Supplementary Information

DPP8/9 inhibition induces pro-caspase-1-dependent monocyte and macrophage pyroptosis

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Supplementary Results

Supplementary Table 1. Compound IC_{50} values

Compound	Recombinant Enzyme IC_{50} (nM)					THP-1 cell viability IC_{50} (nM)			
	DPP4	DPP7	DPP8	DPP9	FAP	*GFP*_sg1	*DPP8/9* KO1	*DPP8/9* KO2	*CASP1* KO
5385 (DPP7 inhibitor)	2,450	1.4	18,032	1,271	>100,000	>60,000	>60,000	>60,000	>60,000
1G244[1,2]	>100,000	>100,000	14	53	>100,000	2,738	21,520	11,780	25,400
L-*allo*-Ile-isoindoline[3]	30,000	14,000	33	55	>100,000	2,496	>60,000	>60,000	>60,000
L-*allo*-Ile-thiazolidine[3]	460	18,000	220	320	>100,000	5,155	>60,000	>60,000	>60,000
Saxagliptin[4,5]	1.3	>33,000	508	98	>33,000	10,770	>60,000	>60,000	>60,000
Sitagliptin[4,5]	18	>33,000	33,780	55,142	>33,000	>60,000	>60,000	>60,000	>60,000
Val-boroPro[3]	<4	310	4	11	560	206	>60,000	>60,000	>60,000
Vildagliptin[4,5]	13	>33,000	5,218	258	>33,000	18,650	>60,000	>60,000	>60,000

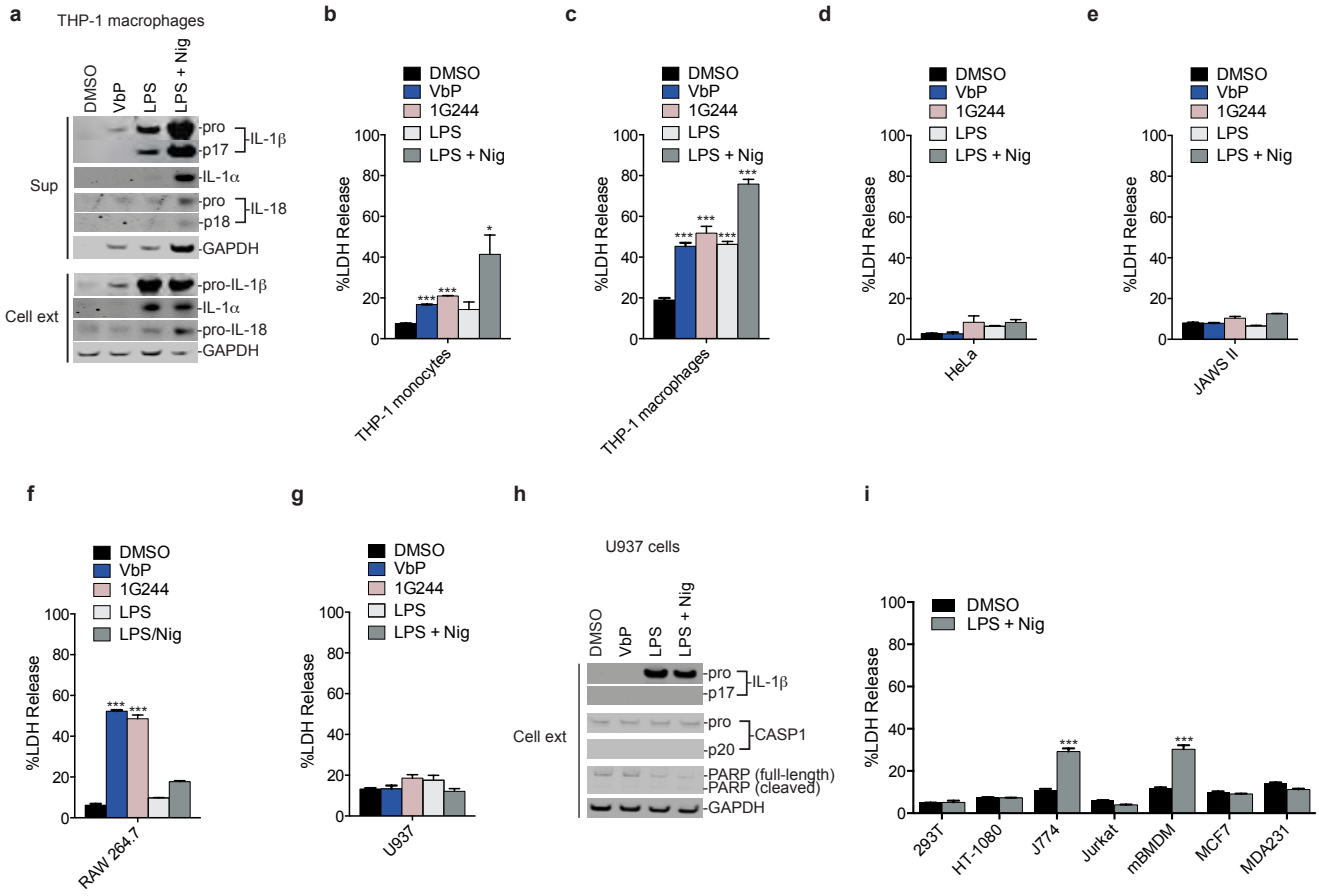
References for Supplementary Table 1:

1. Jiaang, W.T. et al. Novel isoindoline compounds for potent and selective inhibition of prolyl dipeptidase DPP8. *Bioorg. Med. Chem. Lett.* **15**, 687-91 (2005).
2. Wu, J.J. et al. Biochemistry, pharmacokinetics, and toxicology of a potent and selective DPP8/9 inhibitor. *Biochem. Pharmacol.* **78**, 203-10 (2009).
3. Lankas, G.R. et al. Dipeptidyl peptidase IV inhibition for the treatment of type 2 diabetes: potential importance of selectivity over dipeptidyl peptidases 8 and 9. *Diabetes* **54**, 2988-94 (2005).
4. Wang, A. et al. Potency, selectivity and prolonged binding of saxagliptin to DPP4: maintenance of DPP4 inhibition by saxagliptin in vitro and ex vivo when compared to a rapidly-dissociating DPP4 inhibitor. *BMC Pharmacol.* **12**, 2 (2012).
5. Bachovchin, D.A. et al. A high-throughput, multiplexed assay for superfamily-wide profiling of enzyme activity. *Nat. Chem. Biol.* **10**, 656-63 (2014).

