Supporting Information

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SI Materials and Methods

Viruses, Cell Culture, and Transfections. Sendai virus (Cantell strain; Charles River Laboratories) was used at a final concentration of 100 hemagglutinating units/mL. All cells were cultured at 37 °C in an atmosphere of 5% (vol/vol) CO_2. HEK 293T cells and Raw 264.7 cells were cultured in DMEM supplemented with 10% (vol/vol) cosmic calf serum with 100 U/mL penicillin and 100 μg/mL streptomycin. THP-1 cells were cultured in RPMI 1640 medium supplemented with 10% (vol/vol) FBS with 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.05 mM 2-mercaptoethanol.

RNAi and Rescue with Transgenes. The lentiviral shRNA vector, pTY-shRNA-EF1a-puroR-2a-GFP-Flag, was provided by Dr. Yi Zhang (Harvard Medical School). The original vector was modified to pTY- shRNA-EF1a-GFP-IRES-puroR and pTY- shRNA-EF1a-GFP-IRES-hygroR by replacing the 2A peptide sequence with an IRES sequence and also by replacing a puromycin-resistant gene with a hygromycin-resistant gene. The shRNA sequences were cloned into the vectors with U6 promoter. RNAi-resistant cDNA sequences were cloned into the vectors to replace GFP. Lentiviral infection and establishment of stable cell lines were described previously (1). The shRNA sequences were as follows (only the sense strand is shown): human IRF5, 5′-GAGGAAGAGCTGCAGAGGAT-3′; mouse IRF5, 5′-GCAGAGAATAACCCTGATTTA-3′; human IKKβ, 5′-GGGAGAACGAAGTGAAACT-3′; human IKKα, 5′-GTACCAGCATCGGGAACTT-3′; human NEMO, 5′-GGACAAGGCCTCTGTGAAA-3′; human TRAF6, 5′-GGAGAAACCTGTTGTGATT-3′.

Quantitative RT-PCR. Total cellular RNA was isolated using TRIzol. 0.1–1 μg total RNA was used for reverse transcription (RT) using iScript Kit (Bio-Rad). The resulting cDNA served as the template for quantitative RT-PCR analysis using iTaq Universal SYBR Green Supermix (Bio-Rad) and the ViiA7 Real-Time PCR System (Applied Biosystems). Primers for specific genes were as follows: human CXCL10, 5′-GTGGCATTCAAGGAGTACCTC-3′ and 5′-TGATGGCCTTCGATTCTGGATT-3′; human TNF-α, 5′-CCTCTCTCTAATCAGCCCTCTG-3′ and 5′-GAGGACCTGGGAGTAGATGAG-3′; human IFN-β, 5′-ACTGCAACCTTTCGAAGCCTTT-3′ and 5′-TGGAGAAGCACAACAGGAGAGC-3′; human GAPDH, 5′-ATGACATCAAGAAGGTGGTG-3′ and 5′-CATACCAGGAAATGAGCTTG-3′; human IL-12 p40, 5′-AAGGAGGCGAGGTTCTAAGC-3′ and 5′-AAGAGCCTCTGCTGCTTTTG-3′; human IFIT3, 5′-TCAGAAGTCTAGTCACTTGGGG-3′ and 5′-ACACCTTCGCCCTTTCATTTC-3′; human RSAD2, 5′-CAGCGTCAACTATCACTTCACT-3′ and 5′-AACTCTACTTTGCAGAACCTCAC-3′; human ISG15, 5′-CGCAGATCACCCAGAAGATCG-3′ and 5′-TTCGTCGCATTTGTCCACCA-3′.

1. Tanaka Y, Chen ZJ (2012) STING specifies IRF3 phosphorylation by TBK1 in the cytosolic DNA signaling pathway. *Sci Signal* 5(214):ra20.

