TBK1 and IKKε act like an OFF switch to limit NLRP3 inflammasome pathway activation

Fabian A. Fischer[1+], Linda F. M. Mies[1+], Sohaib Nizami[2+], Eirini Pantazi[1], Sara Danielli[1], Benjamin Demarco[1], Michael Ohlmeyer[3,4], Michelle Sue Jann Lee[5], Cevayir Coban[5], Jonathan C Kagan[6], Elena Di Daniel[2*] and Jelena S. Bezbradica[1*]

[1] The Kennedy Institute of Rheumatology, University of Oxford, Oxford, UK

[2] Alzheimer's Research UK Oxford Drug Discovery Institute, NDM Research Building, University of Oxford, Oxford, UK

[3] Icahn School of Medicine at Mount Sinai, New York, NY, USA

[4] Atux Iskay LLC, Plainsboro, NJ, USA

[5] Division of Malaria Immunology, The Institute of Medical Science (IMSUT), University of Tokyo, Japan

[6] Harvard Medical School and Division of Gastroenterology, Boston Children's Hospital, Boston, MA, USA

[+]The authors contributed equally to this work.

*Corresponding authors:

Jelena S. Bezbradica, The Kennedy Institute of Rheumatology, University of Oxford, Oxford, UK. Email jelena.bezbradica@kennedy.ox.ac.uk, phone +44 1865 612 622, ORCID 0000-0003-1441-1727

Elena Di Daniel, Alzheimer's Research UK Oxford Drug Discovery Institute, University of Oxford, Oxford, UK. ORCID 0000-0002-0535-2123

Classification: Biological Sciences; Immunology and Inflammation.

Supplementary methods

<u>Cell stimulation and compound treatment for *in vitro* NLRP3 inflammasome assays</u>

Differentiated primary mouse BMDMs were plated at a density of 10^6 cells/ml CMM media. Differentiated HMDMs were plated at a density of $0.7x10^6$ cells/ml CMM media. Cells were primed for 4 h (or as described in the respective figure legends) with 100 ng/ml *E.Coli* K12 ultrapure LPS (Invivogen, tlrl-peklps), 100 ng/ml sigma LPS (Sigma Aldrich, L4391), 1 µg/ml Pam3CSK4 (Invivogen, tlrl-pms), 100 µg/ml zymosan (Invivogen, tlrl-zyn) or for 1 h with 2.5 µg/ml Poly(IC) (Sigma Aldrich, P1530-25mg). Cells were subsequently stimulated with 2.5 µM, 5 µM or 10 µM nigericin (as described in the respective figure legends) (Sigma Aldrich, N7143-5MG) for 1 h, 150 µg/ml MSU (Invivogen, tlrl-msu) for 5 h or 70 µM R837 (Invivogen, tlrl-imq) for 3 h. The PP2A inhibitor okadaic acid was used at 1 µM (PP2A inh 1: Cambridge Bioscience, 10011490-25 ug-CAY; PP2A inh 2: Sigma Aldrich, 495609-25UG; PP2A inh 3: Sigma Aldrich, 495604-25UG) added 15-30 min before inflammasome activation. The PP2A inhibitor LB-100 was used at 10 µM (Cambridge BioScience, L0400-1 mg) and was added between 30 min to 4 h before inflammasome activation with nigericin. The PP2A activator DBK-1154 (44) was added at 5 µM for 30 min before nigericin. The activity of the PP2A activator was confirmed using a serine/threonine phosphatase assay system (Promega V2460) according to manufacturer specifications. Briefly, $1x10^6$ iBMDMs were treated with DBK-1154 (10 µM) or vehicle alone for 1 h before lysis in non-denaturing buffer (PPase2A buffer containing 1% Triton X-100). Phosphatase activity of the lysates was measured according to manufacturer specifications with the reaction occurring at 30°C for 10 min. TBK1 inhibitors were used at 10 µM (MRT 68601 hydrochloride, Tocris, 5067; MRT 67307 dihydrochloride, Tocris, 5134; BX 795,

Tocris, 4318) and were added 30 min before nigericin. Because many compounds have reversible activity, they were not washed away, i.e. media was not changed, between signal 1 and signal 2, and all stimulations were carried out in the above-described CMM. After cell stimulation, cell supernatants were collected and cells lysed for analysis as described below.

Cell stimulation for *in vitro* AIM2 and NLRC4 inflammasome assays

Primary mouse BMDMs or iBMDMs were isolated and differentiated as described above and plated at a density of $1x10^6$ cells/ml CMM media (for BMDMs), or complete DMEM media (for iBMDMs), in tissue culture-treated 96-well plates the day before the inflammasome assays. Cells were primed for 4 h with 100 ng/ml ultrapure LPS (Invivogen, tlrl-peklps) for BMDMs, or 1 μg/ml for iBMDMs. TBK1 inhibitor MRT 68601 or the PP2A inhibitor okadaic acid were added 30 min before inflammasome activation at a concentration of 10 μM or 1 μM, respectively. AIM2 activation was induced by transfecting cells with 50-200 ng/well calf-thymus DNA (Sigma Aldrich, D4522-5) with 0.25% Lipofectamine-2000 (Thermo Fischer, 11668019). NLRC4 activation was induced by transfecting cells with 100 ng/well ultrapure Flagellin S.T. (Invivogen, tlrl-epstlfla-5) with 0.25% FuGene HD (Promega, E2311). Cells were spun at 1000 g for 10 min to synchronise transfection and then incubated for 1 h or 4 h at 37°C. Supernatants were collected for analysis.

