Supplemental Experimental Procedures

NF-κB restricts NLRP3 inflammasome activation via p62-dependent mitophagy which eliminates mitochondrial signals

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Mice

LysM-Cre mice were purchased from Jackson Laboratories and were crossed with $p62^{F/F}$ and $Atg7^{F/F}$ mice (Komatsu et al., 2005; Muller et al., 2013) to generate $p62^{\Delta Mye}$ and $Atg7^{\Delta Mye}$ mice, respectively. $p62^{\Delta Mye}$ and $Atg7^{\Delta Mye}$ mice and their wild-type littermates ($p62^{F/F}$ and $Atg7^{F/F}$, respectively) were used in all studies. Whole body $p62^{-/-}$ mice were previously described (Rodriguez et al., 2006). $Nlrp3^{A350V/+}CreT$ mice were as previously described (Brydges et al., 2013). All mice were bred and maintained at University of California San Diego (UCSD) and were treated in accordance with guidelines of the Institutional Animal Care and Use Committee of UCSD.

Reagents

Silica and ATP were from Sigma-Aldrich. Ultrapure LPS (*E. coli O111:B4*) was from Invivogen. Monosodium urate crystal (MSU) was from Enzo Life Science. Imject Alum and streptavidin-HRP were from Pierce. MitoSOX and Mitotrackers were from Life Technologies. TMRM was from AnaSpec Inc. (#CA94555). ML120b was from Tocris (#4899). The lactate dehydrogenase (LDH) assay kit was from Roche. 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) liposomes were made by Encapsula NanoSciences as previously described (Zhong et al., 2013). Antibodies used for immunoblot analysis were: anti-mouse IL-1β (#12426S, Cell Signaling Technologies), anti-mouse NLRP3 (#AG-20B-0014-C100, Adipogen), anti-mouse ASC (#AG-25b-0006-C100, Adipogen), anti-mouse caspase-1 p20 (# AG-

20B-0042-C100), anti-mouse OPTN (#10837-1-AP, Proteintech), anti-mouse Tax1bp1 (#14424-A-AP, Proteintech), anti-mouse Calcoco2/Ndp52 (#12229-1-AP, Proteintech), anti-mouse NBR1 (#9891S, Cell Signaling Technologies), anti-mouse Parkin (#sc-30130, Santa Cruz Technologies), anti-VDAC (# 4661S, Cell Signaling Techonologies), anti-p62 (#GP62-C, ProGen), anti-Atg7 (#8558, Cell Signaling Technologies), anti-LC3 (#2775, Cell Signaling Technologies) and anti-tubulin (#T5168, Sigma-Aldrich).

Cell culture and stimulation

Primary bone marrow-derived macrophages (BMDM) were generated by culturing mouse bone marrow cells in the presence of 20% vol/vol L929 conditional medium for 7 days as described (Hornung et al., 2008). BMDMs were seeded in 6-well, 24-well or 48-well plates overnight. On the 2nd day, after pretreatment with ultrapure LPS (200 ng/ml) for 4 hrs, BMDM (1x10^{6} cells/ml) were further stimulated with ATP (4 mM) or Nigericin (10 μM) for 60 min unless otherwise indicated or DOTAP liposomes (100 μg/ml), alum (500 μg/ml), silica (600 μg/ml) and MSU (600 μg/ml) for 6 hrs. For AIM2 inflammasome activation, macrophages were primed with LPS (200ng/ml) followed by transfection of poly(dA:dT) using Lipofectamine 2000 (Life Technologies) according to the manufacture's protocol. For NLRP1b inflammasome activation, BMDM were infected with WT or lethal toxin deleted mutant (ΔLT) B. anthracis at MOI of 2. Culture supernatants were collected 6 hrs post infection and IL-1β release was measured by ELISA. Macrophages with a constitutively active mutant NLRP3 (Nlrp3-A350V) were generated by treating BMDM from *Nlrp3*$^{A350V/+}$*CreT* mice with 1 μM 4-hydroxytamoxifen (Sigma-Aldrich) for 24 hrs. Supernatants and cell lysates were collected for ELISA and immunoblot analyses. Immortalized murine BMDM (iBMDM) from *C57Bl/6 (*WT*)* was generously provided by Dr. Katherine Fitzgerald. Knock down of p62, Atg7, or Parkin was done in WT iBMDM using specific shRNAs (from Sigma shRNA Mission library), and the sequences are as follows: shp62#1 (CCGGGAGGTTGACATTGATGTGGAACTCGAGTTCCACATCAATGTCAACCTCTTTTTG);shp62#2 (CCGGTAGTACAACTGCTAGTTATTTCTCGAGAAATAACTAGCAGTTGTACTATTTTTG); shAtg7#1 (5'-CCGGCCAGCTCTGAACTCAATAATACTCGAGTATTATTGAGTTCAGAGCTGGTTTTTG-3'); shAtg7#2