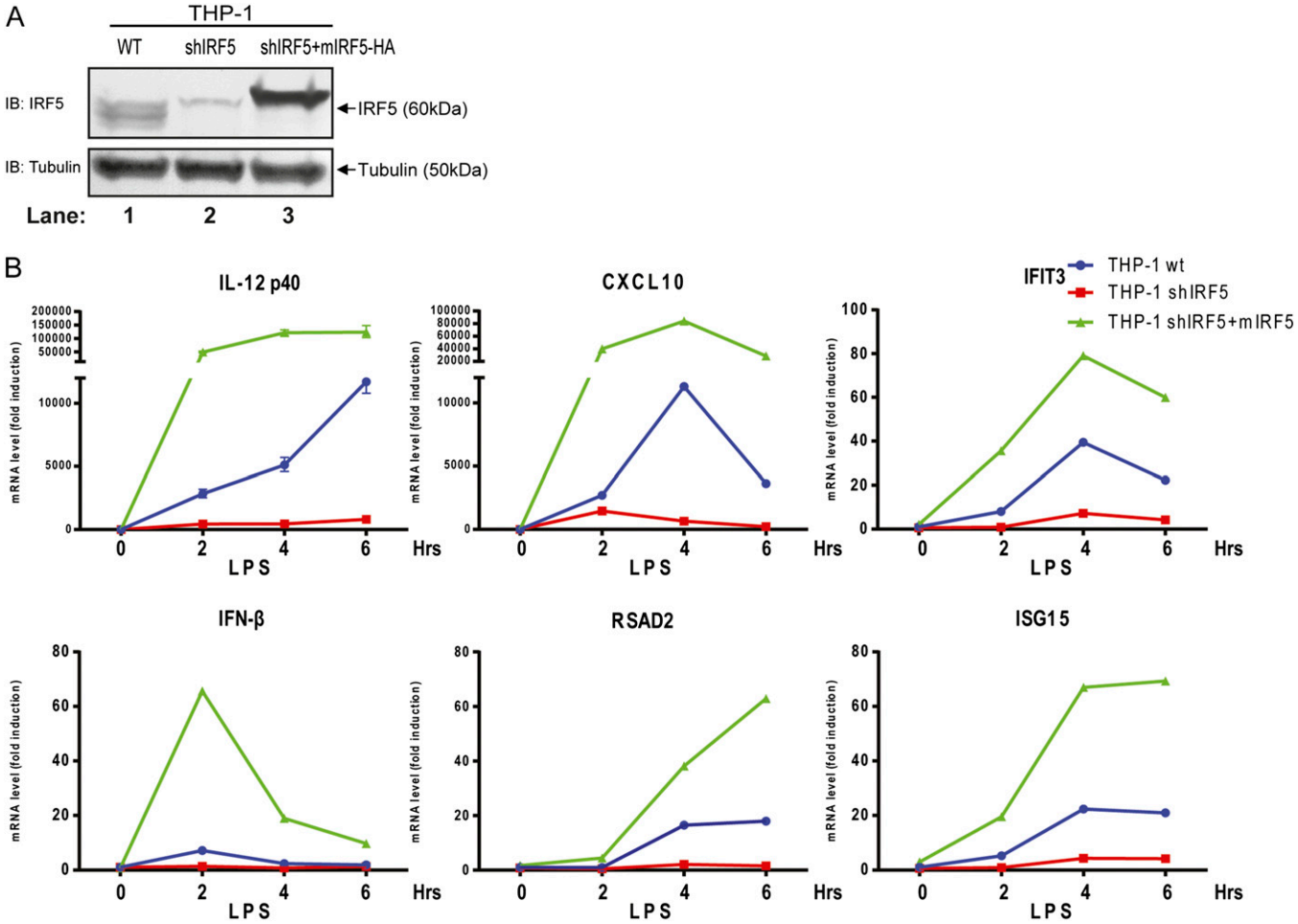
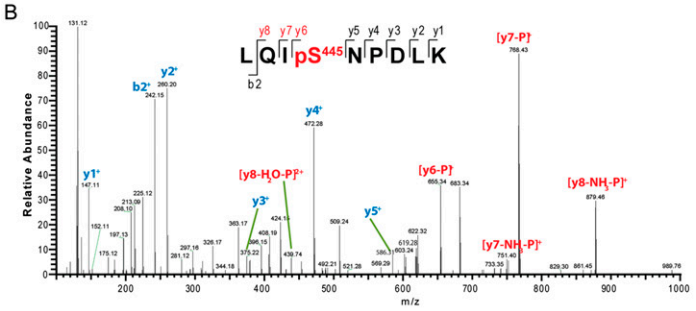
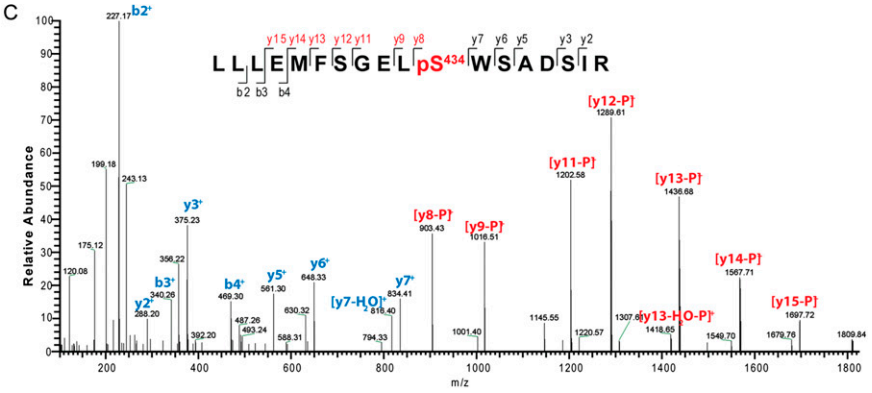


