Supporting Information

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SI Text

Mice. *Myd88*$^{-/-}$ mice were provided by S. Akira (Osaka University, Osaka, Japan). *Sting*$^{-/-}$ mice were provided by G. N. Berber (University of Miami School of Medicine, Miami). *Irf3*$^{-/-}$/*Bcl2l12*$^{-/-}$ (1), *Pycard*$^{-/-}$ (2), *Mkp1*$^{-/-}$ (3), and *Mkp5*$^{-/-}$ (4) mice as described previously were maintained on a C57BL/6 (B6) genetic background. IFN regulatory factor 3 (IRF3) and MyD88 doubly deficient mice were generated by crossing *Irf3*$^{-/-}$/*Bcl2l12*$^{-/-}$ and *Myd88*$^{-/-}$ mice.

Reagents. LPS, peptidoglycan, and poly(dA-dT)·poly(dT-dA) (B-DNA) were purchased from Sigma. Poly(I:C) was purchased from GE Healthcare Biosciences. Cyclic di-GMP was purchased from Invivogen. Other reagents that were purchased are described in each section.

Cells. Peritoneal macrophages, splenocytes, splenic CD11b^{+} cells, and splenic CD11c^{+} cells were prepared as previously described (1, 5, 6). To obtain *Mkp1*$^{-/-}$ macrophages, fetal liver cells were cultured for 2 d in the presence of SCF (20 ng/mL), IL-3 (10 ng/mL), and IL-6 (10 ng/mL) followed by a 6-d culture in the presence of 20 ng/mL macrophage-colony stimulating factor (M-CSF) (Genzyme). SCF, IL-3, and IL-6 were purchased from Peprotech. Bone marrow-derived *Mkp5*$^{-/-}$ macrophages were prepared as previously described (7).

Retinoic Acid-Inducible Gene I-Like Receptor/Stimulator of IFN Gene Stimulation. Nucleic acids were each mixed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol and as described previously (8). Cells were then stimulated with one of these mixtures.

RNA Analysis. Quantitative RT-PCR was performed with a Lightcycler480 instrument and SYBR Green reagents (Roche Molecular Biochemicals).

Primer sequences for *Ifnb1*, *Ifna4*, *Il6*, *Il12b*, *Nos2*, *Ifng*, and *Gapdh* have been described previously (1, 5). Data are presented as relative expression units after normalization to *Gapdh* (mean and SD of triplicates). cDNA microarray analysis was performed using Mouse Genome 430 2.0 Array (Affymetrix) by TAKARA Bio. Data are presented as a fold change in gene expression relative to uninfected WT cells.

Immunoblot Analysis. Cell lysis and immunoblotting were performed as described previously (1, 9). Antibodies for IRF3, MAPK phosphatase 1 (MKP1), TANK-binding kinase-1 (TBK1), β-tubulin, and USF-2 were purchased from Santa Cruz Biotechnology. Anti-FLAG monoclonal antibody was purchased from Sigma-Aldrich. Antibodies for phospho-IRF3, AKT, phospho-AKT, Erk, phospho-Erk, and USF-2 were purchased from Cell Signaling Technology. Antibodies for MKP2, MKP3, MKP5, and phospho-TBK1 were purchased from Abcam. Anti-MKP7 antibody was purchased from Abnova. Nuclear extracts were prepared as described previously (9). Three independent experiments were performed to confirm the reproducibility of the data.

Immunoprecipitation. Immunoprecipitation was performed by using Dynabeads Protein G (Invitrogen) as described previously (5).

