



RAPID COMMUNICATION

GSDMB N-terminal assembles in plasma membrane to execute pyroptotic cell death

Human genome encodes six paralogous gasdermin genes: *GSDMA*, *GSDMB*, *GSDMC*, *GSDMD*, *GSDME* and *DFNB59*.¹ Proteolytic cleavage of these gasdermin proteins liberates an N-terminal (NT) fragment from autoinhibition, which assembles in membrane to form pores and execute pyroptotic cell death in general.¹ In contrast to other gasdermins, *gasdermin B* (*GSDMB*) is the only gasdermin gene that has not been identified in rodents. Zhou et al first shed light on the molecular mechanism by which cytotoxic lymphocyte-derived granzyme A (GZMA) cleaves *GSDMB* to execute pyroptosis in *GSDMB*-positive cells, especially in cancer cells.² In this issue of *Cell*, Hansen et al reported a dynamic host pathogen *S. flexneri* prevents *GSDMB*-mediated lysis by secreting IpaH7.8, which targets and ubiquitinates *GSDMB* for 26S proteasome destruction.³ They showed that *GSDMB* implements bacteriocidal ability by recognition of the phospholipids on Gram-negative bacterial membranes rather than lysing host cells. Although their experimental design and data are clearly presented and straightforward, there are still some doubts to be clarified.

By introducing a tobacco etch virus protease cleavage site into the linker region of *GSDMB*, and incubating with tobacco etch virus protease and liposomes with different lipid components, Hansen et al found neither full-length *GSDMB* nor *GSDMB*-NT interacted with membranes composed of phosphoinositide (PI), phosphatidylinositol phosphates (PIPs), sulfatide, phosphatidylcholine (PC), phosphatidylethanolamine (PE), lysophosphatidylinositol, cholesterol and neutral lipids.³ Remarkably, they identified that cardiolipin (CL), and to a lesser degree phosphatidylglycerol (PG) and di[3-deoxy-D-manno-octulosonyl]-lipid A (KLA), function as binding substrates for *GSDMB*-NT. In fact, the inner leaflet of mammalian cell plasma membrane mainly contains phosphatidylserine, PE and PI, but not CL.⁴ We found that there was an obvious *GSDMB* activation in *GSDMB*-expressing HEK293T cells after 4 h of transfection with GZMA, accompanied by significantly increased lactate

dehydrogenase (LDH) activity in the media (Fig. 1A, B). These results indicate that *GSDMB* can indeed be cleaved by GZMA to induce pyroptosis, which are consistent with the previous finding.² However, according to lipid binding results reported by Hansen et al,³ it seems impossible for *GSDMB*-NT to form pores at cell plasma membrane and execute pyroptosis due to the lack of recognizable phospholipids. In the study of Hansen et al, *GSDMB* activation was only detected in cells infected with *S. flexneri* Δ *ipah7.8*, but not at all in non-infected *GSDMB*-positive HEK293T cells after GZMA transfection (see their Western blot results),³ which is inconceivable. It is difficult to offer a reasonable explanation for the discrepancy between our results and those reported by Hansen et al.

Intriguingly, a previous study⁵ showed almost completely inconsistent results with Hansen et al, that is, *GSDMB*-NT could bind PIPs but weakly with PG, CL and sulfatide. Considering that PI is one of the main phospholipids that make up the inner leaflet of cell membrane, the work of Chao et al seemingly explains why *GSDMB*-NT can form pores on the cell membrane to induce lytic cell death.⁵ Nevertheless, compared with the method of immobilizing lipids with nitrocellulose membrane *in vitro* used by Chao et al, constructing liposomes with a membrane bilayer by Hansen et al to interrogate the membrane binding properties of *GSDMB* can better mimic the real physiological situation, so it seems more convincing.^{3,5} Since Hansen et al uncovered that *GSDMB*-NT obviously interacted with CL, rather than PI or PIPs, and CL is a constituent of the inner mitochondrial membrane, we speculate whether this lytic cell death is secondary to the mitochondrial perforation caused by pore-forming activity of *GSDMB* due to re-localization of CL among membrane domains. Both re-distribution of CL and transmembrane movement lead to movement of CL to the outer mitochondrial membrane. To prove this hypothesis, we transfected *GSDMB*-expressing HEK293T cells with GZMA. At different time points (0, 1, 2 and 4 h), the samples were collected and cell fractionation was performed to isolate cellular compartments, including nucleus, cytoplasm, plasma membrane and mitochondria.

