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Supplemental Information

Gasdermin D Exerts Anti-inflammatory Effects by Promoting Neutrophil Death

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mice

The GSDMD knockout (KO, *Gsdmd*$^{-/-}$) mouse was created by the Model Animal Research Center at Nanjing University (Nanjing, China) on a pure C57BL/6N background using standard CRISPR/Cas9 targeting (**Fig. S1**). CRISPR/Cas9-induced 127bp deletion in exon 3 results in a frame shift and subsequent introduction of a stop codon 32 codons downstream. ELANE KO (*Elane*$^{-/-}$) and Caspase-1/11 DKO mice were purchased from Jackson Laboratories (Bar Harbor, ME). Eight- to twelve-week-old mice were used in all experiments. Corresponding littermates were used as wild-type controls in all the experiments performed with KO mice. The Boston Children's Hospital Animal Care and Use Committee approved and monitored all procedures involving mice.

Cell lines

All cell lines were cultured at 37°C in a humidified atmosphere and 5% CO_2. Lenti-X 293T cells (Takara, Mountain View, CA) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA). HL-60 cells were maintained in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% FCS and differentiated into neutrophil-like cells with 1.25% dimethyl sulfoxide (in DMSO) for 6 days.

Plasmids

cDNA clones of human and mouse *GSDMD* were derived from KBM7 cells and mouse primary neutrophils, respectively. The genes were PCR amplified and inserted into pET SUMO (Thermo Fisher),

pCAG-MCS2-FOS (kindly provided by Dr. Morita), pLVX-TetOne (Takara), pCSII-EF-RfA, and pCSII-CMV-MCS-IRES2-Bsd (kindly provided by Dr. Miyoshi) vectors via sub-cloning. Site-directed mutagenesis was performed by overlap extension PCR using PrimeSTAR GXL DNA polymerase (Takara).

Hematologic analysis

Mice were anesthetized and immediately bled retro-orbitally into an EDTA-coated tube. Total cell counts were determined using a hemocytometer, and differential cell counts were performed using an automated hematology analyzer (Hemavet 850; Drew Scientific, Oxford, CT, or Sysmex, XT2000i). For BM cells, total cell counts were determined using a hemocytometer, and differential cell counts were conducted by microscopic analysis of Wright-Giemsa-stained cytospins or FACS analysis using a CANTOII flow cytometer with FACSDiva software (BD Biosciences, Franklin Lakes, NJ). The absolute number of neutrophils was then determined based on the cytospin or FACS analysis.

Human primary neutrophil isolation

Human primary neutrophils were isolated from apheresis-derived buffy coats provided by the Blood Bank Lab at the Boston Children's Hospital as previously described (Loison et al., 2014). All blood is drawn from healthy blood donors. All protocols have been approved by the Children's Hospital Institutional Review Board (IRB).

Preparation of highly purified neutrophils (HPNs)

To examine GSDMD and ELANE expression in neutrophils, we used highly purified neutrophils (HPNs). Traditional neutrophil isolation using Ficoll or Percoll gradients only reach ~95% purity, and contamination with other cell types (e.g., monocytes) is inevitable. Therefore, we purified neutrophils

from human blood using the traditional Ficoll method followed by negative selection to obtain HPNs (~99.5%). Briefly, human neutrophils were first prepared from apheresis-derived buffy coats using Ficoll as described above. The isolated neutrophils were further purified using the EasySep human neutrophil enrichment kit (StemCell Technologies, Cambridge, UK) according to the manufacturer's protocol. Neutrophil purity was determined by Wright-Giemsa staining.

Murine bone marrow neutrophil isolation

Murine bone marrow neutrophils were isolated from the femur and tibia as previously described (Subramanian et al., 2007). Briefly, bone marrow cells were collected from 8-14 weeks old mice and layered over discontinuous Percoll/HBSS gradients (52%, 64%, 76%) and centrifuged (1060g, 30 min, RT). The interface between the 64% and 76% layers containing neutrophils was harvested. Each preparation yielded about 7-11 million mature neutrophils (>85% purity).

Murine peripheral blood neutrophil isolation

8-14 weeks old mice were sacrificed by euthanizing with CO_2 and immediately bleed by cardiac puncture. The peripheral blood (1-2 ml) was then diluted with 3 ml HBSS with 15 mM EDTA. Cells were spun down (500g, 10 min, RT) and washed once with 4 ml HBSS with 15 mM EDTA. Red blood cells were lysed by resuspension in 5 ml ACK (Ammonium-Chloride-Potassium) Lysing Buffer (Thermo Fisher Scientific) for 5 min at RT. Lysis was stopped by addition of 15 ml RPMI+2% FBS followed by centrifugation at 400g for 5 min. After being washed with isolation buffer (2 mM EDTA, 1% endotoxin-free BSA in HBSS), the cells were resuspended in 6 ml 45% Percoll/HBSS, layered over two other discontinuous Percoll/HBSS gradients (3 ml 81% and 4.5 ml 62%), and centrifuged at 1500*g* at room temperature for 30 minutes (no brake, no acceleration). The interface between the 62% and 81% layers containing neutrophils was harvested and washed once with 10 ml of isolation buffer (1200g, 5

min). The purified neutrophils were resuspended in RPMI 1640 medium containing 10% heat-inactivated FBS and maintained at 37°C. Each preparation routinely yielded >1 million neutrophils (per mouse) with a purity of above 90% by Wright–Giemsa staining and FACS analysis with Gr-1 and CD11b monoclonal antibodies.

Neutrophil death analysis by FACS

Murine or human neutrophils were cultured in RPMI 1640 supplemented with 10 % heat-inactivated low endotoxin (≤ 5 EU/ml, usually ≤ 1 EU/ml) fetal calf serum (FCS, Gibco) and Penicillin-Streptomycin antibiotics at a density of 2×10^6 cells/ml at 37°C in a 5% CO_2 incubator. After indicated time periods, cells were then harvested, washed twice with ice cold PBS and stained with human Annexin V (FITC labeled, Invitrogen) and propidium iodide (PI) following a protocol provided by the manufacturer. FACS was performed using a CANTOII flow cytometer (Becton Dickinson, San Jose, CA). At least ten thousand events were recorded and analyzed using FlowJo software (Tree Star). In this FACS analysis, cell debris were eliminated by appropriate gating on forward and side scatter. Neutrophil death was assessed by the reduction of the percentage of $PI^-Annexin\ V^-$ viable cells ($\%PMN_{viable}$, relative to the input). At each time point (t^n), the total number of neutrophils ($PMN_{total\text{-}t^n}$), including both healthy and apoptotic cells, were counted using a hemocytometer. This number decreased gradually during neutrophil death, particularly at the later stage. The percentage of viable cells ($\%PMN_{viable\text{-}t^n}$) in this population was obtained by FACS analysis. The percentage of viable neutrophils relative to the input ($PMN_{total\text{-}t^0}$, total amount of cells at time "0") at each time point was calculated as follows: $\%PMN_{viable} = (PMN_{total\text{-}t^n} \times \%PMN_{viable\text{-}t^n}) / PMN_{total\text{-}t^0}$.

Mouse peritonitis model

$GSDMD^{-/-}$ or wild-type mice were intraperitoneally injected with $5x10^7$ *E.coli* (strain K12 BW25113) in 200 µl PBS. At indicate times after injection, the mice were sacrificed by euthanizing with CO_2.

Peritoneal exudate cells were then harvested by 3 successive washes with 10 ml HBSS containing 0.2% BSA and 20 mM EDTA. The exudate cell count (total cell number) was determined using a hemacytometer and the differential cell count was determined by FACS analysis. The cells were incubated with FITC anti-Gr1 antibody or anti-rat IgG antibody (as a control) for 15 min at room temperature. After being washed twice with PBS containing 2% BSA, the cells were analyzed by a BD FACSCanto II flow cytometer (BD Biosciences). Total number of neutrophils in the peritoneal exudates was then calculated accordingly as previously described (Bajrami et al., 2016). For measurement of cytokine levels in peritoneal cavity, peritoneal lavage fluids (PLF) were collected using 2 ml PBS/15 mM EDTA. Chemokine and cytokine levels in PLF were determined using ELISA kits.

Bacterial burden

Mouse peritonitis was induced as described above. Peritoneal lavage fluid (PLF) was collected by 3 successive washes with 10 ml HBSS containing 0.2% BSA and 20 mM EDTA. PLFs were then serially diluted in ice-cold sterile PBS, and aliquots were spread on Luria broth (LB) agar plates. After overnight incubation at 37°C, colonies were counted, and bacterial viability was expressed as colony-forming units (cfu) in total lavage fluid.

