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The function of TRADD in signaling via tumor necrosis factor receptor 1 and TRIF-dependent Toll-like receptors

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Abstract

The physiological role of the TRADD adaptor protein remains unclear due to the unavailability of a TRADD-deficient animal model. By generating TRADD-deficient mice, we demonstrated that TRADD plays a significant role in tumor necrosis factor receptor-1 (TNFR1) signaling by orchestrating the formation of TNFR1 signaling complexes. TRADD was essential for TNFR1 signaling in mouse embryonic fibroblasts (MEFs) but partially dispensable in macrophages; abundant expression of the adaptor RIP in macrophages may allow some transmission of TNFR1 signals in the absence of TRADD. Although morphologically normal, TRADD-deficient mice were resistant to TNF-, lipopolysaccharide- and poly(I:C)-induced toxicity. TRADD was also required for TRIF-dependent Toll-like receptor signaling in MEFs but not macrophages. These findings definitively establish the biological role of TRADD in TNF signaling.

INTRODUCTION

The proinflammatory cytokine tumor necrosis factor (TNF) (<http://www.signaling-gateway.org/molecule/query?afcsid=A002291>) is a potent inducer of diverse cellular events including septic shock, production of other cytokines, cell proliferation, differentiation, necrotic cell death and apoptosis^{1,2}. Most TNF-induced processes are mediated by TNF receptor-1 (TNFR1) (<http://www.signaling-gateway.org/molecule/query?afcsid=A002346>), which triggers multiple signaling pathways, including activation of MAP kinases and the transcription factor NF-κB, and in some cases, the engagement of the caspase cascade, which leads to apoptosis³⁻⁷. NF-κB and MAP kinases play a critical role in the induction of many cytokines and immunoregulatory proteins and are pivotal for many inflammatory responses^{3,5,6}. The initiation of these TNF-induced pathways is precisely regulated under physiological conditions and the aberrant commitment or failure of these pathways may be accountable for the pathology of many human diseases, such as cancer, arthritis and AIDS^{2,8}.

The molecular mechanisms of TNFR1 signaling have been intensively studied. Currently, it is believed that the binding of TNF to TNFR1 triggers the trimerization of TNFR1 and subsequent formation of the TNFR1 signaling complex⁵⁻⁷. Several proteins including TRADD (TNFR1-associated death domain protein)

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(<http://www.signaling-gateway.org/molecule/query?afcsid=A002306>), TRAF2 (TNFR associated factor 2) (<http://www.signaling-gateway.org/molecule/query?afcsid=A002308>), and RIP (receptor interacting protein) have been found in the TNFR1 signaling complex and are responsible for mediating downstream signaling events such as the activation of NF- κ B and JNK pathways^{6,7,9-11}. Under some conditions the TRADD-RIP-TRAF2 complex (complex I) dissociates from TNFR1 and recruits another TNF effector protein, FADD (FAS-associated death domain protein), to form an “apoptotic complex” (complex II) which activates caspase-8 and the downstream caspase cascade¹². To better understand the biological functions of the key effectors of TNF signaling, mice deficient in TRAF2, RIP and FADD have been generated¹³⁻¹⁶. Studies with these mice and the cells derived from them have contributed a number of insights to the understanding of the physiological role of each of these proteins in TNF signaling. First, RIP and TRAF2 are required for TNF-induced activation of NF- κ B and MAP kinase pathways^{13,16,17}. Second, FADD is essential for TNF-induced apoptosis^{14, 15}. Third, RIP is critical for TNF-induced necrotic cell death^{18,19}. In addition, studies from several groups suggest that TRAF2 and RIP mediate recruitment of downstream signaling proteins--such as IKK--to the TNFR1 signaling complex to activate specific distal signaling pathways²⁰⁻²². More recently, TRADD and RIP have been found to be necessary for recruiting and activating the NADPH oxidase, NOX1, to initiate TNF-induced necrotic cell death²³. However, due to the lack of the TRADD-deficient mice, the physiological role of TRADD in TNF signaling remains unclear and controversial, although attempts have been made to address this critical issue by overexpression or by knocking down the expression of the TRADD protein^{9,24,25}. For example, it is not known whether TRADD is required for TNF cytotoxicity *in vivo*, and more importantly, it is still unclear as to which TNF-induced signaling pathways are mediated by TRADD, and how TRADD activates such pathways.

In this study, we generated TRADD-deficient (*Tradd*^{-/-}) mice and demonstrated that TRADD plays a critical role in TNFR1 signaling by orchestrating the formation of the TNFR1 signaling complex. We found that TRADD is essential for induction of the four most-studied consequences of TNF-induced TNFR1 signaling in mouse embryonic fibroblasts (MEFs), namely the activation of NF- κ B, the activation of MAP kinases, the induction of necrotic cell death, and the initiation of apoptosis. Our data demonstrate that TRADD is necessary for the recruitment of TRAF2 and the optimal recruitment of RIP to the TNFR1 complex, where TRADD is also responsible for RIP ubiquitination in which TRAF2 and TRAF5 play redundant roles. Surprisingly, TNFR1 signals, albeit to a reduced degree, were transmitted in *Tradd*^{-/-} macrophages, perhaps due to the large amounts of RIP expressed in this cell type. In addition, TRADD played a role in TRIF-dependent Toll-like receptor (TLR) signaling, also in a cell type-specific manner. Taken together, these observations definitively establish the physiological role of TRADD in TNFR1 and TLR signaling.