Supplementary Table 2. sgRNA sequences used in this study

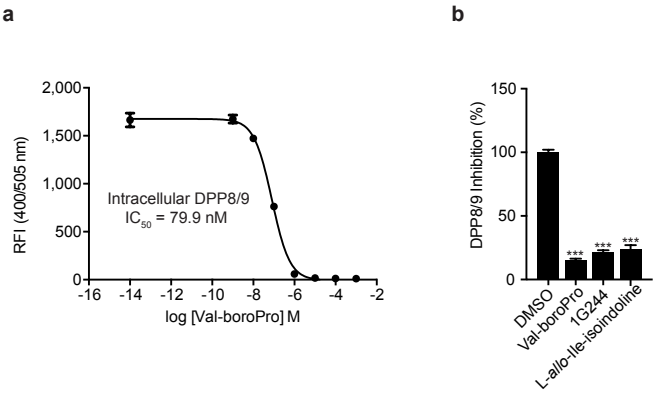
Gene	sgRNA no.	Sequence	Used in complete knockout
h*ASC*	1	CGAGGGTCACAAACGTTGAG	
h*ASC*	2	CCCTCGCGATAAGCGCAGCC	
h*CASP1*	1	CTAAACAGACAAGGTCCTGA	THP-1 *CASP1* KO 1
h*CASP1*	2	AAAGCTGTTTATCCGTTCCA	
m*Casp1*	1	TTAAACAGACAAGATCCTGA	RAW 264.7 *Casp1* KO 1
h*CASP4*	1	AGTTTGACCATCTGCCTCCG	
h*CASP4*	2	AGTTATCCAAAACACCAGTG	
h*CASP4*	3	TGCAGCTCATCCGAATATGG	
h*CASP4*	4	TATGCAAGAGAAGCAACGTA	
h*CASP5*	1	CCACATGCTAAAGAACAACG	
h*CASP5*	2	CTCTTTGCGAAAGAATCGCG	
h*DPP4*	1	CTTACCTGAAATCCATCTTA	
h*DPP4*	2	CTTAGAATACAACTACGTGA	
h*DPP7*	1	CACTTCAACTTCGAGCGCTT	
h*DPP7*	2	CGCGACGAAGGCCGAGTTGT	
h*DPP8*	1	CAGCAGTCTTAATGCTCTCT	THP-1 *DPP8/9* KO 1
h*DPP8*	2	TGGAGCCTTTTTATGTTGAG	
h*DPP8*	5	ATGATTTCATGTTTGTGAAG	THP-1 *DPP8/9* KO 2
h*DPP9*	1	CGGACTCGTATCGGTACCCC	THP-1 *DPP9* KO 2
h*DPP9*	2	GGCCAACATCGAGACAGGCG	THP-1 *DPP9* KO 1; *DPP8/9* KO 1,2
h*FAP*	1	AATGAATTTGAAGAATACCC	
h*FAP*	2	CTGTAGATGTTTCTTCTTCC	
GFP	1	GGGCGAGGAGCTGTTCACCG	
GFP	2	GAGCTGGACGGCGACGTAAA	
h*GSDMD*	1	AGGTTGACACACTTATAACG	
h*GSDMD*	2	TGAGTGTGGACCCTAACACC	THP-1 *GSDMD* KO 1, 2, and 3
h*SCPEP1*	1	GCTTGAACCTACCTGCGTTC	
h*SCPEP1*	2	TTGTCATGGAGCTGGCACTG	
h*SCPEP1*	3	CTTCCATAGGGCGGTCCAGG	

Supplementary Figure 1



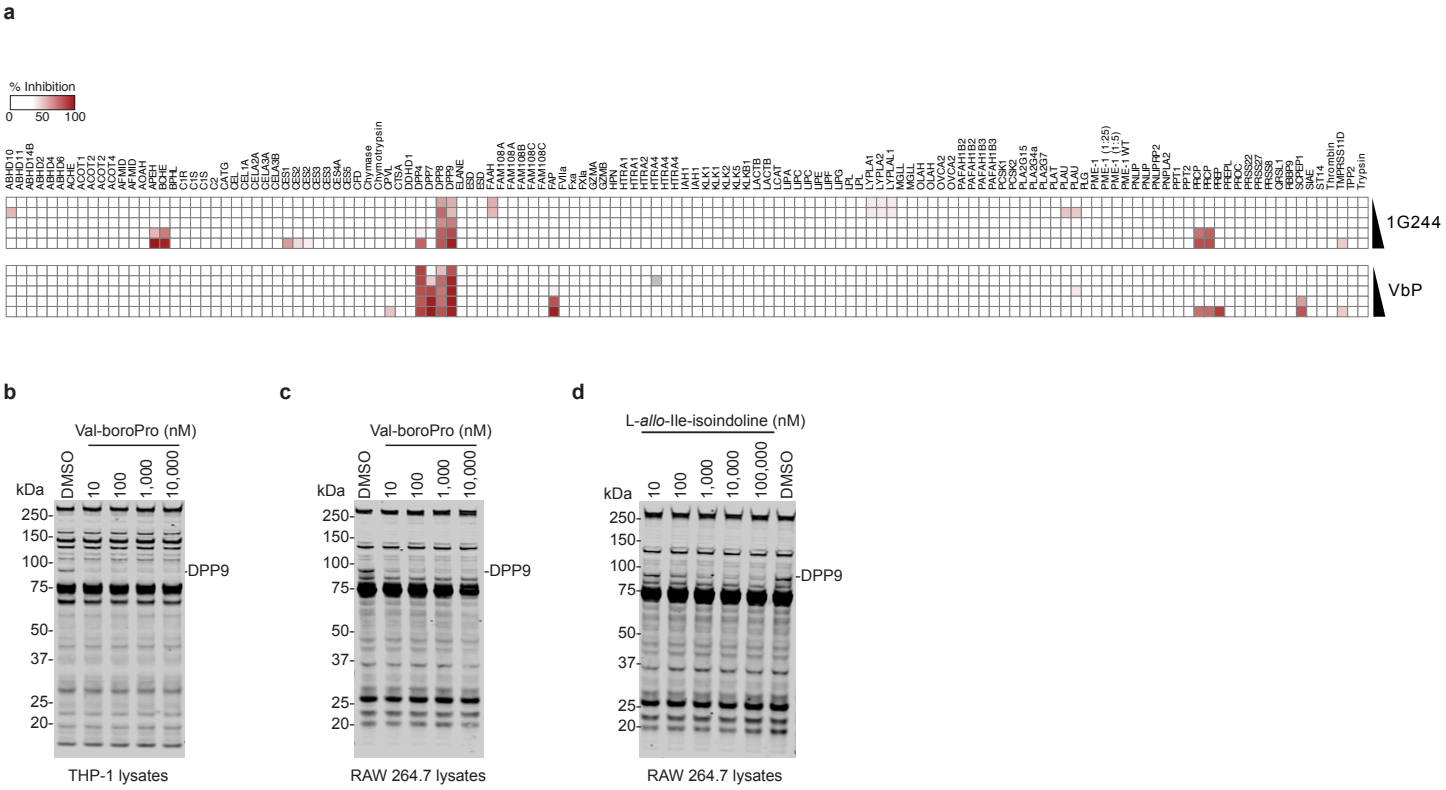
Supplementary Figure 1. Additional LDH and cytokine release results for the cell line panel. a, Immunoblots of cell extracts and supernatants from THP-1 macrophages treated with Val-boroPro (2 μM) for 24 h, or primed with LPS (10 μg/mL) for 24 h followed by stimulation with nigericin (20 μM) for 45 min. Val-boroPro treatment, unlike LPS plus nigericin treatment, releases exclusively pro-IL-18 and pro-IL-1β. Val-boroPro does not stimulate the expression of IL-1α or IL-18. **b-g**, Undifferentiated THP-1 monocytes (**b**), PMA-differentiated THP-1 macrophages (**c**), HeLa (**d**), JAWS II (**e**), RAW 264.7 (**f**), and U937 cells (**g**) were treated with Val-boroPro (2 μM) or 1G244 (10 μM) for 24 h, or primed with LPS (10 μg/mL) for 24 h followed by stimulation with nigericin (20 μM) for 45 min. Only THP-1 and RAW 264.7 cells released LDH after Val-boroPro and 1G244 addition. RAW 264.7 cells, unlike THP-1 cells, are deficient in ASC and therefore do not release high levels of LDH in response to LPS plus nigericin. **h**, Monocytic U937 cells were unexpectedly unresponsive to LPS plus nigericin. Extracts from treated U937 cells were therefore analyzed by immunoblotting. Even though LPS induced expression of IL-1β in U937 cells, LPS plus nigericin did not induce IL-1β cleavage or caspase-1 cleavage, indicating that the NLRP3 inflammasome was not activated. This data is consistent with the data in (**g**) that these cells do not undergo pyroptosis in response to LPS plus nigericin. **i**, Additional cell lines were treated with LPS plus nigericin and analyzed for LDH release. Only the monocytic cell line J774 and mouse bone marrow derived macrophages (mBMDM) responded, as expected. In **b-g** and **i**, data are means ± SEM of three biological replicates. *$p < 0.05$, ***$p < 0.001$ by two-sided Student's *t*-test for vehicle versus treated cells. Full gel images for **a** and **h** are shown in **Supplementary Figure 12**.

