Science Advances
AAAS

Supplementary Materials for

NLRC4 inflammasome–dependent cell death occurs by a complementary series of three death pathways and determines lethality in mice

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Published 22 October 2021, *Sci. Adv.* **7**, eabi9471 (2021)
DOI: 10.1126/sciadv.abi9471

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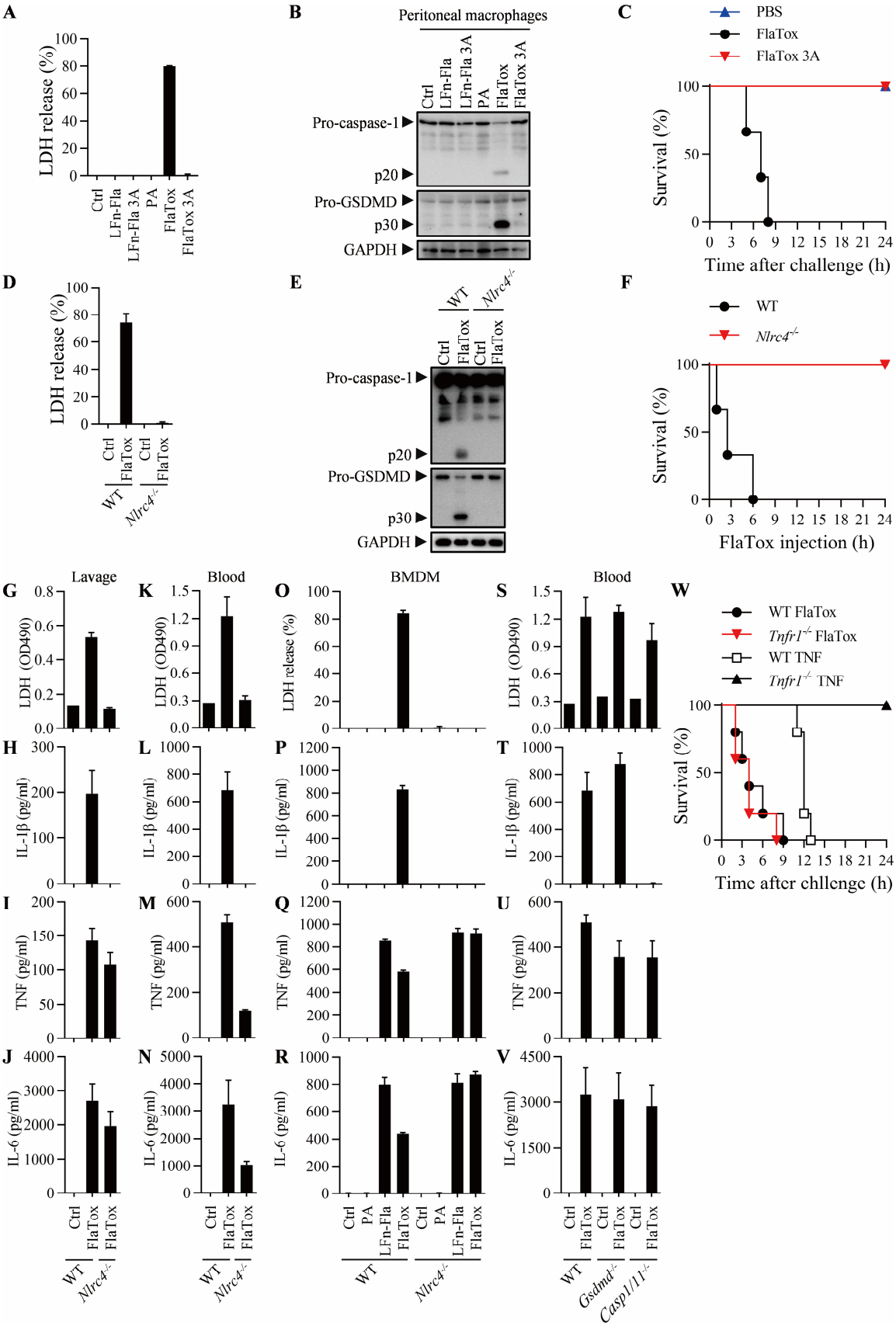

Fig. S1. Cell death and cytokines release by FlaTox mediated NLRC4 inflammasome activation *in vitro* and *in vivo*. (**A** and **B**) Peritoneal macrophages were treated with FlaTox

