Supporting Information

Zhou et al. 10.1073/pnas.1106232108

SI Materials and Methods

Quantitative Real-Time PCR Analysis. Total cellular RNA was prepared using TRIzol (Invitrogen), and reverse transcription was performed with SuperScript First-strand kit (Invitrogen). PCR was run on a 7300 real-time PCR System (Applied Biosystems), using the SYBR Green Master Mix (Applied Biosystems), and each mRNA signal was normalized to cyclophilin (*Cph*) as a housekeeping gene. Values are presented as means ± SD of three independent samples. Primer sequences were used as follows: *Cph*, forward 5′-ATGGTCAACCCCACCGTGT and reverse 5′-TTCTTGCTGTCTTGGAACTTTGTC; CCAAT/enhancer-binding protein β (*Cebpb*), forward 5′-ACGGTGGACAAGCTGAGCG and reverse 5′-CCTTGTGCTGCGTCTCCAGG; arginase, liver (*Arg1*), forward 5′-CTCCAAGCCAAAGTCCTTAGAG and reverse 5′-AGGAGCTGTCATTAGGGACATC; NF-κB polypeptide gene enhancer in B-cells inhibitor α (*Nfkbia*), forward 5′-CTCACGGAGGACGGAGACTC and reverse 5′-CTCTTCGTGGATGATTGCCA; colony-stimulating factor 3 (*Csf3*), forward 5′-ATGGCTCAACTTTCTGCCCAG and reverse 5′-CTGACAGTGACCAGGGGAAC; S100 calcium-binding protein A8 (*S100a8*), forward 5′-AAATCACCATGCCCTCTACAAG and reverse 5′-CCCACTTTTATCACCATCGCAA; *Tnfa*, forward 5-GAGGCCAAGCCCTGGTATG and reverse 5′-CGGGCCGATTGATCTCAGC; *Il6* forward 5′-CTGATGCTGGTGACAACCACA and reverse 5′-AGCCTCCGACTTGTGAAGTG; *Nos2*, forward 5′-CCCTCCTGATCTTGTGTTGGA and reverse 5′-CAACCCGAGCTCCTGGAAC; CCAAT/enhancer-binding protein δ (*Cebpd*), forward 5′-CGACTTCAGCGCCTACATTGA and reverse 5′-CTAGCGACAGACCCCACAC; myeloperoxidase (*Mpo*), forward 5′-AGTTGTGCTGAGCTGTATGGA and reverse 5′-CGGCTGCTTGAAGTAAAACAGG; elastase (*Ela2*), forward 5′-TGCCAGGAATTTCGTCATGT and reverse 5′-GTTGGCGTTAATGGTAGCGGA.

Microarray Analysis. Total cellular RNA was prepared using TRIzol (Invitrogen) and purified using the RNeasy Mini kit (QIAGEN). Samples were processed with the one-cycle cDNA Synthesis kit (Affymetrix) and subjected to Affymetrix expression analysis according to the manufacturer's instructions. Two independent sets of experiments were performed to increase the sample size. In the first set we included cDNA samples of two WT and two TNF-α–induced protein 3 (*TNFAIP3*)-interacting protein 1 double-knockout (*Tnip1*$^{-/-}$) mice, which were analyzed with the Mouse Genome 430 2.0 array (Affymetrix). In the second set we included samples of three WT and three *Tnip1*$^{-/-}$ mice, which were analyzed with the HT 430 PM Array (Affymetrix). The two data sets were summarized using the RMA algorithm, joined, and batch corrected to remove chip effects. Next, a two-way ANOVA model tested the expression of each probe set, and a step-up false-discovery method was applied to assign statistical significance to the probe sets using Partek Genomics Suite 6.4. The two factors were genotype and stimulation. Fold changes were calculated also in STATA/SE 11.1 (StataCorp).

Immunohistochemistry. *Tnip1*$^{-/-}$ mice and littermate controls (5- to 8-wk-old) were perfused with 4% (vol/vol) PBS-buffered formaldehyde and embedded in paraffin. Organs were cut in 5-μm sections, deparaffinized, and stained with the F4/80 antibody (Caltag), followed by detection with secondary biotinylated antibodies and HRP-labeled streptavidin (Thermo Shandon). Slides were incubated with 3,3′diaminobenzidine tetrahydrochloride for 5 min and counterstained with hematoxylin.