Readouts used to monitor inflammasome activity (cytokine, viability, caspase-1)

Secretion of IL-1β, IL-18 and TNF-α were monitored in cell-free supernatants using ELISA (eBioscience, 88-7013-77, 88-7324-77 and BMS618-3TEN or R&D systems, DY401). IL-1β was also measured in cell supernatants and cell lysates by Western

blot using anti-IL-1β antibody (R&D Systems, AF-401-NA) to measure pro-IL-1β, as well as the cleaved p17 fragment. Cellular viability was measured using cell culture supernatants and the Cytox96 nonradioactive cytotoxicity assay (Promega, G1780). Caspase-1 activity was measured in cell lysates and culture media by Western blot using anti-caspase-1 antibody (Casper-1, Adipogen, AG-20B-0042-C100) to detect p46 and p20 cleaved caspase-1. GAPDH (CST, 2118S) or Tubulin served as loading controls. Phosphorylation of TBK1 and AKT were monitored by western blot using antibody against phospho-TBK1 S172 (CST, 5483), phospho-AKT S473 (CST, 4060S) and phospho-AKT substrate (CST, 9614S)

ASC oligomerisation analysis with DSS crosslinking

WT iBMDMs were seeded at $1x10^6$ cells/well in a 12-well plate complete DMEM (cDMEM) containing 10% FBS, 20 mM HEPES and 1x Pen/Strep/Glutamine. On the day of the experiment, medium was replaced with fresh cDMEM and cells were stimulated 1 µg/ml ultrapure LPS (Invitrogen, tlrl-peklps) for 4 h. The Caspase-1 inhibitor VX-765 was added at 10 µM 1 h before Nigericin addition to prevent cell death and therefore protein release. TBK1 inhibitor MRT 68601 was added 30 min before inflammasome activation at a concentration of 10 µM. After 4 h of LPS priming, cells were stimulated with 10 µM Nigericin for 30 min or 1 h. Following stimulation, the medium was aspirated and washed with buffer containing 50 mM HEPES. Cells were then lysed in non-denaturating lysis buffer (50 mM HEPES, 0.5% Triton X-100, containing protease and phosphatase inhibitors) and incubated on ice for 15 min with addition of 2.5 U benzoase. Lysates were then spun for 15 min, 6000g, 4oC and the insoluble fraction was washed three times with 50 mM HEPES buffer. The insoluble pellet was resuspended in 500 µl crosslinking buffer (50 mM HEPES, 150 mM NaCl)

and DSS crosslinker was added to a final concentration of 2 mM and incubated for 45 min at 37oC. Samples were spun again for 15 min, 6000g, 4oC and the remaining pellet was resuspended in SDS/DTT buffer and used for subsequent Western Blot analysis.

Imaging of ASC speck formation in ASC-mCherry reporter iBMDMs

Mouse ASC-mCherry reporter iBMDMs were plated at a density of 100 cells/µl in DMEM media + 10% FBS in 384-well plates. On the day of the experiment, media was replaced with DMEM media with HEPES (Fisher, 21063029) that contains 2 µg/ml Hoechst 33342 (Fisher, H3570) to identify nuclei. Cells were primed with 1 µg/ml LPS (Sigma Aldrich, L2637) for 2 h, and compounds added at the time of priming at the appropriate concentrations as described in the figure legends. Cells were then stimulated with 10 µM nigericin (Sigma Aldrich, SML1779) for 2 h. Cells were fixed with 2 % paraformaldehyde, imaged using InCell 6000 and analysed using IN Cell Developer Toolbox 1.9.2 software. Bright fluorescent specks inside the cells were quantitated and expressed as a percentage of number of specks / number of nuclei, normalised to LPS + nigericin set to 100. MCC950 (Tocris, 5479) was used as the positive control.

Proximity Ligation Assay

WT-ASC-GFP THP-1 cells were seeded at 1.5-2.5x10^5 cells/chamber in 4-well or 8-well microscopy chambers in CMM supplemented with 20 nM PMA to differentiate them into macrophage-like cells. PMA was washed out after 48 h and replaced with fresh CMM. After 72 h, cells were stimulated with 100 ng/ml LPS (Sigma, L4391) for 0.5 h, 1 h and 4 h after which the medium was aspirated and cells were fixed in 4%

PFA in PBS. The cells were permeabilized with 0.1% Triton-X100 in PBS for 10 min and PFA was quenched with 50 mM ammonium chloride for 10 min at RT. Slides were blocked for 30 min with 0.5% BSA in PBS. Primary antibodies against NLRP3 (Enzo Life Sciences) and pTBK1 were diluted 1:50 in 0.5% BSA/PBS and incubated on the slides for 1 h at RT. One sample was incubated with a mouse-IgG1 and anti-pTBK1 only as a negative isotype control (Enzo Life Sciences, ADI-SAB-600-050). Slides were then incubated with an anti-rabbit MINUS and an anti-mouse PLUS probe diluted 1:5 in 0.5% BSA/PBS for 1 h at 37oC . Samples were incubated with a ligase for 0.5 h at 37oC followed by incubation with a polymerase for 100 min at 37oC (both Sigma, Duo-92013). Finally, cells were fixed with DAPI-containing mounting medium (Fisher, P36971) and imaged within a week. Nuclei were imaged in the DAPI channel and PLA signals in the FarRed channel on a Zeiss LSM980 confocal microscope. PLA quantification was performed by counting nuclei in the DAPI channel and using the "Find Maxima" function in Fiji with a threshold set to >17000. Detected PLA foci were subsequently divided by the amount of nuclei in the image to obtain the PLA-to-nuclei ratio.