Results

Generation of *Tradd*^{-/-} mice

TRADD interacts with RIP through its death domain, and with TRAF2 through its N-terminal region¹¹. Therefore, our gene-targeting construct was designed to delete a major portion (exon 3 through 5) of the TRADD protein (51-310 aa), including the RIP- and TRAF2-interacting domains (Supplementary Fig. 1a, online). Southern blotting (Supplementary Fig. 1b, online) and PCR (data not shown) analyses identified 14 correctly targeted mouse embryonic stem (ES) cell clones. Two of them were microinjected into C57Bl/6 blastocysts and successfully transmitted through the germline. These gene-targeted mice were crossed with EIIa-Cre transgenic mice to delete the *neo*^r cassette and exons 3-5 of the *Tradd* gene²⁶. *Tradd*^{+/-} mice were then bred to produce *Tradd*^{-/-} mice. The successful deletion of the *Tradd* gene was clearly demonstrated by PCR analysis of mouse tail DNA (Supplementary Fig. 1c, online) and

immunoblot analysis of TRADD protein in various mouse tissues (Supplementary Fig. 1d, online).

Tradd^{-/-} mice were born at expected Mendelian ratios, indicating no embryonic lethality. The adult null mice appeared, behaved, and reproduced normally. Examination on the development of lymphoid organs in 6 week old *Tradd*^{-/-} mice revealed no obvious abnormalities. All secondary lymphoid organs including Peyer's patches, peripheral lymph nodes, mesenteric lymph nodes and spleen were present. Quantification of Peyer's patches showed no difference with wild-type mice (Supplementary Table 1, online). Histological analysis of spleen demonstrated normal organization of follicles (Supplementary Fig. 2, online). Surface markers on T and B cell populations from spleen, lymph nodes and thymus were examined by FACS analysis. No significant differences in cell numbers or composition of lymphocyte subsets were found (Supplementary Fig. 3, online).

***Tradd*^{-/-} mice are resistant to TNF toxicity**

As TRADD is believed to be a critical mediator of TNFR1-mediated TNF signaling, and TNF cytotoxicity is largely mediated by TNFR1^{6,9}, we first tested whether *Tradd*^{-/-} mice are sensitive to TNF toxicity. To do so, we used a model of TNF-induced hepatitis²⁷. In this model, 7-8 week-old *Tradd*^{-/-} mice and their wild-type littermates bred from heterozygous mice were pretreated with D-galactosamine (GalN), an inhibitor of transcription specifically in the liver²⁸, followed by the administration of TNF. Although all wild-type mice died within 6-8 h after treatment with TNF, *Tradd*^{-/-} littermates were completely protected from TNF-induced liver injury and appeared healthy until being sacrificed at 24 h after treatment (Fig. 1a). TUNEL staining of liver sections of mice sacrificed at 6 h after GalN and TNF treatment demonstrated multiple apoptotic foci in livers of wild-type but not *Tradd*^{-/-} mice (Fig. 1b). Elevated concentrations of circulating alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in wild-type animals confirmed the development of liver injury (Fig. 1c). However, *Tradd*^{-/-} mice treated with TNF and GalN showed similar ALT and AST concentrations as untreated controls. Therefore, the deletion of TRADD rendered mice resistant to TNF cytotoxicity.

TRADD is essential for TNFR1 signaling in MEFs

TNF can induce multiple consequences including activation of NF- κ B and MAP kinases, and induction of necrotic cell death and apoptosis^{5-7,29}. To determine the effects of TRADD-deficiency on TNF signaling and to elucidate which consequence(s) are affected by TRADD deletion, we next performed studies using primary MEFs derived from the wild-type and *Tradd*^{-/-} embryos. MEFs from each embryo were genotyped by PCR and their genotypes were confirmed by immunoblotting with a TRADD-specific antibody (Fig. 2a). Wild-type and *Tradd*^{-/-} MEFs from the same pregnant female were used in our experiments. To examine the activation of MAP kinase and NF- κ B pathways, we examined the phosphorylation of JNK and the degradation of I κ B α following TNF treatment. TNF induced potent phosphorylation of JNK and the degradation of I κ B α in wild-type but not *Tradd*^{-/-} MEFs (Fig. 2b). The protein concentrations of TNFR1 in wild-type and *Tradd*^{-/-} MEFs were similar (data not shown, but see Fig. 4e and Supplementary Fig. 7). Failure of TNF to induce NF- κ B activation in *Tradd*^{-/-} MEFs was further confirmed by NF- κ B DNA binding experiments (Supplementary Fig. 4, online). To be sure that the defect in the activation of these pathways in *Tradd*^{-/-} MEFs was specific to TNF treatment, we treated these cells with another inflammatory cytokine, interleukin (IL)-1 β . The extent of IL-1 β -induced JNK phosphorylation and I κ B α degradation were similar in wild-type and *Tradd*^{-/-} MEFs (Fig. 2c), and the expression of JNK and actin were comparable (Fig. 2b,c). Further examination revealed that TNF- but not IL-1 β -induced activation of the MAP kinases ERK and p38 was also abolished in *Tradd*^{-/-} MEFs.

(Supplementary Fig. 5, online), suggesting that TRADD is required for TNF-induced activation of both NF- κ B and MAP kinases.