Supplementary Figure 2



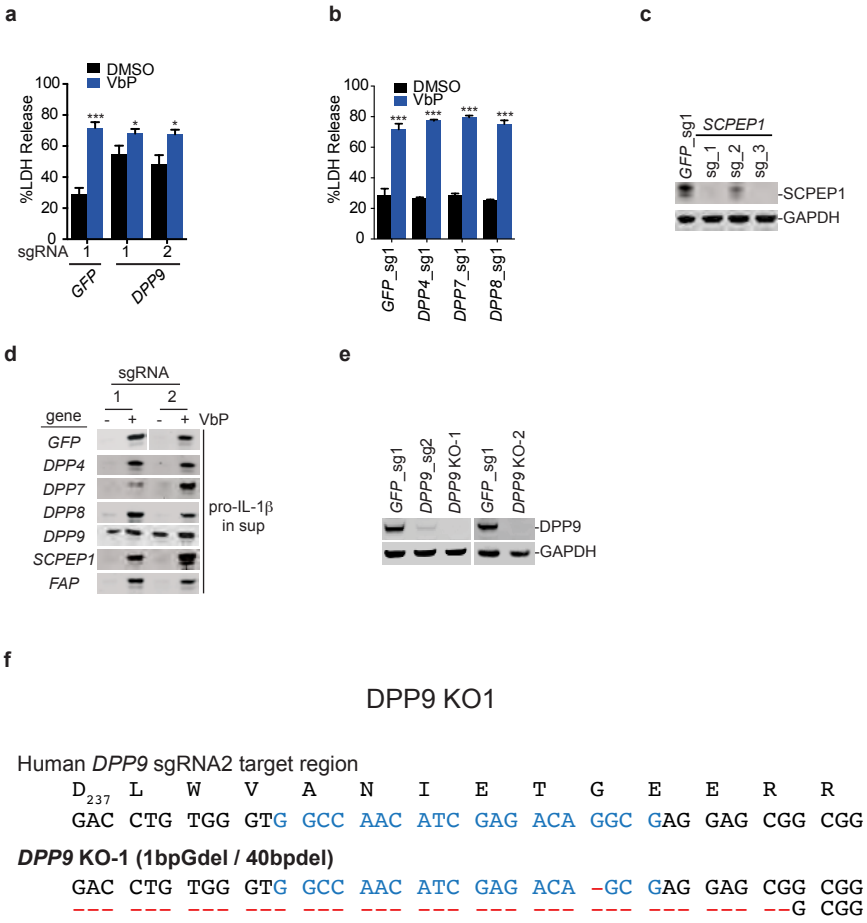
Supplementary Figure 2. Val-boroPro is cell penetrant. a, Val-boroPro inhibits intracellular DPP8/9 in HEK 293T cells with an IC_{50} = 79.9 nM. **b,** Val-boroPro (2 μM), 1G244 (10 μM), and L-*allo*-Ile-isoindoline (20 μM) inhibit intracellular DPP8/9 in THP-1 cells, confirming these compounds enter THP-1 cells. Data are means ± SEM of three biological replicates. ***p < 0.001 by two-sided Student's *t*-test for DMSO versus compound-treated cells.

Supplementary Figure 3



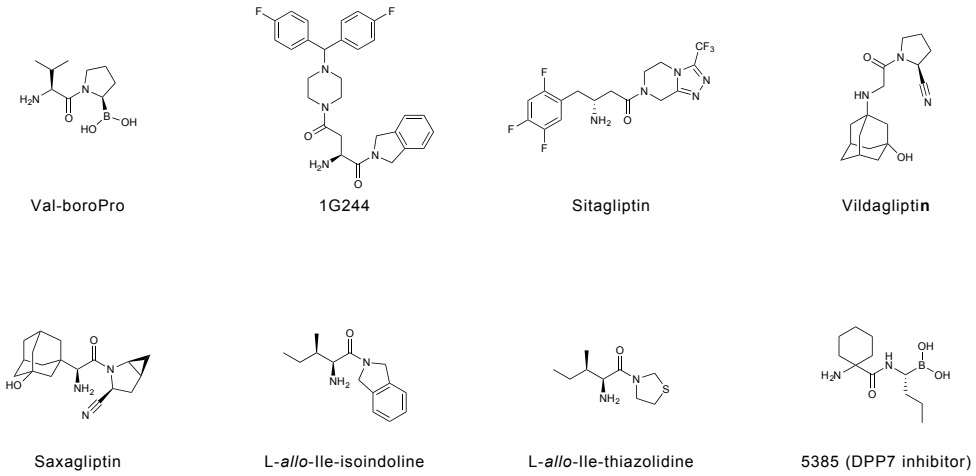
Supplementary Figure 3. Evaluation of DPP8/9 inhibitors by competitive ABPP. **a**, EnPlex serine hydrolase profiling (100 μM top dose, 10-fold dilution series) reveals that Val-boroPro and 1G244 both inhibit DPP8/9, but have otherwise dissimilar selectivity profiles across the serine hydrolases. In particular, 1G244 has little activity against DPP4 and DPP7. **b-d,** Competitive gel-based ABPP of Val-boroPro with the FP-biotin probe in THP-1 lysates (**b**) and RAW 264.7 lysates (**c**), and L-*allo*-Ile-isoindoline in RAW264.7 lysates (**d**). Only inhibition of DPP9 was observed by this method.

