Supporting Information

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SI Materials and Methods

Materials. MRT67307 was synthesized by Natalia Shpiro in the Medical Research Council Protein Phosphorylation Unit. 5Z-7-oxozeaenol was purchased from BioAustralis Fine Chemicals. Pharmacological inhibitors were dissolved in DMSO and stored as 10 mM solutions at −20 °C. LTA, Pam_3CSK_4, poly(I:C), R837, and CpG-type B (ODN1826) were purchased from Invivogen and LPS (*Escherichia coli* strain O55:B5) was from Alexis Biochemicals. Mouse IL-1α was purchased from Sigma-Aldrich. All antibodies used for immunoblotting and immunoprecipitation have been described previously with the exception of anti-IKKβ and anti-NEMO (1, 2). Antibodies were raised in sheep against the entire IKKβ (S189C, bleed 1) and NEMO (S190C, bleed 2) human proteins in sheep and affinity purified by the Division of Signal Transduction Therapy, University of Dundee.

Mice. $MyD88^{-/-}$, TIR-domain-containing adapter-inducing IFNβ $(TRIF)^{-/-}$, and TRAF associated NFκB activator $(TANK)^{-/-}$ mice were described previously (3–5). C57BL/6, $MyD88^{-/-}$, and $TRIF^{-/-}$ mice were bred at the University of Dundee, whereas $TANK^{-/-}$ were bred at Osaka University under specific pathogen-free conditions in accordance with local regulations. C3H/HeJ mice were purchased from Charles River Laboratories. Work was approved by local ethical review and was performed with a UK Home Office project license.

Cell Culture. BMDMs were differentiated for 7 d in DMEM supplemented with 5 ng/mL recombinant M-CSF (R&D Systems) or 20% L929-conditioned medium as a source of M-CSF, 2 mM glutamine, 10% FCS, and the antibiotics penicillin and streptomycin. TBK1- and IKKε-deficient mouse embryonic fibroblasts (MEFs) and control MEFs were cultured in DMEM supplemented with 10% FCS, 2 mM glutamine, penicillin, and streptomycin.

Cell lysis and Immunoblotting. Pharmacological inhibitors dissolved in DMSO, or an equivalent volume of DMSO for control incubations, were added to the culture medium of macrophages or fibroblasts. After 1 h at 37 °C, the cells were stimulated with LTA, Pam_3CSK_4, poly(I:C), LPS, R837, CpG, or IL-1α as described in the figure legends (Figs. 1–5 and Figs. S1–S4). Thereafter, the cells were rinsed in ice-cold PBS and extracted in lysis buffer (50 mM Tris/HCl pH 7.4, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM sodium pyrophosphate, 10 mM sodium β-glycerol 1-phosphate, 1 mM DTT, 1 mM sodium orthovanadate, 0.27 M sucrose, 1% (vol/vol) Triton X-100, 1 mg/mL aprotinin, 1 mg/mL leupeptin, and 1 mM phenylmethylsulphonyl fluoride). Cell extracts were clarified by centrifugation at 14,000 × *g* for 10 min at 4 °C and protein concentrations determined using the Bradford assay. To detect proteins in cell lysates, 20 μg of protein extract was separated by SDS/PAGE. After transfer to PVDF membranes, proteins were detected by immunoblotting and visualized by treating the blots with ECL (Amersham) followed by autoradiography.

Immunoprecipitation. To immunoprecipitate endogenous TBK1, NEMO, and IKKβ, 1 mg of cell protein extract was incubated with 10 μg of antibody for 2 h at 4 °C, followed by the addition of Protein G Sepharose. After mixing for 30 min at 4 °C, the immunocomplexes were washed three times in lysis buffer, denatured in SDS, and subjected to SDS/PAGE followed by immunoblotting.

Statistical Analysis. Data are presented as the mean ± SEM. Statistical significance of differences between experimental groups was assessed using the Student *t* test and were considered significant if $P < 0.05$.

