

# Gasdermin D Protects from Melioidosis through Pyroptosis and Direct Killing of Bacteria

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**Gasdermin D (GSDMD) cleavage by caspase-1 or caspase-11 inflammasomes triggers pyroptosis, a lytic form of cell death protective against intracellular bacteria. In this study, we examine the role of GSDMD in a mouse model of melioidosis. *Gsdmd*<sup>-/-</sup> mice were more susceptible than wild-type mice to intranasal infection with *Burkholderia thailandensis*. Production of IL-18, but not IL-1 $\beta$ , was decreased in *Gsdmd*<sup>-/-</sup> infected mice. Despite lower IL-18, IFN- $\gamma$  was produced in similar amounts in wild-type and *Gsdmd*<sup>-/-</sup> mice. In vitro, secretion of both IL-1 $\beta$  and IL-18 by macrophages or dendritic cells infected with *B. thailandensis* was dependent on GSDMD. Surprisingly, wild-type or GSDMD-deficient neutrophils secreted similar amounts of IL-1 $\beta$ , suggesting these cells may be the source of the GSDMD-independent IL-1 $\beta$  detected in vivo. Recombinant GSDMD was able to directly kill *B. thailandensis* in vitro upon processing by active caspase-1. Moreover, bacteria harvested from wild-type, but not *Gsdmd*<sup>-/-</sup>, macrophages were more susceptible to the microbicidal effect of hydrogen peroxide or human  $\beta$ -defensin-3. Finally, we provide evidence that pyroptosis of in vitro infected macrophages is directly microbicidal. Taken together, these results indicate that the protective action of GSDMD in melioidosis is primarily due to induction of pyroptosis and direct killing of bacteria rather than production of cytokines. *The Journal of Immunology*, 2019, 202: 3468–3473.**

**P**yroptosis is a lytic form of cell death that has been shown to be protective against infection with a variety of intracellular bacteria (1–3). Pyroptosis is triggered by activation of caspase-1 or caspase-11 in the context of canonical and noncanonical inflammasomes (4). Once activated, these caspases cleave the cellular protein gasdermin D (GSDMD) whose N-terminal domain inserts into the cell membrane lipid bilayer, forming pores that lead to osmotic lysis of the cell (5–8). Several mechanisms may account for the protective action of pyroptosis. First, pyroptotic lysis of infected cells eliminates the replicative niche, a response highly effective against obligate intracellular bacteria. Moreover, disruption of cell membrane integrity allows various soluble antimicrobial molecules to gain access to the cell cytoplasm where bacteria replicate. It has also been proposed that during pyroptosis, bacteria remain trapped inside the infected cells and eventually are killed by recruited neutrophils (9). Pyroptosis amplifies the inflammatory response by controlling secretion of IL-1 $\beta$  and IL-18, which depends on GSDMD in several, but not all, settings (10–12), and through the release of a mixture of inflammatory cellular components. Finally, recent reports have shown that GSDMD is able to directly kill

bacteria (7, 13, 14). This outside-in ability to damage bacterial membranes appears to contrast with the exclusively inside-out activity of GSDMD on eukaryotic membranes.

*Burkholderia pseudomallei* is a gram-negative intracellular bacterium that infects various cell types and causes melioidosis, a disease endemic in Southeast Asia and other tropical regions (15). *Burkholderia thailandensis* is a related species not pathogenic for humans that causes a disease similar to melioidosis in mice (16). Although infection can be acquired through different routes, inhalation leads to the most severe form of melioidosis and is associated with high mortality. We and others have shown that the NLRP3 and NLRC4 canonical inflammasomes and caspase-11 play critical protective roles during melioidosis (17–21). NLRP3 controls production of IL-18, a cytokine needed to survive melioidosis, but also IL-1 $\beta$ , a cytokine that is deleterious during infection with *Burkholderia* species. The role of NLRC4 in melioidosis is to trigger pyroptosis in myeloid cells, whereas caspase-11 mediates this response in lung epithelial cells (19).

In this study, we examine the response to infection with *B. thailandensis* in mice lacking GSDMD. Our results show that *Gsdmd*<sup>-/-</sup> mice were more susceptible to the infection not because of decreased production of protective IL-18/IFN- $\gamma$  cytokines but rather because of impaired pyroptosis and the microbicidal activity of GSDMD.

## Materials and Methods

### Ethics statement

All the animal experiments described in the current study were conducted in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. All animal studies were conducted under protocols approved by the Rosalind Franklin University of Medicine and Science Institutional Animal Care and Use Committee. All efforts were made to minimize suffering and ensure the highest ethical and humane standards.

### Mice

C57BL/6J, *Casp1*<sup>-/-</sup>/*Casp11*<sup>-/-</sup>, and *Gsdmd*<sup>-/-</sup> mice [provided by Thirumala Kanneganti, St. Jude Children's Research Hospital (22)], all on

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Received for publication January 15, 2019. Accepted for publication April 5, 2019.

This work was supported by the National Institutes of Health (Grants AI130763 and AI110783 to F.R.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: BALF, bronchoalveolar lavage fluid; BMDC, bone marrow-derived dendritic cell; BMM, bone marrow-derived macrophage; DC, dendritic cell; GSDMD, gasdermin D; LB, Luria-Bertani; MOI, multiplicity of infection; p.i., postinfection.

