Cell

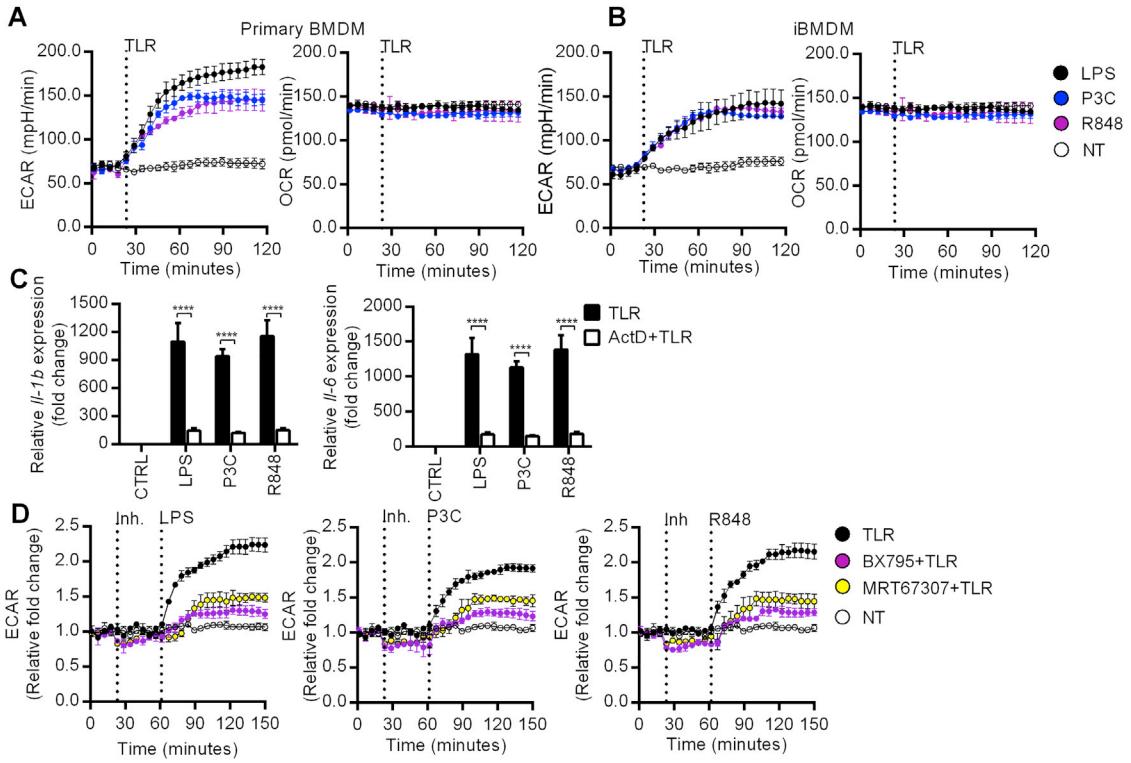

Figure S1. TBK1 Promotes TLR-Induced Rapid Glycolytic Bursts, Related to Figure 1

(A and B) Real-time responses in the ECAR and OCR from BMDMs (A) and iBMDMs (B) treated or not (NT) with indicated TLR ligands. Shown are the un-normalized data from the Seahorse analyzer. Data represent mean ± SEM of triplicate wells. Shown is one representative experiment out of three independent experiments.

(C) BMDMs were pretreated or not with Actinomycin D (ActD) for 40 min, followed by stimulation with indicated TLR ligands for an additional 4 hr. *Il-1b* and *Il-6* transcripts were determined by qPCR.

(D) TLR induced real-time changes in the ECAR of BMDMs stimulated with the TLR ligands indicated, with or without TBK1-IKKε inhibitors (BX795 or MRT67307), or left untreated (NT) were measured by the Seahorse assay. The readout of ECAR is shown as relative fold change in comparison to the basal levels before inhibitor treatment, which is normalized to 1 by the Seahorse analyzer. Data represent mean ± SEM of triplicate wells. Shown is one representative experiment out of three independent experiments.