(2 μg/ml LFn-Fla, 2 μg/ml PA), or FlaTox 3A (2 μg/ml LFn-Fla 3A, 2 μg/ml PA) for 4 h. LDH release was measured at the end of experiments (A), n=3. Cell lysates combined with medium were subjected to Western blot for indicated proteins (B). (**C**) Mice were i.p. injected with FlaTox (4 μg/g LFn-Fla/body weight, 2 μg/g PA/body weight) or FlaTox 3A (4 μg/g LFn-Fla 3A/body weight, 2 μg/g PA/body weight) and monitored for survival rate, n=3 for each group. (**D** and **E**) Peritoneal macrophages from wild-type (WT) or *Nlrc4*$^{-/-}$ mice were treated with FlaTox (2 μg/ml LFn-Fla, 2 μg/ml PA) for 4 h. LDH release was measured at the end of experiments (D), n=3. Cell lysates combined with medium were subjected to Western blot for indicated proteins (E). (**F**) WT or *Nlrc4*$^{-/-}$ mice were i.p. injected with FlaTox (4 μg/g LFn-Fla/body weight, 2 μg/g PA/body weight) and monitored for survival rate, n=3. (**G** to **J**) Mice of indicated genotypes were i.p. injected with FlaTox (4 μg/g LFn-Fla/body weight, 2 μg/g PA/body weight). Peritoneal lavages were isolated by using 1 ml PBS at 2 h after FlaTox challenge. (**K** to **N**) Blood was collected and kept at room temperature for 30 min, and then centrifuged at 4000 rpm for 10 min. Serum was then used in the measurement. Peritoneal lavages and serum were subjected to measurements of LDH (G and K), IL-1β (H and L), TNF (I and M) and IL-6 (J and N), n=1 for PBS group, n=2 for FlaTox treated *Nlrc4*$^{-/-}$ mice, n=3 for FlaTox treated WT. (**O** to **R**) Bone marrow derived macrophages from WT mice or *Nlrc4*$^{-/-}$ mice were treated with PA (2 μg/ml), LFn-Fla (2 μg/ml) or FlaTox (PA 2 μg/ml +LFn-Fla 2 μg/ml) for 4h. LDH (O), IL-1β (P), TNF (Q) and IL-6 (R) were examined. (**S** to **V**) Mice of indicated genotypes were i.p. injected with FlaTox (4 μg/g LFn-Fla/body weight, 2 μg/g PA/body weight). Peritoneal lavages were isolated by using 1 ml PBS at 2 h after FlaTox challenge. Blood was collected and serum was subjected to measurements of LDH (S), IL-1β (T), TNF (U) and IL-6 (V). WT mice were the same as in K to N, n=1 for PBS group, n=3 for FlaTox treated *Gsdmd*$^{-/-}$, *Casp1/11*$^{-/-}$ mice. Column graphs show mean ± SE. (**W**) WT or *Tnfr1*$^{-/-}$ mice were i.p. injected with FlaTox (4 μg/g LFn-Fla/body weight, 2 μg/g PA/body weight) or TNF (1.2μg/g) and monitored for survival rate, n=5 for each group.