TG induced neutrophil recruitment to flamed peritoneal cavity

Peritonitis was induced by intraperitoneal injection of 3% thioglycollate (TG) solution as previously described (Jia et al., 2007). Peritoneal lavage fluids were collected from wild-type and GSDMD knockout mice by 3 successive washes with 10 ml HBSS containing 0.2% BSA and 20 mM EDTA, 7 hr after TG injection. The exudate cell count (total cell number) was determined using a hemacytometer and the differential cell count was determined by FACS analysis. The cells were incubated with FITC anti-Gr1 antibody for 15 min at room temperature. After being washed twice with PBS containing 2%

BSA, the cells were analyzed by a BD FACSCanto II flow cytometer (BD Biosciences). Total number of neutrophils in the peritoneal exudates was then calculated accordingly.

In vitro bactericidal assay

Live *E. coli* particles (ATCC 19138; American Type Culture Collection, 5×10^5 cfu) were opsonized with mouse serum (final concentration 10%) at 37°C for 30 min and then incubated with neutrophils (1×10^5 cells/reaction for bone marrow neutrophils and 1×10^4 cells/reaction for peripheral blood neutrophils) in HBSS (without mouse serum) at 37°C for the indicated times. Samples were then serially diluted and spread on LB agar plates. The number of live *E. coli* cells in each sample was determined after overnight incubation at 37°C. Neutrophil intracellular bactericidal activity was measured using an antibiotic protection assay. Briefly, mouse neutrophils (10^6 cells for bone marrow neutrophils and 1×10^5 cells for peripheral blood neutrophils) were incubated with serum-opsonized live *E. coli* particles at a ratio of 1:5 and incubated at 37°C for 1 h. Kanamycin was then added at the final concentration of 50 μg/ml to kill extracellular bacteria. After an additional 15 min incubation, cells were washed twice with HBSS and lysed with 1 ml sterile water at room temperature. Samples were serially diluted and spread on LB agar plates. Colonies were counted after overnight incubation at 37°C.

In vitro Phagocytosis assay

Fluorescein-conjugated Phrodo™ Green *E.coli* bioparticles (Invitrogen) were reconstituted in HBSS and opsonized with 12.5% mouse serum at 37°C for 30 min. Mouse bone marrow neutrophils were incubated with serum-opsonized bioparticles at a ratio of 1:10 (neutrophils : bioparticles), and incubated at 37°C for 1 hour. Negative controls were incubated on ice for 1 hour. The assays were terminated by cooling the cells on ice. The number of internalized particles was counted under a fluorescence microscope (Olympus lX17, 100X objective). Phagocytosis index was expressed as the number of the

internalized particles per 100 neutrophils. Phagocytosis efficiency was expressed as the percentage of neutrophils that engulfed at least one bioparticle. More than 200 cells were counted from random fields per coverslip for each group.

Recombinant protein expression

Human and mouse pET SUMO-GSDMD plasmids were transformed into BL21(DE3) competent cells for protein overexpression. *E. coli* organisms were pre-cultured in 2 ml LB medium containing 50μg/ml kanamycin at 37ºC overnight. 2 ml bacteria culture was then transferred into 300 ml LB medium containing 50 μg/ml kanamycin and further cultured at 37ºC to an OD 600 of 0.5-0.8. Subsequently, isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1mM was added to the culture medium to induce protein expression. *E. coli* cultures were further incubated at 25ºC overnight. Cells were collected by centrifugation at 5000 rpm for 10 min and stored at -80°C. 6xHis-SUMO fusion GSDMD protein purification was carried out under native conditions using a Ni-NTA Fast Start kit (Qiagen, Limburg, NL). Following elution from the Ni-NTA affinity column with native elution buffer, the proteins were dialyzed in PBS overnight at 4ºC and stored at -80ºC.

Preparation of neutrophil lysate

Purified neutrophils were washed once with ice cold PBS then incubated for 30 min on ice in Triton lysis buffer (20 mM Tris-HCl (pH 7.4), 135 mM NaCl, 1% Triton X-100, 10% glycerol). Samples were centrifuged at 14,000 rpm for 30 min at 4°C. The supernatant was collected and stored at -80°C.

In-gel digestion and LC/MS/MS analysis

N-terminal His-tagged recombinant GSDMD (20 μg) was incubated with recombinant ELANE or neutrophil lysate (7 μg of protein) at 37 °C for 1 h. His-tagged proteins were then enriched by precipitation using Ni-NTA beads in the presence of DFP (100 μM). After three washes, His-tagged proteins were eluted using imidazole and resolved by SDS-PAGE. In-gel digestion was performed manually in 96-well plate format. Gel bands were first cut and diced into ~1mm cubes and transferred into the wells before being de-stained with 50/50 acetonitrile/50mM NH_4HCO_3 solution, dithiothritol (DTT)-reduced and iodoacetamide (IAM)-alkylated, followed by trypsin or AspN digestion overnight at 37°C. Peptides were extracted into 40/60 acetonitrile/0.1% formic acid solution and dried in a SpeedVac. The dry peptide extract was reconstituted in 22 μL 2%/0.2% acetonitrile/formic acid solution and analyzed on a 1D nanoLC-MS/MS platform using a standardized 110 min method (Mass Spec Facility, Broad Institute, MA). Peptides were separated on a C18-AQ column (75 μm × 50 cm, Reprosil-Pur C18-AQ, 1.9 μm) at 275 nl/min and analyzed on a QExactive Plus mass spectrometer in data-dependent acquisition (DDA) mode with MS1 at 35000 and MS2 at 17500 resolution, respectively.

Database search

MS data were first QC checked using in-house developed software (Mass Spec Facility, Broad Institute) and subsequently searched against the modified Swiss-Prot human database using Andromeda integrated into MaxQuant with mass tolerance of 20 ppm (MS1) and 4.5 ppm (MS2). The modified Swiss-Prot human database includes all Gasdermin-D protein sequences derived by sequential C-terminal amino acid removal. Protein identification and concentration were directly reported from MaxQuant and then further processed using an in-house developed pipeline for protein identification and quantitation.

Western blotting

Neutrophils (4 million cells/data point) were centrifuged and lysed immediately with 100 µl boiling protein loading buffer containing protease inhibitor cocktail (Invitrogen, Carlsbad, CA) and DFP (2mM). Samples were incubated at 100ºC for 5 min and transferred to ice. After brief sonication (5-10 s), 25 µl of cell lysate was subjected to 5-20% gradient SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). After blocking the membranes in TBS containing 5% BSA (Sigma) and 0.02% Triton X-100 for 1 h, primary antibodies were added in blocking solution at the following dilutions: mouse anti-GSDMD antibody (Abnova, Taiwan, 1:1000), anti-Caspase-1 (Adipogen, San Diego, CA, 1:1000), anti-human and mouse ELA2 antibodies (R&D Systems, Minneapolis, MN, 1:1000), mouse anti-actin and FLAG antibodies (Sigma, 1:2000). Primary antibody incubations were at 4°C overnight. After washing, goat HRP-conjugated secondary antibodies (Santa Cruz Biotechnology Inc., Dallas, TX) were added (1:10,000) in blocking solution and the membrane incubated at RT for 1 h. The immunocomplexes were detected with Super Signal West Femto substrate (Pierce, Rockford, IL) using an ImageQuant LAS-4000 (GE, Fairfield, CT). Densitometry was performed using ImageJ software Gel Analyzer plug-in (Luo et al., 2002).

Neutrophil elastase activity assay

The ELANE chromogenic substrate MeOSuc-Ala-Ala-Pro-Val-pNA was purchased from Santa Cruz Biotechnology Inc. Cleavage of p-nitroaniline (pNA) from the substrate by ELANE increases absorbance at 405 nm. For mouse ELANE activity, recombinant ELANE (0.1 µg) was incubated with the substrate (500 µM) in 100 ul of MES reaction buffer (50 mM MES pH 5.5, 50 mM NaCl) at 37ºC for 60 min. For human ELANE activity, the indicated amount of recombinant ELANE was incubated with the substrate (500 µM) in 100 ul of PBS at 37ºC for 30 min. Cleavage of the substrate product was detected at OD 405 in a 96-well microplate reader.

Lactate dehydrogenase (LDH) cytotoxicity assay

LDH release was assayed using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI) following the manufacturer's protocol. HEK293T cells were transfected with indicated GSDMD expressing constructs and then incubated at 37ºC for 24 h. The culture media were collected and centrifuged at 400 x g for 5 min to remove cell debris. LDH release in the media was measured at OD 490. The relative LDH release was expressed as the percentage LDH activity in supernatants of cultured cells (medium) compared with total LDH (from media and the cells) and used as an index of cytotoxicity.

