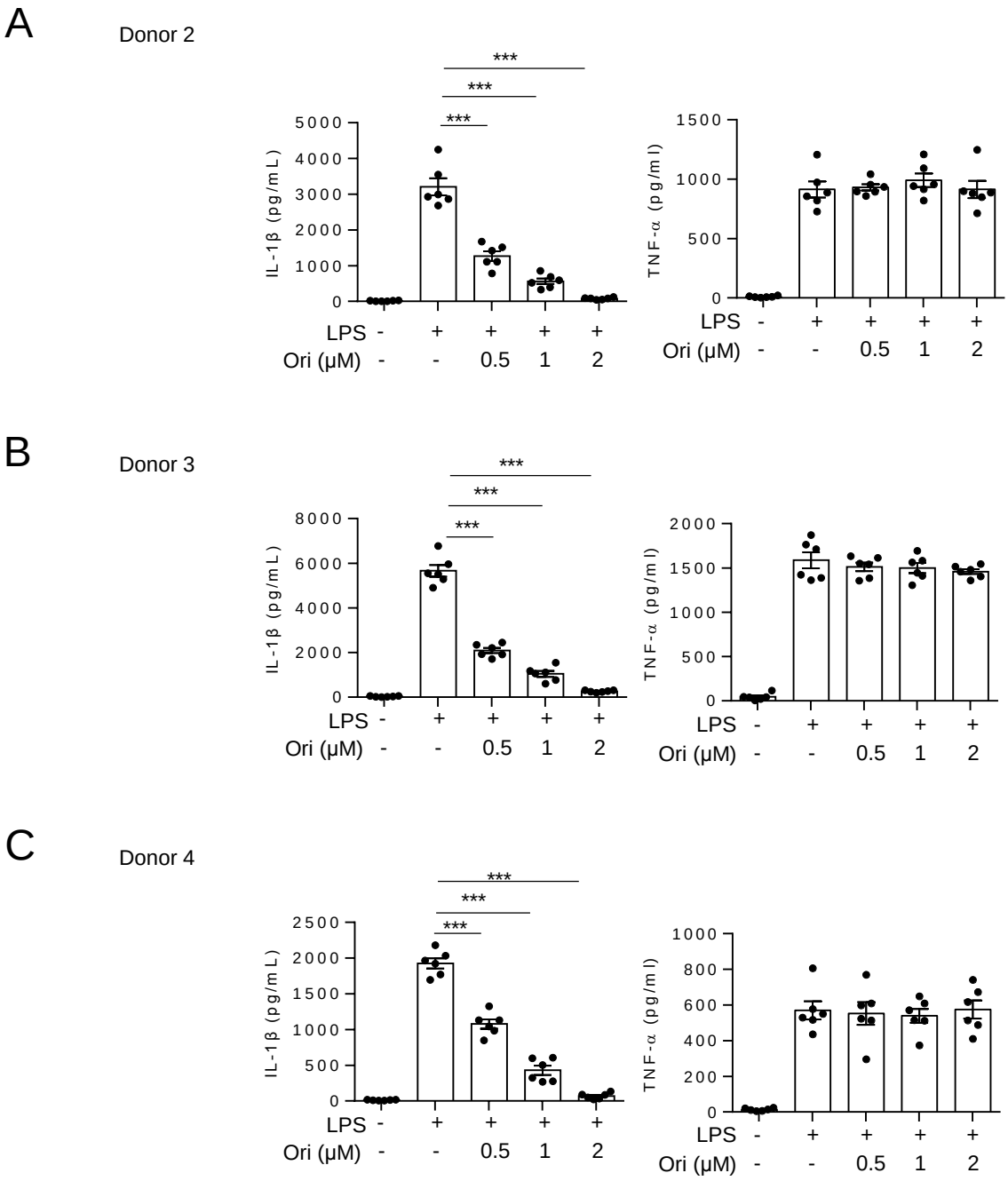
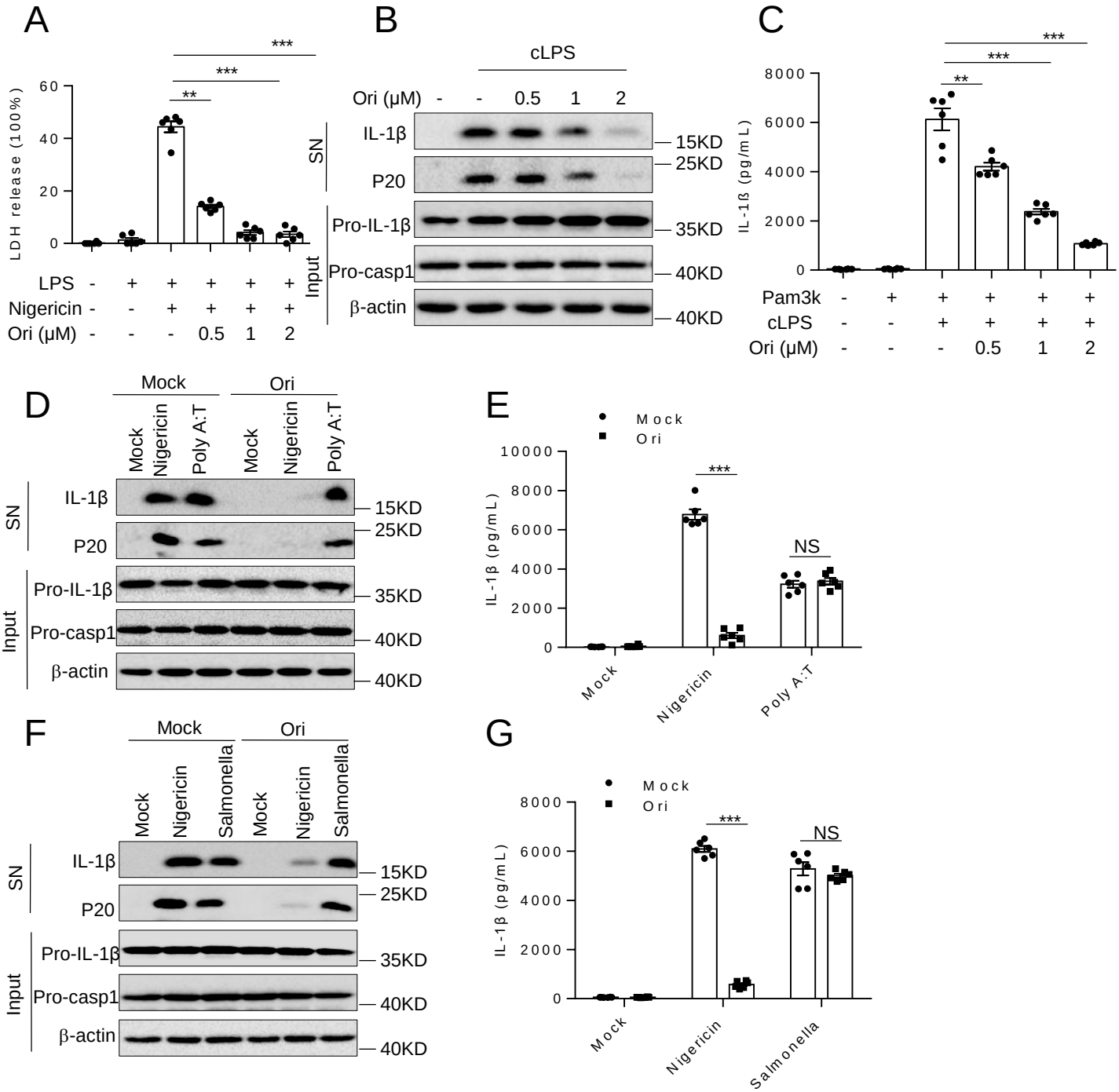
Supplementary Information

Oridonin is a covalent NLRP3 inhibitor with strong anti-inflammasome activity

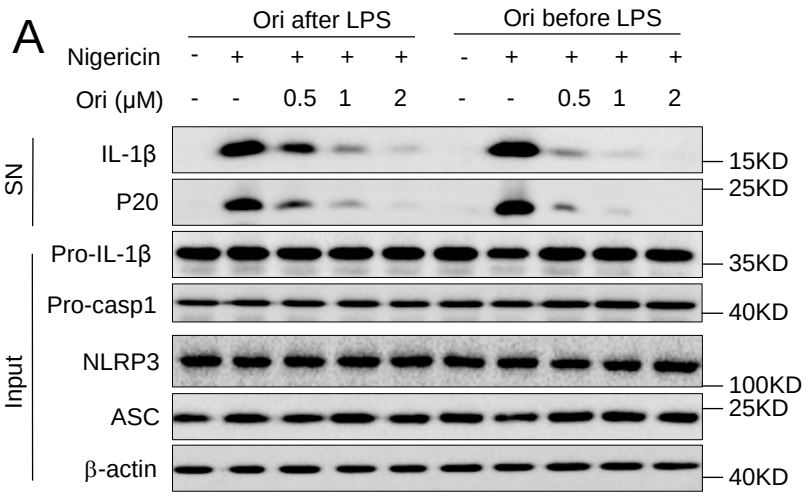
He et al.

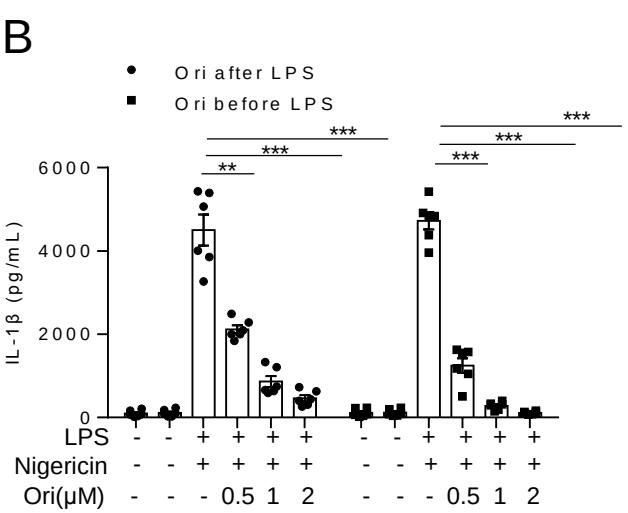


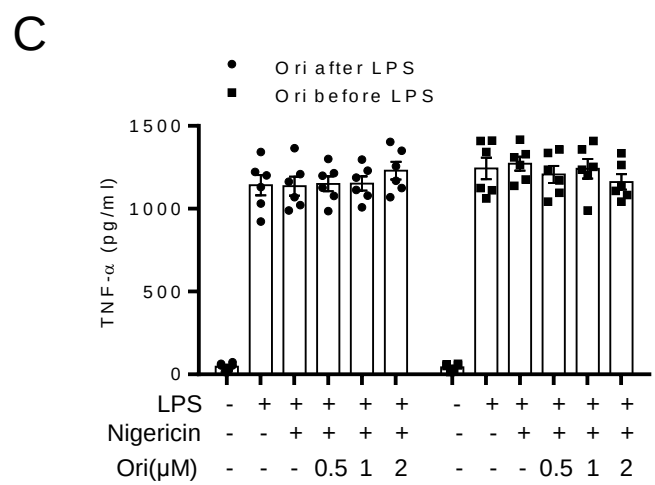
Supplementary Figure. 1. Ori inhibits NLRP3 inflammasome activation in human PBMCs.(A–C) ELISA of IL-1β, TNF-α in supernatants (SN) from PBMCs isolated from three healthy donors, treated with various doses of Ori for 30 min and then stimulated with LPS for 16 hours. Data are from biological triplicates in each (mean and s.e.m of n = 6). Statistics were analyzed using an unpaired Student's t test: ***, $P < 0.001$.