Supplementary Figure 4



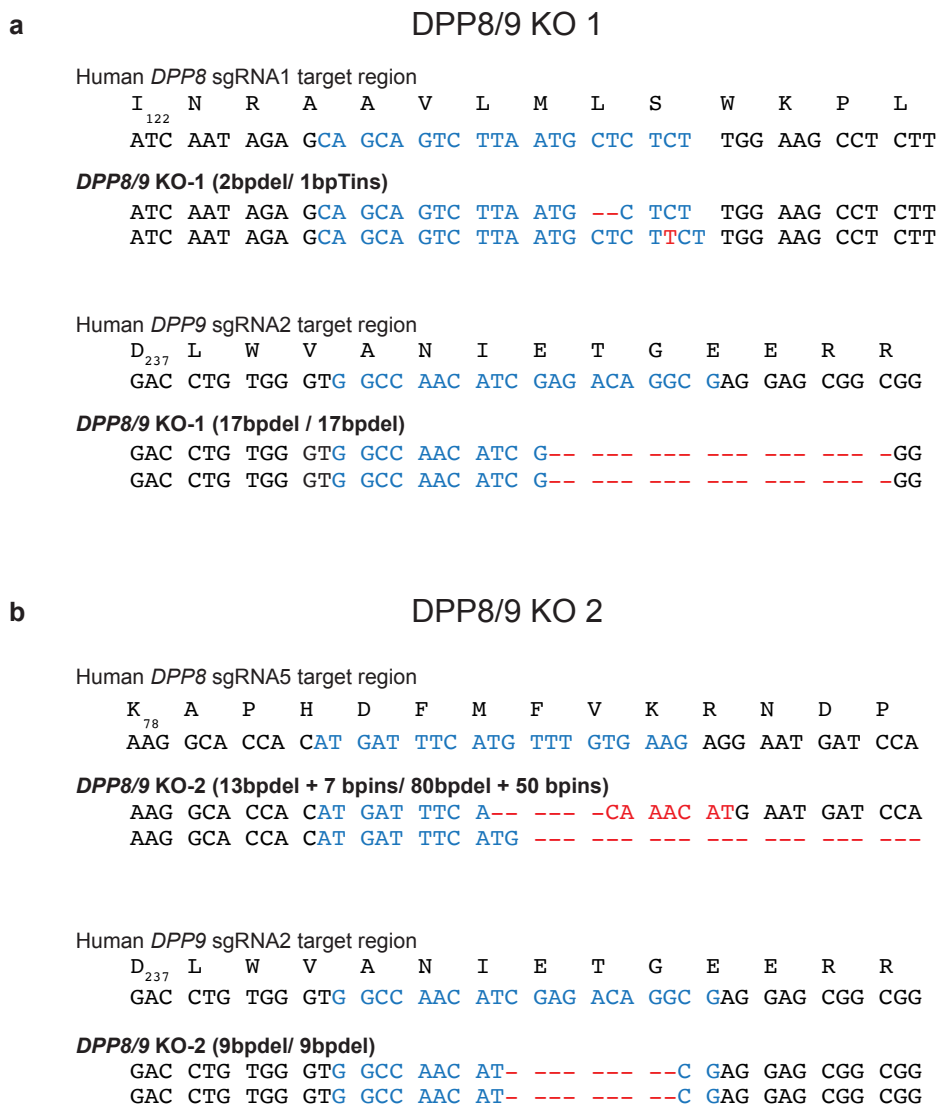
Supplementary Figure 4. Cells treated with sgRNAs to *DPP9* release a small amount of additional LDH after Val-boroPro treatment. a, THP-1 macrophages selected with sgRNAs to *GFP* or *DPP9* were treated with Val-boroPro (2 μM, 24 h) and analyzed for LDH release. Immunoblots of these cells are shown in **Figure 2b**. **b**, THP-1 macrophages infected with sgRNAs to *DPP4, DPP7,* and *DPP8* were treated with Val-boroPro (2 μM, 24 h) and analyzed for LDH release. All of these cell lines behaved similarly to controls. In **a** and **b**, data are means ± SEM of three biological replicates. * $p < 0.05$, ***$p < 0.001$ by two-sided Student's *t*-test for DMSO versus Val-boroPro-treated cells. **c,** Val-boroPro also inhibits FAP and SCPEP1 at 10 μM, as determined by EnPlex. We therefore treated THP-1 cells with sgRNAs to *FAP* and *SCPEP1* as well, and knockout of *SCPEP1* was confirmed by immunoblotting. We were unable to detect FAP protein by Western blotting in THP-1 cells. **d,** THP-1 macrophages treated with the indicated sgRNAs were treated with DMSO or Val-boroPro (2 μM, 24 h), and supernatants were analyzed for levels of pro-IL-1β by immunoblotting. Only cells treated with sgRNAs to *DPP9* secreted higher levels pro-IL-1β into the supernatant before treatment with Val-boroPro, consistent with the LDH results. Moreover, these cells secreted slightly more pro-IL-1β after Val-boroPro treatment, again consistent with the LDH results. **e**, THP-1 cells treated with sgRNAs to *DPP9* were single cell cloned to isolate complete *DPP9* knockouts. Knockout of *DPP9* was assessed by immunoblotting. **f**, DPP9 KO-1 was confirmed by DNA sequencing. Full gel images for **c-e** are shown in **Supplementary Figure 12**.