Lysosomal Membrane Permeabilization (LMP) assay

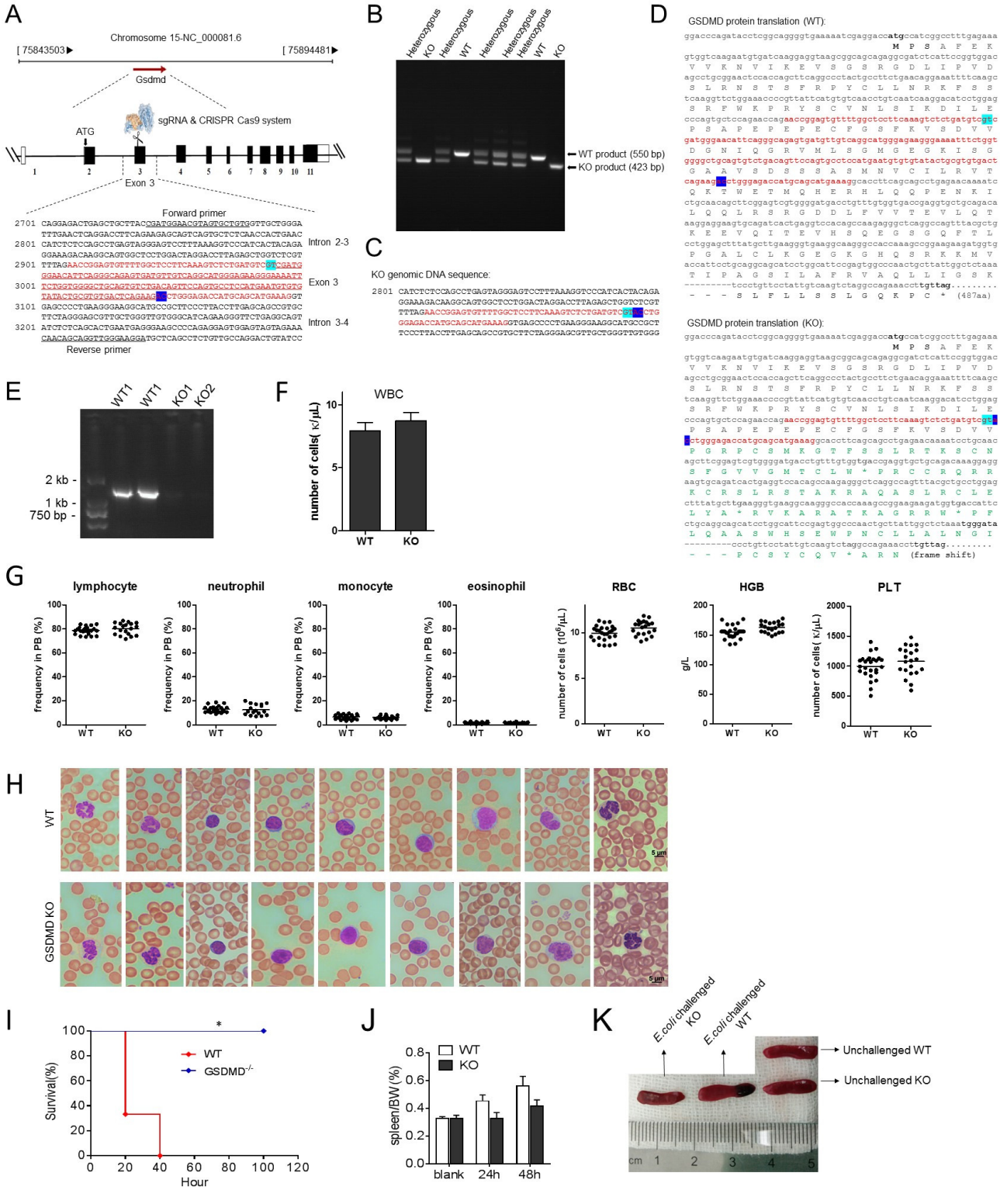
Human neutrophils were isolated as described above and cultured for indicated times. Lysosomal membrane permeability was then assayed as previously described (Loison et al., 2014). Briefly, cells were stained with Acridine Orange (2 ug/ml) in RPMI plus 10 % FCS for 15 min at 37ºC and then washed once in PBS. The red fluorescence was measured by FACS in the PercP/Cy5 channel. The percentages of pale (AO negative) cells were quantified and normalized to the percentage of the control (untreated) cells.

Preparation of neutrophil cytosolic fraction

Purified neutrophils were washed once with ice cold PBS then incubated for 30 min on ice in hypotonic buffer (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM DTT) supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland). Cells were then lysed by 20-30 strokes of a Dounce homogenizer with pestle B. Extracts were then centrifuged at 16,000 x g at 4°C for 30 min. Supernatants were collected as cytosolic fractions.

Immunofluorescent staining

For immunostaining of GSDMD, HEK293T cells were plated onto 18 mm diameter, #1.5 thickness glass coverslips (NeuVitro Corp., El Monte, CA) in 6-well plates overnight. Cells were transfected with 1ug of indicated 3xFLAG-tagged GSDMDs and incubated for 24 h. Cells were fixed for 30 min in 3% paraformaldehyde (PFA) before being washed three times with PBS and permeabilized for 15 min with 0.25% saponin in PBS. Blocking was performed with 2% BSA in PBS for 30 min at RT. The diluted primary anti-FLAG antibodies (1:2000) were added and incubated overnight at 4°C. After three washes with PBS, the Alexa fluor 594 dye-conjugated secondary antibody (1:1000) was added and incubated for 1 h at room temperature. Staining was visualized using the Olympus Fluoview FV1000 confocal system and images were taken using a 60x objective lens.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Gsdmd
sgRNA & CRISPR Cas9 system
ATG
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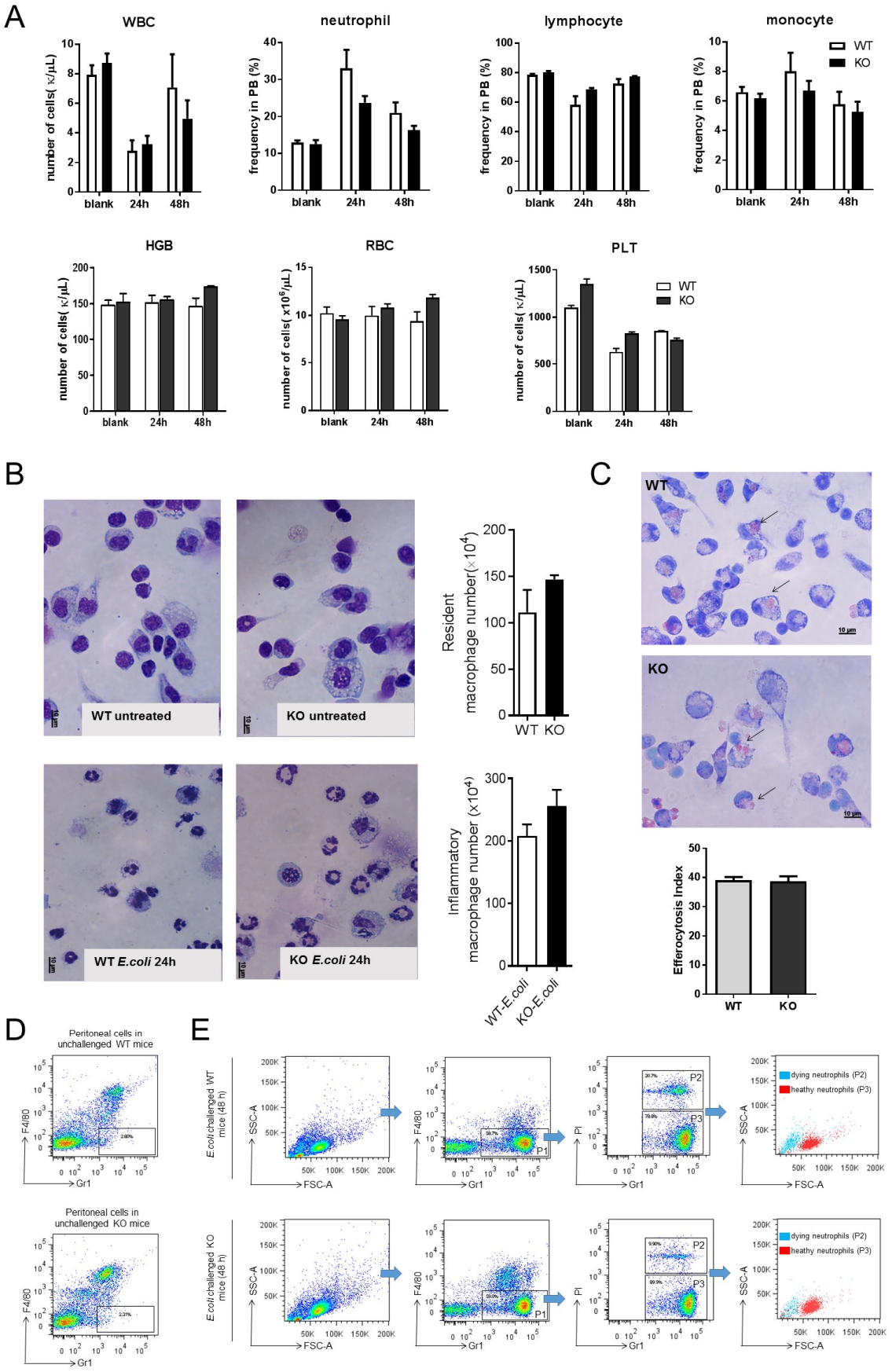
Exon 3
Forward primer
Intron 2-3
Exon 3
Intron 3-4
Reverse primer
B
Heterozygous KO
Heterozygous WT
Heterozygous WT
Heterozygous KO
WT product (550 bp)
KO product (423 bp)
C
KO genomic DNA sequence:
D
GSDMD protein translation (WT):
(487aa)
GSDMD protein translation (KO):
(frame shift)
E
WT1 WT1 KO1 KO2
2 kb -
1 kb -
750 bp -
F
WBC
number of cells(K/μL)
WT KO
G
lymphocyte
neutrophil
monocyte
eosinophil
RBC
HGB
PLT
frequency in PB (%)
number of cells (10^6/μL)
g/L
number of cells(K/μL)
WT KO
H
WT
GSDMD KO
5 μm
I
Survival(%)
WT
GSDMD-/-
Hour
J
spleen/BW (%)
WT
KO
blank 24h 48h
K
E.coli challenged KO
E.coli challenged WT
Unchallenged WT
Unchallenged KO
cm 1 2 3 4 5

