME by corticosteroids and forskolin (21). *GSDME* transcription is reportedly activated by p53, which is inactivated in many cancers (22). As of now, virtually nothing is known about how the GSDM pathways are regulated. Phosphorylation of Thr6 of GSDME by an unknown kinase has been reported to inhibit pore formation (23), but it is likely that other posttranslational modifications and mechanisms to regulate pore formation will be uncovered.

Previous data suggested that GSDMs, especially GSDME, might act as tumor suppressors (24). GSDME is mostly expressed in the brain, female reproductive tract (especially the placenta), kidney, and muscle, but its physiologic role in those tissues is unknown. *GSDME* is epigenetically inactivated by promoter DNA methylation in approximately 90% of human cancer cell lines in the NCI-60 collection, and in gastric, colorectal, and breast cancer, relative to normal tissue, and has loss-of-function mutations in some other cancers (18, 24). The DNA methylase inhibitor 5-aza-2′-deoxycytosine (decitabine) can derepress *GSDME* expression *in vitro*. *GSDME* derepression by decitabine may suppress colony formation and tumor cell proliferation in gastric cancer, melanoma, and colorectal cancer, and may suppress invasiveness of breast cancer (23, 24). Worse 5-year survival and an increase in lymph node metastases are associated with reduced GSDME in breast cancer (25). Moreover, lack of GSDME promotes melanoma cell line resistance to etoposide, which is rescued by *GSDME* overexpression (24). In addition, treatment of lung cancer cells with KRAS, EGFR, or ALK inhibitors leads to caspase-3–mediated GSDME activation, which increases the antitumor effectiveness of these drugs (26). The data for the other GSDMs are less clear. High GSDMD expression predicts poor prognosis in lung adenocarcinoma (27), and GSDMB and GSDMC expression can predict better or worse survival in patients, depending on the cancer type (5).

Inflammation in Cancer: A Double-Edged Sword

The lack of a clear correlation between GSDM expression and cancer prognosis likely reflects the complex role of inflammation in tumorigenesis. Chronic inflammation caused by infection, autoimmunity, and environmental or dietary exposure promotes tumor initiation, progression, angiogenesis, and metastasis through multiple mechanisms that involve not only the tumor, but also TILs and tumor stromal cells (6). Some key transcription factors activated by PAMPs and DAMPs, including NFκB, AP1, and Stat3, promote tumorigenesis. These transcription factors also drive inflammation, and the production and release of proinflammatory cytokines, including IL1β, IL6, TNFα, and IL17, and chemokines. The tumor-promoting effect of inflammation was highlighted by the CANTOS trial, which evaluated a therapeutic blocking antibody to IL1β, the signature proinflammatory cytokine released during pyroptosis, for cardiovascular disease prevention (28). IL1 blockade significantly improved overall cancer survival and reduced the incidence of lung cancer.

However, therapy-induced or tumor-associated acute inflammation also boosts antitumor immunity by promoting antigen presentation by mature dendritic cells and macrophages, the recruitment of immune cells to the tumor microenvironment, and the clonal expansion and development of antitumor functionality and memory of innate and adaptive killer lymphocytes. The role of inflammation in promoting antitumor immunity has been studied in the setting of established tumors, although it is also likely important in immune surveillance of the developing cancer. Full functional activation of killer lymphocytes requires a host cytokine danger signal as a key "adjuvant" third signal in addition to T-cell receptor or natural killer (NK)–activating receptor recognition of tumor antigens or stress signals (signal 1) and costimulation (signal 2). Signal 3 can be provided by interferons, interleukins, or inflammation, either directly by the tumor cell or indirectly by the macrophages or dendritic cells that phagocytose tumor cells and present tumor antigens or are exposed to alarmins released by dying tumor cells. Activation in tumor cells of the innate immune cGAS-STING-type I IFN pathway triggered by sensing cytosolic DNA, released from unstable micronuclei that form as a result of cancer cell DNA damage and chromosomal instability, promotes antitumor immunity (29). Innate immune sensing of cytosolic DAMPs can trigger interferons and/or inflammatory responses. However, because cells die quickly by pyroptosis, pyroptosis inhibits IFN production, which requires gene transcription. What effect IFNs have on inflammation is less clear and may depend on the context. How these two pathways influence each other requires further study, because innate immune pathways typically have considerable cross-talk. Although either IFNs or inflammation promote effective antitumor immunity, it will be important to compare how they do it and whether there are differences in how effective and how sustained immune protection is depending on signal 3.