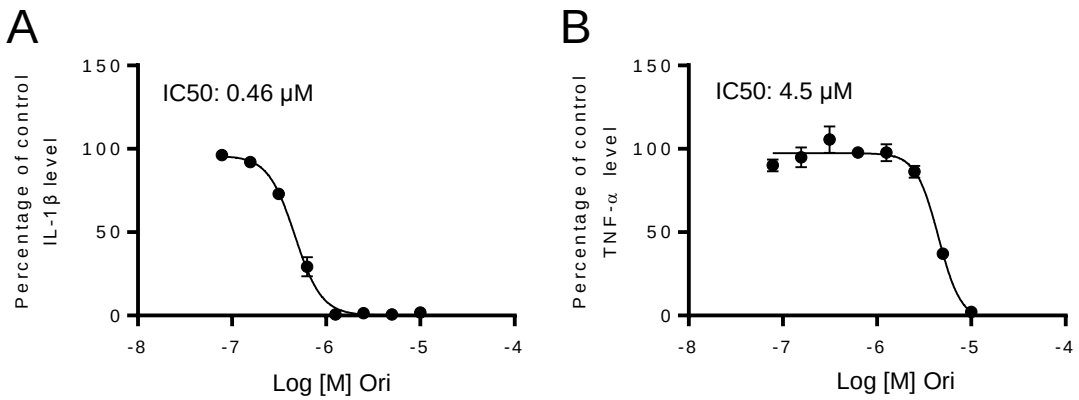
Supplementary Figure. 2. Ori specifically inhibits NLRP3 inflammasome activation. (A) Assay for LDH release in the culture supernatants of LPS-primed BMDMs treated with different doses of Ori and then left stimulated with nigericin. (B, C) Immunoblot analysis (B) of IL-1β and cleaved caspase-1 (p20) or ELISA (C) of IL-1β in culture supernatants of Pam3-primed BMDMs treated with various doses of Ori for 30 min and then stimulated with cLPS. (D, E) Immunoblot analysis (D) of IL-1β and cleaved caspase-1 (p20) or ELISA (E) of IL-1β in culture supernatants of LPS-primed BMDMs treated with of Ori (2 μM) and then stimulated with nigericin and poly A:T. (F, G) Immunoblot analysis (F) of IL-1β and cleaved caspase-1 (p20) or ELISA (G) of IL-1β in culture supernatants of LPS-primed BMDMs treated with of Ori (2 μM) and then stimulated with nigericin and salmonella. Data are from three independent experiments with biological duplicates in each (**A, C, E, G**; mean and s.e.m of $n = 6$) or are representative of three independent experiments (**B, D, F**). Statistics were analyzed using an unpaired Student's t test: **P<0.01, ***P < 0.001, NS, not significant.



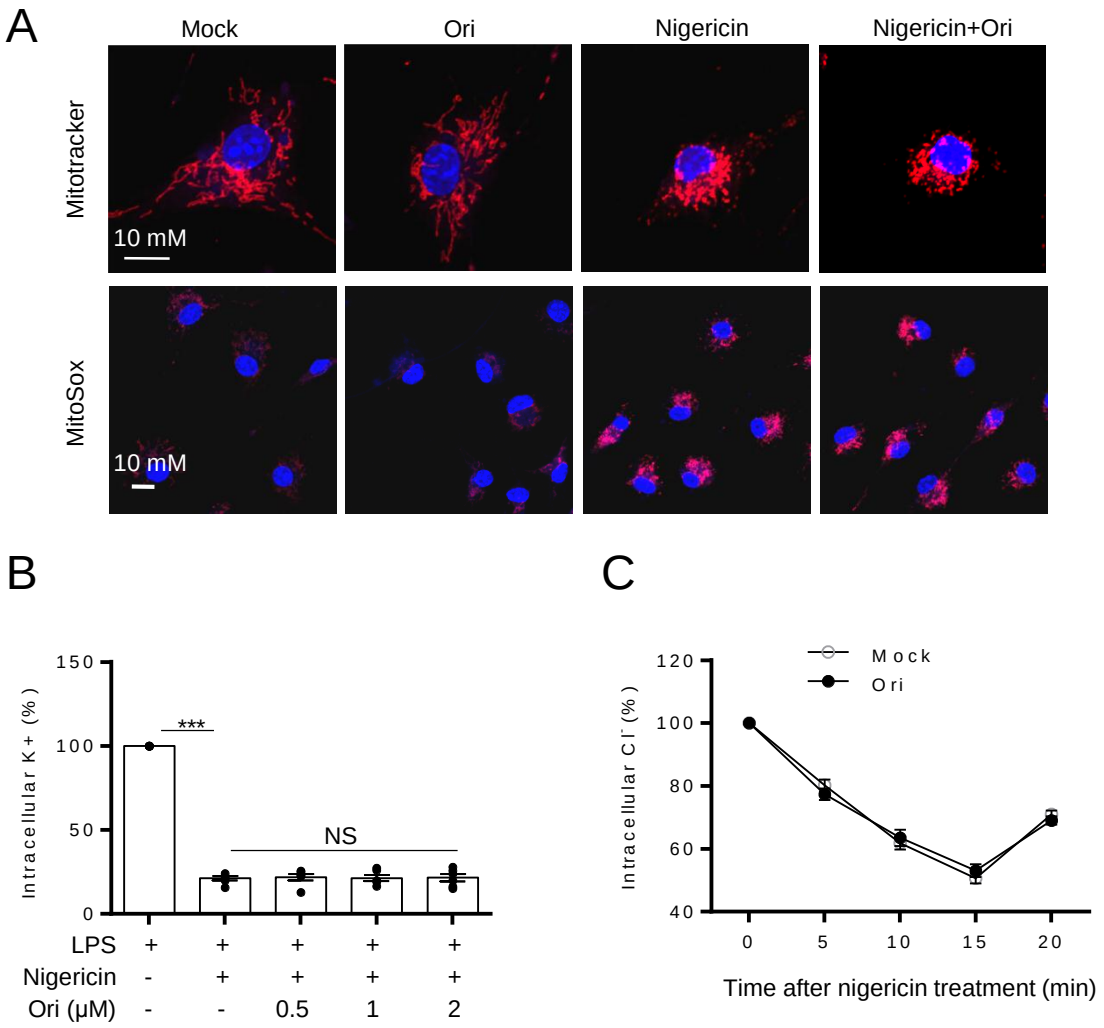




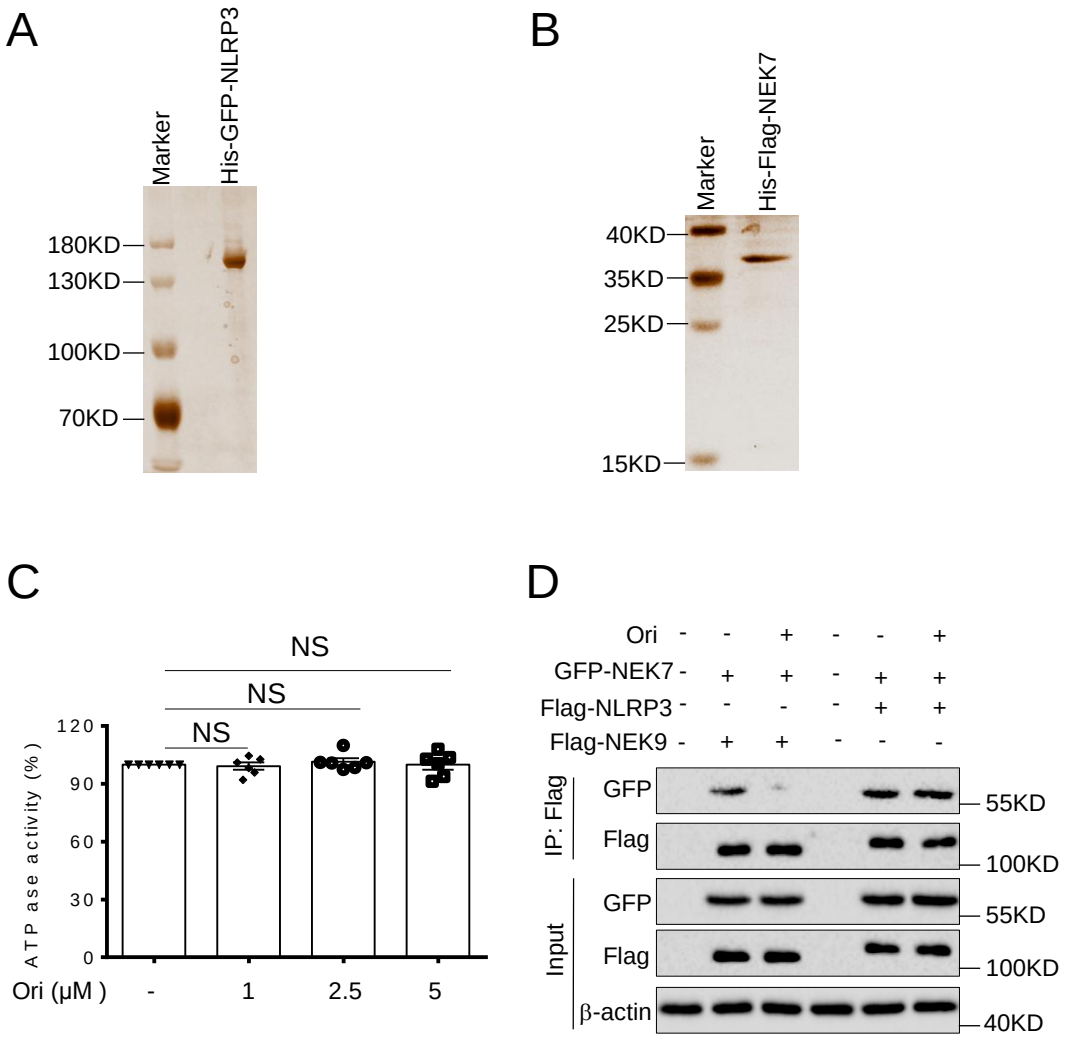
Supplementary Figure. 3. Role of Ori in NLRP3 inflammasome activation and LPS-induced priming. (A) BMDMs were treated with LPS for 3 hours and left stimulated with different doses of Ori for 30 min (Ori after LPS), or BMDMs were treated with different doses of Ori for 30 min and then stimulated with LPS for 3 h (Ori before LPS). After that, the cells were stimulated with nigericin and the indicated proteins in lysates were analyzed by immunoblot. (B, C) ELISA of IL-1β (B) or TNF-a (C) in supernatants from BMDMs described in (A). Data are from three independent experiments with biological duplicates in each (**B, C**; mean and s.e.m of n = 6) or are representative of three independent experiments (**A**). Statistics were analyzed using an unpaired Student's t test: **P<0.01, ***P <0.001.



Supplementary Figure. 4. Effects of Ori on IL-1β or TNF-a production. (A, B) BMDMs were pretreated with different doses of Ori for 30 min and then primed with LPS for 3 hours and the left stimulated with nigericin for another 30 min. Production of IL-1β (A) and TNF-α (B) were measured by ELISA and then the cytokine level is normalized to that of DMSO-treated control cells. Nonlinear regression analysis was performed, and the curve of Log [M] Ori versus the normalized response is presented. Data are from three independent experiments with biological duplicates in each.