Figure S1. Related to Figure 1. Generation of *Gsdmd*-deficient mice using the CRISPR/Cas9 system. **(A)** Schematic of the targeting strategy. Mouse *Gsdmd* gene structure and the location of exon 3 are shown. The sequence deleted in the KO mouse is underlined. The forward and reverse primers used for genotyping are indicated. **(B)** Genome PCR analysis in wild-type (WT), heterozygous, and homozygous (KO) mutant mice using forward and reverse primers. **(C)** Genomic DNA sequence of *Gsdmd*-deficient mice. The desired deletion of the *Gsdmd* gene was confirmed. **(D)** CRISPR/Cas9-induced 127 bp deletion results in a frame shift in exon 3 and subsequent introduction of a stop codon 32 codons downstream. **(E)** Complete depletion of *Gsdmd* mRNA in the KO mice was confirmed by qPCR. **(F)** Total white blood cell count (WBC) in wild-type (WT) and *Gsdmd*-deficient (KO) mice (n >15 mice per genotype). No significant difference ($P < 0.05$) was found between groups. **(G)** Differential white blood cell counts, red blood cell (RBC) counts, platelets (PLT) counts, and hemoglobin (HGB) levels in WT and KO mice (n >15 mice per genotype). No significant difference ($P < 0.05$) was found between groups. **(H)** Peripheral blood smears from WT and KO mice. Shown are representative images. **(I)** Survival rates of mice challenged with lethal sepsis. Age and sex matched (9-week old, n=6) mice were challenged with 30 mg/kg LPS (E.coli 0111:B4). Survival rates were analyzed using the Kaplan-Meier survival curves and log-rank test. * $P < 0.05$ *vs* WT. **(J)** Relative ratio of spleen weight vs. body weight following *E. coli* challenge. The overall weight of each mouse was similar, so correction of spleen weight by body weight did not affect the conclusions. Values are mean ± SD. *$P < 0.01$ (Student's t-test). **(K)** Picture of spleens from unchallenged and *E. coli* challenged WT and KO mice. Experiments were conducted as described in **Fig 1A**.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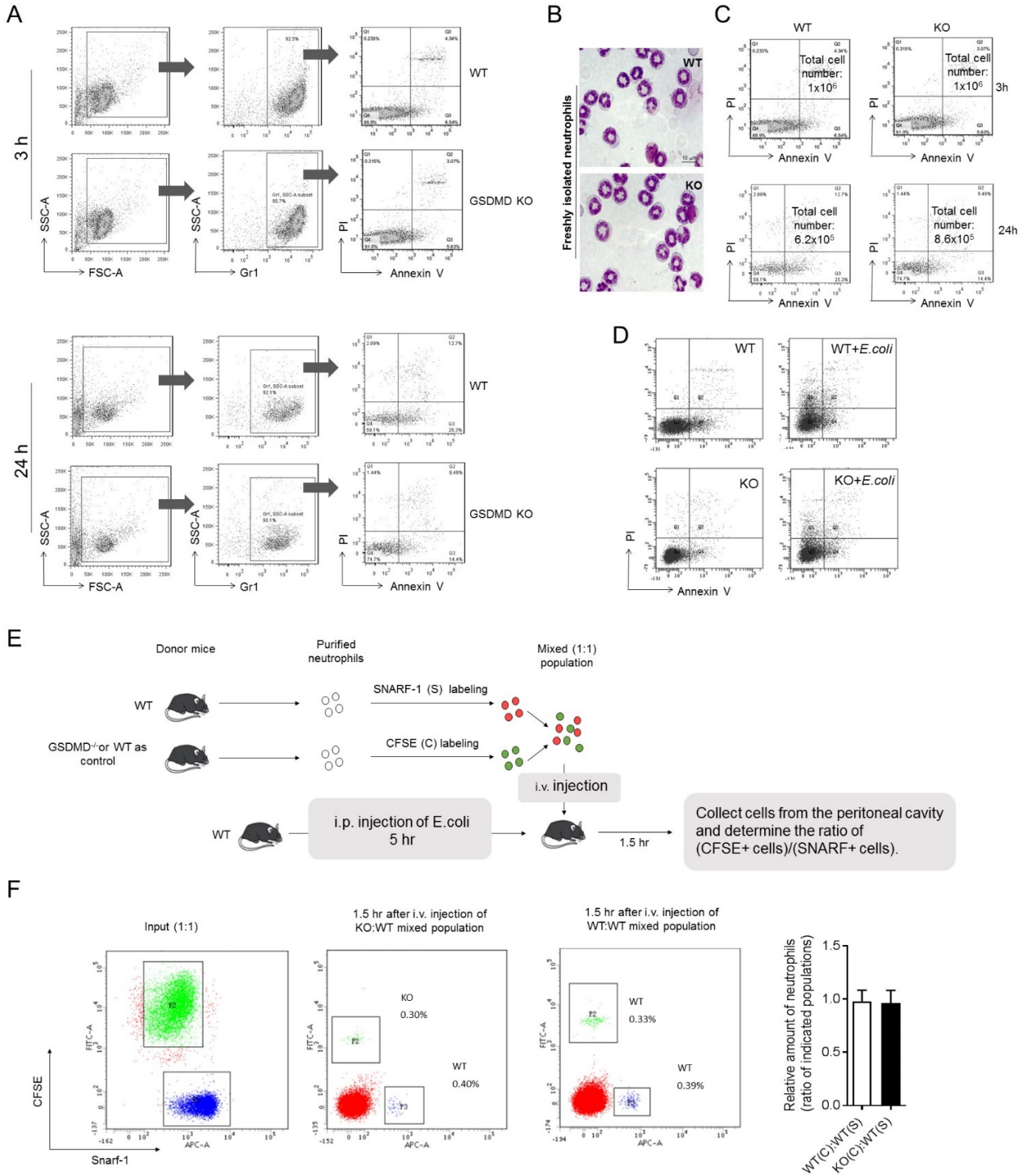
WBC
neutrophil
lymphocyte
monocyte
HGB
RBC
PLT
number of cells(κ/μL)
frequency in PB (%)
number of cells(x10^6/μL)
blank
24h
48h
WT
KO
B
WT untreated
KO untreated
WT E.coli 24h
KO E.coli 24h
Resident macrophage number(×10^4)
Inflammatory macrophage number (×10^4)
WT-E.coli
KO-E.coli
C
WT
KO
Efferocytosis Index
D
Peritoneal cells in unchallenged WT mice
Peritoneal cells in unchallenged KO mice
F4/80
Gr1
E
E.coli challenged WT mice (48 h)
E.coli challenged KO mice (48 h)
SSC-A
FSC-A
PI
P1
P2
P3
dying neutrophils (P2)
heathy neutrophils (P3)