Sample Preparation for MS Analysis. The gel lanes containing the immunopurified samples (Fig. 1*A*) were excised into 100 bands, each of which was cut into small plugs, washed with 50% (vol/vol) acetonitrile, and destained by repeated incubations in 100 mM ammonium bicarbonate (pH 8.0) containing 50% acetonitrile. Gel plugs were reduced (10 mM DTT, 1 h at 37 °C), alkylated (50 mM iodoacetamide, 45 min at room temperature in the dark), washed twice with 50% acetonitrile in 50 mM ammonium bicarbonate, dried in a SpeedVac (Savant), and rehydrated for 10 min in 10 μL of 0.2 μg/μL trypsin. Then 25 μL of ammonium bicarbonate (25 mM, pH 8.0) was added and incubated for ∼12 h at 37 °C, followed by peptide extraction using 20–30 μL of 0.2% formic acid. The peptide-containing solution then was transferred to a sample vial for liquid chromatography-tandem MS (LC-MS/MS) analysis.

Electrospray Ionization Ion Trap MS Analysis. LC-MS/MS analysis was performed using a ThermoFisher LTQ XL linear ion trap mass spectrometer in line with a nanoAcquity ultra-performance LC system (Waters Corporation). Tryptic peptides were loaded onto a "precolumn" (Symmetry C18, 180-μm i.d. × 20 mm, 5-μm particle) (Waters Corporation) which was connected through a zero dead-volume union to the analytical column (BEH C18, 75-μm i.d. × 100 mm, 1.7-μm particle) (Waters Corporation). The peptides were eluted over a gradient (0–70% B in 60 min, 70–100% B in 10 min, where B = 70% (vol/vol) acetonitrile, 0.2% formic acid) at a flow rate of 250 nL/min and were introduced online into the linear ion trap mass spectrometer (ThermoFisher) using electrospray ionization. Data-dependent scanning was incorporated to select the 10 most abundant ions (one microscan per spectrum; precursor isolation width 3.0 Da; 35% collision energy; 30-ms ion activation; exclusion duration, 30 s; repeat duration, 15 s; repeat count, 2) from a full-scan mass spectrum for fragmentation by collision-activated dissociation.

Database Search. Product ions (b/y-type ions) were queried in an automated database search against a UniProt database [Sprot 53.0 (269293 sequences; 98902758 residues), *Mus musculus* subset)] by the Mascot search algorithm (1). The following residue modifications were allowed in the search: carbamidomethylation on cysteine (fixed modification) and oxidation on methionine (variable modification). Mascot was searched with a precursor ion tolerance of 1.5 Da and a fragment ion tolerance of 0.6 Da and using the automatic decoy database-searching tool in Mascot. The identifications from the automated search were verified by manual inspection of the raw data.

ChIP. Control or CpG-DNA–treated bone marrow-derived macrophages (BMM) were fixed by adding formaldehyde to the cell culture medium (final concentration 1%, vol/vol) for 10 min at room temperature, washed with ice-cold PBS, lysed with buffer containing 50 mM Tris HCl (pH 8.0), 10 mM EDTA, 1% (wt/vol) SDS, and a protease inhibitor mixture (Roche), followed by sonication in an ice-water bath for 5 × 10 s at 15-μm amplitude (Sanyo 150 sonicator) and centrifugation at 20,000 × *g* for 10 min. The obtained supernatants were diluted 10 times with dilution buffer [20 mM Tris HCl (pH8.0), 150 mM NaCl, 1 mM EDTA, 1% (wt/vol) Triton X-100, 0.01% SDS, protease inhibitor mixture (Roche)] and incubated with antibodies against cREL (C), DNA-dependent RNA polymerase II (Pol II, CTD4H8), C/EBPβ (H-7),

