**RESEARCH ARTICLE SUMMARY**

**IMMUNOLOGY**

**Granzyme A from cytotoxic lymphocytes cleaves GSDMB to trigger pyroptosis in target cells**

Zhiwei Zhou*, Huabin He*, Kun Wang, Xuyan Shi, Yupeng Wang, Ya Su, Yawei Wang, Di Li, Wang Liu, Yongliang Zhang, Lianjun Shen, Weidong Han, Lin Shen, Jingjin Ding, Feng Shao†

**INTRODUCTION:** In cellular immunity, cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells use perforin to deliver serine protease granzymes into target cells to kill them. Gasdermins are pore-forming proteins that execute pyroptosis, a form of proinflammatory cell death. Gasdermin D (GSDMD) is cleaved by caspase-1/4/5/11 upon inflammasome activation, releasing the pore-forming domain for plasma membrane disruption. Gasdermin E (GSDME) is similarly cleaved by caspase-3, converting apoptosis to pyroptosis. The functional mechanism for other gasdermins is unknown.

**RATIONALE:** The view that granzymes induce target-cell apoptosis was proposed two decades ago, when apoptosis was thought to be the dominant form of programmed cell death and assays to ascertain apoptosis were insufficiently accurate. Furthermore, granzyme cytotoxicity was only assessed in a few cell types. Discovery of the gasdermin family, which are true cell death executors, has altered our understanding of programmed cell death. In this work, we explored whether members of the gasdermin family might respond to granzymes and induce pyroptosis.

**RESULTS:** The expression of gasdermin B (GSDMB) but no other gasdermins in human embryonic kidney (HEK) 293T cells induced pyroptotic killing by NK cells, accompanied by an interdomain cleavage of GSDMB. These processes were blocked by inhibiting the perforin–granzyme pathway. In vitro profiling of all five human granzymes identified granzyme A (GZMA), which readily cleaved GSDMB, predominantly at Lys244 within the interdomain linker. This cleavage unmasked the pore-forming activity of GSDMB. GZMA, delivered into GSDMB-reconstituted cells by electroporation or perforin, induced extensive pyroptosis with interdomain cleavage of GSDMB. These effects were diminished by a K229A/K244A (KK/A) mutation of GSDMB (in which lysine (K) was replaced by alanine (A) at positions 229 and 224, respectively). In cells normally undergoing apoptosis upon GZMA delivery, the additional expression of GZMA-cleavable GSDMB converted apoptosis into pyroptosis. Pyroptotic killing by NK cells was blocked by both the KK/AA mutation and a knockdown of GZMA expression. Among 39 cell lines, three, including the esophageal carcinoma (OE19 cells), expressed GSDMB and underwent pyroptosis upon GZMA delivery. Knockout experiments revealed that pyroptosis in OE19 cells required the interdomain cleavage of GSDMB. Furthermore, GSDMB expression was up-regulated by interferon-γ (IFN-γ). Approximately one-third of GSDMB-negative cell lines showed IFN-γ–induced GSDMB expression. IFN-γ promoted GZMA- or NK cell-induced pyroptosis in several target cells. Primary T cells, including anti-CD19 chimeric-antigen receptor (CAR) T cells and NY-ESO-1–specific T cell receptor (TCR)–engineered T cells (TCR T cells), also induced pyroptosis in GSDMB-reconstituted cells through cleavage of GSDMB by GZMA. Introducing GZMA-cleavable GSDMB into mouse cancer cells promoted tumor clearance in mice. GSDMB was highly expressed in certain tissues, particularly digestive tract epithelia, including the derived tumors. GSDMB appeared to be silenced in gastric and esophageal cancers. The Cancer Genome Atlas database recorded a strong positive correlation between GSDMB expression and patient survival for bladder carcinoma and skin cutaneous melanoma.

**CONCLUSION:** GZMA from cytotoxic lymphocytes cleaves and activates GSDMB to induce target cell pyroptosis. This immune effector mechanism promotes CTL-mediated tumor clearance in mice. High GSDMB expression in the digestive system suggests the importance of GSDMB-mediated immunity in these tissues and will guide immunotherapy for related cancers. Our findings suggest that substrates such as gasdermins, rather than their upstream proteases, determine the nature of cell death.