GSDME Spontaneously Activates Antitumor Immunity

Research suggests a critical role of GSDMs and pyroptosis in activating antitumor immunity and suppressing tumor growth. To investigate the hypothesis that GSDME is a tumor-suppressor protein, we studied the effect of GSDME expression on mouse tumor growth and antitumor immunity in immunocompetent mice using a variety of tumors in which we manipulated *Gsdme* expression (4). As expected, in *Gsdme*-expressing tumors, activation of caspase-3 triggered pyroptosis, while in *Gsdme*-nonexpressing tumors, caspase-3 activation caused apoptosis. Tumor *Gsdme* expression strongly suppressed *in vivo* growth of mouse melanoma and breast and colon tumors. Tumor suppression was mediated by killer lymphocytes because it was abrogated in mice genetically deficient in perforin or lymphocytes, or in mice depleted of NK and $CD8^+$ T cells. GSDME expression enhanced tumor-associated macrophage phagocytosis and the

number and functions of tumor-specific TILs. Moreover, vaccination with GSDME-overexpressing B16 melanoma suppressed the subsequent outgrowth of B16 tumors that only weakly express endogenous GSDME (4). In another study, knocking out *Gsdme* in B16 melanoma expressing low levels of GSDME, significantly reduced survival of tumor-implanted mice (23). Thus, GSDME expression by the tumor turns immunologically "cold" tumors, such as B16 melanoma, into "hot" tumors, actively under immune control.

It is not clear what features of pyroptotic tumor cells are responsible for activating antitumor immunity. Immunostimulatory alarmins released during pyroptosis, including ATP and HMGB1, are prime suspects. Extracellular ATP triggering of purinergic P2X7 receptor signaling in cancer cells or infiltrating immune cells would enhance pyroptosis (30), creating a positive feedback loop. However, in one study of GSDME$^+$ tumor–bearing mice, no expression of *Il1b* was detected in tumor cells, and IL1β was not detected in the sera (4), making it unlikely that the tumors secreted inflammatory cytokines. It is likely that dendritic cells and macrophages in the tumor are activated by pyroptotic tumor cells to both better present tumor antigens and secrete inflammatory cytokines and chemokines, enhancing T-cell recruitment and activation. The lack of detection of IL1β in the sera of these mice does not imply that IL1β was not released from tumor-associated macrophages and dendritic cells, because IL1β is so rapidly neutralized *in vivo* that it is often not detected in the sera even during extensive systemic inflammation.

Activation of other GSDMs expressed in tumor cells should also cause pyroptosis. Although this has not been studied much, GSDMD-mediated pyroptosis has been shown to occur in human acute myelogenous leukemia (AML) cell lines that express caspase-1 and GSDMD (31). Pyroptosis in these cells could be triggered by a serine dipeptidase inhibitor (Val-boroPro), which activated the Nlrp1b inflammasome and caspase-1 to trigger GSDMD cleavage and activation. Val-boroPro treatment of mice bearing these leukemias strongly reduced AML proliferation *in vivo* and prolonged survival in a *CASP1*-dependent manner. However, because these were human AML cells, and these experiments were done in immunodeficient mice, tumor cell death rather than an immune response was responsible for better tumor control. It would be interesting to evaluate the immune consequences of GSDMD activation in the tumor by comparing the effect of Val-boroPro on progression of mouse AML tumors in immune-deficient and -competent mice.

Granzyme B Cleaves GSDME and Activates Pyroptosis

Killer lymphocytes have previously been thought to eliminate their target cells by noninflammatory caspase-dependent and -independent programmed cell death (1). Dying cells externalize phosphatidyl serine (PS), which is recognized by phagocytes. The phagocytic cells rapidly engulf the dying cell before it undergoes secondary necrosis. Alarmins that might be released from necrotic cells are degraded within phagosomes and not released into the extracellular milieu. Thus, killer cell–mediated death has been thought to be noninflammatory, minimizing bystander cell damage. However, newer studies show that killer lymphocytes activate pyroptosis in GSDME-expressing tumor cells even when caspase-3 is inhibited or knocked out (4). Tumor pyroptosis depends on release of cytotoxic granules, perforin, and granzymes. Indeed, granzyme B, which cleaves after Asp residues like the caspases, but not the other abundant granzyme (granzyme A), which cleaves after Arg or Lys, directly cleaves GSDME at the same residue as caspase-3, Asp270. Although caspase-3 was not required for pyroptosis, it amplifies killer cell–induced pyroptosis by granzyme B because granzyme B also activates caspase-3, which can cleave more GSDME (**Fig. 1**). Tumors expressing a D270A granzyme B and caspase-3 noncleavable mutant GSDME grew as well as *Gsdme*-deficient tumors, indicating that caspase-3 or granzyme B cleavage of GSDME was responsible for antitumor immunity. Because inflammation in the tumor increases TIL recruitment and cytotoxic TIL function, once tumor pyroptosis is triggered, recruited TIL will further amplify inflammation within the tumor. It is not clear how tumor pyroptosis is triggered in the first place. The trigger could be spontaneous death in poorly perfused regions of the tumor due to hypoxia or inadequate nutrients, or due to tumor attack by innate or innate-like lymphocytes that recognize stressed tumor cells. A key feature is that this "immunogenic cell death" of the tumor occurs spontaneously without any exogenous therapy.

