Beyond tumor necrosis factor receptor: TRADD signaling in toll-like receptors


*The Campbell Family Institute for Breast Cancer Research, Ontario Cancer Institute, University Health Network and Department of Medical Biophysics, University of Toronto, Toronto, ON, Canada MSG 2C1; †Institute of Microbiology and Immunology, School of Life Science, National Yang-Ming University, Taipei, Taiwan 112, Republic of China; and ‡Department of Immunology, University of Toronto, Sunnybrook and Women’s College Health Sciences Centre, 2075 Bayview Avenue, Toronto, ON, Canada M4N 3M5

Contributed by Tak W. Mak, July 9, 2008 (sent for review June 24, 2008)

Tumor necrosis factor receptor 1-associated death domain protein (TRADD) is the core adaptor recruited to TNF receptor 1 (TNFR1) upon TNFα stimulation. In cells from TRADD-deficient mice, TNFα-mediated apoptosis and TNFα-stimulated NF-κB, JNK, and ERK activation are defective. TRADD is also important for germinal center formation, DR3-mediated costimulation of T cells, and TNFα-mediated inflammatory responses in vivo. TRADD deficiency does not enhance IFNγ-induced signaling. Importantly, TRADD has a novel role in TLR3 and TLR4 signaling. TRADD participates in the TLR4 complex formed upon LPS stimulation, and TRADD-deficient macrophages show impaired cytokine production in response to TLR ligands in vitro. Thus, TRADD is a multifunctional protein crucial both for TNFR1 signaling and other signaling pathways relevant to immune responses.

TNF | innate immunity

Tumor necrosis factor alpha (TNFα) is a pleiotropic cytokine involved in a broad range of biological activities, including inflammation and cell differentiation, survival, and death (1). TNFα mediates these activities by engaging two distinct cell surface receptors: TNFR1 and TNFR2. Activation of TNFR1 leads to the recruitment of the intracellular death domain (DD)-containing adaptor TNFR-associated DD protein (TRADD) through a homotypic interaction with DD of TNFR1. On one hand, the recruitment of TRADD can promote the association of the TNFR1 complex with Fas-associated DD (FADD), which induces caspase activation and cell death. On the other hand, TRADD can also recruit receptor-interacting protein kinase 1 (RIP1) and TNFR-associated factor-2 (TRAF2), which trigger NF-κB activation, leading to cell survival and proinflammatory responses (1).

TRADD was originally identified in a yeast two-hybrid screen performed to identify TNFR1-interacting proteins (2). Intriguingly, TRADD is the first adaptor protein identified that binds directly to the DD of TNFR1 but transduces signals resulting in either apoptosis or NF-κB activation (2–4). Indeed, TRADD overexpression in 293T cells activates both apoptotic and cell-survival signaling pathways (2). In addition to TNFR1, TRADD may mediate signaling downstream of the death receptors (and TNFR superfamily members) DR3 and DR6 (5, 6). As well, recent TRADD knockdown studies have indicated that TRADD may be involved in signaling mediated by receptors unrelated to the TNFR superfamily, such as in IFNγ receptor (7–9). These findings give tantalizing hints of the potential breadth of TRADD functions.

To date, a knockout animal model has yet to be reported for TRADD, even though this important adaptor was identified more than ten years ago. In this study, we generate TRADD knockout mice by conditional gene targeting and show that not only is TRADD indispensable for TNFα-induced NF-κB activation and apoptosis in vitro and TNFα-induced inflammatory responses in vivo, but also that this molecule is involved in germinal center (GC) formation, T cell costimulation, and TLR signaling. Our TRADD knockout mice represent a very useful tool for extending the ever-increasing list of TRADD functions in vitro and in vivo.