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GZMA from cytotoxic lymphocytes cleaves GSDMB in target cells, predominantly at Lys244 within the interdomain linker. The cleavage allows GSDMB pore-forming domain (GSDMB-N) to perforate plasma membrane and induce pyroptosis. Expression of GSDMB wild type (WT) but not its GZMA-resistant K/A mutant in mouse cancer cells promotes cytotoxic T lymphocyte–mediated tumor clearance when the inhibitory checkpoint is blocked by antibody to programmed cell death 1 (PD-1). IFNγ, IFN-γ receptor.
**RESEARCH ARTICLE**

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**Granzyme A from cytotoxic lymphocytes cleaves GSDMB to trigger pyroptosis in target cells**

Zhiwei Zhou,1,3, Huabin He,2,3, Kun Wang,3, Xuyan Shi,3, Yupeng Wang,1,3, Ya Su,3, Yao Wang,4, Da Li,3, Wang Liu,1,3, Yongliang Zhang,5, Lianjun Shen,5, Weidong Han,4, Lin Shen,6, Jingjin Ding,2,3, X. Shen1,3, X. Li1

Cytotoxic lymphocyte–mediated immunity relies on granymes. Granymes are thought to kill target cells by inducing apoptosis, although the underlying mechanisms are not fully understood. Here, we report that natural killer cells and cytotoxic T lymphocytes kill gasdermin B (GSDMB)–positive cells through pyroptosis, a form of proinflammatory cell death executed by the gasdermin family of pore-forming proteins. Killing results from the cleavage of GSDMB by lymphocyte-derived granzyme A (GZMA), which unleashes its pore-forming activity. Interferon-γ (IFN-γ) up-regulates GSDMB expression and promotes pyroptosis. GSDMB is highly expressed in certain tissues, particularly digestive tract epithelia, including derived tumors. Introducing GZMA-cleavable GSDMB into mouse cancer cells promotes tumor clearance in mice. This study establishes gasdermin-mediated pyroptosis as a cytotoxic lymphocyte–killing mechanism that may enhance antitumor immunity.

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**REFERENCES AND CITATIONS**

1. Correlation coefficient.
2. Analysis of variance.
4. Probability level.
5. Significance level.
6. Student’s t-test.
7. Fisher’s exact test.
8. Chi-squared test.
10. One-way analysis of variance.
11. Two-way analysis of variance.
14. Linear regression analysis.
15. Partial correlation analysis.
16. Multiple regression analysis.
17. Multilevel modeling.
18. Principal component analysis.
19. Factor analysis.
20. Confirmatory factor analysis.
21. Path analysis.
22. Structural equation modeling.
23. Network analysis.
24. Bayesian network analysis.
32. Reinforcement learning with deep Q-learning.
33. Policy gradient methods.
34. Actor-critic methods.
35. Q-learning.
37. Inter-domain cleavage.
38. Pyroptosis.
40. Caspase-dependent cell death.
41. Caspase-independent cell death.
42. Gasdermin-mediated cell death.
43. Pyroptosis executor.
44. Lymphocyte–killing mechanism.
45. Antitumor immunity.
46. Cytotoxic T lymphocytes (CTLs).
47. Natural killer (NK) cells.
49. Gasdermin D (GSDMD).
50. Caspase-1.
51. Caspase-11.
52. Monocyte death.
53. Interleukin-18 (IL-18).
54. GSDMB-dependent pyroptosis.
55. Mouse GZMA.
56. IFN-γ.
57. GSDMB expression.
58. GSDMB-dependent pyroptosis.
59. Nonapoptotic killing.
60. Caspase-dependent cell death.
61. Caspase-independent cell death.
63. Pyroptosis executor.
64. Lymphocyte–killing mechanism.
65. Antitumor immunity.
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106. Mouse GZMA.
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109. Pyroptosis executor.
110. Lymphocyte–killing mechanism.
111. Antitumor immunity.
Fig. 1. GSDMB expressed in target cells confers pyroptotic killing by NK cells and undergoes interdomain cleavage. (A) Immunoblotting of gasdermin-Flag (GSDM-Flag) expression in 293T cells. (B) Representative images of gasdermin-expressing 293T cells after a 6-hour incubation with NK-92M1 cells. 293T cells were preloaded with a fluorescent dye calcein AM. Arrowheads indicate pyroptotic cells. Scale bar, 25 μm. (C) Quantification of pyroptosis by measuring calcein release from the gasdermin-expressing 293T cells after a 6-hour incubation with NK-92M1 cells. (D) Effect of the pan-caspase inhibitor zVAD on NK-92M1 cell-induced calcein release from GSDM-expressing 293T cells. (E) Representative flow cytometry pseudocolor dot-plots of PI- and annexin V-stained GSDM-expressing 293T cells after incubation with NK-92M1 cells in the presence or absence of zVAD. (F) Effect of EGTA and a pan-serine protease inhibitor DCI on NK-92M1 cell-induced calcein release from GSDMB-expressing 293T cells. (G and H) Immunoblotting of GSDMB-Flag cleavage in NK-92M1 cell–treated 293T cell and inhibition by EGTA and DCI. The antibodies to GSDMB and Flag recognize the N- and C-terminal cleavage products, respectively. Data are means ± SD from three replicates [(C), (D), and (F)]. ***P < 0.001; two-tailed unpaired Student’s t test. Data are representative of three [(A to D) and (F)] or two [(E), (G), and (H)] independent experiments.

