Supplementary Materials

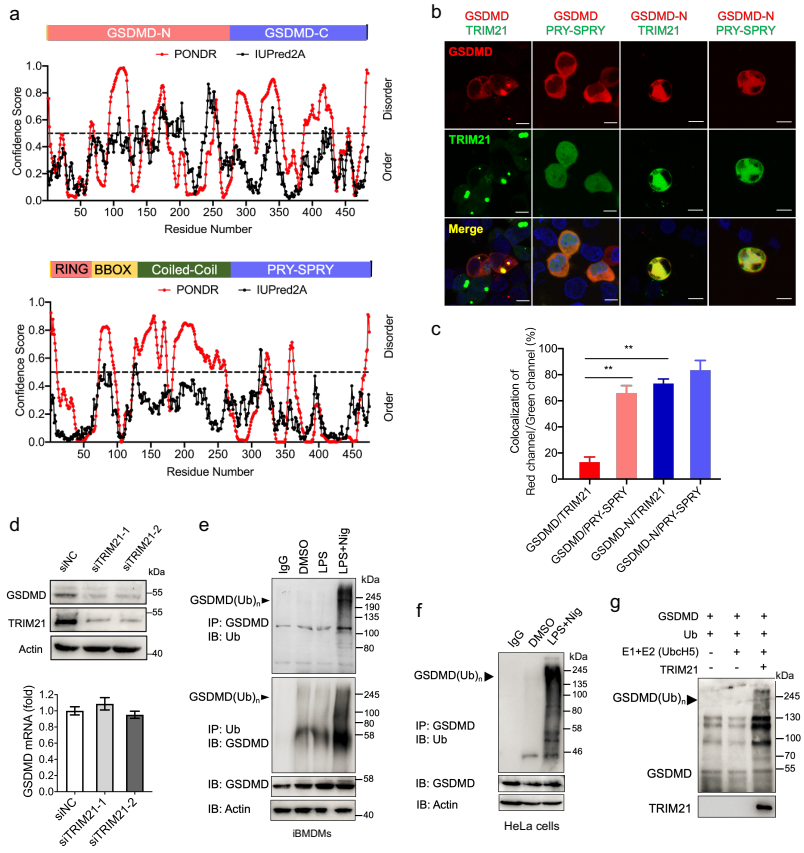

Figure S1. GSDMD interacts with TRIM21, related to Figure 1-3. a, Overlays of the predicted disordered regions in GSDMD and TRIM21 from the PONDR (http://www.pondr.com/) and IUPred2A (https://iupred2a.elte.hu/plot) servers. **b**, HEK293T cells were transfected with Orange-GSDMD (full-length GSDMD or GSDMD-N with the Orange tag) and TRIM21-GFP (full-length TRIM21 or its PRY-SPRY domain with the GFP tag). Nuclei were labeled with the live-cell DNA stain Hoechst 33342 (Beyotime) at RT for 10 min. Scale bar, 10 μm. **c**, Quantification of the

colocalization efficiency between GSDMD (the full-length GSDMD or its N-terminus) and TRIM21 (the full-length TRIM21 or its PRY-SPRY domain). The data are the means ± SD of triplicate samples from a representative experiment. $**P < 0.01$. **d**, Depletion of *TRIM21* in human HeLa cells caused decreased levels of GSDMD as determined by western blot, whereas mRNA levels remained unchanged. Data are shown as mean ± s.d. **e**, GSDMD underwent robust ubiquitination upon inflammatory stimulation. iBMDM cells were primed with LPS for 2 h and stimulated without or with 20 μM Nig for 30 min. Total cell lysates were subjected to GSDMD or ubiquitin pull-down and immunoblotting. **f**, HeLa cells were transfected with LPS followed by nigericin treatment or no treatment for 2 h. Cells were subjected to GSDMD pull-down and immunoblotting analysis. **g**, *In vitro* ubiquitination assay was performed in the presence of ubiquitin, E1, E2, GSDMD, and TRIM21. All data are representative of three independent experiments.