Figure S2. Related to Figure 3. The elevated neutrophil accumulation in GSDMD-deficient mice was due to delayed neutrophil death. (A) Total white blood cell count (WBC), differential white blood cell counts, red blood cell (RBC) counts, platelets (PLT) counts, and hemoglobin (HGB) levels in wild-type (WT) and *Gsdmd*-deficient (KO) mice (male, 8-12 week old) after *E.coli* challenge. The experiments were conducted as described in **Fig. 2E**. No significant difference ($P < 0.05$) was found between groups. (**B**) The numbers of resident and inflammatory macrophages in the peritoneal cavity in WT and *Gsdmd*-deficient (KO) mice. The macrophages were identified by morphology examination of Wright-Giemsa stained peritoneal cells (large size, large cytoplasmic region, and single, round nucleus). All values represent mean ± SD of three independent experiments. **(C)** Clearance of dead neutrophils by macrophages (efferocytosis). The assay was conducted as previously described (Mondal et al., 2011). Briefly, wild-type or *Gsdmd*-deficient (KO) peritoneal macrophages were incubated with one-day cultured bone marrow neutrophils at a density of (1:10) for 90 minutes at 37°C. Cells were washed and efferocytosis was analyzed by HEMA3 staining. Number of macrophages containing one or more apoptotic cell was scored as % efferocytosis. Arrowheads indicate macrophage containing engulfed apoptotic cells. All values represent mean ± SD of three independent experiments. **(D)** Representative flow cytometry plots for peritoneal neutrophils in unchallenged WT and *Gsdmd*-deficient (KO) mice. Numbers denote the frequency of each population among live singlets. Very few neutrophils were detected in the peritoneal cavity of WT or KO mice. (**E**) Gating strategy to analyze neutrophil death in inflamed peritoneal cavities in WT and KO mice. The experiments were conducted as described in **Fig. 3C**.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3 h
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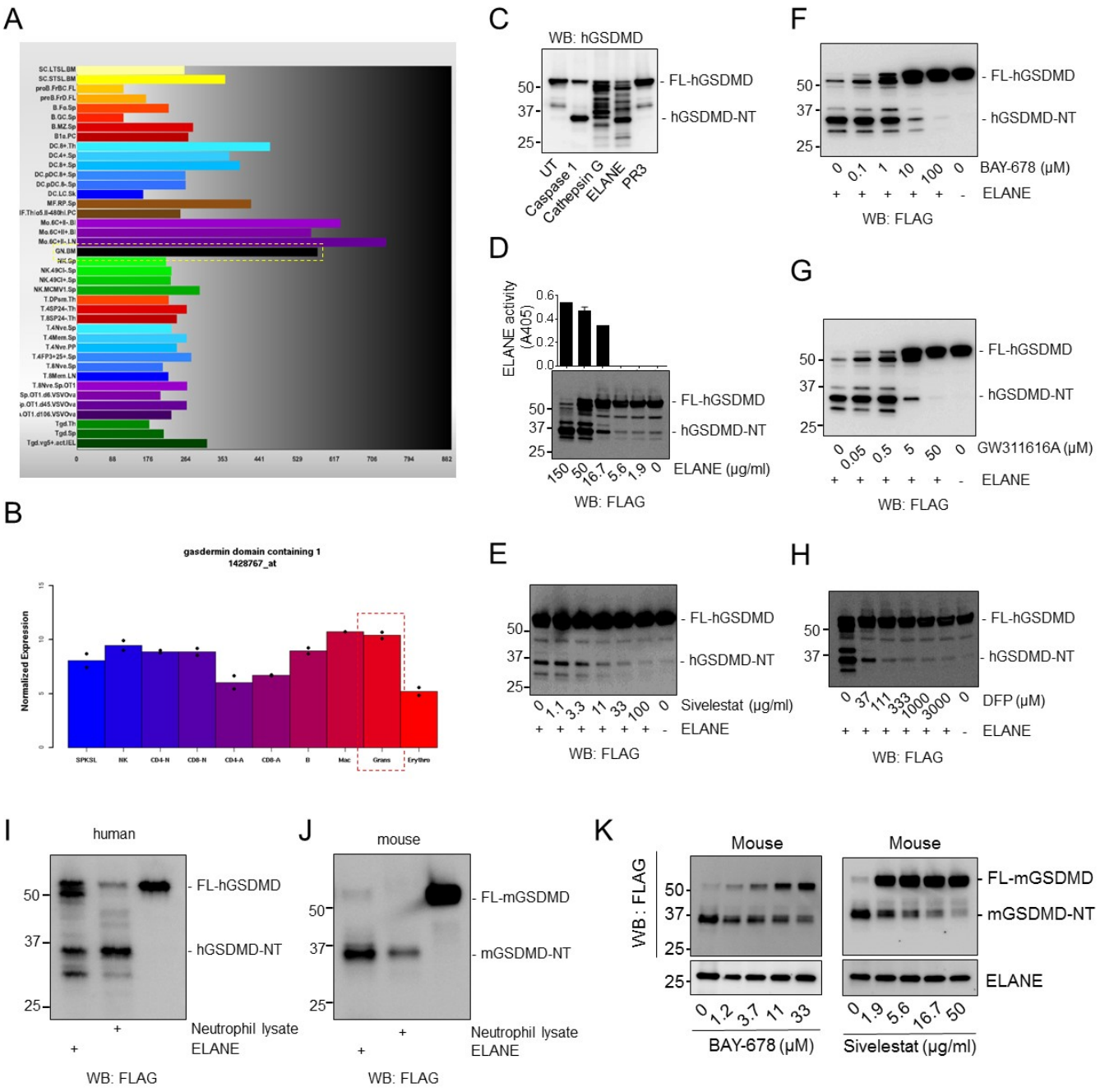
FSC-A
Gr1
PI
Annexin V
WT
GSDMD KO
24 h
B
Freshly isolated neutrophils
WT
KO
C
WT
KO
Total cell number: 1x10^6
Total cell number: 6.2x10^5
Total cell number: 8.6x10^5
3h
24h
PI
Annexin V
D
WT
WT+E.coli
KO
KO+E.coli
E
Donor mice
Purified neutrophils
Mixed (1:1) population
WT
GSDMD-/- or WT as control
SNARF-1 (S) labeling
CFSE (C) labeling
i.v. injection
i.p. injection of E.coli 5 hr
1.5 hr
Collect cells from the peritoneal cavity and determine the ratio of (CFSE+ cells)/(SNARF+ cells).
F
Input (1:1)
1.5 hr after i.v. injection of KO:WT mixed population
1.5 hr after i.v. injection of WT:WT mixed population
CFSE
Snarf-1
FITC-A
APC-A
KO
0.30%
WT
0.40%
WT
0.33%
WT
0.39%
Relative amount of neutrophils (ratio of indicated populations)
1.5
1.0
0.5
0.0
WT(C):WT(S)
KO(C):WT(S)