Wild-type MEF cells undergo apoptosis if treated with TNF plus the protein synthesis inhibitor cycloheximide (CHX), but undergo necrotic cell death when both CHX and z-VAD-fmk, a membrane-permeable pan-caspase inhibitor, are present^{29,30}. To assess the role of TRADD in TNF-induced apoptosis and necrotic cell death, we treated wild-type and *Tradd*^{-/-} MEFs with CHX or both CHX and z-VAD, and then treated with TNF. As we reported previously³⁰, wild-type MEF cells were sensitive to TNF-induced apoptosis and necrotic cell death. In contrast, neither apoptosis nor necrotic cell death was detected in *Tradd*^{-/-} MEFs (Fig. 2d). These results indicated that TRADD is essential for both types of TNF-induced cell death and are consistent with our *in vivo* toxicity experiments. The resistance of *Tradd*^{-/-} MEFs to apoptosis is specific to TNF as Fas-induced apoptosis was normal in *Tradd*^{-/-} MEFs and thymocytes (Supplementary Fig. 6, online). Therefore, we conclude that TRADD is pivotal for all examined TNFR1-mediated TNF signaling events in MEFs.

Previous studies have attempted to address the role of TRADD in TNFR1-mediated TNF signaling^{9,24,25}. Using an overexpression approach, TRADD was thought to recruit RIP and TRAF2 to the TNFR1 signaling complex⁹. More recent studies with an siRNA knockdown approach suggested that TRADD and RIP compete for binding to TNFR1^{24,25}. To understand the role of TRADD in the formation of the TNFR1 signaling complex, we performed immunoprecipitation experiments with a TNFR1-specific antibody in wild-type and *Tradd*^{-/-} MEFs and then examined the recruitment of RIP and TRAF2 proteins. As previously reported²⁰, both TRAF2 and RIP co-precipitated with TNFR1 following TNF treatment in wild-type MEFs (Fig. 3a). However, in *Tradd*^{-/-} MEFs, TNF-induced RIP recruitment was markedly decreased and no TRAF2 recruitment was detected, though total input protein amounts of RIP and TRAF2 were similar in both wild-type and *Tradd*^{-/-} MEFs (Fig. 3a). As a control, the amount of TNFR1 protein precipitated by anti-TNFR1 was similar in wild-type and *Tradd*^{-/-} MEFs (Supplementary Fig. 7, online). Importantly, no modified RIP was observed in *Tradd*^{-/-} MEFs, whereas TNFR1-associated RIP was heavily modified in wild-type MEFs (Fig. 3a). Previous reports have indicated that RIP is modified by ubiquitination in the TNFR1 complex^{21,31}. A difference in RIP ubiquitination in TNFR1 immunoprecipitants from wild-type and *Tradd*^{-/-} MEFs was confirmed by immunoblotting with a ubiquitin-specific antibody (Supplementary Fig. 8, online). These results suggested that TRAF2 recruitment is TRADD-dependent, that RIP recruitment to TNFR1 does not absolutely require but is enhanced by TRADD, and that ubiquitination of RIP requires the presence of TRADD in the TNFR1 complex. The above data implies that TRADD is required for forming a functional TNFR1 signaling complex in MEFs.

Non-canonical ubiquitination of RIP is critical for relaying a TNF signal to activate IKK^{22,31}. A previous report suggested that TRAF2 is required for non-canonical RIP ubiquitination³¹. As TRAF2 recruitment is TRADD-dependent, the absence of TRAF2 in TNFR1 complex may explain why RIP is not ubiquitinated in *Tradd*^{-/-} MEFs. To confirm this possibility, we examined RIP recruitment and ubiquitination in *Traf2*^{-/-} MEFs. Surprisingly, we found that RIP was recruited and ubiquitinated normally in *Traf2*^{-/-} MEFs (Fig. 3b). Because TRAF5 has a redundant role with TRAF2 in TNF signaling³², we then examined TNF-induced RIP recruitment and ubiquitination in *Traf5*^{-/-} and *Traf2*^{-/-}*Traf5*^{-/-} (double knockout) MEFs. Whereas TNF-induced RIP recruitment was normal in both *Traf5*^{-/-} and *Traf2*^{-/-}*Traf5*^{-/-} MEFs, TNF-induced RIP ubiquitination was detected in *Traf5*^{-/-} MEFs, but not in the *Traf2*^{-/-}*Traf5*^{-/-} MEFs (Fig. 3b). In *Traf5*^{-/-} MEFs, TRAF2 was recruited to the TNFR1 complex similarly in comparison to wild-type cells (Fig. 3b). Protein expression of TRAF2 and TRAF5 in these cells was verified by immunoblotting (Fig. 3c). These results

suggested that TRAF2 and TRAF5 play a redundant role in mediating TNF-induced RIP ubiquitination.

Modest TNF signaling in *Tradd*^{-/-} macrophages

To investigate whether TRADD is essential for TNFR1 signaling in cells other than MEFs, we treated peritoneal macrophages isolated from wild-type and *Tradd*^{-/-} mice with TNF. Surprisingly, unlike in MEFs, where no signaling was observed, JNK phosphorylation and IκBα degradation were detected in *Tradd*^{-/-} macrophages, although in lower amounts when compared to wild-type cells (Fig. 4a). To be sure that the TNF-induced activation of NF-κB and JNK in *Tradd*^{-/-} macrophages is mediated by TNFR1 and not TNFR2, we treated macrophages with human TNF, which binds only to TNFR1. Again, low amounts of JNK phosphorylation, IκBα degradation and p38 phosphorylation were observed in *Tradd*^{-/-} macrophages (Fig. 4b), suggesting that TNF induced activation of NF-κB and MAP kinases in *Tradd*^{-/-} macrophages mainly through TNFR1. This observation implies that deletion of TRADD has different effect on TNFR1 signaling in MEFs than in macrophages.