Supplementary Figure 5



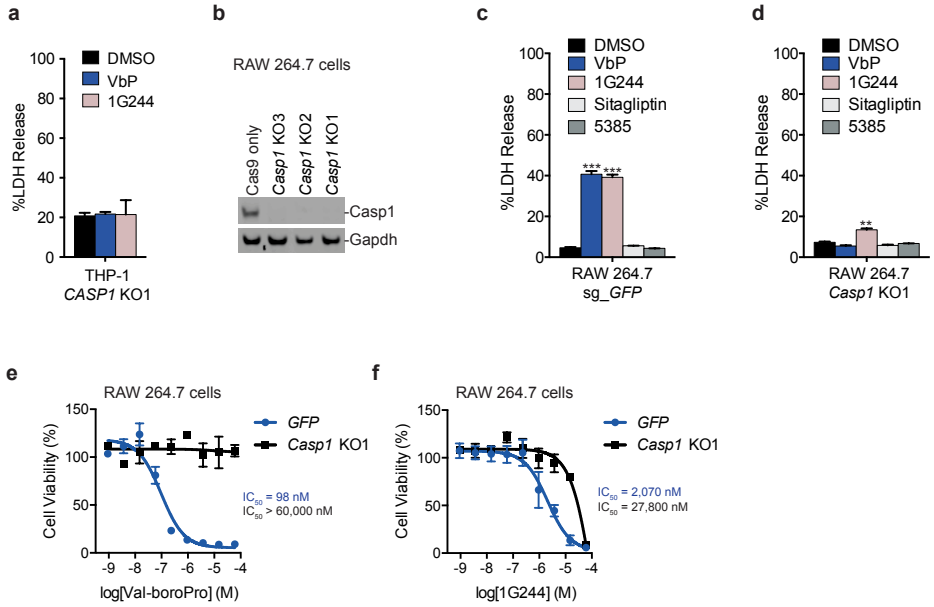
Supplementary Figure 5. Compounds used in this study. IC_{50} values against recombinant enzymes and against THP-1 cells are shown in **Supplementary Table 1**.

Supplementary Figure 6



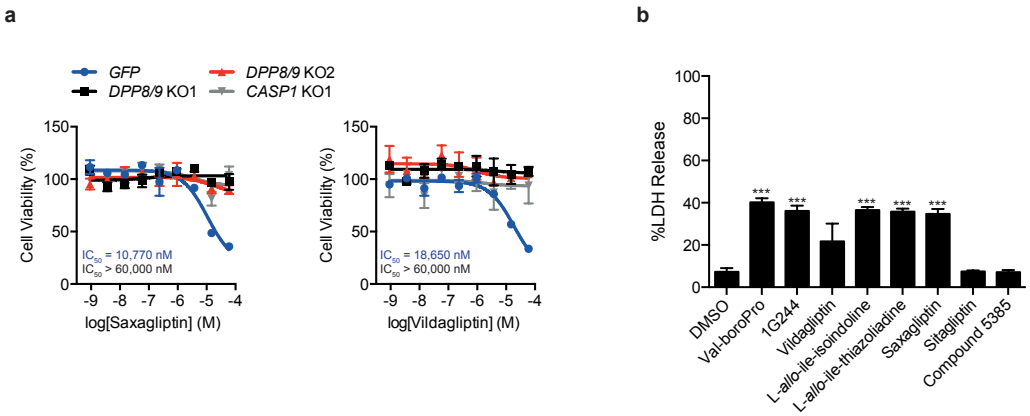
Supplementary Figure 6. *DPP8* and *DPP9* alleles in double knockout cell lines. a,**b**, *DPP8* and *DPP9* alleles were analyzed by sequencing to confirm THP-1 *DPP8/9* KO1 (**a**) and *DPP8/9* KO2 (**b**).

Supplementary Figure 7



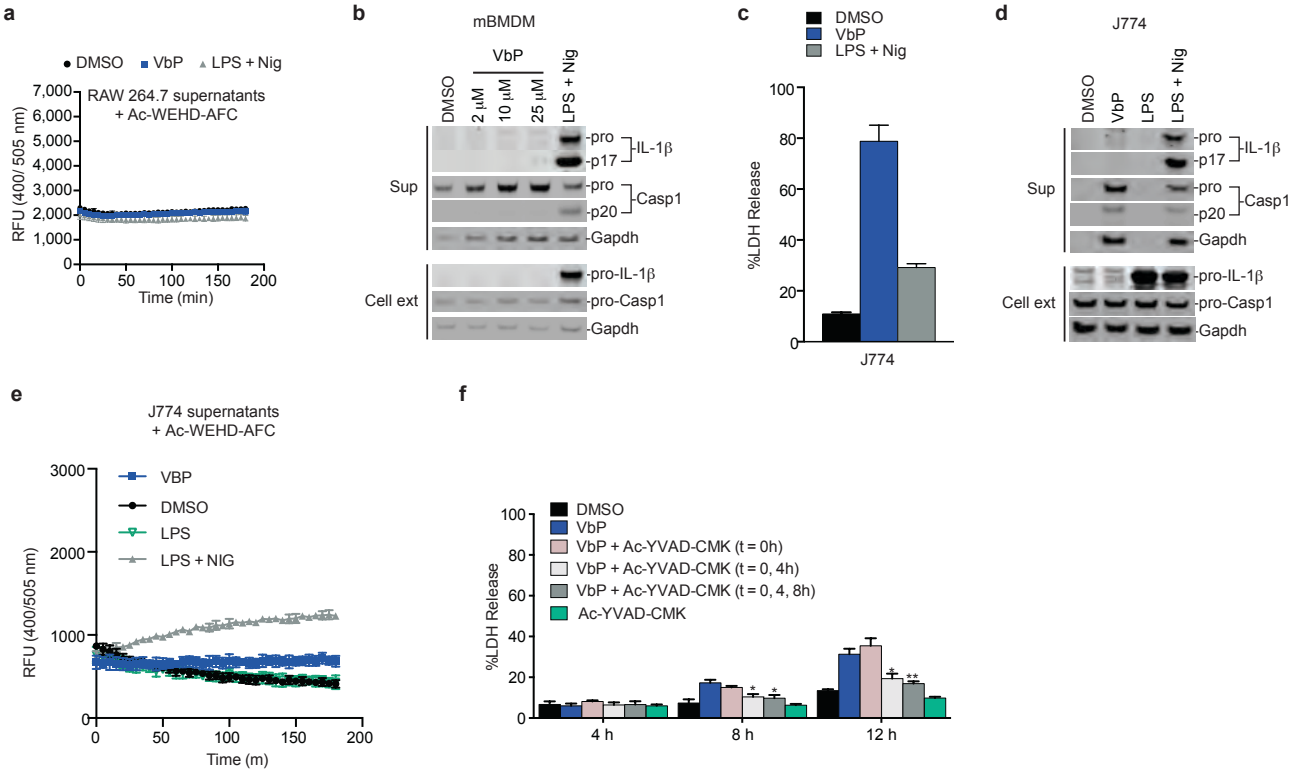
Supplementary Figure 7. Val-boroPro is not cytotoxic to caspase-1 knockout cells. a, Caspase-1 knockout THP-1 macrophages do not release LDH after treatment with Val-boroPro (2 μM) or 1G244 (10 μM) for 24 h. **b**, Confirmation of caspase-1 knockout in RAW 264.7 cells by immunoblotting. Full gel images are shown in **Supplementary Figure 12**. **c**,**d**, LDH release induced by treatment with Val-boroPro (2 μM), 1G244 (10 μM), sitagliptin (20 μM), and the DPP7 inhibitor 5385 (20 μM) for 24 h in control (**c**) and caspase-1 knockout (**d**) RAW 264.7 cells. Caspase-1 knockout cells undergo some cell death after 1G244 treatment, indicating some engagement of the 1G244 off-target. Sitagliptin and 5385 do not release LDH in either of these lines. **e**,**f**, Cell viability of the GFP control and caspase-1 knockout RAW 264.7 cells after treatment with Val-boroPro (**e**) or 1G244 (**f**) for 24 h as determined by CellTiter-Glo. Caspase-1 knockout RAW 264.7 cells are completely resistant to Val-boroPro, but are killed by 1G244 at doses ≥ 10 μM. Data are means ± SEM of three biological replicates. ** $p < 0.01$, ***$p < 0.001$ by two-sided Student's *t*-test for DMSO versus compound-treated cells.