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C57BL/6J genetic background, were bred under specific pathogen-free conditions in the Rosalind Franklin University of Medicine and Science animal facility. Age- (8- to 12-wk-old) and sex-matched animals were used in all experiments. Experimental groups were composed of at least five mice, unless stated otherwise.

#### Bacteria strains, intranasal infections, and treatments

*B. thailandensis* E64 (obtained from the American Type Culture Collection) was grown in Luria broth to midlogarithmic phase, the titer was determined by plating serial dilutions on Luria-Bertani (LB) agar, and stocks were maintained frozen at  $-80^{\circ}\text{C}$  in 20% glycerol. For mouse infections, frozen stocks were diluted in sterile PBS to the desired titer. Mice were anesthetized using isoflurane, and the infectious doses were applied to the nares in 50  $\mu\text{l}$  total volume PBS.

#### Determination of bacterial growth in organs

Organs aseptically collected were weighed and homogenized in 1 ml of PBS. Serial dilutions were plated on LB agar plates containing streptomycin (100  $\mu\text{g}/\text{ml}$ ) using the Eddy Jet Spiral Plater (Neutec). Bacterial colonies were counted 24 h later using the Flash & Grow Automated Bacterial Colony Counter (Neutec).

#### Bronchoalveolar lavage fluid collection, flow cytometry, and cytokine measurements

Bronchoalveolar lavage fluid (BALF) was collected from euthanized mice by intratracheal injection and aspiration of 1 ml of PBS. Cells obtained from BALF were counted and stained for 30 min with anti-CD11b (clone M1/70; BioLegend), anti-CD11c (clone N418; BioLegend), anti-F4/80 (clone BM8; BioLegend), and anti-Ly6G (clone 1A8; BD Pharmingen) and acquired with a LSRII BD flow cytometer. Data were analyzed using FlowJo (TreeStar, Ashland, OR) software. Cytokine levels in tissue culture conditioned supernatants, BALF, and sera were measured by ELISA using the following kits: IL-1 $\beta$ , IFN- $\gamma$ , IL-12p70 (eBioscience), and IL-18 (MBL, Nagoya, Japan).

#### Bone marrow-derived macrophages, bone marrow-derived dendritic cells, and neutrophils, pyroptosis, and intracellular bacterial growth

Bone marrow-derived macrophages (BMM) and bone marrow-derived dendritic cells (BMDC) were derived in RPMI 1640–10% FCS from bone marrow in presence of conditioned media of L929 fibroblasts or

HEK293T cells expressing GM-CSF. Neutrophils were purified from bone marrow using StemCell Technologies reagents (Mouse Neutrophil Enrichment Kit). The purity of each cell preparation was determined by flow cytometry staining and routinely found to be more than 90% for macrophages (defined as CD11b $^{+}/\text{F4}/80^{+}$ ) or neutrophils (CD11b $^{+}/\text{Ly6G}^{+}$ ) and 70% for dendritic cells (DC) (CD11b $^{+}/\text{CD11c}^{+}$ ). Release of LDH in tissue culture media, a reflection of pyroptosis, was measured using the Roche Cytotoxicity Detection Kit (11644793001; Roche Applied Science). Cells were plated in 48-well plates. Bacteria were added to the cell culture, and the plates were centrifuged at  $300 \times g$  for 10 min to maximize and synchronize infection and incubated for 30 min at  $37^{\circ}\text{C}$ . Cells were washed with PBS to remove extracellular bacteria, and medium containing kanamycin and gentamicin (200  $\mu\text{g}/\text{ml}$  each) was added to inhibit extracellular bacteria growth. Media were collected at different time points for LDH and cytokine measurement. Cells were lysed in PBS plus 2% saponin plus 15% BSA, and serial dilutions of the lysates were plated on LB agar plates containing streptomycin (100  $\mu\text{g}/\text{ml}$ ).

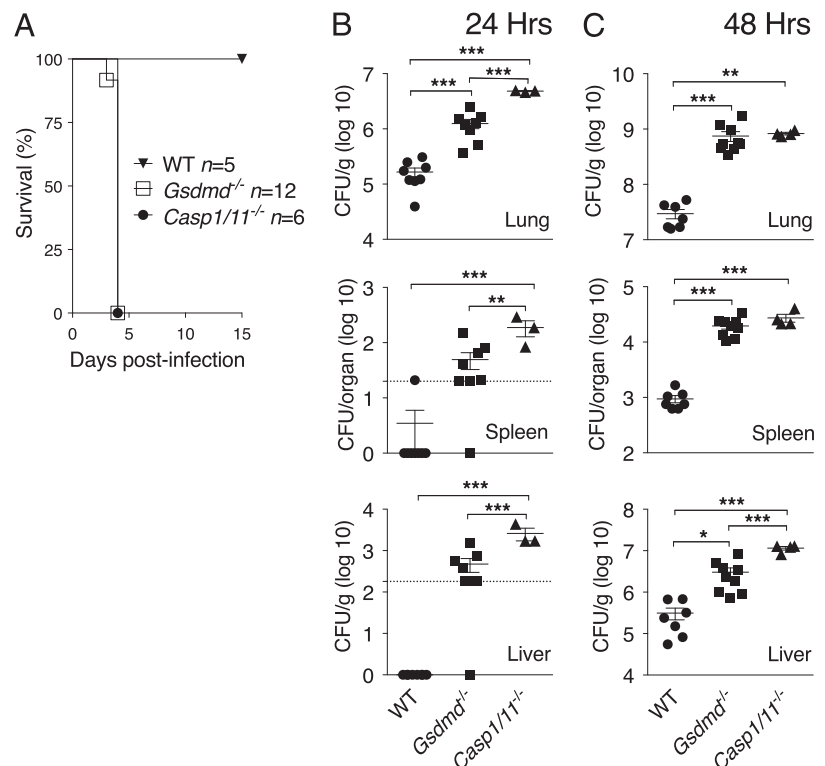
#### Recombinant GSDMD expression and purification

