Gasdermin E suppresses tumour growth by activating anti-tumour immunity

Cleavage of the gasdermin proteins to produce pore-forming amino-terminal fragments causes inflammatory cell death (pyroptosis)\(^3\). Gasdermin E (GSDME, also known as DFNAS)—mutated in familial ageing-related hearing loss\(^2\)—can be cleaved by caspase 3, thereby converting noninflammatory apoptosis to pyroptosis in GSDME-expressing cells\(^1-5\). GSDME expression is suppressed in many cancers, and reduced GSDME levels are associated with decreased survival as a result of breast cancer\(^2,6\), suggesting that GSDME might be a tumour suppressor. Here we show that 20 of 22 tested cancer-associated GSDME mutations reduce GSDME function. In mice, knocking out Gsdme in GSDME-expressing tumours enhances, whereas ectopic expression in Gsdme-repressed tumours inhibits, tumour growth. This tumour suppression is mediated by killer cytotoxic lymphocytes; it is abrogated in perforin-deficient mice or mice depleted of killer lymphocytes. GSDME expression enhances the phagocytosis of tumour cells by tumour-associated macrophages, as well as the number and functions of tumour-infiltrating natural-killer and CD8\(^+\) T lymphocytes. Killer-cell granzyme B also activates caspase-independent pyroptosis in target cells by directly cleaving GSDME at the same site as caspase 3. Uncleavable or pore-defective GSDME proteins are not tumour suppressive. Thus, tumour GSDME acts as a tumour suppressor by activating pyroptosis, enhancing anti-tumour immunity.

Evidence that GSDME might act as a tumour suppressor (including epigenetic GSDME inactivation by promoter DNA methylation in many cancer lines and primary cancers\(^2,6\); suppression by GSDME of colony formation and cell proliferation in gastric cancer, melanoma and colorectal cancer and of breast-cancer invasiveness; and worse five-year survival and increased metastases from breast cancers that poorly express GSDME\(^2,6\)) prompted us to probe whether and how GSDME might function as a tumour suppressor. Induction of inflammatory cell death in GSDME-expressing cancers subjected to intrinsic stresses (hypoxia or endoplasmic-reticulum stress) or extrinsic challenges (chemotherapy, radiation or attack by cytotoxic lymphocytes) that activate caspase 3 could have a marked effect on the tumour microenvironment, immune-cell recruitment and function, and tumour growth. Here we show that GSDME in tumours suppresses tumour growth by increasing the anti-tumour functions of tumour-infiltrating natural-killer (NK) and CD8\(^+\) T killer lymphocytes.

**GSDME converts apoptosis to pyroptosis**

We first found that Gsdme messenger RNA and/or protein in seven mouse tumour cell lines was within the range seen in primary breast cancers and colorectal cancer in The Cancer Genome Atlas (TCGA) database (https://www.cancer.gov/tcga) (Extended Data Fig. 1a–c). Gsdme was knocked out in two highly expressing mouse lines, namely EMT6 triple negative breast cancer and CT26 colorectal cancer (Extended Data Fig. 1d–g), and one human neuroblastoma line, namely SH-SYSY (Extended Data Fig. 1h). Moreover, Gsdme or GSDME was stably expressed in two poorly expressing mouse cancer cell lines—B16-F10 melanoma (hereafter referred to as B16) and 4T1E triple negative breast cancer (Extended Data Fig. 1i, j)—and in a human cervical carcinoma cell line (HeLa) (Extended Data Fig. 1k).

To examine the effect of GSDME on cell death, we treated B16 cells that were expressing empty vector or overexpressing mouse GSDME (mGSDME) with raptinal, a rapid caspase 3 activator\(^7\). We found that
mGSDME did not alter cell death kinetics and extent, as measured by annexin V/propidium iodine staining. Ectopic expression of GSDME (n = 6 mice per group) in B16 cells (Fig. 1b) did not trigger pyroptosis in B16 cells expressing empty vector (with pyroptosis being assessed by uptake of SYTOX green and release of lactate dehydrogenase (LDH)), but did so in mGSDME-overexpressing cells after roughly 40 min (Extended Data Fig. 2b, c). Numerous cell membrane and SYTOX green uptake were detected by time-lapse fluorescence microscopy in mGSDME-overexpressing cells only (Extended Data Fig. 2d and Supplementary Videos 1, 2). Empty-vector cells instead became apoptotic (detached and shrunken, with membrane blebbing). After adding rapamycin, caspase-3-mediated cleavage was detected independently of mGSDME overexpression, beginning within 20 min, and mGSDME cleavage was detected coincidentally if mGSDME was expressed (Extended Data Fig. 2e). Although rapamycin did not appreciably change cellular levels of the nuclear protein HMGB1, only mGSDME-overexpressing B16 cells released HMGB1. Similarly, rapamycin or tumour-necrosis-factor (TNF)-related apoptosis-inducing ligand (TRAIL) converted apoptosis to pyroptosis only in HeLa cells that overexpressed human GSDME (hGSDME) (Extended Data Fig. 2f–i and Supplementary Videos 3, 4). Thus, GSDME is cleaved rapidly after caspase 3 activation and then permeabilizes the cell membrane, converting apoptosis to pyroptosis, as previously reported.3,4.

**Loss-of-function GSDME mutations in cancer**

If GSDME is a tumour suppressor, then some GSDME expressing cancers might have loss-of-function (LOF) mutations. We examined the TCGA database of single-nucleotide polymorphisms in primary cancers for GSDM mutations (Extended Data Fig. 3a). GSDME and GSDMC had the most mutations, and there were more GSDME mutations around the caspase 3 cleavage site. Single-nucleotide polymorphisms in the N terminus were mapped onto the GSDME N-terminal pore, modelled on the basis of the mouse GSDMA3 N terminus.18 We expressed 18 GSDME N-terminal conserved-site mutants in HEK293T cells and tested the cells for pyroptosis. All of the mutant proteins were well-expressed (Extended Data Fig. 4c, f), and 16 of 18 cancer-associated single-nucleotide polymorphisms substantially reduced LDH release compared with the wild-type GSDME N terminus (Extended Data Fig. 3d, g), suggesting that some cancer-associated GSDME N-terminal mutations cause LOF. Mutations in the globular domain close to the oligomerization and cell-membrane-binding sites had the largest effect. Four premature stop mutants (GSDME 1–46, 1–210, 1–451 or 1–491) also did not cause pyroptosis in HEK293T cells, although some of the mutated proteins (1–451 and 1–491) may have been unstable (Extended Data Fig. 3h–i). We verified a previously described19 LOF F2A mutation (single-letter amino-acid code) (Extended Data Fig. 3m, n). F2A, D18 and P212 are conserved between human and mouse GSDME. Expression of mGSDME N-termini with an F2A, D18V or P212L mutation in HEK293T cells, or of F2A or P212L full-length mGSDME mutants in 4T1E cells, markedly reduced spontaneous or rapamycin-induced pyroptosis, respectively, compared with unmutated mGSDME (Extended Data Fig. 3o, r). Thus, 20 of 22 (91%) studied cancer-related GSDME mutations cause LOF.