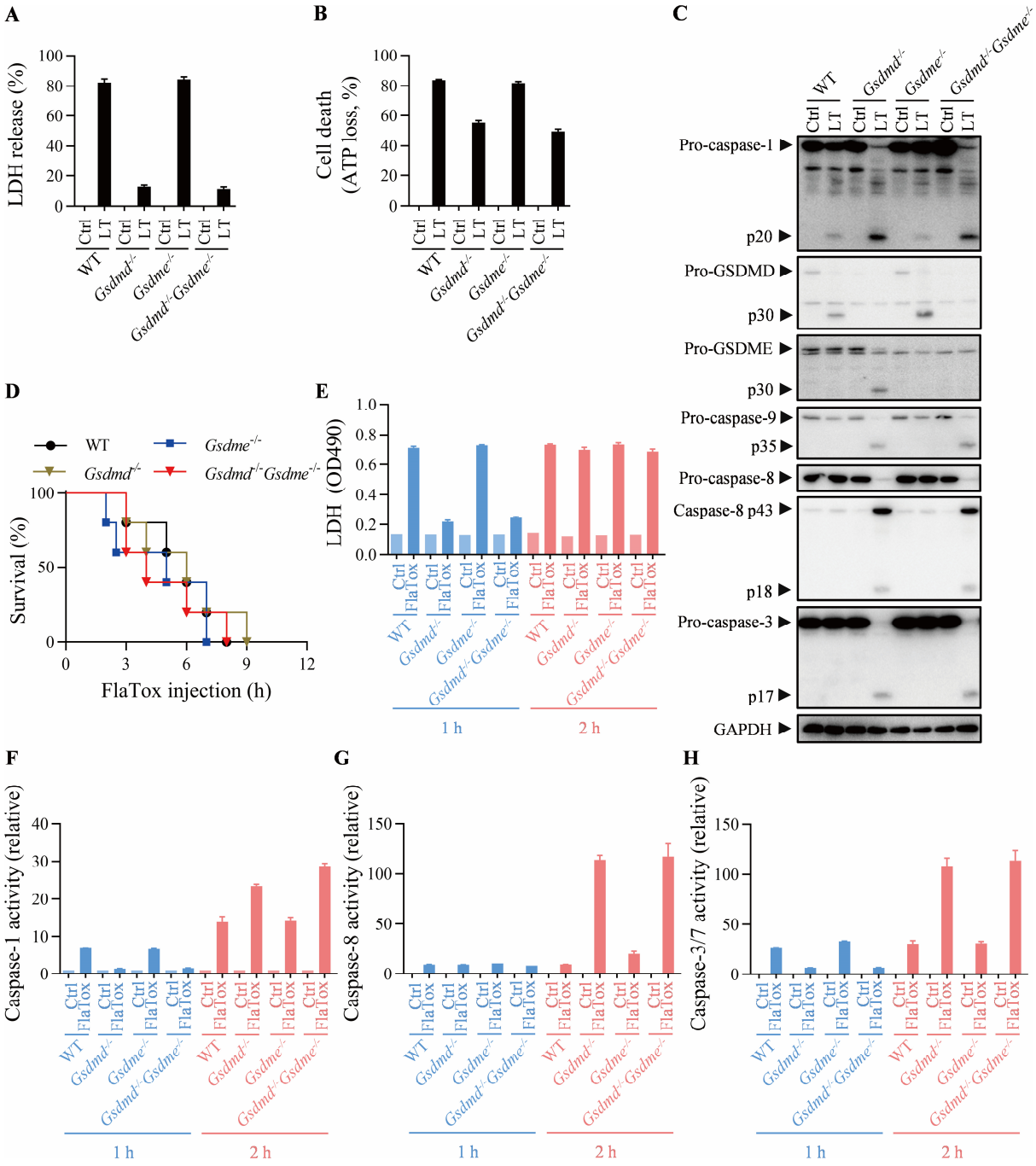

Fig.S2. Additional *Gsdme* deletion cannot block FlaTox-induced death of *Gsdmd* knockout cells and mice. (**A** to **C**) Peritoneal macrophages from mice of indicated genotypes were treated with LT (2 μg/ml LF, 2 μg/ml PA) for 4 h. The LDH release (A) and ATP loss (B) were measured. Cell lysates combined with medium were subjected to Western blot for indicated proteins (C). Graphs show mean ± SE from three independent experiments. (**D** to **H**) Mice of indicated genotypes were i.p. injected with FlaTox (4 μg/g LFn-Fla/body weight, 2 μg/g PA/body weight) and monitored for survival (D) at the indicated times, n=5. Peritoneal

lavages were isolated by using 1 ml PBS at 1 h and 2 h after FlaTox challenge and then subjected to measurements of LDH (E), caspase-1 activity (F), caspase-8 activity (G), or caspase-3/7 activity (H) (n=1 for PBS group, n=2 for FlaTox treated group). Graphs show mean ± SE.