Results

TRADD Is Indispensable for TNFα-Mediated Cell Death. To dissect the role of TRADD in TNFα-induced signaling, we generated E14.5 mouse embryonic fibroblast cells (MEFs) from traddWT/WT and traddKO/KO mice (supporting information (SI) Figs. S1 and S2), treated them with TNFα plus cycloheximide (CHX) for 24 h, and assessed cell death by 7-AAD staining and flow cytometry. More than 70% of traddWT/WT MEFs underwent apoptosis after stimulation with TNFα plus CHX (Fig. 1 A and B). In contrast, traddKO/KO MEFs were completely resistant to TNFα-induced cell death under the same conditions. MEFs of both genotypes showed equivalent sensitivity to other cell-death-inducing agents, including sorbitol, etoposide, doxorubicin, anisomycin, and UV irradiation (data not shown). Similarly, TRADD deficiency did not alter the susceptibility of CD4+ T cells to Fas-mediated activation-induced cell death (10), indicating that TRADD plays no role in Fas-mediated apoptosis (data not shown). Taken together, our results show that TRADD is indispensable for the cell-death arm of TNFR1 signaling but is not involved in the death triggered by many other agents.

TRADD Is Essential for TNFα-Mediated NF-κB Activation. NF-κB activation is a key signaling event required for TNFα-induced production of inflammatory cytokines (1–3). We compared NF-κB activation in MEFs and bone-marrow-derived macrophages (BMMacs) (data not shown) obtained from traddWT/WT and traddKO/KO mice and found that TNFα-induced NF-κB activation could not be triggered in the absence of TRADD (Fig. 1 C and D); meanwhile, LPS or IL-1β-induced NF-κB activation remained intact (Fig. 1 D).

The authors declare no conflict of interest.

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‡Present address: Toronto Western Hospital, Toronto, ON, Canada MST 2S8.


¶Present address: Academia Sinica, Taipei 11529, Taiwan.

**To whom correspondence should be addressed. E-mail: tmak@uhnres.utoronto.ca.

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Gel mobility shift assay. MEFs from B and NF-αng/ml), and RelA/p65 translocation was assessed. 12430/H20841

MEFs were stimulated with 10 ng/ml TNF

Fig. 2. Alterations to TNFR1 signaling and complex formation in the absence of TRADD. (A) Representative flow cytometric data with 1 μg/ml CHX. (B) Bar graph summary of all flow cytometric data. Results shown are the mean viability ± SD of triplicate determinations. **, P < 0.01, Student’s t test. For A and B, data are representative of at least three independent experiments. (C) Gel mobility shift assay. MEFs from traddWT/WT and traddKO/KO mice were stimulated with 10 ng/ml TNFα for the indicated times, and NF-κB activation was evaluated by EMSA. (D) RelA/p65 translocation. The MEFs in A were subjected to immunofluorescent staining to detect RelA/p65 translocation into the nucleus. MEFs were also treated with LPS (500 ng/ml) or IL-1β (10 ng/ml), and RelA/p65 translocation was assessed.

Fig. 1. TRADD is indispensable for TNFα-induced cell death and TNFα-mediated NF-κB activation. Primary MEFs from traddWT/WT and traddKO/KO mice were treated for 24 h with TNFα alone (10 ng/ml), CHX alone (1–10 μg/ml), or TNFα plus CHX. Viability was assessed by T-AdA staining and flow cytometry. (A) Representative flow cytometric data with 1 μg/ml CHX. (B) Bar graph summary of all flow cytometric data. Results shown are the mean viability ± SD of triplicate determinations. **, P < 0.01, Student’s t test. For A and B, data are representative of at least three independent experiments. (C) Gel mobility shift assay. MEFs from traddWT/WT and traddKO/KO mice were stimulated with 10 ng/ml TNFα for the indicated times, and NF-κB activation was evaluated by EMSA. (D) RelA/p65 translocation. The MEFs in A were subjected to immunofluorescent staining to detect RelA/p65 translocation into the nucleus. MEFs were also treated with LPS (500 ng/ml) or IL-1β (10 ng/ml), and RelA/p65 translocation was assessed.