Supplementary Figure 8



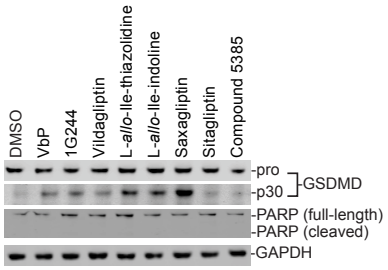
Supplementary Figure 8. Profiling additional inhibitors for pyroptosis induction. a, Cell viability of THP-1 monocytes after treatment with saxagliptin or vildagliptin for 48 h relative to DMSO as determined by CellTiter-Glo. The IC_{50} values for control THP-1 cell lines (treated with an sgRNA to GFP, values in blue) and for *DPP8/9* KO1 THP-1 (values in black) cells are shown. **b**, LDH release by the indicated compounds from THP-1 monocytes after 48 h. All compounds were used at 20 μM, except Val-boroPro (2 μM) and 1G244 (10 μM). In **a** and **b**, data are means ± SEM of three biological replicates. ***$p < 0.001$ by two-sided Student's *t*-test for DMSO versus compound-treated cells.

Supplementary Figure 9



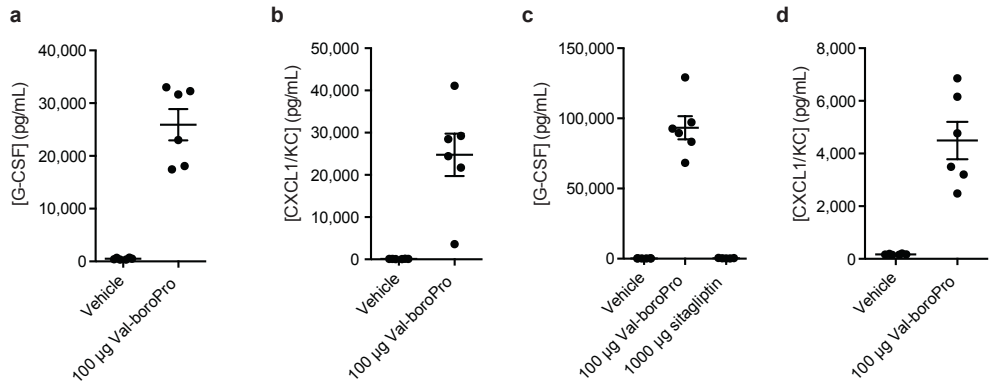
Supplementary Figure 9. Val-boroPro releases pro-caspase-1 from macrophages. a, Cleavage of Ac-WEHD-AFC (50 μM) was monitored in supernatants from treated RAW 264.7 macrophages. Data are means ± SEM of four independent experiments. **b,** Immunoblots of cell extracts and supernatants from mBMDMs treated with Val-boroPro or LPS plus nigericin. mBMDMs secrete exclusively pro-caspase-1 after Val-boroPro treatment. These cells do not express detectable IL-1β endogenously or after Val-boroPro treatment, and therefore no IL-1β is observed in the cell extract or supernatant in DMSO or Val-boroPro-treated samples. In striking contrast, LPS strongly stimulates the expression of IL-1β, and LPS plus nigericin induces the release of both cleaved IL-1β and cleaved caspase-1. **c**, J774 cells undergo significantly more LDH release after Val-boroPro treatment than LPS plus nigericin treatment. Data are means ± SEM of three biological replicates. **d**, Immunoblots of cell extracts and supernatants from treated J774 cells. Like mBMDMs, J774 cells do not express detectable IL-1β endogenously or after Val-boroPro treatment, and therefore no IL-1β is observed in the cell extract or supernatant in DMSO or Val-boroPro-treated samples. LPS plus nigericin stimulates the expression of IL-1β and induces the release of both cleaved IL-1β and cleaved caspase-1. Unlike the other cell lines tested, we were able to detect a small amount of what appeared to be cleaved caspase-1 in the supernatant after Val-boroPro treatment, although the vast majority of caspase-1 remained in the pro form. **e**, Supernatants from Val-boroPro-treated J774 cells have detectable, albeit very low, Ac-WEHD-AFC cleavage activity. Supernatants of J774 cells treated with LPS plus nigericin, which underwent significantly less pyroptosis than Val-boroPro treated cells, have a much greater amount of Ac-WEHD-AFC cleavage activity. This indicates that, even though we appeared to detect cleaved caspase-1, very little active mature active caspase-1 is produced in Val-boroPro-treated J774 cells. Data are means ± SEM of three biological replicates. **f**, Re-administration of Ac-YVAD-CMK after 4 and 8 h extended the blockade of Val-boroPro-induced LDH release from RAW 264.7 cells. Data are means ± SEM of three biological replicates. * $p < 0.05$, ** $p < 0.01$ by two-sided Student's *t*-test for these cells versus those treated once with Ac-YVAD-CMK. Full images for **b** and **d** are in **Supplementary Figure 12**.

Supplementary Figure 10



Supplementary Figure 10. Profiling inhibitors for effects on GSDMD and PARP cleavage. Immunoblots of cell extracts from THP-1 monocytes treated with the indicated compounds for 48 h. All compounds were used at 20 μM, except Val-boroPro (2 μM) and 1G244 (10 μM). We observed cleavage of GSDMD, but not PARP, in extracts from cells that released LDH in **Supplementary Figure 8b**, consistent with pyroptosis and not apoptosis. Full gel images are shown in **Supplementary Figure 12**.

Supplementary Figure 11



Supplementary Figure 11. Val-boroPro, but not sitagliptin, induces cytokines in mice. a,**b** Val-boroPro (100 μg/mouse) induces higher levels of serum G-CSF (**a**) and CXCL1/KC (**b**) after 6 h in wild-type C57BL/6 mice as measured by ELISA. Data are means ± SEM; n = 6 mice/group. $p < 0.001$ for G-CSF and $p < 0.00001$ for CXCL1/KC by two-sided Student's *t*-test for Val-boroPro versus control-treated mice. **c**,**d** Similarly, Val-boroPro (100 μg/mouse) induces higher levels of serum G-CSF after 6 h (**c**) and CXCL1/KC after 2 h (**d**) in wild-type BALB/c mice. Sitagliptin (1000 μg/mouse) has no effect on serum G-CSF (**c**). Data are means ± SEM; n = 6 mice/group. $p < 0.00001$ for G-CSF and $p < 0.001$ for CXCL1/KC by two-sided Student's *t*-test for Val-boroPro versus control-treated mice.