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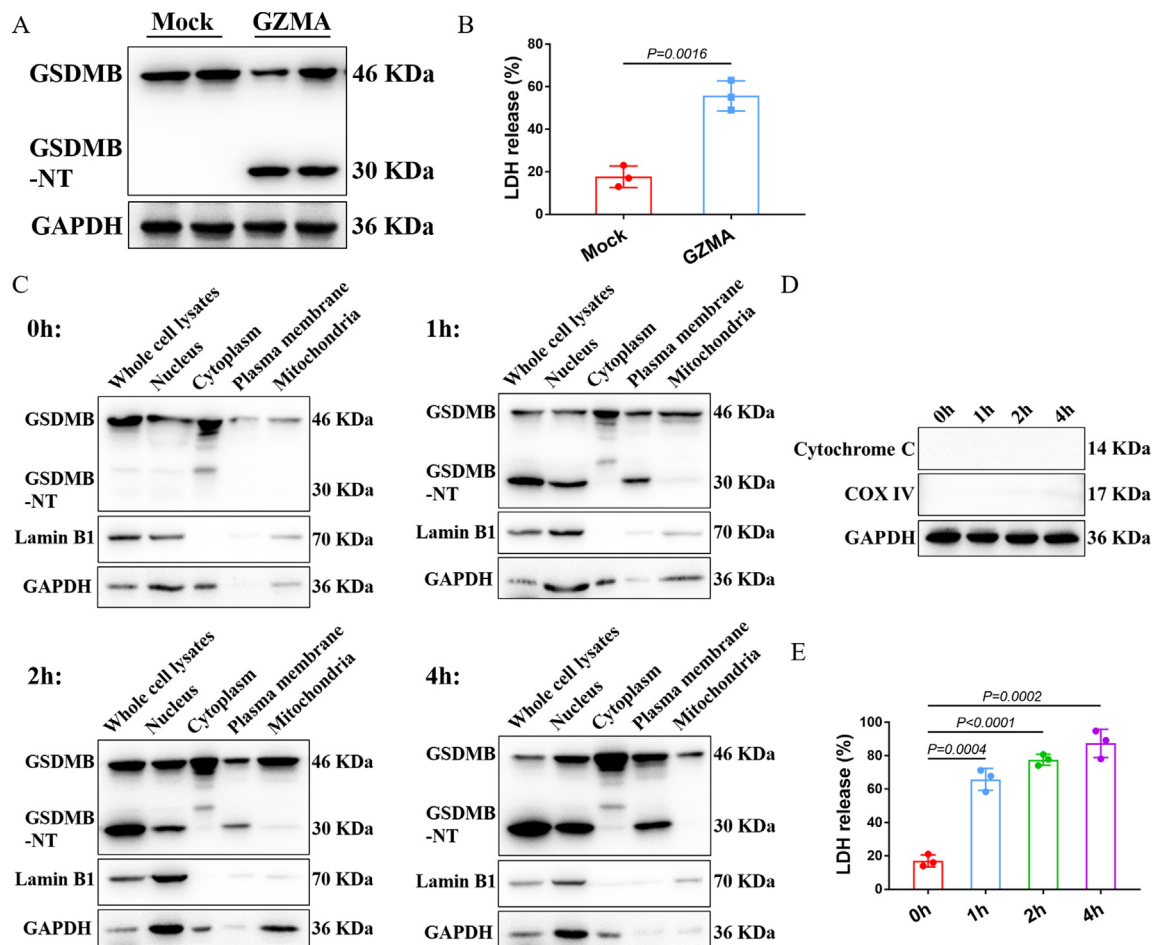