A stationary phase culture of *Escherichia coli* BL21 (DE3) transformed with the pDB.His.MBP vector encoding full-length human GSDMD [kindly provided by Dr. Wu (7)] was diluted 10 times in LB broth, and protein expression was induced with 1 mM IPTG for 4 h. The bacteria pellet was lysed by four freeze-thaw and sonication cycles in 20 mM Tris-HCl (pH 7.4), 0.2 M NaCl, 1 mM EDTA, 1% Nonidet P-40, and 0.1 mg/ml egg white lysozyme. Crude lysate was passed on an amylose resin (New England Biolabs) column followed by wash with 10 column volumes of 20 mM Tris-HCl (pH 7.4), 0.2 M NaCl, and 1 mM EDTA. His-MBP-GSDMD protein was eluted with 10 mM maltose and concentrated with Microcon centrifugal filters with a 30-kDa cutoff (Millipore). To obtain full-length GSDMD, the His-MBP tag was removed by digestion with AcTEV protease (100 U/ml; Invitrogen) for 6 h at  $16^{\circ}\text{C}$ . AcTEV protease, which carries a 6His tag at the N terminus, was removed from the cleavage reaction by Nickel resin chromatography.

#### In vitro GSDMD cleavage and bactericidal assay

*B. thailandensis* ( $10^4$  CFU) were incubated for 2 h at  $37^{\circ}\text{C}$  with full-length GSDMD (5  $\mu\text{g}/\text{ml}$ ) in presence or absence of 0.5 U of active caspase-1 (Enzo) in a final volume of 10  $\mu\text{l}$ . Bacteria dilutions were then plated on LB agar/streptomycin.

**FIGURE 1.** GSDMD is protective in melioidosis. (A) Mice were infected intranasally with *B. thailandensis* ( $10^5$  CFU), and their survival was monitored. (B and C) Mice infected with *B. thailandensis* ( $5 \times 10^5$  CFU) were sacrificed at the indicated time points p.i., and the bacterial burden in organs was measured. For (B) and (C), data were pooled from two independent experiments. Dotted line indicates the bacterial titer detection limit. Data are expressed as mean  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , (A) log rank Kaplan–Meier test, (B and C) one-way ANOVA.



### Bacteria harvest from infected BMM and treatment with H<sub>2</sub>O<sub>2</sub> or $\beta$ -defensin-3

Wild-type or *Gsdmd*<sup>-/-</sup> BMM were infected with *B. thailandensis* (multiplicity of infection [MOI] 50) for 4 h in presence of glycine (5 mM) to prevent cell lysis. BMM were washed in PBS twice and lysed in PBS plus 2% saponin plus 15% BSA to harvest intracellular bacteria. Lysates were treated for 30 min with PBS, H<sub>2</sub>O<sub>2</sub> (2 mM), or human  $\beta$ -defensin-3 (50  $\mu$ g/ml) (Preprotech) before plating on LB agar/streptomycin.

### Statistical analysis

All data were expressed as mean  $\pm$  SD. Survival curves were compared using the log rank Kaplan–Meier test. One-way ANOVA and Tukey posttest or unpaired *t* test was used for analysis of data, as specified in the figure legends. Significance was set at *p* < 0.05.