Chimeric Antigen Receptor T–cell Activation of Pyroptosis Causes Cytokine Storm

Chimeric antigen receptor (CAR) T cells, like native killer lymphocytes, transfer granzymes into target cells. A recent study shows that during CAR T–cell attack, granzyme B leads to activation of both GSDME and caspase-3, causing pyroptosis (32). CAR T–cell therapies, currently approved for treating certain types of lymphoma and acute lymphoblastic leukemia, which often express GSDME, all use anti-CD19 to target cells. Because the CAR has much higher affinity for its ligand than conventional T-cell receptors have for MHC-peptide antigens and CAR T–cell killing has been optimized by engineering costimulatory domains on the receptor, CAR T cells much more efficiently induce pyroptosis than non-engineered cytotoxic T lymphocytes. How much pyroptosis contributes to the effectiveness of CAR T–cell therapy has not been evaluated. Cytokine-release syndrome (CRS), a serious toxicity of current CAR T–cell therapy, is a consequence of extensive pyroptosis. In this study (32), CRS in mice was blocked by knocking out *GSDME* in the tumor. Moreover, because the tumor burden in these hematologic malignancies is often high and these tumors often express GSDME, the numbers of killed target cells undergoing pyroptosis is very large. It is also possible (although this has not been reported) that the inflammatory conditions of CRS might induce GSDME expression in normal B cells, which do not ordinarily express GSDME, causing a switch from apoptotic death to pyroptosis in these abundant CAR T–cell targets, enhancing CRS. Moreover, the culture supernatants from leukemia cell lines or primary leukemias cocultured with CAR T cells activate caspase-1–GSDMD–mediated pyroptosis in macrophages, further increasing cytokine release (**Fig. 1**). ATP was the alarmin in these supernatants responsible for macrophage inflammasome activation. ATP is recognized by the macrophage P2X7 receptor, leading to NLRP3 inflammasome activation and GSDMD pores (30). In CAR T cell–treated patients, the clinical severity of CRS correlated with GSDME expression in the tumor. Currently, CRS is generally well suppressed by blocking signaling of the inflammatory cytokine IL6 using anti-IL6R (tocilizumab), sometimes given prophylactically. CAR T–cell therapy can also cause neurotoxicity, which can be severe and is not responsive to blocking IL6. Little is known about the mechanism behind it, except that it is inflammatory, and the blood–brain barrier is disrupted. Given the high expression of GSDME by neurons and of GSDMD by microglia, pyroptosis might be involved in neurotoxicity caused by CAR T–cell therapy.