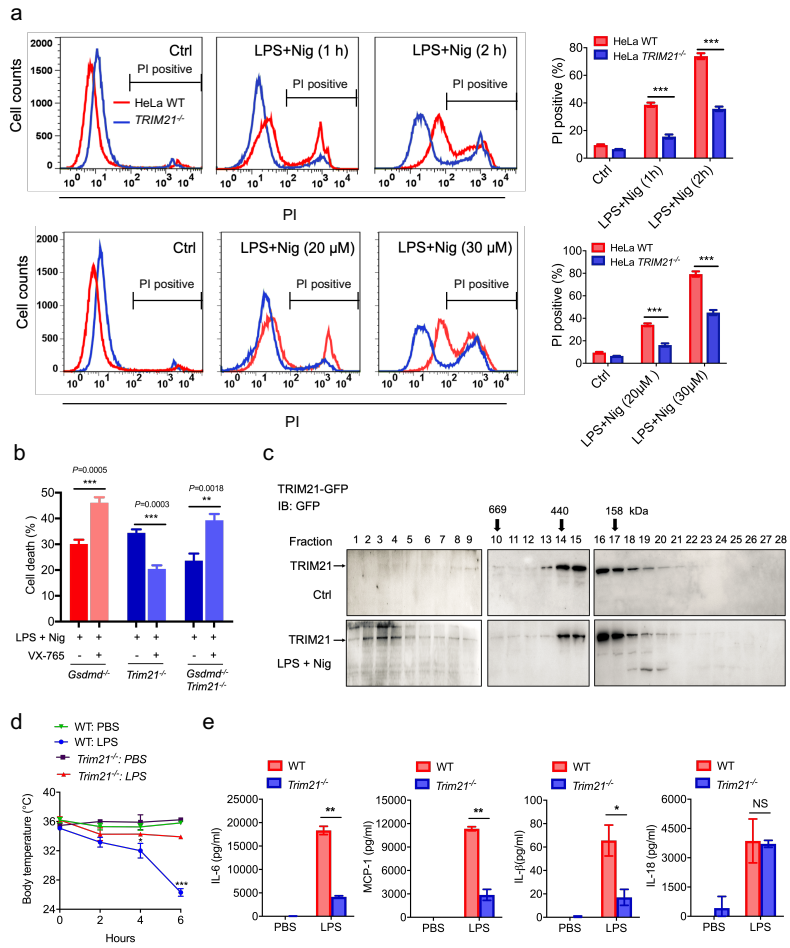

Figure S2. TRIM21 positively regulates cell pyroptosis, related to Figures 5 and 6.

a, WT and *TRIM21*$^{-/-}$ HeLa cells were transfected with LPS (1 μg/mL) and treated with Nig at the indicated doses and times, followed with the FACS analysis. The PI representative plots of data are shown in the left panels. **b**, *Gsdmd*$^{-/-}$, *Trim21*$^{-/-}$, and *Gsdmd*$^{-/-}$*Trim21*$^{-/-}$ iBMDM cells were pretreated with VX-765, an inhibitor of Caspase-1, for 30 min at 37°C, then cells were stimulated with LPS plus Nig for another 2 h. Cell death was determined by the CytoTox96 assay. **c**, TRIM21-GFP-reconstituted HeLa cells were treated with or without LPS transfection plus Nig stimulation. Cells

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were lysed by the RIPA lysis buffer (containing 1% Triton X-100 and 0.1% SDS), and lysates were fractionated by gel-filtration chromatography (Superpose 6 10/300 column) followed by immunoblotting analysis. **d**, Body temperature plot over time in WT or *Trim21*$^{-/-}$ mice intraperitoneally injected with PBS or LPS (5 mg/kg). **e**, Cytokine concentrations in the serum from *Trim21*$^{-/-}$ and WT mice challenged as in **d**. The data are the means ± SD of triplicate samples from a representative experiment. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. All data are representative of three independent experiments.