## Results

### GSDMD is protective in melioidosis

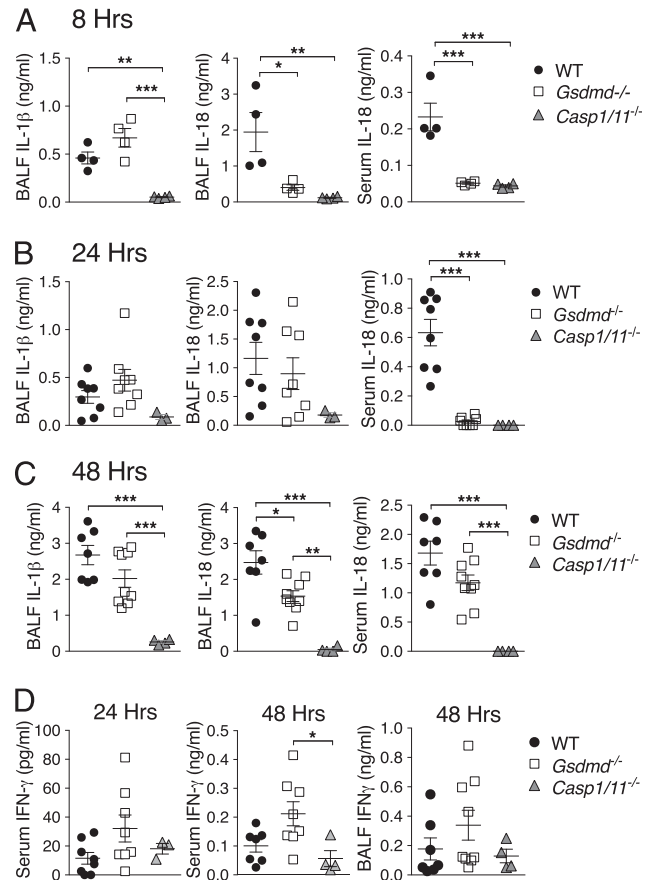
Previous works from our group and others have shown that canonical and noncanonical inflammasomes play a protective role during lung infection with *B. thailandensis*, indicating a critical role for pyroptosis (17–20). As shown in Fig. 1A, mice deficient in GSDMD, whose cleavage by caspase-1 or caspase-11 triggers pyroptosis, are more susceptible to *B. thailandensis* intranasal infection than wild-type mice and succumb synchronously 4 d postinfection (p.i.), similarly to *Casp1*<sup>-/-</sup>/*Casp11*<sup>-/-</sup> mice. Compared with wild-type mice, *Gsdmd*<sup>-/-</sup> mice had higher bacterial burdens in organs 24 and 48 h p.i. (Fig. 1B, 1C). Interestingly, bacterial burden in *Casp1*<sup>-/-</sup>/*Casp11*<sup>-/-</sup> mice was significantly higher than *Gsdmd*<sup>-/-</sup> mice, suggesting the existence of GSDMD-independent protective mechanisms downstream of caspase-1/11.

### GSDMD controls in vivo production of IL-18 but not IL-1 $\beta$

Levels of IL-1 $\beta$  and IL-18 were measured in wild-type and *Gsdmd*<sup>-/-</sup> mice 8, 24, and 48 h p.i. (Fig. 2). Although IL-1 $\beta$  levels in BALF were not significantly different between these mouse strains at any time point, IL-18 was detected in reduced amount in BALF or serum of *Gsdmd*<sup>-/-</sup> mice. Despite reduced IL-18 production in *Gsdmd*<sup>-/-</sup> mice, the level of IFN- $\gamma$ , the cytokine that mediates the protective action of IL-18 in melioidosis (19), was comparable in sera or BALF of wild-type or *Gsdmd*<sup>-/-</sup> mice (Fig. 2D). IL-12p70 was also decreased in *Gsdmd*<sup>-/-</sup> mice (Supplemental Fig. 1), indicating that it does not compensate for the reduced level of IL-18 for induction of IFN- $\gamma$ . As expected, the levels of IL-1 $\beta$  and IL-18 were drastically reduced in *Casp1*<sup>-/-</sup>/*Casp11*<sup>-/-</sup> mice. Taken together, these results suggest that GSDMD protects mice from *B. thailandensis* infection and that it partially contributes to secretion of IL-18. Surprisingly, secretion of IL-1 $\beta$  in vivo appeared to be only modestly affected by absence of GSDMD.

### GSDMD controls in vitro production of IL-1 $\beta$ and IL-18 in macrophages and DC but not in neutrophils

The observation that IL-18, but not IL-1 $\beta$ , production in vivo requires GSDMD is quite surprising as secretion of both cytokines is primarily dependent on processing by caspase-1. To better understand this phenomenon, we examined IL-1 $\beta$ /IL-18 secretion and pyroptosis in wild-type and GSDMD-deficient BMDC or BMM infected in vitro with *B. thailandensis*. As shown in Fig. 3A and 3B, LDH release, a measure of pyroptosis, was drastically decreased in BMDC or BMM lacking GSDMD, as previously shown (5, 6). Concomitantly, intracellular replication of *B. thailandensis* was more robust in GSDMD-deficient cells than wild-type cells. Secretion of IL-1 $\beta$  and IL-18 was also significantly impaired in BMDC or BMM lacking GSDMD. This result contrasts with the secretion pattern observed in vivo and suggests