TBK1 and PP2A siRNA knockdown in iBMDMs

For TBK1 knockdown, WT or IKKε KO iBMDMs were seeded at $5x10^4$ cells/well in a 24-well plate format and transfected the following day using a final concentration of 10 nM of 4 individual siRNA against TBK1 (Dharmacon/Perkin Elmer, J-063162-05-0002, J-063162-06-0002, J-063162-07-0002, J-063162-08-0002) or a scrambled control (Dharmacon/Perkin Elmer, D-001810-10-05) and 3 µl Lipofectamine RNAiMAX (Invitrogen, 13778075) per well in DMEM (Fisher, 31053028) + 10% FBS + 1% GlutaMax + 1% Sodium Pyruvate. After 72 hours incubation, without further replating,

the cells were primed with ultrapure LPS (1 µg/ml, Invivogen, tlrl-peklps) for 30 min and stimulated with nigericin (10 µM) for 1 h. The supernatants were collected for IL-18 ELISA and cell lysates collected for Western blot. For PP2A knockdown, WT iBMDMs were seeded at 2.5-3x10^6 cells per well in a TC-treated 6-well plate and transfected the following day with 3 individual siRNAs against PP2A at a final concentration of 50 nM and 15 µl of Lipofectamine RNAiMax (Dharmacon/Perkin Elmer, J-040657-05-0002, J-040657-07-0002, J-040657-08-0002). After 48 hours incubation, cells were harvested, counted, re-seeded at 1x10^6 cells/ml in a 96-well plate and left to attach for 2 h. Cells were then primed for 4 h with 1 µg/ml ultrapure LPS (Invivogen, tlrl-peklps) and then stimulated for 1 h with 10 µM nigericin. The supernatants were collected for IL-1β ELISA, and cell lysates for Western blot. Protein levels for TBK1 (CST3504) or PP2A (BD Biosciences, 610556) were analysed by western blot. Tubulin (Sigma, T5168) and GAPDH (CST, 2118S) served as loading controls.

TBK1-NLRP3 co-immunoprecipitation in HEK293T cells

HEK293T cells (Sigma Aldrich, 12022001-1VL) were plated the day before transfection in 6-well dishes in RPMI. Cells were transiently transfected using Lipofectamine-2000 (Thermo Fischer, 11668019) with 2 µg of an empty control vector or 2 µg of the NLRP3 and/or 2 µg of the TBK1-expressing constructs. Plasmid pcDNA3.1 constructs were generated commercially (VectorBuilder, Michigan, USA) containing FLAG-tagged mouse WT NLRP3, FLAG-tagged mouse S3A or S3D NLRP3 mutants and HA-tagged mouse WT TBK1. 24 h post-transfection, cells were lysed for 30 min in TNT lysis buffer (50 mM TRIS, 150 mM NaCl, 1 % Triton) containing a phosphatase inhibitor cocktail (10 mM sodium fluoride, 1 mM sodium orthovanadate,

1 mM sodium pyrophosphate, 1x Roche complete protease inhibitor cocktail). Lysates were spun at 4°C for 5 min and supernatants were transferred to fresh tubes. A fraction of the supernatants was collected as the input control and the remaining supernatants were incubated with G Protein Dynabeads (Thermo Fisher, 10003D) for preclearing 30 min at 4°C. Samples were placed on a magnet and the pre-cleared lysates were transferred to new reaction tubes. Lysates were then further incubated with 2 µg anti-FLAG antibody (Sigma Aldrich, F7425-2MG) or 2 µg isotype control (Santa Cruz, SC-2027) for 2 h at 4°C. Then, 30 µl G Protein Dynabeads were added and incubated for additional 1 h at 4°C. The beads were washed multiple times with TNT lysis buffer and one final time with TBS buffer. 25 µl of Elution buffer (0.1 M Glycine, pH 2.5) was added to each tube and incubated for 10 min rocking at room temperature. Samples were placed on a magnet, supernatants were transferred to fresh tubes and neutralized with an equal volume of TRIS, pH 8.0. Samples were then mixed with SDS/DTT sample buffer, boiled at 95°C for 5 minutes and used for Western blot analysis and ECL detection (Bio-Rad, 1705061).

Endogenous NLRP3-NEK7 co-immunoprecipitation in iBMDMs

WT or NLRP3-KO iBMDMs were seeded at $2x10^6$ cells per well in a TC-treated 6-well plate the day before the experiment. Medium was aspirated with fresh medium and cells were then treated with 1 µg/ml ultrapure LPS (Invitrogen, tlrl-peklps) for 0.5 h or 4 h. TBK1 inhibitor MRT 68601 was added 30 min before harvest for non-stimulated and 4 h LPS-treated cells or 30 min before LPS for the 0.5 h LPS time point at a concentration of 10 µM. Cells were subsequently lysed in TNT lysis buffer and the immunoprecipitation protocol was performed as described above by immunoprecipitating NLRP3 with an anti-NLRP3 antibody at 1 µg/ml (Cryo-2, Caltag

Medsystem, AG-20B-0014-C100). IP and whole cell lysate samples were used for further Western blot analysis.

Statistical analysis

Unless stated otherwise, *in vitro* data were analysed using Prism Software V10 and two-way ANOVA with multiple column comparisons. *In vivo* data were analysed using Prism Software V10 and one-way ANOVA with multiple column comparisons. In all experiments * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Supplementary figures