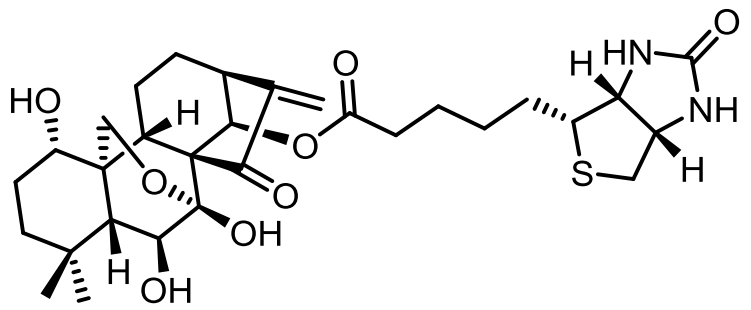


Supplementary Figure. 5. Ori has no effects on mitochondrial damage, potassium or chloride efflux. (A) Confocal microscopy analysis in LPS-primed BMDMs treated with Ori (2 μM) and then left stimulated with nigericin, followed by staining with Mitosox, Mitotracker red and DAPI. (B) Qualification of potassium efflux in LPS-primed BMDMs treated with different doses of Ori and then left stimulated with nigericin. (C) Qualification of chloride efflux in LPS-primed BMDMs treated with Ori(2 μM) and then left stimulated with nigericin at different time points. Data are from three independent experiments with biological duplicates in each (**B, C**; mean and s.e.m of $n = 6$) or are representative of three independent experiments (**A**). Statistics were analyzed using an unpaired Student's t test: ***P <0.001, NS, not significant.



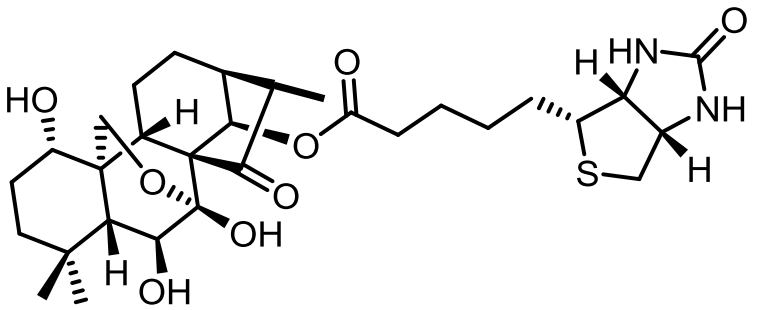
Supplementary Figure. 6. Ori has no effects on NLRP3 ATPase activity and NEK7-NEK9 interaction. (A, B) Silver staining of the purified His-GFP-NLRP3 (A) or His-Flag-NEK7 (B) protein. (C) ATPase activity assay for purified human NLRP3 in the presence of different concentrations of Ori. (D) IP and immunoblot analysis of the interaction of GFP-NEK7 and Flag-NEK9 in the lysates of HEK-293T cells. Data are from three independent experiments with biological duplicates in each **(C**; mean and s.e.m of $n = 6$) or are representative of three independent experiments (**A, B, D**). Statistics were analyzed using an unpaired Student's t test: NS, not significant.

A



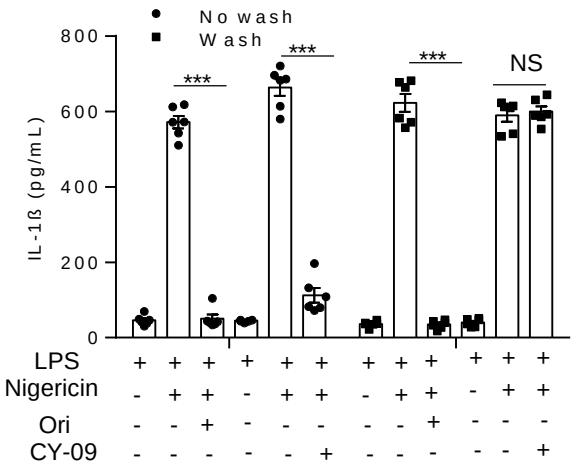
Bio-Ori

B

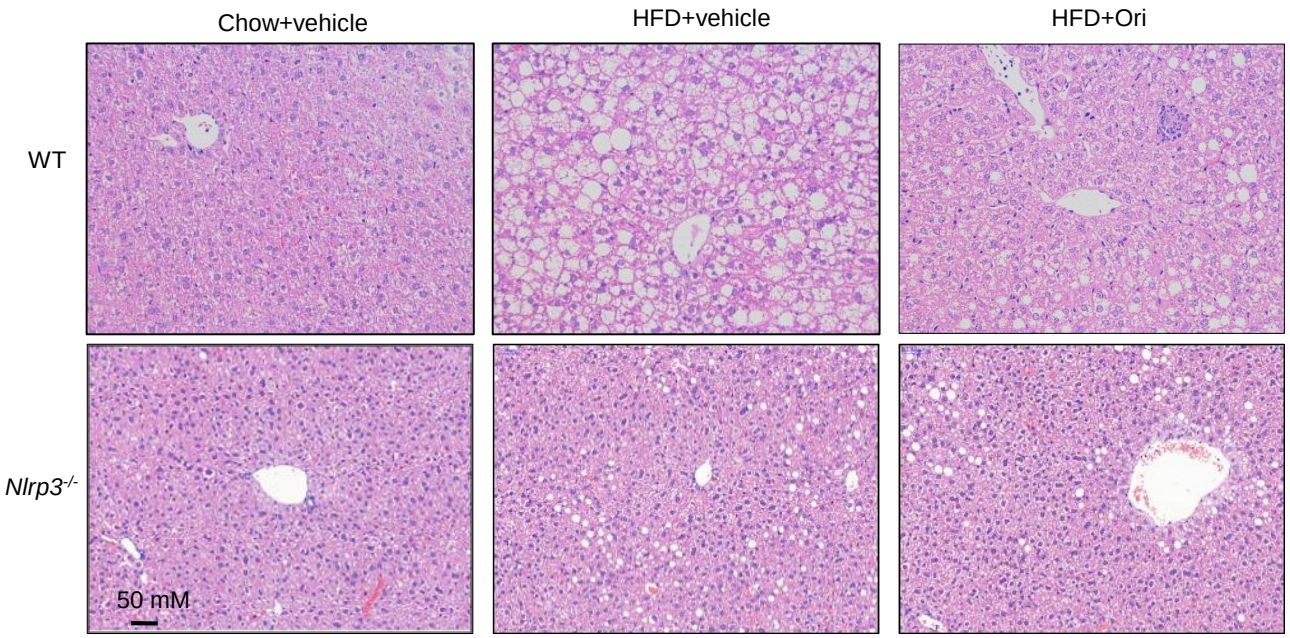


Bio-R-Ori

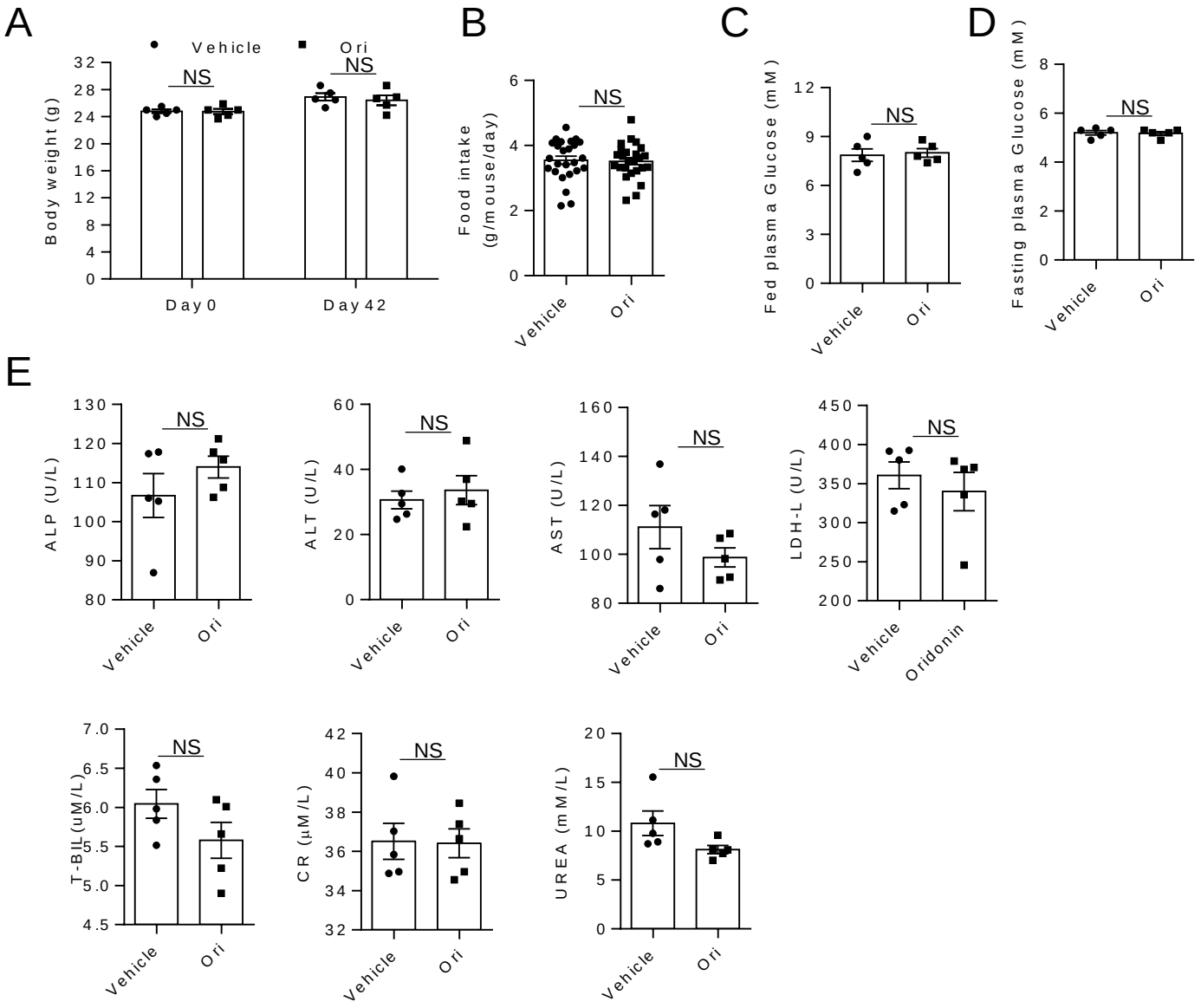
Supplementary Figure. 7. Structure of biotinylated compounds. (A, B) Structure of Bio-Ori (A) and Bio-R-Ori (B).



