Supporting Information

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To create TRADD knockout mice, we first generated TRADD-deficient ES cells by using a conditional gene targeting strategy (Fig. S1*a*). Recombinant ES cells bearing a WT *tradd* allele and a floxed *tradd* allele were microinjected into C57BL/6 mouse blastocysts to generate mice heterozygous for the floxed mutation ($tradd^{WT/flox}$). These mice were then crossed to Deleter CRE mice (1) to remove *tradd* coding exons 3–5 and/or the TK/Neo selection markers ($tradd^{WT/KO}$ or $tradd^{WT/fl3–5}$). Removal of exons 3–5 does not remove the entire coding region but the known functional domain of TRADD (death domain) is eliminated. The remaining sequences only encode a very short fragment of TRADD N terminus. $tradd^{WT/KO}$ mice were then intercrossed to generate completely TRADD-deficient ($tradd^{KO/KO}$) mice. Southern blotting confirmed the floxing of the *tradd* gene in ES cells (Fig. S1*b*) and the successful deletion of the floxed allele in $tradd^{KO/KO}$ mice (Fig. S1 *c* and *d*). TRADD protein depletion in $tradd^{KO/KO}$ cells was confirmed by Western blotting (shown later in Fig. S6 *a–c*). Surprisingly, $tradd^{KO/KO}$ mice were healthy and showed no obvious abnormalities, suggesting that TRADD plays little or no exclusive role in embryogenesis.

Most studies of TRADD deficiency reported to date have been based on siRNA interference, shRNA knockdown, or dominant negative mutant expression in transformed tumor cell lines (2–4). Our conditional targeting strategy resulted in the insertion of the Neo/TK cassette in the 3′ UTR of the *tradd* gene, which generated a hypomorphic allele (5) characterized by reduced mRNA expression. We found that, by breeding our $tradd^{WT/KO}$ mice with $tradd^{WT/flox}$ mice, we obtained offspring whose mouse embryonic fibroblasts (MEFs) expressed various levels of TRADD protein (Fig. S2*a*). Specifically, $tradd^{WT/KO}$ MEFs expressed half the amount of TRADD protein present in $tradd^{WT/WT}$ MEFs, and $tradd^{KO/flox}$ MEFs expressed a very low level of TRADD protein that mimicked the level present in TRADD knockdown cells (2–4).

Interestingly, $tradd^{KO/flox}$ cells were only partially resistant to TNFα plus CHX treatment (Fig. S2*b*), indicating that even a minute amount of TRADD protein in a cell is sufficient to mediate a nearly normal level of TNFα-induced cell death. As well, $tradd^{KO/flox}$ cells produced comparable amounts of IL-6 in response to TNFα stimulation (Fig. S2*c*). Thus, even the very low level of TRADD protein present in $tradd^{KO/flox}$ cells is sufficient to mediate normal levels of TNFα-induced inflammatory cytokine production.

SI Methods

Generation of TRADD Knockout Mice. The strategy used to generate the *tradd* conditional allele is shown in Fig. S1*a*. The targeting construct used to generate the *tradd* conditional allele contained a thymidine kinase (TK)/neomycin (Neo) cassette, a short arm, a long arm, and *tradd* exons 3–5 flanked by loxP sites. All *tradd* gene fragments were PCR-amplified from genomic DNA prepared from IB10 ES cells (129Sv/J). The linearized targeting vector was electroporated into IB10 ES cells, and G418-resistant clones were screened. Southern blotting using a flanking probe was used to detect the $tradd^{WT}$ and $tradd^{flox}$ alleles (Fig. S1*a*). Recombinant ES clones were injected into C57BL/6 blastocysts and chimeric mice showing germline transmission were backcrossed with B6 mice to produce TRADD-deficient mice of the B6 background. We also backcrossed the chimeras into the FVB background in case we needed to rescue the line from embryonic lethality. All mice used in this study were of the B6.F3 or FVB.F3 generations. To remove *tradd* exons 3–5 and the Neo/TK cassette, we backcrossed $tradd^{WT/flox}$ mice with Deleter CRE mice (a CMV-Cre recombinase transgenic strain) to generate $tradd^{WT/KO}$ ES cells. $tradd^{KO/KO}$ mice were generated by intercrossing $tradd^{WT/KO}$ mating pairs.