GSDMB-expressing 293T cells than that in cells expressing other gasdermins or the empty vector (fig. S1, A to C).

To specifically examine target cell lysis in the coculture killing assay, we preloaded 293T cells with the calcein AM (acetoxyethyl) dye and performed the classical calcein release assay. Despite low background killing by NK-92M1 cells in control 293T cells, this more sensitive assay confirmed that the expression of GSDMB, but not any of the other four gasdermins, induced extensive lysis of 293T cells (Fig. 1, B and C, and fig. S1D). Consistent with the inability of GSDMD and GSDME to sensitize 293T cells to killing by NK-92M1 cells, these two gasdermins were not cleaved (fig. S1, E to G). Compared with 293T cells, HeLa and human melanoma A375 cells expressed higher amounts of caspase-3 and were more sensitive to NK-92M1 cells owing to caspase-3 cleavage of GSDME. However, additional expression of GSDMB in these two cell lines still promoted killing by NK-92M1 cells (fig. S1, H to K). Both flow cytometry and calcein release assays further revealed that GSDMB-rendered pyroptotic killing by NK cells was completely resistant to the pan-caspase inhibitor zVAD (carbobenzoxy-valyl-alanyl-aspartyl-O-methyl-fluoromethylketone) (Fig. 1, D and E). Thus, NK cells can induce target cells to undergo GSDMB-dependent but caspase-independent pyroptosis.

We next examined whether GSDMB-mediated pyroptosis is downstream of cytotoxic granule-derived perforin and granzyme activity. The Ca²⁺ chelator EGTA, an inhibitor of perforin and NK cell degranulation (39), blocked NK-92M1–induced pyroptosis in GSDMB-expressing 293T cells (Fig. 1F). A similar effect was observed with the pan-granzyme inhibitor DCI (3,4-dichloroisocoumarin) (Fig. 1F) (40). Consistent with the requirement of granzyme activity, immunoblotting by using an antibody that recognizes the N-terminal domain of GSDMB revealed a ~30-kDa (p30) cleavage fragment (Fig. 1G). The presence of a Flag tag at the carboxyl terminus of GSDMB allowed identification of another ~16-kDa (p16) fragment in NK-92M1–treated 293T cells (Fig. 1H). The appearance of these two cleavage fragments, whose combined molecular weight equaled that of full-length GSDMB (~47 kDa), was diminished by the addition of EGTA or DCI to the coculture killing assay (Fig. 1, G and H). Thus, GSDMB-dependent pyroptosis is due to intrinsic killing activity of NK cells and requires the specific cleavage of GSDMB.

Site-specific cleavage of GSDMB by GZMA releases its pore-forming activity

The above results strongly indicate that a granzyme mediates the interdomain cleavage of GSDMB and induces target cell pyroptosis. To test this hypothesis, we purified all five human granzymes from 293F cells whose activities were confirmed through in vitro cleavage
Fig. 2. GZMA cleaves GSDMB to induce pyroptosis in GSDMB-reconstituted target cells. (A) In vitro cleavage assay of GSDMB by five human granzymes. Shown is a representative Coomassie Blue-stained gel. (B) Diagram of GSDMB structure and the GZMA cleavage sites (Lys\(^{244}\) and Lys\(^{229}\)). (C) Equal amounts of full-length (FL) GSDMB or GSDMB-(N+C) proteins were electroporated into or added directly to 293T cells. ATP-based cell viability is shown. (D to F) GZMA was delivered into 293T cells expressing GSDMB WT or K229A/K244A (KK/AA) mutant by perforin. (D) Representative cell images (arrowheads indicate pyroptotic cells). Scale bar, 25 μm. (E) LDH release-based cell death. (F) Immunoblotting of GSDMB cleavage. (G) 293T cells expressing GSDMB WT or KK/AA double mutant were preloaded with calcein AM and then cocultured with NK-92MI cells. Quantification of calcein AM release and immunoblotting of GSDMB cleavage are shown. (H to J) Effect of siRNA knockdown of GZMA on pyroptotic killing of GSDMB-expressing 293T cells by NK-92MI cells. (H) Anti-GZMA immunoblot in NK-92MI cells. (I) Immunoblotting of GSDMB cleavage. (J) Quantification of calcein AM release. Data are means ± SD from three replicates [(C), (E), (G), and (J)]. ***p < 0.001; two-tailed paired Student’s t test. Data are representative of three [(A) and (C) to (F)] or two [(G) to (J)] independent experiments.
resistant to pyroptosis. Furthermore, complete resistance was observed with 293T cells expressing GSDMB K229A/K244A (fig. S3, E and F). For simplicity, the double mutant was used in subsequent analyses. In a human colon cancer cell line (SW480) that naturally expressed GSDMD but no other gasdermins (fig. S4A), electroporation of GZMA triggered extensive apoptotic death (fig. S4, B and C). Although the physiological relevance of this cell death requires further investigation, GZMA-induced apoptosis was converted to pyroptosis by expressing GSDMB wild type (WT) but not its K229A/K244A double mutant in SW480 cells (fig. S4, B and C). In a correlation with the cell death response, the p30 fragment of GSDMB was detected in cells expressing GSDMB WT but not the K229A/K244A double mutant (fig. S4D). Thus, the cleavage of GSDMB at Lys324 by GZMA is necessary and sufficient to trigger pyroptosis.