or control rabbit IgG serum for 18 h at 4 °C. All antibodies were obtained from Santa Cruz. Immune complexes were precipitated with Protein A or Protein G Sepharose beads (GE Healthcare) and washed sequentially with low-salt buffer [20 mM Tris HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100], high-salt buffer [20 mM Tris HCl (pH 8.0), 500 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100], and LiCl buffer [10 mM Tris HCl (pH 8.0), 250 mM LiCl, 1 mM EDTA, 0.1% SDS, 1% Nonidet P-40], followed by two wash cycles with TE buffer [10 mM Tris HCl (pH 8.0), 1 mM EDTA]. Protein/chromatin complexes were eluted from the beads with 500 μL of buffer containing 0.1 M $NaHCO_3$ and 1% SDS, and crosslinking was reversed by treatment with 20 μL of 5-M NaCl and incubation at 65 °C for 4 h. DNA was extracted with phenol/chloroform and quantified by quantitative PCR (qPCR). The sequences of the promoter-specific primers were *Nfkbia* (−191 to −86), 5′-GGGCCTGGTCGGGAGGACTT and 5′-GGGCCGGGGTTCATCGGAGA; *Nos2* (−338 to −232), 5′-CAGCAGCTGCAAGCCAGGGT and 5′-AAGGCAGTGGCCCCTAGGCA; *S100a8* (−51 to 91), 5′-GGCAGCTGGCCAAGCTTTCC and 5′-TCCCCCAACCCTCGCTAAGGA; *Csf3* (−22 to 102), 5′-TCCCGGGGCCTTGATGGCTT and 5′-AGCTTCATGCGCCTCTGGGC.

1. Perkins DN, Pappin DJ, Creasy DM, Cottrell JS (1999) Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 20:3551–3567.

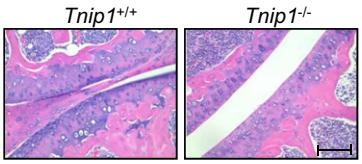

Fig. S1. Absence of joint inflammation in *Tnip1*$^{-/-}$ mice. Eight-week-old *Tnip1*$^{+/+}$ and *Tnip1*$^{-/-}$ mice were killed, and tissue sections of knee joints were stained with H&E and analyzed by microscopy. Images shown are representative of six mice of each genotype. (Scale bar: 40 μM.)

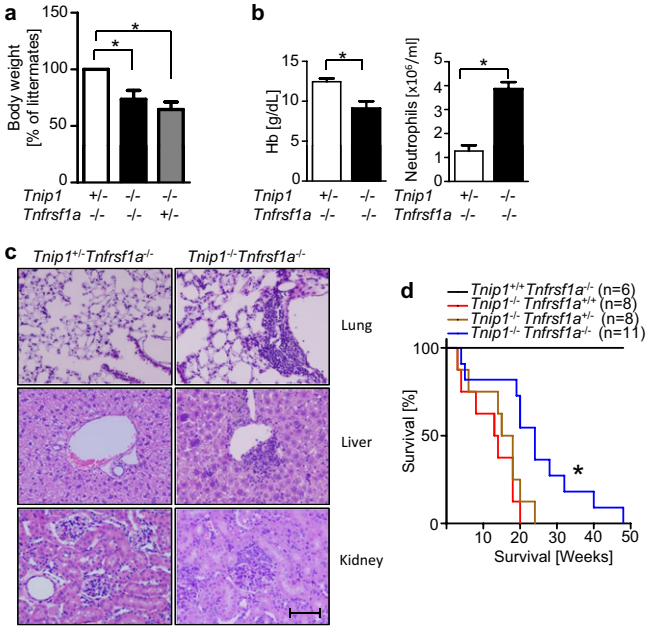

Fig. S2. Inflammatory disease in *Tnip1*$^{-/-}$ mice proceeds independently of TNFRI. (*A* and *B*), *Tnip1*$^{+/-}$ mice were intercrossed with Tnf receptor superfamily member 1A (*Tnfrsf1a*)$^{+/-}$ mice, and body weight (*A*) and blood hemoglobin concentration (Hb) and neutrophil counts in the peripheral blood (*B*) of 8- to 12-wk-old mice were determined (n = 6). (*C*) Microscopic image of H&E-stained tissue sections of lung, liver, and kidney of 12-wk-old *Tnip1*$^{+/-}$*Tnfrsf1a*$^{-/-}$ and *Tnip1*$^{-/-}$*Tnfrsf1a*$^{-/-}$ mice. (Scale bar: 80 μM.) (*D*) Survival of live-born pups of indicated genotypes from intercrossing of *Tnip1*$^{+/-}$*Tnfrsf1a*$^{+/-}$ mice. The number of mice of each genotype is indicated in parentheses. The asterisk indicates a significant increase in survival of *Tnip1*$^{-/-}$*Tnfrsf1a*$^{-/-}$ mice versus *Tnip1*$^{-/-}$*Tnfrsf1a*$^{+/-}$ and *Tnip1*$^{-/-}$*Tnfrsf1a*$^{+/+}$ mice ($P < 0.01$).