Fig. S1. IRF5 is essential for cytokine induction by LPS. (*A*) Efficiency of IRF5 knockdown and rescue in THP-1 cells. Cell extracts from WT THP1 (lane 1) and the cells stably expressing an shRNA against IRF5 with (lane 3) or without (lane 1) reconstitution with mIRF5-HA were immunoblotted with an IRF5 antibody. (*B*) IRF5 is essential for LPS-induced expression of cytokines and IFN-stimulated genes. THP-1 cell lines as described in *A* were stimulated with LPS (5 μg/mL) for the indicated time. Total RNA was isolated to measure the expression of the indicated genes by qRT-PCR.

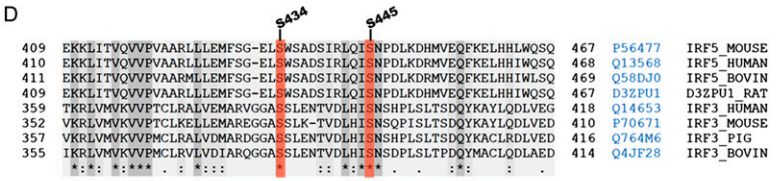
A

	Condition 1 (IRF5+BSA)	Condition 2 (IRF5+IKKβ)
Coverage	96%	96%
Score	25151	25420
Matches	588	691
Sequences	44	46





D



E

Peptide	Condition	Non-phosphorylated peptide	Phosphorylated peptide
R.LLLEMFpS430GELSWSADSIR.L	BSA	55	0
	IKK2	62	1
R.LLLEMFSGELSWpS436ADSIR.L	BSA	55	0
	IKK2	58	5

Fig. S2. Identification of IRF5 phosphorylation sites by mass spectrometry and functional analyses. (*A*) Summary of mass spectrometry data indicating the coverage of the IRF5 protein and the total numbers of peptide sequences in the experiment described in Fig. 3 *B* and *C*. The data were analyzed by Mascot (Matrix Science). (*B*) Tandem mass (MS2) spectrum after HCD fragmentation of the ion with m/z = 554.28 ($z = 2^+$), indicating S445 phosphorylation. (*C*) MS2 spectrum after HCD fragmentation of the ion with m/z = 1,067.50 ($z = 2^+$), indicating S434 phosphorylation. (*D*) Sequence alignment of the C-termini of IRF5 and IRF3 from different species as indicated. Conserved serine residues in IRF5 and IRF3, including S434 and S445 of mouse IRF5, are highlighted. (*E*) IRF5 protein incubated with IKKβ or BSA was analyzed by tandem mass spectrometry as described in Fig. 3 *A* and *B*. The total numbers of the indicated phosphorylated and nonphosphorylated peptides are shown.

