

Article

GSDMB promotes non-canonical pyroptosis by enhancing caspase-4 activity

Qin Chen^{1,†}, Peiliang Shi^{1,†}, Yufang Wang^{1,†}, Dayuan Zou¹, Xiuwen Wu², Dingyu Wang¹, Qiongyuan Hu², Yujie Zou¹, Zan Huang^{1,3}, Jianan Ren², Zhaoyu Lin^{1,*}, and Xiang Gao^{1,*}

¹ State Key Laboratory of Pharmaceutical Biotechnology and Nanjing Drum Tower Hospital, Model Animal Research Center, Nanjing University, Nanjing 210061, China

² Department of Surgery, Jinling Hospital, Medical School of Nanjing University, Nanjing 210061, China

³ Jiangsu Province Key Laboratory of Gastrointestinal Nutrition and Animal Health, Nanjing Agriculture University, Nanjing 210095, China

[†] These authors contributed equally to this work.

* Correspondence to: Zhaoyu Lin, E-mail: linzy@nju.edu.cn; Xiang Gao, E-mail: gaoxiang@nju.edu.cn

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Gasdermin B (GSDMB) has been reported to be associated with immune diseases in humans, but the detailed molecular mechanisms remain unsolved. The N-terminus of GSDMB by itself, unlike other gasdermin family proteins, does not induce cell death. Here, we show that GSDMB is highly expressed in the leukocytes of septic shock patients, which is associated with increased release of the gasdermin D (GSDMD) N-terminus. GSDMB expression and the accumulation of the N-terminal fragment of GSDMD are induced by the activation of the non-canonical pyroptosis pathway in a human monocyte cell line. The downregulation of GSDMB alleviates the cleavage of GSDMD and cell death. Consistently, the overexpression of GSDMB promotes GSDMD cleavage, accompanied by increased LDH release. We further found that GSDMB promotes caspase-4 activity, which is required for the cleavage of GSDMD in non-canonical pyroptosis, by directly binding to the CARD domain of caspase-4. Our study reveals a GSDMB-mediated novel regulatory mechanism for non-canonical pyroptosis and suggests a potential new strategy for the treatment of inflammatory diseases.

Keywords: GSDMB, GSDMD, pyroptosis, sepsis

Introduction

Pyroptosis, a pro-inflammatory form of cell death, is critical for host defense against infection and danger signals. However, excessive pyroptosis may cause septic shock and other immunological diseases (Strowig et al., 2012). The signature terminal events of pyroptosis are the activation of inflammatory caspases and the release of the GSDMD N-terminus (1–275 aa) to form pores in the plasma membrane (Broz, 2015; Rühl and Broz, 2016). In the presence of various microbial and endogenous stimuli, the inflammatory caspases (caspase-1/4/5 in humans and caspase-1/11 in mice) are activated to cleave GSDMD and then trigger cell membrane rupture in macrophages and neutrophils (Schroder and Tschopp, 2010; Shi et al., 2014). This process leads to the massive release of cellular contents including damage-associated molecular patterns (DAMPs) and cytokines, which triggers a robust local or systemic inflammatory response.

Pyroptosis is defined as either canonical or non-canonical pyroptosis based on the different stimuli and inflammatory caspases. In canonical pyroptosis, a range of DAMPs from the cell membrane activate caspase-1 through canonical inflammasomes, such as the NLRP3-ASC complex, and the GSDMD N-terminus is then released by caspase-1 to initiate pyroptosis. For non-canonical pyroptosis, cytoplasmic LPS activates different inflammatory caspases (human caspase-4/5 and mouse caspase-11) to cleave GSDMD, independent of inflammasomes and caspase-1 (Man and Kanneganti, 2015; Aglietti and Dueber, 2017; Man et al., 2017). A GSDMD deficiency exhibits a protective effect on an LPS-induced lethal septic shock mouse model and improves the survival of mice (Kayagaki et al., 2015). Thus, GSDMD N-terminus-activated pyroptosis may play a major role in the pathogenesis and mortality of inflammatory diseases.

GSDMD belongs to the gasdermin family. These gene family members share conserved N- and C-terminal domains with ~45% overall sequence homology (Tamura et al., 2007). In humans, the gasdermin family is composed of five paralogous subfamily genes, *GSDMA*, *GSDMB*, *GSDMC*, *GSDMD*, and *GSDME*.

However, there is no counterpart to the human *GSDMB* gene in the mouse genome, while other subfamily genes are conserved (Tanaka et al., 2013; Kovacs and Miao, 2017). *GSDMB* has a much broader expression pattern compared to other GSDM family members that show highly tissue-specific expression in the skin epithelium, gastrointestinal tract and immune cells (Saeki et al., 2009). Interestingly, we and others found the N-terminus of this family of proteins, with the exception of *GSDMB*, has the ability to induce cell death (Shi et al., 2015a, b). It remains unclear whether *GSDMB* functions by a similar mechanism as other GSDM family proteins.

Genome-wide association studies (GWAS) reveal a strong linkage between *GSDMB* and susceptibility to inflammatory diseases such as Crohn's disease, asthma, and type I diabetes (Saleh et al., 2011; Zhao et al., 2015; Chao et al., 2017). A study on transgenic mice expressing *GSDMB* has suggested the promoting role of *GSDMB* in the pathogenesis of asthma with increasing airway responsiveness and remodeling (Das et al., 2016). However, few studies have defined how *GSDMB* participated in the inflammatory regulation mechanistically. In this study, we found that *GSDMB* is upregulated in sepsis and Crohn's disease patients. Furthermore, *GSDMB* was involved in non-canonical pyroptosis through promoting the cleavage of *GSDMD* by enhancing the enzyme activity of caspase-4. Our study identified the functional mechanism of *GSDMB* in non-canonical pyroptosis and provided new insights into the pathogenesis of immune diseases.

Results

Sepsis and Crohn's disease are accompanied with GSDMB upregulation

In this study, we examined the expression of *GSDMD* and the *GSDMD* N-terminal fragment in leukocytes from septic patients ($n = 4$) and healthy volunteers ($n = 5$) by western blot analysis. We found that the *GSDMD* level was elevated, along with increased release of the *GSDMD* N-terminal fragment (Figure 1A and B). Thus, *GSDMD* N-terminus-activated pyroptosis may play a major role in the pathogenesis and mortality of sepsis in humans, which is consistent with the previous reports (Strowig et al., 2012). Since *GSDMD* and *GSDMB* both belong to the gasdermin family and *GSDMB* is also expressed in macrophages (Saeki et al., 2009), we detected the expression of *GSDMB* in leukocytes from sepsis patients. Interestingly, we found that the *GSDMB* expression level was significantly upregulated in sepsis patients (Figure 1A and C).

Among the phylogenetic analysis, *GSDMD* and *GSDMB* belong to the same protein family and share conserved protein sequences (Shi et al., 2015b). Previous works proposed that the *GSDMB* N-terminus could induce cell death in 293T cells (Ding et al., 2016; Panganiban et al., 2018), but we did not find any cleavage of *GSDMB* in sepsis patients (Supplementary Figure S1). To clarify the relationship between pyroptosis and the cleavage of *GSDMB*, we identified the *GSDMB* N-terminus (1–236 aa) and *GSDMB* C-terminus (237–417 aa) based on

previous reports (Shi et al., 2015b; Panganiban et al., 2018) (Figure 1D). Since the *GSDMD* N-terminus (1–275 aa) induced cell death, we examined whether the *GSDMB* N-terminus (1–236 aa) caused cell death. We cloned full-length *GSDMB* (1–417 aa), the *GSDMB* N-terminal domain (1–236 aa), the *GSDMB* C-terminal domain (237–417 aa), and the *GSDMD* N-terminal domain (1–275 aa) into pEGFP-N1 plasmids. Constructed plasmids were transfected into 293T cells, respectively. Western blotting showed that the expected proteins were normally expressed in 293T cells (Figure 1E). After 16 h of transfection, we found that only the *GSDMD* N-terminus (1–275 aa) induced significant cell death, and neither the *GSDMB* N-terminus nor *GSDMB* C-terminus caused cell death (Figure 1F and G). These results suggested that the *GSDMB* N-terminus did not work like the *GSDMD* N-terminus to cause cell death. In addition, immunohistochemistry analyses of colon tissues also showed that the level of *GSDMB* was significantly increased in the Crohn's disease samples ($n = 6$) compared with the para-carcinoma colon samples (considered to be normal tissues, $n = 6$) (Figure 1H). These results indicated that a high level of *GSDMB* was associated with inflammatory diseases without the release of the N-terminus.