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2. Clark K, et al. (2011) Novel cross-talk within the IKK family controls innate immunity. *Biochem J* 434:93−104.
3. Adachi O, et al. (1998) Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity* 9:143−150.
4. Yamamoto M, et al. (2003) Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* 301:640−643.
5. Kawagoe T, et al. (2009) TANK is a negative regulator of Toll-like receptor signaling and is critical for the prevention of autoimmune nephritis. *Nat Immunol* 10:965−972.

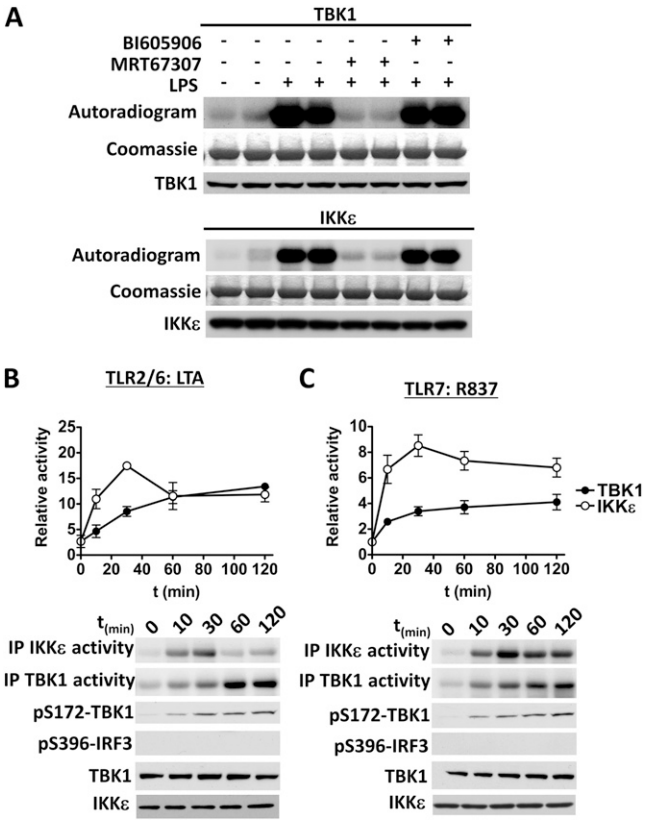

Fig. S1. Activation of TBK1 and IKKε by TLR ligands. (*A*) BMDMs were stimulated for 30 min without (–) or with (+) 100 ng/mL LPS and the cells lysed. TBK1 and IKKε were immunoprecipitated separately from the cell extracts, washed and assayed for kinase activity without (–) or with (+) 1 μM of MRT67307 or 1 μM of the IKKβ inhibitor BI605906 as described under *Materials and Methods*. Reactions were terminated in SDS, the proteins resolved by SDS/PAGE, stained with Coomassie Blue (*Middle*), and the gels autoradiographed (*Top*). An aliquot of each immunoprecipitation was also immunoblotted for TBK1 and IKKε as a loading control (*Bottom*). (*B* and *C*) BMDMs were stimulated for the times indicated with 2 μg/mL LTA (*B*) or 2 μg/mL R837 (*C*). The catalytic activities of TBK1 and IKKε were then measured as described in *A* and quantitated by phosphorimager analysis (mean ± SEM, *n* = 3). Cell extract (20 μg protein) was also immunoblotted with the antibodies indicated (bottom three panels).