Proximity Ligation Assay. Proximity ligation assay (PLA) was performed with a Duolink In Situ Proximity Ligation Assay Kit (Olink Bioscience) according to the manufacturer's protocol. Quantitative analyses of the PL spots were done as follows. The PL spot numbers per cell were counted in the inspected microscopic field (each containing about 50–80 cells), and the mean number of the spots per cell in the field was calculated. In all cases examined, the figure represents the mean PL spot numbers per cell detected in three independent microscopic fields. Endoplasmic reticulum, mitochondria, and endosome were stained with anticalnexin antibody (Abcam), anti-TOMM20 antibody (Sigma-Aldrich), and anti-EEA1 antibody (BD Bioscience), respectively. Other antibodies used for PLA are described above. Three independent experiments were performed to confirm the reproducibility of the data.

Infection. *Listeria monocytogenes* and *Salmonella typhimurium* were prepared as described previously (1). Peritoneal macrophages were infected with bacteria [multiplicity of infection (MOI) = 0.2–3.0] for the indicated time periods in RPMI supplemented with 10% (vol/vol) FCS without antibiotics. For in vivo infection, mice were i.p. infected with *L. monocytogenes* (10^5 cfu); 2.5 d later, spleens were isolated from infected mice, and *Ifnb1* mRNA expression was measured by quantitative RT-PCR.

In Vitro Kinase Assay. TBK1 was first immunoprecipitated by an anti-TBK1 antibody in the lysates of peritoneal macrophages. The precipitate was then incubated with myelin basic protein as a substrate in kinase buffer (20 mM Hepes, pH 7.4, 20 mM $MgCl_2$, 2 mM DTT, 20 μM ATP) in the presence of ^{32}P-labeled ATP for 30 min at 30 °C. The reaction products were resolved by SDS/PAGE, and the incorporation of ^{32}P was detected by autoradiography.

Retrovirus Production and Transduction. Murine IRF7 cDNA (10) was cloned into pBabe-puro retroviral vector, and the IRF7-expressing virus was prepared as described previously (8). Raw 264.7 cells were infected with the virus in the presence of 8 μg/mL polybrene (Sigma) for 4 h and selected by puromycin (4.5 μg/mL; Wako) after 3 d.

Plasmid Construction. Murine IRF3 cDNA described in ref. 8 was cloned into pCNX2-FLAG (10). Expression vectors for FLAG-tagged murine TBK1 and myelocytomatosis oncogene (Myc)-tagged JAB were described previously (11, 12). Expression vectors for Myc-tagged human MKPs were described previously (13, 14).

Colony Formation Assay. Spleens from *L. monocytogenes*-infected mice were homogenized in PBS, serially diluted, and plated on LB agar. Colonies were counted after incubation for 12 h at 37 °C.

Statistical Analysis. Differences between control and experimental groups were evaluated by Student *t* test or Dunnett's multiple comparison test.