Activation of non-canonical pyroptosis promotes GSDMB expression in THP-1 cells

The functional study of *GSDMB* *in vivo* is hindered by the lack of a *GSDMB* gene in mice. To explore the function of *GSDMB* in inflammatory diseases, we analyzed the role of *GSDMB* in pyroptosis in THP-1 cells, a human monocyte cell line. A previous study demonstrated that mice lacking either *caspase-11* or *Gsdmd* exhibited a protective effect against LPS-induced lethal septic shock (Kayagaki et al., 2015), suggesting that non-canonical pyroptosis played an important role in sepsis. In the present study, when the non-canonical pyroptosis pathway was activated by transfecting THP-1 cells with LPS, we found that *GSDMB* and *GSDMD* protein levels were both significantly elevated 8 h after LPS transfection (Figure 2A). The transcription levels of *GSDMB* and *GSDMD* were also upregulated (Figure 2B). LDH release and the *GSDMD* N-terminal fragment level, indicators of terminal pyroptosis events, were comparable between LPS-transfected cells and control cells at 8 h post-treatment (Figure 2A and C). At 16 h post-treatment, LDH release and *GSDMD* cleavage were both significantly increased, along with the upregulation of *GSDMB* and *GSDMD* transcription (Figure 2D–F). In the meantime, we did not detect any *GSDMB* cleavage band (Supplementary Figure S2). The above results suggested that the *GSDMB* response is quicker than that of *GSDMD* in cytoplasmic LPS-induced non-canonical pyroptosis.

Consistent with a previous report (Kayagaki et al., 2015), we found that LPS-transfected THP-1 cells showed higher caspase-4 expression and a significant increase in caspase-4 enzyme activity at 16 h (Figures 2D and 1G). Meanwhile, after LPS transfection, THP-1 cells exhibited a signature morphology of pyroptosis with cell swelling and the rupture of the membrane (Figure 2H). When canonical pyroptosis was activated with Nigericin (Chen et al., 2016) along with THP-1, *GSDMB* expression did not

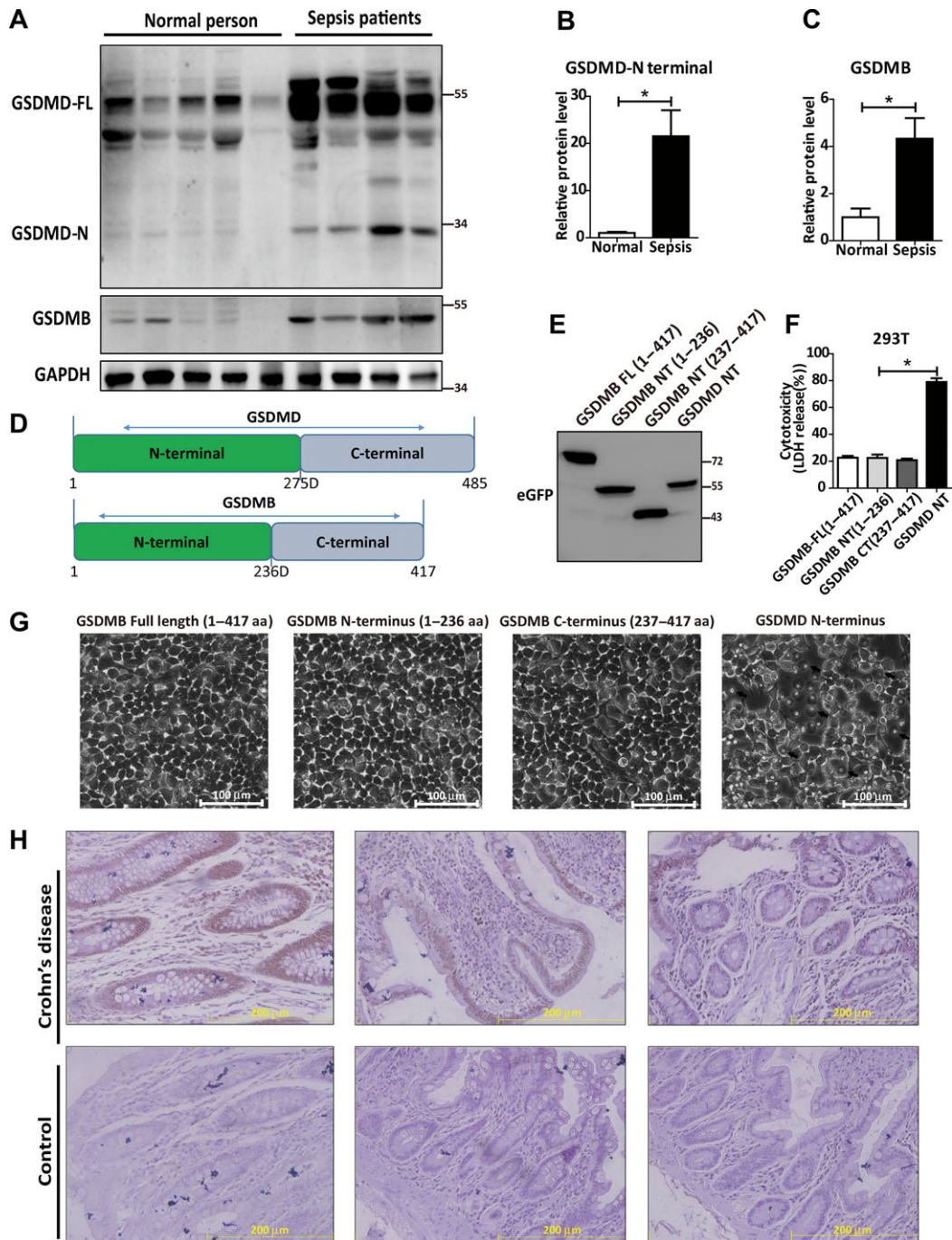


Figure 1 Sepsis and Crohn's disease are accompanied with GSDMB upregulation. **(A)** We collected blood from sepsis patients and healthy individuals and examined the GSDMB protein expression as well as the GSDMD cleavage level in white blood cells using western blotting. GAPDH was used as the loading control. **(B and C)** The intensities of the GSDMD N-terminus **(B)** and GSDMB protein **(C)** bands were quantified using the ImageJ software program and normalized to that of GAPDH. **(D)** Graphic representation of GSDMB and GSDMD proteins. **(E–G)** 293T cells were transfected with full-length GSDMB (1–417 aa), GSDMB N-terminus (1–236 aa), GSDMB C-terminus (237–417 aa), and GSDMD N-terminus (1–275 aa) plasmids. **(E)** Western blotting analysis of the protein expression levels in cells transfected with plasmids after 12 h of transfection. **(F)** LDH was released from 293T cells after 16 h of transfection. **(G)** The cell morphology of 293T cells after 16 h of transfection. Only the GSDMD N-terminus (1–275 aa) showed distinct cell death. **(H)** Human colon tissues from Crohn's disease patients and colon cancer patients (para-carcinoma colon tissues) were examined via immunohistochemistry with an anti-GSDMB antibody ($n = 6$ subjects per group). Data are presented as mean \pm SD. * $P < 0.05$ between groups as indicated.

change significantly, although the releases of GSDMD and GSDMD N-terminus were both upregulated (Figure 2I–K). Collectively, these data demonstrated that GSDMB is associated with non-canonical pyroptosis but not with canonical pyroptosis.

GSDMB expression is important for non-canonical pyroptosis in THP-1 cells

To determine whether GSDMB was required for cytoplasmic LPS-induced non-canonical pyroptosis, we knocked down GSDMB