Figure 1 GSDMB is activated in HEK293T cells after 4-h transfection of GZMA and GSDMB-NT assembles in the plasma membrane rather than mitochondria to execute pyroptotic cell death. (A) GSDMB-expressing HEK293T cells were transfected with GZMA or empty vector (Mock group), and Western blots were performed to detect GSDMB activation in cell lysates after 4 h. (B) LDH activity in cell culture supernatant collected from experiments in (A) was elevated strikingly after GZMA transfection. (C) GSDMB-expressing HEK293T cells were transfected with GZMA, and the cell fractionation was performed including nucleus, cytoplasm, plasma membrane and mitochondria after 0, 1, 2 and 4 h. GSDMB-NT localization of different cell fractions was identified by Western blots at indicated timepoints. (D) The release of cytochrome c in cytoplasm without mitochondria was evaluated after transfection with GZMA for the indicated timepoints. (E) LDH activity in cell culture supernatant collected from experiments in (A, B) was detected.

Remarkably, consistent with the results in whole cell lysates, an obvious GSDMB-NT localization at plasma membrane was observed after 1, 2 and 4 h of transfection with GZMA (Fig. 1C). However, almost no GSDMB-NT was found in mitochondria (Fig. 1C). In addition, there was no release of cytochrome C in the whole time in the cytoplasm where mitochondria were removed (Fig. 1D), indicating the integrity of mitochondria was not damaged after GZMA transfection. Elevated LDH activity in cell culture supernatant further confirmed the lytic cell death after transfection with GZMA, and the notable increase of LDH release at 1 h after transfection suggested that the pyroptosis could occur rapidly in the early stage (Fig. 1E). Collectively, these results suggest that, like other gasdermin family proteins, GSDMB-mediated pyroptotic cell death is due to GSDMB-NT assembles in plasma membrane instead of mitochondria to form pores, which disrupts cell membrane integrity and results in the release of cellular contents into extracellular

space. Interestingly, in addition to assembling in plasma membrane, we also observed the comparable accumulation of GSDMB-NT in the nucleus (Fig. 1C), which may further promote the process of this lytic cell death.

While we do not dispute the membrane binding data of GSDMB from Hansen et al,³ their conclusion that GSDMB-NT does not interact with mammalian cell plasma membrane is indeed contradictory to our experimental evidence. There are several potential explanations for the conflicting results. Possibly, unlike other gasdermins, there may be a unique binding profile for GSDMB that can recognize some other types of substrates in plasma membrane, such as glycolipids or glycoproteins. Previous studies^{3,5} only focused on the binding properties of phospholipids. In addition, there are four GSDMB isoforms in human (1–4; Ensemble: ENSG00000073605), which differ in the length and sequence of the linker between the N- and C-terminal domains.¹ Whether different isoforms of GSDMB possess

distinct lipid binding activity is currently unknown. Our study analyzed the longest GSDMB isoform 3 (416 residues, National Center for Biotechnology Information Reference Sequence: NM_001165958.2), and we could not find which isoform of GSDMB was used for lipid binding assay in Hansen et al.³ (GSDMB isoform 1 used in their transgenic mouse model). More *in vitro* and *in vivo* evidence is required to understand whether the binding profile of GSDMB depend on its isoforms.

In conclusion, our data provide the evidence that GSDMB-NT, like other gasdermin family members, assembles in the mammalian cell plasma membrane to execute pyroptosis. However, whether GSDMB-NT recognizes other kinds of substrates except phospholipids to form pores or whether the lipid binding characteristics of different GSDMB isoforms are consistent still needs further study.

Conflict of interests

The authors declare no competing interests.

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