We also examined GZMA cleavage of GSDMB under physiological settings. Similar to electroporation of GZMA, cytosolic delivery of GZMA by purified perforin caused extensive pyroptosis in GSDMB-expressing 293T cells. This did not occur with perforin alone and was diminished in cells expressing the GSDMB K229A/K244A double mutant (Fig. 2, D and E). Accordingly, GSDMB WT but not K229A/K244A was cleaved into the p30 fragment in response to perforin-delivered GZMA (fig. 2F). In the NK cell-killing assay, blocking GZMA cleavage by the K229A/K244A double mutation in GSDMB also reduced pyroptosis in 293T cells (Fig. 2G). Accordingly, small interfering RNA (siRNA) knockdown of GZMA in NK-92MI cells inhibited the cleavage of GSDMB as well as pyroptosis in GSDMB-expressing 293T cells (Fig. 2, H to J). YTS is another NK cell line that expresses little GZMA endogenously (fig. S4E). When GZMA WT but not S212A mutant was reconstituted into YTS cells, the cells gained the ability to induce pyroptosis in GSDMB-expressing 293T cells, accompanied by interdomain cleavage of GSDMB (fig. S4, E to G). Thus, cleavage of GSDMB by GZMA causes pyroptotic killing of target cells by NK cells.

**Endogenous GSDMB is sufficient to mediate GZMA-induced pyroptosis**

To further probe the physiological role of GZMA cleavage of GSDMB, we sought to examine cells that express GSDMB endogenously. Among a panel of 39 different human cancer cell lines, three of them—OE19 (esophageal carcinoma), SW837 (rectum adenocarcinoma), and SKCO1 (colorectal adenocarcinoma)—showed apparent GSDMB expression, whereas the remaining 36 cell lines expressed little or no GSDMB (fig. S5A). When active GZMA was electroporated into the three GSDMB+ cells, around 40% of SW837 and OE19 cells and 20% of SKCO1 cells underwent lytic death, featuring pyroptotic morphologies (Fig. 3, A and B). Furthermore, robust interdomain cleavage of endogenous GSDMB—evident by the appearance of the p30 fragment—appeared concurrently (Fig. 3C).
The knockdown of GSDMB expression in OE19 cells by any of the three independent GSDMB-specific siRNAs effectively blocked GZMA electroperoration–induced pyroptosis (fig. S6A). GSDMB-knockout (KO) OE19 cells generated by using the CRISPR-Cas9 method also resisted pyroptotic killing by GZMA (fig. S6B). The reexpression of GSDMB WT but not the K229A/K244A double mutant in the GSDMB−/− OE19 cells restored GZMA electroperoration–induced pyroptosis (fig. 3D). When GZMA was delivered into OE19 cells by perforin, endogenous GSDMB underwent interdomain cleavage with the appearance of the p30 fragment (fig. S6C). Perforin-delivered GZMA also induced pyroptosis in OE19 cells, which was diminished in all three GSDMB KO clones (fig. S6, D and E). Thus, GSDMB+ cells undergo pyroptosis in response to interdomain cleavage by GZMA.