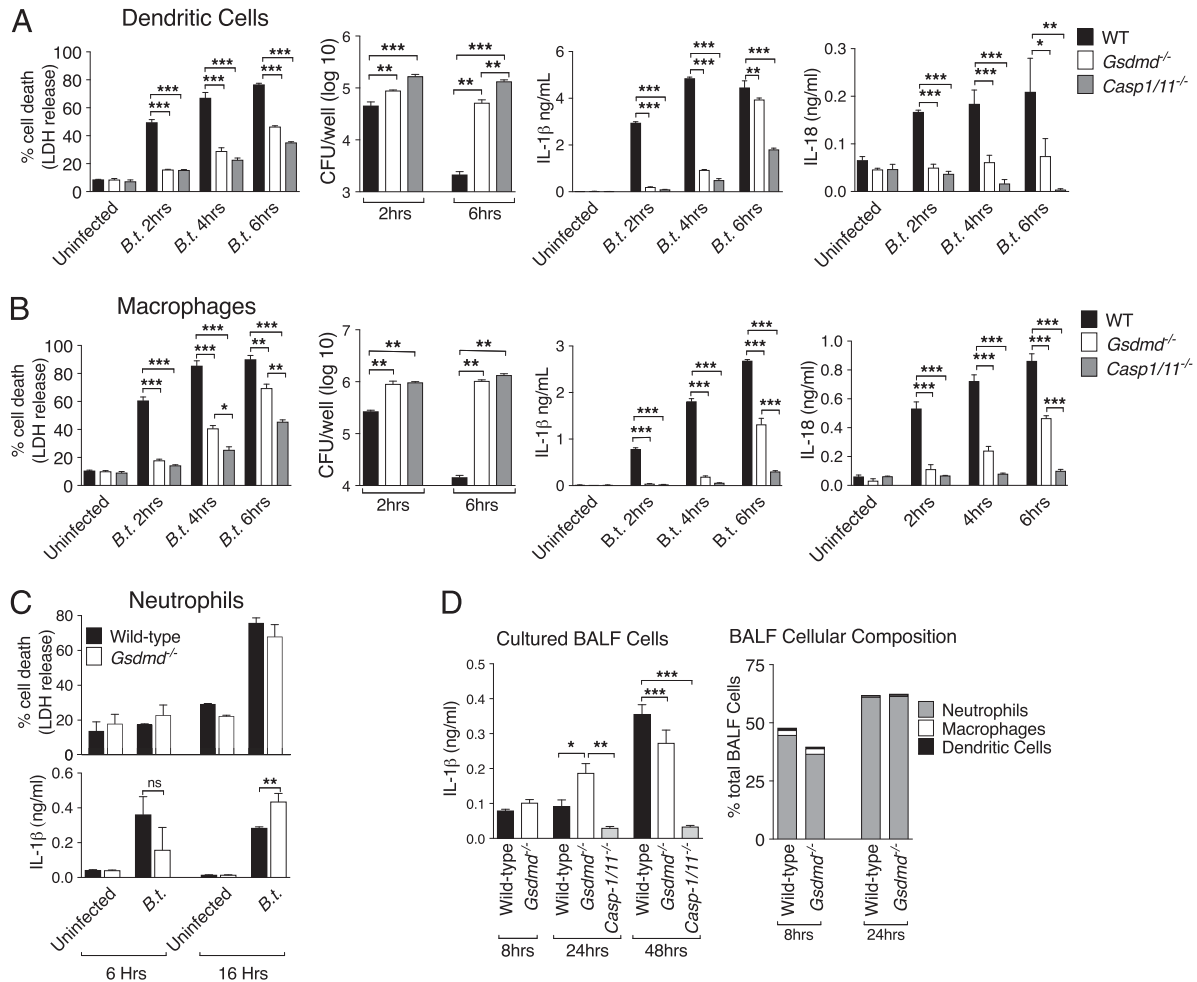


**FIGURE 2.** GSDMD controls in vivo production of IL-18 but not IL-1 $\beta$ . Mice infected with *B. thailandensis* [(A)  $5 \times 10^6$  CFU; (B–D)  $5 \times 10^5$  CFU] were sacrificed at the indicated time points p.i., and cytokines in BALF and serum were measured. (B–D) Data were pooled from two independent experiments. Data are expressed as mean  $\pm$  SD. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001, one-way ANOVA.

that a cell type other than macrophages or DC may be responsible for the GSDMD-independent production of IL-1 $\beta$  observed in vivo. For this reason, we focused our attention on neutrophils, the most abundant leukocytes that are capable of secreting IL-1 $\beta$ . As shown in Fig. 3C, secretion of IL-1 $\beta$  by neutrophils was not affected by absence of GSDMD and was caspase-1 dependent (Supplemental Fig. 2). IL-18 was not detected in neutrophil culture supernatants (data not shown). Cell death of infected neutrophils was GSDMD independent. Moreover, cells purified from the BALF of wild-type or *Gsdmd*<sup>-/-</sup> mice 8, 24, or 48 h p.i. secreted similar amounts of IL-1 $\beta$  when cultured in vitro for 6 h (Fig. 3D). Flow cytometry analysis confirmed that the composition of the BALF cells was comparable in wild-type and *Gsdmd*<sup>-/-</sup> mice and was found to be constituted primarily by neutrophils. Taken together, these results indicate that GSDMD controls production of IL-1 $\beta$  and IL-18 in macrophages and DC, whereas neutrophils secrete IL-1 $\beta$  in a GSDMD-independent fashion and may be the cellular source of the GSDMD-independent IL-1 $\beta$  detected in vivo. These results also indicate that the protective action of GSDMD in melioidosis is not due to impaired IL-18/IFN- $\gamma$  production but rather may be related to GSDMD induction of pyroptosis.