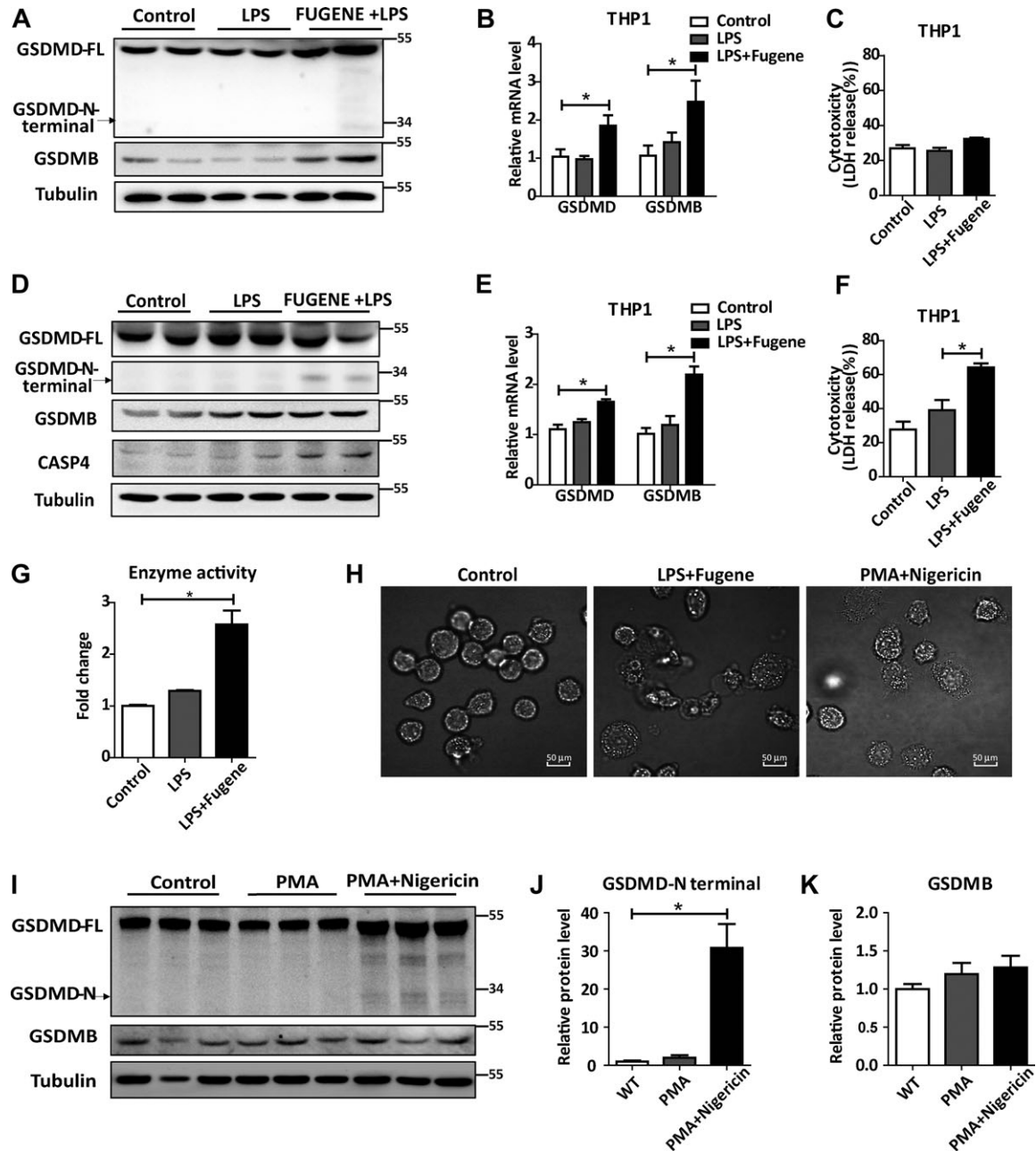


Figure 2 Activation of non-canonical pyroptosis promotes GSDMB expression in THP-1 cells. (A–G) THP-1 cells were treated with LPS or transfected with LPS using FuGENE HD, and analyzed for protein levels of GSDMB, full-length GSDMD, and the GSDMD N-terminus in THP-1 cells after 8 h (A) or 16 h (D), expression profiles of GSDMB and GSDMD in THP-1 cells after 8 h (B) or 16 h (E), LDH release from THP-1 cells after 8 h (C) or 16 h (F), and the protein level of caspase-4 (D) and enzyme activity of caspase-4 (G) in THP-1 cells after 16 h of treatment. (H) The morphology of THP-1 cells in canonical and non-canonical pyroptosis. For non-canonical pyroptosis, images of THP-1 cells were taken after 16 h of transfection with LPS using FuGENE HD. For canonical pyroptosis, images of THP-1 cells were taken after PMA pretreatment for 24 h, followed by Nigericin treatment for 4 h. (I–K) THP-1 cells were pre-treated with PMA and stimulated with Nigericin or without Nigericin. (I) Protein levels of GSDMB, full-length GSDMD, and GSDMD N-terminus in THP-1 cells were analyzed after 4 h of stimulation. The intensities of GSDMD N-terminus (J) and GSDMB protein (K) bands were quantified using the ImageJ software program and normalized to that of Tubulin. The data are presented as mean ± SD. **P* < 0.05 between groups as indicated.

expression by short hairpin RNA (shRNA) in THP-1 cells (Figure 3A and Supplementary Figure S3A). The decrease in GSDMB protein expression was confirmed by western blot, which showed a significant knockdown efficiency (Figure 3B and Supplementary Figure S3B). When GSDMB was knocked down, the cleavage of GSDMD and release of LDH were both significantly reduced in non-canonical pyroptosis (Figure 3C–E and Supplementary Figure S3C). Consistently, the overexpression of GSDMB promoted the cleavage of GSDMD and significantly increased the LDH release in non-canonical pyroptosis (Figure 3F–I). These results indicated that GSDMB is crucial for the activation of non-canonical pyroptosis and upstream of GSDMD cleavage.

GSDMB enhances the enzyme activity of caspase-4 by binding to caspase-4

Previous studies indicated that the activation of caspase-4 was required for the cleavage of GSDMD during non-canonical pyroptosis (Zhao et al., 2018). Therefore, we examined whether caspase-4 mediates GSDMB and promotes the cleavage of GSDMD in non-canonical pyroptosis. To sort out the functional relationships of these proteins, we co-expressed GSDMB, GSDMD, and caspase-4 in 293T cells. As expected, GSDMB promoted the cleavage of GSDMD by caspase-4 (Figure 4A and B). Since mouse Gsdmd and caspase-11 are the orthologues to human GSDMD and caspase-4, we also detected the effect of GSDMB on the cleavage of mouse Gsdmd by caspase-11 and found similar results (Figure 4C and D). In contrast, GSDMB had no influence on the cleavage of GSDMD by caspase-1 (Figure 4E and F), which was consistent with the above results that GSDMB is involved in non-canonical pyroptosis rather than canonical pyroptosis.

Initially, we suspected that GSDMB might interact directly with GSDMD to influence the cleavage of GSDMD, because we previously reported that the C-terminal domain of GSDMA3 could bind to its N-terminal domain and block the activity of the N-terminal domain (Shi et al., 2015b). However, we failed to detect the interaction between GSDMB and GSDMD in a co-immunoprecipitation assay (Figure 4G). Surprisingly, we found that GSDMB strongly interacted with caspase-4 (Figure 4H and I). In addition, the co-expression of GSDMB and caspase-4 in 293T cells led to higher caspase-4 enzyme activity than that observed in cells only expressing caspase-4 (Figure 4J). These results suggested that GSDMB promoted the cleavage of GSDMD by increasing the enzyme activity of caspase-4.

Full-length GSDMB promotes the enzyme activity of caspase-4 through the GSDMB N-terminus (1–83 aa) binding with the CARD domain of caspase-4

We further probed the specific regions involved in the binding between GSDMB and caspase-4. Expression vectors were generated to produce truncated GSDMBs, deleting 1–83 aa, 84–167 aa, 168–251 aa, 252–335 aa, and 336–417 aa, respectively (Figure 5A). Co-immunoprecipitation assays were performed to examine the interaction of these truncated GSDMBs with caspase-4. The results showed that fragment 1 (1–83 aa) of GSDMB was essential for binding with caspase-4 (Figure 5B). To identify the crucial region in

caspase-4 for binding with GSDMB, we generated caspase-4 truncation constructs (Figure 5C). We deleted the CARD domain, subunit 1, and subunit 2 from full-length caspase-4. We found that the CARD domain of caspase-4 was the target domain that bound with GSDMB (Figure 5D). Taken together, we demonstrated that GSDMB interacts with the CARD domain of caspase-4 through the N-terminal domain (1–83 aa).

Chao et al. (2017) have shown that GSDMB can be cleaved by caspase-3/6/7 to release a short GSDMB N-terminus (1–91 aa), and the cleavage site is 88DNVD91. We investigated whether GSDMB was also a substrate of the other human caspase family members and found that GSDMB was also specifically cleaved by human caspase-1/4/8/9 in addition to caspase-3/6/7 (Figure 5F). Previous works demonstrated that the release of the GSDMD N-terminus (1–275 aa) by caspase-1/4/5/11 cleavage could induce cell death (Kayagaki et al., 2015; Shi et al., 2015a), and we wondered whether the release of a short GSDMB N-terminus (1–91 aa) by caspase-1/3/4/6/7/8/9 induced cell death. We constructed plasmids encoding the GSDMB N-terminus (1–91 aa) and GSDMB C-terminus (92–417 aa) (Figure 5E) and then transfected the GSDMB N-terminus (1–91 aa), GSDMB C-terminus (92–417 aa), and full-length GSDMB into 293T cells for 16 h. In fact, we found neither the GSDMB N-terminus nor GSDMB C-terminus caused cell death (Figure 5H and I). Following these results, we examined whether the cleavage of GSDMB was required for the function of GSDMB in the promotion of caspase-4 enzyme activity. We overexpressed caspase-4 with either the GSDMB N-terminus (1–91 aa), GSDMB C-terminus (92–417 aa), or full-length GSDMB in 293T cells. We found that only the full-length GSDMB protein promoted caspase-4 enzyme activity (Figure 5G). All of the results above indicated that full-length GSDMB promoted the enzyme activity of caspase-4 through the GSDMB N-terminus (1–83 aa) binding with the CARD domain of caspase-4.