**Interferon-γ up-regulates GSDMB expression and promotes GZMA-induced pyroptosis**

Given the absence of detectable GSDMB protein in many cell lines (fig. S5A), we investigated whether GSDMB expression could be transcriptionally induced. SW837 cells were individually treated with a panel of cytokine receptor agonists or kinase inhibitors (fig. 3E and fig. S5B). Interferon-α (IFN-α), IFN-β, IFN-γ, and to a lesser extent tumor necrosis factor–α (TNF-α) increased the expression of endogenous GSDMB. Although the action of TNF-α was specific to certain cells, IFN-γ exhibits broad effects: 11 of the 36 GSDMB−/− cell lines, including two esophageal carcinoma cell lines (OE19 and OE33) and a breast cancer cell line (HCC1584). HCC1584 and OE33 cells normally expressed little to no GSDMB (fig. 3E).

Both IFN-γ and TNF-α are released by activated cytotoxic lymphocytes, and the former critically promotes or drives cell-mediated immunity. Up-regulation of GSDMB by IFNs and TNF-α underscores its role in lymphocyte cytotoxicity. IFN-γ priming of HCC1584 and SW837 cells markedly promoted pyroptosis induced by the cytosolic delivery of GZMA (fig. 3F and fig. S6F). In the NK-92MI cell–killing assay, pretreating SKO1 or OE19 cells with IFN-γ also caused more extensive pyroptotic death (fig. 3G). In both assays (fig. 3, F and G), possible cell death caused by IFN-γ alone (fig. S6G) was excluded from the analyses by extensive washing performed between IFN-γ priming and GZMA-mediated killing. Both basal and IFN-γ–primed pyroptosis in SKO1 or OE19 cells were inhibited by siRNA knockdown of GZMA in the NK-92MI cells (fig. 3, H and I). Knockdown of GZMA also reduced the interdomain cleavage of IFN-γ–primed endogenous GSDMB (fig. 3, H and J). Thus, GSDMB expression can be induced by IFN-γ, which promotes the GZMA-mediated pyroptotic killing of target cells.

**GZMA cleavage of GSDMB mediates pyroptotic killing by CTLs**

Similar to NK cells, CTLs use the perforin–granzyme pathway to confer cytotoxicity on target cells and also express high amounts of GZMA (2). To determine whether GZMA cleavage of GSDMB–induced pyroptosis contributes to CTL cytotoxicity, we first assayed the killing of CD19-expressing 293T cells by human anti-CD19 chimeric-antigen receptor (CAR) T cells. In a calcine release assay, the CAR T cells caused severe leakage of calcine fluorescence from the 293T cells only when they expressed GSDMB (fig. 4, A and B). Cells that released calcine exhibited evident pyroptotic morphologies. CAR T cell–induced pyroptosis was not detected in 293T cells expressing the K229A/K244A double mutant of GSDMB (fig. 4, A and B). Accordingly, interdomain cleavage in response to incubation with the CAR T cells occurred only with WT but not the K229A/K244A double mutant of GSDMB (fig. 4B). Conversely, siRNA knockdown of GZMA in CAR T cells inhibited calcine release from GSDMB-expressing 293T cells (fig. 4, C and D).

We also assayed primary human T cells engineered to express a specific T cell receptor (TCR) that recognizes the immunogenic tumor antigen NY-ESO-1. These TCR-engineered T cells (TCR T cells) induced pyroptosis in NY-ESO-1−/− A375 cells when GSDMB was coexpressed (fig. 4, E and F). These TCR T cells also stimulated the interdomain cleavage of GSDMB but did not activate the caspase-3–GSDME pathway because of the low amounts of GZMB in the particularly primary T cells (fig. 4, G and H). The K229A/K244A mutation in GSDMB not only blocked its interdomain cleavage but also rendered A375 cells resistant to pyroptotic killing by TCR T cells (fig. 4, E to G).

Unlike other human gasdermins, GSDMB has no orthologs in mice. However, mouse GZMA (mGZMA), sharing ~89% sequence similarity with human GZMA, could also cleave human GSDMB into the p30 and p16 fragments, albeit with less activity than that of human GZMA (fig. S7A). The cleavage by mGZMA was also diminished by the K229A/K244A double mutation in GSDMB. Consistently, the cytosolic delivery of mGZMA induced pyroptosis in 293T cells that express GSDMB WT but not the K229A/K244A double mutant (fig. S7B). This correlated with the interdomain cleavage of the expressed GSDMB (fig. S7C). We then isolated splenocytes from OT-1 transgenic mice and stimulated them with the SIINFEKL (OVA257–264) peptide to generate CTLs. In a coculture killing assay, these splenic CTLs triggered evident GSDMB-dependent pyroptosis in mouse colon adenocarcinoma MC38 cells that had been pulsed with the OVA257–264 peptide (fig. 4I). This pyroptosis response was not observed in MC38 cells expressing the K229A/K244A double mutant of GSDMB (fig. 4I). Thus, CTLs can also use GZMA to cleave GSDMB in target cells and therefore kill them through pyroptosis.