Screening of TRADD Knockout Mice. Three PCR primer pairs (see Table S1) were used to screen for TRADD-deficient mice. With primer pair A plus B, we amplified a 230bp fragment from the WT allele by using the following PCR program: 1 cycle at 95°C for 5 min; 35 cycles at 94°C for 45 sec, 62°C for 45 sec, and 72°C for 1 min 20 sec; 1 cycle at 72°C for 10 min for the last extension. With primer pair A plus C, we amplified a 600-bp fragment from the KO allele by using the following program: 1 cycle at 95°C for 5 min; 40 cycles at 94°C for 45 sec, 60°C for 45 sec, and 72°C for 1 min 20 sec; 1 cycle at 72°C for the last 10 min extension. Primer pair A plus C also amplified a 1.85-kb fragment from the WT allele under low stringency conditions. The recombination of the *tradd* locus was also verified by Southern blotting in which the flanking probe (FP), the middle arm probe (MP), or the long arm probe (LAP) was hybridized to *Bgl*II/*Kpn*I-digested genomic DNA (Fig. S1 *c* and *d*).

^{3}H-thymidine Incorporation. For T cell proliferation studies, 96-well round-bottom plates were precoated with either anti-CD3 antibody (0.3 μg/ml, 145–2C11, BD Biosciences) or with anti-CD3 plus anti-CD28 (5 μg/ml, 37.51, BD Biosciences). Purified T cells were seeded in these plates at 10^5 cells per well in a total volume of 200 μl of RPMI containing 10% FBS and 2-ME. Stimulation was allowed to proceed for 48 h before the addition of a pulse of 1 μCi ^{3}H-thymidine per well followed by incubation for an additional 8 h. The amount of ^{3}H-thymidine incorporated by the proliferating T cells was measured using a Topcount (Perkin–Elmer).

Thymocyte Killing and Activation-Induced Cell Death. For thymocyte killing experiments, thymocytes were seeded at 10^6 cells per well in 24-well plates and treated for 24 h with anti-CD3 (3 μg/ml), dexamethasone (1 or 3 μM), etoposide (1 or 3 μM), anisomycin (1 or 10 μM), or staurosporine (1 or 3 μM). Viability was assessed by 7-AAD staining. For activation-induced cell death experiments, $CD4^+$ T cells (10^6 cells per well) were stimulated with plate-bound anti-CD3 plus anti-CD28 and soluble IL-2 (20 ng/ml) for 5 days. After resting for 24 h, surviving cells were purified by using the Histopaque 1083 density-separating method (Sigma). Cells (10^6 per well) were washed and restimulated for 24 h in 24-well plates precoated with anti-CD3. Cells were harvested, stained with 7-AAD, and analyzed by flow cytometry to determine cell viability.

Real-Time RT-PCR. MEFs (2×10^6) were plated in 10-cm plates and cultured for 24 h before stimulation with 10 ng/ml TNFα in 4 ml of RPMI medium for 1 or 4 h. Stimulated cells were washed, lysed with 1 ml TRIzol solution, and transferred into 1.5 ml Eppendorf tubes for total RNA purification according to the manufacturer's protocol. Total RNA was reverse-transcribed and assayed by quantitative real-time PCR. All reactions were performed in an ABI-7900HT Fast Real-Time PCR system using Power SYBR Green PCR reagents according to the manufacturer's instructions (Applied Biosystems). Primer sequences are listed in Table S1.

Gel Mobility Shift Assay. Nuclear and cytoplasmic extracts were prepared from MEFs and used in standard gel mobility shift assays. The NF-κB oligonucleotide probes for EMSA were end-labeled with [^{32}]ATP by using T4 polynucleotide kinase (New England BioLabs). Nuclear extracts (10 μg of protein) were prepared in EMSA buffer [10 mM Hepes (pH 7.9), 6% (vol/vol) glycerol, 2% (vol/vol) Ficoll, 100 mM KCl, 0.5 mM EDTA, 2.5 mM $MgCl_2$, and 1 mM DTT] and incubated with the DNA probes for 15 min at room temperature. Protein-DNA complexes were analyzed on a 5% native polyacrylamide gel.