**GSDME affects tumour growth and immunity**

EMT6 and CT26 tumours knocked out for Gsdme grew much faster in immunocompetent mice than did tumours expressing endogenous Gsdme (Extended Data Fig. 4a, f). The tumour microenvironment of Gsdme−/− EMT6 tumours had fewer CDS T and NK cells and a trend towards fewer tumour-associated macrophages (Extended Data Fig. 4b). Tumour-infiltrating lymphocytes (TILs: CDS T and NK cells) from Gsdme−/− EMT6 and CT26 tumours also expressed less granzyme B (GzmB) and perforin (PFN) (Extended Data Fig. 4c, d, g, h), and produced less interferon-γ (IFNγ) and TNF after stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Extended Data Fig. 4e, i, j). Thus, endogenous GSDME suppresses tumour growth and promotes TIL function. Conversely, ectopic mGSDME expression in 4T1E tumours (Extended Data Fig. 5) and B16 cells (Extended Data Fig. 6) markedly inhibited tumour growth (Fig. 1a and Extended Data Fig. 6a). GSDME-expressing 4T1E tumours had substantially more tumour-infiltrating NK cells and tumour-associated macrophages, but not CDS TILs (Extended Data Fig. 5a). More NK and CDS TILs expressed GzmB and PFN (Extended Data Fig. 1b, c) and more CDS TILs produced IFNγ and TNF (Extended Data Fig. 1d). 4T1E tumours overexpressing F2A or P212L LOF mutants of GSDME did not have more functional TILs (Extended Data Fig. 1a–d, Extended Data Fig. 5a and data not shown). Similarly, increased TIL numbers and function were observed in B16 tumours overexpressing wild-type but not F2A GSDME (Extended Data Fig. 6b–d). Thus, GSDME pores were required for tumour-suppressive immune enhancement. Ectopic mGSDME expression in 4T1E tumours producing enhanced green fluorescent protein (eGFP) (Fig. 1e–g) and induced more functional TILs per milligram of tumour. Ectopic expression of pore-forming, but not inactive, GSDME reduces tumour growth and enhances tumour immune responses.
Similarly, depletion of either killer-cell subset markedly increased substantially in mice lacking both CD8+ T and NK cells, indicating that tumour inhibition, but GSDME expression did not alter tumour growth CD8+ T or NK cells modestly but noticeably reduced GSDME-mediated control antibodies (Fig. 2b and Extended Data Fig. 7). Depletion of either Gsdme+/+ tumour inhibition, we implanted T o determine whether killer cells are important for GSDME-mediated in the anti-tumour effect of GSDME was also observed by comparing Gsdme−/− mice even in the absence of GSDME.

Both diabetic (NOD), severe combined immunodeficient (SCID), interleukin-12 (IL-12) and PG-PyO-depleted (PG-PyO−/−) mice were 2.5× more likely to be GFP-positive, indicating increased tumours expressed substantially more PFN (Extended Data Fig. 5e), tumour-specific CD8+ TILs could be examined using GFP tetramers. Also, both depleted tumours were twice as likely to be GFP-positive, indicating increased in vivo phagocytosis of tumour cells (Fig. 1g).

Killer lymphocytes mediate tumour suppression

Enhanced immune function in mGSDME-expressing tumours suggests that tumour inhibition might be immune-mediated. To investigate this hypothesis, we compared GSDME-expressing and Gsdme−/− EMT6 tumours in wild-type BALB/c mice and in NSG mice (for ‘non-obese diabetic (NOD), severe combined immunodeicient (SCID), interleukin-2-receptor-γ-null’), the latter of which lack mature lymphocytes (Fig. 2a). Both Gsdme−/− and Gsdme−/− EMT6 tumours grew more rapidly in NSG than in wild-type mice, indicating immune protection in the wild-type mice even in the absence of GSDME. Gsdme deficiency did not markedly affect tumour growth in NSG mice, whereas Gsdme−/− EMT6 tumours grew faster in immunocompetent mice. A requirement for lymphocytes in the anti-tumour effect of GSDME was also observed by comparing empty-vector and mGSDME-overexpressing B16 tumours (Fig. 2c).

To determine whether killer cells are important for GSDME-mediated tumour inhibition, we implanted Gsdme−/− and Gsdme−/− EMT6 tumours in mice lacking CD8+ T cells and/or NK cells and in mice treated with control antibodies (Fig. 2b and Extended Data Fig. 7). Depletion of either CD8+ T or NK cells modestly but noticeably reduced GSDME-mediated tumour inhibition, but GSDME expression did not alter tumour growth substantially in mice lacking both CD8+ T and NK cells, indicating that both types of killer cell are responsible for tumour suppression (Fig. 2b). Similarly, depletion of either killer-cell subset markedly increased the growth of only GSDME-expressing and not empty-vector B16 cells (Extended Data Fig. 8). Thus, both CD8+ T and NK cells mediate the tumour-suppressive effects of GSDME.

This killer-cell dependence of protection suggests that pyroptosis causes immunogenic cell death (ICD).9 The gold-standard criterion of ICD is protection from secondary-tumour challenge after vaccination with tumour cells undergoing ICD. To determine whether pyroptosis causes ICD, we vaccinated mice with either wild-type or GSDME-overexpressing B16 cells subcutaneously in the left flank, and further challenged the mice ten days later with wild-type B16 on the right flank (Fig. 2d–f). At the vaccination site, GSDME and caspase 3 cleavage were easily detected by immunoblot only in GSDME-overexpressing tumours (Fig. 2d), indicating that pyroptosis occurred spontaneously in vivo and that cell death increased greatly in GSDME-overexpressing tumours. Moreover, vaccination with GSDME-overexpressing, compared with wild-type, B16 cells substantially reduced the growth of challenge wild-type B16 tumours (Fig. 2e) and improved tumour-free survival (Fig. 2f). Five of eight mice vaccinated with wild-type B16 cells developed palpable tumours, whereas only one of eight mice vaccinated with GSDME-overexpressing B16 cells did (P = 0.039, χ² test). Thus, pyroptosis is a form of ICD that occurs spontaneously in GSDME-overexpressing tumours.

Recognition by CD8+ T and NK cells triggers both cytokine secretion and cytotoxic granule-mediated, PFN-dependent cell killing, but the latter is generally considered key to anti-tumour immunity. To determine whether killing mediates tumour suppression, we compared the growth of empty-vector and mGSDME-overexpressing 4T1E tumours in wild-type and Prf1−/− (PFN-deficient) mice (Fig. 2g). Both tumours grew much faster in PFN-deficient than in wild-type mice. Although GSDME markedly reduced 4T1E growth in wild-type mice, GSDME conferred no noticeable advantage in PFN-deficient mice, indicating that granule-dependent cytotoxicity was responsible for GSDME’s tumour suppression. Given that GSDME had no substantial effect on tumour growth in mice lacking killer lymphocytes or PFN, GSDME’s tumour suppression
must be primarily mediated by killer lymphocytes. GSDM-negative tumours tended to be larger than GSDM-positive tumours at later time points even in NSG and lymphocyte-depleted mice, suggesting that cell-intrinsic mechanisms of GSDM-mediated tumour suppression may exist.