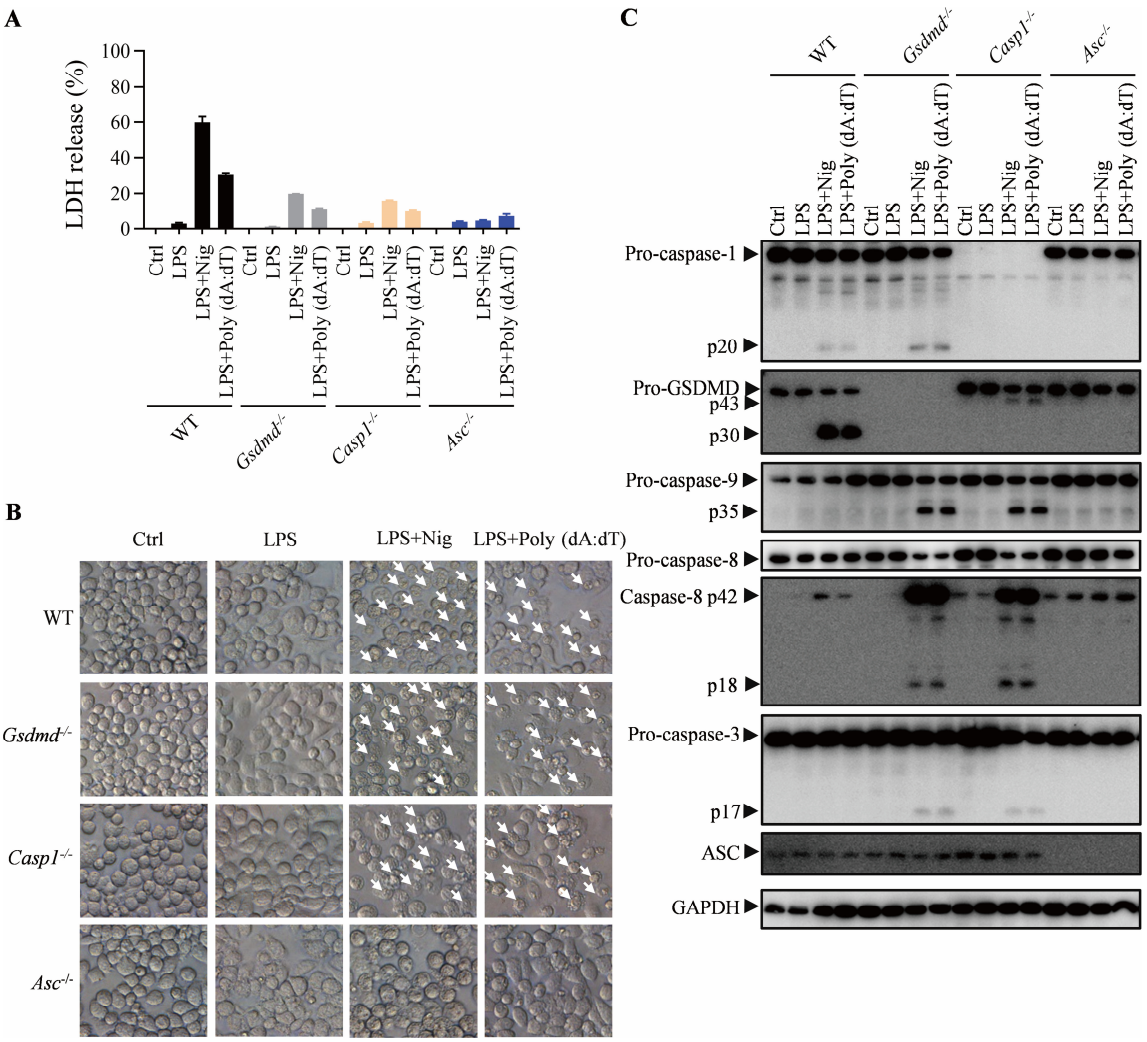

Fig. S3. NLRP3 and AIM2 inflammasome activation induces apoptosis in *Gsdmd* or *Casp1* deficient J774 cells. (**A** to **C**) J774 cells of indicated genotypes were treated with LPS (1 μg/ml) and then stimulated with nigericin (10 μM) or Poly (dA:dT) (2 μg/ml) for 3 h. LDH release was measured (A). Cell death was analyzed by microscopy. Arrows indicate pyroptotic or apoptotic cells (B). Cell lysates combined with medium were subjected to Western blot for indicated proteins (C). Graphs show mean ± SE from three independent experiments.

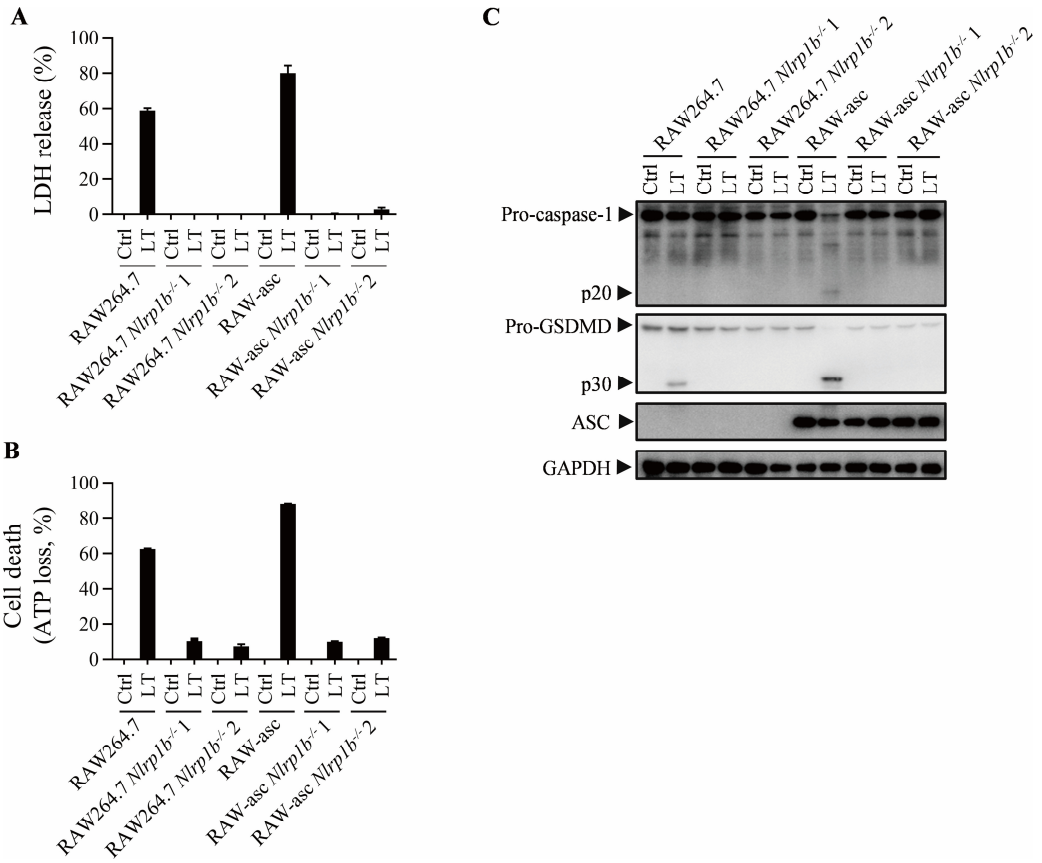

Fig. S4. LT induces NLRP1b dependent pyroptosis in RAW264.7 and RAW-asc cells. (**A** to **C**) RAW264.7 or RAW-asc cells with or without *NLRP1b* deletion were treated with LT (2 μg/ml LF, 2 μg/ml PA) for 4 h. The LDH release (A) and ATP loss (B) were measured. Cell lysates combined with medium were subjected to Western blot for indicated proteins (C). Graphs show mean ± SE from three independent experiments. Note: RAW264.7 cell line is in a genetic background of lethal toxin sensitive NLRP1b, and is an Asc null cell line.