Figure S1: PP2A phosphatase acts as ON switch to licence NLRP3 inflammasome responses in primary mouse macrophages. It has a modest effect on AIM2 and no effect on NLRC4 inflammasome. (a) BMDMs were primed with 100 ng/ml LPS for 4 h, followed by stimulation with 5 μM nigericin for 1 h, after which supernatants were collected and cells lysed. Cells were treated with three different batches of PP2A inhibitor okadaic acid at 1 μM (PP2A inh 1: okadaic acid, Cambridge BioScience; PP2A inh 2: InSolution okadaic acid, Sigma Aldrich; PP2A inh 3: okadaic acid Prorocentrum sp., Sigma Aldrich) 30 min before adding nigericin. **(b)** IL-1β cytokine secretion by BMDMs was measured in the supernatants using ELISA; **(c)** cytotoxicity of BMDMs was measured by LDH release into the supernatant and is presented as percentage of total cellular LDH (100% cell lysis control); **(d)** TNF cytokine secretion by BMDMs was measured in the supernatants using ELISA; Data are shown as mean + standard error of the mean from pooled results of four in (b, c)

or three independent experiments in (d) **(e)** TNF cytokine secretion by iBMDMs, after PP2A deletion using siRNA, was measured in the supernatants using ELISA. Data are mean + SD for one representative of three independent experiments **(f)** Lysates were analysed for PP2A knockdown by Western Blot. One representative blots of three independent experiments is shown **(g,h)** BMDMs were primed with 100 ng/ml LPS for 4 h, followed by transfection with calf-thymus DNA to activate AIM2 inflammasome for 4 h. IL-1β cytokine secretion and cytotoxicity were measured as described above. Data are mean + SD of triplicates of one experiment, representative of four. **(i,j)** BMDMs were primed with 100 ng/ml LPS for 4 h, followed by transfection with Salmonella Typhimurium Flagellin to activate NLRC4 inflammasome for 4 h. IL-1β cytokine secretion and cytotoxicity were measured as described above. Data are mean + SD of triplicates of one experiment, representative of four. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Figure S2: PP2A inhibition with LB-100 limits NLRP3 inflammasome responses in macrophages. (a) BMDMs were primed with 100 ng/ml LPS for 4 h, followed by stimulation with 5 μM nigericin for 1 h. LB-100 (10 μM) was added for 4 h (with LPS), 2 h, 1 h or 30 min before adding nigericin. Okadaic acid (PP2A inhibitor 1) was used at 1 μM added 30 min before nigericin. After stimulation, supernatants were collected and cells lysed. **(b)** IL-1β cytokine secretion was measured in the supernatant using ELISA; **(c)** cellular viability was measuring LDH release into the supernatant and is presented as percentage of total cellular LDH (100% cell lysis control); **(d)** TNF cytokine secretion was measured in the supernatant using ELISA; **(e)** IL-1β and caspase-1 were measured in supernatant (SN) and cell lysates (XT) using Western blot. Data are shown as mean + standard error of the mean from pooled results of four

(b, c) or three independent experiments (d, e) with one representative Western blot shown. (**f,g,h**) Mice were injected with LPS and ATP to activate NLRP3 inflammasome, in the presence or absence of LB-100, as described in Figure 2. Peritoneal myeloid cells were analysed using flow cytometry. Gating strategy and representative plots are shown. Bars are means of n=3 mice, n=4 mice (LPS+ATP) and n=4 mice (LPS+ATP+LB-100).

Figure S3: The PP2A activator DBK-1154 does not activate nor modulate the NLRP3 inflammasome pathway. (a) BMDMs were primed with 100 ng/ml LPS for 4 h, followed by stimulation with 2.5 μM or 5 μM nigericin for 1 h, after which supernatants were collected for cytokine analysis. Cells were treated with 5 μM of the PP2A activator DBK-1154 30 min before adding nigericin. **(b)** IL-1β cytokine secretion was measured in the supernatants using ELISA. **(c)** cellular viability was measured by LDH release into the supernatant and is presented as percentage of total cellular LDH (100% cell lysis control). **(d)** TNF cytokine secretion was measured in the supernatants using ELISA. Data are shown as mean + standard deviation from one representative of four independent experiments in (b,c,d). (**e**) PP2A phosphatase activity was assessed in iBMDMs using a phosphatase assay kit (Promega, V2460) with a vehicle control or 10 μM DBK-1154 for 1 h. Data is shown as mean + SD from one experiment.