**Killer cells activate pyroptosis**

The strong dependence of GSDM-mediated tumour suppression on cytotoxicity suggested that killer lymphocytes might cause pyroptosis in GSDM-expressing targets. To determine whether killer cells induce pyroptosis, we incubated the human NK line YT with empty-vector and hGSDM-overexpressing HeLa cells. Although cell death was comparable (Fig. 3a), pyroptosis occurred only in GSDM-expressing HeLa cells and increased with more NK cells (Fig. 3b, c). Using time-lapse microscopy, we found that both empty-vector and GSDM-positive HeLa cells began to detach about an hour after adding YT cells (Fig. 3d and Supplementary Videos 5, 6). Empty-vector HeLa cells showed progressive apoptotic morphology and did not take up SYTOX green over 160 min, whereas GSDM-positive HeLa cells began to take up SYTOX green after 15–20 min and underwent increasing pyroptotic membrane ballooning. Caspase 3 was cleaved in YT-cocultured empty-vector and GSDM-expressing HeLa cells collected 4 h after adding YT cells, but GSDM was cleaved only in GSDM-positive tumours, which released much more HMGB1 (Fig. 3e). Treatment of GSDM-positive HeLa cells with YT cells or another human NK line, NK-92, or with raptinal or TRAIL produced a GSDME much more HMGB1 (Fig. 3e).

**Granzyme B cleaves GSDME after D270**

Because NK-induced pyroptosis in GSDM-expressing cells does not require caspases, we speculated that Gzm proteases might cleave GSDM. Gzm-mediated cell death is caspase-independent, but is amplified by caspases because GzmB cuts and activates caspase 3 (ref. 1). To test whether Gzms cleave GSDM, we incubated lysates from GSDM-expressing or GSDMD-overexpressing HEK293T cells with purified human Gzms or Gzmb, the most abundant Gzms. Gzmb, but not Gzma, led to GSDM but not GSDMD cleavage (Fig. 4e). Given that the caspase-activated and killer-cell-activated GSDME N termini were of similar sizes (Fig. 4a), Gzmb either activates caspase 3 to cleave GSDM and/or directly cleaves GSDM at the same residue. In fact, Gzmb did not activate cleavage of the GSDME D270A mutant, but wild-type GSDME was still cleaved when all caspases were inhibited (Fig. 4f) or in lysates of CASP3−/− GSDM-expressing HeLa cells (Fig. 4g), suggesting that Gzmb directly cleaves GSDME after D270. We verified this direct cleavage by showing that Gzmb cleaved recombinant GSDME (Fig. 4h).

To determine whether Gzmb cleaves GSDM in cells, we treated SH-SY5Y, a GSDM-positive human neuroblastoma, with PFN with or without Gzmb (Fig. 4i and Extended Data Fig. 10a–c). The combination, but not PFN alone, caused pyroptotic morphology (Extended Data Fig. 10a). Within 30 min of adding Gzmb and PFN, GSDM and caspase 3 cleavage were detected by immunoblot (Fig. 4i). GSDM cleavage and LDH release from SH-SY5Y required Gzmb and PFN, and were partially inhibited by inhibiting caspase 3 or all caspases (Extended Data Fig. 10b, c). Knocking out GSDME by CRISPR–Cas9 in SH-SY5Y (Extended Data Fig. 1h) did not change overall Gzmb- and PFN-mediated cell death (Fig. 4j), but blocked pyroptosis (Fig. 4k and Extended Data Fig. 10d). Thus, Gzmb induces GSDME-dependent pyroptosis in tumour targets both directly by cleaving GSDM and indirectly by activating caspase 3.

**D270 cleavage mediates tumour suppression**

Because both Gzmb and caspase 3 use cleavage at D270 to activate GSDME, mutation of this residue in tumours should abrogate tumour suppression. To test this hypothesis, we compared the growth of B16 (Extended Data Fig. 1a–d) and 4T1 (Extended Data Fig. 1e–h) cells stably overexpressing wild-type or D270A mGSDM or empty vector. Although wild-type GSDME reduced tumour growth as before (Fig. 1...
Fig. 4 | GzmB directly cleaves GSDME to cause pyroptosis. a, Immunoblot of hGSDME-positive HeLa cells after no treatment or treatment with YT or NK92 cells or with TRAIL for 4 h or raptinal for 1 h. b, Effect of EGTA, zVAD-fmk and zDEVD-fmk on YT-induced SYTOX green uptake in empty vector and hGSDME-positive HeLa cells. c, d, Expression of GSDME and caspase 3 in hGSDME-positive (control) and hGSDME-positive caspase 3−/− HeLa cells (c) and YT-induced SYTOX green uptake (d) in empty vector and hGSDME-positive and hGSDME-negative caspase 3−/− HeLa cells. e, f, Immunoblots, probed for Flag–GSDME, of cell lysates of HEK293T cells expressing Flag-tagged wild-type or D270A (f, right lane) hGSDME after 1 h incubation with phosphate-buffered saline (PBS) or recombinant GzmA or GzmB (800 nM). g, Immunoblots, probed for GSDME, of cell lysates of hGSDME-positive HeLa cells, knocked out or not (control) for caspase 3, after 1 h incubation with GzmB. h, Coomassie-stained SDS–PAGE gel of in vitro reaction of recombinant GzmB incubated with recombinant hGSDME for 1 h. NT and CT, N-terminal and C-terminal GSDME cleavage products; FL, full-length GSDME. i, SH-SYSY cells treated with PFN plus or minus GzmB or with medium (‘Untreated’), for 2 h or for the indicated times, were analysed by immunoblot of cell lysates probed for caspase 3, GSDME or actin. j, k, Effects of GSDME knockout (KO) on the cell death and pyroptosis of SH-SYSY cells treated with PFN plus or minus GzmB, assessed after 1 h by CellTiter-Glo (j) or SYTOX green uptake (k). Differences among multiple groups in b, d, j, k were analysed by one-way ANOVA using the Holm–Sidak method for multiple comparisons. Data are mean ± s.d. of biological triplicates and are representative of three (a–h) or two (i–k) independent experiments. ***P < 0.0001.

Discussion

Here we have shown in melanoma, triple negative breast cancer and colorectal cancer tumours that GSDME expression acts as a tumour suppressor by inducing pyroptosis, which enhances anti-tumour killer-cell cytotoxicity. The anti-tumour activity of GSDME was abrogated in mice lacking killer lymphocytes or PFN. Mutations abolishing GSDME pore formation or cleavage by GzmB/caspase 3 also blocked tumour suppression. Tumour suppression occurred without any extrinsic treatment. We consider what it is that initially triggers in vivo pyroptosis. It may have been initiated by spontaneous apoptosis of hypoxic or stressed regions of the tumour or by immune-mediated killing. Our working model is that GSDME expression in spontaneously dying tumour cells provides inflammatory danger signals that recruit immune cells to the tumour microenvironment and promote their functionality. GSDME expression increased not only the number and function of TILs, but also macrophage-mediated phagocytosis, which is predicted to enhance anti-tumour adaptive immunity. Some tumours evade immunity by resisting phagocytosis25,26; tumour GSDME may help to overcome this immune evasion strategy.

Killer-cell-mediated death was previously thought of as noninflammatory. Here we have shown, however, that killer lymphocytes activate pyroptosis when GzmB cleaves GSDME at the caspase 3 site. Caspase-resistant cancer cells should be susceptible to killer-cell-mediated apoptosis (because Gzm-activated death is mostly caspase-independent) as well as pyroptosis, provided that the cancer cells express GSDME. Pyroptosis may augment killer-cell immunity by providing adjuvant-like danger signals. Our results indicate that pyroptosis, similar to necroptosis25, is a form of ICD. Implanted GSDME-positive B16 melanoma cells, but not GSDME-negative cells, spontaneously underwent pyroptosis and protected mice from challenge with wild-type B16 cells. Protection by such vaccination—the gold standard for ICD27—did not require chemotherapy or radiation, suggesting that GSDME-expressing tumours, even otherwise ‘immunologically cold’ tumours such as B16, are spontaneously undergoing pyroptotic ICD in vivo. It is worth noting that the GSDME-positive tumours studied here do not release interleukin (IL)-1β or require it for immune protection, because Il1b was expressed by only one of the mouse cell lines studied (Extended Data Fig. 9f, g) and IL-1β was not detected in sera of GSDME-positive tumour-bearing mice (data not shown).