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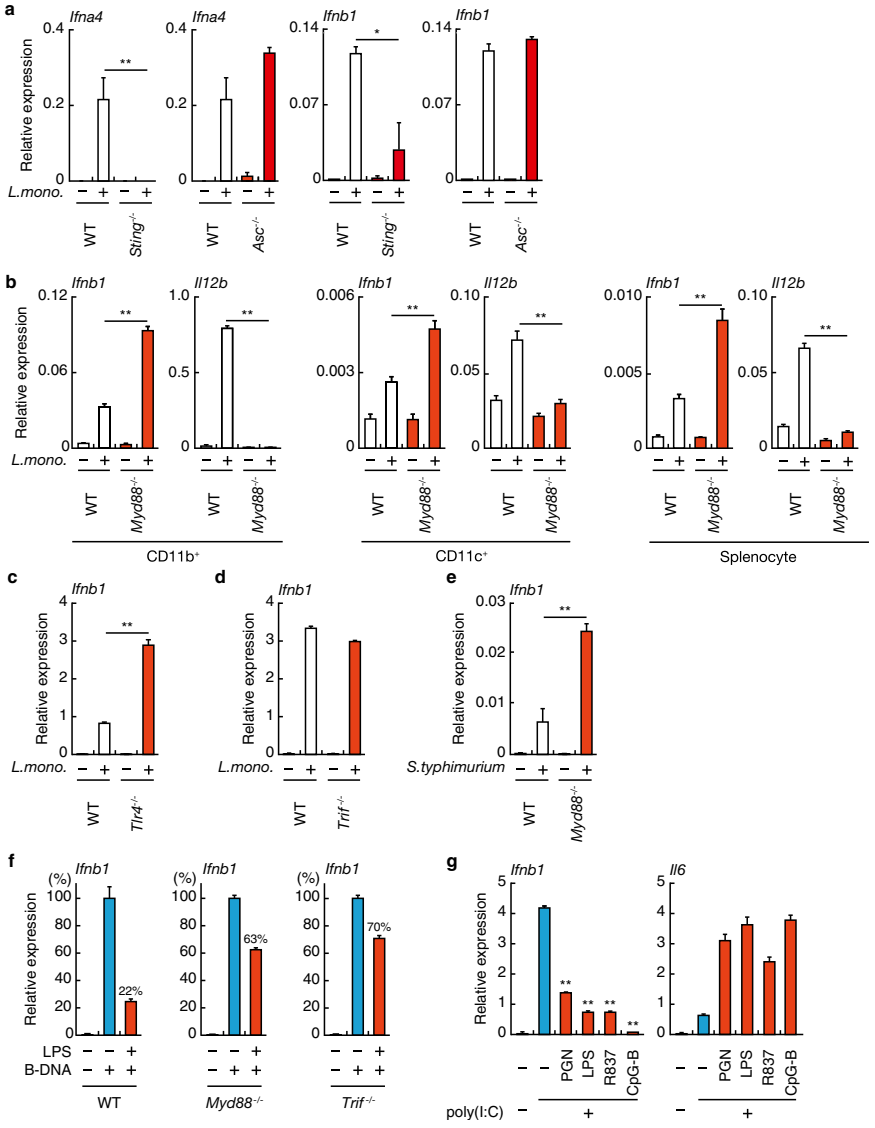

Fig. S1. Toll-like receptor (TLR)-induced suppression of stimulator of IFN gene (STING) / retinoic acid-inducible gene I-like receptor (RLR) -mediated type I IFN gene expression. (*A*) Quantitative RT-PCR analysis of *Ifna4* and *Ifnb1* mRNA in WT, STING-deficient (*Sting*$^{-/-}$), and apoptosis-associated speck-like protein containing a carboxy-terminal CARD (ASC)-deficient (*Asc*$^{-/-}$) peritoneal macrophages infected for 6 h with *L. monocytogenes*. $^{**}P < 0.01$; $^{*}P < 0.05$. For clarity, results are shown in separate graphs with data from same WT cells and data from each mutant cells. (*B*) Quantitative RT-PCR analysis of *Ifnb1 and Il12b* mRNA in WT and MyD88-deficient (*Myd88*$^{-/-}$) splenocytes, splenic CD11b^{+} cells, and splenic CD11c^{+} cells infected for 6 h with *L. monocytogene*. $^{**}P < 0.01$. (C) Quantitative RT-PCR analysis of *Ifnb1* mRNA in WT and TLR4-deficient (*Tlr4*$^{-/-}$) peritoneal macrophages infected for 6 h with *L. monocytogenes*. $^{**}P < 0.01$. (*D*) Quantitative RT-PCR analysis of *Ifnb1* mRNA in WT and Toll/IL-1R domain-containing adaptor inducing IFNb (TRIF)-deficient (*Trif*$^{-/-}$) peritoneal macrophages infected for 6 h with *L. monocytogenes*. (*E*) Quantitative RT-PCR analysis of *Ifnb1* mRNA in WT and *Myd88*$^{-/-}$ peritoneal macrophages infected for 6 h with *S. typhimurium*. $^{**}P < 0.01$. (*F*) Quantitative RT-PCR analysis of *Ifnb1* mRNA in WT, *Myd88*$^{-/-}$, and *Trif*$^{-/-}$ peritoneal macrophages stimulated for 6 h with indicated combinations of B-DNA and LPS. (*G*) Quantitative RT-PCR analysis of *Ifnb1* and *Il6* mRNA in peritoneal macrophages stimulated for 6 h with indicated combinations of poly(I:C) and TLR ligands. $^{**}P < 0.01$ compared with cells stimulated with poly(I:C).