To extend this finding, we examined the TNFR1 recruitment of RIP and TRAF2 in *Tradd*^{-/-} macrophages as described in Fig. 3. Following TNF treatment, slightly less RIP was recruited to the TNFR1 signaling complex in *Tradd*^{-/-} than in wild-type macrophages, but RIP recruitment was much more efficient when compared to *Tradd*^{-/-} MEFs (Fig. 4c). However, RIP was ubiquitinated much less efficiently in *Tradd*^{-/-} macrophages than in wild-type cells (Fig. 4c). Notably, a small amount of TRAF2 was recruited to TNFR1 following TNF treatment in *Tradd*^{-/-} macrophages (Fig. 4c); this finding further confirms a differential requirement for TRADD in TNFR1 signaling in MEFs and macrophages. Consistent with this conclusion, TNF-induced cell death occurred in *Tradd*^{-/-} macrophages (Fig. 4d) but not *Tradd*^{-/-} MEFs (Fig. 2d).

To explore the potential mechanism underlying this apparent tissue specificity in TNFR1 signaling, we examined the protein expression of RIP and TNFR1 in macrophages and MEFs. Whereas similar amounts of TNFR1 protein were detected in these cells, the amount of RIP protein was markedly higher in macrophages than that in MEFs (Fig. 4e). As RIP can interact with TRAF2¹¹, our data suggests that, in the absence of TRADD, the abundant RIP protein in macrophages may be sufficient to recruit TRAF2 and to form a functional TNFR1 signaling complex in response to TNF, albeit less efficiently than in wild-type cells.

TRADD in TRIF-dependent TLR signaling

Previous studies suggested that LPS and poly(I:C) toxicity in the presence of GalN is TNFR1-dependent^{33,34}. Therefore, we tested whether *Tradd*^{-/-} mice are resistant to LPS and poly(I:C) toxicity using 7-8 week old wild-type and *Tradd*^{-/-} littermates born from breedings of *Tradd*^{+/-} mice. Treatment with LPS plus GalN resulted in 100% mortality of wild-type mice within 9-10 h, but *Tradd*^{-/-} littermates remained unaffected and were sacrificed after 24 h (Fig. 5a). TUNEL staining of liver sections displayed massive apoptosis in hepatocytes of wild-type but not *Tradd*^{-/-} mice (data not shown). Elevated concentrations of serum ALT and AST in wild-type animals confirmed the process of liver damage (Supplementary Fig. 9, online). High doses of LPS alone (1 mg/mouse) were lethal to both wild-type and *Tradd*^{-/-} mice (data not shown).

To determine whether the resistance of *Tradd*^{-/-} mice to GalN plus LPS treatment was the result of a defect in LPS signaling or TNFR1 signaling, we examined LPS signaling in peritoneal macrophages isolated from 7 week old wild-type and *Tradd*^{-/-} littermates. Amounts of LPS-induced JNK and p38 phosphorylation and IκBα degradation were similar in wild-type and *Tradd*^{-/-} macrophages (Fig. 5b). Similarly, *Tradd*^{-/-} mice were also resistant to toxicity

induced by treatment with poly(I:C) plus GalN; *Tradd*^{-/-} mice were healthy until sacrificed at 72 h after treatment, while the wild-type littermates were dead within 30 h following treatment (Fig. 5c). Nevertheless, we observed no difference in poly(I:C)-induced JNK and p38 phosphorylation and IκBα degradation in wild-type and *Tradd*^{-/-} macrophages (Fig. 5d). These results indicated that deletion of TRADD does not disrupt TLR3 and TLR4 signaling in macrophages. Therefore, the resistance of *Tradd*^{-/-} mice to LPS and poly(I:C) treatment in the presence of N-galactosamine is most likely due to a defect in TNFR1 signaling.

In addition, we also measured LPS- and poly(I:C)-induced production of different inflammatory cytokines in *Tradd*^{-/-} mice (*in vivo*) and macrophages (*in vitro*). TRADD-deficient mice showed no difference in TNF production after LPS plus GalN or poly(I:C) plus GalN treatment, but a slight decrease in LPS or poly(I:C)-induced IL-6 production was observed in *Tradd*^{-/-} mice (Fig. 5e,f). Whereas *Tradd*^{-/-} macrophages produced similar amounts of TNF, IL-6 and IL-12 in response to LPS, the production of all these three cytokines in response to poly(I:C) treatment was notably reduced in these cells (Fig. 5g). However, poly(I:C)-induced production of IFNβ was not altered in *Tradd*^{-/-} macrophages (Supplementary Fig. 10, online). These data suggest that deletion of TRADD did not affect TLR4 and TLR3 signaling, but may have some effect on the production of some cytokines. This may be due, in part, to a secondary effect from the defect of TNFR1 signaling as the cytokines were measured at different times after treatments.