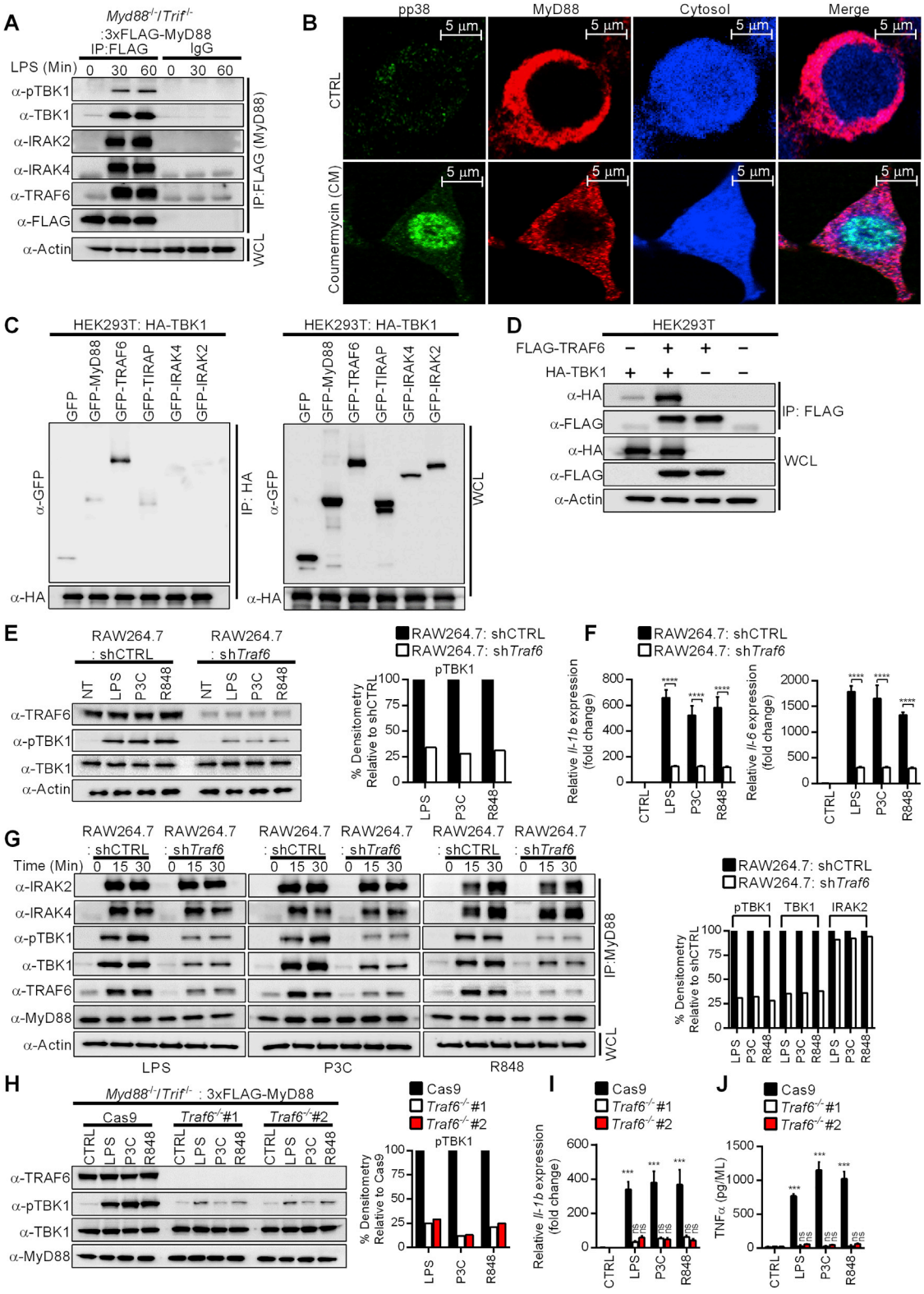

Figure S2. TRAF6 Recruits TBK1 to the Myddosome, Related to Figure 2

(A) 3xFLAG-MyD88-expressing *Myd88*$^{-/-}$/*Trif*$^{-/-}$ iBMDMs were stimulated with LPS for the times indicated. Components of the myddosome were determined by western analysis of M2 anti-FLAG antibody immunoprecipitates. Mouse IgG1 was used as IgG control.