Importantly, the abrogation of TNFα-induced NF-κB activation observed in traddKO/KO cells was not because of up-regulation of NF-κB inhibitors such as IκB and A20 (11–14) (Fig. S3a, Center and Right). These observations corroborate with cytoplasmic signaling events upstream of NF-κB activation in TNFα-stimulated traddWT/WT and traddKO/KO MEFs and BM-Macs. In contrast to the situation in traddWT/WT cells, TNFα treatment failed to induce significant degradation of IκB in traddKO/KO MEFs (Fig. 24) or BM-Macs (Fig. 2B). To bolster the above findings, we also evaluated IL-6 production in TNFα-stimulated traddWT/WT and traddKO/KO MEFs by ELISA. We showed that IL-6 was not induced in the absence of TRADD (Fig. S3b), confirming our real-time RT-PCR results (Fig. S3a Left). In contrast, IL-6 production in response to IL-1β and LPS stimulation was unaltered in the absence of TRADD (Fig. 3B).

TRADD Is Essential for TNFR1-Mediated MAP Kinase Activation. MAP kinase (MAPK) activation is reportedly involved in transducing TNFα signaling (15). We therefore analyzed the effect of TRADD deficiency on TNFα-induced ERK and JNK activation. Surprisingly, traddKO/KO cells still exhibited some degree of JNK and ERK activation in response to murine TNFα stimulation, albeit with altered kinetics (Fig. 2 A and B). To eliminate potential confounding effects because of MAPK activation downstream of TNFR2, we stimulated traddWT/WT and traddKO/KO cells with human TNFα, which is thought to preferentially activate TNFR1 in murine cells (16). Under these conditions, MAPK activation was completely abolished in the absence of TRADD (Fig. 2C), confirming that TRADD is essential for TNFα-mediated MAPK activation.

The Composition of the TNFR1 Complex Is Altered in TNFα-Stimulated TRADD-Deficient Cells. TRADD is believed to be the core adaptor that recruits RIP, TRAF2, and FADD into the TNFR1 complex in response to TNFα stimulation (3, 4). To determine whether deletion of TRADD would affect TNFR1 complex formation, we treated traddWT/WT and traddKO/KO MEFs with TNFα and carried out immunoprecipitation (IP) experiments by using an antibody specific for TNFR1. Peak binding of RIP and TRAF2 to TNFR1 was detected in the WT after 5 min of TNFα stimulation, with RIP exhibiting a ladder-like pattern of ubiquitination (Fig. 2D). Surprisingly, the association of RIP with TNFR1 occurs even without receptor triggering, and RIP (but

Fig. 2. Alterations to TNFR1 signaling and complex formation in the absence of TRADD. (A–C) ERK, JNK, and IκB. traddWT/WT and traddKO/KO MEFs (A) and BM-Macs (B) were stimulated with 10 ng/ml murine TNFα (A and B) or 10 ng/ml human TNFα (C) for the indicated times, and ERK and JNK phosphorylation and IκB degradation were assessed by Western blotting. β-tubulin and actin were used as loading controls. (D) TNFR1 complex composition. traddWT/WT and traddKO/KO MEFs were stimulated with 10 ng/ml TNFα for the indicated times, and the TNFR1 complex was isolated by IP. TRAF2, RIP, and ubiquitinated RIP (Ub-RIP) within the TNFR1 complex were detected by Western blotting. Data shown are representative of at least three independent experiments.
TRADD Outside TNFR1. In addition to TNFR1 signaling, TRADD may mediate signaling by the TNF receptor superfamily member DR3 (5, 6), which is thought to provide costimulatory signals during T cell activation (5). We validated this hypothesis by showing that WT CD4⁺ T cells, but not TRADD-deficient CD4⁺ T cells, exhibited increased proliferation after anti-CD3 plus TL1a stimulation (Fig. S5). Thus, TRADD is indeed indispensable for DR3-mediated T cell costimulation.

Recently, TRADD was implicated in the cross-talk between TNFR1 signaling and IFNγ signaling. In particular, in RAW cells, TRADD knockdown increases IFNγ-induced STAT1 activation and function (7). To validate these observations in primary TRADD-deficient cells, we examined IFNγ-induced STAT1 phosphorylation in traddKO/KO and traddKO/KO MEFs (Fig. S6a), BM-Macs (Fig. S6b) and CD4⁺ T cells (Fig. S6c). Although RIP and IκB levels appeared normal in mutant MEFs, no enhancement of IFNγ-induced STAT1 activation was detected in any traddKO/KO cell type. In fact, if anything, the TRADD-deficient cells of a tested pair sometimes showed a slight reduction in STAT1 activation after IFNγ stimulation. Further analysis of IFNγ signaling needs to be performed both in primary and transformed TRADD-deficient cells to resolve this controversy.