RelA/p65 Immunofluorescent Staining. MEFs (2×10^4) were plated on cover slips overnight and stimulated with TNFα (10 ng/ml), IL-1β (10 ng/ml), or LPS (500 ng/ml) for 30 min. Stimulated cells were fixed in methanol and stained with mouse anti-NF-κB (RelA/p65; sc-8008, Santa Cruz Biotechnology) followed by Cy3-conjugated sheep F(ab′)$_2$ anti-mouse IgG (Sigma). The stained slides were mounted in DAPI-containing mounting solution and observed under a fluorescence microscope.

***In Vivo* TNFα Injection.** *tradd*$^{WT/WT}$, *tradd*$^{KO/KO}$, and *tnfr1*$^{-/-}$ mice were injected intravenously in the tail vein on each of 5 days with 3.0 μg of murine TNFα in 100 μl PBS. Serum samples were collected just before TNFα injection and at 6 and 24 h after injection, and IL-6 concentrations were evaluated by ELISA. Skin samples around the injection site were collected for H&E staining.

Immunization and FDC/GC Detection. Male mice (6–12 weeks of age) of the *tradd*$^{WT/WT}$ and *tradd*$^{KO/KO}$ genotypes were injected i.p. on day 0 with 10^8 washed sheep red blood cells (SRBC) (HemoStat Laboratories). At 10 days after immunization, spleens were isolated from immunized mice, fixed for 24 h in zinc-buffered formalin (Fisher Scientific), and transferred to 70% ethanol before processing through paraffin. For frozen sections, spleens were frozen in OCT embedding medium and sections (6-μm thick) were cut by using a cryostat microtome. The sections were mounted onto slides coated with polyL-lysine and air-dried. Detection of FDCs was carried out by incubating frozen spleen sections with biotinylated anti-CR1 (clone 8C12) (BD Biosciences). Detection of GCs was carried out by incubating formalin-fixed spleen sections with biotinylated peanut agglutinin (PNA; Vector Labs) and biotinylated anti-B220 (BD Biosciences).

1. Schwenk F, Baron U, Rajewsky K (1995) A cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. *Nucleic Acids Res* 23:5080–5081.
2. Wesemann DR, Qin H, Kokorina N, Benveniste EN (2004) TRADD interacts with STAT1-alpha and influences interferon-gamma signaling. *Nat Immunol* 5:199–207.
3. Zheng L, *et al.* (2006) Competitive control of independent programs of tumor necrosis factor receptor-induced cell death by TRADD and RIP1. *Mol Cell Biol* 26:3505–3513.
4. Sethi G, Ahn KS, Chaturvedi MM, Aggarwal BB (2007) Epidermal growth factor (EGF) activates nuclear factor-kappaB through IkappaBalpha kinase-independent but EGF receptor-kinase dependent tyrosine 42 phosphorylation of IkappaBalpha. *Oncogene* 26:7324–7332.
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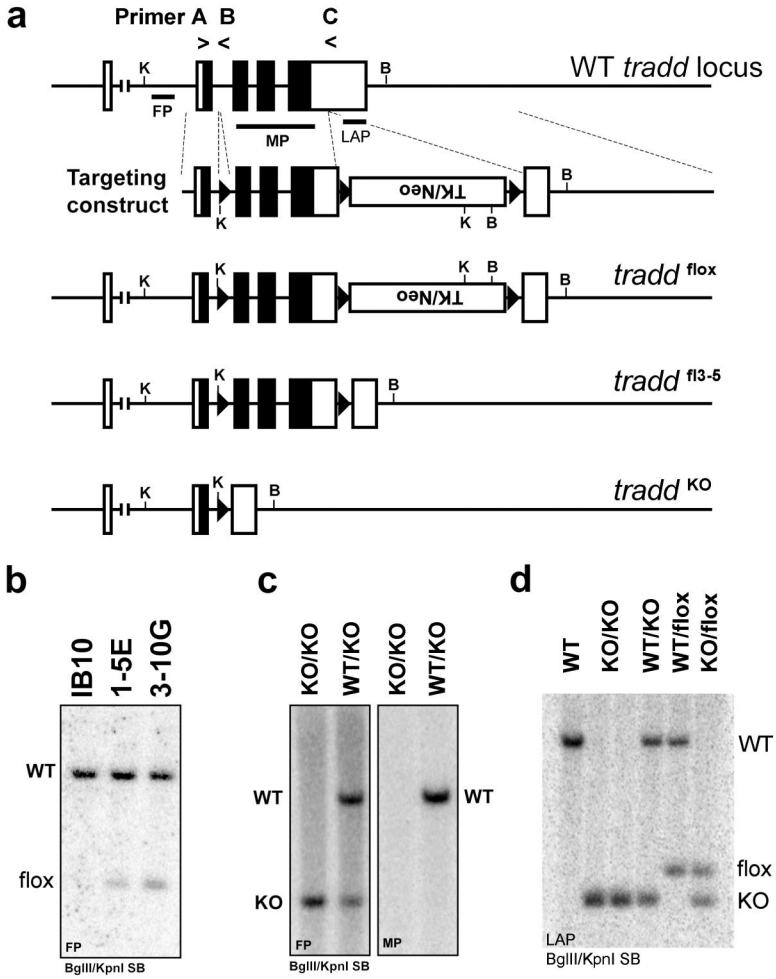