As TRADD deletion had different effect on TNF signaling in MEFs and macrophages, we next tested whether TLR4 and TLR3 signaling were altered in *Tradd*^{-/-} MEFs. Due to the weaker activation of NF-κB by LPS and poly(I:C) in primary MEFs, we examined IκBα phosphorylation instead of its degradation. Whereas LPS-induced phosphorylation of JNK and IκBα were normal in *Tradd*^{-/-} MEFs (Fig. 6a), poly(I:C)-induced IκBα and JNK phosphorylation were impaired in these cells (Fig. 6b). Poly(I:C)-induced signaling was normal in *Tnfr1*^{-/-} MEFs (Fig. 6c), implicating TRADD directly in TLR3 signaling.

It is known that LPS-induced NF-κB activation can be mediated by two redundant pathways, the TRIF pathway (which is in part RIP-dependent) and the MyD88-dependent pathway^{35, 36}. In contrast, TLR3 signals exclusively through the TRIF pathway³⁶. Therefore, the unaffected TLR4 signaling in *Tradd*^{-/-} MEFs may be due to the presence of an intact MyD88-dependent pathway. To test this possibility, *Tradd*^{-/-} MEFs were transfected with an siRNA construct specific for MyD88 (MyD88-siRNA) and then treated with LPS. Both IκBα and JNK phosphorylation were reduced in *Tradd*^{-/-} MEFs transfected with the MyD88-siRNA, compared to those transfected with a control siRNA (Fig. 6d). Residual phosphorylation of JNK and IκBα were likely due to incomplete knockdown of MyD88. In contrast, LPS-induced phosphorylation of JNK and IκBα were not affected in wild-type MEFs transfected with the MyD88-siRNA, presumably due to the presence of an intact TRIF-dependent pathway (Fig. 6d). We also analyzed cytokine production in response to LPS and poly(I:C) in MEFs. TRADD-deficiency did not affect TNF and IL-6 production in LPS-treated cells, but poly(I:C)-induced production of these cytokines was notably decreased in *Tradd*^{-/-} cells (Fig. 6e). Thus, TRADD appears to be involved in TLR3 and TLR4-mediated MyD88-independent activation of NF-κB and MAP kinases in MEFs.

Discussion

TNF signaling has been one of the most intensively studied research fields for almost two decades. Particularly, a wealth of literature on TNFR1 signaling has been generated because TNFR1 mediates most of the TNF-induced diverse cellular responses. Our understanding of the molecular mechanisms of TNFR1-mediated TNF signaling has been greatly advanced by studies of mice lacking TNFR1 effector proteins, such as TRAF2, RIP and FADD¹³⁻¹⁶.

However, even though TRADD was identified as a component of the TNFR1 complex in 1995, the physiological role of TRADD in TNF signaling has remained vague and debatable due to the unavailability of a TRADD-deficient animal model. In this report, we utilized *Tradd*^{-/-} mice to show that TRADD plays a significant role in TNFR1 signaling by mediating the formation of TNFR1 signaling complexes. Our study indicates that the essential role of TRADD in TNFR1 signaling is tissue-specific, as deletion of TRADD resulted in the complete failure of all TNF-mediated signaling events in MEFs, but only partial impairment in macrophages. We conclude that the presence of abundant RIP protein in macrophages may be sufficient to overcome the absence of TRADD and allow transmission of TNF signals to some extent.

It was speculated that TRADD deletion in the mouse may lead to early embryonic lethality. To avoid this potential obstacle and to be sure that TRADD-deficient cells would be available for us to study the role of TRADD in TNFR1 signaling, we initially decided to generate TRADD conditional knockout mice. However, surprisingly we found that *Tradd*^{-/-} mice are viable and developmentally normal.

The existing literature on the role of TRADD in TNFR1-mediated signaling is confusing and controversial. Earlier studies suggested that TRADD mediates the activation of NF- κ B and the induction of apoptosis through recruiting downstream effector proteins such as TRAF2, RIP and FADD⁹⁻¹¹. More recent reports using siRNA to knock down the expression of TRADD indicated that TRADD is dispensable for TNF-induced apoptosis²⁴ or necrotic cell death²⁵ and that RIP recruitment and ubiquitination is enhanced when TRADD expression is decreased²⁵. In our current study with *Tradd*^{-/-} mice, MEFs and macrophages, we found that TRADD is essential for TNF cytotoxicity *in vivo* and for all known TNFR1-mediated consequences in MEFs, but plays a less critical role in transducing TNFR1 signals in macrophages. Based on our study, it is clear that TRADD promotes the recruitment of RIP and TRAF2 to the TNFR1 complex even though RIP can directly interact with TNFR1 in its absence. This finding is likely explained by the higher binding affinity of TRADD, compared to RIP, for TNFR1^{9,11}. Therefore, in many types of cells such as MEFs in which RIP protein abundance is relatively low, TRADD is necessary for adequate recruitment of RIP and its ubiquitination by TRAF2 or TRAF5. More importantly, without TRADD, the trivial amount of RIP recruited to TNFR1 in these types of cells is not sufficient to transduce any TNFR1 signals. In these types of cells, TRADD appears necessary to recruit other effectors, such as TRAF2 and FADD, to the signaling complexes. This conclusion is consistent with previous findings that FADD, which is recruited by TRADD, is required for TNF-induced apoptosis^{14,15} and that TRADD is needed for TNF-induced, NOX1-mediated necrotic cell death²³.

In contrast, in cells such as macrophages, in which RIP protein abundance is quite high, TRADD was less critical for RIP recruitment to TNFR1. The presence of high amounts of RIP protein in these cells may be a factor in allowing RIP to be recruited to TNFR1, and—as RIP interacts with TRAF2 and FADD directly^{11,21}—to recruit other effector proteins to TNFR1 in the absence of TRADD. Nevertheless, abundant RIP cannot completely overcome TRADD deficiency, as TNFR1 signaling was much weaker in *Tradd*^{-/-} than wild-type macrophages.