Supplementary Figure 12

Figure 1b

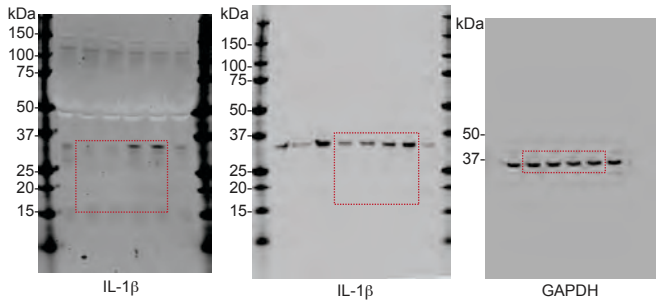


Figure 2b

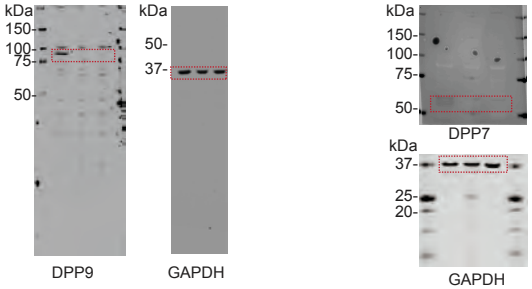


Figure 2c,e,g

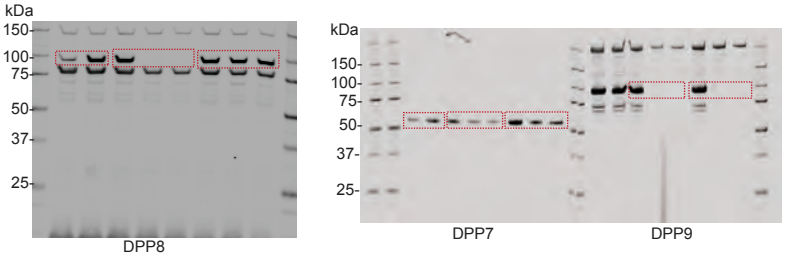


Figure 2c

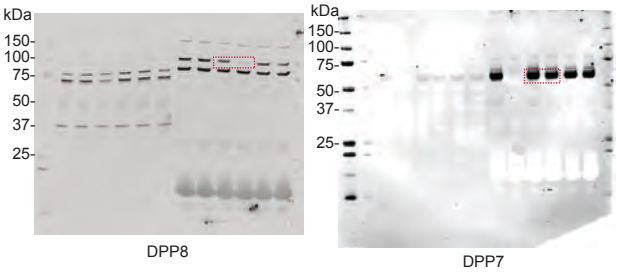


Figure 3a

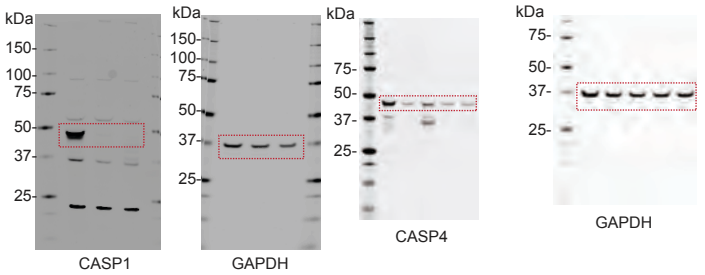


Figure 4a

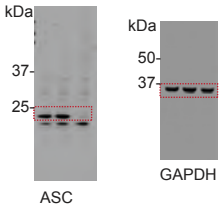


Figure 4c

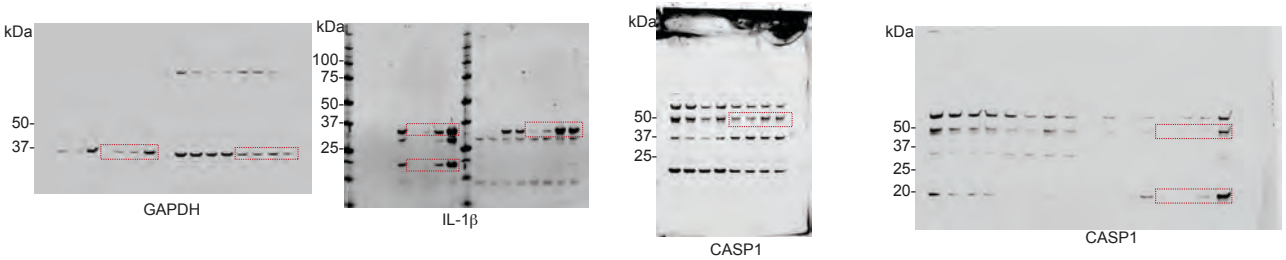


Figure 4d

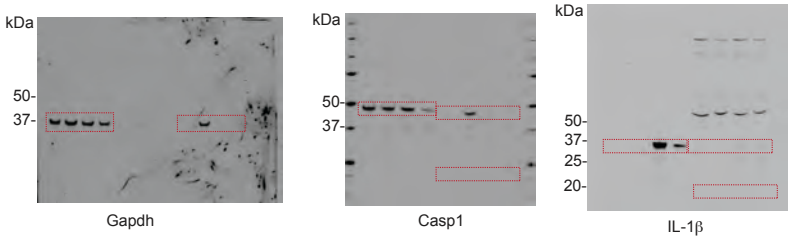


Figure 5a

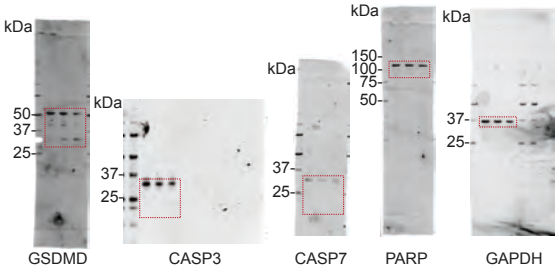


Figure 5b

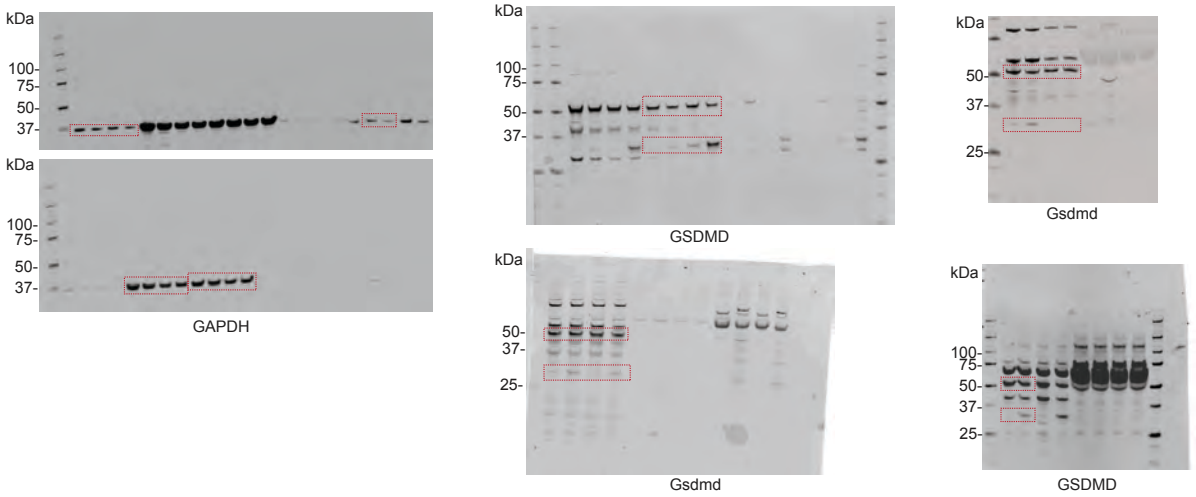
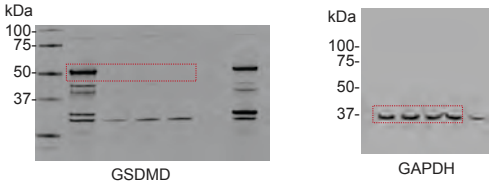
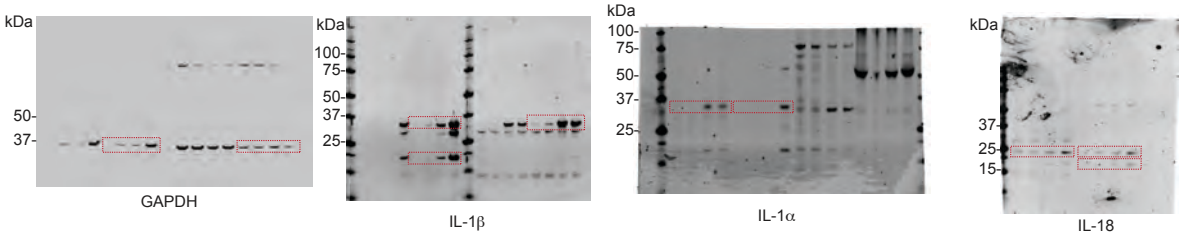


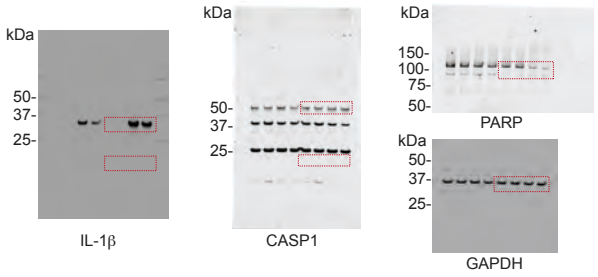