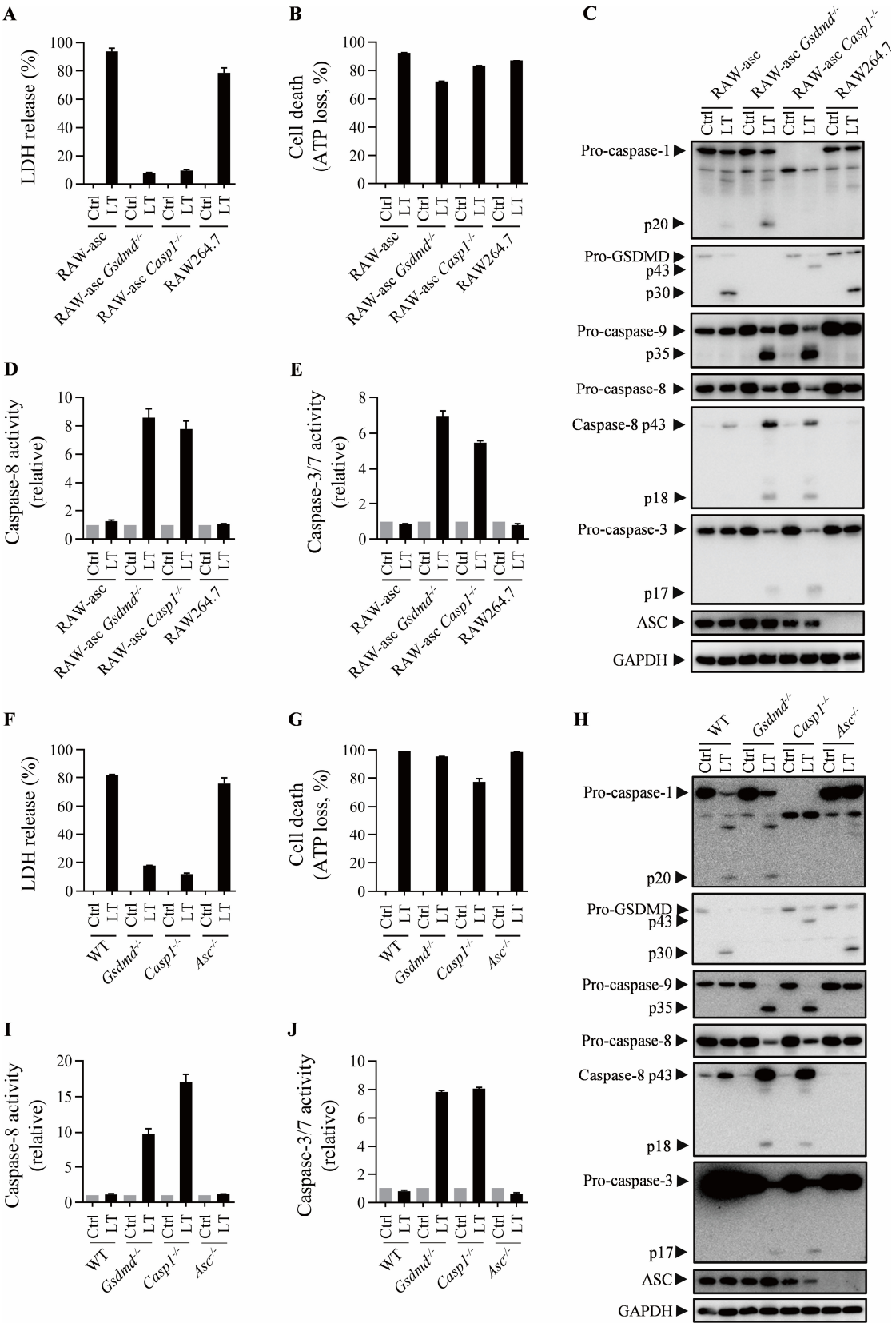

Fig. S5. NLRP1b inflammasome-induced pyroptosis is not affected by *Asc* deficiency but switches to apoptosis in *Gsdmd*$^{-/-}$, *Casp1*$^{-/-}$ RAW-asc cells, or J774 cells. (**A** to **E**) RAW-asc cells of indicated genotypes or RAW264.7 cells (*Asc null*) were treated with LT (2

μg/ml LF, 2 μg/ml PA) for 4 h. The LDH release (A) and ATP loss (B) were measured. Cell lysates combined with medium were subjected to Western blot for indicated proteins (C). The caspase-8 activity (D) and caspase-3/7 activity (E) were measured. (**F** to **J**) J774 cells of indicated genotypes were treated with LT (2 μg/ml LF, 2 μg/ml PA) for 4 h. The LDH release (F) and ATP loss (G) were measured. Cell lysates combined with medium were subjected to Western blot for indicated proteins (H). The caspase-8 activity (I) andcaspase-3/7 activity (J) were measured. Graphs show mean ± SE from three independent experiments.