Direct Gzm-mediated induction of pyroptosis provides a simple mechanism for triggering inflammatory death—much simpler than canonical inflammasome activation, which requires at least four molecules (a sensor, an adaptor, an inflammatory caspase and GSDMD), or even the noncanonical pathway, which requires an inflammatory caspase and GSDMD. GSDME and other GSDMs may sense other mislocalized cytosolic proteases as danger signals. The GSDM linker region is an unstructured loop, making it a good protease substrate. Consistent with this, neutrophil serine proteases, which are homologous to Gzms, can cleave GSDMD to induce neutrophil netosis28,29.

Cancer cells have developed two strategies—epigenetic suppression of GSDME expression and LOF mutations—to avoid GSDME-mediated tumour suppression. Epigenetic suppression of GSDME is more common than GSDME mutation24,40. We have shown here that many cancer-related GSDME mutations reduce pyroptosis, and that mutations of D270, the shared GzmB/caspase 3 cleavage site and a prominent cancer mutation, have enabled tumours to evade tumour suppression by GSDME. Therapeutic strategies to induce GSDME—such as use of the DNA methylation inhibitor decitabine4, an approved leukaemia and myelodysplasia drug—are worth exploring.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information,
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Methods

Data reporting
No statistical methods were used to predetermine sample size. The experiments were not randomized and the investigators were not blinded to outcome assessment.

Cell lines and cell culture conditions
HeLa, HEK293T and EMT6 cells were purchased from ATCC. 4TIE and 4TIE–eGFP cells were generated in our laboratory by sorting 4T1 cells for high E-cadherin expression, as previously reported48. 4T1 and 4T07 cells were gifted from F. Miller. B16F10 and CT26 cells were provided by G. J. Freeman. SH-SY5Y cells were a gift from Y. Shi. The YT-Indy NK cell line was a gift from Z. Brahimi. HeLa, HEK293T, 4T1, 4TIE, 4TIE–eGFP and B16F10 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). 6 mM HEPES, 1.6 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 U ml⁻¹ penicillin G and 100 μg ml⁻¹ streptomycin sulfate. EMT6, CT26, SH-SY5Y and YT-Indy NK cells were cultured in RPMI medium with 10% FBS and the same supplements. All cells were verified to be free of mycoplasma by PCR and were authenticated by morphology only.

Plasmids
Full-length human GSDMD and GSDME and mouse Gsdme were cloned into pFlag-CMV4 plasmids. GSDME and Gsdme point mutation plasmids were generated by quick-change PCR (Stratagene). GSDME truncations were amplified by PCR from pFlag-CMV4-GSDME. pLVX-Puro empty vector was a gift from C. Bowman-Colin. Full-length wild-type GSDME and mutant GSDMEm were cloned into pLVX-Puro using a HiFi one-step kit (Gibson Assembly). LentiCRISPR-v2 puro and LentiCRISPR-v2 hygro vectors were obtained from Addgene and guide RNAs were cloned into the vectors as previously described19,20. All plasmids were verified by sequencing.

Stable cell lines
To generate lentiviruses, pLVX-puro Gsdme plasmid was transfected into HEK293T cells with pSPAX2 and pCMV-VSV-G at a 1/1/2 ratio. Supernatants collected 2 days later were used to transduce B16 and 4TIE cells for 48 h. Puromycin (Sigma, 3 μg ml⁻¹) was then added to select GSDME-expressing cells. pLVX-puro GSDME was used to generate HeLa cells stably expressing GSDME, and pLVX-puro empty vector was used to generate control cells.

CRISPR–Cas9 knockout cells
Gsdme gRNAs (5’-TTTCTTAAGAAGTTGATGC-3’ and 5’-CAAGCTGCAACATTCAAGTC-3’) and GSDME gRNAs (5’-TAAGTACAGCTTCTAAGT-3’ and 5’-TGACAAAAAGAAGAAGATCTG-3’) were cloned into LentiCRISPR-v2 puro and Casp3 gRNA (5’-GGAGAACACTGAATACCTCTAAAGTC-3’) was cloned into LentiCRISPR-v2 hygro, as previously described39,20. The resulting plasmids were transfected into HEK293T cells with pSPAX2 and pCMV-VSV-G at a 1/1/2 ratio. Supernatants from HEK293T cells transfected with plasmids that express mouse Gsdme, human GSDME or Casp3 guide RNAs were collected 2 days later and used to transduce mouse EMT6 and CT26 cell lines, human SH-SY5Y cells or GSDME-positive HeLa cells, respectively. Two days later, 3 μg ml⁻¹ (8 μg ml⁻¹ for CT26 cells) of puromycin or 200 μg ml⁻¹ hygromycin was added to select for positive cells for 5 days or 14 days, respectively. Cells were then subcloned into 96-well plates and screened for GSDME expression by immunoblot. Selected knockout clones were analysed and verified by DNA sequencing. LentiCRISPR-v2 empty vector was used to generate control cells.

Reagents
Raptinal, EGTA, necrostatin-1s (Nec-1s), ferrostatin-1 (Fer-1), α-tocopherol (vitamin E) and desferoxamine (DFO) were from Sigma. TRIzol was from Life Technologies. TRAIL was from Enzo Life Sciences. α-tocopherol (vitamin E) and desferoxamine (DFO) were from Sigma. TRIzol was from Life Technologies. TRAIL was from Enzo Life Sciences. zVAD-fmk and zDEVD-fmk were from BD Biosciences. SYTOX green was from Invitrogen. Vybrant DiD dye was from ThermoFisher Scientific.

Gene-expression assays
RNA was extracted using TRIzol reagent according to the manufacturer’s instructions and was subject to reverse transcription using the SuperScript III system (Invitrogen). Gsdme expression was assayed by qRT–PCR using SsoFast Supermix (Bio-Rad). Breast cancer (BRCA) and colon cancer (COAD) RNA-sequence expression data were obtained from TCGA using the University of California Santa Cruz (UCSC) Xena bioinformatic tool17. The log2 difference between GSDME and GAPDH expression was calculated for both tumour and normal tissue and plotted using Prism software.

Immunoblot
Cells were lysed in lysis buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% Triton-X100) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) before use. Cell lysate in SDS loading buffer (50 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 1% 2-mercaptoethanol, 0.1% bromophenol blue) was boiled and analysed by SDS–PAGE (Bio-Rad) and transfer to polyvinylidene fluoride (PVDF) membranes (MilliporeSigma), which were then blocked with 2% milk in TBST buffer (150 mM NaCl, 20 mM Tris-HCl pH 7.6, 0.1% Tween-20) for 30 min before incubation with primary antibodies for 2 h at room temperature or 4 °C overnight. After washing with TBST, membranes were incubated with secondary antibody for 40 min and then washed three times with TBST before adding the West Femto substrate (ThermoFisher Scientific) for detection. Antibodies were anti-GSDME (ab215191, Abcam), anti-Flag (F1804, Sigma), anti-caspase 3 (9662S, Cell Signaling), anti-β-MHC (ab18236, Abcam), anti-α-tubulin (T5168, Sigma), and anti-β-actin (JLA20, Developmental Studies Hybridoma Bank).