Fig. S1. Targeting strategy and germline confirmation. (*a*) *tradd* coding exons 3–5 and a TK/Neo selection cassette were flanked by loxP sequences and subcloned into the targeting vector. After treatment with CRE, the TK/NEO cassette and/or exons were removed, producing the floxed ($tradd^{fl3-5}$) or KO ($tradd^{KO}$) alleles, respectively. (*b*) Genomic Southern blotting (SB) performed by using the flanking probe (FP) to detect recombinant ES clones. (*c*) Genomic Southern blotting performed by using the FP or middle arm probe (MA) to detect KO ES cells. (*d*) Genomic Southern blotting performed by using the long arm probe (LAP) and tail DNA from mice of the indicated genotypes to identify WT and TRADD-deficient (KO) mice. For *b*–*c*, DNA was digested with BglII and KpnI.

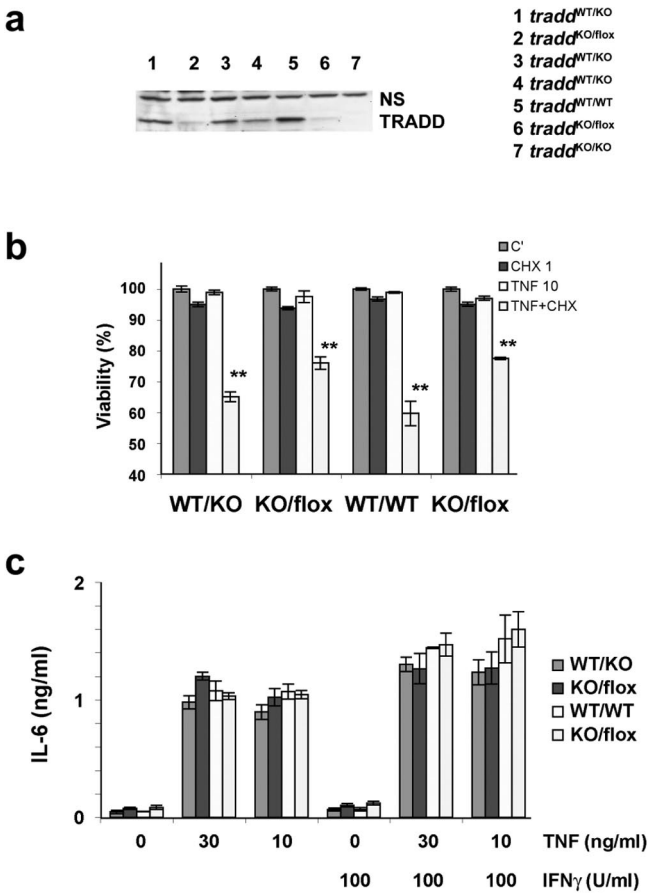

Fig. S2. TRADD expression in *tradd*$^{WT/WT}$, *tradd*$^{WT/KO}$, and *tradd*$^{KO/flox}$ MEFs. (*a*) Western blot of TRADD protein levels in *tradd*$^{WT/WT}$, *tradd*$^{WT/KO}$, and *tradd*$^{KO/flox}$ MEFs. NS, nonspecific band that served as an internal control for protein loading. (*b*) TRADD knockdown mimics. MEFs from *tradd*$^{WT/WT}$, *tradd*$^{WT/KO}$, and *tradd*$^{KO/flox}$ mice were analyzed as in Fig. 1*B*. **, $P < 