(5'-CCGG-GCCTGGCATTTGATAAATGTACTCGAGTACATTTATCAAATGCCAGGCTTTTTG-3'); shPark2#1(5'-CCGGCGGAGGATGTATGCACATGAACTCGAGTTCATGTGCATACATCCTCCGTTTTTG-3'); shPark2#2(5'-CCGGCGTGATCTGTTTGGACTGTTTCTCGAGAAACAGTCCAAACAGATCACGTTTTTG-3'). Mouse neutrophils were isolated from bone marrows of *p62*$^{F/F}$ and *p62*$^{\Delta Mye}$ mice using EasySep™ Mouse Neutrophil Enrichment Kit (Stem Cell Technologies), and stimulated the same way as BMDM described above. Knocking down of p62 expression in NLRP3 mutant (Nlrp3-A350V) macrophages was achieved by lentiviral infection of mutant BMDM at day 3 after differentiation. Cells were analyzed after puromycin selection at day 7.

Enzyme-linked immunosorbent assay

Paired (capture and detection) antibodies and standard recombinant mouse IL-1β (from R&D Systems) and TNF (from eBioscience) were used to determine cytokines concentrations in cell culture supernatants and mouse sera according to manufacturer's instructions.

Measurement of active caspase-1 by FLICA assay

The levels of active caspase-1 were quantified using an inhibitor-based, fluorescent probe (FLICA™ FAM-YVAD-FMK, #98, ImmunoChemistry Technologies) that specifically recognized active caspase-1. Briefly, macrophages were primed with LPS (100 ng/ml) followed by treatment with ATP or nigericin for 45 min or alum, silica, MSU or DOTAP liposomes for 5 hrs. Then the cells were washed twice with PBS and loaded with 4 μM of FLICA™ FAM-YVAD-FMK for 40 min and washed twice with PBS. Fluorescence intensity was determined using a FilterMax F5 multimode plate reader (Molecular Devices) and the data were normalized to LPS-primed but NLRP3 agonist-untreated controls.

Immunoblot analysis

Cells were lysed in RIPA buffer (25 mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Roche, #11836153001) and phosphatase inhibitor cocktail (Sigma-Aldrich, #P5726). Protein concentrations were quantified using BCA Protein Assay Kit (Pierce, #23225). Equal amounts of protein were loaded into SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were then incubated with antibodies against p62, NBR1, NDP52, OPTN, TAX1BP1, ATG7, Parkin, ASC, NLRP3, VDAC, α-Tubulin, caspase-1 and IL-1β (as described above), followed by appropriate secondary HRP–conjugated antibodies, and then developed with ECL.

Mitochondrial ROS detection

Mitochondrial ROS were measured using MitoSOX. Briefly, macrophages were first primed with LPS (100 ng/ml) followed by treatment with ATP or nigericin for 45 min or alum, silica, MSU or DOTAP liposomes for 3 hrs, after which the cells were washed twice with PBS and loaded with 4 μM of MitoSOX for 20 min and washed twice with PBS. Fluorescence intensity was determined using a FilterMax F5 multimode plate reader (Molecular Devices), and the data were normalized to LPS-primed but NLRP3 agonist-untreated controls.