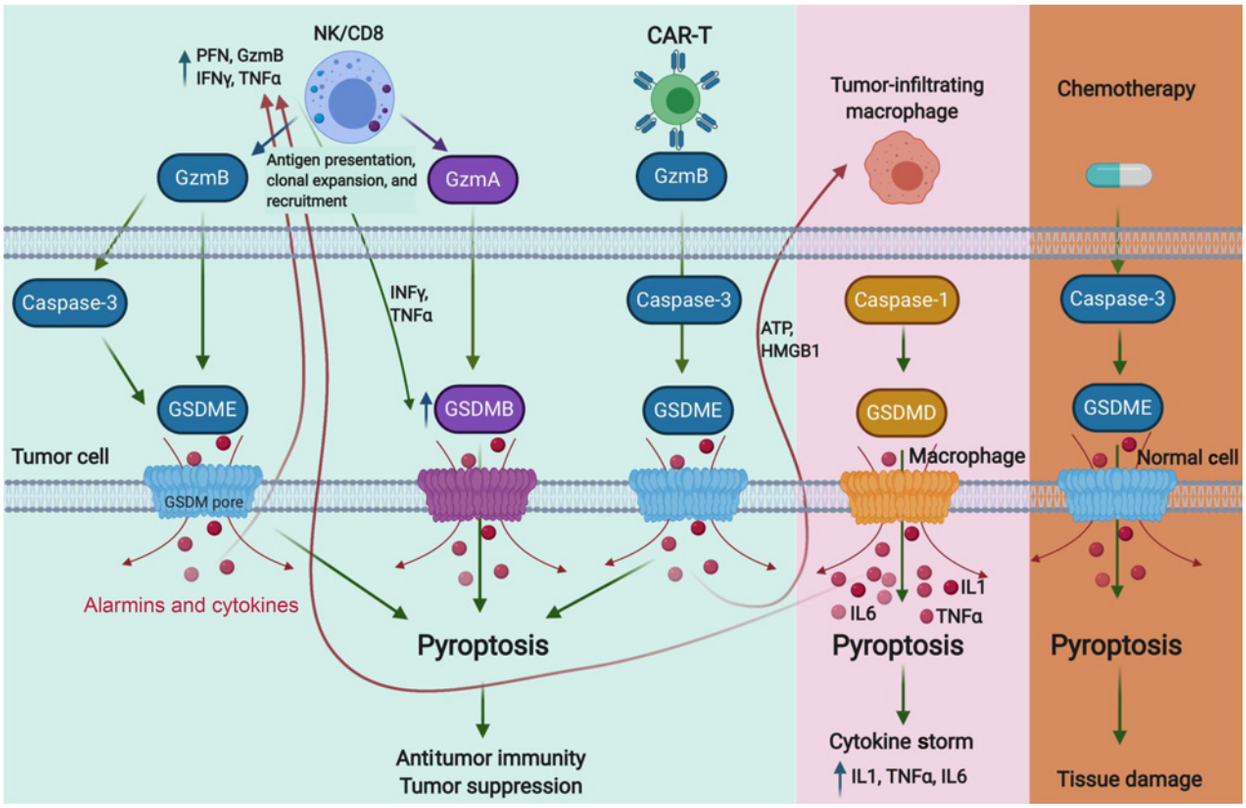

Figure 1.

Pyroptosis in GSDM-expressing tumors activates antitumor immunity. Pyroptosis can be triggered by caspase-3 activation or killer cell attack in tumors expressing GSDME to suppress tumor growth and promote antitumor immunity. NK and $CD8^+$ cytotoxic lymphocytes and CAR T cells activate pyroptosis in GSDM-expressing tumors directly when granzyme B (GzmB) cleaves and activates GSDME or granzyme A (GzmA) activates GSDMB. Alarmins released from tumor cells undergoing pyroptosis can also activate pyroptosis in tumor-infiltrating macrophages and dendritic cells to enhance antigen presentation and functional activation of TILs. Secondary activation of macrophages can mediate CRS during CAR T–cell therapy. In addition, pyroptosis triggered by chemotherapy drugs in GSDME-expressing normal gastrointestinal and lung epithelial cells and hematopoietic cells contributes to their toxicity. Green arrows indicate direct pathways leading to pyroptosis, and red arrows indicate cytokine and alarmin release and their secondary effects. PFN, perforin.

Granzyme A Activates Pyroptosis in GSDMB-Expressing Tumors

Another study showed that cytotoxic lymphocytes also induce pyroptosis in GSDMB-expressing tumor cells when granzyme A selectively cleaves and activates GSDMB independently of any caspase (**Fig. 1**; ref. 5). Like GSDME, GSDMB is not expressed by most cancer cell lines—only 3 of 39 human cancer cell lines tested expressed GSDMB and were sensitive to granzyme A–induced pyroptosis. However, GSDMB is induced in some tumors by type I IFNs and by IFNγ and TNFα, suggesting that GSDMB might be induced in tumors under immune attack to amplify pyroptosis. Although ectopic expression of GSDMB did not have any effect on growth of colorectal cancer or melanoma tumors in mice, GSDMB expression synergized with anti–PD-1 checkpoint blockade to suppress tumor growth. The lower *in vivo* potency of granzyme A against GSDMB-expressing tumors, compared with granzyme B against GSDME-expressing tumors, could be due to caspase-3 amplification of granzyme B–mediated pyroptosis. Aside from caspases and granzymes, the only other proteases known to cleave GSDMs are neutrophil elastase and cathepsin G (33–35), neutrophil serine proteases, which cleave GSDMD and are highly homologous to the granzymes. GSDMD activation is also needed for neutrophil netosis (34), which strongly promotes breast and colon cancer metastasis (36).