0.01$, Student's *t* test. (*c*) IL-6 production. MEFs from *tradd*$^{WT/WT}$, *tradd*$^{WT/KO}$, and *tradd*$^{flox/KO}$ mice were treated for 24 h with TNFα (10 ng/ml) and IFNγ (100 U/ml). IL-6 in the culture supernatants was measured by ELISA. For *b* and *c*, the results shown are the mean viability ± SD of triplicate determinations. For *a*–*c*, data are representative of at least two independent experiments.

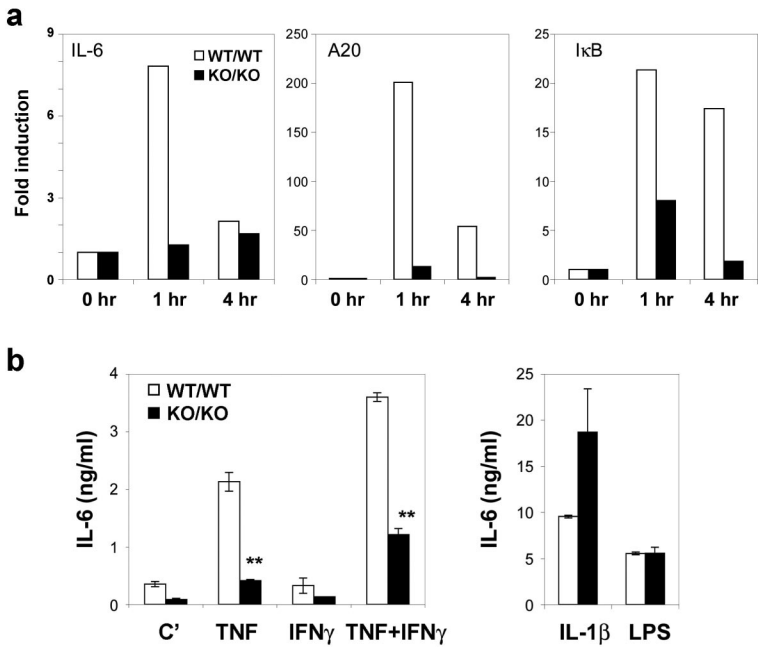

Fig. S3. TRADD deficiency results in inhibited IL-6 production and normal NF-κB inhibitors. (*a*) MEFs from *tradd*$^{WT/WT}$ and *tradd*$^{KO/KO}$ mice were treated for 1 or 4 h with 10 ng/ml TNFα and the induction of IL-6 (positive control), and the NF-κB inhibitors A20 and IκB were evaluated by real-time RT-PCR. The results shown are the average fold induction over background. For *a*–*c*, data are representative of at least two independent experiments. (*b*) MEFs from *tradd*$^{WT/WT}$ and *tradd*$^{KO/KO}$ mice were treated for 24 h with TNFα (10 ng/ml), IFNγ (100 U/ml), IL-1β (10 ng/ml), and/or LPS (100 ng/ml). IL-6 in the culture supernatants was measured by ELISA. The results shown are the mean IL-6 level ± SD of triplicate determinations. **, $P < 0.01$, Student's *t* test. C', control (no stimulus).