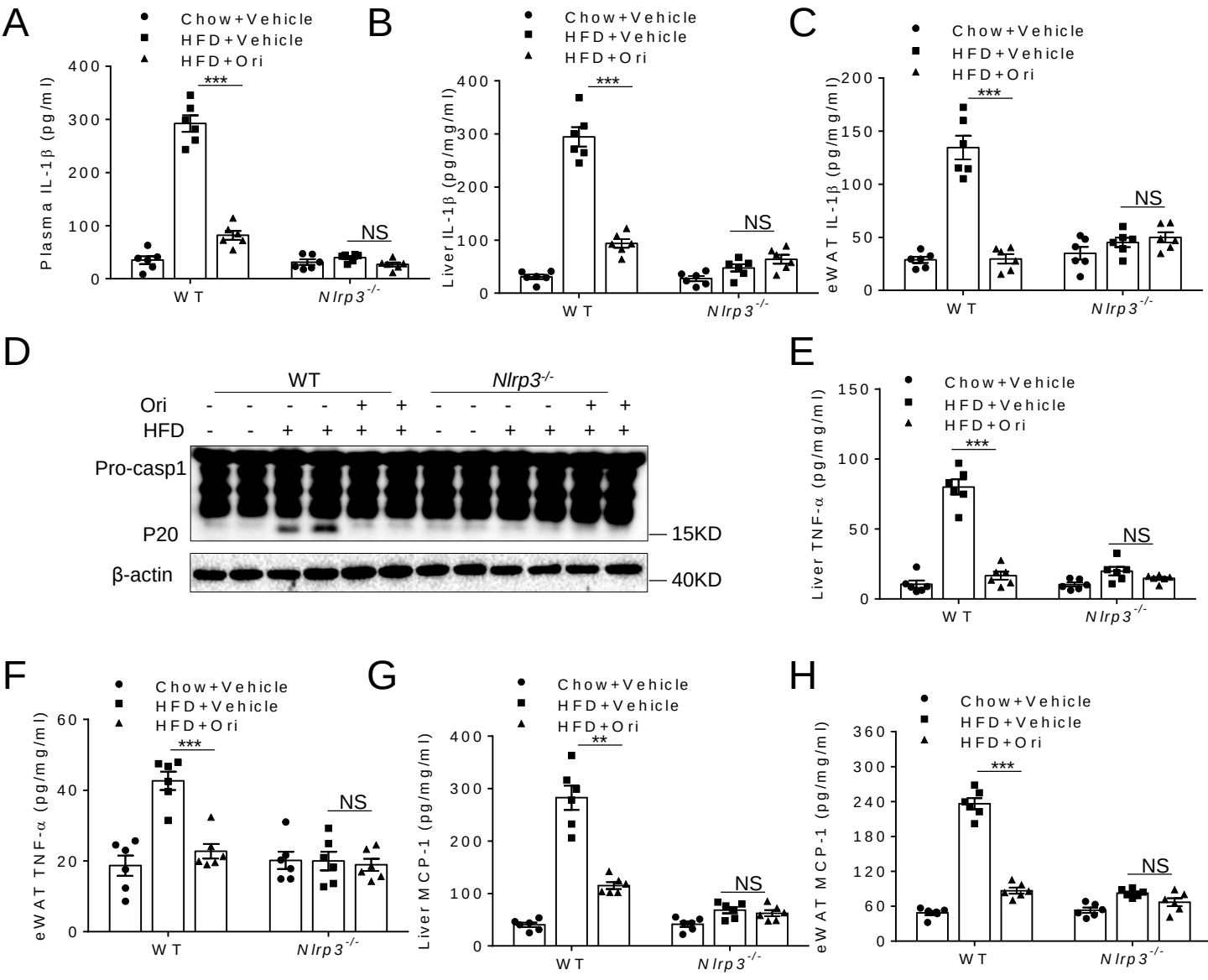
Supplementary Figure. 8. Inhibitory effects of Ori is not reversible. ELISA of IL-1β in supernatants from LPS-primed BMDMs that were treated Ori (2 μM) or CY-09 (5 μM) for 15 min and washed 3 times, then left stimulated with nigericin. Mean and s.e.m of $n = 6$， Statistics were analyzed using an unpaired Student's t test: ***P <0.001, NS, not significant.



Supplementary Figure. 9. The role of Ori in HFD-induced hepatic steatosis. Representative H&E staining of liver sections of WT or *Nlrp3*$^{-/-}$ mice that were first fed with HFD for 12 weeks and then treated with Ori for 6 weeks. Data are representative of two independent experiments.



Supplementary Figure. 10. Long-term Ori treatment has no effects on the metabolic parameters and serum chemistry of healthy mice. (A-D) Body weights (A), food intake (B), fed plasma glucose (C) or fasting plasma glucose (D) of WT mice which were treated with Ori once a day at the dose of 3 mg/kg for 6 weeks. Mean and s.e.m of $n = 5$. (E) Qualification of alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), creatinine, urea (UREA) and total bilirubin (T-BIL) in the serum of WT mice which were treated with Ori once a day at the dose of 3 mg/kg for 6 weeks. Mean and s.e.m of n = 5. Statistics were analyzed using an unpaired Student's t test: NS, not significant.



Supplementary Figure. 11. Ori suppresses NLRP3-dependent chronic inflammation in diabetic mice. (A-H) WT or *Nlrp3*$^{-/-}$ mice were first fed with HFD for 12 weeks and then treated with Ori for 6 weeks. Plasma IL-1β (A) were assessed by ELISA. Liver (B, E, G) and adipose tissue (WAT) (C, F, H) were isolated and cultured for 24 hours and supernatants were analyzed by ELISA for IL-1β (B, C), TNF-a (E, F) or MCP-1 (G, H). Caspase-1 activation in WAT was analyzed by immunoblot as indicated (**D**). n = 6 per group. Data are shown as mean and s.e.m. and are representative of two independent experiments. Statistics were analyzed using an unpaired Student's t test: **P <0.01, ***P <0.001, NS, not significant.

Supplementary Figure. 12. Scans of the full films used to generate Western blot data for figure 1B,1E,1G

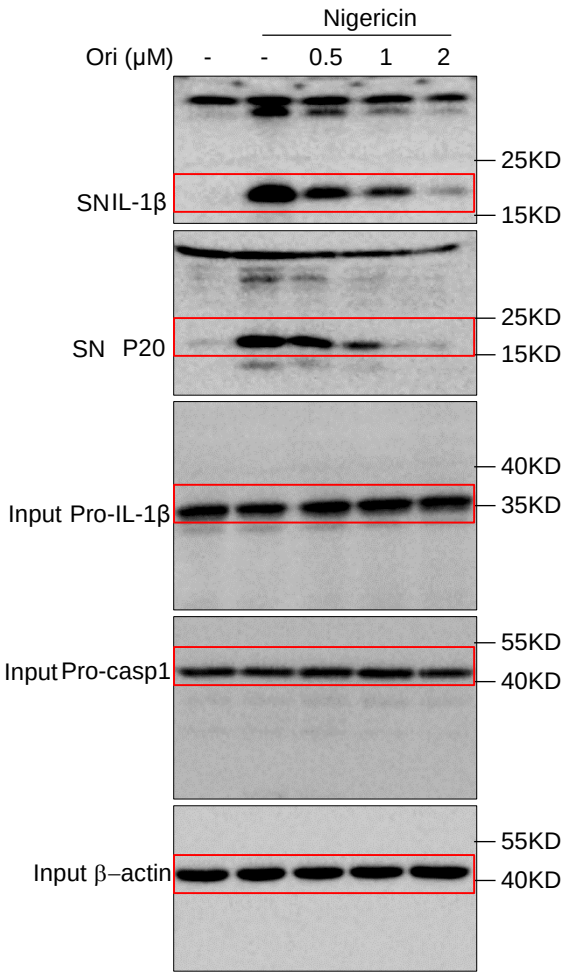


Figure 1B

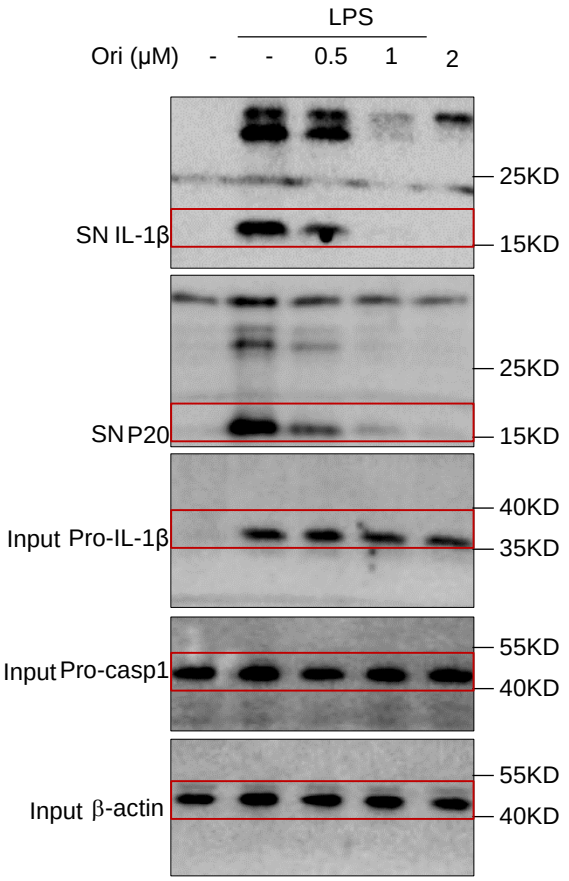


Figure 1G

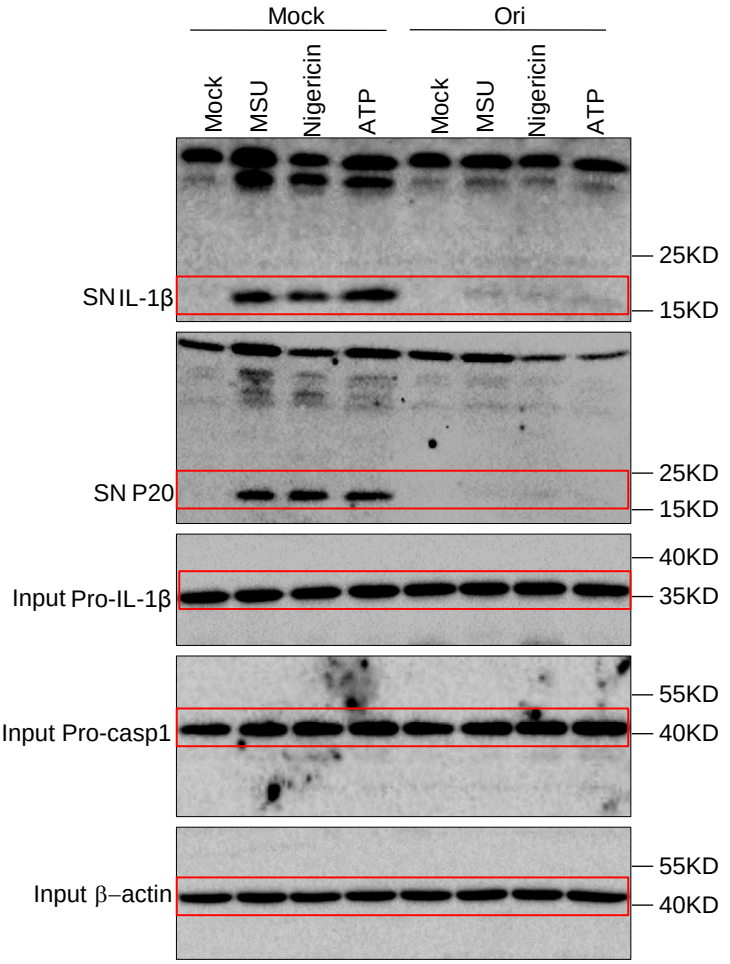


Figure 1E

Supplementary Figure. 13. Scans of the full films used to generate Western blot data for figure 2A,2B,2C