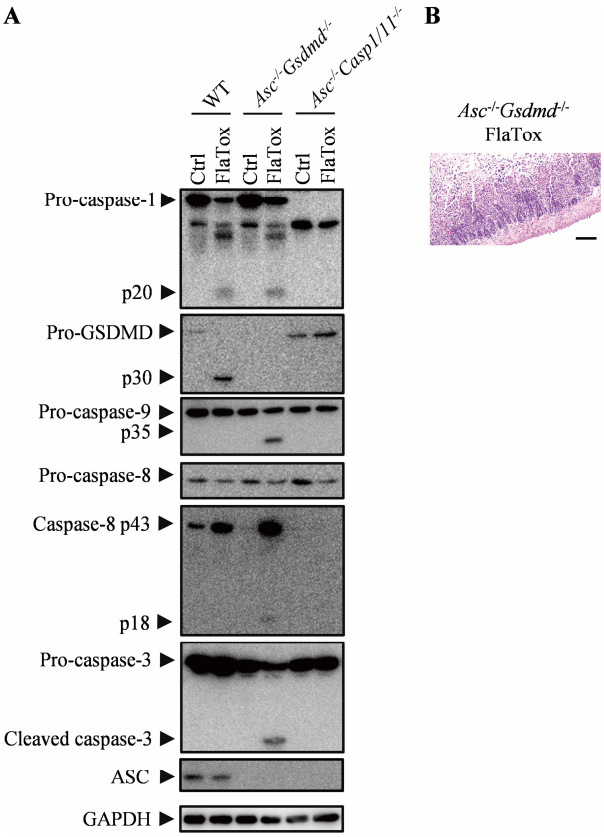

Fig. S6. Double knockout of *Casp1/11* and *Asc* completely blocks FlaTox-induced cell death. (**A**) Peritoneal macrophages from wild-type (WT) or *Asc*$^{-/-}$*Gsdmd*$^{-/-}$ or *Asc*$^{-/-}$*Casp1/11*$^{-/-}$ mice were treated with FlaTox (2 μg/ml LFn-Fla, 2 μg/ml PA) for 4 h. Cell lysates combined with medium were subjected to Western blot for indicated proteins. (**B**) H&E staining of small intestinal tissue of mouse treated as indicated for 3 h. The scale bar represents 100 μm.

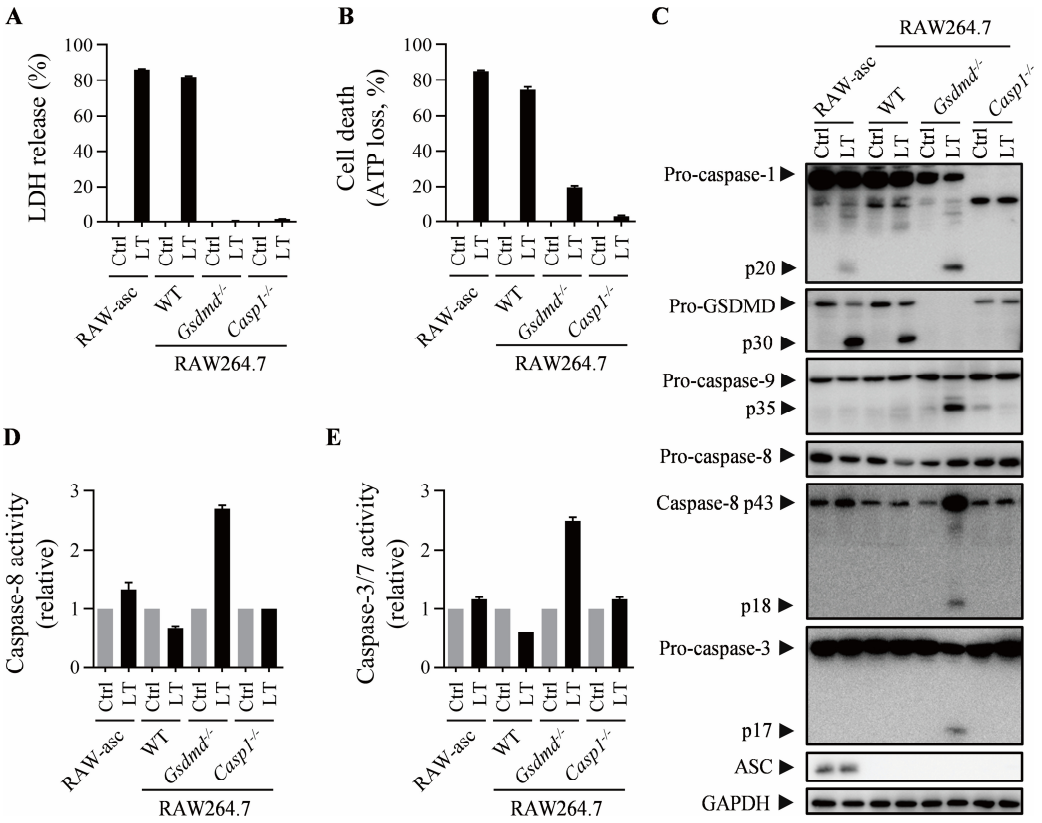

Fig. S7. LT induces caspapse-1 dependent apoptosis in RAW264.7 cells with *Gsdmd*$^{-/-}$ and *Asc* null genetic background. (**A** to **E**) RAW-asc or RAW264.7 (*Asc null*) cells of indicated genotypes were treated with LT (2 μg/ml LF, 2 μg/ml PA) for 4 h. The LDH release (A) and ATP loss (B) were measured. Cell lysates combined with medium were subjected to Western blot for indicated proteins (C). The caspase-8 activity (D) and caspase-3/7 activity (E) were measured. Graphs show mean ± SE from three independent experiments.