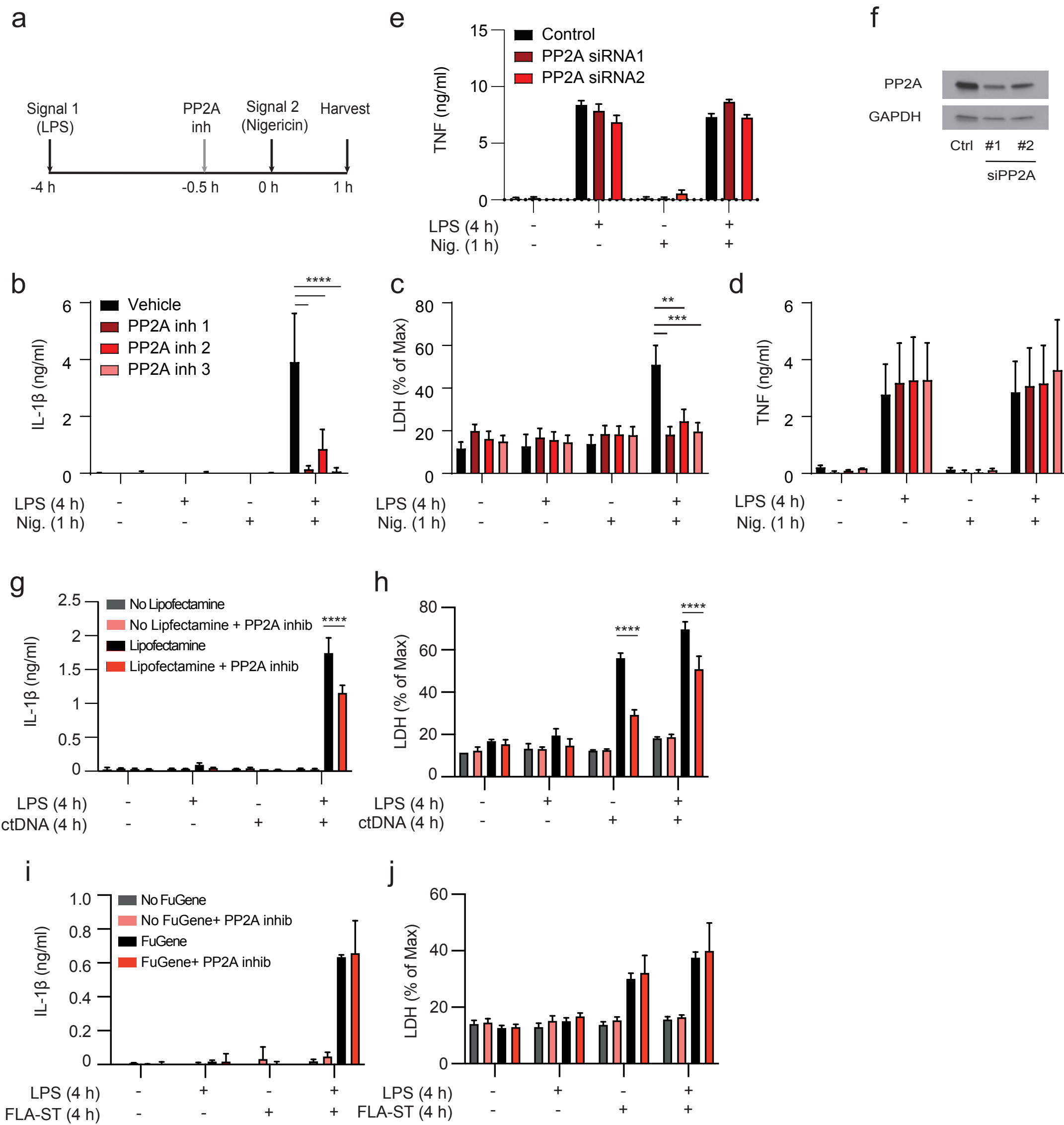
Figure S4: TBK1 inhibitors do not affect AIM2 and NLRC4 responses. (a,b,c) BMDMs were primed with 100 ng/ml LPS for 4 h, and iBMDMs were primed with 1 μg/ml LPS for 4 h followed by transfection with calf-thymus DNA (ctDNA) to activate AIM2 inflammasome for 1 h or 4 h. TBK1 inhibitor (MRT 68601 at 10 μM) was added 30 min before transfection of DNA. IL-1β cytokine and cellular viability were measured.

Data are mean + SD of triplicates of one experiment, representative of two. (**d,e**) BMDMs were primed with 100 ng/ml LPS for 4 h, and iBMDMs were primed with 1 μg/ml LPS for 4 h followed by transfection with Salmonella Typhimurium Flagellin (FLA-ST) to activate NLRC4 inflammasome for 1 h or 4 h. TBK1 inhibitor (MRT 68601at 10 μM) was added 