Phosphorylation of NF- κ B is required for the expression of GSDMB and GSDMD in THP-1 cells

The RNA-seq and ChIP analysis by Liu et al. (2017) revealed that NF- κ B elevated GSDMD transcription by binding to the GSDMD upstream promoter region. We found that the expression of both GSDMB and GSDMD was upregulated upon the activation of non-canonical pyroptosis in THP-1 (Figure 2B and E). Thus, we wondered whether NF- κ B can also regulate GSDMB expression. Indeed, phosphorylated NF- κ B (p-NF- κ B) was significantly upregulated, while the total NF- κ B level remained unchanged in THP-1 cells with non-canonical pyroptosis (Figure 6A–C). To further assess whether NF- κ B was involved in the regulation of cytoplasmic LPS-induced GSDMB and GSDMD expression, THP-1 cells were treated with the NF- κ B inhibitors JSH-23 (10 μ M) or QNZ (10 μ M) for 20 h. Then, the cells were transfected with LPS to induce non-canonical pyroptosis, and the protein levels of GSDMB and GSDMD were examined after 16 h. Cytoplasmic LPS-induced GSDMB and GSDMD expression were both significantly attenuated by either JSH-23 or QNZ (Figure 6D, G, and H), which was consistent with the p-NF- κ B level (Figure 6D and F), while the total NF- κ B did not show any significant changes (Figure 6E).

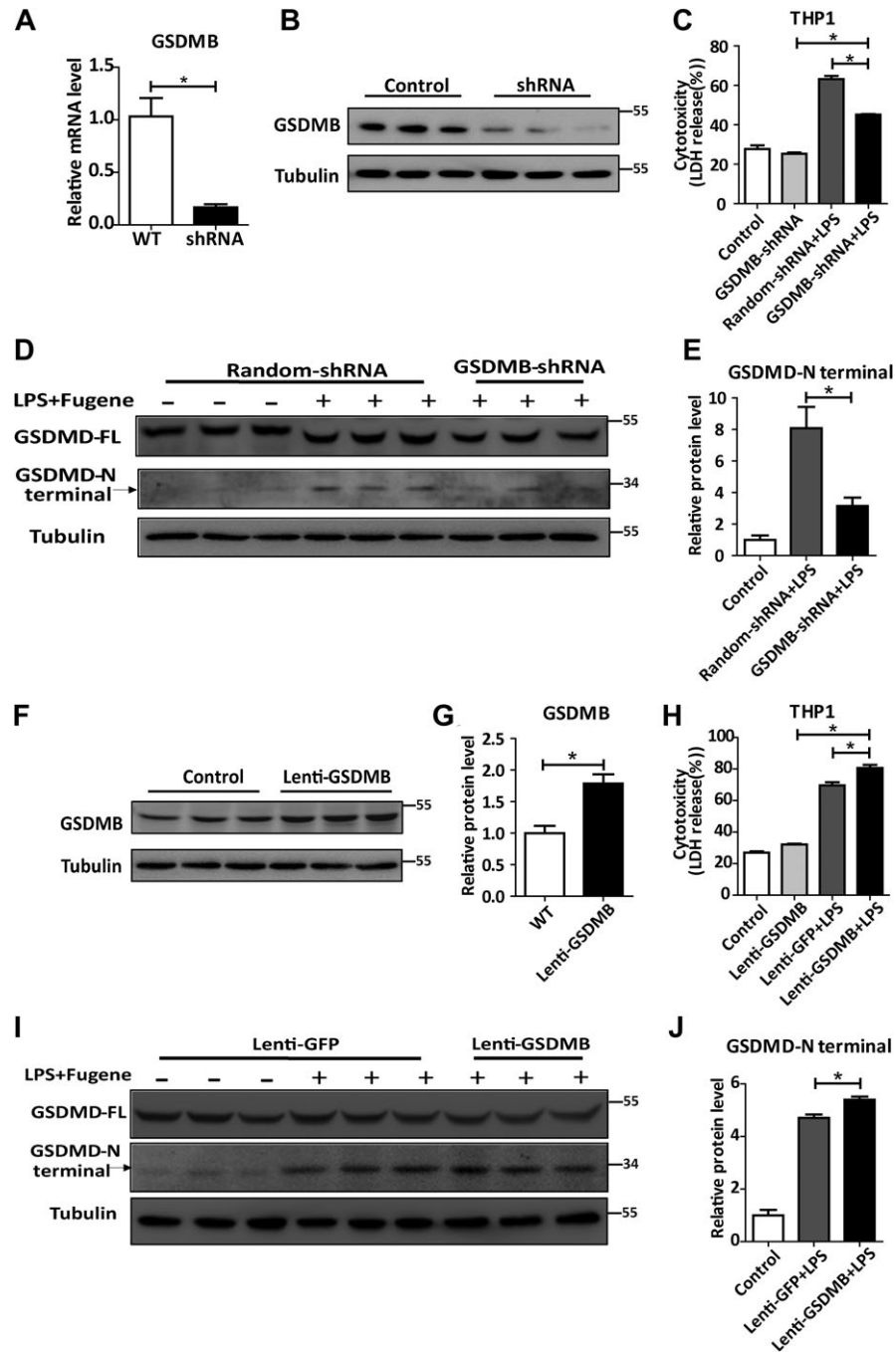


Figure 3 GSDMB expression is important for non-canonical pyroptosis in THP-1 cells. (A–E) THP-1 cells were infected with lentiviruses (pLKO puro) driving the expression of shRNAs targeting GSDMB or control shRNAs and analyzed for GSDMB transcript levels (A) and GSDMB protein levels (B) in THP-1 cells upon GSDMB shRNA knockdown, as well as LDH released from GSDMB-knockdown THP-1 cells after 16 h of transfection with LPS (C). (D and E) The cleavage of GSDMD protein was measured in GSDMB-knockdown THP-1 cells after 16 h of transfection with LPS (D) and GSDMD N-terminus protein bands were quantitatively analyzed using the ImageJ software program (E). (F–J) THP-1 cells were infected with lentivirus (pTRIPZ puro) driving the expression of GSDMB or eGFP. (F and G) GSDMB protein levels were measured in THP-1 cells upon GSDMB overexpression and quantitatively analyzed. (H) LDH released from GSDMB-overexpressing THP-1 cells was measured after 16 h of transfection with LPS. (I and J) The cleavage of GSDMD protein was measured in GSDMB-overexpressing THP-1 cells after 16 h of transfection with LPS (I) and GSDMD N-terminus bands were quantitatively analyzed (J). The data are presented as mean \pm SD. * $P < 0.05$ between groups as indicated.

