Title: Gasdermin D inhibition prevents multiple organ dysfunction during sepsis by blocking NET formation

Short title: Gasdermin D is a target to improve sepsis outcomes

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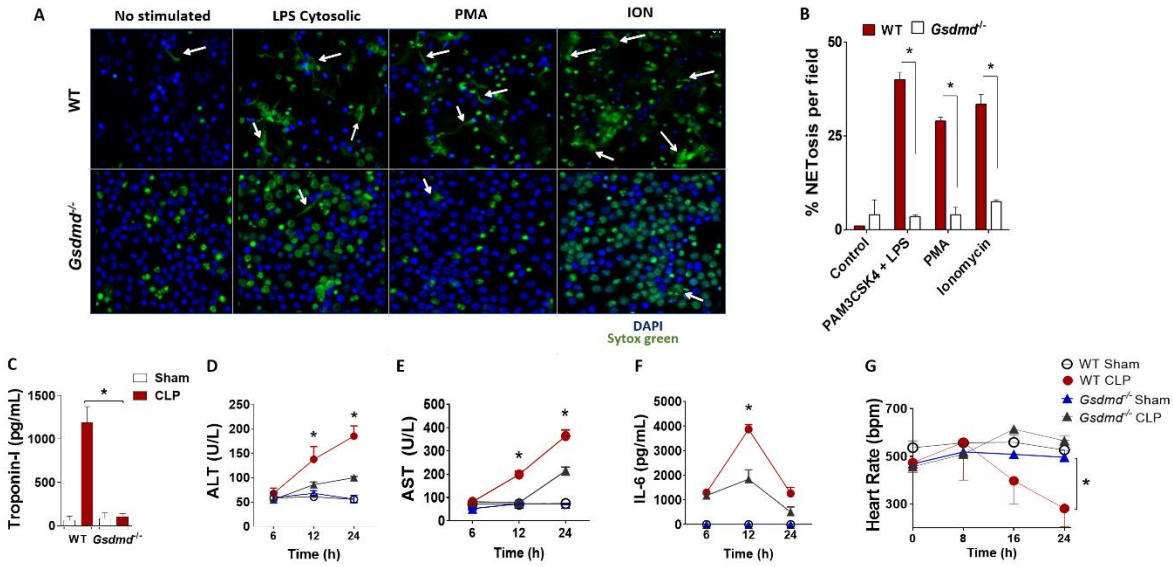
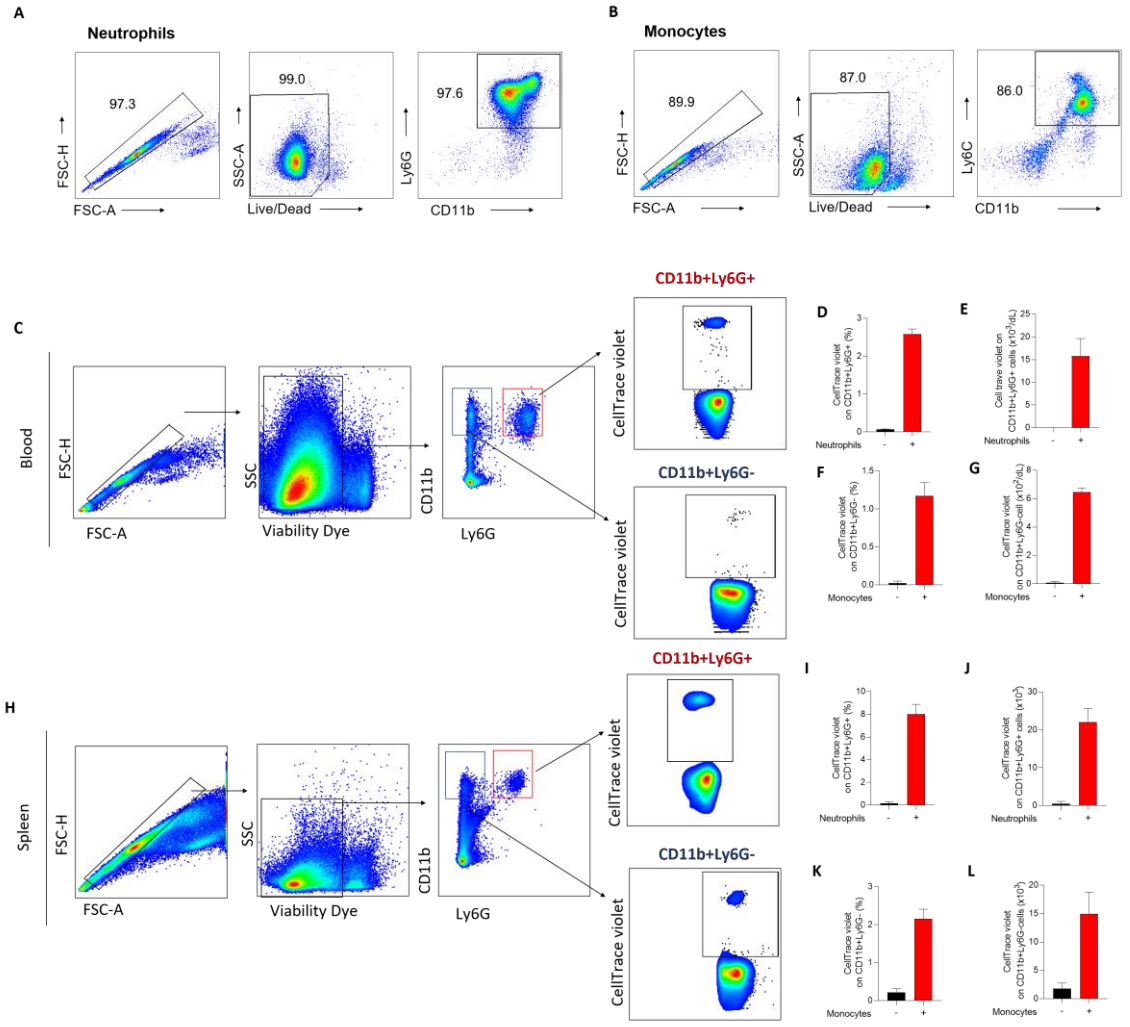
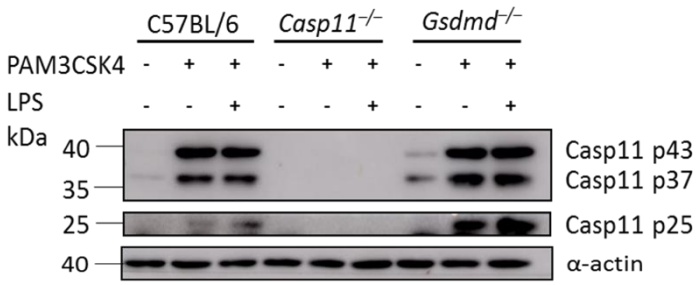
Supplemental figures