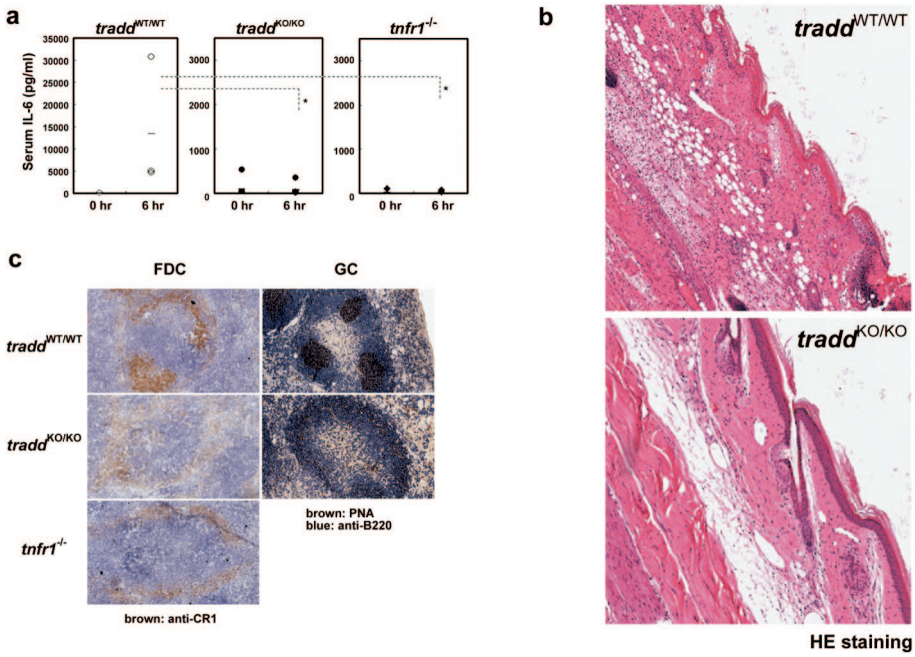

Fig. S4. Abolition of TNFα-induced inflammatory responses and absence of FDC clusters and SRBC-induced GCs in *tradd*$^{KO/KO}$ mice. (*a*) Reduced IL-6 production. *tradd*$^{WT/WT}$ ($n = 4$), *tradd*$^{KO/KO}$ ($n = 5$), and *tnfr1*$^{-/-}$ ($n = 3$) mice were injected in the tail vein with TNFα (3 μg per mouse) and serum IL-6 levels were determined by ELISA 6 h later. Horizontal bar, mean value. *,$P < 0.05$, Student's *t* test. (*b*) Reduced inflammatory tissue damage associated with TNFα injection. *tradd*$^{WT/WT}$ and *tradd*$^{KO/KO}$ mice were intravenously injected with 3.0 μg of TNFα on each of 5 days. Samples of tail skin around the injection site were acquired on day 5 and stained with HE. Inflammatory damage can be seen in the WT but not in the mutant. (*c*) Decreased FDC and impaired GC formation. (*Left*) FDC clusters in nonimmunized mice of the indicated genotypes were determined by anti-CR1 staining. (*Right*) GC formation in splenic follicles of SRBC-immunized *tradd*$^{WT/WT}$ and *tradd*$^{KO/KO}$ mice was determined by anti-B220 and PNA staining. Results shown in *b* are representative of three mice examined per group.