**GZMA cleavage of GSDMB promotes tumor clearance in mice**

Granzyme-mediated cell death is a critical mechanism for cytotoxic lymphocytes to eliminate malignant cells in antitumor immunity. We therefore hypothesized that GZMA– and GSDMB–dependent pyroptosis may have an important function in CTL-mediated tumor clearance, particularly given the proinflammatory nature of pyroptosis. To test this hypothesis, mouse colon carcinoma CT26 cells reconstituted with human GSDMB WT, GSDMB K229A/K244A, or an empty vector were engrafted subcutaneously into BALB/c mice (fig. 5A). Growth of the CT26 tumors was then followed continuously for 3 weeks. However, no statistically significant differences in tumor growth were observed among the three groups of mice; GSDMB+ and GSDMB− tumor grafts showed comparable increases in their volume and weight (fig. 5, B to E). CT26 tumors are known to respond well to blockade of the programmed cell death 1 (PD-1)–programmed death ligand 1 (PD-L1) pathway (46–49). The PD-1–PD-L1 interaction inhibits T cell recognition of target cells. Thus, the presence of the PD-1–PD-L1 checkpoint should prevent downstream granzyme functioning in the intact CT26 model. We therefore intraperitoneally injected antibody to PD-1 at days 8, 11, and 14 into the tumor-bearing mice (fig. 5A). Under this condition, a partial inhibition of tumor growth was observed with the control CT26 tumor grafts (fig. 5, B to E, and fig. S8A). Expression of GSDMB in CT26 cells nearly completely suppressed the tumor growth, and at day 22, the tumor burden was almost negligible (fig. 5, B to E, and fig. S8B). Expression of the GZMA-resistant K229A/K244A double mutant of GSDMB in CT26 cells showed no such effect. Moreover, tumors that express GSDMB WT but not the K229A/K244A double mutant contained increased numbers of PD−/− necrotic cells (fig. S8B).

We also examined the antitumor immune function of GSDMB in a more aggressive B16-F10 tumor model. For this, B16-F10 cells expressing an empty vector, GSDMB WT, or GSDMB K229A/K244A were engrafted subcutaneously into C57BL/6 mice, which was also followed by treatment with antibody to PD-1. Compared with the vector control, expression of GSDMB resulted in much slower increases in the tumor volume and the tumor weight, which were not observed with GSDMB K229A/K244A double mutant (fig. 5, F and G, 29 May 2020)
and fig. S8A). Theoretically, the antigenicity difference between GSDMB WT and GSDMB K229A/K244A may account for the contrasting tumor clearance effect, but the probability is extremely low. Thus, pyroptosis mediated by the GZMA–GSDMB pathway could potentially serve as an important effector mechanism in antitumor immunity.

**Insights into cancer and cancer immunotherapy**

Our initial untargeted profiling only identified a few cancer cell lines that were positive for GSDMB expression (fig. S5A). Among a panel of 27 diverse human tissue samples, we recorded high amounts of GSDMB expression in the esophageal epithelium, the tongue, the gastric mucosa, the duodenal mucosa, the jejunal mucosa, the ileal mucosa, the appendix, the colonic mucosa, the rectal mucosa, the trachea, and the bladder (fig. S9A).

This suggests that GSDMB may play important pathophysiological functions in these tissues. Upon realizing this tissue tropism, we were able to identify 15 more cancer cell lines with high GSDMB expression (fig. S9B). Among these, six were derived from esophageal and gastric tissues—echoing a previous report (50)—eight were from intestinal tissues, and one was ovarian in origin.

We further profiled a collection of diverse clinical tumor samples and found that GSDMB was prevalently expressed in colon, rectal, pancreatic, and cervical cancers but showed little or no expression in breast, lung, and liver cancers (fig. S10, A to D). Amounts of GSDMB expression in the four positive tumors did not significantly differ from those observed in corresponding tumor-adjacent normal tissues.

We identified 15 more cancer cell lines that were positive for GSDMB expression in 34 out 75 gastric and 44 out of 80 esophageal tumor tissues (fig. S10, B and C). By contrast, the vast majority of normal tissues corresponding to the 75 gastric and 80 esophageal tumor tissues were GSDMB−, suggesting the possible silencing of GSDMB in these two cancer types.