30 min before transfection of Flagellin. IL-1β cytokine and cellular viability were measured. Data are mean + SD of triplicates of one experiment, representative of two.

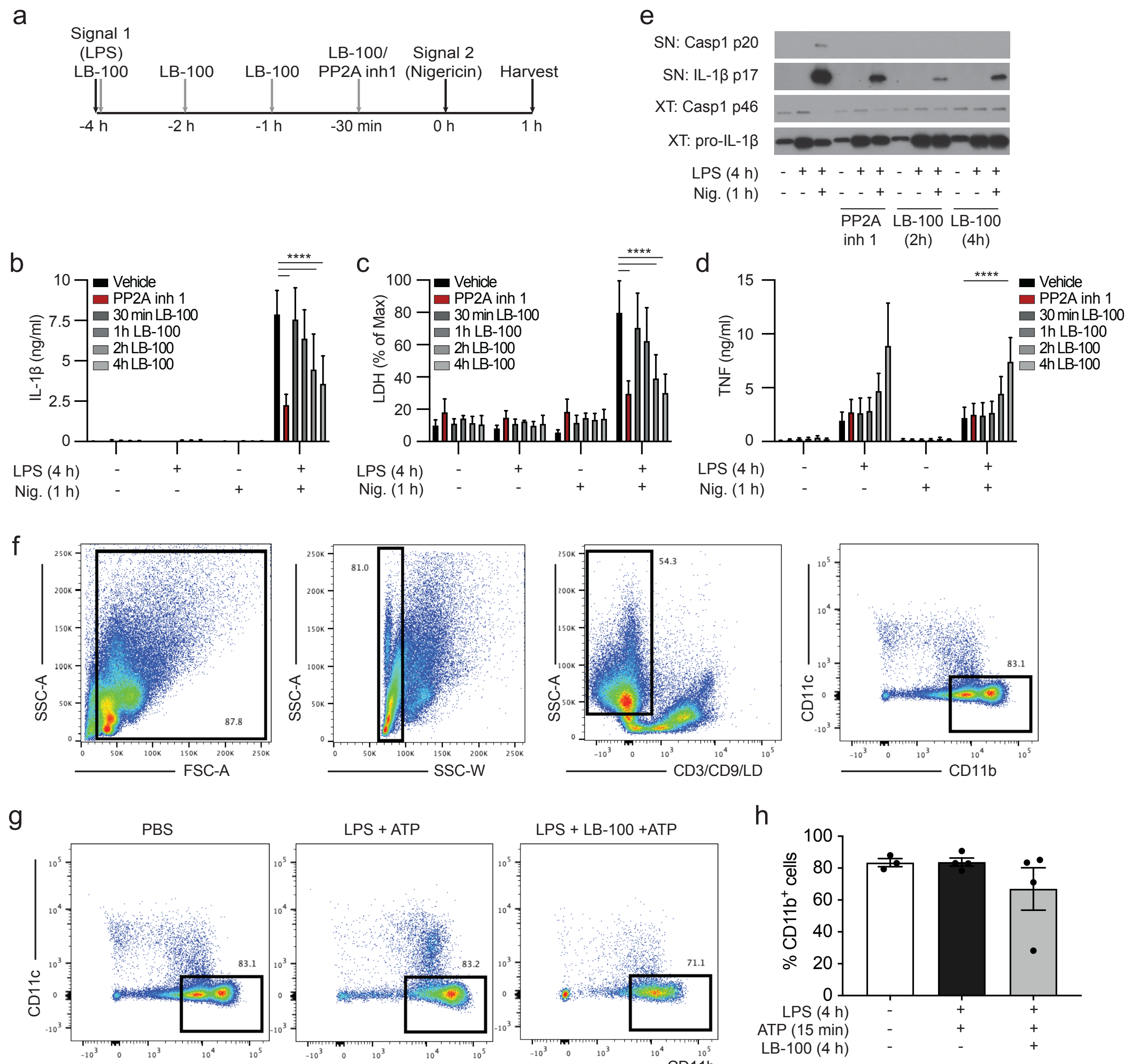
Figure S5: TBK1 deletion alone does not boost NLRP3 responses. (a,b) WT, TNF/TBK1 HET or TNF/TBK1 DKO BMDMs were primed with 100 ng/ml LPS for 4 h and stimulated with 2.5 μM nigericin for 1 h. Inflammasome responses were monitored by Caspase-1 cleavage or LDH release assay. One representative experiment of two is shown. Bars are means of triplicates + SD. **(c)** WT or IKKε KO iBMDMs were treated with TBK1 siRNA to generate single TBK1 KO or TBK1/IKKε double KO. Inflammasome responses were monitored by Caspase-1 cleavage. One representative experiment of three is shown.