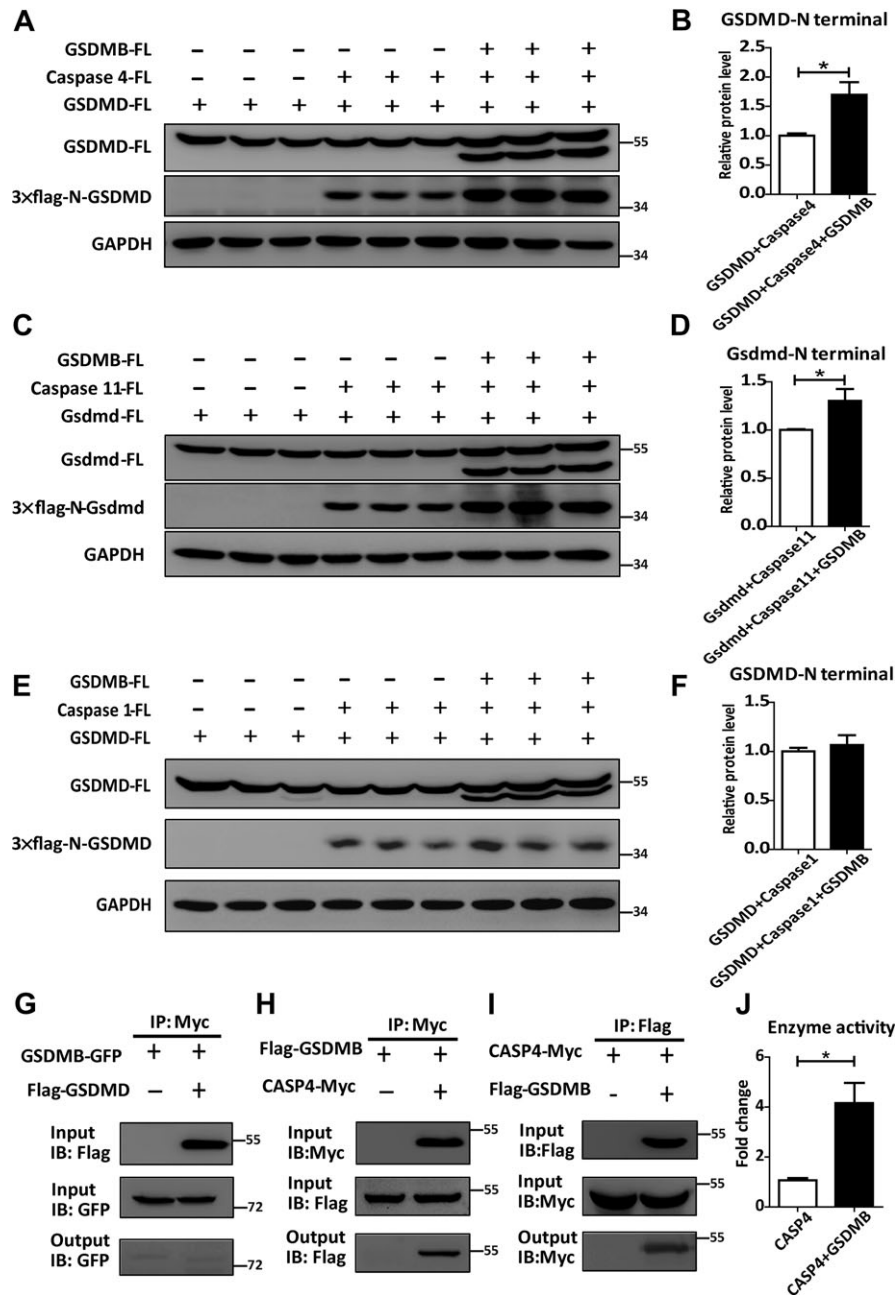


Figure 4 GSDMB enhances the enzyme activity of caspase-4 by binding to caspase-4. (**A**, **C**, **E**) Western blot analyses show the cleavage of GSDMD in 293T cells. (**B**, **D**, **F**) Graphs show GSDMD N-terminus levels normalized to GAPDH expression using the ImageJ software program. (**A** and **B**) 293T cells were co-transfected with plasmids encoding 3×FLAG-full-length GSDMD and either pcs2 (+) vector, pcs2 (+)-caspase-4, or pcs2 (+)-caspase-4 plus 3×FLAG-full-length GSDMB. (**C** and **D**) 293T cells were co-transfected with plasmids encoding 3×FLAG-full-length GSDMD and either pcs2 (+) vector, pcs2 (+)-caspase-11, or pcs2 (+)-caspase-11 plus 3×FLAG-full-length GSDMB. (**E** and **F**) 293T cells were co-transfected with plasmids encoding 3×FLAG-full-length GSDMD and either pcs2 (+) vector, pcs2 (+)-caspase-1, or pcs2 (+)-caspase-1 plus 3×FLAG-full-length GSDMB. (**G**) GSDMB did not interact with GSDMD. Co-IP analysis was performed in GSDMB-GFP- and FLAG-GSDMD-transfected 293T cells. (**H** and **I**) GSDMB interacted with caspase-4. Co-IP analysis was performed in caspase-4-Myc- and FLAG-GSDMB-transfected 293T cells. (**J**) The enzyme activity of caspase-4 in 293T cells. 293T cells were co-transfected with plasmids encoding pcs2 (+)-caspase-4 and either pcs2 (+) vector or pcs2 (+)-GSDMB. Graphs show mean \pm SD of triplicate wells and represent three independent experiments. * $P < 0.05$ between groups as indicated.