Figure S3. Related to Figure 3. The role of GSDMD in regulating neutrophil death and recruitment. (A) Gating strategy to analyze the death of *in vitro* cultured WT and *Gsdmd*-deficient (KO) neutrophils. In this FACS-based analysis, the characteristic phosphatidylserine exteriorization was detected by annexin V, an anticoagulant protein that has high affinity and selectivity for phosphatidylserine. The membrane integrity of the dying neutrophils was monitored using propidium iodide (PI), a membrane impermeable dye. Experiments were conducted as described in **Fig 3E**. **(B)** Freshly isolated WT and GSDMD-deficient neutrophils. Shown are representative images. **(C)** Death of *in vitro* cultured WT and GSDMD-deficient neutrophils. Neutrophils were cultured in RPMI 1640 medium containing 10% heat-inactivated FBS at a density of 2 x 10^6 cells per ml. Dying cells were detected by annexin V-FITC and propidium iodide (PI) staining. *Shown are representative FACS* plots depicting healthy (PI^-/annexin V^-) and dying (PI^+ or annexin V^+) peritoneal neutrophils. Experiments were conducted as described in **Fig 3E**. **(D)** Death of *in vitro* cultured WT and GSDMD-deficient neutrophils in the presence of *E.coli.* Neutrophils were cultured with *E.coli* (1:5 ratio) for 2 hours. *Shown are representative FACS* plots depicting healthy (PI^-/annexin V^-) and dying (PI^+ or/and annexin V^+) neutrophils. Experiments were conducted as described in **Fig 3G. (E-F)** Disruption of GSDMD in neutrophils did not affect neutrophil recruitment during infection and inflammation. **(E)** Recruitment of adoptively transferred neutrophils. WT and GSDMD deficient neutrophils were labeled with intracellular fluorescent dye intracellular fluorescent dye 5-(and -6)-carboxyfluorescein diacetate succinimidyl esters (CFSE) or 5-(and -6)-chloromethyl SNARF-1 acetate (SNARF-1). Labeled cells were mixed (1:1) and intravenously injected to WT mice challenged with 3% thioglycollate (TG). The cells accumulated in the peritoneal cavity were collected at indicated time point and analyzed using a FACSCanto II flow cytometer. **(F)** The ratios of adoptively transferred WT and GSDMD-deficient neutrophils. All values represent mean ± SD of three experiments.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Grans
Erythro
C
WB: hGSDMD
FL-hGSDMD
hGSDMD-NT
UT
Caspase 1
Cathepsin G
ELANE
PR3
D
ELANE activity (A405)
ELANE (µg/ml)
WB: FLAG
E
Sivelestat (µg/ml)
ELANE
WB: FLAG
F
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WB: FLAG
G
GW311616A (µM)
ELANE
WB: FLAG
H
DFP (µM)
ELANE
WB: FLAG
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FL-mGSDMD
mGSDMD-NT
ELANE
BAY-678 (µM)
Sivelestat (µg/ml)

Figure S4. Related to Figure 4. GSDMD cleavage in neutrophils is mediated by ELANE. **(A)** GSDMD mRNA expression in hematopoietic cells from the ImmGen Database. BM neutrophil population is highlighted in yellow. **(B)** GSDMD mRNA expression in hematopoietic cells from the Gene Expression Across Multiple Hematopoietic Lineages Database. Neutrophil population is highlighted in red. **(C-H)** ELANE-mediated cleavage of human GSDMD. **(C)** FLAG-hGSDMD was overexpressed in HEK293T cells. Cell lysates containing recombinant FLAG-hGSDMD were incubated with recombinant human caspase 1 (100 units), PR3 (2 μg), ELANE (2 μg), or cathepsin G (2 μg) at 37°C for 30 min in a 6-well plate. The protein samples were subjected to SDS-PAGE followed by western blotting with an anti-GSDMD antibody. The positions of the full-length (FL-hGSDMD) and ELANE-cleaved N-terminal hGSDMD (hGSDMD-NT) are indicated. **(D)** The degree of GSDMD cleavage was positively correlated with the amount of ELANE enzymatic activity in the reaction. FLAG-hGSDMD was incubated with different doses of ELANE. ELANE enzymatic activity was determined using an ELANE-specific substrate following the manufacturer's protocol. **(E)** ELANE-mediated GSDMD cleavage conducted in the presence of indicated amount of sivelestat. **(F)** ELANE-mediated GSDMD cleavage conducted in the presence of indicated amount of BAY-678. **(G)** ELANE-mediated GSDMD cleavage conducted in the presence of indicated amount of GW311616A. **(H)** ELANE-mediated GSDMD cleavage conducted in the presence of indicated amount of pan-serine protease inhibitor DFP. **(I-J)** GSDMD N-terminal fragment produced by neutrophil lysate was the same size as the one cleaved by recombinant ELANE. **(I)** Cleavage of human GSDMD. **(J)** Cleavage of mouse GSDMD. The positions of the full-length and ELANE-cleaved GSDMD are indicated. **(K)** Mouse GSDMD cleavage by neutrophil lysate was inhibited by ELANE-specific protease inhibitors in a dose dependent manner. FLAG-mGSDMD was overexpressed in HEK293T cells. Cell lysates containing recombinant FLAG-hGSDMD were incubated with mouse neutrophil lysate in the presence of indicated amount of ELANE inhibitors BAY-678 or sivelestat at 37°C for 30 min. The full-length and ELANE-cleaved GSDMD were detected with an anti-FLAG antibody.

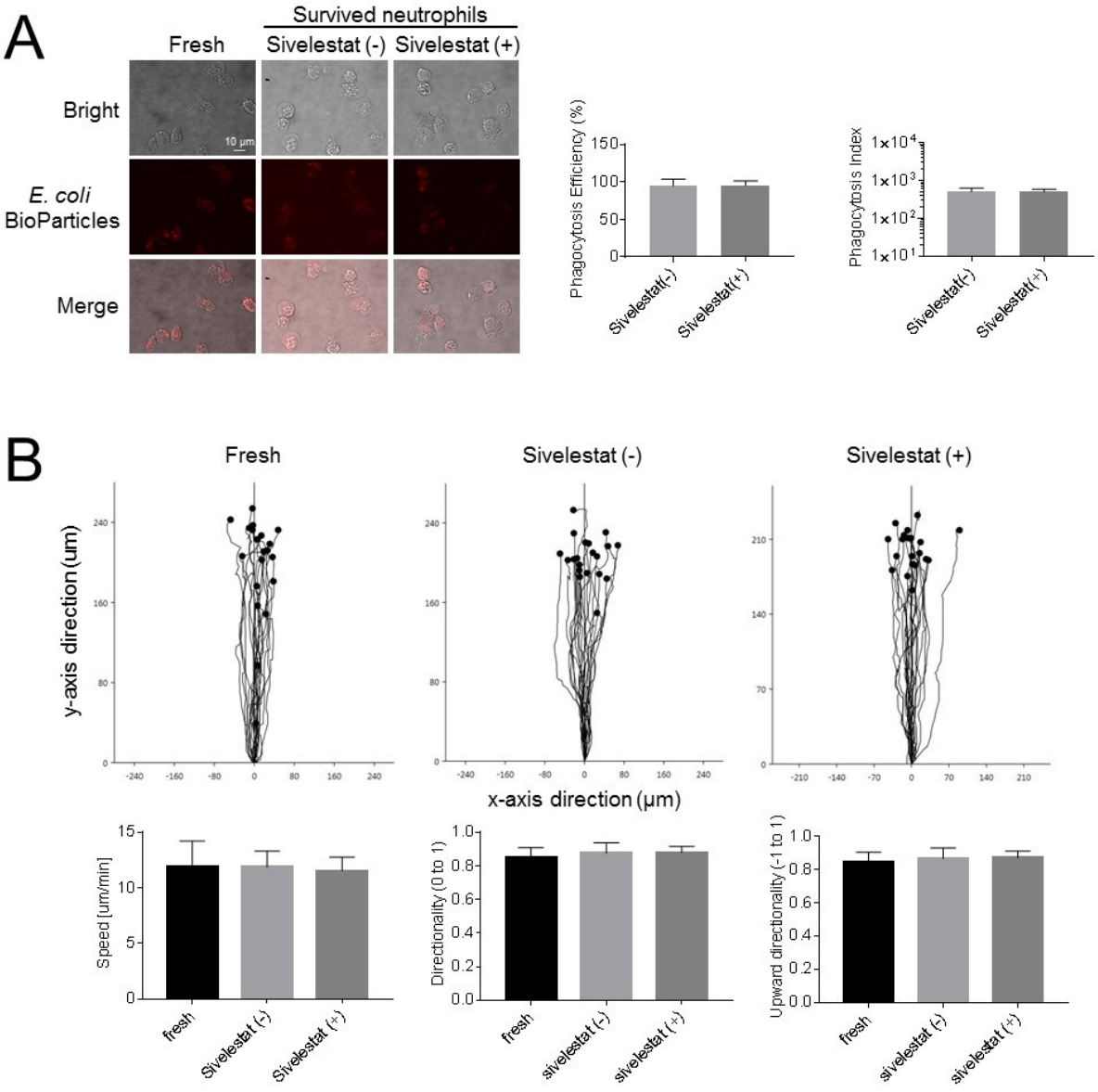
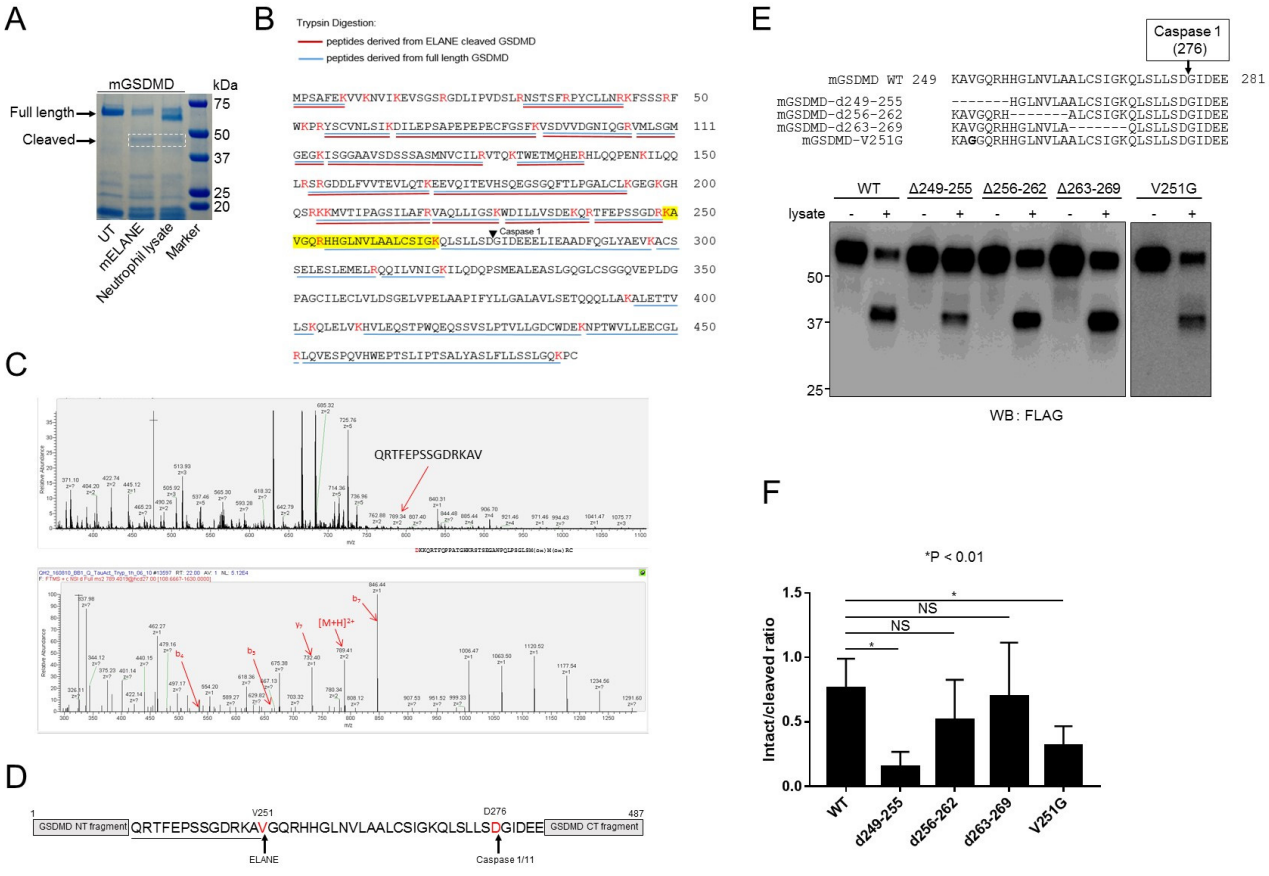