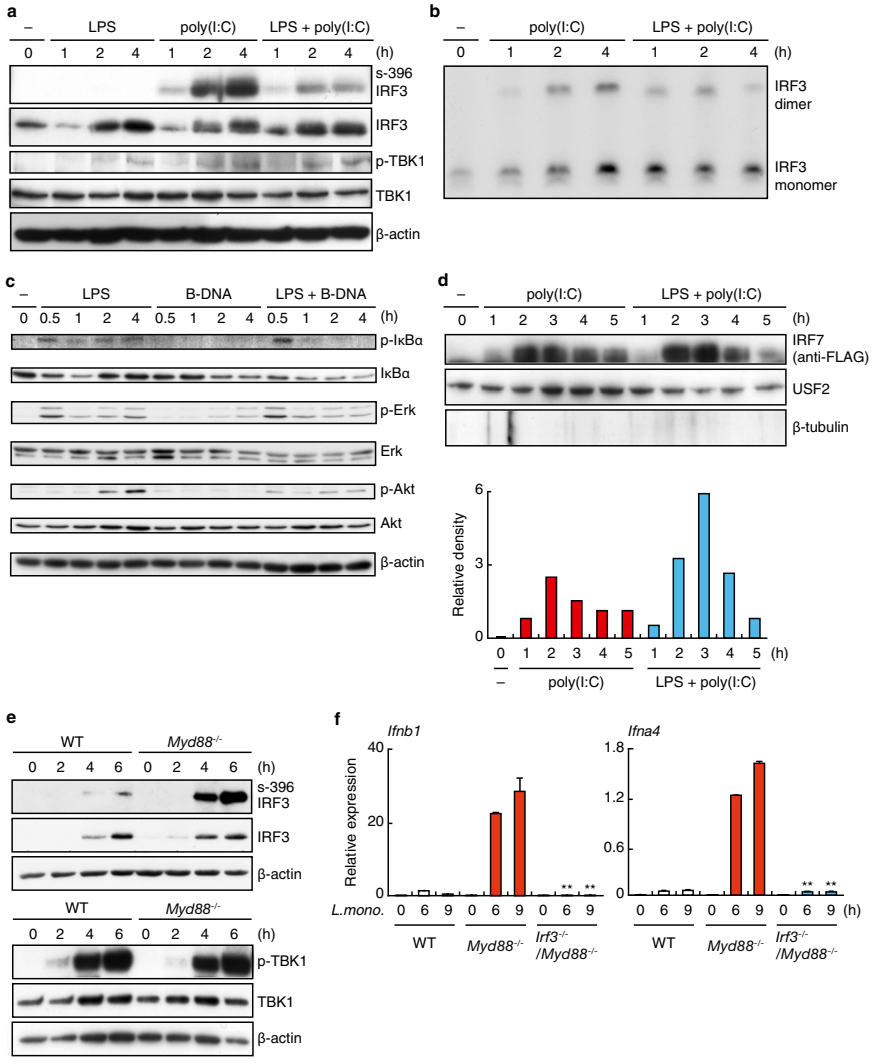

Fig. S2. TLR-induced suppression of RLR/STING-mediated IRF3 activation. (*A*) Immunoblot analysis of IRF3, TBK1, and β-actin in peritoneal macrophages stimulated for indicated times with LPS, poly(I:C), or poly(I:C) plus LPS as assessed by SDS/PAGE. (*B*) Immunoblot analysis of IRF3 in peritoneal macrophages stimulated for indicated times with poly(I:C) or poly(I:C) plus LPS as assessed by native PAGE. (*C*) Immunoblot analysis of IκBα, Erk, AKT, and β-actin in peritoneal macrophage stimulated for indicated times with B-DNA or B-DNA plus LPS as assessed by SDS/PAGE. (*D*, *Upper*) Immunoblot analysis of the nuclear translocation of Flag-tagged IRF7 in Raw 264.7 cells stimulated for indicated times with poly(I:C) or poly(I:C) plus LPS as assessed by SDS/PAGE. USF-2 and β-tubulin serve as markers of nuclear content and cytosolic content, respectively. Relative band