### GSDMD directly kills bacteria and renders them more susceptible to attack by H<sub>2</sub>O<sub>2</sub> and $\beta$ -defensin-3

GSDMD has been proposed to be directly bactericidal (7, 13, 14). To test whether *B. thailandensis* is susceptible to this activity,



**FIGURE 3.** GSDMD controls in vitro production of IL-1 $\beta$  and IL-18 in macrophages and DC but not in neutrophils. BMM (A), BMDC (B), and neutrophils (C) were infected with *B. thailandensis* (MOI 50). LDH release, intracellular bacteria replication, and cytokine secretion were measured at the indicated time points p.i. (D) Cells obtained from BALF of mice ( $n = 3$ ) infected ( $5 \times 10^5$  CFU) for the shown amount of time were cultured in vitro for 6 h, and IL-1 $\beta$  was measured in conditioned supernatants. Right graph shows cellular composition of BALF. One representative experiment of two (D), three (C), and four (A and B) is shown. Data are expressed as mean  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , unpaired  $t$  test, one-way ANOVA.

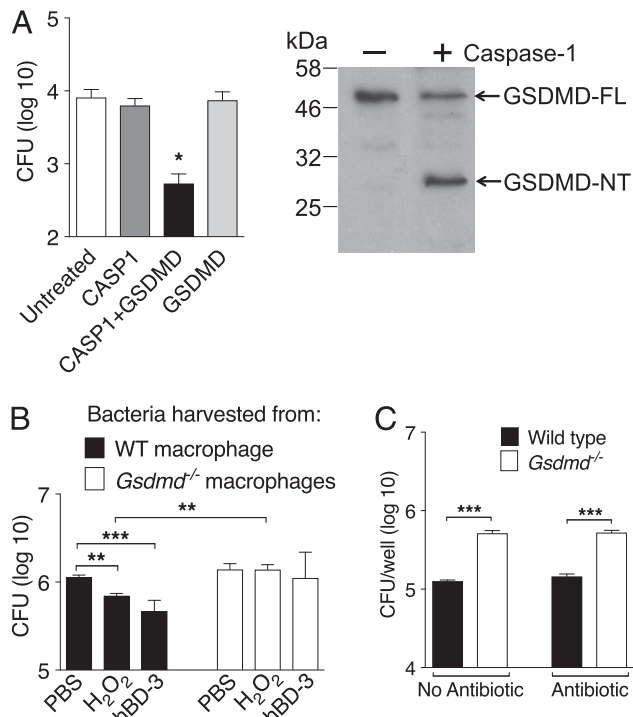
bacteria were incubated with recombinant full-length GSDMD in the presence or absence of purified active caspase-1. As shown in Fig. 4A, bacteria viability was not affected by incubation with caspase-1 or full-length GSDMD. However, when both proteins were present, bacteria viability was severely impaired. Importantly, recombinant caspase-1 was found to cleave full-length GSDMD generating the N-terminal fragment responsible for executing pyroptosis (7, 14). Although it is still unclear how GSDMD may damage eukaryotic or bacterial membranes, it has been shown that bacteria recovered from wild-type macrophages are more susceptible to attack by various damaging agents than bacteria recovered from GSDMD-deficient macrophages, likely because of destabilization of bacterial membrane by GSDMD (9). In agreement with this notion, *B. thailandensis* obtained from wild-type BMM were significantly more susceptible to the microbicidal activity of hydrogen peroxide or human  $\beta$ -defensin-3 than bacteria obtained from GSDMD-deficient BMM (Fig. 4B). Finally, supporting a direct bactericidal activity of GSDMD, significantly more bacteria could be recovered from GSDMD-deficient than wild-type BMM even when the infection occurred in the presence of glycine, to prevent cell lysis, but in the absence of gentamicin/kanamycin (Fig. 4C). These results are consistent with the notion that pyroptosis is protective against intracellular bacteria not just because cell lysis allows penetration of various

extracellular antimicrobial agents but also because GSDMD directly damages bacterial membranes.

## Discussion

Previous works from our and others laboratories have shown the critical role of inflammasome and pyroptosis during infection with *Burkholderia* species (21). In agreement with those results and a recent paper, we report in this study that *Gsdmd*<sup>-/-</sup> mice are more susceptible to melioidosis and succumb to infection with significantly higher bacterial burden than wild-type mice. Surprisingly, although GSDMD-deficient BMM or BMDC secreted drastically lower amounts of IL-1 $\beta$  or IL-18, absence of GSDMD in vivo significantly decreased production of IL-18 but not IL-1 $\beta$ . This contrasts with the nearly complete inability of *Casp1*<sup>-/-</sup>/*Casp11*<sup>-/-</sup> mice to produce mature IL-1 $\beta$  or IL-18 and suggests the existence of caspase-1-dependent but GSDMD-independent pathways for IL-1 $\beta$  secretion, as previously proposed (11, 23). Our data indicate that neutrophils are able to release IL-1 $\beta$  in a GSDMD-independent fashion and may be the cellular source of the IL-1 $\beta$  detected in vivo. It should be noted that in our experiments with neutrophils, both pyroptosis and IL-1 $\beta$  secretion depended on caspase-1. Interestingly, IFN- $\gamma$ , the cytokine that mediates the protective action of IL-18 in melioidosis, was detected in similar amounts in wild-type or *Gsdmd*<sup>-/-</sup> mice, suggesting that the