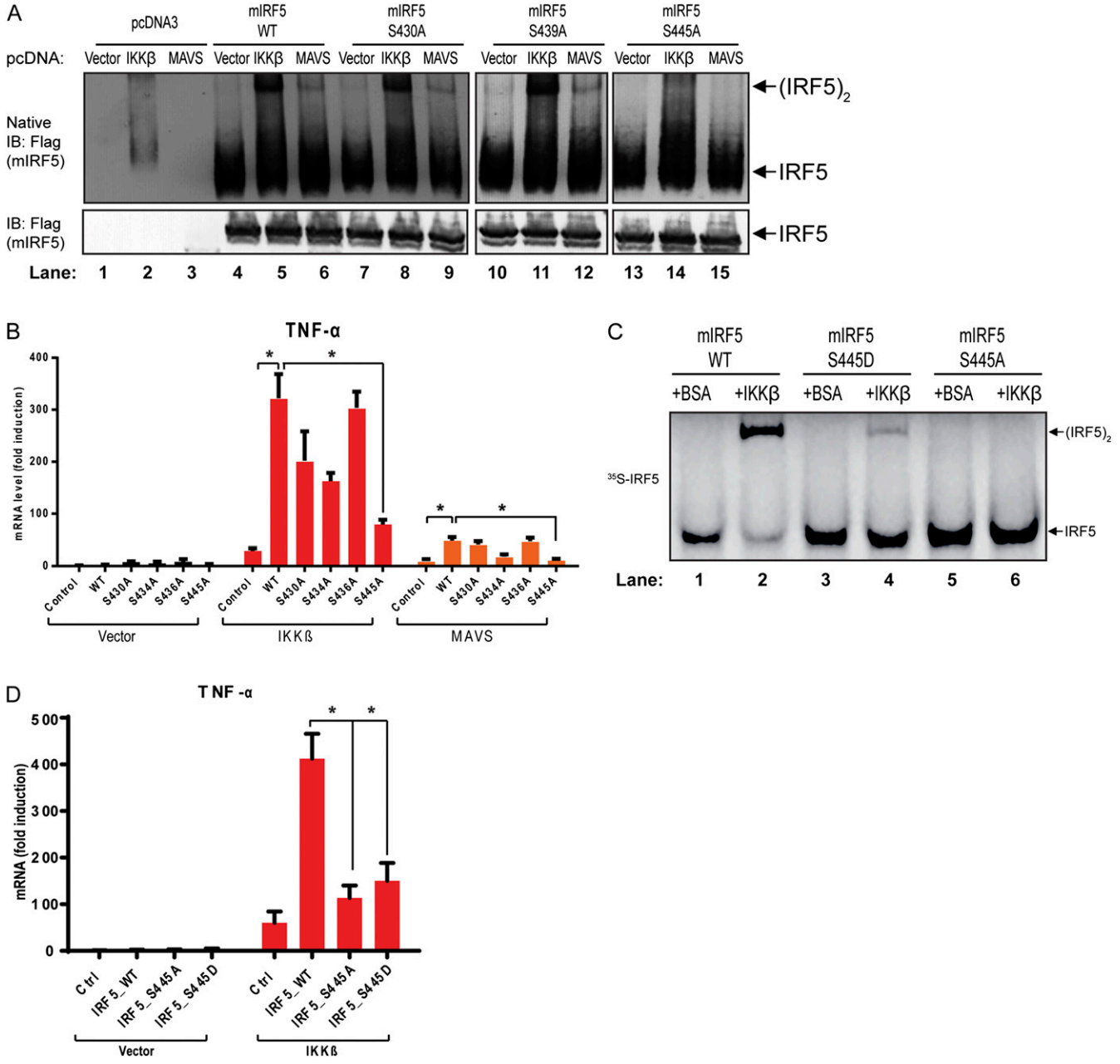


Fig. S3. Serine 445 is essential for IRF5 activation and TNF-α induction. (*A*) S445A mutation of IRF5 prevented its activation by IKKβ and MAVS in cells. 293T cells were transfected with the expression vectors for WT or mutant Flag-mIRF5 as indicated for 12 h before another transfection with the expression vector encoding IKKβ or MAVS for 24 h. IRF5 dimerization (*Upper*) and expression (*Lower*) were analyzed by immunoblotting after native PAGE and SDS/PAGE, respectively. (*B*) S445A mutation of IRF5 significantly inhibited TNF-α induction. Total RNA was isolated from cells described in *A* to measure TNF-α levels by qRT-PCR. (*C*) S445D mutation inhibited IRF5 dimerization. WT and mutant ^{35}S-IRF5 (S445D and S445A) proteins were translated in vitro and incubated with IKKβ or BSA