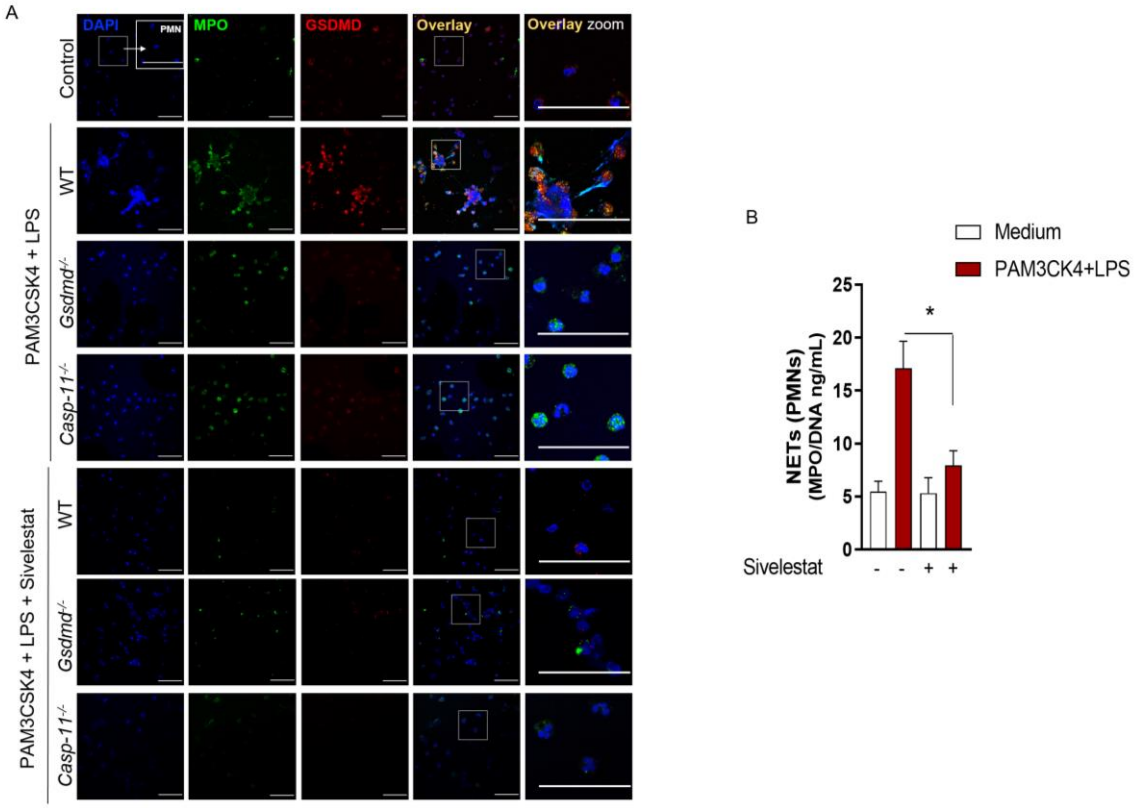
Supplemental figure 1. Gasdermin D deficiency reduces NETs release and circulating levels of organ-injury markers and inflammatory cytokines. (A) Bone marrow neutrophils from WT and *Gsdmd*$^{-/-}$ mice were primed with PAM3CSK4 (1 µg/ml) for 4 h and then transfected with ultrapure LPS (10 µg/ml) or stimulated with PMA (50 nM) or ionomycin (5 µM) for 4 h. NETosis of mice neutrophils was detected by the extracellular DNA marker SYTOX green. (B) Microscopy images were blinded and quantified for the percentage of NETosis per field, where cells with delobulated nuclei and diffuse/spread DNA were counted as the cells undergoing NETosis. Quantitative analysis of NETs, PAM3CSK4+LPS, PMA, or ionomycin stimuli. (C) The circulating levels of troponin-I in *Gsdmd*$^{-/-}$. (D and E) The plasma levels of ALT and AST and (F) IL-6 were determined at 6, 12 and 24 h after sepsis induction by CLP in *Gsdmd*$^{-/-}$ mice. (G) Heart rate was continuously monitored by telemetry, through which we calculated the mean arterial pressure for 24 h after sepsis induction. The data are the mean ± SEM (*p<0.05 one-way ANOVA, followed by Tukey B and C; D-G WT CLP vs *Gsdmd*$^{-/-}$ CLP t-test in D-G). Data are representative of at least two independent experiments, each including 5-7 animals per group.