It has been suggested that TRADD is involved in signaling pathways besides TNFR1 signaling. For instance, it has been reported that nuclear TRADD forms a complex with STAT1- α and affects IFN- γ signaling³⁷. In our study, we found that *Tradd*^{-/-} mice are resistance to LPS and poly(I:C) toxicity, but this resistance likely results from a defect in TNFR1 signaling, as TRADD deletion did not affect TLR4 and TLR3 signaling in macrophages. However, we did find that deletion of TRADD impairs TLR3 signaling and the TRIF-dependent arm of TLR4 signaling in MEFs. The different effects of TRADD deletion on TLR signaling in macrophages

and MEFs may be due to the different amounts of RIP in these cells, as RIP is required for TRIF-dependent TLR3 and TLR4 signaling^{38,39}. In MEFs, TRADD may be required for the ubiquitination of RIP in response to poly(I:C) or LPS treatment.

Recently, TRADD was found to be a component of RIG-like helicase antiviral pathway⁴⁰. Clearly, TRADD plays a much broader role in mediating different biological processes than just as an effector protein of TNFR1. The availability of *Tradd*^{-/-} mice will allow us to provide a better understanding of the physiological functions of TRADD.

Methods

Animals

C57Bl/6 mice were purchased from NCI Frederick, B6.FVB-Tg(EIIa-Cre)C5379Lmgd/J transgenic mice and TNFR1-KO mice were purchased from The Jackson Laboratory. All animal experiments were performed under protocols approved by the National Cancer Institute Animal Care and Use Committee and followed NIH guidelines

Generation of *Tradd*^{-/-} mice

pKO Scrambler 917 TK vector was used to construct the targeting vector. The *Tradd* gene was amplified from a BAC clone isolated from 129/Sv RPC122 mouse BAC DNA-library in three fragments: 3.5 kb *HpaI-HindIII* first fragment, 2.2 kb *SacII-XmaI* second fragment and 6.7 kb *SrfI-NotI* third fragment. A *neo* resistance gene cassette flanked by *FRT* and *loxP* sites was excised from pLTM260 vector with *HindIII* and *SacII* restriction enzymes and was cloned between the first and the second fragments. A third *loxP* site was inserted after the exon 5 of *Tradd* between the second and the third fragments. The targeting vector was linearized with *PvuI* and electroporated into the CMT-1 mouse ES cell line derived from the 129/S6SvEv mouse strain following standard procedure. One hundred ninety-two G418- and ganciclovir-resistant ES clones were picked and screened for the gene-targeting event. The 3' homologous recombination was screened by Southern blot analysis using a DNA probe lying just outside of the targeting construct. The 5' homologous recombination event was screened by PCR with the primers 5RecF (5'-CACTCGGTTTGGAAACGAAT-3') and 5RecR (5'-CTACCCGGTAGAATTTCATA-3'). The 55 positive ES clones were further checked for the presence of the third *loxP* site (after exon 5) by PCR using the primers: loxPF (5'-TTTCAGCCAGGTGGTGGTGACGC-3') and loxPR (5'-GCAATCCTCCTGCCTCAACATCCT-3'). Among the 14 positive clones, two independent ES clones were microinjected into blastocysts collected from C57BL/6 mice to generate chimeras. Male chimeric mice were bred to C57BL/6 females for germline transmission of the targeted gene. The gene-targeted mice were crossed to EIIa-Cre transgenic mice to delete the *neo*^r cassette and a portion of the *Tradd* gene. Successful gene deletion was confirmed by PCR using primers deltaF (pr1) 5'-TGCACATGTGTCCTCGAGTG-3' and deltaR (pr2) 5'-GGAGAGCTTGGCTGTCTTGG-3'.

Southern blot

ES cell genomic DNA was digested with *HindIII*, separated on agarose gels and transferred to nitrocellulose membranes. Hybridization was performed using ULTRAHyb solution (Ambion) with ³²P-labeled probe (Strip-EZ DNA kit, Ambion).

Reagents

Anti-phospho-JNK (# 44-682G) was purchased from Biosource. Anti-IκBα (C-21), anti-TRADD (H-278), anti-TRAF5 (E-4) and anti-TRAF2 (N-19) antibodies were purchased from Santa Cruz Biotechnology. Anti-JNK (G151-666) and anti-RIP (G322-2) were from

Pharmingen. Anti-actin (AC-40) was obtained from Sigma. Anti-TNFR1 (# AF-425-PB) and anti-TRAF2 (324522) were from R&D. Anti-phospho-ERK (#9101), anti-phospho-p38 (3D7, 28B10), anti-ERK (#9106), anti-p38 (#9212) and anti-phospho-I κ B α (14D4) were purchased from Cell Signaling. GalN, LPS and poly(I:C) were obtained from Sigma. mTNF was from Biosource; hTNF and IL-1 β were from R&D. zVAD-fmk was from MP Biomedicals.