Figure S6: TBK1 interacts with NLRP3 in LPS-stimulated macrophages. (a) PMA-differentiated THP1 macrophages were stimulated with LPS for 30 min, 60 min or 4 h. Interaction between endogenous phospho-TBK1 and NLRP3 was analysed by PLA. Unstimulated macrophages, or LPS stimulated macrophages stained with mouse IgG1 isotype control antibodies or PLA probes only served as specificity controls. **(b)** PLA signals were quantified using Fiji and the ratio between nuclei and PLA was calculated. One representative experiment of two is shown.

Figure S7: Serine 3 mutants are equally expressed in retrovirally transduced NLRP3 KO BMDMs. BMDMs from NLRP3 KO mice were transduced with GFP-labelled viruses containing only a FLAG-tag (control) or FLAG-tagged NLRP3 constructs. Transduction efficiency was measured by FACS analysis of GFP-positive BMDMs at day 6 of differentiation.

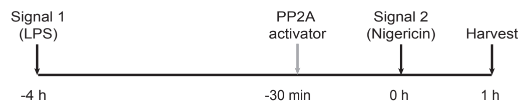


Suppl. FIGURE 1

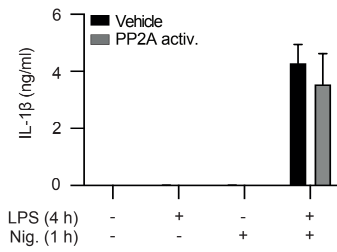


Suppl. FIGURE 2

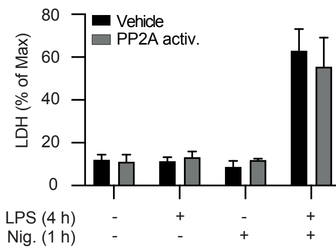
a



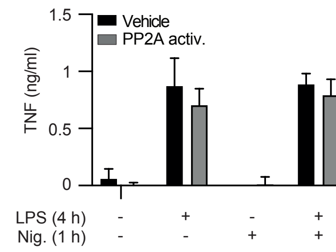
b



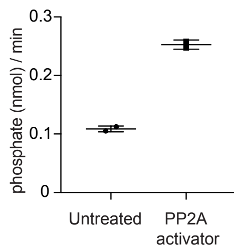
c



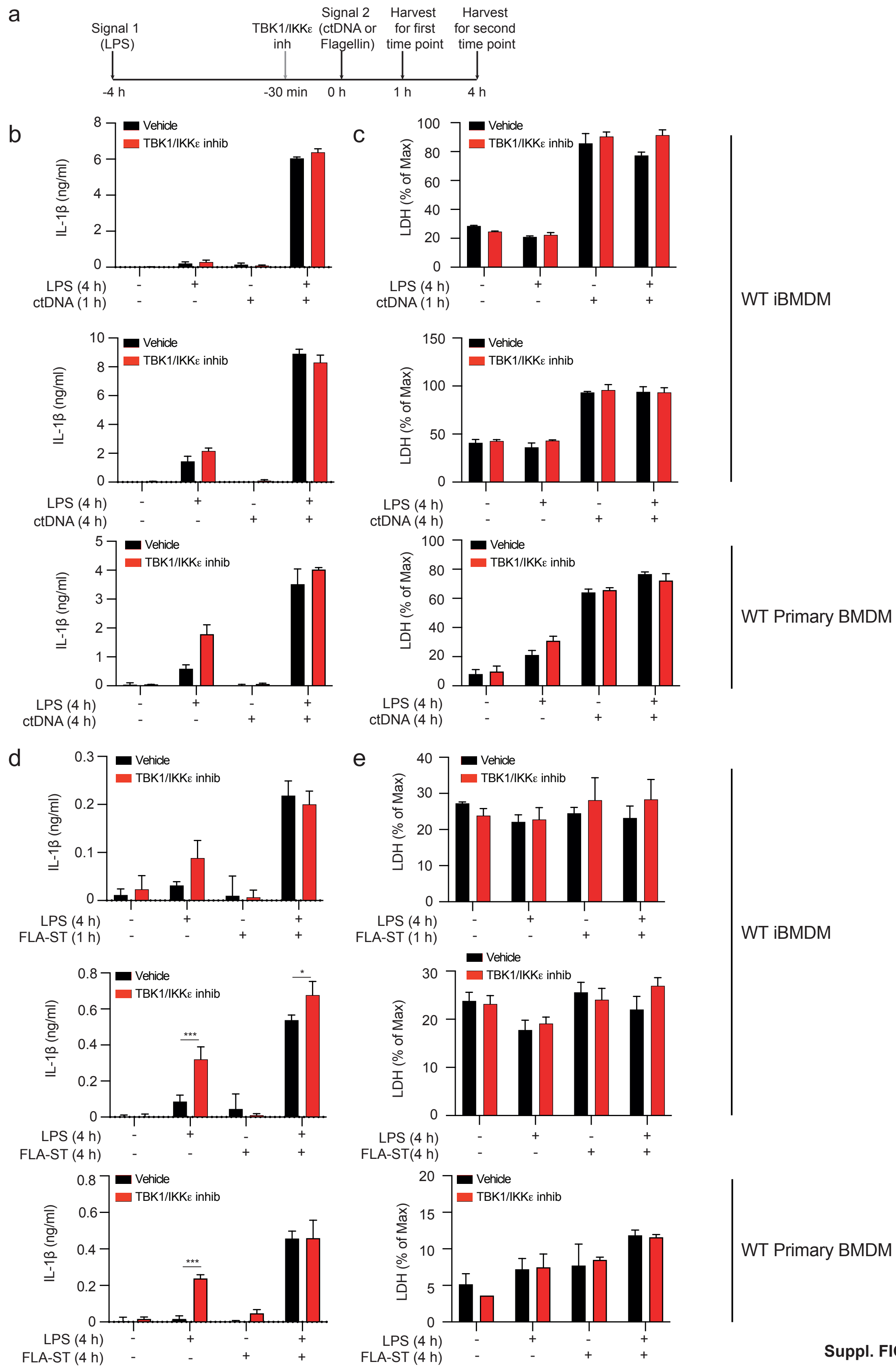
d



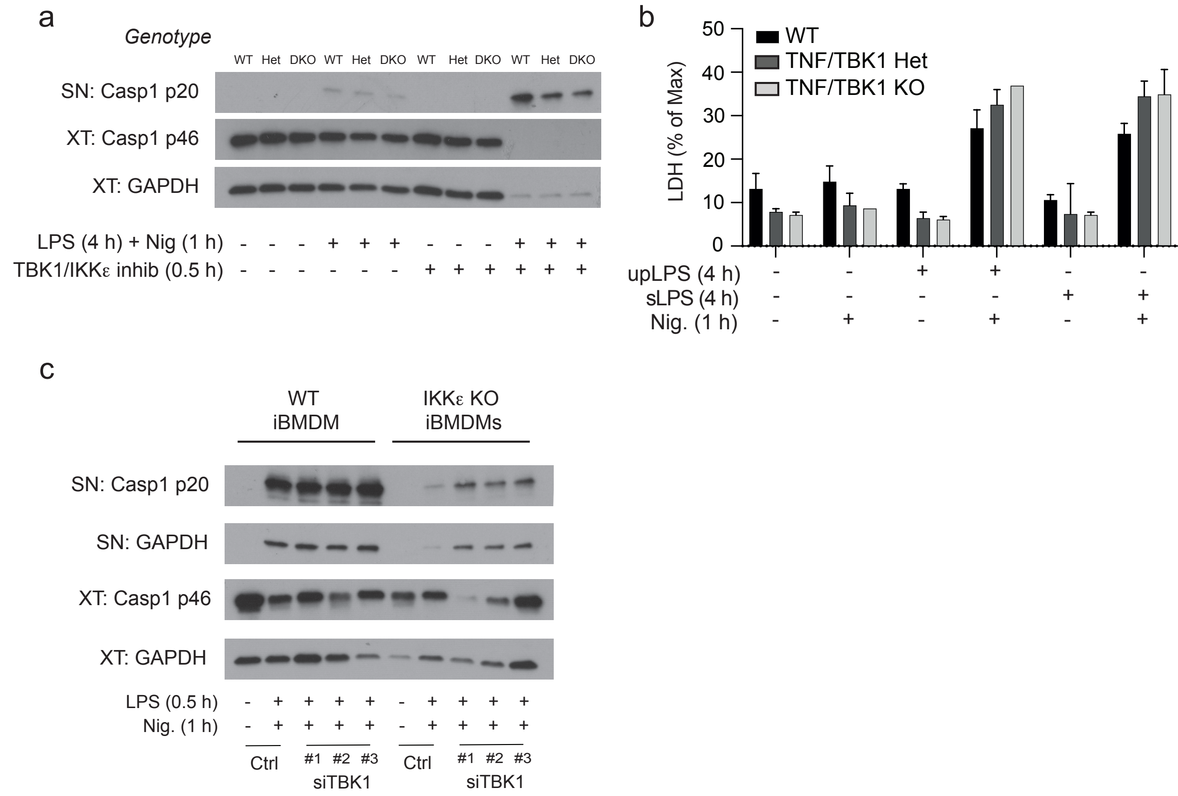
e



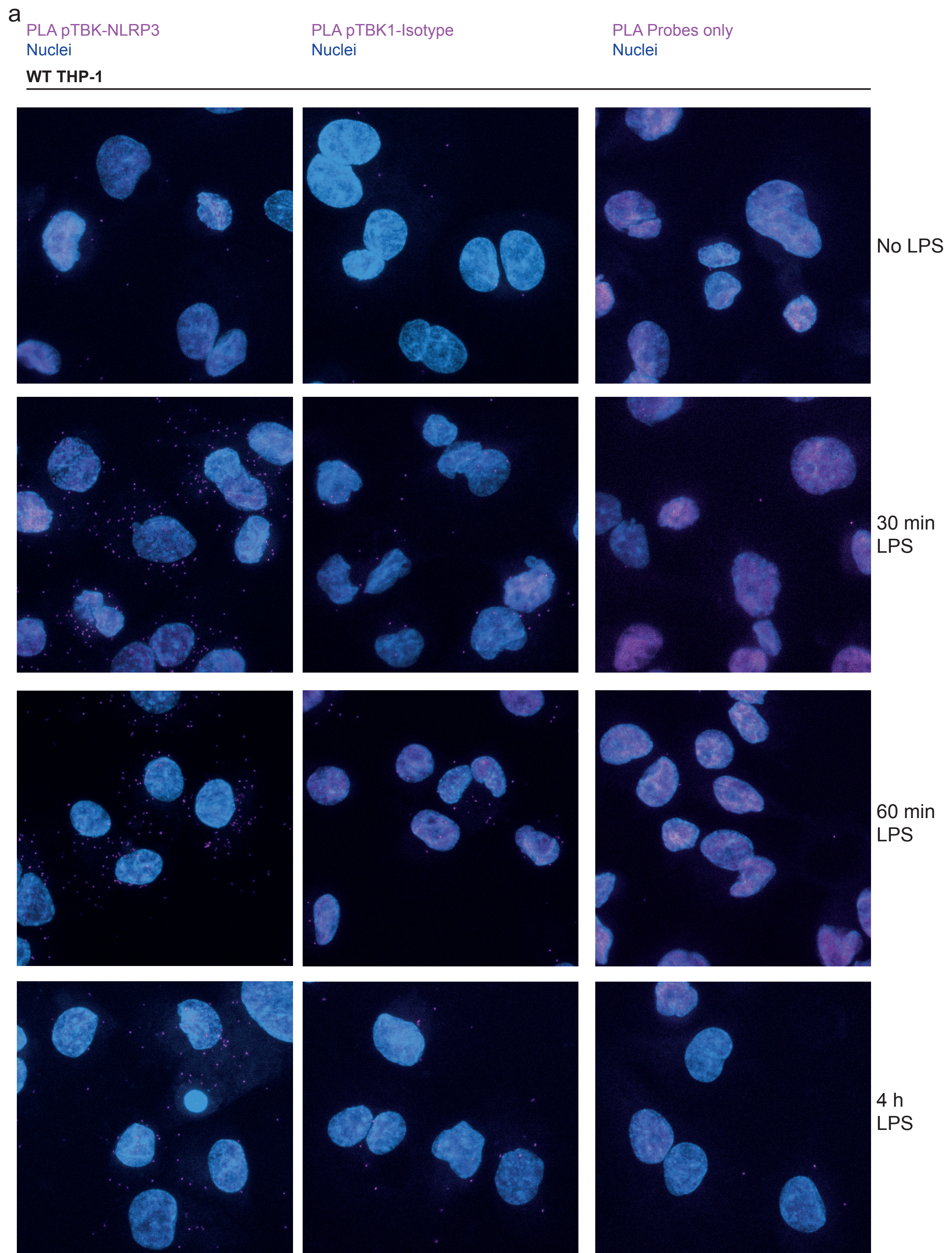
Suppl. FIGURE 3



Suppl. FIGURE 4

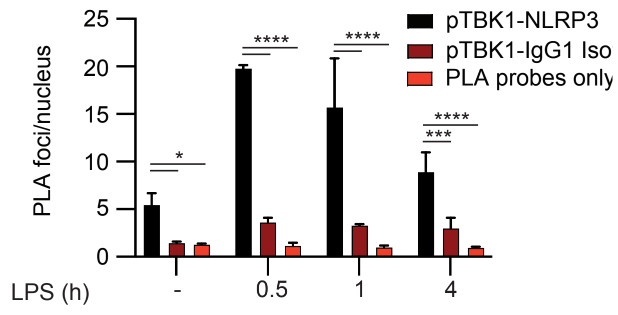


Suppl. FIGURE 5

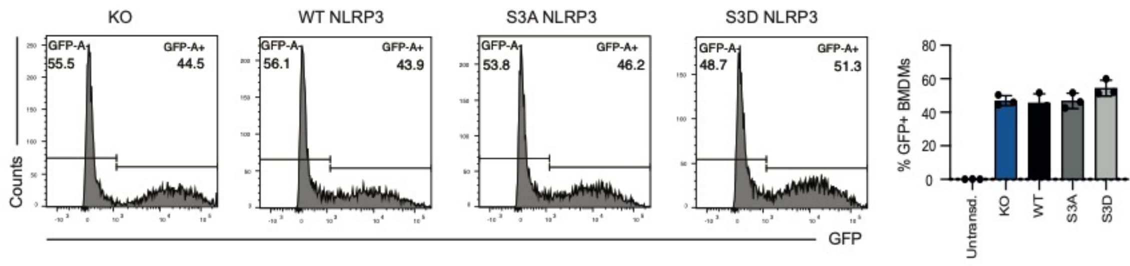


Suppl. FIGURE 6 part 1

b



Suppl. FIGURE 6 part 2



Suppl. FIGURE 7