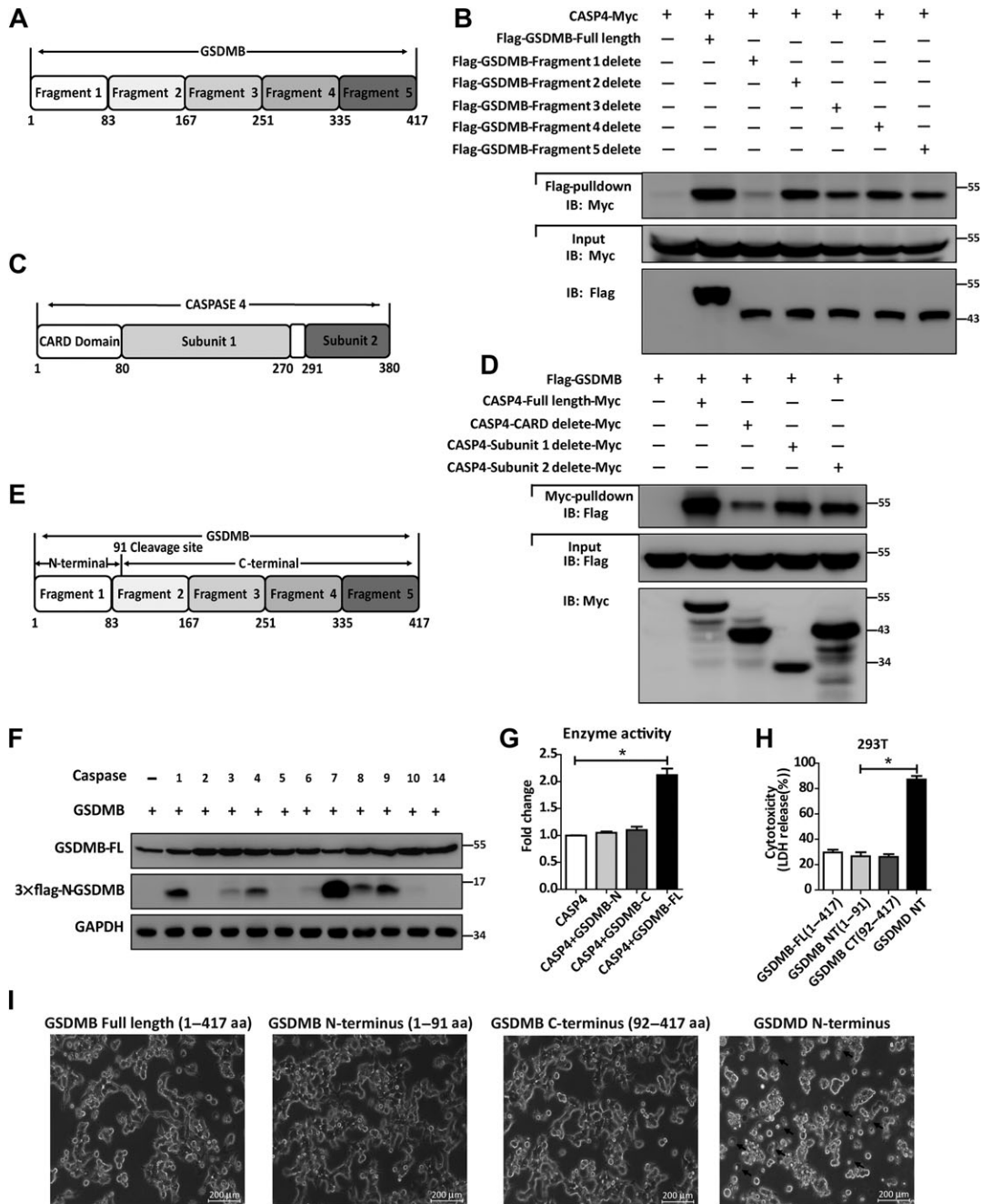


Figure 5 Full-length GSDMB promotes the enzyme activity of caspase-4 through the GSDMB N-terminus (1–83 aa) binding with the CARD domain of caspase-4. **(A)** Schematic of the GSDMB protein and the truncation fragments: fragment 1 (1–83 aa), fragment 2 (84–167 aa), fragment 3 (168–251 aa), fragment 4 (252–335 aa), and fragment 5 (336–417 aa). **(B)** 293T cells were transfected with the caspase-4-Myc expression plasmid and plasmids expressing the indicated FLAG-tagged GSDMB truncation form. Immunoprecipitation was performed using a monoclonal FLAG antibody, and samples were analyzed with a monoclonal Myc antibody via western blotting. **(C)** Graphic representation of the caspase-4 protein and the functional domains: CARD domain (1–80 aa), subunit 1 (81–270 aa), and subunit 2 (291–380 aa). **(D)** 293T cells were transfected with FLAG-GSDMB expression plasmid and plasmids expressing the indicated caspase-4-Myc truncation form. Immunoprecipitation was performed using a monoclonal Myc antibody, and the samples were analyzed with a monoclonal FLAG antibody via western blotting. **(E)** Graphic representation of GSDMB protein and the caspase cleavage site. **(F)** Assays of GSDMB cleavage by members of the human caspase family overexpressed in cells. 293T cells were co-transfected with plasmids encoding 3×FLAG-mouse GSDMB and the indicated pcs2 (+)-human caspase constructs. Total cell lysates were analyzed via anti-FLAG and anti-GAPDH immunoblotting. **(G)** The enzyme activity of caspase-4 in 293T cells. 293T cells were co-transfected with plasmids encoding pcs2 (+)-caspase-4 and either pcs2 (+) vector, pcs2 (+)-GSDMB N-terminus, pcs2 (+)-GSDMB C-terminus, or pcs2 (+)-full-length GSDMB. **(H and I)** 293T cells were transfected with full-length GSDMB (1–417 aa), GSDMB N-terminus (1–91 aa), GSDMB C-terminus (92–417 aa), and GSDMD N-terminus (1–275 aa) plasmids. **(H)** LDH released from 293T cells after 16 h of transfection. **(I)** The cell morphology of 293T cells after 16 h of transfection. The graphs show mean \pm SD of triplicate wells and represent three independent experiments. * $P < 0.05$ between groups as indicated.

These results demonstrated that the p-NF- κ B positively regulated the expression of GSDMB and GSDMD in the non-canonical pyroptosis of macrophages.

Discussion

The expression of GSDMB had been detected in esophagus, stomach, liver, colon, and lung tissues as well as in lymphocytes (Wu et al., 2009). A substantial amount of bioinformatics analysis supported that GSDMB was associated with the susceptibility to immune diseases, including asthma, Crohn's disease, and type I diabetes (Verlaan et al., 2009; Pal and Moul, 2015; Chao et al., 2017). However, the direct relationship between GSDMB and these inflammatory diseases had not been determined. In the current study, we demonstrated that GSDMB was significantly elevated in sepsis and Crohn's disease patients. Our data demonstrated that GSDMB promoted non-canonical

pyroptosis through binding with caspase-4 to increase the cleavage of GSDMD (Figure 7). These results are consistent with earlier reports suggesting that GSDMB may play an important role in several inflammatory diseases through regulating non-canonical pyroptosis (Simon and van der Meer, 2007; Hagar et al., 2013; Williams et al., 2015).

The absence of the *GSDMB* gene in the rodent genome makes it impossible to dissect GSDMB function using a gene target approach in mice. In this study, we found that human GSDMB can enhance mouse Gsdmd cleavage by caspase-11, the mouse orthologue of human caspase-4. This indicated that the components for mouse pyroptosis are comparable with human GSDMB function. This is consistent with the report that GSDMB-transgene mice are prone to the asthma phenotype by inducing 5-LO to increase TGF- β 1 expression in the bronchial epithelium (Das et al., 2016). Nevertheless, animals naturally expressing

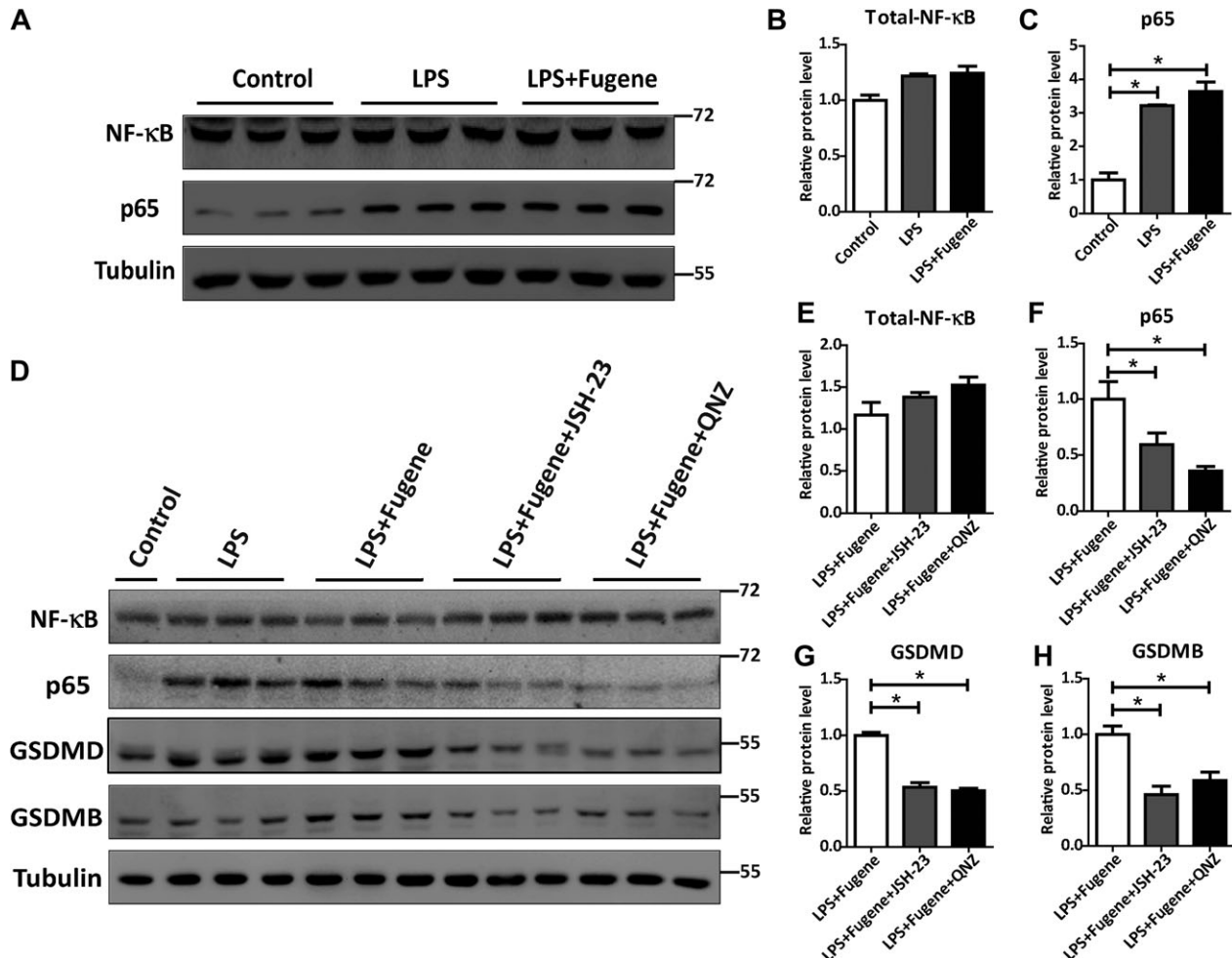


Figure 6 p-NF- κ B positively regulates the expression of GSDMB and GSDMD in THP-1 cells. (A–C) THP-1 cells were treated with LPS or transfected with LPS using FuGENE HD. (A) Protein levels of NF- κ B and p65 in THP-1 cells after 8 h of treatment. NF- κ B (B) and p65 (C) protein bands were quantified using the ImageJ software program and normalized to that of tubulin. (D–H) NF- κ B inhibitor (JSH-23 or QNZ) was used to pre-treat THP-1 cells at a final concentration of 10 μ M for 20 h, and cells were further stimulated with LPS or transfected with LPS using FuGENE HD for 8 h. (D) Western blot analysis of NF- κ B, p65, GSDMD, and GSDMB protein levels. Graphs show NF- κ B (E), p65 (F), GSDMD (G), and GSDMB (H) protein levels normalized to Tubulin using the ImageJ software program. Graphs show mean \pm SD of triplicate wells and represent three independent experiments. * $P < 0.05$ between groups as indicated.