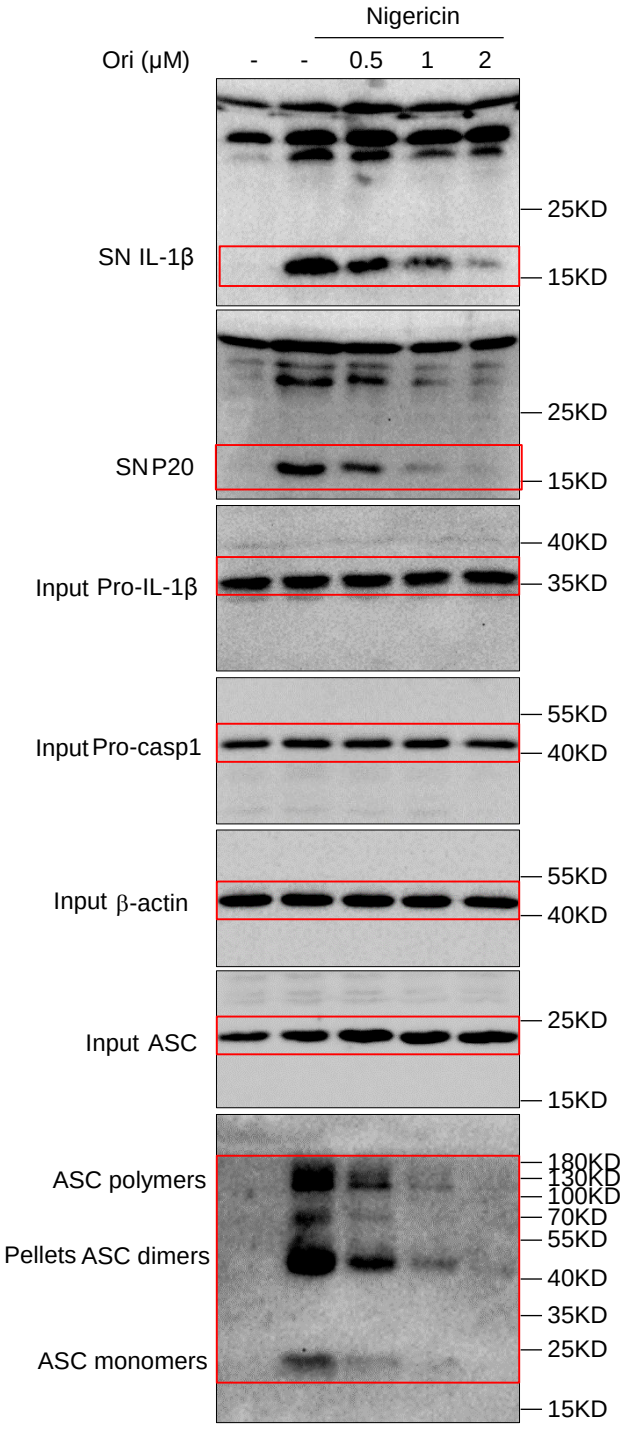


Figure 2A

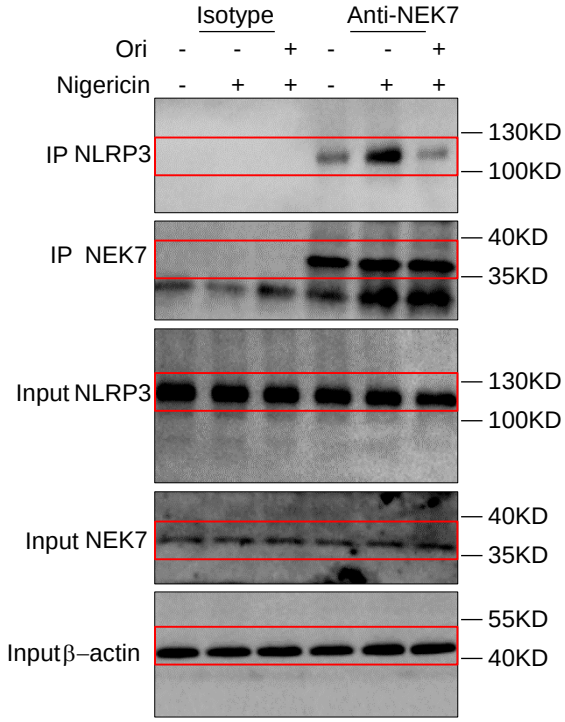


Figure 2B

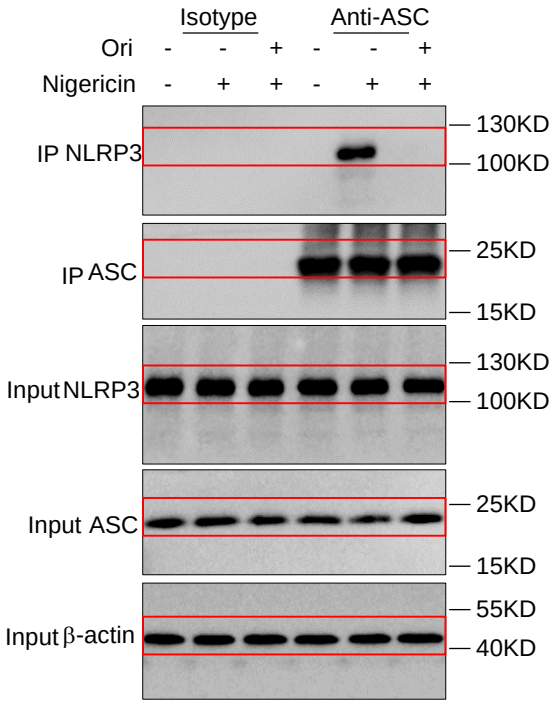


Figure 2C

Supplementary Figure. 14. Scans of the full films used to generate Western blot data for figure 2D,2E,2F,2G

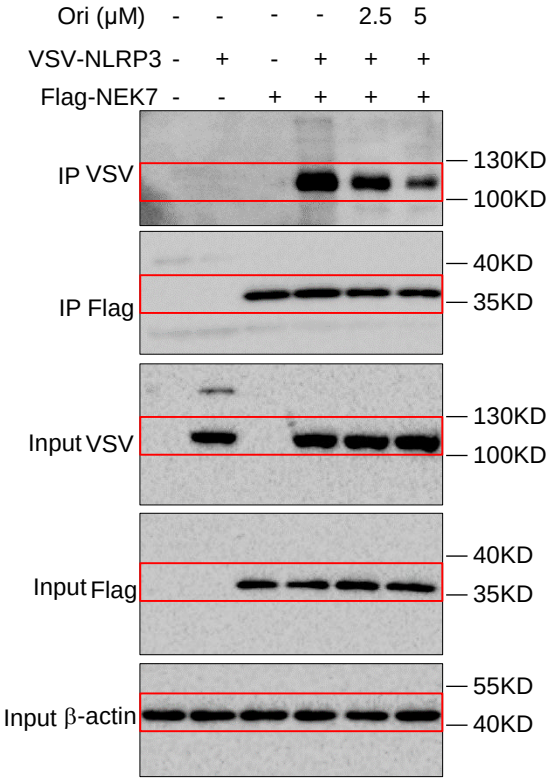


Figure 2D

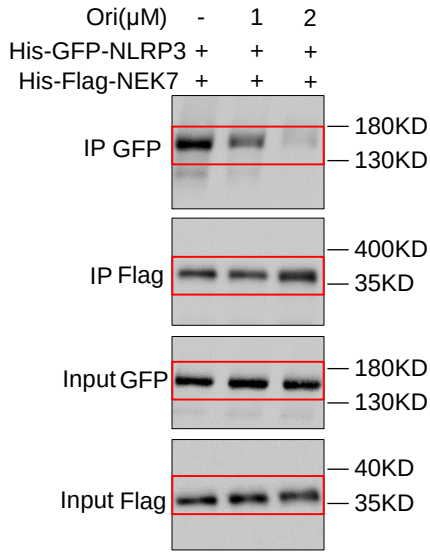


Figure 2E

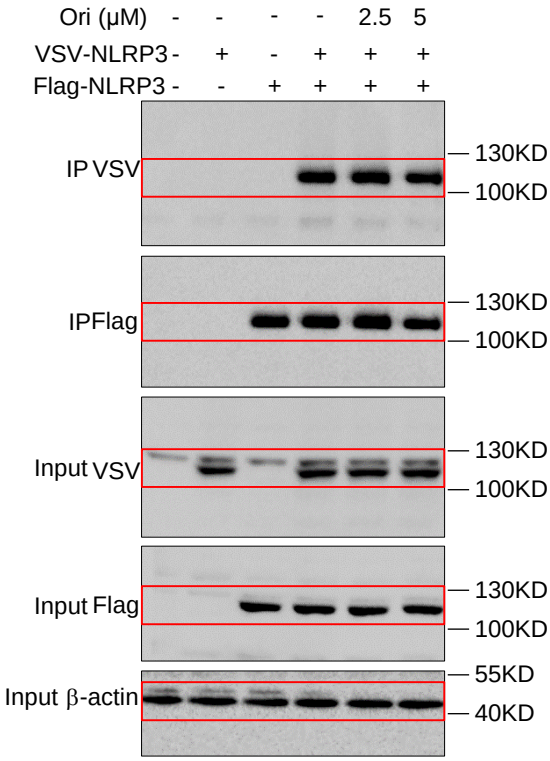


Figure 2F

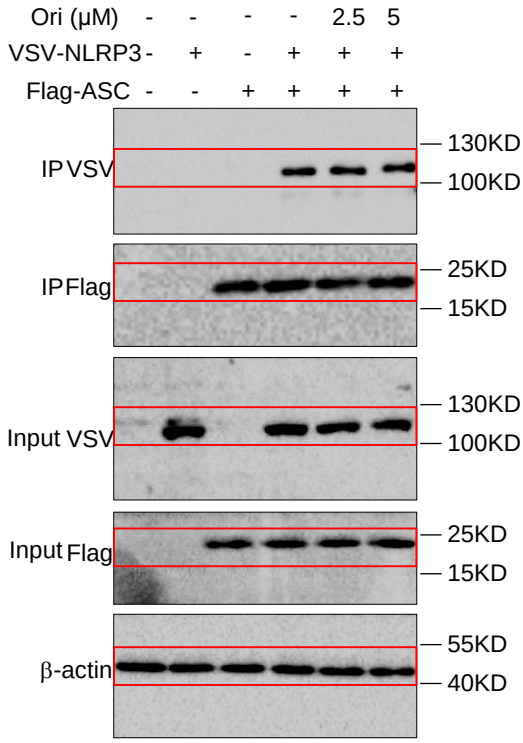


Figure 2G

Supplementary Figure. 15. Scans of the full films used to generate Western blot data for figure 3A,3B,3C,3D