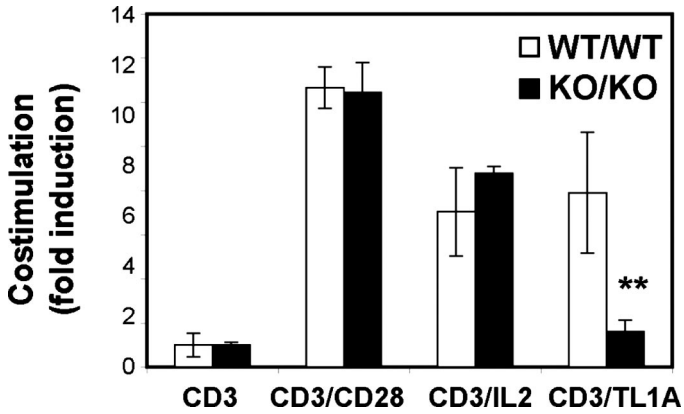

Fig. S5. TRADD is required for DR3 mediated CD4$^+$ T cell costimulation. CD4$^+$ T cells were purified from *tradd*$^{WT/WT}$ and *tradd*$^{KO/KO}$ mice and stimulated for 56 h with plate-bound anti-CD3 (0.3 μg/ml) plus anti-CD28 (5 μg/ml), IL-2 (10 ng/ml), and/or mouse TL1a (3 μg/ml). Costimulation was assessed in terms of increased T cell proliferation as measured by ^{3}H-thymidine incorporation. Results shown are the mean fold induction (over anti-CD3 stimulation alone) $\pm$ SD of triplicate determinations. **, $P < 0.01$, Student's *t* test. Data shown are representative of at least three independent experiments.

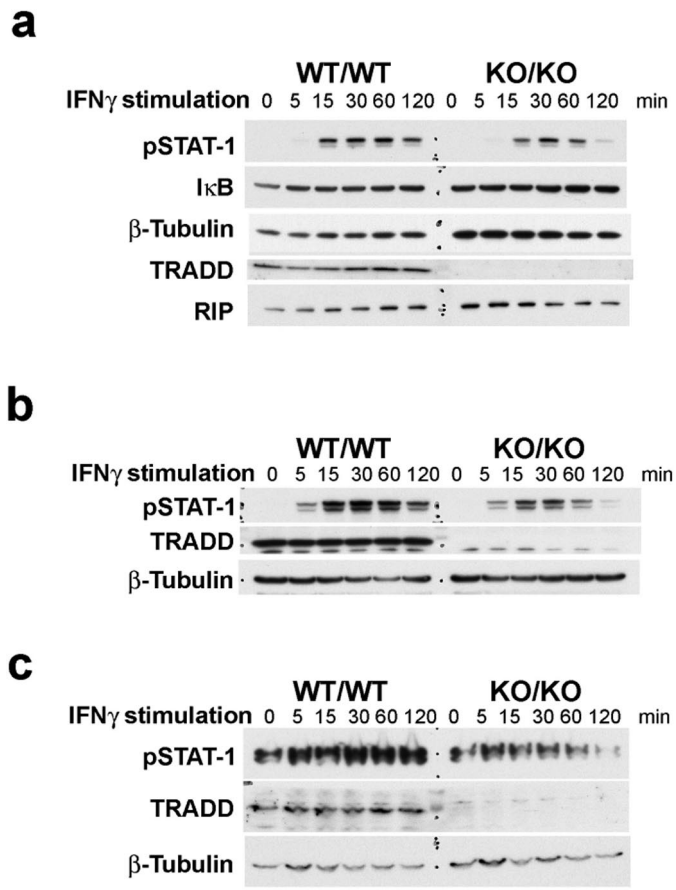

Fig. S6. TRADD deficiency does not enhance IFNγ signaling. (*a*–*c*) IFNγ signaling. *tradd*$^{WT/WT}$ and *tradd*$^{KO/KO}$ MEFs (*a*) ($n = 3$ pairs), BM-Macs (*b*) ($n = 7$ pairs), and CD4^{+} T cells (*c*) ($n = 3$ pairs) were stimulated with 10 U/ml IFNγ for the indicated times, and STAT1 phosphorylation was assessed by Western blotting. IκB and RIP were also examined in MEFs (*b*). For *a*–*c*, results shown are representative of at least three independent experiments.

Table S1. Primer sequences

Mouse Screening	
Primer A: Common primer	5′-TGGAGCCTTTGCTTATTGCACATGT-3′
Primer B: WT primer	5′- GTTGTCCCCCAAACAGGACT-3′
Primer C: KO primer	5′-CACTATTCCACTGAAGGTGACTAAC-3′
Real-time RT-PCR	
IL-6 forward	5′-AGTTGCCTTCTTGGGACTGA-3′
IL-6 reverse	5′-TCCACGATTTCCCAGAGAAC-3′
A20 forward	5′-TCGTGGCTCTGAAAACCAATG-3′
A20 reverse	5′-GATGGGTCTTCTGAGGATGTTGC-3′
IκBα forward	5′-AACCTGCAGCAGACTCCACT-3′
IκBα reverse	5′-GACACGTGTGGCCATTGTAG-3′