in the presence of ATP. Dimerization of IRF5 was analyzed by native PAGE, followed by autoradiography. (*D*) S445D mutation of IRF5 inhibited TNF-α induction. Similar to *B*, except that the IRF5 S445D mutant was tested.

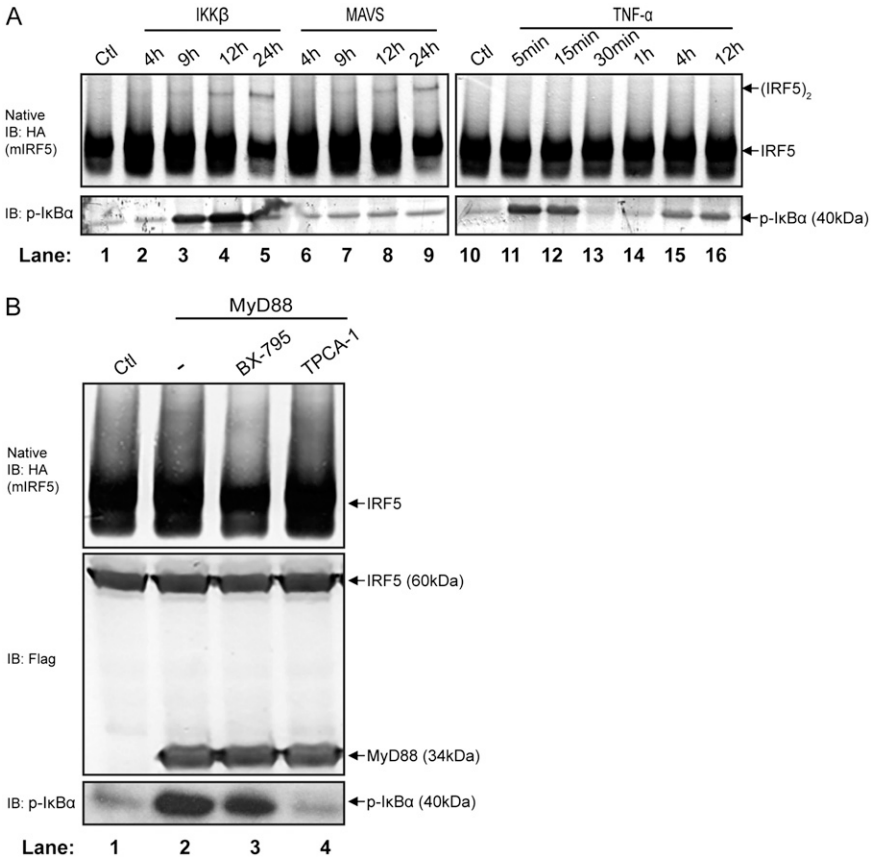


Fig. S4. TNF-α and MyD88 activate IKKβ, but not IRF5. (*A*) 293T Flag-mIRF5-HA cells were transfected with pcDNA-Flag-IKKβ (IKKβ) or pcDNA-Flag-MAVS (MAVS) or treated with recombinant TNF-α protein (TNF-α) for the indicated time. (*Upper*) Activation of IRF5 was analyzed by native PAGE, followed by immunoblotting with an IRF5 antibody. (*Lower*) Activation of IKKβ was monitored by immunoblotting with a phospho-IκBα antibody. (*B*) Similar to *A*, except that the cells were transfected with pcDNA-Flag-MyD88 (MyD88) and incubated with the indicated inhibitors for 24 h.