Supplementary Methods

Mice

Mice were treated in accordance with the Swiss Federal Veterinary Office guidelines (Switzerland) and under conditions approved by the Walter and Eliza Hall Institute Animal Ethics Committee (Australia). All mice were generated on or backcrossed onto a C57BL/6-WEHI background. H2K-Bcl-2 transgenic[1], Vav-Bcl-2 transgenic[2], Nlrp3−/−[3], Caspase-1−/−[4], Pycard−/−[5], MAVS−/−[6], Park2−/− (Jackson Laboratories[6]), Cyclophilin D−/−[7], Ripk3−/−[8], Mlk−/−[9], Caspase-8fl/Y/lo[10] and Bok−/−[11] mice have all been previously described. Non-transgenic littermates (WT) were used as controls in studies with Bcl-2 transgenic mice. To generate Bak−/−Bax−/−[12] or Caspase-9−/−[13] BMDMs, lethally irradiated C57BL/6-Ly5.1 mice were reconstituted with Bak−/−Bax−/− or Caspase-9−/− fetal liver cells. At 8-10 weeks post-reconstitution, peripheral blood was stained for Ly5.1 and Ly5.2 to assess the extent of hematopoietic reconstitution by donor-derived (Ly5.2) cells, which consistently was >95%.

Reagents

The primary antibodies used were as follows: Actin (Sigma), Parkin (Cell Signaling), MAVS (Cell Signaling), phospho-p65 (Cell Signaling), IκBα (Cell Signaling), Cyclophilin D (MitoSciences), Caspase-1 (Adipogen), IL-1β (RnD systems), PARP (Cell Signaling), Caspase-3 (Cell Signaling), Bax (Cell Signaling), Bak (Millipore), Cytochrome C (Pharmingen), RIPK3 (Axxora), Smac/DIABLO, Caspase-8 (clone 3B10: Enzo Life Sciences), MLKL (all in house). Other reagents used were ultra-pure LPS, poly(I:C), poly(dA:dT), R837 (all from Invivogen), Alum (thermo scientific), MSU and ABT-737 (both in house), MitoTracker Red and MitoTracker Green (Life technologies, Invitrogen). Staurosporine, cycloheximide, Cyclosporin A, ATP and nigericin were purchased from Sigma.
**Western blot analysis**

Cells were lysed on the plate with reducing sample buffer. Reduced and boiled cell supernatants and lysates were run on 8%, 12% or 4-12% gradient polyacrylamide gels (Invitrogen) and proteins transferred to nitrocellulose (Amersham) membranes for detection. Membranes were blocked with 5% skim milk in PBST (PBS containing 0.05% Tween 20) for 0.5-1 h and all primary antibody incubations performed overnight and secondary HRP-conjugated antibody incubations performed for 1-2 h (diluent 5% skim milk PBST). Membranes were washed 4-6 times (5-30 min/wash) in PBST after all antibody incubations.

**IFNβ RT-PCR**

BMDMs (1x10^6) were transfected with 5 µg/mL poly(I:C) (lipofectamine) for 6 h. Total RNA was purified and RT-PCR performed to measure IFNβ mRNA levels. All values were normalized against 18sRNA.

**Mitochondrial membrane and cytosolic fractionation**

BMDMs were washed in ice-cold PBS and harvested in isotonic mitochondrial buffer (MB: 210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM HEPEs, pH 7.5) supplemented with a complete protease inhibitor cocktail (Roche). Cells were homogenized for ~80 strokes with a Dounce homogenizer, transferred to Eppendorf tubes and centrifuged at 500g for 5 min at 4°C to eliminate nuclei and intact cells. The resulting supernatant was centrifuged at 10,000g for 30 min at 4°C to obtain a heavy membrane pellet enriched for mitochondria. The supernatant (cytosol) and pellet (mitochondria) were subsequently analysed by Western blot.

**Measurements of mitochondrial number and respiration**

MitoTracker Green was used to measure mitochondrial numbers as previously described [14]. Wild type and Parkin-KO (Park2) BMDMs (1x10^6) were treated with 20 ng/mL LPS for 3 h and stimulated for 1 h with CCCP (10 µM), as indicated in the figure legend. Cells were then incubated with 200 nM Mitotracker green (detects total mitochondria) or Mitotracker red (detects respiring mitochondria) for 15 min and analysed by flow cytometry. The gating strategy is presented in Fig. S2.
References


cell-survival-or-death-/flow-cytometric-measurement-of-cell-organelle-autophagy.