Cell-death assays
To measure membrane lysis, culture medium was collected and LDH release was measured using the CytoTox 96 cytotoxicity assay (Promega) according to the manufacturer’s instructions. To assess pyroptosis induced by the GSDME N terminus in HEK293T cells, we carried out HEK293T transfection using the calcium-phosphate method and measured LDH release 20 h after transfection. Pyroptotic cells were also visualized using an Olympus IX70 inverted microscope, and protein expression was analysed by immunoblot. For raptinal-induced pyroptosis, B16 GSDME cells, 10 μM raptinal was used to treat B16 cells for 2 h and LDH release was assessed at indicated time points. For TRAIL-induced pyroptosis in HEa GSDME cells, LDH release was measured 16 h after treatment with 100 ng ml⁻¹ TRAIL. For YT-induced pyroptosis in HeLa GSDME cells, LDH release was measured 4 h after treatment (E/T ratio = 2/1). For PFP/GzmB-induced pyroptosis in SH-SY5Y cells, LDH was measured 1 h after treatment in GSDME-knockout cells or 2 h after treatment in the presence of caspase inhibitors.

Overall cell death due to pyroptosis or apoptosis was measured at the same time as LDH release. To measure overall cell death in raptinal-treated B16 cells, we stained samples with allopseudocyanin (APC)-conjugated annexin V (Invitrogen) and propidium iodide (PI) (Sigma) according to the manufacturer’s instructions and analysed the results by BD FACSCanto II (BD Biosciences) using FlowJo V.10 (TreeStar) software. Cell death was determined by counting annexin V- and/or PI-positive cells. To measure overall cell death in HEa or SH-SY5Y cells, we assessed cell viability by measuring ATP levels using a CellTiter-Glo kit (Promega). The untreated cells were considered as a 100% viability control and cell death was inferred as a reduction in the number of viable cells.

SYTOX green uptake and time-lapse microscopy
To assess raptinal-induced cell death, we seeded cells in 96-well plates overnight and treated them with 10 μM raptinal for 2 h in the presence of...
Isolation of tumour-infiltrating immune cells

Tumours were collected, cut into small pieces and treated with 2 mg ml⁻¹ collagenase D, 100 μg ml⁻¹ DNase I (both from Sigma) and 2% FBS in RPMI with agitation for 30 min. Tumour fragments were homogenized and filtered through 70-μm strainers, and immune cells were purified by Percoll-gradient centrifugation and washed with Leibovitz’s L-15 medium.

Antibody staining and flow cytometry

To detect eGFP-tetramer-specific CD8+ TILs, we stained cells with phycoerythrin-labelled eGFP-specific MHC class I (H2Kb) tetramer carrying the peptide HYLSTQSA22 (NIAID Tetramer Facility). Immune cells isolated from tumours were stained with CD45 PerCPCy5.5 (clone 30-F11), CD8 PacBlue, PerCPCy5.5, Alexa700, fluorescein isothiocyanate (FITC) or -APC (clone 55-6.7), CD4 PE-Cy7, APC or PerCPCy5.5 (clone GK1.5), CD49d-FITC, PacBlue or PerCPCy5.5 (clone DX5), NKp46-APC (clone 29A1.4), CD11b-Alexa700 (clone M170), F4/80-PE-Cy7 (clone BM8) and CD44-PerCPCy5.5 (clone IM7) (BioLegend). Dead cells were excluded using the Aqua dead-cell stain kit (ThermoFisher Scientific) added with cell-surface antibodies. For intracellular staining, cells were first stained with antibodies to cell-surface markers for 30 min, then fixed and permeabilized with fixation/permeabilization buffer (BD Phamingen) and stained with Gzm-PacBlue (clone GB11, ThermoFisher Scientific) and perforin-phycoerythrin (clone S6600B, BioLegend). For intracellular cytokine staining (ICS), roughly 10⁶ cells per sample were cultured in RPMI medium containing 2% FBS and stimulated with either PMA (50 ng ml⁻¹), ionomycin (2 μg ml⁻¹), and Golgiplug (1.5 μl ml⁻¹, ThermoFisher Scientific) for 4 h, or with eGFP200–208 (HYLSTQSA peptide; 10 μg ml⁻¹, Genscript) and Golgiplug for 6 h. Cells were then stained with IFNy-PacBlue (clone clone XMGL1.2) and TNF-phycoerythrin-Cy7 (clone MP6-XT22) after fixation/permeabilization.

Protein expression and purification

The full-length coding sequence of human GSDME was cloned into the pDB.His MBA vector to generate a recombinant construct with an N-terminal polyhistidine–maltose-binding protein (His, MBP) tag followed by a tobacco etch virus protease (TEV) cleavage site. The plasmid was verified by DNA sequencing and transformed into Escherichia coli BL21 (DE3) cells. Successful transformants were selected on an LB plate supplemented with 50 μg ml⁻¹ kanamycin, transferred to LB medium with the same antibiotic, and grown at 37 °C with vigorous shaking until the optical density reached 1.0. Protein expression was then induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 26 °C overnight. Cells were collected by centrifugation and frozen in liquid nitrogen for long-term storage at −80 °C. To purify human GSDME, we resuspended thawed E. coli pellets in buffer A (50 mM Tris-HCl at pH 8.0, 150 mM NaCl) and sonicated the cells to lyse them. The recombinant protein was captured on nickel-charged (Ni)-NTA beads (Qiagen) using a gravity-flow column and eluted with buffer A supplemented with 500 μM imidazole. The His-MBP tag was removed by overnight incubation with TEV at 4 °C followed by Ni-NTA affinity chromatography. The flow-through containing GSDME was concentrated and further fractionated using a Superdex 200 gel filtration column (GE Healthcare Life Sciences) equilibrated with buffer A. Monomer fractions of GSDME were pooled and frozen at −80 °C for further use.

Recombinant GzmA and GzmB were purified from HEK293T cells and PFN was purified from YT-Indy NK cells as previously described [32, 33].

PFN/GzmB killing assay

The PFN/GzmB-mediated killing assay in SH-SYSY cells was performed as previously described [34]. In brief, 500 nM GzmB and/or sublytic PFN in buffer P (10 mM HEPES pH 7.5 in Hanks’ balanced salt solution (HBSS)) was added to SH-SYSY cells in buffer C (10 mM HEPES pH 7.5, 4 mM CaCl₂, 0.4% bovine serum albumin (BSA) in HBSS). The sublytic concentration of PFN was determined as a concentration that caused 5−15% cytolysis of the target cell on its own. After 2 h incubation, LDH release, cell death, SYTOX green uptake and GSDME cleavage were assayed as described above.
In vitro cleavage assay
For in vitro cleavage in cell lysates, HEK293T cells transiently expressing Flag–hGSDMD or Flag–hGSDME for 48 h were lysed in lysis buffer containing 50 mM Tris–HCl pH 7.4, 150 mM NaCl and 1% Triton X100 (2 × 10^6 cells per millilitre), and cell lysates (20 μl) were incubated with GzmA or GzmB at 37 °C for 1 h. Cleavage products were detected by anti-Flag immunoblot. Cleavage of recombinant GSDME protein by recombinant GzmB was analysed by SDS–PAGE and Coomassie staining after incubation in buffer A at 37 °C for 1 h.