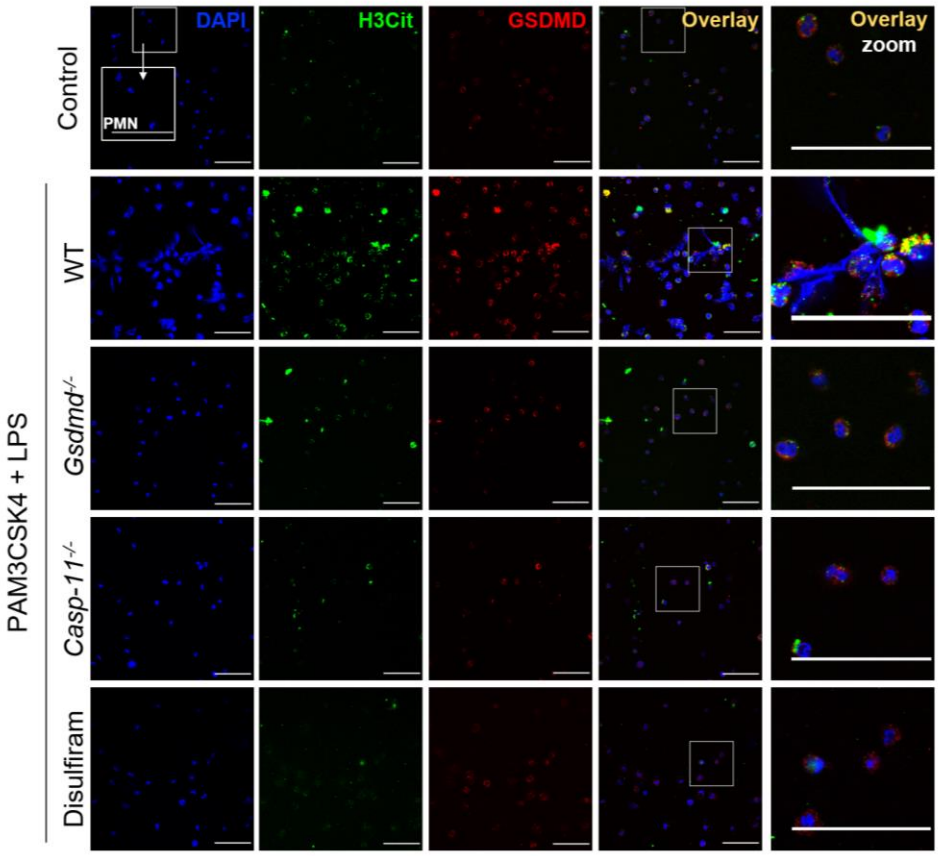
Supplemental figure 2. Viability of WT-neutrophils and -monocytes 12 hours after transfer to *Gsdmd*$^{-/-}$ mice. (A-B) Mouse bone marrow neutrophils (97.6% of viable CD11b+Ly6G+ cells) and monocytes (86% of viable CD11b+Ly6C+ cells) were isolated from WT-mice. Further, these cells were stained with 5 uM CellTrace^{TM} dye and injected (1×10^7 cells/mouse, i.v) in *Gsdmd*$^{-/-}$ mice. Then, 12 h after transfer, blood and spleen samples were collected to analyze the viability of transferred neutrophils and monocytes. (C) Gating strategy: after gating for singlets (FSC-H vs. FSC-A) viable cells were gated for viability dye negative. Next the neutrophils (CD11b+/Ly6G+) and monocytes (CD11b+/Ly6G-) were gated to determine the population stained with CellTracer Dye. (D-E) Quantification of transfer neutrophils in the blood. (F-G) Quantification of transfer monocytes in the blood. (H) Gating strategy: after gating for singlets (FSC-H vs. FSC-A), viable cells were gated for viability dye negative, next the neutrophils (CD11b+/Ly6G+) and monocytes (CD11b+/Ly6G-) were gated to determine the population stained with CellTracer Dye. (I-J and K-L) Quantification of transfer WT neutrophils and monocytes in the spleen, respectively. Data are representative of at least two independent experiments, each including 4-6 animals per group.