Figure S5. Related to Figure 5. Chemotaxis and phagocytosis capability of the survived cells. (A) *In vitro* phagocytosis assay. Human neutrophils were cultured in the presence or absence of Sivelestat (1μg/ml) for 24 hr. Fluorescein-conjugated *E. coli* bioparticles (10 μg/ml) were opsonized with 20% human serum and incubated with survived neutrophils at 37°C for 90 min. Cells were fixed with 4% PFA and phagocytosed bioparticles were manually counted to determine phagocytosis efficiency and index. **(B)** Neutrophil chemotaxis assay. Neutrophil chemotaxis was assessed using EZ-Taxiscan chemotaxis device (Effector Cell Institute, Tokyo, Japan) as previously described (Hattori et al., 2010; Sakai et al., 2012). Chemotaxis was triggered by fMLP (1 μl of 0.1 μM human fMLP, Sigma: F3506). Chemotaxis speed, directionality and upward directionality were calculated using Gradientech Tracking Tool™ PRO v2.1. All values represent mean ± SD (n=20 cells).


A
mGSDMD
kDa
Full length
Cleaved
75
50
37
25
20
UT
mELANE
Neutrophil lysate
Marker
B
Trypsin Digestion:
peptides derived from ELANE cleaved GSDMD
peptides derived from full length GSDMD
MPSAFEKVVKNVIKEVSGSRGDLIPVDSLRNSTSFRPYCLLNRKFSSSRF 50
WKPRYSCVNLSIKDILEPSAPEPEPECFGSFKVSDVVDGNIQGRVMLSGM 111
GEGKISGGAAVSDSSSASMNVCILRVTQKTWETMQHERHLQQPENKILQQ 150
LRSRGDDLFVVTEVLQTKEEVQITEVHSQEGSGQFTLPGALCLKGEGKGH 200
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SELESLEMELRQQILVNIGKILQDQPSMEALEASLGQGLCSGGQVEPLDG 350
PAGCILECLVLDSGELVPELAAPIFYLLGALAVLSETQQQLLAKALETTV 400
LSKQLELVKHVLEQSTPWQEQSSVSLPTVLLGDCWDEKNPTWVLLEECGL 450
RLQVESPQVHWEPTSLIPTSALYASLFLLSSLGQKPC
Caspase 1
C
QRTFEPSSGDRKAV
D
1
GSDMD NT fragment
QRTFEPSSGDRKAVGQRHHGLNVLAALCSIGKQLSLLSDGIDEE
GSDMD CT fragment
487
V251
D276
ELANE
Caspase 1/11
E
Caspase 1
(276)
mGSDMD WT 249 KAVGQRHHGLNVLAALCSIGKQLSLLSDGIDEE 281
mGSDMD-d249-255 -------HGLNVLAALCSIGKQLSLLSDGIDEE
mGSDMD-d256-262 KAVGQRH-------ALCSIGKQLSLLSDGIDEE
mGSDMD-d263-269 KAVGQRHHGLNVLA-------QLSLLSDGIDEE
mGSDMD-V251G KAGGQRHHGLNVLAALCSIGKQLSLLSDGIDEE
WT
Δ249-255
Δ256-262
Δ263-269
V251G
lysate
- + - + - + - + - +
50
37
25
WB: FLAG
F
*P < 0.01
Intact/cleaved ratio
1.5
1.0
0.5
0.0
NS
NS
*
*
WT
d249-255
d256-262
d263-269
V251G
G
hGSDMD MGSAFERVVRRVVQELD-HGGEFIPVTSLQSSTGFQPYCLVVRKPSSSWFWKPRYKCVNL
mGSDMD MPSAFEKVVKNVIKEVSGSRGDLIPVDSLRNSTSFRPYCLLNRKFSSSRFWKPRYSCVNL
hGSDMD SIKDILEPDAAEPDVQRGRSFHFYDAMDGQIQGSVELAAPGQAKIAGGAAVSDSSSTSMN
mGSDMD SIKDILEPSAPEPEPECFGSFKVSDVVDGNIQGRVMLSGMGEGKISGGAAVSDSSSASMN
hGSDMD VYSLSVDPNTWQTLLHERHLRQPEHKVLQQLRSRGDNVYVVTEVLQTQKEVEVTRTHKRE
mGSDMD VCILRVTQKTWETMQHERHLQQPENKILQQLRSRGDDLFVVTEVLQTKEEVQITEVHSQE
hGSDMD GSGRFSLPGATCLQGEGQGHLSQKKTVTIPSGSTLAFRVAQLVIDSDLDVLLFPDKKQRT
mGSDMD GSGQFTLPGALCLKGEGKGHQSRKKMVTIPAGSILAFRVAQLLIGSKWDILLVSDEKQRT
hELANE
Caspase 1/11
hGSDMD FQPPATGHKRSTSEGAWPQLPSGLSMMRCLHNFLTDGVPAEG-AFTEDFQGLRAEVETIS
mGSDMD FEPSSGDRKAVGQRHHGLNVLAALCSIGKQLSLLSDGIDEEELIEAADFQGLYAEVKACS
mELANE
hGSDMD KELELLDRELCQLLLEGLEGVLRDQLALRALEEALEQGQS-LGPVEPLDGPAGAVLECLV
mGSDMD SELESLEMELRQQILVNIGKILQDQPSMEALEASLGQGLCSGGQVEPLDGPAGCILECLV
hGSDMD LSSGMLVPELAIPVVYLLGALTMLSETQHKLLAEALESQTLLGPLELVGSLLEQSAPWQE
mGSDMD LDSGELVPELAAPIFYLLGALAVLSETQQQLLAKALETTVLSKQLELVKHVLEQSTPWQE
hGSDMD RSTMSLPPGLLGNSWGEGAPAWVLLDECGLELGEDTPHVCWEPQAQGRMCALYASLALLS
mGSDMD QSSVSLPTVLLGDCWDEKNPTWVLLEECGLRLQVESPQVHWEPTSLIPTSALYASLFLLS
hGSDMD GLSQEPH
mGSDMD SLGQKPC