Statistics
A Student’s t-test (two-tailed) or Mann–Whitney test was used to determine differences between two groups. Multiple comparisons between two groups were performed by multiple t-test with type I error correction. One- or two-way ANOVA was used to calculate differences among multiple populations. Differences between tumour growth curves and SYTOX green uptake curves were compared by first calculating the area-under-curve values for each sample and then comparing different groups using the Student’s t-test or one-way ANOVA. Type I errors were corrected by the Holm–Sidak method. P values of less than 0.05 were considered significant.

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability
All relevant data are available in the Source Data (for Figs. 1–4 and Extended Data Figs. 1–11) or Supplementary Information associated with this paper.

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Author contributions
Z.Z. conceived the study. Z.Z., Y.Z. and J.L. designed experiments and analysed data. Z.Z. and Y.Z. performed the majority of the experiments, assisted by S.X., Q.K., S.L., X.L., C.J., K.F.M.-S., T.M.Y.M. and J.A. S.S., Y.Y. and H.W. analysed the locations of GSDME mutations, helped to prepare recombinant proteins and provided valuable comments. J.L., Z.Z. and Y.Z. wrote the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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Correspondence and requests for materials should be addressed to Z.Z. or J.L.
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Extended Data Fig. 1 | GSDME expression in human and mouse tumour cell lines. a, b, Gsdme messenger RNA (a) and protein (b) levels in the indicated mouse cancer cell lines, assessed by qPCR, relative to Gapdh (a), and by immunoblot (b). c, GSDME expression relative to GAPDH in normal tissue and tumours from breast invasive carcinoma (BRCA) and colon adenocarcinoma (COAD) patients from TCGA, compared with the qRT–PCR values for mouse cancer cell lines used here. P values comparing normal tissue and cancer tissues were calculated using unpaired two-tailed Student's t-test.

d, f, Expression of mGSDME in EMT6 (d) and CT26 (f) clones knocked out for Gsdme, or in control (Ctl) cells treated with nontargeting vector, assessed by immunoblot for GSDME. Actin serves as a loading control. e, g, Cell proliferation determined by CellTiter 96 in EMT6 (e) or CT26 (g) control and Gsdme knockout cells (n = 6 samples per group). h, Expression of hGSDME in SH-SY5Y clones knocked out for GSDME or in control cells treated with nontargeting vector, assessed by immunoblot for GSDME. i, j, Expression of mGSDME in GSDME-overexpressing (OE) and empty-vector-transduced B16 (i) or 4T1E (j) cells, assessed by immunoblot. k, Expression of hGSDME in GSDME-overexpressing and empty-vector-transduced HeLa cells, assessed by immunoblot. Differences among multiple groups in e, g were analysed by one-way ANOVA, using the Holm–Sidak method for multiple comparisons. P values in e, g compare knockout and control cells. ***P < 0.0001. Data are mean ± s.d. of three technical (a) or six biological (e, g) replicates. Data are representative of at least two independent experiments.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Raptinal and/or TRAIL induces pyroptosis in B16 and HeLa cells overexpressing GSDME. 

a–e, Comparison of cell death after adding raptinal to empty vector and mGSDME-overexpressing B16 cells. 

a, Kinetics of overall cell death, assayed by counting annexin-V-positive and/or PI-positive cells by flow cytometry. 

b, c, Pyroptosis assessed by SYTOX green uptake (b) and LDH release (c). 

d, Time-lapse microscopy images showing morphological changes and SYTOX green uptake. 

e, Kinetics of caspase 3 and GSDME cleavage and HMGB1 release by immunoblot of cell lysates and culture supernatants. 

f, g, SYTOX green uptake assessed by plate reader (f) and time-lapse confocal microscopy (g) after treatment of empty vector and hGSDME-overexpressing HeLa cells with raptinal. 

h, i, TRAIL-mediated induction of cell death at 16 h post treatment, assessed by CellTiter-Glo (h), and LDH release in HeLa cells transduced with an empty vector or in hGSDME-overexpressing HeLa cells in the presence or absence of the pan-caspase inhibitor zVAD-fmk (i). UNT, untreated. The areas under the curve in a–c, f and data in h, i were compared by two-tailed Student’s t-test. Data are means ± s.d. of biological triplicate wells. ***P < 0.0001. Scale bar, 20 μm. Data are representative of two independent experiments.
Extended Data Fig. 3 | See next page for caption.
**Extended Data Fig. 3 | GSDME mutations in tumours are mostly LOF.**
a, GSDME N-terminal (NT) mutations in primary cancer cells in TCGA (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga). Asterisks denote stop codons; red, truncations; blue, mutants analysed here. b–g. Seventeen GSDME-NT cancer-associated mutations are mapped onto GSDME-NT (amino acids 1–241)—modelled here on the basis of the cryo-electron microscopy structure of the pore conformation of the GSDMA3-NT (Protein DataBank identification code 6CB8)8. These mutations were analysed in two arbitrarily assigned groups (b, e). These 17 mutations and the E245K mutation were analysed, after transient expression of wild-type Flag-tagged GSDME (WT FL GSDME) or wild-type or mutated GSDME-NT (amino acids 1–270) in HEK293T cells, for GSDME expression by immunoblot (c, f) and for LDH release by CytoTox 96 assay (d, g). h–l. Effect of four cancer-related GSDME truncation mutations (resulting in shortened proteins comprising amino acids 1–46, 1–210, 1–451 and 1–491) on GSDME-mediated cell death. GSDME expression of truncated proteins or FL or GSDME-NT (1–270) in HEK293T cells (h, k) and their effect on HEK293T cell death, assessed by morphology using microscopy (i) and by LDH release (j, l). The NT46 truncated protein is too small to be detected in k. Red arrows indicate ballooning pyroptotic cells. m, n. Effect of F2A mutation on GSDME-NT-induced pyroptosis in HEK293T cells. Protein expression is detected by anti-Flag immunoblot (m) and pyroptosis is assessed by LDH release (n). o, p. Effect of transient expression of mouse WT FL GSDME or wild-type or mutated GSDME-NT (mNT270, amino acids 1–270) in HEK293T cells (o) and on LDH release (p). q, r. Effect in 4T1E cells of expression of wild-type or mutated FL mGSDME (q) on rapaltn-induced SYTOX green uptake (r). Differences among multiple groups in d, g, j, l, n, p, r were analysed by one-way ANOVA, using the Holm–Sidak method for multiple comparisons. Data are mean ± s.d. of three technical (d, g, j, l, n, p) or biological (r) replicates. P values compare unmutilated with mutated constructs. ***P < 0.0001. Data are representative of three independent experiments.
Extended Data Fig. 4 | Gsdme knockout in EMT6 and CT26 cells increases tumour growth and reduces immune responses within tumours.