TRADD Is Involved in TLR Signaling and Responses. Several signaling mediators involved in TNFR downstream signaling have also been reported to play roles in modulating TLR signaling (18–20). To determine whether TRADD is one of these mediators, we treated RAW cells with the TLR4 ligand LPS for 5–90 min and carried out IP with anti-TLR4 antibody to isolate the TLR4 receptor complex. We found that both TRADD and RIP coimmunoprecipitated with TLR4 (Fig. 3A). To further assess the interaction between TRADD and components of the TLR4 complex, we cotransfected 293T cells with a TRADD-expressing vector plus vectors expressing the indicated Myc-tagged mediators. Proteins associating with TRADD were identified by TRADD-IP and anti-Myc Western blotting. IRAK4KO was used as a negative control; pCDNA3 and pBABE were used as empty vector controls. (D) TLR4-TIR associates with TRADD; reciprocal IP involving anti-Myc IP and anti-TRADD Western blotting. For A–D, data are representative of at least two independent experiments.

TRADD Deficiency Compromises TNFα-Induced Responses in Vivo. To verify that the defects we observed in TRADD-deficient cells in vitro were also relevant in vivo, we intravenously infected groups of traddWT/WT, traddKO/KO, and tnfα-/− (negative control) mice with 3.0 μg of recombinant TNFα and evaluated serum IL-6 at 0 and 6 h after infection. One traddWT/WT mouse was found dead at 4 h after infection. However, all other WT mice, but none of the traddKO/KO or tnfα−/− mice, responded to the TNFα treatment by exhibiting a dramatic increase in serum IL-6 at 6 h after injection (Fig. S4a). Histological analyses of the TNFα injection sites in the mutant strains confirmed that TRADD and TNFR1 are essential for the local tissue damage associated with TNFα injection (Fig. S4b).

In addition to inflammatory cytokine production, engagement of TNFR1 is also required for the formation of follicular dendritic cell (FDC) clusters in resting animals and splenic GC formation in immunized animals (17). We found that in contrast to WT mice, TRADD-deficient mice and TNFR1-deficient mice showed no organized FDC clusters in the spleen (Fig. S4c Left). As well, upon sheep RBC immunization, explicit GC formation at the junction of the T cell and B cell regions was observed in splenic follicles of traddWT/WT mice but not in traddKO/KO mice (Fig. S4c Right). These results demonstrate that the development of normal FDC clusters and GC formation in the spleen require TRADD.
TRADD deficiency on LPS-stimulated NF-κB and MAPK activation in BM-Macs. There were no obvious alterations in MAPK activation (Fig. 4C, STAT1 activation (Fig. 4D), or IκB degradation (Fig. 4D and E) in the absence of TRADD. However, we did observe a reduction in p65 phosphorylation status in traddKO/KO cells, particularly at later timepoints (Fig. 4C, first row). TLR4 engagement leads to activation of the Myd88-dependent pathway that mediates early NF-κB and MAPK activation, as well as the MyD88-independent, TRIF-dependent pathway that mediates late NF-κB and IRF3 activation (21, 22). Given the reductions in serum TNFα production and late stage p65 phosphorylation in LPS-stimulated TRADD-deficient cells, we suspect that it is the TRIF-dependent pathway that is compromised in the absence of TRADD.