(legend continued on next page)

(B) *Myd88*$^{-/-}$/*Trif*$^{-/-}$ iBMDMs expressing 3xFLAG-MyD88-GyrB were stimulated with CM for 30 min and fixed. Cells were stained with antibodies specific for FLAG (for MyD88) and pp38. Cytosol was visualized by expression of the IRES-GFP from the retroviral vector and was pseudo colored in blue. Images are representative of at least three independent experiments where more than 100 cells were examined per condition. The scale bar represents 5 μm.
(C) 293T cells were co-transfected with plasmids encoding HA-TBK1 and the indicated GFP-tagged myddosome components in a pairwise manner. 24 hr after transfection, cells were lysed. TBK1 was isolated via an HA-specific antibody, and the immunoprecipitates were analyzed by western blot.
(D) 293T cells were transfected with plasmids encoding HA-TBK1 and/or FLAG-TRAF6 in the combinations indicated. 24 hr after transfection, cells were lysed. TRAF6 was isolated via the M2 FLAG antibody and the immunoprecipitates were analyzed by western blot.
(E) RAW264.7 cells expressing shTRAF6 and shCTRL were stimulated with TLR ligands for 15 min and lysed. pTBK1, TBK1, TRAF6 and Actin were detected by western analysis (left). Quantification of TLR-induced TBK1 activation was performed by ImageJ (right).
(F) RAW264.7 cells expressing shTRAF6 and shCTRL were stimulated with TLR ligands for 4 hr. mRNA was extracted. *Il-1b* and *Il-6* transcripts were analyzed by qPCR.
(G) RAW264.7 cells expressing shTRAF6 and shCTRL were stimulated with TLR ligands for the times indicated. MyD88 was immunoprecipitated and myddosome components were determined by western analysis (left). Quantification of myddosome-associated pTBK1, TBK1 and IRAK2 was performed by ImageJ (right).
(H) *Traf6*-sufficient (Cas9) and *Traf6*-deficient (*Traf6*$^{-/-}$#1 and *Traf6*$^{-/-}$ #2) iBMDMs were stimulated with TLR ligands (or not) for 15 min and lysed. The proteins indicated were detected by western analysis (left). Quantification of TLR-induced TBK1 activation was analyzed by ImageJ (right).
(I) *Traf6*-sufficient (Cas9) and *Traf6*-deficient (*Traf6*$^{-/-}$#1 and *Traf6*$^{-/-}$ #2) iBMDMs were stimulated with TLR ligands (or not) for 4 hr. mRNA was extracted. The induction of *Il-1b* transcript was analyzed by qPCR.
(J) *Traf6*-sufficient (Cas9) and *Traf6*-deficient (*Traf6*$^{-/-}$#1 and *Traf6*$^{-/-}$ #2) iBMDMs were stimulated with TLR ligands (or not) for 4 hr. Secreted TNFα was determined by ELISA.
For western analysis, each panel is a representative experiment of at least 3 independent repeats.

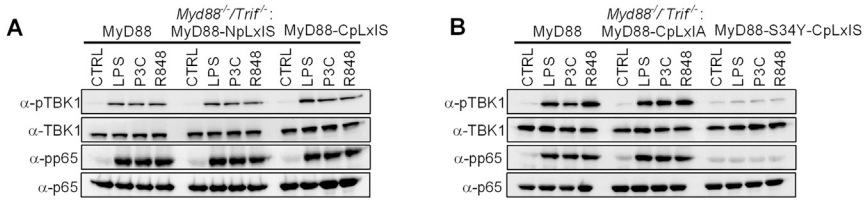

Figure S3. Synthetic Myddosomes Activate TBK1 and p65, Related to Figure 3

(A) *Myd88*$^{-/-}$/*Trif*$^{-/-}$ iBMDMs expressing MyD88, MyD88-NpLxIS and MyD88-CpLxIS were stimulated with TLR ligands for 90 min and lysed. The proteins indicated were examined by western blot.

(B) *Myd88*$^{-/-}$/*Trif*$^{-/-}$ iBMDMs expressing MyD88-CpLxIS and its mutant alleles were treated with TLR ligands for 90 min and lysed. The proteins indicated were examined by western blot.

For western analysis, each panel is a representative experiment of at least 3 independent repeats.