Harnessing Tumor Cell Pyroptosis for Immunotherapy

Because pyroptosis in the tumor activates antitumor immunity, devising therapeutic strategies to induce pyroptosis could ignite protective antitumor immune responses or broaden immunotherapy responses to checkpoint inhibitors or other immunotherapies in immunologically "cold" and unresponsive tumors. Potentially this could be done by inducing *GSDME* in tumors in which it is epigenetically silenced and taking advantage of spontaneous events or killer cells or chemotherapy to activate pyroptosis and inflammation. Decitabine, which upregulates *GSDME* and converts apoptosis to pyroptosis in tumor cells *in vitro* (18), or other epigenetic drugs could be one way to do this, although they would not be specific because other epigenetically silenced tumor-suppressor genes and genes that promote antitumor immunity could be induced. Consistent with this idea, *RIPK3*, the central mediator of necroptosis, another form of inflammatory and immunogenic cell death, is also suppressed in multiple tumor cells by DNA methylation (37). It is worth evaluating whether

one of the mechanisms behind the therapeutic effectiveness of decitabine in human myelodysplasia and leukemia might be induction of GSDME. A study showed that pretreatment with decitabine upregulated GSDME and enhanced the potency of nanoparticle-delivered cisplatin in a mouse triple-negative breast cancer model (38). Another study showed that combination treatment with a BRAF inhibitor and a MEK inhibitor induces GSDME-mediated pyroptosis to activate antitumor immunity in GSDME-expressing melanoma (39). These studies indicate that chemotherapy can be potentiated by converting apoptosis to pyroptosis.

An alternative therapeutic strategy would be to introduce an N-terminal GSDM fragment or a full-length GSDM together with a GSDM activator selectively into tumor cells. In a mouse triple-negative breast cancer model, intravenously injected nanoparticles linked to GSDMA3 (a mouse isoform of human GSDMA) were preferentially taken up into tumors and released the pore-forming fragment, suppressing tumor growth in a T cell–dependent manner. These nanoparticles also potentiated tumor suppression by checkpoint inhibition (40). Almost the entire tumor regressed even when only a small fraction (10%–30%) of the tumor cells were transduced, consistent with a vaccination-like immune-mediated effect. Some of the immune effects in this study may have been secondary to induction of pyroptosis in macrophages in addition to tumor cells, because macrophages took up the nanoparticles and died, and IL1β was increased in the serum of these mice.

Contribution of Pyroptosis to Chemotherapy Toxicity

At the same time that pyroptosis is potentiating various types of tumor therapy, GSDM expression in normal cells also increases the toxicity of cancer therapy (**Fig. 1**). GSDMs are highly expressed in the gastrointestinal tract and hematopoietic cells, which are important sites of chemotherapy toxicity. *Gsdme*$^{-/-}$ mice tolerate chemotherapy drugs, such as cisplatin, 5-FU, and bleomycin, much better than *Gsdme*$^{+/+}$ wild-type mice (18); weight loss, gastrointestinal, hematopoietic, and pulmonary toxicities are strongly reduced and splenic lymphocyte depletion attenuated. GSDME is also highly expressed in cardiac muscle and might exacerbate anthracycline cardiotoxicity, although this has not been studied. CD34^{+} hematopoietic stem cells also express caspase-1 and GSDMD. Although Val-boroPro induces pyroptosis in AML cells, it also activates pyroptosis in normal B cells and CD34^{+} hematopoietic stem cells (31). Because of potential toxicity to normal tissue, antitumor benefits of triggering GSDM activation will be most effective if GSDM activation is restricted to the tumor, either by targeted cancer therapy that does not kill noncancerous cells and activate caspase-3–GSDME-pyroptosis in normal cells and/or by selective GSDM expression or activation in the tumor.

Concluding Remarks

Pyroptosis has a profound effect on tumors. In tumor cells, it enhances antitumor immunity, but in tumor-infiltrating macrophages and in normal mucosal cells or blood cells, it can exacerbate chemotherapy toxicity or CRS as a result of CAR T–cell therapy. As we learn more about pyroptosis, we may be able to harness our knowledge to improve outcomes for patients with cancer.

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