TRIF is crucial for the MyD88-independent signaling pathway activated by engagement of TLR3 or TLR4 (21). To elucidate the role of TRADD in the TRIF-dependent pathway while eliminating any potentially compensatory effects of the Myd88-dependent pathway (23), we examined the role of TRADD in TLR3 signaling. We first examined IL-6 production in response to polyinosine-polycytidylic acid [poly(I:C)], a synthetic TLR3 ligand. We used TRADD/TNF double knockout mice for these experiments to ensure that autocrine TNFα could not indirectly regulate the TLR3 response. We found that BM-Macs and lung fibroblasts from the double mutants produced significantly less IL-6 than did WT cells after 24 h poly(I:C) stimulation (Fig. 5A and B). Next, we examined TNFα production in response to poly(I:C) and found that peritoneal macrophages from traddKO/KO mice produced significantly less TNFα at 24 h after treatment than did peritoneal macrophages from WT mice (Fig. 5C). These data corroborate the deficit in TLR signaling that we noted in LPS-treated TRADD-deficient cells and implicate TRADD in the TRIF-dependent pathway.

Signaling through the TRIF pathway results in the activation of NF-κB and IRF3 (22). To further define TRADD’s role in TRIF signaling, we carried out luciferase reporter assays in which 293T cells were transfected with either an NF-κB or IRF3 luciferase reporter in the presence of TRADD and/or TRIF expression vectors. Results are expressed as luciferase activity relative to β-galactosidase activity ± SD. For A–E, *, P < 0.05, Student’s t test. Data are representative of at least two independent experiments.

Discussion

Although TRADD has been identified for over a decade, the TRADD knockout mouse has been generated only recently. Our analyses of various cells from these mutant animals have confirmed the answers to some questions while raising others. Our results show that TRADD is essential for TNFα-induced apoptosis and TNFα-induced TNFR1-mediated NF-κB activation. TRADD deficiency did not completely abolish murine TNFα-mediated ERK and JNK activation. However, human (TNFR1-specific) TNFα-mediated ERK and JNK activation was completely abolished in the absence of TRADD. Our result indicates that TRADD is necessary for TNFR1 but not TNFR2-mediated MAPK activation.
Our analyses of the TNFR1 signaling complex also revealed an essential role for TRADD in the ubiquitination of RIP that supports NF-κB activation. The most intriguing result emerging from this analysis was the association of unubiquitinated RIP with TNFR1 is TRADD-independent. Given that ubiquitination of RIP has been proposed as an essential step during TNFα-induced NF-κB activation (24), the role of TRADD in mediating TNFα-induced NF-κB activation may occur through the induction of RIP ubiquitination. Nonetheless, our data suggest that the identity and association sequence of the various signaling mediators and adaptors that participate in the TNFR1 receptor complex in response to TNFα may need to be reconsidered.

Several lines of evidence point to roles for TNFR1 downstream signal adaptors (including FADD, RIP, and TRAF2) in lymphopoesis and immune responses. In the current study, we found TRADD to be crucial for the formation of FDC clusters in splenic primary follicles and for GC formation after immunization. This defect is reminiscent of that observed in TNFR1 KO mice (17). Our histological result served as an in vivo confirmation that TNF-TNFR1-TRADD signaling is essential for FDC and subsequent GC formation in the spleen. Whether TRADD is further involved in TNFR1-independent adaptive responses or in T cell and B cell development and functions remains an open question and mandates further investigation.

Previous reports have suggested that TRADD interacts with STAT1 and down-regulates IFNγ signaling and bioactivity. This hypothesis arises chiefly from work done by Chen et al. (25). However, our investigation does not support a negative regulatory role for TRADD in IFNγ-induced responses. In fact, we sometimes observed a slight reduction in STAT1 phosphorylation in TRADD-deficient MEFs, BM-Macs, and T cells. TRADD’s role in IFNγ signaling thus remains an unresolved controversy. Studies of pathogen infection in vivo and Th1/Th2 differentiation analysis will help to fully characterize the role of TRADD in IFNγ signaling. It will also be interesting to determine whether TRADD is involved in cytokine signaling mediated by other STATs.