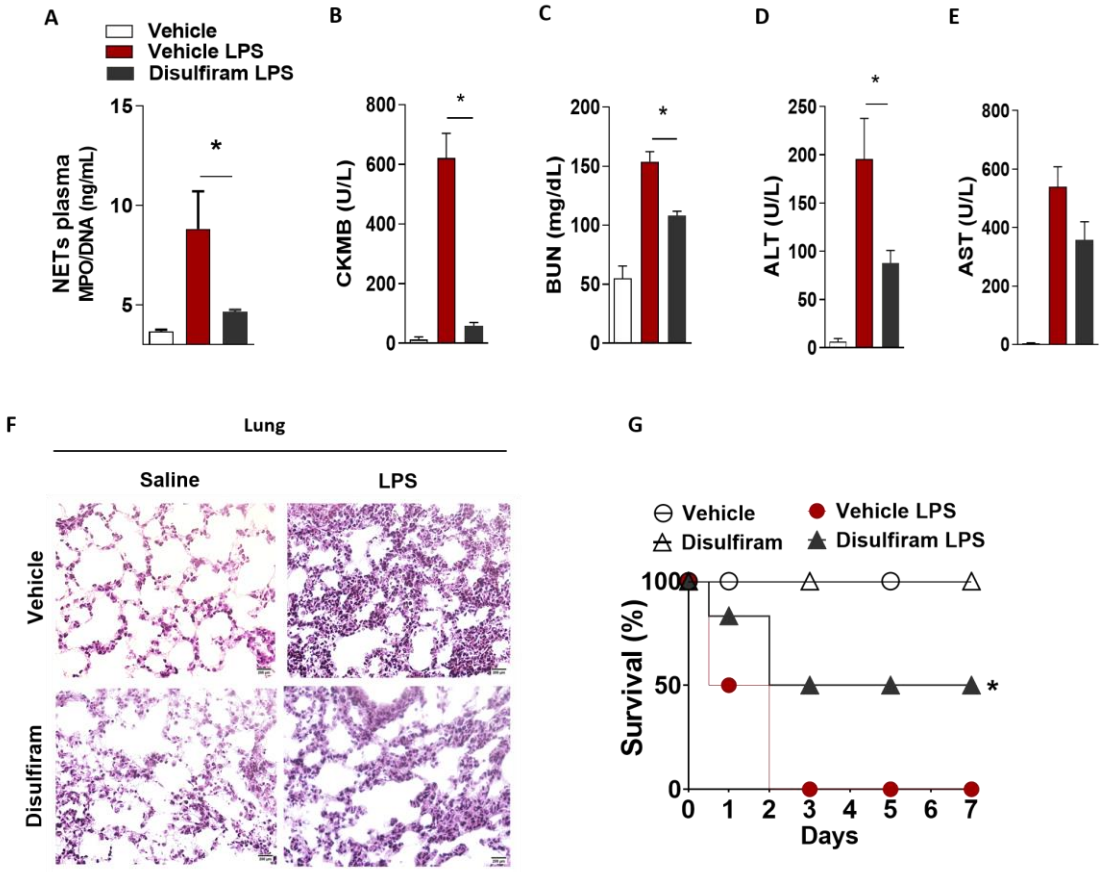
Supplemental figure 3. Caspase 11 activation is not affected in neutrophils with gasdermin D deficiency. Bone marrow neutrophils from WT *Casp11*$^{-/-}$ and *Gsdmd*$^{-/-}$ mice were primed with PAM3CSK4 (1 µg/ml) for 4 h and then transfected with ultrapure LPS (10 µg/ml) for 4h. The cell lysates were harvested for immunoblot analysis of pro-caspase-11 (p43 and p37) and its cleaved fraction caspase-11 (p25). Actin (α-actin) was used as a loading control. Data are representative of at least two independent experiments.