intensity (IRF7/USF2) is shown in *D*, *Lower*. (*E*) Immunoblot analysis of IRF3, TBK1, and β-actin in WT and *Myd88*$^{-/-}$ peritoneal macrophages infected for indicated times with *L. monocytogenes* as assessed by SDS/PAGE. (*F*) Quantitative RT-PCR analysis of *Ifnb1* and *Ifna4* mRNA in WT, *Myd88*$^{-/-}$, and IRF3- and MyD88-deficient (*Irf3*$^{-/-}$*Myd88*$^{-/-}$) peritoneal macrophages infected for 6 or 9 h with *L. monocytogenes*. $^{**}P < 0.01$ compared with *Myd88*$^{-/-}$ cells.

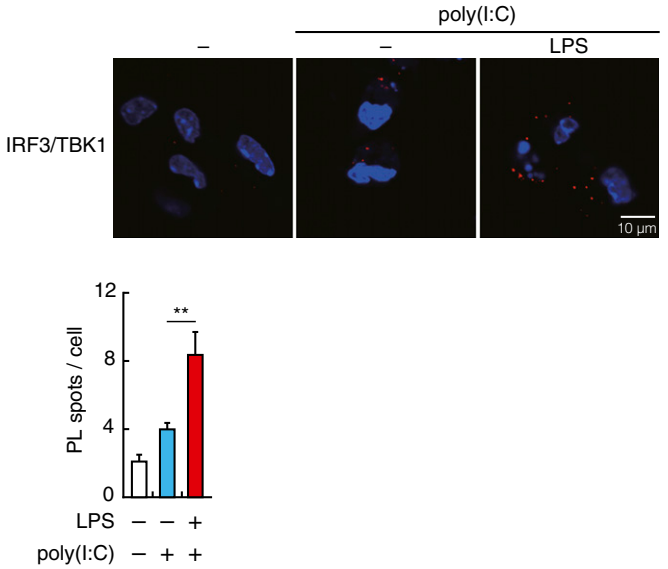

Fig. S3. TLR-induced suppression of IRF3–TBK1 dissociation. (*Upper*) PLA for the IRF3–TBK1 complex in peritoneal macrophages stimulated for 5 h with poly(I:C) or poly(I:C) plus LPS. Red spots indicate the IRF3–TBK1 complex, whereas nuclei are stained in blue (DAPI staining). In *Lower*, data for quantitative analysis for PL spots in a cell are shown. $^{**}P < 0.01$.