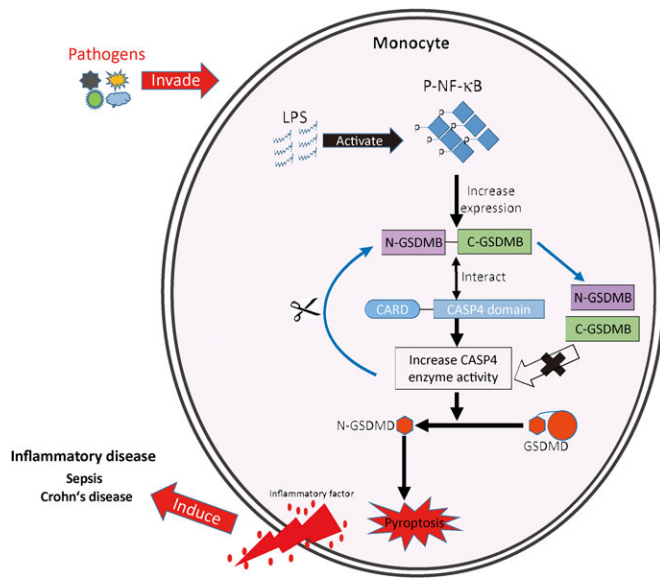


Figure 7 GSDMB promotes pyroptosis through the caspase-4-mediated non-canonical pathway. Schematic diagram shows GSDMB in non-canonical pyroptosis by influencing GSDMD cleavage. Cytoplasmic LPS activates NF- κ B to induce GSDMB expression in THP-1 cells. Then, GSDMB promotes the caspase-4 activity, which is required for the cleavage of GSDMD in non-canonical pyroptosis, by directly binding to the CARD domain of caspase-4. Additionally, the positive effect of GSDMB on caspase-4 in non-canonical pyroptosis can be terminated by a negative feedback mechanism, in which GSDMB is cleaved by caspase-4. Then, the release of the GSDMD N-terminus leads to the pore formation and cell membrane rupture, which in turn causes a massive secretion of inflammatory cytokines.

the GSDMB protein, such as monkeys, dogs, and cattle, may provide more direct evidence on the physiological function of GSDMB *in vivo*.

The binding of GSDMB with caspase-4 may result in the oligomerization of the caspase-4 proteins, which further drive conformational changes in caspase-4 that lead to higher activity. Under the above situations, multiple caspase-4 molecules are brought into close proximity, which may induce an active enzyme conformation. Shi et al. (2014) demonstrated that LPS directly binds to the CARD domain of caspase-4/11 with high specificity and affinity, suggesting that the CARD domain may serve as a key regulatory domain for caspase-4 activity. This was consistent with our findings that GSDMB bound to the CARD domain of caspase-4 and regulated its enzymatic activity. A commonly believed model is that caspase-1 or caspase-9 requires oligomerization for auto-processing and the release of the active form (Riedl and Shi, 2004; Yang et al., 2015). It has been reported that caspase-4/11 can also form oligomers, although this auto-process is not necessary for activating caspase-4/11 (Yang et al., 2015). A detailed analysis is required in the future to test this hypothesis.

GSDMB can be cleaved by the apoptotic executioners caspase-3/6/7 at the 88DNVD91 site, releasing the GSDMB N-terminus

(1–91 aa) and GSDMB C-terminus (91–417 aa) (Chao et al., 2017). Unlike the N-terminus of GSDMD and GSDMA/Gsdma33, which were affiliated with cardiolipin and showed pyroptotic activity, the GSDMB N-terminus was affiliated with sulfatide and did not cause cell death (Zhou et al., 2012; Shi et al., 2015b; Chao et al., 2017). Consistently, in this work, we demonstrated that neither the GSDMB N-terminus (1–91 aa) nor the GSDMB N-terminus (1–236 aa) caused cell death (Figures 1F, G and 5H, I). Moreover, our results demonstrated that the GSDMB N-terminus lost the ability of full-length GSDMB to promote the enzyme activity of caspase-4 (Figure 5G). This conclusion is inconsistent with Ronald's work, which proposed that GSDMB was cleaved by caspase-1 at the 236 aa site and the GSDMB N-terminus (1–236 aa) induced cell death (Panganiban et al., 2018). In fact, as found in other reports, only GSDMB cleaved by caspase-1 at the 91 aa site can produce a 10-kDa N-terminus (Chao et al., 2017). Taken together, we believe that the GSDMB N-terminus does not have a pore-forming ability, and the enhanced cell death caused by GSDMB is actually through enhancing the enzymatic activity of caspase-4.

Interestingly, while GSDMB can bind to caspase-4 to promote caspase-4 enzymatic activity, it is also a *bona fide* substrate for caspase-4, with the same digestion position as caspase-3/6/7 (Figure 5F). Therefore, we believe that the positive effect of GSDMB on caspase-4 in non-canonical pyroptosis can be terminated by a negative feedback mechanism. This mechanism may serve an important protective function, as excessive or prolonged pyroptosis in response to infectious pathogens can be detrimental. In particular, caspase-7 displayed the highest cleavage activity for GSDMB, implying potential cross-talk between the apoptosis pathway and the non-canonical pyroptosis pathway.

Moreover, the NF- κ B signaling pathway, which was correlated with the inflammatory response, had been considered to exist in connection with pyroptosis (Takagi, 2011; Simard et al., 2013). Several studies had determined that NF- κ B could bind to NLRP3 and the GSDMD upstream promoter region, and they suggested the transcriptional regulation of NF- κ B on NLRP3, GSDMD, and their downstream targets (Qiao et al., 2012; Liu et al., 2017). In the current study, we observed an enhanced p-NF- κ B expression in THP-1 cells under non-canonical pyroptosis and found similarly elevated expression of GSDMB and GSDMD. Pre-treating THP-1 cells with NF- κ B inhibitors (JSH-23 and QNZ), we found that GSDMB and GSDMD expression both declined in non-canonical pyroptosis, which was consistent with the reduction of p-NF- κ B. Based on these findings, we suggested that NF- κ B is involved in the regulation of non-canonical pyroptosis by modulating the expression of GSDMD and GSDMB in THP-1 cells. Among the many known pattern recognition receptors, toll-like receptors (TLRs) are one of the characterized sensing molecular families located on the surface of cells (TLR1, 2, 4, 5, 6, and 10) and endosomes (TLR3, 7, 8, 9, 11, 12, and 13) to sense exogenous and endogenous danger signals (Moresco et al., 2011). It may be possible that the cytoplasmic LPS, which induced the non-canonical pyroptosis, could be recognized by intracellular TLRs and activated the NF- κ B pathway to regulate GSDMD and GSDMB expression. For example, previous studies demonstrated that

TLR-9 regulated the NF- κ B–NLRP3–IL-1 β pathway to mediate intestinal inflammation, and CD14 (a co-receptor with TLR4 for LPS recognition) was also required for the TLR-9-dependent induction of pro-inflammatory cytokines (Baumann et al., 2010; Zanoni et al., 2011; Li et al., 2017). TLR-9 may recognize cytoplasmic LPS with a CD14 assistant to activate the NF- κ B pathway and then regulated GSDMD/GSDMB transcriptional expression. Future research will focus on dissecting the recognition mechanism between cytoplasmic LPS and NF- κ B.

In general, humans are more susceptible to inflammatory disease than rodents, which we use for mechanistic studies. Our data provided a potential explanation for this interesting phenomenon because of the lack of the GSDMB gene in rodent genomes. Our results also support the previous bioinformatics studies suggesting a strong link between GSDMB and susceptibility to inflammatory diseases. We believe further detailed analysis will shed more important insight into the mechanisms of GSDMB-regulated non-canonical pyroptosis and provide potential targets for treating inflammatory diseases.

Materials and methods

Blood samples and leukocyte isolation

Blood samples from sepsis patients were procured by Jinling Hospital, Medical College of Nanjing University. All samples were obtained with informed consent from patients according to the Declaration of Helsinki. In addition, the acquisition of blood samples was approved by the Institutional Review Board of Jinling Hospital. Blood was also drawn from 5 healthy volunteers for the isolation of leukocytes. Up to 5 ml of blood was collected in heparinized tubes. The protocol for the separation of leukocytes from human blood was based on a previous study (Hirsch and Cohn, 1960). Briefly, blood samples were centrifuged at 1000 \times g for 10 min at 4°C, and the plasma supernatant was removed. Then, cells were incubated in Gey's solution (4.15 g NH₄Cl and 0.5 g KHCO₃ in 500 ml of ddH₂O) for 5 min at room temperature to remove the red blood cells. Leukocytes were isolated and prepared for protein extraction and western blotting.

Immunohistochemistry

This research was approved by the Ethics Committee of Jinling Hospital, and all patients signed written consent that their surgical specimens could be used for scientific research. Human colon specimens were obtained from six patients with Crohn's disease and six patients without Crohn's disease (control, para-carcinoma colon tissue) undergoing surgical resection. The colon samples were fixed in 10% buffered formalin and then cut into paraffin sections. Immunohistochemistry staining was performed following the manufacturer's protocol (MXB Biotechnologies). Briefly, 5- μ m paraffin sections were deparaffinised, rehydrated, submerged in antigen retrieval solution, and then treated with endogenous peroxidase blocker for 10 min, followed by incubation with normal goat serum. The sections were incubated at 4°C overnight with the primary antibody for GSDMB (Abgent) and then visualized with a 3,3'-diaminobenzidine tetrahydrochloride detection kit (MXB) and counterstained

with hematoxylin. Images were taken with a high-resolution digital camera (Olympus DP 50).