The most exciting result of our study is the uncovering of an important role for TRADD in TLR signaling. We found that TRADD participates in the TLR4 signaling complex formed in LPS-stimulated cells. Our biochemical analyses indicate that this involvement of TRADD occurs very far upstream in the signaling pathway and serves the form of a direct association between TRADD and TLR4-TIR and/or an indirect association between TRADD and RIP in the TRIF-dependent pathway. The latter proposition is supported by our studies of the effects of TRADD deficiency on the TLR3 pathway because signaling downstream of TLR3 engagement relies solely on TRIF. Upon TLR3 stimulation by poly(I:C), we noted a significant decrease in cytokine production by TRADD-deficient cells compared with controls. Detailed analyses of TLR3 signaling, as well as assessments of TLR4 signaling in TRADD/MyD88 double-knockout mice, will be crucial for the delineation of the precise involvement of TRADD in the TRIF pathway. Nevertheless, our study is seminal in that it has revealed a new role for TRADD in TLR signaling that is independent of its functions in the TNFR1 pathway. A recent paper by Michallet et al. (26) has revealed TRADD’s participation in the RIG-like helicase antiviral pathway. This finding, in combination with our current observations, suggests that TRADD may be a critical player in the host antiviral and antibacterial response. This uncoupling of TRADD as a player in antiviral immunity may contribute to the development of new therapeutic strategies against viral diseases.

### Methods

**Cells and Reagents.** MEFs, bone-marrow-derived macrophages, lung fibroblasts, and T cells were prepared as described (21, 27). Mouse TNFR1, human TNFR1, mouse TNFR2, human IFNα, mouse IFNβ, and human TNFα were purchased from Invitrogen. Poly(I:C) was from Invivogen. Mouse TLR1a was the kind gift of Ping Wei (Human Genome Science, Rockville, Maryland). LPS, LTA, PGN, 7-AD, CHX, sorbitol, etoposide, doxorubicin, anisomycin, dexamethasone, and staurosporine were all from Sigma. The anti-CD3 and anti-CD28 antibodies used for T cell stimulation were from BD Bioscience.

**Western Blotting.** MEFs and BM-Macs were resuspended in serum-free DMEM or RPMI, respectively, for 2 h before stimulation with 10 ng/ml TNFα, 10 ng/ml IFNγ, 100 ng/ml LPS, or 100 μg/ml poly(I:C). Cells were lysed in RIPA buffer [150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 0.5% DOC, 0.1% SDS, and complete protease inhibitor mixture from Roche], and protein concentrations were determined by the BCA protein assay (Thermo Scientific). Samples containing equal amounts of protein were fractionated on a precast 4–20% SDS/PAGE gel (Invitrogen) and transferred onto a PVDF membrane. TRADD levels were determined by incubating the blot with rabbit anti-TRADD polyclonal antibody (Santa Cruz Biotechnology). For signaling studies, rabbit anti-pERK1/2, anti-pp38, anti-pAKT, and anti-p65 (from Cell Signaling), anti-pJNK (from Santa Cruz Biotechnology), and anti-pSTAT1 (from Upstate) were used as primary antibodies to determine levels of phosphorylated kinases. Anti-λ (from Cell Signaling) was used to determine IκB degradation. To visualize proteins, blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (GE Health), and processed with the ECL Plus system (GE Health).

**Immunoprecipitation.** Cells cultured on 15-cm tissue culture plates were treated with TNFα (10 ng/ml) or LPS (100 ng/ml) as indicated in the figures. Cells were washed, harvested, and lysed in IP buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.2% Nonidet P-40, 10% glycerol, and a complete protease mixture]. Each IP binding sample contained 500–1000 μg of total lysate protein. Anti-TNFR1 or anti-TLR4 antibody (both from BD Biosciences) was mixed first with protein G beads (GE Health) and then with the lysates and incubated overnight. The beads plus the antibodies and associated proteins were washed five times in IP buffer and resuspended in SDS/PAGE sample buffer. Samples were fractionated by SDS/PAGE, and protein components of receptor complexes were determined by Western blotting.

**Cytokine Levels.** Levels of IL-6 and TNFα in serum samples or culture supernatants were measured by using ELISA kits from BD Biosciences, following the manufacturer’s instructions.