***In vivo* toxicity assays**

The experiments were conducted in accordance with ACUC guidelines. *Tradd*^{-/-} and wild-type siblings at 7-8 weeks of age were sensitized with intraperitoneal administration of 700mg/kg of Galactosamine (GalN). After 15 min of GalN treatment, a sub-lethal dose of recombinant mouse TNF diluted with pyrogen-free saline (20 μ g/Kg) or a lethal dose of LPS (100 μ g/kg) or poly(I:C) (50 μ g/kg) was administered intravenously (TNF) or intraperitoneally (LPS, poly(I:C)). Blood was collected by tail bleeding. Serum was separated and analyzed for transaminases on a Vitros 250 chemistry analyzer.

TUNEL assay

For terminal deoxynucleotidyltransferase nick-end labeling (TUNEL) assay (*In situ* cell death detection kit, Roche) mice were sacrificed at 6 h after treatment. The livers were excised, fixed in 10% buffered formalin, sectioned at a thickness of 5 μ m and were analyzed by the TUNEL assay according to manufacturer's protocol.

Preparation of mouse embryonic fibroblasts

Mouse embryonic fibroblasts were prepared according to the previously published protocol⁴¹ with some modifications. Briefly, 13.5 day-old embryos were isolated from pregnant mice. Each embryo was incubated in a separate eppendorf tube in Cellstripper (Cellgro) at 37°C for 30 min. Afterwards cells were mashed up by pipetting, filtered through the cell strainers and cultured in DMEM with 10% FBS.

Preparation of peritoneal macrophages

Mice were injected intraperitoneally with 1.5 ml of 3% Thioglycolate (BD Bioscience). Peritoneal exudates were isolated 3 days post-injection by lavage of the peritoneal cavity with 10 ml of ice-cold HBSS. Cells were then plated in cell culture plates, washed twice with HBSS 2 h after plating and incubated overnight in DMEM with 10% FBS prior to experiments. For the immunoprecipitation experiments, cells from three mice of each group, wild-type or *Tradd*^{-/-}, were pooled together.

Immunoblot and co-immunoprecipitation

After treatments as described in the figure legends, cells were collected and lysed in M2 buffer (20mM Tris at pH 7, 0.5 % NP-40, 25mM NaCl, 3mM EDTA, 3mM EGTA, 2mM DTT, 0.5mM PMSF, 20mM β -glycerol phosphate, 1mM sodium vanadate, 1 μ g/ml leupeptin). Cell lysates were fractionated by SDS-polyacrylamide gel and immunoblotted. The proteins were visualized by enhanced chemiluminescence (ECL), according to the manufacturer's instruction (Amersham). For immunoprecipitation assays, cells were treated with mTNF (30ng/ml) as indicated in the legend and then collected in lysis M2 buffer. The lysates were mixed and precipitated with anti-TNFR1 (R&D) and protein G-Agarose beads by overnight incubation at 4 °C. The beads were washed five times with lysis buffer, and the bound proteins were resolved in 4-20 % SDS-polyacrylamide gels and detected by immunoblot.

Cytotoxicity assay

TNF-induced cell death was determined using tetrazolium dye colorimetric test (MTT test). The MTT absorbance was then read using a plate reader at 570 nm.

ELISA

Production of TNF, IL-6 and IL-12 was measured by ELISA according to the kit manufacturer's instructions (R&D Systems).

siRNA Knockdown of MyD88

MEFs were plated in 6-well plates, and cells were transfected with 50 pmol of MyD88-specific siRNA (Santa Cruz, SC-35987) or Lamin A&C-specific control siRNA (Dharmacon, 5'-GGUGGUGACGAUCUGGGCU-3') using Lipofectamine 2000 reagent (Invitrogen). After 48 h cells were treated as indicated in the figure legend and analyzed by immunoblotting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Resistance of *Tradd*^{-/-} mice to TNF cytotoxicity *in vivo*. **(a)** Survival curves of *Tradd*^{+/+} (*n*=8) and *Tradd*^{-/-} (*n*=4) after treatment with GalN (700mg/kg) and TNF (20μg/kg). **(b)** TUNEL analysis of liver tissue isolated 6 h after administration of GalN and mTNF or GalN only. The results represent the independent staining of 6 mice from each group **(c)** Serum concentrations of liver enzymes at 6 h after treatment with GalN and TNF. Results are averages ± s.d (6 mice for each group).

Figure 2.

TNFR1-mediated signaling is abolished in *Tradd*^{-/-} MEFs. **(a)** PCR (left) and immunoblot analyses (right) of MEFs prepared from *Tradd*^{+/+} and *Tradd*^{-/-} embryos. **(b, c)** *Tradd*^{+/+} and *Tradd*^{-/-} MEFs were treated with mTNF (30ng/ml) **(b)** or recombinant IL-1β (20ng/ml) **(c)** for indicated time periods and protein lysates were analyzed by immunoblotting with antibodies specific for the indicated proteins. **(d)** *Tradd*^{+/+} and *Tradd*^{-/-} MEFs were treated with CHX (10μg/ml) alone (white bars), CHX and mTNF (30ng/ml) (black bars) or CHX, zVAD-fmk (20μM) and mTNF (grey bars) for 24h and cell viability was analyzed by MTT assay. All cells were pretreated with CHX or CHX and zVAD-fmk for 30 min before mTNF treatment. Results are averages ± s.e.m. All experiments were repeated at least three times.

Figure 3.