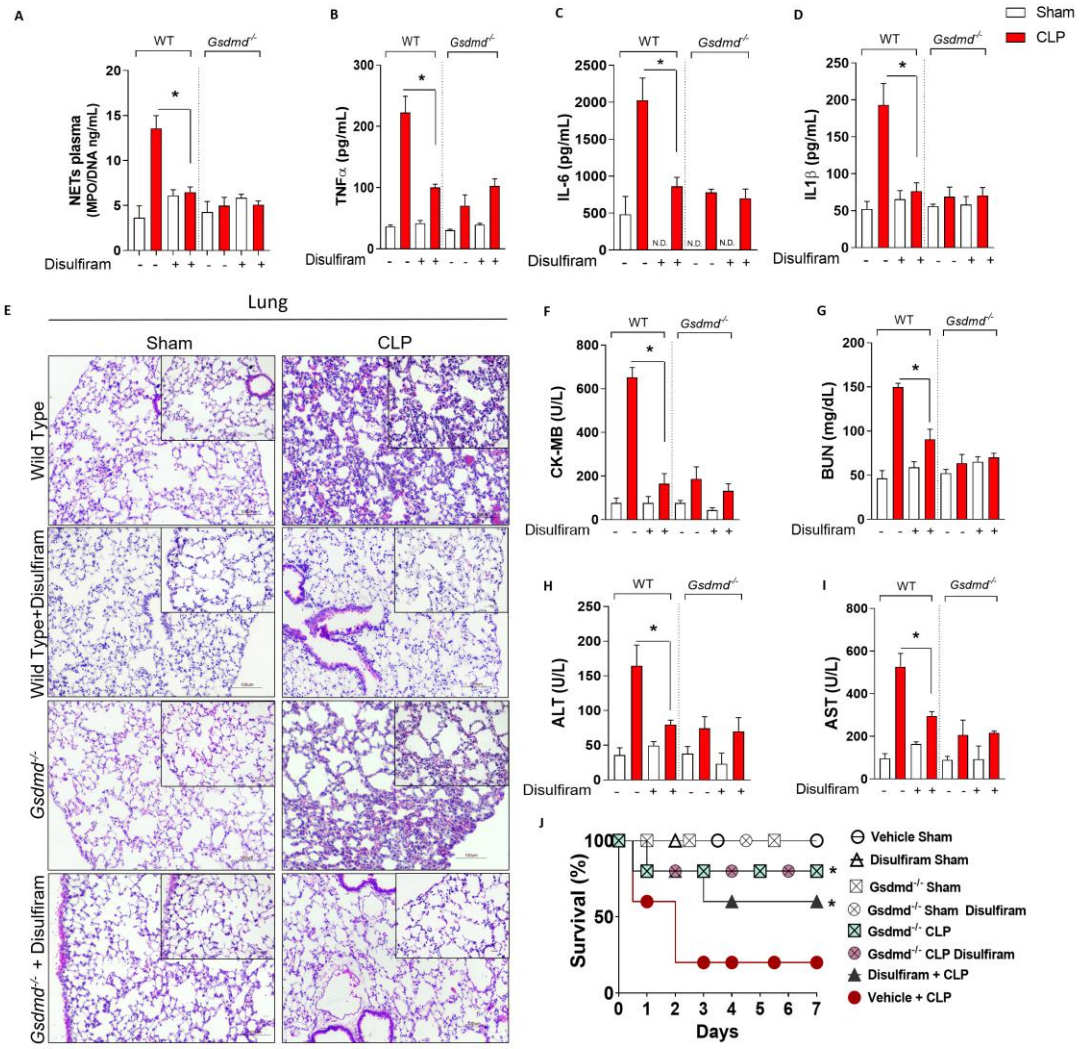
Supplemental figure 4. Neutrophil elastase mediates activation of gasdermin D and NETs production (A) Bone marrow neutrophils from WT, *Gsdmd*$^{-/-}$ and *Casp11*$^{-/-}$ mice were treated with Sivelestat (Neutrophil elastase inhibitor, 10 µM) and primed with PAM3CSK4 (1 µg/ml) for 4h and then transfected with ultrapure LPS (10 µg/ml) for 4h. Representative fluorescence images of NETs stained for DNA (DAPI, blue), myeloperoxidase (MPO, green), and gasdermin D cleaved fraction (GSDMD, red) are shown. The scale bar indicates 50 µm at a 630x magnification. (B) The concentrations of MPO/DNA-NETs in the neutrophil culture supernatant after 4h of stimuli were determined by a picogreen test. Data are representative of at least two independent experiments.