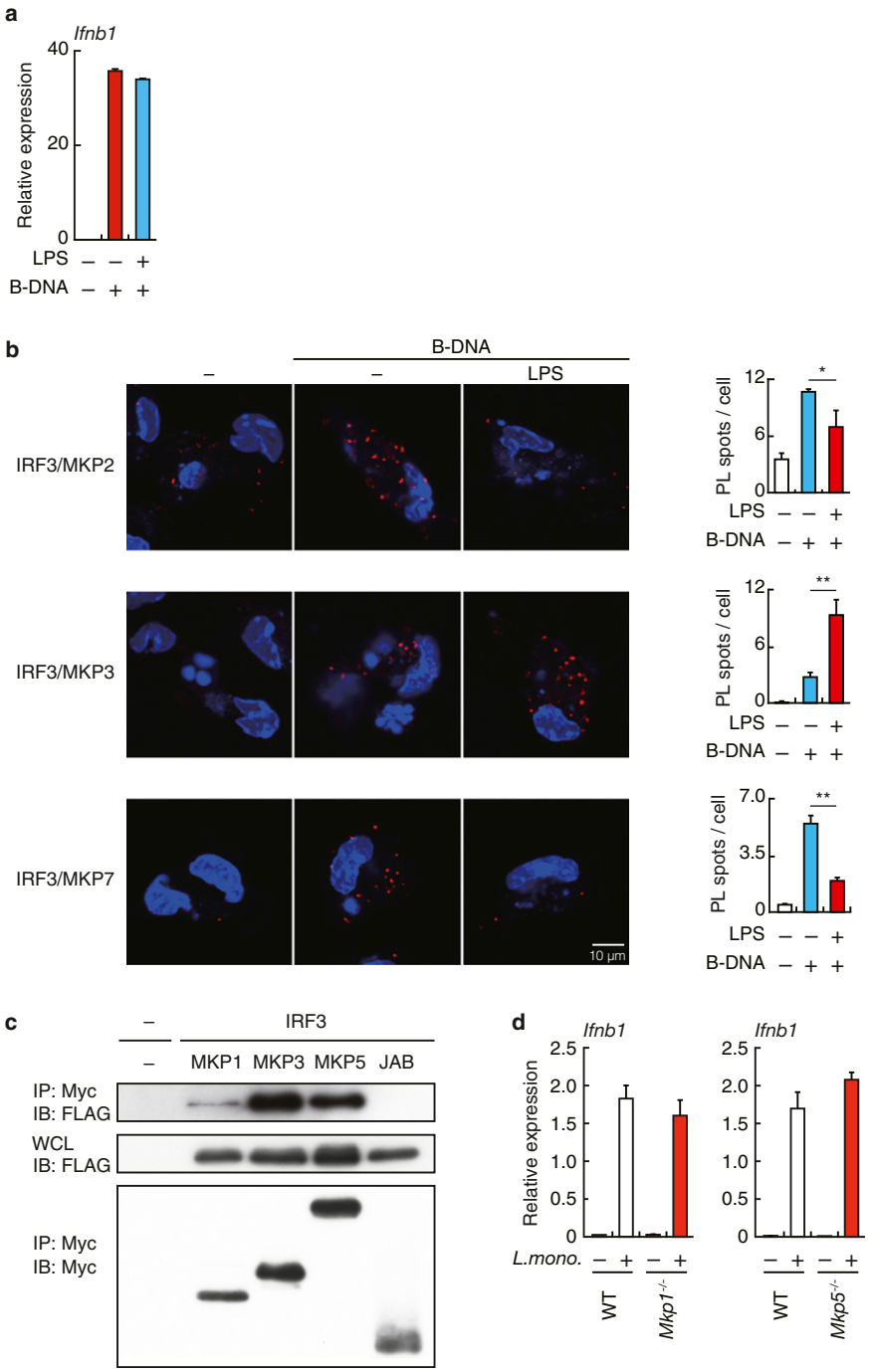

Fig. S4. MKP-dependent suppression of IRF3–TBK1 dissociation. (*A*) Quantitative RT-PCR analysis of *Ifnb1* mRNA in WT peritoneal macrophages stimulated for 6 h with B-DNA or B-DNA plus LPS in the presence of sodium fluoride (20 μM). (*B*, *Left*) PLA for the IRF3–MKP complex in peritoneal macrophages stimulated for 4 h with B-DNA or B-DNA plus LPS. Red spots indicate the IRF3–MKPs complex, whereas nuclei are stained in blue (DAPI staining). In *B*, *Right*, data for quantitative analysis for PL spots in a cell are shown. $^{**}P < 0.01$; $^{*}P < 0.05$. (*C*) Cell lysates prepared from HEK293T cells transiently transfected with indicated combinations of FLAG-tagged IRF3 and Myc-tagged MKPs were immunoprecipitated (IP) with the anti-Myc antibody and subjected to immunoblot (IB) analysis using the anti-Myc or anti-FLAG antibody as indicated. WCL, whole-cell lysate. (*D*) Quantitative RT-PCR analysis of *Ifnb1* mRNA in WT, MKP1-deficient (*Mkp1*$^{-/-}$), and MKP5-deficient (*Mkp5*$^{-/-}$) M-CSF–derived macrophages infected for 6 h with *L. monocytogenes*.