TRADD is essential for the formation of a functional TNFR1 signaling complex in MEFs. (a) *Tradd*^{+/+} and *Tradd*^{-/-} MEFs were treated with mTNF for indicated time periods and cell extracts were immunoprecipitated with anti-TNFR1 overnight. Immunoprecipitants were resolved by SDS-PAGE, and analyzed by immunoblotting with anti-RIP, and anti-TRAF2. One percent of cell extracts from each sample was used as a control for protein input. (b) *Tradd*^{+/+}, *Traf2*^{-/-}, *Traf5*^{-/-} and *Traf2*^{-/-} *Traf5*^{-/-} MEFs were treated with 30ng/ml of mTNF for indicated times and cell extracts were immunoprecipitated with anti-TNFR1 overnight. Immunoprecipitants were resolved by SDS-PAGE, and analyzed by immunoblotting with anti-RIP and anti-TRAF2. (c) *Tradd*^{+/+}, *Traf2*^{-/-}, *Traf5*^{-/-} and *Traf2*^{-/-} *Traf5*^{-/-} MEF lysates were

probed with antibodies specific for the indicated proteins. All experiments were repeated at least three times.

Figure 4.

TRADD contributes to TNF signaling in macrophages. (a,b) *Tradd*^{+/+} and *Tradd*^{-/-} peritoneal macrophages were treated with mTNF (30ng/ml) (a) or hTNF (30ng/ml) (b) for indicated time periods and protein lysates were analyzed by immunoblotting with indicated antibodies. * non-specifically cross-reacted band, ** JNK band from previous probing with anti-JNK. (c) *Tradd*^{+/+} and *Tradd*^{-/-} peritoneal macrophages (2×10⁷ cells) were treated with mTNF for 5 min and cell extracts were immunoprecipitated with anti-TNFR1 overnight. Immunoprecipitants were resolved by SDS-PAGE, and analyzed by immunoblotting with anti-RIP and anti-TRAF2. One percent of cell extracts from each sample was used as a control of protein input. (d) *Tradd*^{+/+} (white bars) and *Tradd*^{-/-} (black bars) peritoneal macrophages were

pretreated with CHX (10 μ g/ml) or CHX and zVAD-fmk (20 μ M) for 30 min before mTNF (30ng/ml) treatment (8 h), and cell viability was analyzed by MTT assay. Results are averages $\pm$ s.e.m. (e) Equal amounts of lysates from *Tradd*^{+/+} and *Tradd*^{-/-} MEFs and macrophages were resolved by SDS-PAGE and protein expression was analyzed by immunoblotting with indicated antibodies. All experiments were repeated at least three times except for immunoprecipitation experiment, which was repeated twice.

Figure 5.

Resistance of *Tradd*^{-/-} mice to LPS and poly(I:C) toxicity in the presence of N-galactosamine. (a) Survival curves of *Tradd*^{+/+} (*n*=8) and *Tradd*^{-/-} (*n*=6) after treatment with GalN (700mg/kg) and LPS (100μg/kg). (b) Peritoneal macrophages isolated from *Tradd*^{+/+} and *Tradd*^{-/-} mice were treated with LPS (100ng/ml) for indicated time periods and protein lysates were analyzed by immunoblotting with indicated antibodies. * IκBα band from previous probing with anti-IκBα antibody, ** nonspecific band (c) Survival curves of *Tradd*^{+/+} (*n*=3) and *Tradd*^{-/-} (*n*=3) after treatment with GalN (700mg/kg) and poly(I:C) (50μg/kg) (d) Peritoneal macrophages isolated from *Tradd*^{+/+} and *Tradd*^{-/-} mice were treated with poly(I:C) (20μg/ml) for indicated time periods and protein lysates were analyzed by immunoblotting with indicated antibodies. (e) Serum concentrations of TNF at 1 h and IL-6 at 4 h after GalN plus LPS treatment of *Tradd*^{+/+} (black bars) and *Tradd*^{-/-} (white bars) mice. (f) Serum concentrations of TNF at

1 h and IL-6 at 2 h after GalN plus poly(I:C) administration in *Tradd*^{+/+} (black bars) and *Tradd*^{-/-} (white bars) mice. (g) Peritoneal macrophages from *Tradd*^{+/+} (black bars) and *Tradd*^{-/-} (white bars) mice were stimulated with LPS (10µg/ml) or poly(I:C) (100µg/ml) for 24 h, and the supernatants were subjected to measurement of TNF, IL-6 and IL-12 by ELISA. All experiments were repeated at least three times. 6 mice from each group (*Tradd*^{+/+} or *Tradd*^{-/-}) were used for ELISA (e,f).

Figure 6.

TRADD has a role in TLR3 and TLR4 signaling in MEFs. (**a,b,c**) *Tradd*^{+/+} and *Tradd*^{-/-} (**a,b**) and *Tnfr1*^{-/-} (**c**) MEFs were treated with LPS (10μg/ml) (**a**) or poly(I:C) (100μg/ml) (**b,c**) for indicated time periods. Cell lysates were analyzed by immunoblotting with indicated antibodies. (**d**) *Tradd*^{+/+} and *Tradd*^{-/-} MEFs were plated in 6-well plates and transfected with 50 pmol of MyD88-specific siRNA or control siRNA as indicated. After 48 h cells were treated with 10 μg/ml LPS for indicated time periods and protein lysates were analyzed by immunoblotting with indicated antibodies. (**e**) *Tradd*^{+/+} (black bars) and *Tradd*^{-/-} (white bars) MEFs were stimulated with LPS (10μg/ml) or poly(I:C) (100μg/ml) for 24 h, and supernatant TNF and IL-6 were measured by ELISA. All experiments were repeated at least three times.