**FIGURE 4.** GSDMD directly kills bacteria and renders them more susceptible to attack by  $H_2O_2$  and  $\beta$ -defensin-3. **(A)** *B. thailandensis* ( $10^4$  CFU) was incubated with recombinant GSDMD in the presence or absence of active caspase-1 for 2 h. Bacteria were then plated on LB agar/streptomycin. Left panel shows cleavage of full-length GSDMD to generate the N-terminal domain. **(B)** Wild-type or *Gsdmd*<sup>-/-</sup> BMM were infected with *B. thailandensis* (MOI 50) for 4 h in the presence of glycine (5 mM). Intracellular bacteria were harvested from lysed BMM and treated for 30 min with  $H_2O_2$  (2 mM) or human  $\beta$ -defensin-3 (50  $\mu$ g/ml) before plating dilutions on LB agar/streptomycin. **(C)** BMM were infected with *B. thailandensis* (MOI 50) as in (B) in the presence or absence of gentamicin/kanamycin (400  $\mu$ g/ml) for 4 h. Intracellular bacteria replication was measured. One representative experiment of two (A and B) or three (C) is shown. Data are expressed as mean  $\pm$  SD. \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001, (A and B) one-way ANOVA, (C)  $t$  test.

amount of IL-18 produced by *Gsdmd*<sup>-/-</sup> mice, although lower than that of wild-type mice, is sufficient to stimulate protective amounts of IFN- $\gamma$ . Thus, the susceptibility of *Gsdmd*<sup>-/-</sup> mice to *B. thailandensis* does not appear to be due to inability to produce IL-18/IFN- $\gamma$ . Rather, the protective response absent in *Gsdmd*<sup>-/-</sup> mice may be related to pyroptosis of infected cells.

Recent work by the Miao laboratory has shown that, in addition to exposing intracellular bacteria to extracellular antimicrobial molecules, the pyroptotic lytic cell death immobilized bacteria into intracellular traps (9). Our data point to an additional mechanism responsible for the microbicidal action of pyroptosis. We have shown in this study that recombinant GSDMD was able to kill *B. thailandensis* in vitro after processing into the N-terminal fragment by active caspase-1. Moreover, bacteria recovered from GSDMD-deficient macrophages were more resistant than those recovered from wild-type cells to the toxic action of hydrogen peroxide or  $\beta$ -defensin-3, suggesting that the GSDMD-dependent sublethal damaging of bacterial membrane synergizes with the action of antimicrobial agents. These findings are in agreement with previous works (7, 13, 14) and expand the repertoire of the antimicrobial mechanisms deployed by induction of pyroptosis.

It is interesting to note that our study refined our understanding of the dynamic of the gentamicin protection assay. The accepted interpretation of this assay is that the integrity of the cellular

membrane excludes gentamicin and therefore allows measurement of the replication of intracellular bacteria. Induction of pyroptosis or other forms of lytic cell death, in wild-type but not inflammasome or GSDMD-deficient cells, would allow the penetration of antibiotics and killing of bacteria. Our results show that regardless of the presence of gentamicin/kanamycin, wild-type cells restrict *B. thailandensis* intracellular replication much more efficiently than GSDMD-deficient cells. This indicates that, at least in the case of *B. thailandensis*, the restriction of intracellular bacteria replication by pyroptotic wild-type cells observed in the gentamicin protection assay is due to the toxic effect of GSDMD rather than that of the antibiotic (to which *B. thailandensis* is highly resistant). The importance of this result is that it demonstrates that pyroptosis can be directly microbicidal, an activity previously unappreciated.

## Acknowledgments

We thank Thirumala Kanneganti (St. Jude Children's Research Hospital) for providing *Gsdmd*<sup>-/-</sup> mice, Hao Wu (Harvard Medical School) for the pDB.His.MBP-GSDMD vector, and Bob Dickinson for help with flow cytometry.

## Disclosures

The authors have no financial conflicts of interest.