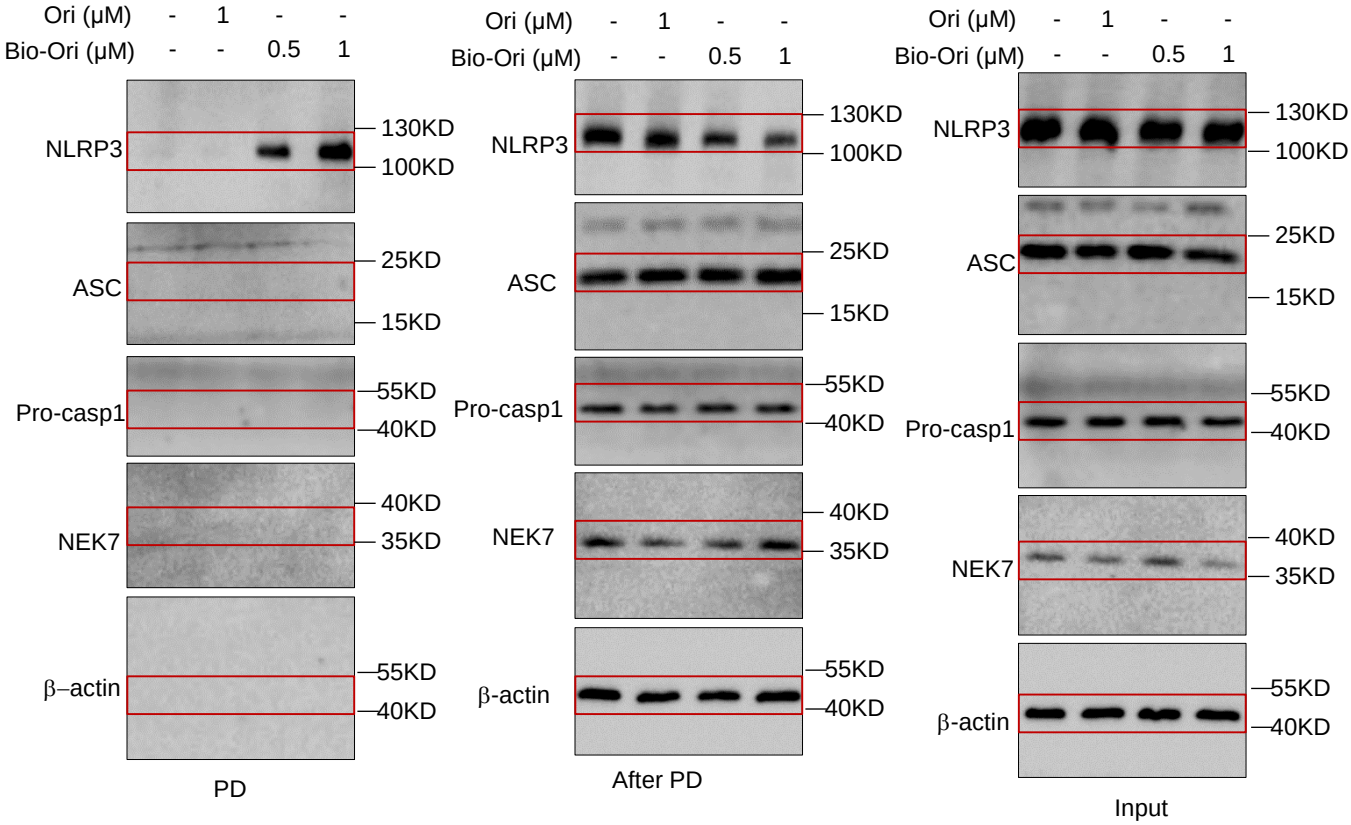


Figure 3A

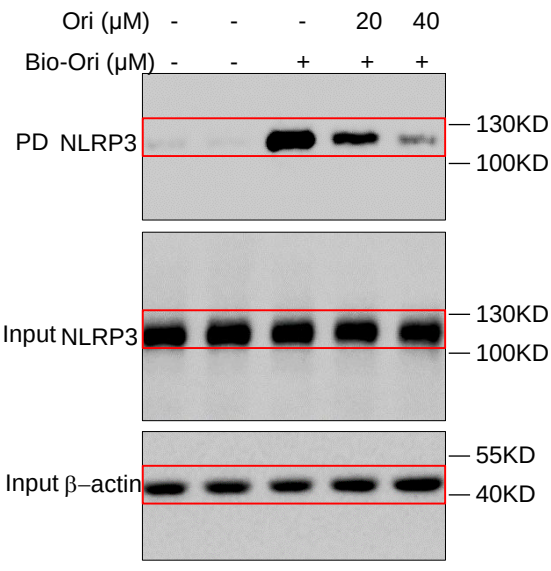


Figure 3B

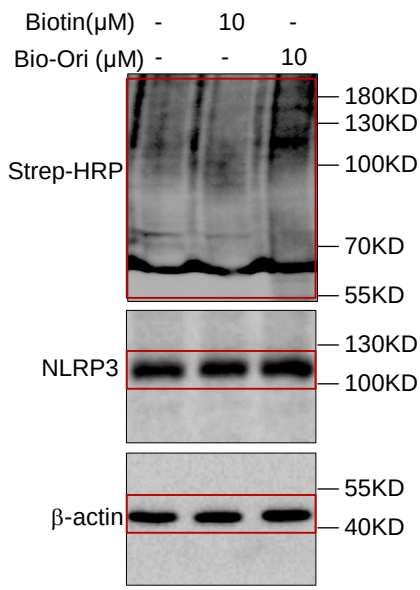


Figure 3C

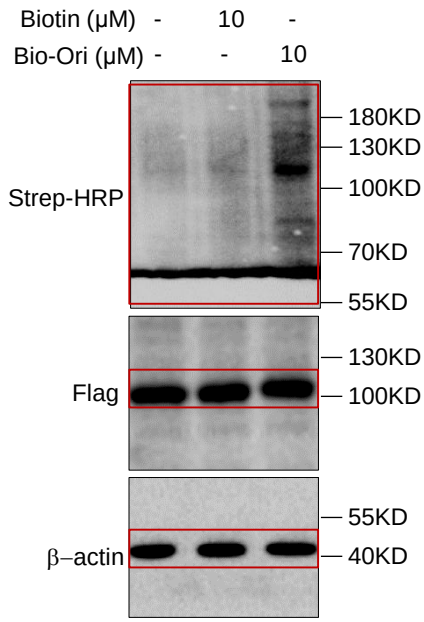


Figure 3D

Supplementary Figure. 16. Scans of the full films used to generate Western blot data for figure 3E,3G,3H,4B

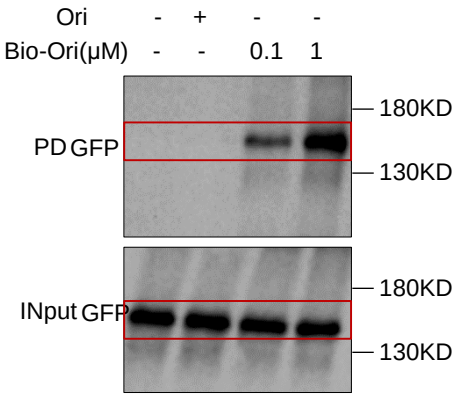


Figure 3E

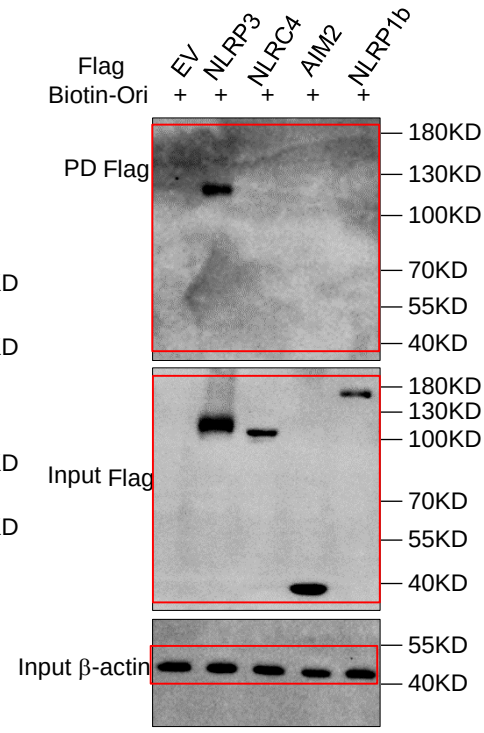


Figure 3G

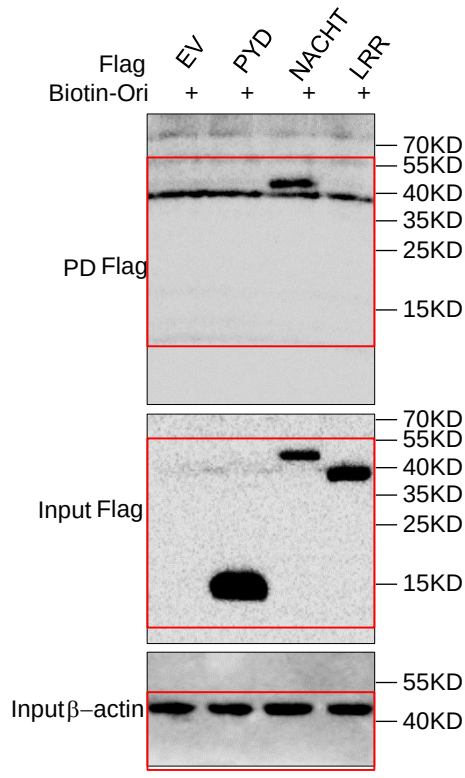


Figure 3H

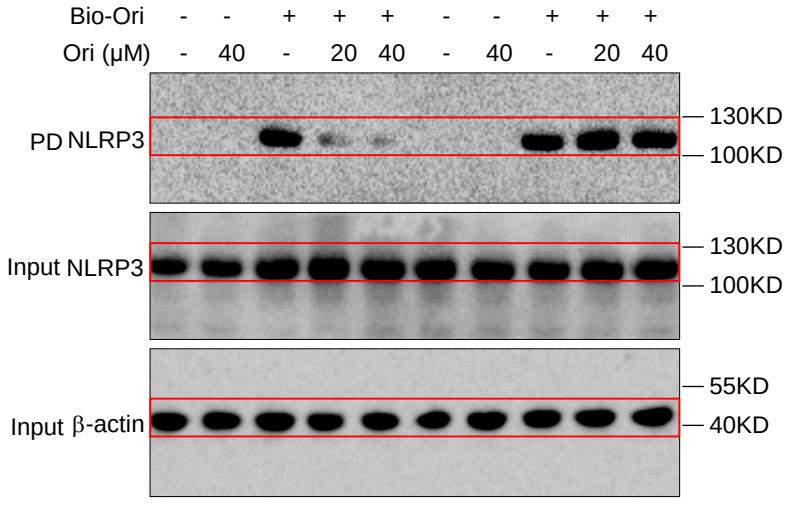


Figure 4B

Supplementary Figure. 17. Scans of the full films used to generate Western blot data for figure 4C,4D,4E,5B