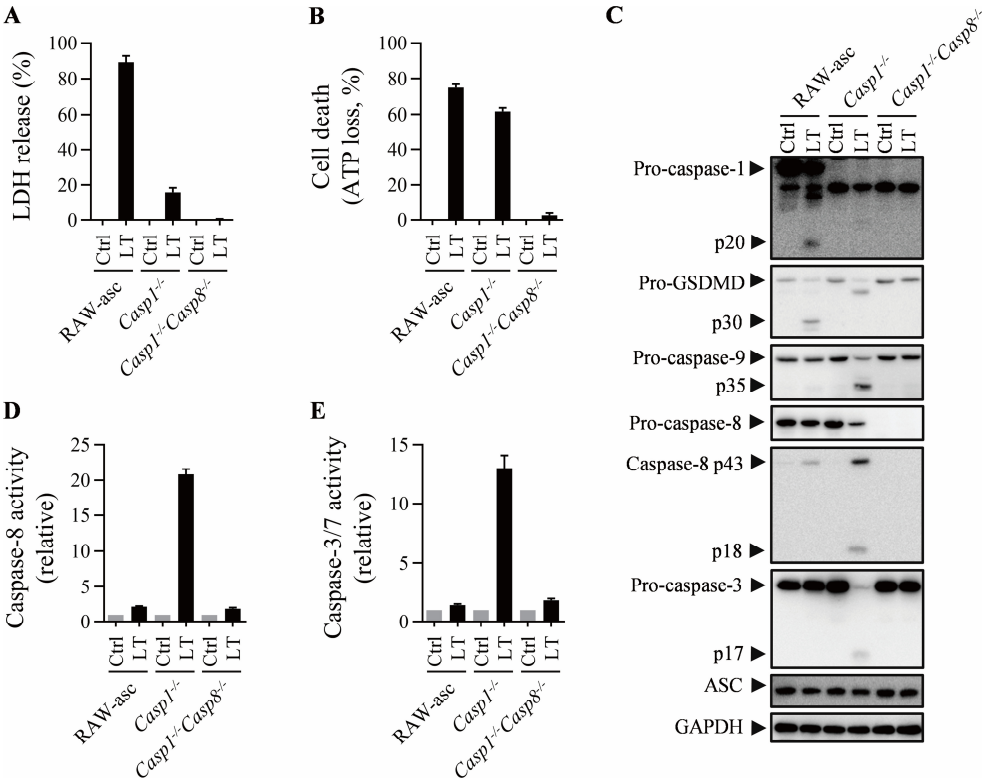

Fig. S8. Double knockout of *Casp1* and *Casp8* completely blocks NLRP1b inflammasome-mediated cell death. (**A** to **E**) RAW-asc cells of indicated genotypes were treated with LT (2 μg/ml LF, 2 μg/ml PA) for 4 h. The LDH release (A) and ATP loss (B) were measured. Cell lysates combined with medium were subjected to Western blot for indicated proteins (C). The caspase-8 activity (D) and caspase-3/7 activity (E) were measured. Graphs show mean ± SE from three independent experiments.

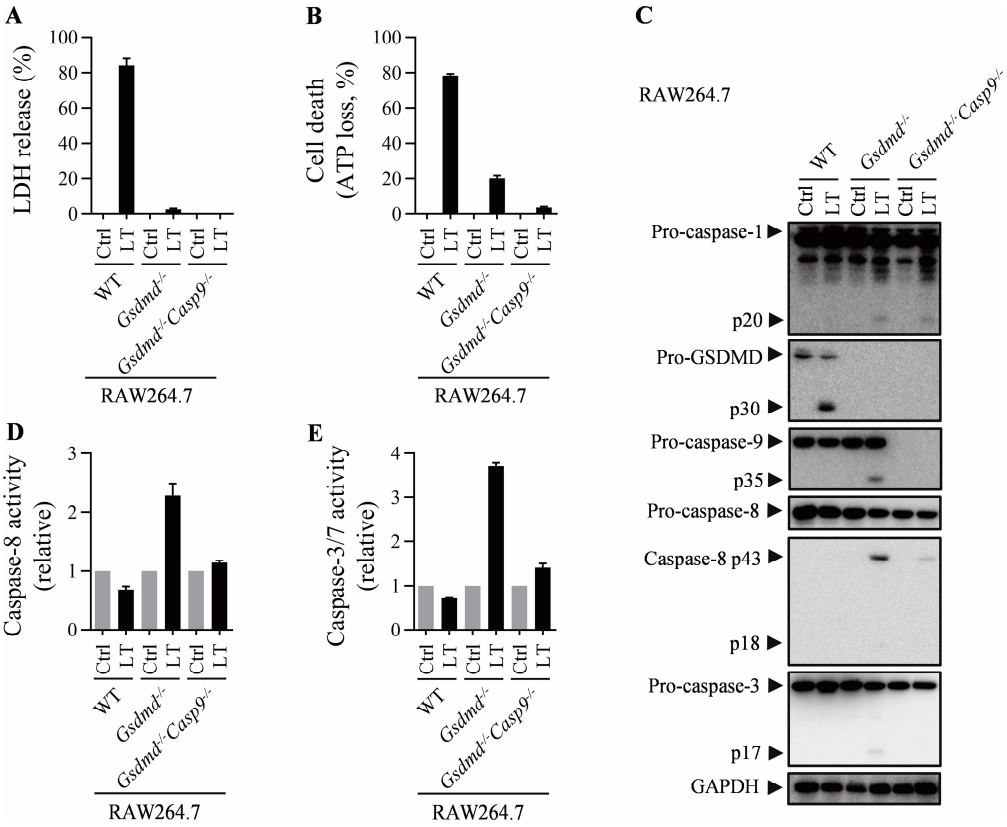

Fig. S9. LT mediated caspase-1 dependent apoptosis requires caspase-9. (**A** to **E**) RAW264.7 cells of indicated genotypes were treated with LT (2 μg/ml LF, 2 μg/ml PA) for 4 h. The LDH release (A) and ATP loss (B) were measured. Cell lysates with medium were subjected to Western blot for indicated proteins (C). The caspase-8 activity (D) and caspase-3/7 activity (E) were measured. Graphs show mean ± SE from three independent experiments.