a–j. Comparison of control and Gsdme−/− orthotopic EMT6 tumours (control n = 6, Gsdme−/− n = 7) (a–e) and subcutaneous CT26 tumours (control n = 6, Gsdme−/− n = 5) (f–j) in BALB/c mice. The effects of knockout are demonstrated in Extended Data Fig. 1. Shown are tumour growth (a, f); numbers of CD8+ TILs (left), NK cells (middle) and tumour-associated macrophages (right), normalized to tumour weight (b); representative flow plots (left) and mean percentage of CD8+ TILs expressing GzmB or PFN (right) (c, g); mean percentages of NK cells expressing GzmB or PFN (d, h); and representative flow plots (left) and mean percentage of CD8+ TILs (right, e, i) and CD4+ TILs (j) producing IFNγ or TNF after PMA and ionomycin stimulation. The area under the curve in a, f and differences between two groups in b–e, g–j were compared by two-tailed Student’s t-test. Data are mean + s.e.m. Data are representative of at least two independent experiments.
Extended Data Fig. 5 | Effects of GSDME expression on tumour growth and immune responses within 4T1E tumours. a, 4T1E cells stably expressing wild-type mGSDME (n = 6 mice per group), the inactive F2A mutant of mGSDME (n = 6 mice per group) or an empty vector (n = 7 mice per group), implanted in mammary fat pads, were analysed for tumour-infiltrating immune-cell numbers. b–e, 4T1E cells, stably expressing eGFP and then stably transduced to express mGSDME or empty vector (b), were compared for raptinal-induced SYTOX green uptake in vitro (c), tumour growth after orthotopic implantation (n = 7 mice per group) (d), and percentage of CD8+ TILs expressing GzmB or PFN (e). Comparisons in a were calculated by one-way ANOVA using the Holm–Sidak method for multiple comparisons; comparisons in e were calculated by two-tailed Student’s t-test; comparisons in c, d were calculated by comparing the difference between the areas under the curve by two-tailed Student’s t-test. Data shown are mean ± s.e.m. ***P < 0.0001. All data are representative of two independent experiments.
Extended Data Fig. 6 | GSDME overexpression in B16 cells reduces tumour growth and increases immune responses within tumours. a, Tumour growth in C57BL/6 mice that were implanted subcutaneously with B16 cells stably transduced with empty vector (n = 6 mice per group) or expressing mGSDME (n = 8 mice per group) or inactive F2A GSDME (n = 5 mice per group). b, Numbers of CD8+ (left) and NK (middle) TILs and tumour-associated macrophages (right) in tumours, normalized to tumour weight. c, Percentage of CD8+ TILs expressing GzmB or PFN (left), and IFNγ or TNF after PMA and ionomycin stimulation (right). d, Percentage of NK cells expressing GzmB or PFN. The area under the curve of tumour growth curves in a was compared by one-way ANOVA with Holm–Sidak correction for type I errors. Comparisons in b–d were calculated by one-way ANOVA using the Holm–Sidak method for multiple comparisons. Data are mean ± s.e.m. All data are representative of at least two independent experiments.
Extended Data Fig. 7 | Depletion of CD8+ T and NK cells in mice bearing EMT6 tumours. a–c, Experimental scheme (a) and representative flow plots of CD4+ and CD8+ T cells and NK cells in the peripheral blood (b) and tumours (c) of mice bearing EMT6 tumours treated with isotype control antibody or anti-CD8 and/or anti-asialo-GM1 antibodies in Fig. 2b. Samples were obtained on day 3 (blood) and day 11 (tumours) after tumour challenge. Data are representative of at least two independent experiments.
Extended Data Fig. 8 | B16 tumour growth in mice depleted of CD8⁺ or NK cells. **a**, Experimental scheme and growth of empty vector or mGSDME-positive B16 tumours in mice depleted of CD8⁺ T cells (n = 6 mice per group) or NK cells (n = 6 mice per group) or treated with an isotype control antibody. Empty vector, n = 6 mice per group; mGSDME-positive, n = 7 mice per group. **b, c**, Antibody depletion was verified by flow cytometry using peripheral blood mononuclear cells (PBMCs) on day 7 after tumour challenge or using TILs at the time of necropsy. Representative flow plots show depletion of CD8⁺ T cells (top) or NK cells (bottom) in the peripheral blood (b) and tumours (c) of mice bearing B16 empty vector or B16 GSDME tumours treated with isotype control, anti-CD8 or anti-NK1.1 antibodies. The area under the curve of tumour growth curves was compared by one-way ANOVA with Holm–Sidak correction for type I error. Data are mean ± s.e.m. All data are representative of two independent experiments.
Extended Data Fig. 9 | Necroptosis and ferroptosis are not involved in GSDME-mediated pyroptosis. a, Effect of EGTA, necrostatin-1s (Nec-1s), α-tocopherol (Vit. E), ferrostatin-1 (Fer-1) and desferoxamine (DFO) on YT-cell-induced SYTOX green uptake in EV and hGSDME-positive HeLa cells. b–g, Cell-death-related gene expression in mouse and human cancer cell lines. Ripk3 (b), RIPK3 (c), Mlkl (d), MLKL (e), Il1b (f) and IL1B (g) mRNA levels, relative to Gapdh or GAPDH, respectively, of the indicated mouse (b, d, f) or human (c, e, g) cancer cell lines were assayed by qRT–PCR. Data are mean ± s.d. of biological (a) or technical (b–g) triplicates and are representative of two independent experiments.
Extended Data Fig. 10 | PFN plus GzmB induce pyroptosis in SH-SY5Y cells. 

**a–c**, SH-SY5Y cells treated with PFN with or without GzmB or medium for 2 h were analysed by microscopy (**a**), immunoblot of cell lysates probed for GSDME and actin (**c**) or LDH release (**b**). In **b, c**, treatment was in the presence of 30 μM of caspase inhibitors as indicated. 

**d**, Effects of GSDME knockout on pyroptosis induced by PFN with or without GzmB, assessed after 1 h of treatment by LDH release. Differences among multiple groups in **b, d** were analysed by one-way ANOVA using the Holm–Sidak method for multiple comparisons. Data are mean ± s.d. of biological triplicate wells. ***P < 0.0001. Data are representative of two independent experiments.
Extended Data Fig. 11 | Uncleavable D270A mutation blocks GSDME-mediated tumour protection and induction of anti-tumour immunity. 

a–d, B16 cells stably expressing empty vector (n = 7 mice per group), wild-type GSDME (n = 8 mice per group) or D270A uncleavable GSDME (n = 7 mice per group) were implanted in C57BL/6 mice and followed for tumour growth (a) and the functional phenotype of CD8+ and NK TILs (b–d). Shown are the percentages of CD8+ or NK TILs expressing GzmB or PFN (b, c) and of CD8+ TILs expressing IFNγ or TNF induced by PMA and ionomycin (d).

e–h, 4T1E cells stably expressing empty vector, wild-type, F2A or D270A GSDME were implanted in syngeneic (BALB/c, n = 6 mice per group) mice and followed for tumour growth (e) and the functional phenotype of CD8+ and NK TILs (f–h). Shown are the percentages of CD8+ TILs expressing GzmB or PFN (f) or induced by PMA and ionomycin to express IFNγ or TNF (h) and of NK TILs expressing GzmB or PFN (g).

i, j, Gsdme−/− EMT6 cells rescued by transduction with lentiviruses expressing empty vector, wild-type, F2A or D270A GSDME were examined by immunoblot for GSDME expression (i) and for tumour growth after orthotopic implantation in BALB/c mice (n = 8 mice per group) (j). Areas under the curve of tumour growth curves or differences among multiple groups were compared by one-way ANOVA with Holm–Sidak correction for type I error. Data are mean ± s.e.m. All data are representative of two independent experiments.
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

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Data collection SWISS-MODEL was used to model the GSDME-NT structure. Breast cancer and colon cancer RNA-seq expression data were obtained from The Cancer Genome Atlas (TCGA) using the University of California Santa Cruz (UCSC) Xena bioinformatic tool.