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential (Ψm) was measured using TMRM. Briefly, macrophages were primed with LPS (100 ng/ml) followed by treatment with ATP or nigericin for 30 min or alum, silica, MSU or DOTAP liposomes for 3 hrs, after which the cells were washed twice with PBS and loaded with 200 nM of TMRM for 30 min and washed twice with 50 nM TMRM. The cells were resuspended in PBS and fluorescence intensity was measured with a FilterMax F5 multimode plate reader (Molecular Devices). The data were normalized to LPS-primed but NLRP3 agonist-untreated controls.

Cellular fractionation and measurement of cytosolic mtDNA

Macrophages were first primed with LPS (100 ng/ml) followed by treatment with ATP or nigericin for 60 min or other NLRP3 agonists for 5 hrs. Cellular fractionation was then performed using a mitochondrial isolation kit for culture cells (#89874, ThermoScientific) according to manufacturer's instructions. Measurement of cytosolic mtDNA was as previously described (Nakahira et al., 2011) by using the mitochondrial isolation kit above. DNA was isolated from 300 µl of the cytosolic fractions and mitochondrial DNA encoding cytochrome c oxidase 1 was measured by quantitative real-time PCR with same volume of the DNA solution. Nuclear DNA encoding 18S ribosomal RNA was used for normalization. Primers' sequences are as previously described (Nakahira et al., 2011).

Immunofluorescent staining and confocal microscopy

BMDM were seeded at 0.2 x 10^6 cells per well in 8 well glass slides, and rested overnight for proper attachment. Then, the cells were primed with LPS (10 ng/ml) for 2 hrs, and treated with ATP or nigericin for 30 min, or other NLRP3 agonists for 2 hrs. After the treatment, the cells were washed twice with sterile PBS and fixed with 4% paraformaldehyde (PFA), permeabilized with 0.01% Triton X-100 and blocked in 2% BSA and 1% donkey serum. The cells were then incubated overnight with primary antibodies, including anti-p62 (#GP62-C, ProGen), anti-TOM20 (#sc-11415, Santa Cruz Technologies), anti-poly-ubiquitin (#D071-3, MBL), anti-GFP (#ab13970, Abcam). For experiments that used mitotracker instead of Tom20 antibody, cells were stained with mitotracker red (#M-7512, Life Technologies) prior to PFA fixation. Secondary fluorescent antibodies (Alexa-488, -594 or -647, from Life Technologies or Jackson Laboratories) were added for 1 hr and DAPI was used for nuclear counterstaining. Samples were imaged through a SP5 confocal microscope (Leica) 24 hrs after mounting.

Electron microscopy

After LPS priming and NLRP3 agonist stimulation, macrophages were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 pre-warmed up at 37°C for 5 minutes at room temperature followed by 1 hr over ice. All solutions and steps from this point on were utilized and performed at 4°C. After fixation cells

were rinsed 5 times for 1 minute each in 0.1 M cacodylate buffer, then post-fixed in 2% OsO_4 in 0.1 M cacodylate buffer for 30 minutes. After 2 washes for 1 minute each in 0.1 M cacodylate buffer and 3 washes for 1 minute each in double-distilled water, cells were *en bloc* stained with 2% aqueous uranyl acetate overnight at 4°C. After 5 rinses for 1 minute each in double-distilled water cells were dehydrated in a cold graded ethanol series (20%, 50%, 70%, 90%, 100%) for 3 minutes each on ice, then rinsed once in room temperature 100% ethanol, and embedded in Durcupan ACM resin (Electron Microscopy Sciences). Sections were cut with a diamond knife at a thickness of 70-90 nm. The sections were examined using a JEOL 1200 EX at 80 kV. Images were recorded on film at either 4000 x or 10,000 x magnification. The film negatives were digitized using a FlexTight scanner at either 1000 or 1500 dpi. To avoid bias, the entire population of mitochondria in each image (number of images = 10) was examined to count the number of abnormal mitochondria. The percentage of abnormal mitochondria was determined by dividing the number of abnormal mitochondria by the total number of mitochondria per image. The mean, standard error of the mean (SEM) and Student's t-test p values are reported.

p62 reconstitution in *p62$^{\Delta My e}$* macrophages

p62$^{\Delta My e}$ macrophages were transduced with viral supernatants corresponding to the pMigR1-p62 and pMigR1-p62ΔUBA retroviral vectors obtained as previously described(Wooten et al., 2005). Viral supernatants were filtered through a 0.45-µm-pore-size filter (Millipore) and supplemented with Polybrene (8 µg/ml) before adding to cells. Four days after viral transduction, cells were analyzed for p62 and p62ΔUBA localization by confocal microscopy after LPS priming followed by NLRP3 agonist stimulation.