Given that many cancers were positive for GSDMB to varying degrees, we mined the public cancer gene expression and prognosis database using the newly developed GEPIA2 webserver that analyzes the data of 9736 tumors from The Cancer Genome Atlas (TCGA) database (51). Among 33 different tumors analyzed (fig. S11A), GSDMB mRNA expression and its corresponding prognosis value showed a statistically highly significant correlation (P < 0.001) for bladder carcinoma (BLCA), skin cutaneous melanoma (SKCM), and kidney renal clear carcinoma (KIRC) (fig. S11B). In BLCA and
Fig. 5. GZMA cleavage of GSDMB promotes antitumor immunity. (A to E) BALB/c mice were implanted subcutaneously (s.c.) with mouse colon cancer CT26 cells harboring an empty vector or expressing GSDMB WT or the K229A/K244A (KK/AA) double mutant (n = 7 or 8 mice per group). (A) The experimental design. (B) and (E) Photographs and weights of the CT26 tumors on day 22, respectively. (F and G) B16-F10 cells harboring an empty vector or expressing GSDMB WT or KK/AA mutant (n = 5 mice per group) were subcutaneously implanted into C57BL/6 mice. The mice were treated or not treated with antibody to PD-1 (aPD-1) same as that in the CT26 model. [(C) and (F)] Average tumor volumes of each group of mice. [(D and G)] The tumor size/weight of individual mouse at indicated time points. Data are means ± SD [(C), (E), and (F)], *P < 0.05, ***P < 0.001; two-tailed unpaired Student’s t test in (E), two-way analysis of variance (ANOVA) in (C) and (F). Data are representative of three [(B) to (E)] or two [(F) and (G)] independent experiments.

SKCM patients, high GSDMB expression was associated with an overall survival benefit, whereas KIRC patients showed a negative correlation. We also examined other gasdermins using the same parameters and observed a positive correlation with low-grade glioma (LGG) and SKCM for GSDMC and GSDMD, respectively (fig. S11, C and D). Considering the crucial function of GSDMB in cytotoxic lymphocyte–mediated tumor clearance, these analyses may prove instructive for future cancer immunotherapy approaches.

Discussion

We have demonstrated that GZMA from cytotoxic lymphocytes cleaves and activates GSDMB to induce target cell pyroptosis. The proinflammatory nature of pyroptosis, which distinguishes it from apoptosis, suggests that this type of lymphocyte killing is immunogenic and augments immunity. This ensures a sufficiently robust immune response. The presence of GSDMB in various healthy tissues, particularly those composing the digestive system, indicates that the GZMA–GSDMB pyroptotic axis may also play important roles in immunity to microbial infections in addition to cancers shown here. GZMA exhibits low cytotoxicity in vitro but considerable proinflammatory properties in vivo (32, 33). This seeming contradiction may be well explained by our finding that GZMA-mediated pyroptotic killing requires GSDMB, but that GSDMB is not expressed in cells previously used to study GZMA. Moreover, the function of GSDMB in cytotoxic lymphocyte–mediated killing is supported by its transcriptional priming by IFN-γ. This sheds new light on our understanding of the multifarious roles played by IFN-γ in immunity, including against tumors.

Last, our finding that GSDMB acts downstream of a granzyme reinforces our view that proteolytic enzymes such as caspases as well as granzymes are not cell death executor proteins per se. Rather, these enzymes cleave various downstream substrates, including gasdermins, which in turn can both initiate and implement particular cell death programs (24, 25, 33). Various target cells, which express a diversity of death executor molecules, may respond differently to the same initiating proteolytic activity, including that of granzymes. This further suggests that different target cells, even when they are similarly recognized by the same type of cytotoxic lymphocyte, may undergo dissimilar types of cell death. Thus, future studies of lymphocyte-mediated immunity, particularly in antitumor immunity, should be more focused on death execution–related events inside target cells rather than surface molecules (such as PD-1).

Methods summary

To explore the possible role played by gasdermins in lymphocyte cytotoxicity, we generated HEK 293T cells that stably expressed each of the five human gasdermins with a C-terminal Flag tag. They were then subjected to a 6-hour incubation with NK-92MI cells. HeLa, A375, and SW480
cells were also used to analyze pyroptosis functions of exogenous GSDMB and its cleavage by GZMA. SW837, OE19, and SKCO1 cells were subjected to GZMA or lymphocyte killing to examine endogenous GSDMB function. HCC1954, SW837, OE19, and SKCO1 cells were used to demonstrate the effect of IFN-γ priming of GSDMB expression and the increased pyroptotic killing by GZMA or cytotoxic lymphocytes. Primary CTCs—including anti-CD19 CAR T cells, NY-ESO-1–specific TCR T cells, and OT-1 mouse-derived CTCs—were prepared to assess pyroptotic killing of target cells. To evaluate GZMA cytotoxicity, purified GZMA was delivered into target cells either by means of electroporation or perfusion. To measure killing as well as to investigate the type of cell death (such as pyroptosis or apoptosis), morphological examination with microscopy, calcine release from target cells, adenosine triphosphate (ATP)-based cell viability and lactate dehydrogenase (LDH) release assays, as well as flow cytometry of annexin V– and PI-stained cells were used when appropriate. siRNA knockdown or CRISPR-Cas9 KO of GSDMB in OE19 cells and siRNA knockdown GZMA in NK-92MI or CAR T cells were performed to probe the genetic role of GZMA cleavage of GSDMB.