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. S1a). 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 flox/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/WT flox). 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. S1b) and the successful deletion of the floxed allele in tradd KO/KO mice (Fig. S1c 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/WT flox mice, we obtained offspring whose mouse embryonic fibroblasts (MEFs) expressed various levels of TRADD protein (Fig. S2a). Specifically, tradd WT/WT MEFs expressed half the amount of TRADD protein present in tradd WT/WT WT/KO and tradd KO/WT flox MEFs expressed a very low level of TRADD protein that mimicked the level present in TRADD knockdown cells (2–4).

Interestingly, tradd KO/WT cells were only partially resistant to TNFα plus CHX treatment (Fig. S2b), 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/WT cells produced comparable amounts of IL-6 in response to TNFα stimulation (Fig. S2c). Thus, even the very low level of TRADD protein present in tradd KO/WT 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. S1a. 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 (129SvJ). 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. S1a). 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/WT flox mice with Deleter CRE mice (a CMV-Cre recombinase transgenic strain) to generate tradd WT/WT KO/KO ES cells. tradd KO/KO mice were generated by intercrossing tradd WT/WT KO/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 230-bp 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 BglII/KpnI-digested genomic DNA (Fig. S1c and d).

3H-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 10⁶ 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 3H-thymidine per well followed by incubation for an additional 8 h. The amount of 3H-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 10⁶ 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 (10⁶ 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 (10⁶ 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 × 10⁶) 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 [32P]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 MgCl₂, 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 × 10⁵) 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. traddWT/WT, traddKO/KO, and infri−/− 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 traddWT/WT and traddKO/KO genotypes were injected i.p. on day 0 with 10⁶ 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).

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<sup>fl3–5</sup>*) or KO (*tradd<sup>KO</sup>* ) 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 traddWT/WT, traddWT/KO, and traddKO/flox MEFs. (a) Western blot of TRADD protein levels in traddWT/WT, traddWT/KO, and traddKO/flox MEFs. NS, nonspecific band that served as an internal control for protein loading. (b) TRADD knockdown mimics. MEFs from traddWT/WT, traddWT/KO, and traddKO/flox mice were analyzed as in Fig. 1B. **, P < 0.01, Student’s t test. (c) IL-6 production. MEFs from traddWT/WT, traddWT/KO, and traddKO/flox mice were treated for 24 h with TNF (10 ng/ml) and IFN-γ (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 \textit{tradd}^{WT/WT} and \textit{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 \textit{tradd}^{WT/WT} and \textit{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\textsuperscript{KO/KO} mice. (a) Reduced IL-6 production. tradd\textsuperscript{WT/WT} (n = 4), tradd\textsuperscript{KO/KO} (n = 5), and trfr\textsuperscript{−/−} (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\textsuperscript{WT/WT} and tradd\textsuperscript{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\textsuperscript{WT/WT} and tradd\textsuperscript{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 \textit{tradd}^WT/WT and \textit{tradd}^KO/KO mice and stimulated for 56 h with plate-bound anti-CD3 (0.3 \(\mu\)g/ml) plus anti-CD28 (5 \(\mu\)g/ml), IL-2 (10 ng/ml), and/or mouse TL1a (3 \(\mu\)g/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) ± 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<sup>WT/WT</sup> and tradd<sup>KO/KO</sup> MEFs (a) (n = 3 pairs), BM-Macs (b) (n = 7 pairs), and CD4<sup>+</sup> 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’- GTTGTCCCCAAAACAGGACT-3’ |
| Primer C: KO primer 5’-CACTATCCACTGAAGGTGACTAAC-3’ |
| Real-time RT-PCR | IL-6 forward 5’-AGTTGCCCTTTGGGACTGA-3’ |
| IL-6 reverse 5’-TCCACGATTTCCAGAAAC-3’ |
| A20 forward 5’-TCGTGGCCTGAAAAACCAATG-3’ |
| A20 reverse 5’-GATGGGTCTTCTGAGGATGTTGC-3’ |
| lxBa forward 5’-AACCTGCAGCAGACTCCACT-3’ |
| lxBa reverse 5’-GACACGTGTGGCCATTTGAG-3’ |