Supplemental figure 5. Inhibition of gasdermin D reduces histone H3 citrullination. Bone marrow neutrophils from WT, *Casp11*$^{-/-}$, *Gsdmd*$^{-/-}$ mice or WT neutrophils treated with disulfiram were primed with PAM3CSK4 (1 µg/ml) for 4h and transfected with ultrapure LPS (10 µg/ml) for more than 4h. Representative fluorescence images of NETs stained for DNA (DAPI, blue), citrullinated histone H3 (H3Cit, green), and gasdermin D cleaved fraction (GSDMD, red) are shown. The scale bar indicates 50 µm at a 630x magnification. Data are representative of at least two independent experiments.



Supplemental figure 6. Gasdermin D pharmacological inhibition reduces the deleterious effects of endotoxemia in mice. Mice were pretreated with disulfiram (80 mg/kg) or vehicle by subcutaneous injection 24 and 4 h before intraperitoneal challenge with LPS (10 mg/kg). Subsequently, the mice were treated at 6 h and 18 h after LPS injection. (A) The MPO/DNA-NETs concentration in the plasma was determined 24 h post-challenge with LPS. (B-E) The systemic levels of organ injury markers were determined 24 h after LPS injection. (F) Representative images of H&E staining of the lung tissue sections 24 h after LPS injection are shown. The scale bar indicates 400x magnification. (G) The mice were pretreated with disulfiram (80 mg/kg) or vehicle by subcutaneous injection 24 and 4 h before endotoxemia, 6 h after LPS, and every 12 h for 2 days and followed for survival. The data are the mean ± SEM (*p<0.05, one-way ANOVA, followed by Tukey in A- E; H-Mantel-Cox log-rank test in G). Data are representative of at least two independent experiments, each including 5-7 animals per group.



Supplemental figure 7. The effects of disulfiram on sepsis are dependent on gasdermin D. The WT or *Gsdmd*$^{-/-}$ mice were pretreated with disulfiram (80 mg/kg) or vehicle by subcutaneous injection 24 and 4 h before CLP-induced sepsis, 6 h after CLP and every 12 h for 2 days and followed for survival analysis. (A) The MPO/DNA-NETs concentration in the plasma was determined 24 h post-CLP-induced sepsis. (B-D) The systemic levels of TNF-α, IL-6, and IL-1β were measured by ELISA 24 h post-CLP-induced sepsis. (E) Representative images of the histological staining of the lung sections performed 24h after sepsis induction are shown at a 200x magnification. The square insets represent the image at a 400x magnification. (F-I) The circulating levels of organ injury markers were determined 24h after sepsis induction by CLP. (J) Overall survival. The data are the mean ± SEM (*p<0.05, one-way ANOVA, followed by Tukey in A-D and F-I; H-Mantel-Cox Log-rank test in J). Data are representative of at least two independent experiments, each including 5-7 animals per group.

Supplemental material and methods

Blood pressure measurement by telemetry

A Data Sciences International (St. Paul, MN) telemetry system was used for the measurement of cardiovascular parameters. The mice were anesthetized with isoflurane (2% for induction and 1% for maintenance), after which the left carotid was exposed for implantation surgery of a radio-telemeter transmitter (TA11PA-C10; Data Sciences, St. Paul, MN, USA). Seven days after the implantation surgery, the mice were ready to be recorded, which occurred while they were awake in their cages and in a continuous way, starting after the electromagnetic command. The data were acquired and analyzed using the Labchart 8 (Ad Instruments) software. The data were expressed as mean arterial pressure in millimeters of mercury (MAP, mmHg) and heart rate (HR) in beats per minute (bpm).

NETs quantification by High Content Analysis

Isolated neutrophils from WT, Casp11, and GSDMD mice were cultured in RPMI medium supplemented with SYTOX green, and DAPI stimulated with cytosolic LPS, PMA, ionomycin, or culture medium (as negative control) and incubated for 4 h. Fluorescent and bright-field images (356.4 × 356.4 µm) were acquired using an ImageXpress Micro XLS Widefield High-Content Analysis System with a 0.60 numerical aperture 40× objective. Fluorescent images were acquired using a DAPI filter with excitation of 377/50 nm and an emission of 447/60 nm, with an exposure time of 100 ms, and a FITC filter with excitation and emission wavelengths of 482/35 nm and 536/40 nm, with an exposure time of 10 ms (adapted from 1). After the acquisition, the images positive for SYTOX green were quantified as NETs.

References

1. Colon DF, Wanderley CW, Franchin M, Silva CM, Hiroki CH, Castanheira FVS, Donate PB. Neutrophil extracellular traps (NETs) exacerbate severity of infant sepsis. Crit Care 2019; 23: 113.