Data analysis Pymol was used to analyze the modeled GSDME-NT structure. Graph design and statistical analysis was performed using Prism V6.0. Protein and DNA sequence analysis were performed by Blast. Images and videos were processed and analyzed by ImageJ Fiji. Flow cytometry data were analyzed by FlowJo v.10.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size           | We chose sample size based on pilot experiments and literature reports in the field to achieve at least 80% power and a two-sided type I error of 5%. The chosen sample size was sufficient to determine statistical significance in our established tumor model. |
|-----------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions       | No data was excluded from our analysis.                                                                                                                                                           |
| Replication           | All experiments were performed at least two or three times with similar results.                                                                                                                     |
| Randomization         | Mice were randomly allocated to each experimental group.                                                                                                                                           |
| Blinding              | Blinding was not performed due to personnel availability. Random allocation and quantitative measurement using instruments and kits in our experiments minimized biased assessments.                          |

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|                                 | Clinical data |
|                                 | ChIP-seq |
|                                 | Flow cytometry |
|                                 | MRI-based neuroimaging |

Antibodies

- **Antibodies used**
  - anti-GSDME (clone EPR19859, Abcam, Cat#ab215191, Lot#GR3187594-23, 1:1000)
  - anti-FLAG (clone M2, Sigma, Cat# F1804, Lot# SLCB0524, 1:1000)
  - anti-caspase-3 (Cell Signaling, Cat#96625, Lot# 18, 1:1000)
  - anti-HMGB1 (Abcam, Cat#ab18256, Lot# GR3237475-1, 1:1000)
  - anti-α-tubulin (clone B-5-1-2, Sigma, Cat#T5168, Lot# B38M4813V, 1:2000)
  - anti-β-actin (clone JLA20, Developmental Studies Hybridoma Bank, Cat# JLA20, 1:1000)
  - CD45-PerCPCy5.5 (clone 30-F11, BioLgend, Cat#103132, Lot# B276559, 1:100)
  - CD8-PacBlue (clone 53-6.7, BioLgend, Cat#100728, Lot# B25982, 1:100)
  - CD8-PerCPCy5.5 (clone 53-6.7, BioLgend, Cat#100734, Lot# B268252, 1:100)
  - CD8-Alexa700 (clone 53-6.7, BioLgend, Cat#100730, Lot# B246153, 1:100)
  - CD8-FITC (clone 53-6.7, BioLgend, Cat#100706, Lot# B269214, 1:100)
  - CD8-APC (clone 53-6.7, BioLgend, Cat#100712, Lot# B257513, 1:100)
  - CD4-PE-Cy7 (clone 4G1, BioLgend, Cat#100422, Lot# B280301, 1:100)
  - CD4-APC (clone 4G1, BioLgend, Cat#100412, Lot# B280301, 1:100)
  - CD4-PerCPCy5.5 (clone 4G1, BioLgend, Cat#100434, Lot# B248434, 1:100)
  - CD49b-FITC (clone DX5, BioLgend, Cat# 108906, Lot# B271046, 1:100)
  - CD49b-PacBlue (clone DX5, BioLgend, Cat# 108918, Lot# B285512, 1:100)
  - CD49b-PerCPCy5.5 (clone DX5, BioLgend, Cat# 108915, Lot# B245454, 1:100)
  - Nkp46-APC (clone 29A1.4, BioLgend, Cat# 137628, Lot# B252578, 1:100)
  - CD11b-Alexa700 (clone M1170, BioLgend, Cat# 10122, Lot# B259438, 1:100)
  - F4/80-PE-Cy7 (clone BM8, BioLgend, Cat# 123114, Lot# B280917, 1:100)
  - CD44-PerCPCy5.5 (clone IM7, BioLgend, Cat# 103032, Lot# B213817, 1:100)
  - granzyme B-PacBlue (clone GB11, ThermoFisher Scientific, Cat# 515408, Lot# B271129, 1:10)
  - Perforin-PE (clone 5160098, BioLgend, Cat# 154406, Lot# B271129, 1:20)
IFN-γ-PacBlue (clone XMG1.2, BioLegend, Cat# 505818, Lot# B234191, 1:100), TNF-α-PE-Cy7 (clone MP6-XT22, BioLegend, Cat# 506324, Lot# B279986, 1:100), anti-mouse CD8 (clone 2.43, BioXCell, Cat# BE0061, Lot# 7051183), anti-NK1.1 (clone PK136, BioXCell, Cat#BE0036, Lot# 693118J3), anti-Asialo-GM1 (clone Poly21460, BioLegend, Cat# 146002, Lot# B288357), InVivoMAb rat IgG2b isotype control, anti-keyhole limpet hemocyanin (clone LTF-2, BioXCell, Cat# BE0090, Lot# 5101/0141).

Validation
All antibodies used in this study are commercially available and are validated by previous publications. [Links to antibody validation sources]

Eukaryotic cell lines
Policy information about cell lines

Cell line source(s)
HeLa, HEK293T and EMT6 were purchased from ATCC. 4T1E and 4T1E-eGFP cells were generated in our lab by sorting 4T1 for high E-cadherin expression. 4T1 and 4TO7 cells were kind gifts of Dr. Fred Miller (Wayne State University). B16F10 and CT26 were kindly provided by Dr. Gordon J. Freeman (Dana-Farber Cancer Institute). SH-SY5Y cell was a generous gift of Dr. Yang Shi (Boston Children’s Hospital). The YT-Indy NK cell line was a gift of Dr. Zacharie Brahmi (Indiana University).

Authentication
All the cell lines were authenticated by their morphology and functions according to ATCC instructions and published studies.

Mycoplasma contamination
All cells were verified to be free of mycoplasma by PCR.

Commonly misidentified lines
No commonly misidentified lines were used.

Animals and other organisms
Policy information about studies involving animals. ARRIVE guidelines recommended for reporting animal research

Laboratory animals
Female C57BL/6, BALB/c and NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ (NSG) mice (6-8 weeks) were purchased from Jackson Laboratories. Prf1-/- mice (Jackson Laboratories) in the BALB/c background were bred on site.

Wild animals
This study did not involved wild animals.

Field-collected samples
This study did not involved samples collected from the field.

Ethics oversight
All procedures were conducted using protocols approved by the Harvard Medical School IACUC.

Note that full information on the approval of the study protocol must also be provided in the manuscript.
Flow Cytometry

Plots

Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

- Tumors were harvested from different groups of mice at the time of necropsy. Tumors were cut into small pieces and treated with 2 mg/ml Collagenase D, 100 μg/ml DNase I (both from Sigma) and 2% FBS in RPMI with agitation for 30 min. Tumor fragments were homogenized, filtrated through 70 μm strainers and immune cells were purified by Percoll-gradient centrifugation and washed with Leibovitz’s L-15 medium.
- Peripheral blood samples were collected via facial vein bleeding. Peripheral blood mononuclear cells were purified by Histopaque and washed with Leibovitz’s L-15 medium.

Instrument

- Flow cytometry was performed by BD FACSCanto II.

Software

- Data was acquired by BD FACSDiva software, then analyzed using FlowJo software V.10 (Tri-Star).

Cell population abundance

- N/A

Gating strategy

- FSC/SSC gates were used to select mononuclear cells. FSC/FSH gates were then used to gate on single cells. Cells were then gated on live cells. Live cells were further gated on CD45+ leukocytes and then different populations of immune cells based on their expression of distinct markers. When needed, isotype control antibody staining was used to define positive/negative cell populations.

- Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.