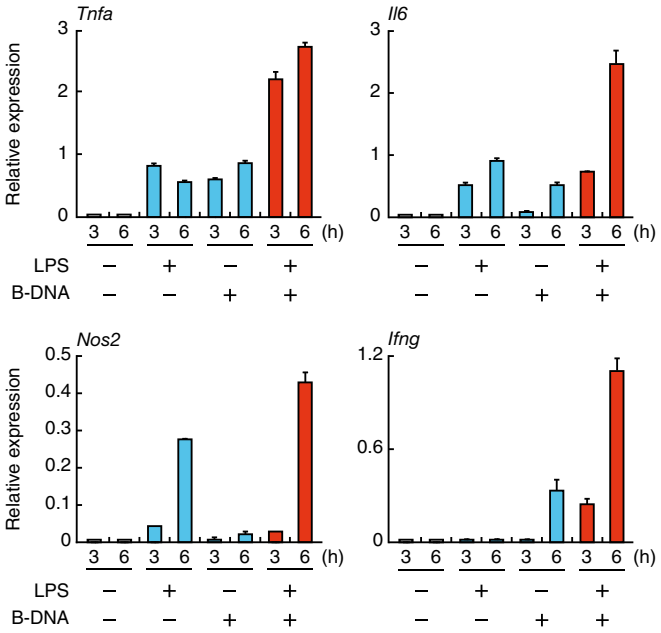

Fig. S5. Cooperative gene induction between STING and TLR pathways. Quantitative RT-PCR analysis of *Tnfa*, *Il6*, *Nos2*, and *Ifng* mRNA in WT peritoneal macrophages stimulated for 6 h with B-DNA, LPS, or B-DNA plus LPS.

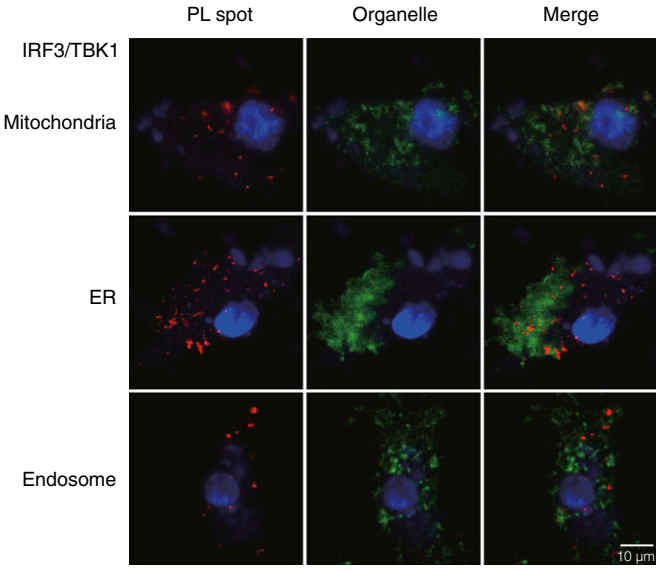

Fig. S6. Subcellular localization of the IRF3–TBK1 complex. PLA for the IRF3–TBK1 complex in peritoneal macrophages stimulated for 5 h with B-DNA plus LPS. Red spots indicate the IRF3–MKPs complexes, whereas nuclei are stained in blue (DAPI staining). Each organelle [mitochondria, endoplasmic reticulum (ER), endosome] stained with a specific antibody as described is shown in green.