For in vitro biochemical experiments, granymes were expressed and purified from 293F cells. Recombinant gasdermins purified from E. coli were incubated with the granyme to assess possible cleavage, and the cleavage products were visualized on SDS–polyacrylamide gel electrophoresis gels. Throughout the study, a rabbit monoclonal antibody to GSDMB (EPR28041) that recognizes an epitope in the pore-forming domain, developed in collaboration with Abcam, was used for the immunoblot of GSDMB expression in cell lines, immunohistochemistry of GSDMB expression in normal or tumor tissues, as well as immunoblotting of GZMA-catalyzed cleavage during cell killing. To examine GSDMB’s antimtumor function, GSDMB WT, the K229A/K244A double mutant, or an empty vector were transduced into B7-positive targets. Antibody to PD-1 or anti-CD19 CAR T cells, adenosine triphosphate (ATP) release assays, as well as flow cytometry and PI-stained cells were used for the immunoblot of GSDMB expression.

K244A double mutant, or an empty vector were transduced into B7-positive targets. Antibody to PD-1 or anti-CD19 CAR T cells, adenosine triphosphate (ATP) release assays, as well as flow cytometry and PI-stained cells were used for the immunoblot of GSDMB expression.


ACKNOWLEDGMENTS

We thank Z. Shen for providing antibody to PD-1 and NK-92MI and A375 cells, Z. Dong for YTS cells, X. Zhao for SW837 and SKG01 cells, W. Shen for Edman sequencing, Y. Sun for assistance with microscopy, and Y. Xu for assistance with mouse experiments. We also thank members of the Shao laboratory for their assistance and helpful discussions.

Funding: The work was supported by the Basic Science Center Project of the National Natural Science Foundation of China (81788104), the National Key Research and Development Program of China (2017YFA0505900 and 2016YFA0501500), the Chinese Academy of Medical Sciences Innovation Fund for Medical Sciences (2019-2M-S-084), the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB37030202), and a fund from Hoffmann-La Roche AG (ROADS027).

Author contributions: Z.Z. and F.S. conceived the study; Z.Z., together with H.H. and Y.S., performed most of the experiments; K.W. contributed to the finding of GSDMB transcriptional priming; X.S., Yu.W., D.L., W.L., and J.D provided technical supports and insights. Ya.W. and W.H. prepared CAR T cells; Y.Z. and Lia.S. provided TCR T cells; Lin.S. provided tumor tissue samples; and Z.Z and F.S. analyzed the data and wrote the manuscript. F.S. obtained the funding and supervised the study. Competing interests: The authors declare no competing interests. Data and materials availability: All data are available in the main text or the supplementary materials. All the plasmids and cell lines generated in this study are available from the authors under a materials transfer agreement with the National Institute for Biological Sciences.

SUPPLEMENTARY MATERIALS

science.sciencemag.org/content/368/6494/eaaz7548/suppl/DC1

Materials and Methods

Figs. S1 to S11

References (54–59)

Movies S1 and S2

View/request a protocol for this paper from Bio-protocol.
Granzyme A from cytotoxic lymphocytes cleaves GSDMB to trigger pyroptosis in target cells

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Science 368 (6494), eaaz7548.
DOI: 10.1126/science.aaz7548Originally published online April 16, 2020

Granzyme A lights a fire

Cytotoxic T cells and natural killer cells use several strategies to kill infected or transformed cells. One such pathway entails the delivery of a family of serine proteases called granzymes to target cells through perforin-mediated pores to induce a form of programmed cell death called apoptosis. Zhou et al. show that granzyme A cleaves and activates gasdermin B (GSDMB), a central player in the highly inflammatory cell death process known as pyroptosis (see the Perspective by Nicolai and Raulet). GSDMB expression was highly expressed in some tissues and could be up-regulated by interferon-γ. Enforced expression of GSDMB in cancer cells enhanced tumor clearance in a mouse model, suggesting that this pathway may be a target for future cancer immunotherapies.

Science; this issue p. eaaz7548; see also p. 943