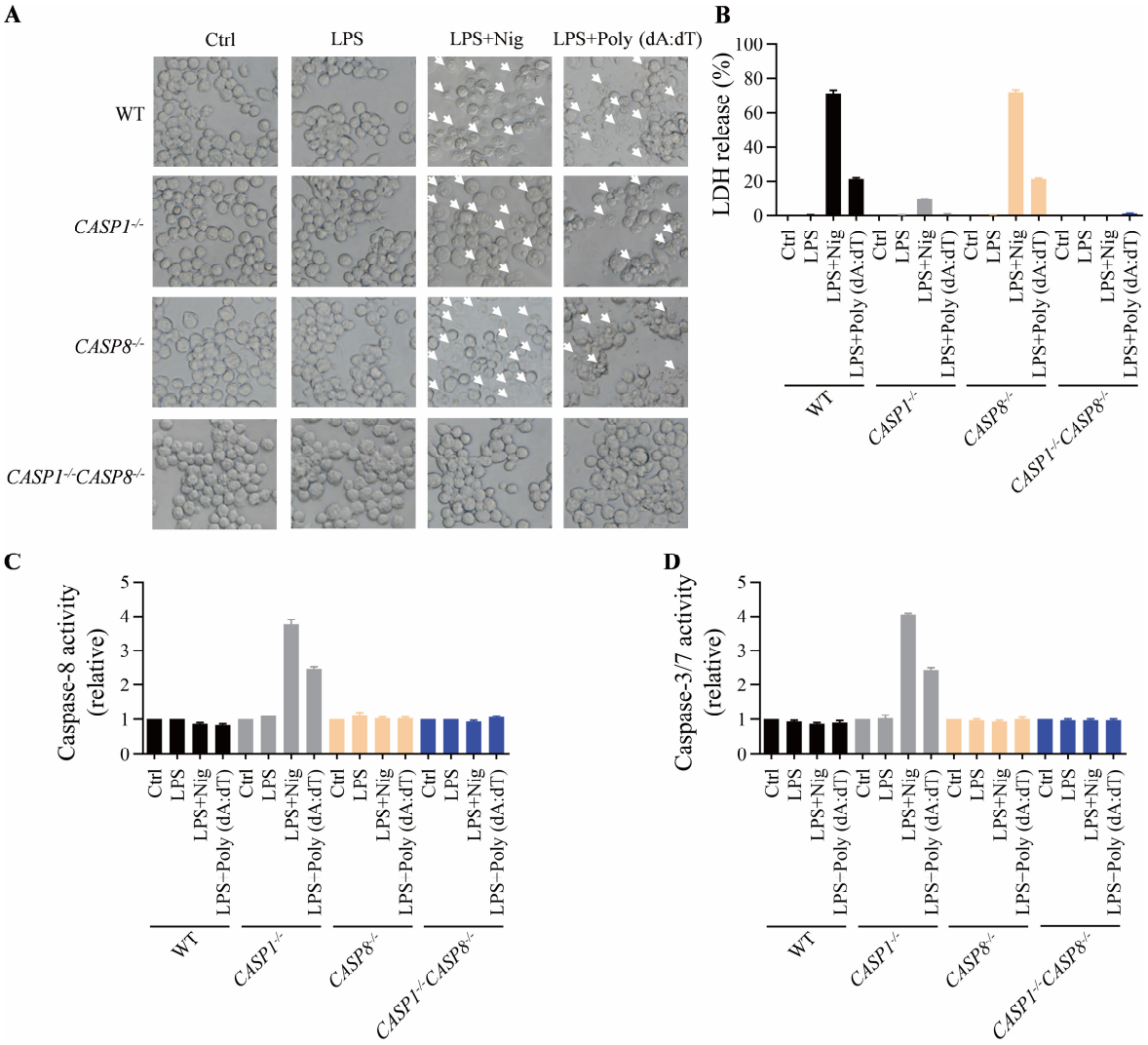

Fig. S10. Double knockout of caspase-1 and caspase-8 completely blocks cell death induced by NLRP3 and AIM2 inflammasomes in human cells. (**A** to **D**) PMA differentiated THP1 cells of indicated genotypes were treated with LPS (200 ng/ml) and then stimulated with nigericin (10 μM) for 3 h, or transfected with Poly (dA:dT) (4 μg/ml) for 6 h. Morphology of THP1 cells was recorded by microscopy. Arrows indicate pyroptotic or apoptotic cells (A). LDH release was measured at the end of the experiment (B). The caspase-8 activity (C) and caspase-3/7 activity (D) were measured. Graphs show mean ± SE from three independent experiments.