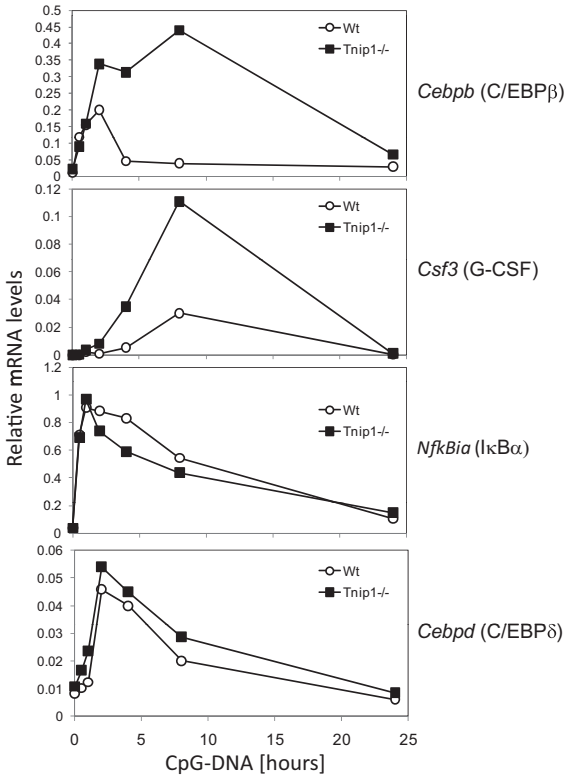

Fig. S3. Kinetics of mRNA expression during CpG-DNA stimulation in *Tnip1*$^{+/+}$ and *Tnip1*$^{-/-}$ BMM. *Tnip1*$^{+/+}$ and *Tnip1*$^{-/-}$ BMM were stimulated with CpG-DNA, and mRNA levels of indicated genes were determined at different time points by qPCR.

Table S1. Peptides identified and manually verified from LC-MS/MS analysis in the constitutive and activated MyD88-dependent protein complexes described in Fig. 1*A*

	Control	Coumermycin		Control	Coumermycin
MyD88 (bait)	RLSLFLNPR	RLSLFLNPR	TRAF6	Not detected	EILSLTVK
	VGSGSLDSFMFSIPLVALNVGVR	LSLFLNPR			REILSLTVK
	LSLFLNPR	ELETRPDPTR			LHLQLPTAQR
	TPVAADWTLLAEEMGFEYLEIR	SLLDAWQGR			LTILDQSEALIR
	ELETRPDPTR	LLELLALLDR			KEGFQPRSTDAGV
	SLLDAWQGR	LLELLALLDREDILK			YECPICLMALR
	SGASVGR	QQNQESEKPLQVAR			METQSMYVGELK
	LLELLALLDR	QLEQTDYR			
	LLELLALLDREDILK	LKLCVSDR			
	LLELLALLDREDILKELK	DVLPGTCVWSIASELIEK			
	IEEDCQK	RMVVVVSDDYLQSK			
	QLEQTDYR	MVVVVSDDYLQSK			
	LKLCVSDR	FALSLSPGVQQK			
	LCVSDRDVLPGTCVWSIASELIEK	KDFPSILR			
	DVLPGTCVWSIASELIEK	DFPSILR			
	RMVVVVSDDYLQSK	FITICDYTNPCTK			
	MVVVVSDDYLQSK	SWFWTR			
	ECDFQTKFALSLSPGVQQK	RLIPIK			
	FALSLSPGVQQK	LAKALSLP			
	FALSLSPGVQQKR				
	KDFPSILR				
	DFPSILR				
	FITICDYTNPCTK				
	SWFWTR				
IRAK1	not detected	IGEGGFGCVYR	TRAF3	Not detected	VSLLQNESVEKNK
		QSFLTEVEQLSR			VTELESVDK
		LDILLGTAR			NTGLLESQLSR
		SSNVLLDER			
		LGDFGLAR			
		GTLAYLPEEYIK			
		DLIEDEAEEAGVTLK			
		APAQAAQQLQR			
		AIQFLHQDSPSLIHGDIK			
IRAK2	Not detected	VQGVSITR	ABIN1	Not detected	MALEFNR
		SLLDTGPIMAGAQR			IFEEDFQR
		ISEGTFADIYQGQR			VPEAGAFGAAEK
		NGVAFAFK			MNEEKEELKK
		FLQAEMQLCLR			YLQDQLSPLTR
		SANVLLDQHLNPK			QENEALKAKLDK
		DLLLSEIPNSTSSVCSR			LQAQVTLTNAQLK
		VSLAGVEEQLR			KQELVTQNELLK
		KTSMGKAVVK			TSILQTLCEQLR
		DLLLSEIPNSTSSVCSRKTSMGK			
IRAK4	RFEALLQTGK	NLNVGILR			
	ISDFGLAR	RFEALLQTGK			
	DRTSVMPMPK	FEALLQTGK			
	GCVNNTIVAVKK	SITNNFDEQPASAGGNR			
		MGEGGFGVVYK			
		GCVNNTIVAVK			
		LGAMVEISTEELK			
		SANILLDKDFTAK			
		ISDFGLAR			
		IVGTTAYMAPEALR			
		TIEDYTDEK			