## References

- Miao, E. A., I. A. Leaf, P. M. Treuting, D. P. Mao, M. Dors, A. Sarkar, S. E. Warren, M. D. Wewers, and A. Aderem. 2010. Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria. *Nat. Immunol.* 11: 1136–1142.
- Jorgensen, I., and E. A. Miao. 2015. Pyroptotic cell death defends against intracellular pathogens. *Immunol. Rev.* 265: 130–142.
- Andrade, W. A., and D. S. Zamboni. 2018. Inflammasome-dependent mechanisms involved in sensing and restriction of bacterial replication. *Curr. Issues Mol. Biol.* 25: 99–132.
- Jiménez Fernández, D., and M. Lamkanfi. 2015. Inflammatory caspases: key regulators of inflammation and cell death. *Biol. Chem.* 396: 193–203.
- Kayagaki, N., I. B. Stowe, B. L. Lee, K. O'Rourke, K. Anderson, S. Warming, T. Cuellar, B. Haley, M. Roose-Girma, Q. T. Phung, et al. 2015. Caspase-1 cleaves gasdermin D for non-canonical inflammasome signalling. *Nature* 526: 666–671.
- Shi, J., Y. Zhao, K. Wang, X. Shi, Y. Wang, H. Huang, Y. Zhuang, T. Cai, F. Wang, and F. Shao. 2015. Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature* 526: 660–665.
- Liu, X., Z. Zhang, J. Ruan, Y. Pan, V. G. Magupalli, H. Wu, and J. Lieberman. 2016. Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores. *Nature* 535: 153–158.
- Shi, J., W. Gao, and F. Shao. 2017. Pyroptosis: gasdermin-mediated programmed necrotic cell death. *Trends Biochem. Sci.* 42: 245–254.
- Jorgensen, I., Y. Zhang, B. A. Krantz, and E. A. Miao. 2016. Pyroptosis triggers pore-induced intracellular traps (PITs) that capture bacteria and lead to their clearance by efferocytosis. *J. Exp. Med.* 213: 2113–2128.
- Evavold, C. L., J. Ruan, Y. Tan, S. Xia, H. Wu, and J. C. Kagan. 2018. The pore-forming protein gasdermin D regulates interleukin-1 secretion from living macrophages. *Immunity* 48: 35–44.e6.
- Semino, C., S. Carta, M. Gattorno, R. Sitia, and A. Rubartelli. 2018. Progressive waves of IL-1 $\beta$  release by primary human monocytes via sequential activation of vesicular and gasdermin D-mediated secretory pathways. *Cell Death Dis.* 9: 1088.
- Monteleone, M., A. C. Stanley, K. W. Chen, D. L. Brown, J. S. Bezbradica, J. B. von Pein, C. L. Holley, D. Boucher, M. R. Shakespear, R. Kapetanovic, et al. 2018. Interleukin-1 $\beta$  maturation triggers its relocation to the plasma membrane for gasdermin-D-dependent and -independent secretion. *Cell Rep.* 24: 1425–1433.
- Qiu, S., J. Liu, and F. Xing. 2017. 'Hints' in the killer protein gasdermin D: unveiling the secrets of gasdermins driving cell death. *Cell Death Differ.* 24: 588–596.
- Ding, J., K. Wang, W. Liu, Y. She, Q. Sun, J. Shi, H. Sun, D. C. Wang, and F. Shao. 2016. Pore-forming activity and structural autoinhibition of the gasdermin family [Published erratum appears in 2016 *Nature* 540: 150.]. *Nature* 535: 111–116.
- Wiersinga, W. J., T. van der Poll, N. J. White, N. P. Day, and S. J. Peacock. 2006. Melioidosis: insights into the pathogenicity of *Burkholderia pseudomallei*. *Nat. Rev. Microbiol.* 4: 272–282.
- West, T. E., C. W. Frevert, H. D. Liggitt, and S. J. Skerrett. 2008. Inhalation of *Burkholderia thailandensis* results in lethal necrotizing pneumonia in mice: a surrogate model for pneumonic melioidosis. *Trans. R. Soc. Trop. Med. Hyg.* 102(Suppl. 1): S119–S126.

17. Ceballos-Olvera, I., M. Sahoo, M. A. Miller, L. Del Barrio, and F. Re. 2011. Inflammasome-dependent pyroptosis and IL-18 protect against *Burkholderia pseudomallei* lung infection while IL-1 $\beta$  is deleterious. *PLoS Pathog.* 7: e1002452.
18. Sahoo, M., L. Del Barrio, M. A. Miller, and F. Re. 2014. Neutrophil elastase causes tissue damage that decreases host tolerance to lung infection with burkholderia species. *PLoS Pathog.* 10: e1004327.
19. Wang, J., M. Sahoo, L. Lantier, J. Warawa, H. Cordero, K. Deobald, and F. Re. 2018. Caspase-11-dependent pyroptosis of lung epithelial cells protects from melioidosis while caspase-1 mediates macrophage pyroptosis and production of IL-18. *PLoS Pathog.* 14: e1007105.
20. Aachoui, Y., I. A. Leaf, J. A. Hagar, M. F. Fontana, C. G. Campos, D. E. Zak, M. H. Tan, P. A. Cotter, R. E. Vance, A. Aderem, and E. A. Miao. 2013. Caspase-11 protects against bacteria that escape the vacuole. *Science* 339: 975–978.
21. Sahoo, M., L. Lantier, and F. Re. 2016. Role of canonical and non-canonical inflammasomes during burkholderia infection. *Curr. Top. Microbiol. Immunol.* 397: 199–214.
22. Karki, R., E. Lee, D. Place, P. Samir, J. Mavuluri, B. R. Sharma, A. Balakrishnan, R. K. S. Malireddi, R. Geiger, Q. Zhu, et al. 2018. IRF8 regulates transcription of naips for NLRC4 inflammasome activation. *Cell* 173: 920–933.e13.
23. Schneider, K. S., C. J. Groß, R. F. Dreier, B. S. Saller, R. Mishra, O. Gorka, R. Heilig, E. Meunier, M. S. Dick, T. Čiković, et al. 2017. The inflammasome drives GSDMD-independent secondary pyroptosis and IL-1 release in the absence of caspase-1 protease activity. *Cell Rep.* 21: 3846–3859.