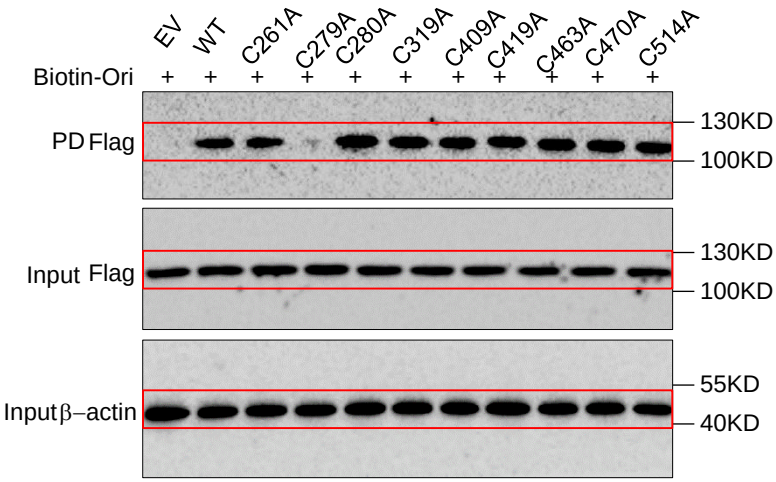


Figure 4C

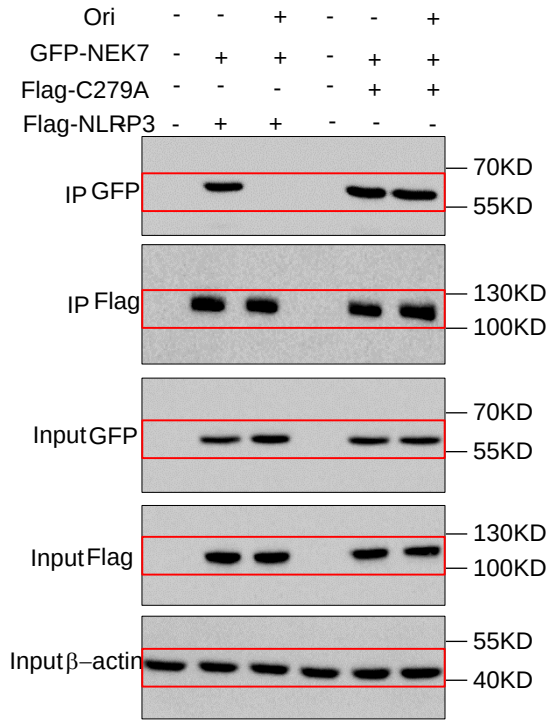


Figure 4D

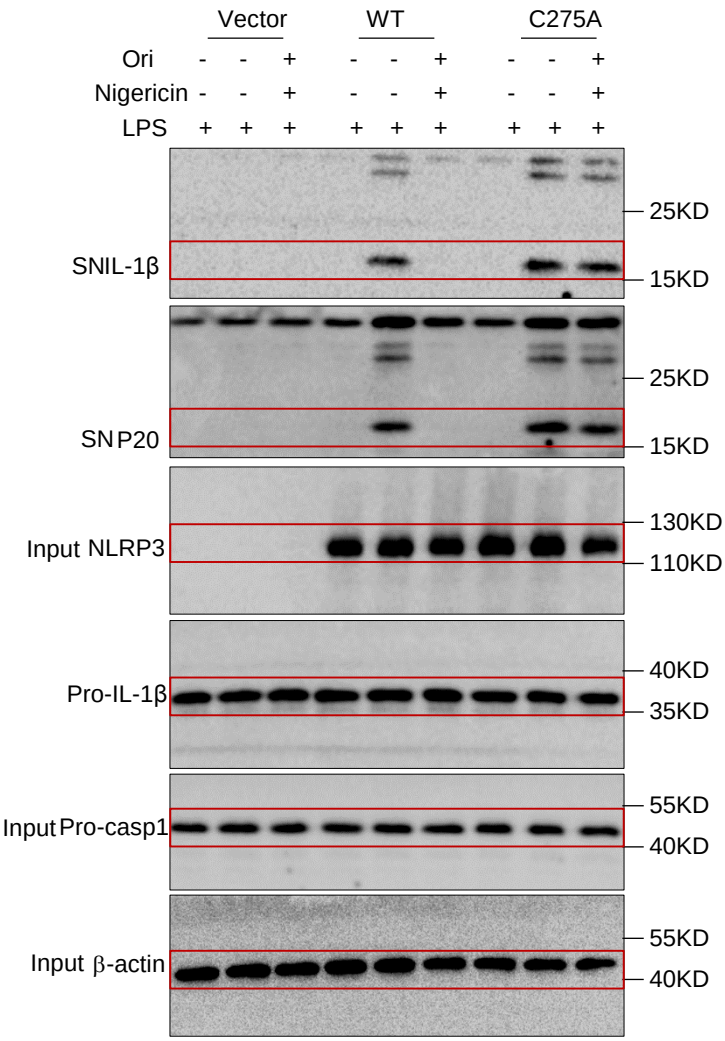


Figure 4E

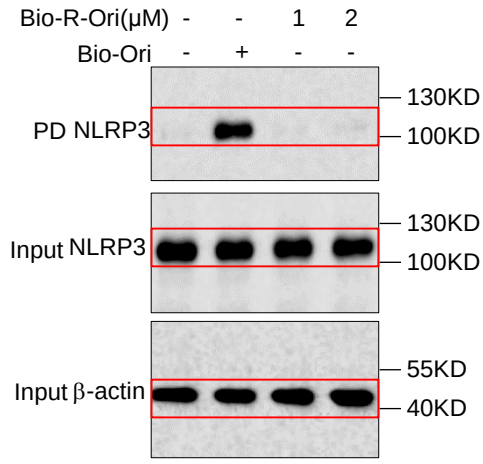


Figure .5B

Supplementary Figure. 18. Scans of the full films used to generate Western blot data for figure 5C, Supplementary Figure. 2B,2D

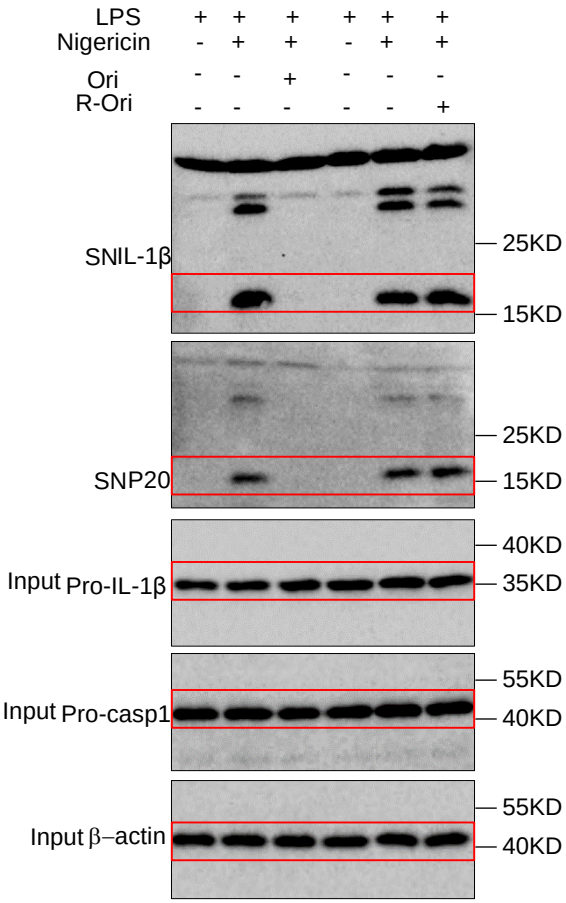
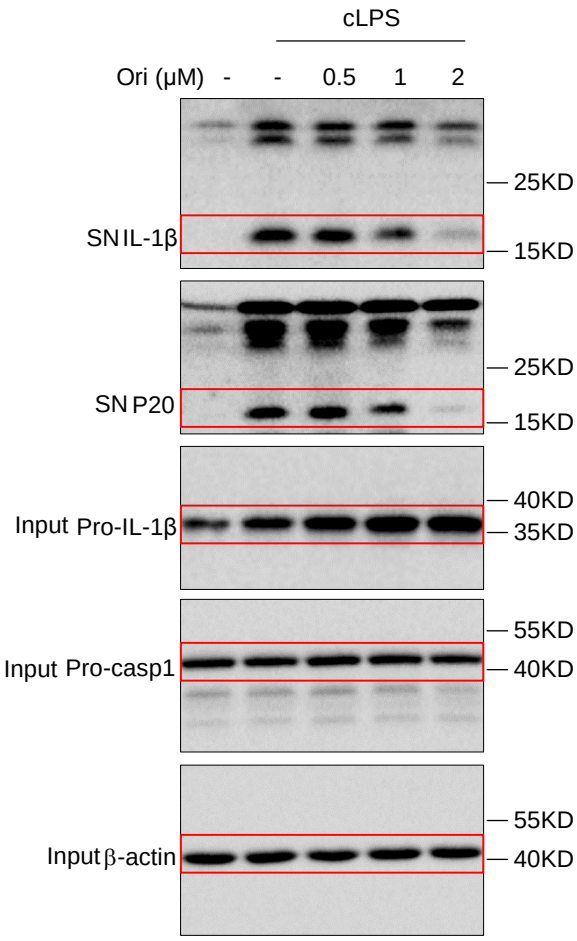
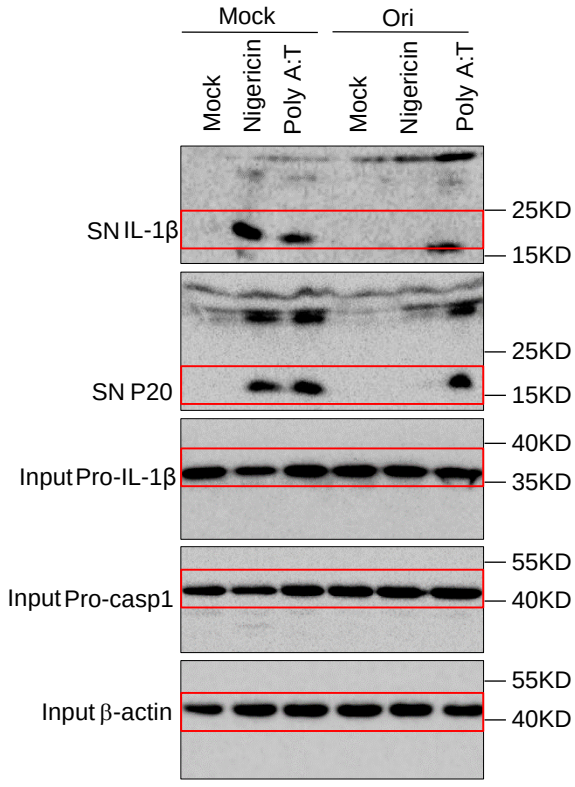