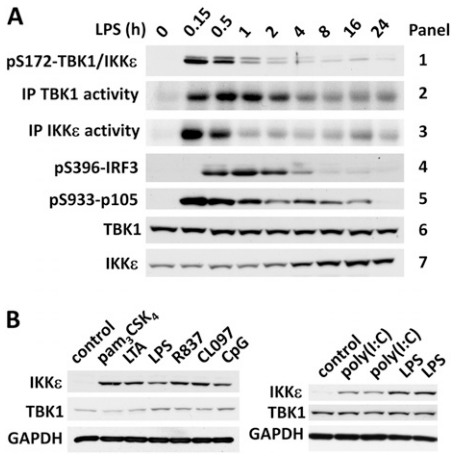

Fig. S2. Effects of prolonged TLR signaling on the IKK-related kinases. (*A*) RAW264.7 cells were stimulated with 100 ng/mL LPS for the times indicated. TBK1 and IKKε were immunoprecipitated and their catalytic activities were measured by incubating the immunoprecipitated kinases with GST-IRF3 and Mg[γ-^{32}P]-ATP as described in *Materials and Methods* (*Upper*, second and third panels). Cell extract (20 μg protein) was also immunoblotted with antibodies that recognize TBK1 phosphorylated at Ser172 to monitor activation by a second independent method (*Upper*). (*Lower* four panels) A total of 20 μg cell extract protein was immunoblotted with the antibodies indicated. (*B*) RAW264.7 cells were stimulated for 16 h with 1 μg/mL Pam_3CSK_4, 2 μg/mL LTA, 10 μg/mL poly(I:C), 100 ng/mL LPS, 2 μg/mL R837, 1 μg/mL CL097, or 2 μM CpG. Cell extracts (20 μg protein) were immunoblotted for IKKε or TBK1 and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a loading control.

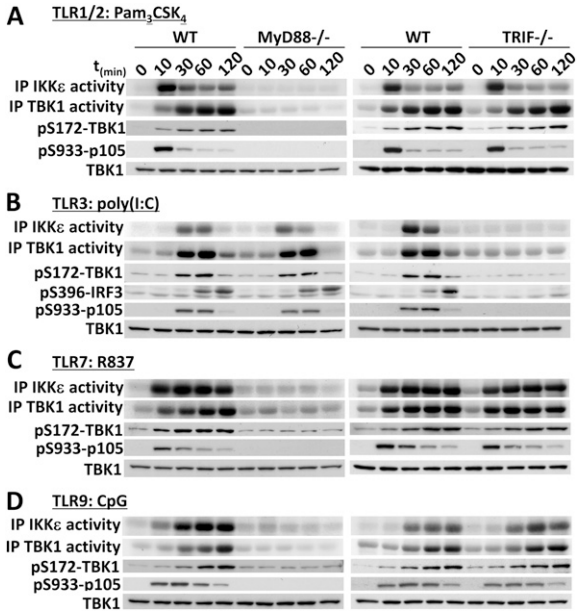

Fig. S3. Activation of TBK1 and IKKε in MyD88- and TRIF-deficient macrophages. BMDMs from MyD88$^{-/-}$ or TRIF$^{-/-}$ mice or WT littermates were stimulated for the times indicated with (*A*) 1 μg/mL Pam_3CSK_4, (*B*) 10 μg/mL poly(I:C), (*C*) 2 μg/mL R837, or (*D*) 2 μM CpG. The catalytic activities of TBK1 and IKKε were measured as described in Fig. S1 (top two panels) and *Materials and Methods*. Cell extracts (20 μg protein) were also immunoblotted with the indicated antibodies (*A*, *C*, and *D*, bottom three panels and *B*, bottom four panels).

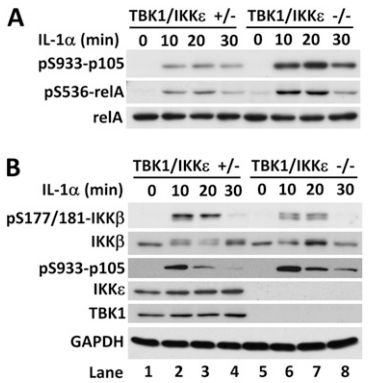

Fig. S4. Ablation of the IKK-related kinases enhances the IL-1–stimulated activation of the canonical IKKs in MEFs. Control and TBK1/IKKε DKO MEFs were stimulated with 0.5 ng/mL (*A*) or 5 ng/mL (*B*) IL-1α for the times indicated and the cell extracts were immunoblotted with the indicated antibodies. In *B*, note that the IL-1α–stimulated decrease in the electrophoretic mobility of IKKβ was reduced in TBK1/IKKε DKO MEFs (compare lanes 2–4 with 6–8).