Cell culture

Human embryonic kidney (HEK) 293T cells and 293FT cells were obtained from the ATCC. These two cell lines were cultured in Dulbecco's-modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. THP-1 (human monocyte) cells were obtained from the Shanghai Institute of Biochemistry and Cell Biology and maintained in RPMI-1640 medium supplemented with 10% FBS (PAN Biotech) and penicillin/streptomycin. Cells were grown at 37°C in a 5% CO₂ incubator. To activate the canonical caspase-1 inflammasome pathway, THP-1 cells were pre-treated with 50 ng/ml PMA for 24 h and then stimulated with 10 μ M Nigericin (Chen et al., 2016). For activation of the non-canonical caspase-4/11 inflammasome pathway, THP-1 cells were transfected with 5 μ g/ml LPS using 0.25% (v/v) FuGENE HD (Promega) as described in a previous study (Kayagaki et al., 2015). To inhibit the NF- κ B pathway, the NF- κ B inhibitors JSH-23 and QNZ were purchased from MedchemExpress and used to pre-treat THP-1 cells for 20 h.

Plasmid construction

Complementary DNA (cDNA) for mouse *Gsdmd* and mouse *caspase-11* was amplified from reverse-transcribed cDNA from mouse tissue. cDNA for human *GSDMB* and *GSDMD* was amplified as previously described (Shi et al., 2015b). cDNA for human *caspase-2*, *caspase-3*, *caspase-4*, *caspase-5*, *caspase-7*, *caspase-9*, and *caspase-10* was kindly provided by Prof. Jiahuai Han from Xiamen University, China, and cDNA for human *caspase-1*, *caspase-6*, *caspase-8*, and *caspase-14* was synthesized by a gene synthesis facility (GenScript). The human and mouse gasdermin cDNA was inserted into the *NotI* and *XbaI* sites of separate p3 \times FLAG vectors. The encoded gasdermin proteins had a 3 \times FLAG tag at the N-terminus. The human and mouse caspase cDNA was inserted into the *Clal* and *XbaI* sites of separate pcs2 (+) expression vectors. Moreover, a pEGFP vector containing human GSDMB was constructed, as described in our previous work, and the encoded proteins had a GFP tag at the C-terminus. The Myc-tagged caspase-4 truncation constructs and FLAG-tagged GSDMB truncation constructs were generated by PCR amplification and standard recombinant DNA techniques. The GSDMB N-terminus (1–91 aa), GSDMB C-terminus (91–417 aa), and full-length GSDMB proteins were amplified into the *Clal* and *XbaI* sites of pcs2 (+) vectors. The above constructed plasmids were confirmed by PCR amplification, restriction enzyme digestion, and DNA sequencing.

shRNA knockdown and lentiviral overexpression

For GSDMB knockdown, pLKO-1 plasmids containing shRNA-targeting human GSDMB were constructed to target the GSDMB ORF (GCCTTGTTGATGCTGATAGAT, CCGGATATCCCAGCAGTAT, and GGAGACGGTAAAGGAGGAA). This shRNA-expression lentiviral plasmid was co-transfected with the plasmids pSPAX2 and pMD2G into 293FT cells. Viral-containing media were collected, filtered, and concentrated by ultracentrifugation. THP-1 cells

were infected with the lentivirus and selected for puromycin resistance. To construct the lenti-GSDMB overexpression system, GSDMB was cloned into the modified vector pTRIPZ to drive transgene expression with an IRES eGFP for visualization in cells. The pTRIPZ-GSDMB expression plasmid, pSPAX2 plasmid, and pMD2G plasmid were co-transfected overnight using Lipofectamine 2000 into 293FT cells. The lentivirus particles were harvested as described above. The GSDMB mRNA and protein levels in cells were measured 48 h post-infection.

Western blotting

The total cell protein was extracted on ice using lysis buffer (50 mM Tris-HCl, pH = 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.1 mM EDTA, 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, 0.1 mM Na₃VO₄, 0.1 mM NaF, and cocktail protein inhibitor). The protein concentrations were determined with a Bradford bioassay using a Bradford protein assay kit (Sangon). Protein samples were electrophoresed in 4% stacking and 10% resolving SDS-PAGE gels, and the fractionated proteins were transferred to Hybond-P polyvinylidene difluoride membranes (Amersham Bioscience). Blots were blocked with 5% fat-free milk at room temperature for 1 h and were incubated with primary antibody overnight at 4°C. After being washed with TBST, the blots were incubated with secondary antibody for 1 h at room temperature. Proteins were visualized using enhanced chemiluminescence substrate (Tanon) and then quantified using a Tanon Chemiluminescent Imaging System. Anti-GSDMD, anti-FLAG, anti-GAPDH, anti-tubulin, anti-mouse IgG, and anti-rabbit IgG antibodies were purchased from Sigma-Aldrich. Other antibodies used in this study included anti-GSDMB (Abgent), anti-caspase-4 (Santa Cruz Biotechnology), anti-total NF-κB (Cell Signaling Technology), anti-p-NF-κB (Cell Signaling Technology), and anti-Myc (Abcam).

Real-time quantitative PCR analysis

To measure mRNA (GSDMB and GSDMD) expression, relative standard real-time PCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems). The PCR primers were designed using the software Primer Express (Applied Biosystems), and the housekeeping gene *36B4* was used as an endogenous control. The total RNA was isolated from THP1 cells with RNAiso Plus (TaKaRa) according to the manufacturer's instructions. A PrimeScriptTM RT reagent kit with gDNA Eraser (TaKaRa) was used to synthesize first-strand complementary DNA from equivalent amounts of RNA. Then, quantitative RT-PCR was performed with SYBR Premix EX TaqTM (TaKaRa). Each sample was amplified in quadruplicate. Relative gene expression was calculated using the relative standard curve and was normalized to the expression of the housekeeping gene *36B4*.

Co-immunoprecipitation studies

HEK293 cells were transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen). After 24 h of transfection, cells were lysed with a non-denaturing co-immunoprecipitation lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1 mM EDTA,

1% NP-40, pH = 8.0). Cells were then sonicated three times for 10 sec and centrifuged at 12000 rpm for 15 min at 4°C. Then, the whole-cell lysate was precleared with Protein G-agarose beads (GE Healthcare) for 4 h and incubated with 2 μg of the indicated antibody overnight at 4°C. The following day, protein complexes were pulled down with Protein G-agarose for 2 h with shaking at 4°C. Beads were washed three times with lysis buffer and four times with PBS and then eluted by boiling in protein loading buffer, followed by detection via western blotting, as described above.

Measurement of caspase-4 activity

Caspase-4 activity was determined using a Caspase-4 Assay (Colorimetric) System (Abcam) according to the manufacturer's instructions. In brief, the culture medium was removed, and the cells were collected and incubated in chilled cell lysis buffer for 10 min on ice. The cytosolic extract was obtained by centrifugation at 10000× *g* for 1 min at 4°C. The protein concentration was determined with a Bradford protein assay kit (Sangon). Cytosol containing 200 μg of protein was combined with 5 μl of 4 mM LEVD-p-NA substrate (200 μM final concentration) in 2× reaction buffer (containing 10 mM DTT), and the reaction was conducted for 1 h at 37°C. Cytosolic caspase-4 activity was assayed by measuring the increase in absorbance at 405 nm.

Cytotoxicity assay

LDH activity in the supernatant was determined to evaluate cell death. LDH release was measured using a CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega) according to the manufacturer's guidelines. In addition, the absorbance of the supernatant was examined at 490 nm. All values represent the percentage of LDH release compared with a maximum lysis control (1% Triton X-100-lysed cells).

Statistical analysis

All of the results are expressed as mean ± SD. Differences between groups were evaluated for statistical significance using Student's *t*-test. *P*-values <0.05 were considered statistically significant (**P* < 0.05, ***P* < 0.01).

Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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Author contributions: Q.C. and P.S. conducted most of the experiments, analyzed the data, and prepared the figures; Y.W., D.Z., and D.W. constructed the plasmids; X.W., Q.H., and J.R. prepared the patient samples; Z.H. and Y.Z. contributed valuable discussion during the study; Q.C., P.S., Z.L., and X.G. contributed to the experimental design and drafted the paper; and X.G. and Z.L. designed the study and edited and revised the paper. All authors reviewed the results and approved the final version of the manuscript.

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