IRAK: IL-1 receptor–associated kinase; TRAF: TNF receptor-associated factor.

Table S2. Embryonic lethality in ABIN1-deficient mice

Genotype	Number	Percentage	Expected percentage
F1 × F1, Tnip1$^{+/-}$ × Tnip1 $^{+/-}$			
Tnip1$^{+/+}$	29	42	25
Tnip1$^{+/-}$	37	53	50
Tnip1$^{-/-}$	3	4.3	25
F5 × F5, *Tnip1*$^{+/-}$ × *Tnip1*$^{+/-}$			
Tnip1$^{+/+}$	21	26.9	25
Tnip1$^{+/-}$	49	62.8	50
Tnip1$^{-/-}$	8	10.3	25

Tnip1$^{+/-}$ mice were backcrossed to C57BL/6 mice (from 129S2 ES-cell background), and the genotypes of pups born alive from the F1 generation and the F5 generation were determined. Expected Mendelian ratios are shown for comparison. Note increased survival of *Tnip1*$^{-/-}$ pups from the F5 interbreeding, indicating some influence of the genetic background on embryonic lethality.

Table S3. Embryonic lethality in Tnip1$^{+/-}$ Tnfrsf1a$^{+/-}$ × Tnip1$^{+/-}$ Tnfrsf1a$^{+/-}$ mice

	Tnip1$^{+/+}$	*Tnip1*$^{+/-}$	*Tnip1*$^{-/-}$	*Tnip1*$^{-/-}$ (%)	Expected percentage
Tnfrsf1a$^{+/+}$	12	27	4	9.3	25
Tnfrsf1a$^{+/-}$	26	47	10	12	50
Tnfrsf1a$^{-/-}$	12	31	13	23.2	25

Tnip1$^{+/-}$ mice (F4 backcrossed to C57BL/6) were crossed with *Tnfrsf1a*$^{-/-}$ mice (C57BL/6 background), and heterozygous *Tnip1*$^{+/-}$*Tnfrsf1a*$^{+/-}$ mice were further intercrossed, and the genotypes of pups born alive were determined. Expected Mendelian ratios are shown for comparison.

Table S4. Clover analysis of promoter sequences of genes deregulated (up-regulated) by CpG-DNA in *Tnip1*$^{-/-}$ BMM versus *Tnip1*$^{+/+}$ BMM

Transcription factor	Matrix (TRANSFAC)	Identifier (TRANSFAC)	*P* value
C/EBPβ	M00109	V$CEBPB_01	0.001
C/EBP	M00912	V$CEBP_Q2_01	0.001
NF-κB	M00774	V$NFKB_Q6_01	0.022
NF-κB	M00194	V$NFKB_Q6	0.036
AP-1	M00172	V$AP1FJ_Q2	0.013

Five-kilobase promoter regions of genes deregulated (up-regulated) in *Tnip1*$^{-/-}$ BMM upon CpG-DNA treatment were analyzed by Cis-element overrepresentation (Clover) for overrepresentation of transcription factor binding sites (cis-elements) compared with genes comparably induced in *Tnip1*$^{+/+}$ and *Tnip1*$^{-/-}$ BMM upon CpG-DNA treatment. *P* values <0.01 were considered significant. AP-1, activator protein 1.