Figure 5c



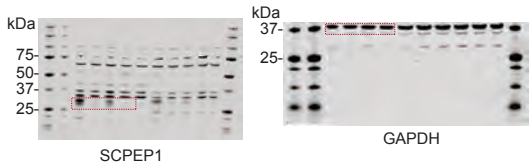
Supplementary Figure 1a



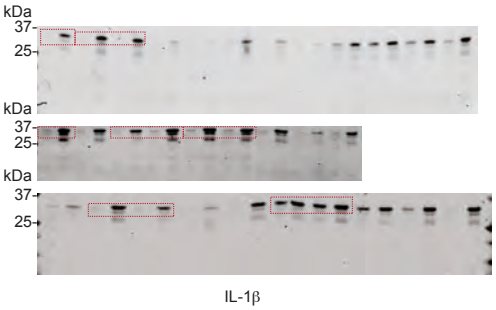
Supplementary Figure 1h



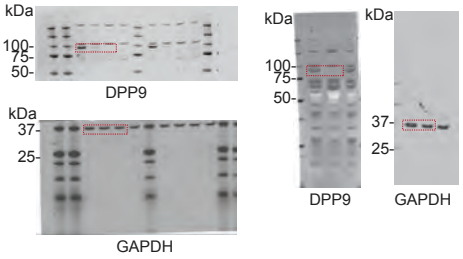
Supplementary Figure 4c



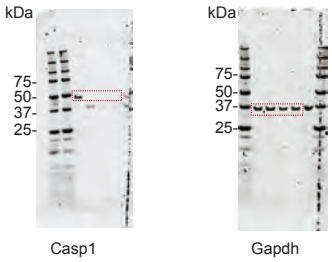
Supplementary Figure 4d



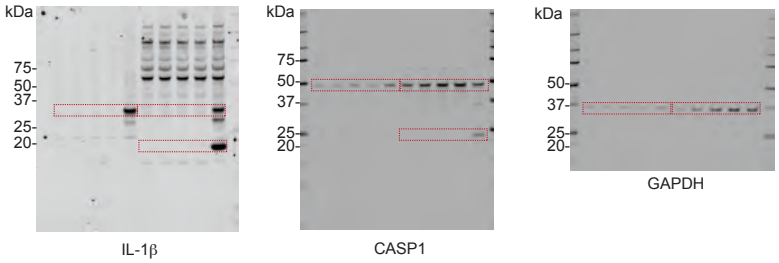
Supplementary Figure 4e



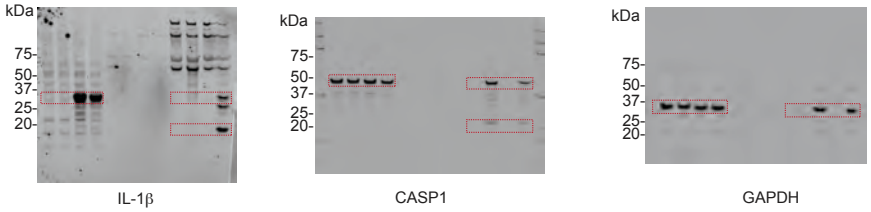
Supplementary Figure 7b



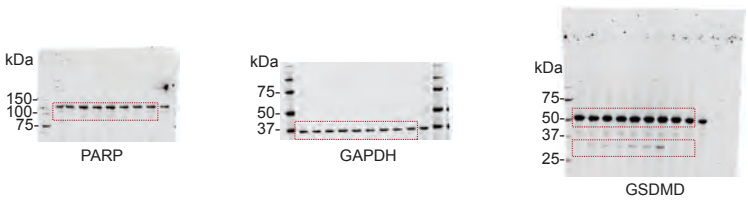
Supplementary Figure 9b



Supplementary Figure 9d



Supplementary Figure 10



Supplementary Figure 12. Full gel images for those cropped in the paper figures. The specific bands shown in the figures are highlighted by red boxes.