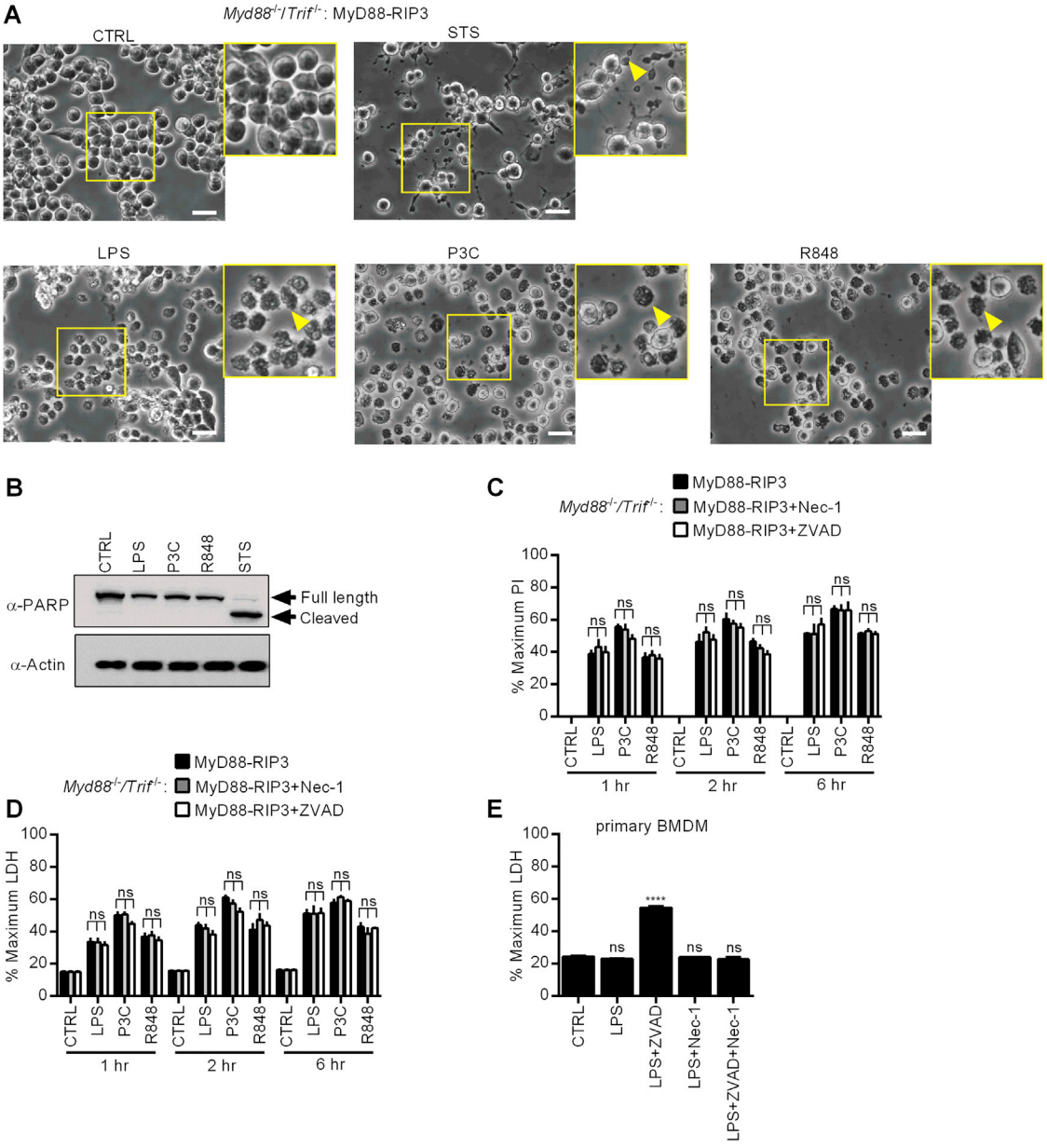

Figure S4. Cell Death Induced by MyD88-RIP3 Is Distinct from Apoptosis and Is Independent of RIP1, Related to Figure 4

(A) *Myd88*$^{-/-}$/*Trif*$^{-/-}$ iBMDMs expressing MyD88-RIP3 were treated with TLR ligands or staurosporine (STS) for 1 hr. Images of cell morphology were taken 1 hr post-stimulation. The arrow head highlights a dead cell. The scale bar represents 10 μm. Images are representative of at least three independent experiments.

(B) *Myd88*$^{-/-}$/*Trif*$^{-/-}$ iBMDMs expressing MyD88-RIP3 were treated with TLR ligands or staurosporine (STS) for 6 hr and lysed. PARP and Actin were detected by western analysis.

(C) BMDMs were stimulated with LPS (or not) in the presence of indicated inhibitors (or not) (Nec-1 5 μM; ZVAD 10 μM) for 18 hr. Membrane rupture was determined by PI staining.

(D and E) The cells indicated were treated with TLR ligands (or not) and inhibitors (Nec-1 5 μM; ZVAD 10 μM) (or not). Extracellular LDH in the culture media was quantified.