Figure S6. Related to Figure 6. Determination of ELANE cleavage site in mouse GSDMD. (A) Recombinant mouse His-SUMO-GSDMD was incubated with mELANE or mouse neutrophil lysates at 37°C for 30 min and subjected to SDS-PAGE followed by colloidal blue staining. The full length and ELANE-cleaved GSDMD N-terminal fragment were trypsin digested and analyzed by mass spectrometry (MS). **(B)** MS analysis of full length and ELANE-cleaved GSDMD. Trypsin digested peptides derived from the full length (blue lines) and ELANE-cleaved (red lines) mGSDMD are underlined. The putative ELANE cleavage sites are highlighted. **(C)** MS analysis of the ELANE cleavage site in mGSDMD was conducted using partially trypsin-digested samples. Top, mass spectrum of mGSDMD peptides generated through the cleavage. Bottom, MS/MS spectrum of the corresponding peptide showing the fragment ions detected/used for protein identification. MS analysis identifies V251 as the ELANE cleavage site in mGSDMD. **(D)** ELANE- and caspase-1-cleaved mGSDMD at different sites. The ELANE and caspase-1 cleavage sites as well as the peptide identified from the partially trypsin-digested sample are indicated. **(E-F**) Confirmation of ELANE cleavage site in mouse GSDMD. **(E)** The cleavage of mGSDMD deletion or point mutants by ELANE. The FLAG-tagged mGSDMD mutants were overexpressed in HEK293T cells. ELANE-mediated mGSDMD cleavage was carried out and analyzed as described in **Fig. S4J**. **(F)** Results of densitometry. Relative amounts of protein in **(E)** were quantified using NIH Image software as previously described. ELANE-elicited cleavage of mouse GSDMD was expressed as ratio of cleaved mGSDMD (mGSDMD-NT) and uncleaved full length mGSDMD (FL-mGSDMD). Results are the means (± SD) of three independent experiments. *, $P<0.01$ versus wild-type mGSDMD by Student's t test. **(G)** Comparison of the ELANE cleavage sites in human and mouse GSDMD. Human and mouse GSDMD amino acid sequences were aligned using Clustal Omega. Cleavage sites of Caspase 1 and ELANE are indicated with black arrow and red arrows, respectively.

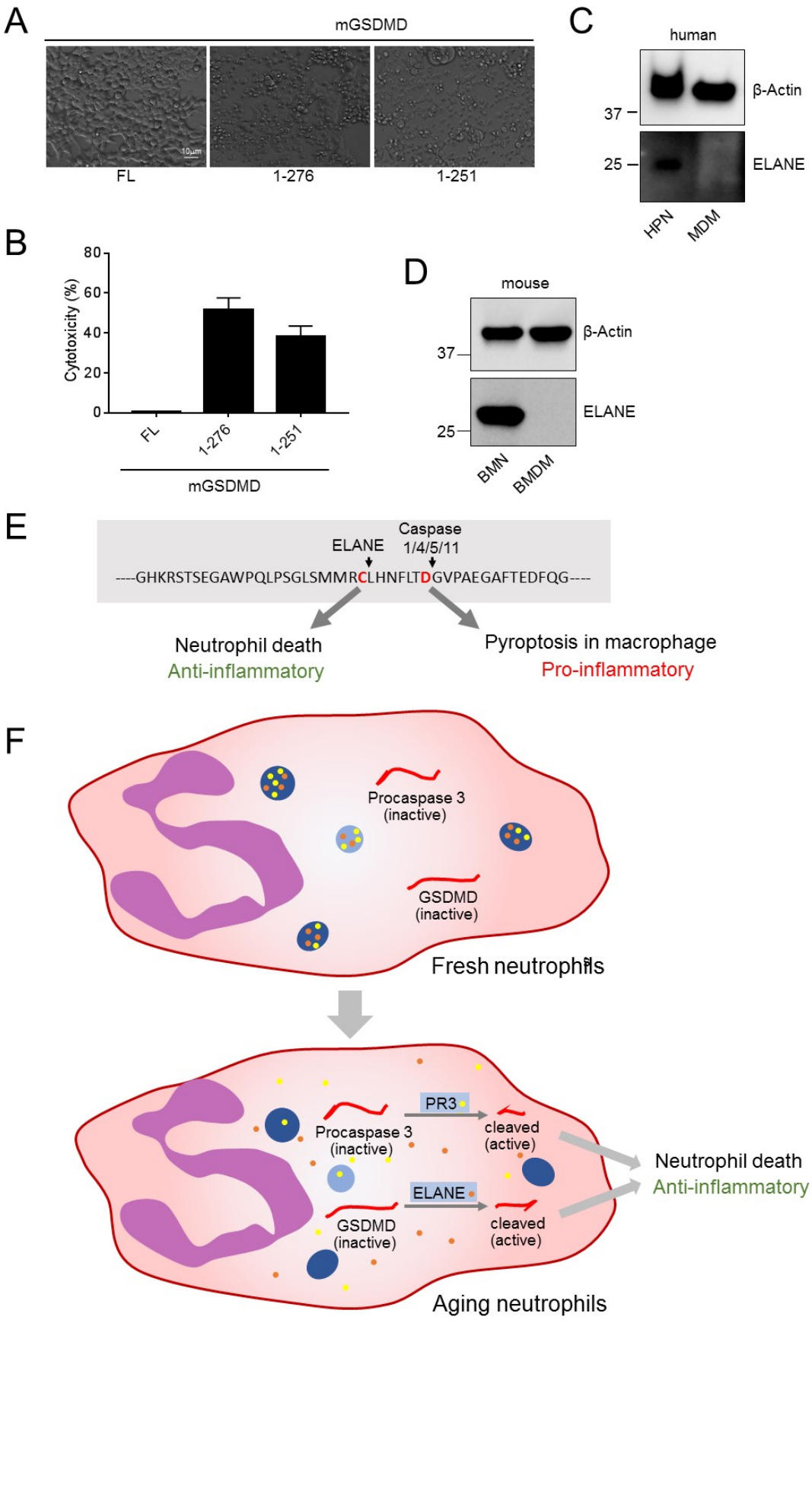
A
mGSDMD
FL
1-276
1-251
C
human
β-Actin
ELANE
37
25
HPN
MDM
B
Cytotoxicity (%)
80
60
40
20
0
FL
1-276
1-251
mGSDMD
D
mouse
β-Actin
ELANE
37
25
BMN
BMDM
E
ELANE
Caspase 1/4/5/11
----GHKRSTSEGAWPQLPSGLSMMRCLHNFLTDGVPAEGAFTEDFQG----
Neutrophil death
Anti-inflammatory
Pyroptosis in macrophage
Pro-inflammatory
F
Procaspase 3 (inactive)
GSDMD (inactive)
Fresh neutrophils
PR3
Procaspase 3 (inactive)
cleaved (active)
ELANE
GSDMD (inactive)
cleaved (active)
Neutrophil death
Anti-inflammatory
Aging neutrophils

Figure S7. Related to Figure 7. ELANE-induced cleavage of GSDMD is a key mechanism controlling neutrophil death. (A-B) ELANE-cleaved mouse GSDMD (mGSDMD-eNTs) are active and can lead to lytic cell death. HEK293T cells were transfected with the full-length mGSDMD or ELANE-cleaved N-terminal fragment (mGSDMD 1-251) expressing constructs. The analyses were conducted 24 h after transfection. **(A)** Cell morphology was assessed by brightfield microscopy. **(B)** Cytotoxicity was measured based on death-associated release of lactate dehydrogenase (LDH) according to the manufacturer's protocol. **(C-D)** Monocyte-derived macrophages do not express ELANE. **(C)** ELANE expression in human highly purified neutrophils (HPNs) and monocyte-derived macrophages (MDMs) was assessed by western blotting using an anti-ELANE antibody. **(D)** ELANE expression in mouse bone marrow neutrophils (BMNs) and bone marrow-derived macrophages (BMDMs) was assessed by western blotting. **(E)** GSDMD can exert context-dependent pro-inflammatory and anti-inflammatory effects. **(F)** Lysosomal membrane permeabilization (LMP) induced release of serine proteases from the granules to the cytosol mediates neutrophil death.

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