Figure 5C

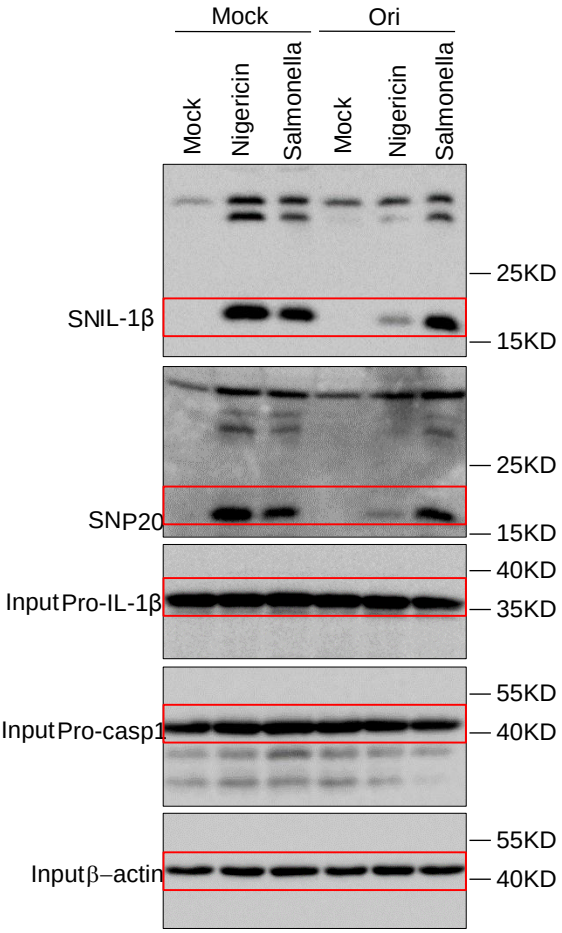


Supplementary Figure. 2B

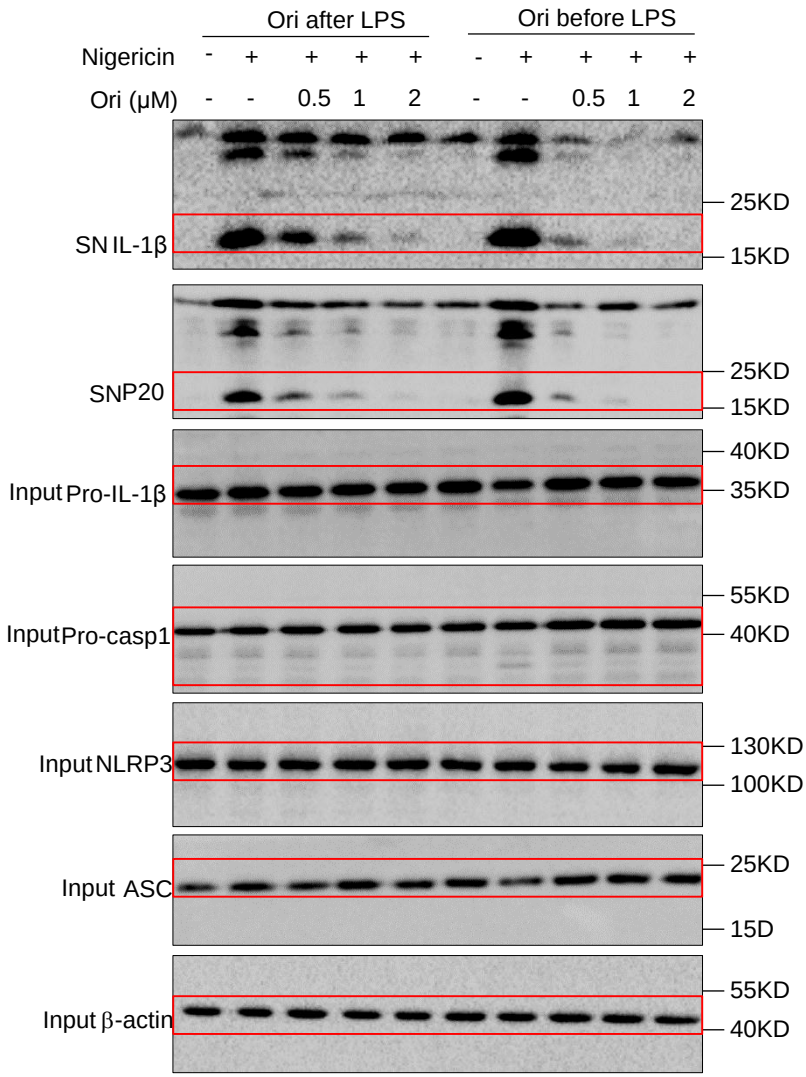


Supplementary Figure. 2D

Supplementary Figure. 19. Scans of the full films used to generate Western blot data for Supplementary Figure. 2F,3A

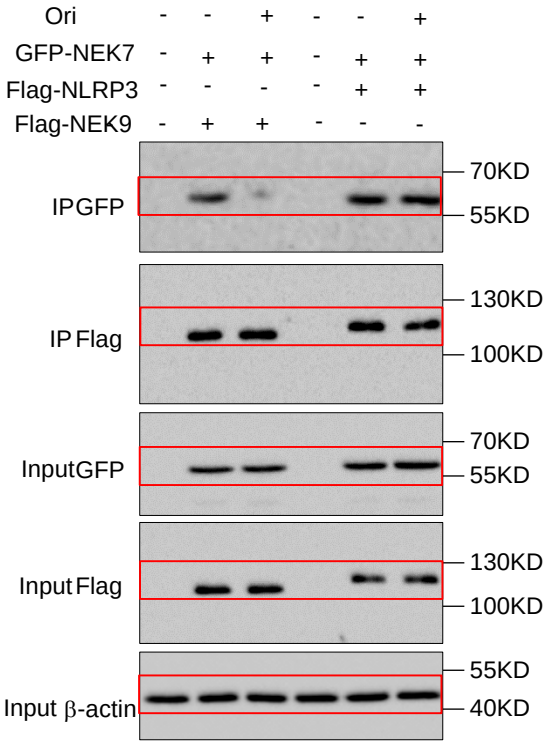


Supplementary Figure. 2F

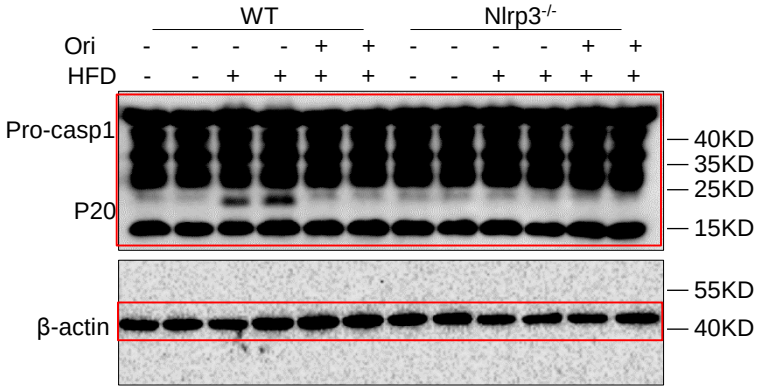


Supplementary Figure. 3A

Supplementary Figure. 20. Scans of the full films used to generate Western blot data for Supplementary Figure. 6D,11D



Supplementary Figure. 6D



Supplementary Figure. 11D

Supplementary Table 1. Sequences of primers used for Plasmid constructions

Flag-NEK9	1Forward: GATTACAAAGACGATGACGATAAATCGGTGCTGGGCGAGTACGA 1Reverse: GATCTAGAGTCGCGGCCGCTCTAGAGGCTGGGTCTACAGG 2Forward: AGCGGCCGCGACTCTAGATCGCCCTATTCTATAGTGTCAC 2Reverse: TTTATCGTCATCGTCTTTGTAATC
His-GFP-NLRP3	1Forward: ATGCATCACCATCACCATCATCACCATATGGTGAGCAAGGGCGAGGA 1Reverse: TCATTTTTCGAACTGCGGATGGCTCCACCAAGAAGGCTCAAAGACGA 2Forward: TGGAGCCATCCGCAGTTCGAAAAATGAGATCCACTAGTCCAGTGTGG 2Reverse: ATGGTGATGATGGTGATGGTGATGCATCGAGCTCGGTACCAAGCTTA
His-Flag-NEK7	1Forward: ATGCATCACCATCACCATCATCACCATGATTACAAAGACGATGACGATAAA 1Reverse: TCATTTTTCGAACTGCGGATGGCTCCAGCTGCTTGCAGTGCATGCAT 2Forward: TGGAGCCATCCGCAGTTCGAAAAATGAGATCCACTAGTCCAGTGTGG 2Reverse: ATGGTGATGATGGTGATGGTGATGCATCGAGCTCGGTACCAAGCTTA
NLRP3（C261A）	1Forward: CTGTTCTATATCCACGCTCGGGAGGTGAGCCTTGT 1Reverse: GGCTACCCGTGATATTGCTGAAGAGCTTGG 2Forward: CCAAGCTCTTCAGCAATATCACGGGTAGCC 2Reverse: ACAAGGCTCACCTCCCGAGCGTGGATATAGAACAG
NLRP3（C279A）	1Forward: GACCTGATCATGAGCGCTTGCCCCGACCCAAAC 1Reverse: GGCTACCCGTGATATTGCTGAAGAGCTTGG 2Forward: CCAAGCTCTTCAGCAATATCACGGGTAGCC 2Reverse: GTTTGGGTCGGGGCAAGCGCTCATGATCAGGTC
NLRP3（C280A）	1Forward: GACCTGATCATGAGCTGCGCTCCCGACCCAAAC 1Reverse: GGCTACCCGTGATATTGCTGAAGAGCTTGG 2Forward: CCAAGCTCTTCAGCAATATCACGGGTAGCC 2Reverse: GTTTGGGTCGGGAGCGCAGCTCATGATCAGGTC
NLRP3（C319A）	1Forward: CACATAGGACCGCTCGCTACTGACTGGCAGAAG 1Reverse: GGCTACCCGTGATATTGCTGAAGAGCTTGG 2Forward: CCAAGCTCTTCAGCAATATCACGGGTAGCC 2Reverse: CTTCTGCCAGTCAGTAGCGAGCGGTCCTATGTG
NLRP3（C409A）	1Forward: GTCCTCTTCACCATGGCTTTCATCCCCCTGGTC 1Reverse: GGCTACCCGTGATATTGCTGAAGAGCTTGG 2Forward: CCAAGCTCTTCAGCAATATCACGGGTAGCC 2Reverse: GACCAGGGGGATGAAGCACATGGTGAAGAGGAC
NLRP3（C419A）	1Forward: GTCTGCTGGATCGGCTGCACTGGACTGAAACAG 1Reverse: GGCTACCCGTGATATTGCTGAAGAGCTTGG 2Forward: CCAAGCTCTTCAGCAATATCACGGGTAGCC 2Reverse: CTGTTTCAGTCCAGTGCAGCCGATCCAGCAGAC
NLRP3（C463A）	1Forward: CAGGAGCACGGCCTCGCTGCCCACCTCTGGGGG 1Reverse: GGCTACCCGTGATATTGCTGAAGAGCTTGG 2Forward: CCAAGCTCTTCAGCAATATCACGGGTAGCC 2Reverse: CCCCCAGAGGTGGGCAGCGAGGCCGTGCTCCTG
NLRP3（C470A）	1Forward: CACCTCTGGGGGCTCGCTTCTTTGGCTGCAGAT 1Reverse: GGCTACCCGTGATATTGCTGAAGAGCTTGG 2Forward: CCAAGCTCTTCAGCAATATCACGGGTAGCC 2Reverse: ATCTGCAGCCAAAGAAGCGAGCCCCCAGAGGTG
NLRP3（C514A）	1Forward: CAAAAGGAAGTGGACGCTGAGAAGTTCTACAGC 1Reverse: GGCTACCCGTGATATTGCTGAAGAGCTTGG 2Forward: CCAAGCTCTTCAGCAATATCACGGGTAGCC 2Reverse: GCTGTAGAACTTCTCAGCGTCCACTTCCTTTTG
Plex-NLRP3	Forward: CTACTAGAGGATCGACTAGTATGACGAGTGTCCGTTGCAA Reverse: GGGCCCTCTAGACTCGAGCTACCAGGAAATCTCGAAGA
Plex-NLRP3(C275A)	1Forward: GACCTGATTGTCAGCGCATGGCCTGACCCAAAC 1Reverse: TTAACGATCCGAGCTCGGTA 2Forward:TACCGAGCTCGGATCGTTAA 2Reverse: GTTTGGGTCAGGCCATGCGCTGACAATCAGGTC