Depletion of mitochondria-derived NLRP3 inflammasome-activating signals

For mtDNA depletion, *p62$^{F/F}$* and *p62$^{\Delta My e}$* BMDM were treated with 450 ng/ml of ethidium bromide (EtdBr) for 4 days as previously described (Rongvaux et al., 2014). For inhibition of mitochondrial protein synthesis, the above cells were treated with chloramphenicol (200 µg/ml) as previously described

(Shimada et al., 2012). Cells were then primed with LPS followed by stimulation with NLRP3 agonists. After treatment, cells and culture supernatants were collected for measuring active caspase-1 by FLICA assay and IL-1β and TNF by ELISA, respectively.

Septic shock model

Septic shock was induced by the intraperitoneally (i.p.) injecting 8-12 weeks old sex-matched *p62*$^{\Delta Mye}$ and *Atg7*$^{\Delta Mye}$ mice or their wild-type littermates (*p62*$^{F/F}$ and *Atg7*$^{F/F}$, respectively) with LPS (*E. coli O111:B4,* Sigma-Aldrich) at 30 mg/kg body weight. Mouse survival was monitored every 6 hrs post injection for a total of 72 hrs. In separate experiments, mice were treated with the same dose of LPS and immune sera were collected 3 hrs post injection. Serum IL-1β and TNF were measured by ELISA.

Peritonitis model

Peritonitis was induced by i.p. injection of PBS or 300 μg alum (dissolved in 0.2 ml sterile PBS) into 8-12 weeks old sex-matched *p62*$^{\Delta Mye}$ and *Atg7*$^{\Delta Mye}$ mice and their wild-type littermates (*p62*$^{F/F}$ and *Atg7*$^{F/F}$, respectively). 15-18 hrs after injection, mice were euthanized and the peritoneal cavities were washed with 6 ml cold sterile PBS. Total recruited peritoneal exudate cell (PEC), neutrophils ($CD11b^+Ly6G^+F4/80^-$) and monocytes ($CD11b^+Ly6C^+Ly6G^-$) present in the peritoneal lavage fluid were quantified by flow cytometry. For blocking of Fc-mediated interactions, mouse cells were pre-incubated with 0.5-1 μg of purified anti-mouse CD16/CD32. Isolated cells were stained with labeled antibodies in PBS with 2% FCS and 2 mM EDTA or cell staining buffer (Biolegend). Dead cells were excluded based on staining with Live/Dead fixable dye (eBioscience). Antibodies specific for the following antigens were used: CD45(hOKT4), CD11b(M1/70), CD11c(N418), MHCII(M5/114.15.2), Gr-1(1A8-l66g), Ly6C(HK1.4), and Ly6G(1A8-Ly6g) (all from eBioscience). Cells were analyzed on a Beckman Coulter Cyan ADP flow cytometer. Data were analyzed using FlowJo software (Treestar).

Fulminant hepatitis model

10-12 weeks old mice were i.p. administered with LPS (Sigma-Aldrich; 0.5 μg/mouse) and D-galactosamine hydrochloride (D-Gal, from Sigma-Aldrich; 20 mg/mouse). D-Gal is an inhibitor of hepatic RNA synthesis, and induces hepatic damage and macrophage infiltration. When given with a low dose of LPS, D-Gal strongly sensitizes mice to produce apoptotic liver injury with severe hepatic congestion, resulting in fulminant hepatitis. Mice were euthanized at 6 hrs after LPS/D-Gal injection, blood and liver samples were then collected for pathological and immunological analyses. 6-10 mice per each group were used for this experiment.

Statistics

All data are shown as means ± s.d. or means ± s.e.m. as indicated. Statistical analysis was performed using a two-tailed Student's t-test or log-rank test (for survival analysis). For all tests, P-values lower than 0.05 were considered statistically significant.

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