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The Adaptor Protein TRADD Is Essential for TNF-Like Ligand 1A/Death Receptor 3 Signaling

Yelena L. Pobezinskaya, Swati Choksi, Michael J. Morgan, Xiumei Cao, and Zheng-gang Liu

TNFR-associated death domain protein (TRADD) is a key effector protein of TNFR1 signaling. However, the role of TRADD in other death receptor (DR) signaling pathways, including DR3, has not been completely characterized. Previous studies using overexpression systems suggested that TRADD is recruited to the DR3 complex in response to the DR3 ligand, TNF-like ligand 1A (TL1A), indicating a possible role in DR3 signaling. Using T cells from TRADD knockout mice, we demonstrate in this study that the response of both CD4⁺ and CD8⁺ T cells to TL1A is dependent upon the presence of TRADD. TRADD knockout T cells therefore lack the appropriate proliferative response to TL1A. Moreover, in the absence of TRADD, both the stimulation of MAPK signaling and activation of NF-κB in response to TL1A are dramatically reduced. Unsurprisingly, TRADD is required for recruitment of receptor interacting protein 1 and TNFR-associated factor 2 to the DR3 signaling complex and for the ubiquitination of receptor interacting protein 1. Thus, our findings definitively establish an essential role of TRADD in DR3 signaling.

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Death receptor (DR) 3 (also known as TNFRSF25, WSL-1, LARD, TRAMP, Apo3) is a member of the TNFR superfamily and a member of the DR subfamily that has seven additional members, including TNFR1, FAS, DR4, DR5, DR6, nerve growth factor receptor, and ectodysplasin 1 anhidrotic receptor (1). All of the receptors of this subfamily contain a death domain (DD) as a part of their intracellular domain, and of these seven, DR3 has the highest homology to TNFR1 (2–5). However, in contrast to the ubiquitous expression of TNFR1, DR3 expression is restricted to lymphocyte-enriched tissues such as thymus, spleen, small intestine, and PBL (2–6) and has been shown to be especially upregulated in activated T cells (5, 6). The ligand to DR3 receptor is a member of the TNF superfamily and is called TNF-like ligand 1A (TL1A or TNFSF15). Originally thought to be predominantly expressed by endothelial cells (7), later studies have shown that TL1A can be produced by variety of other cells including monocytes, macrophages, plasma cells, dendritic cells, and T cells (8–10).

TNFR1 signaling has been very well characterized. The TNF-α–dependent activation of TNFR1 leads to formation of a signaling complex that contains several important molecules, including TNFR-associated death domain protein (TRADD), receptor interacting protein (RIP), and TNFR-associated factor (TRAF) 2 (11–13). These molecules are responsible for mediating the activation of MAPK and NF-κB. In some cases, a secondary complex (complex II) dissociates from the receptor and associates with FADD (14), and this complex is responsible for caspase activation and apoptosis. This is in contrast to other death receptors, such as Fas, DR4, and DR5, which are capable of binding directly to FADD in the primary receptor complex (1). The generation of RIP1 and TRAF2 knockout mice did much to unveil the important functions of these molecules in MAPK and NF-κB activation downstream of TNFR1 (15, 16). However, TRADD has always been considered to be the first adaptor molecule that is recruited to TNFR1 (17), and TRADD-deficient mice generated recently by our laboratory (18) and others (19, 20) made it possible to definitively establish the role of TRADD in TNFR1 signaling that occurs mainly through recruitment of these molecules.

A majority of studies on the mechanisms of DR3 signaling have been conducted previously using overexpression systems. Similar to TNFR1, DR3 induces both NF-κB activation and apoptosis when ectopically overexpressed in cell lines (2–5). Of the adaptor proteins tested in overexpression studies, TRADD was the only protein that had a strong interaction directly with DR3 (2, 4), whereas the association of RIP and TRAF2 with DR3 was very weak and greatly enhanced in the presence of transfected TRADD (4). In these systems, FADD was also shown to be recruited to DR3 upon coexpression of TRADD, and association of FADD directly with DR3 was observed only at very high expression levels (3). In addition, DR3-mediated apoptosis was dependent on the presence of FADD and caspase-8 in embryonic mouse fibroblasts (15, 21). These studies do suggest that TRADD is the primary adaptor recruited to DR3 upon TL1A stimulation. However, a detailed examination of the physiological role of TRADD in DR3 signaling pathways has not yet been undertaken.

We therefore sought to examine DR3 signaling events in cells derived from TRADD knockout mice. In this study, we show that in the absence of TRADD, TL1A cannot induce proper JNK and NF-κB activation. This is largely due to the inability of DR3 to effect the recruitment of the critical adaptor proteins TRAF2 and RIP1 in TRADD-deficient cells, thus leading to a nonfunctional DR3 signaling complex. Moreover, TL1A does not promote T cell pro
liferation in TRADD-deficient cells. We also confirm that TL1A-induced DR3 signaling does not result in T cell apoptosis.

Materials and Methods

Reagents

Anti–phospho-JNK Ab was purchased from Biosource International. Anti–TRADD and TRAF2 Abs were purchased from Santa Cruz Biotechnology. Anti–JNK, RIP, Fas, CD28, CD3ε, anti–CD4–PE, and anti–CD8–allophycocyanin Abs were from BD Pharmingen. Anti–phospho-IκBα, anti–phospho-p38, anti–p38, anti–phospho-ERK, and anti–ERK were purchased from Cell Signal
ing Technology. Recombinant murine TL1A, TNF-α, IL-2, and anti–DR3 Ab were from R&D Systems. PMA was from Alexis Biochemicals. SMAC mimic (SM-164) was a kind gift from Dr. Shaomeng Wang.

Animals

TRADD knockout mice were described previously (18). Three- to 4-wk-old TRADD knockout and wild-type littermates were used to isolate T cells.

T cell purification

Purification of CD4+ and CD8+ lymph node T cells or pan T cells was performed using MACS CD4+ and CD8+ T cell or pan T cell isolation kits (Miltenyi Biotec), respectively, according to the manufacturer’s instructions. Briefly, lymph node cell populations were depleted of non-T cells by incubation with biotin-conjugated Ab mixture and antibiotin magnetic microbeads followed by separation on MACS columns. Purity of isolated T cells was >97%.

Treatment of cells

Before any treatment, T cells were preactivated with plate-bound anti-CD3 (1 μg/ml) Abs overnight.

In vitro cell proliferation assay

T cells were washed with PBS twice, resuspended at 10^6/ml in PBS, warmed in 37°C water bath for 10 min, mixed with 1 μl 5 mM CFSE stock (Vybrant CFDA SE cell tracer kit; Molecular Probes Invitrogen), and incubated in 37°C water bath for 10 min. Reaction was stopped with ice-cold complete RPMI 1640 media. Cells were then plated in 24-well plates at 0.5 × 10^6/well, harvested after 72 h, stained with anti-CD4 or CD8 Ab, and analyzed on an FACSCalibur (BD Biosciences) using FlowJo software (Tree Star).

Western blot analysis and coimmunoprecipitation

After treatments as described in the figure legends, cells were collected and lysed in M2 buffer (20 mM Tris [pH 7], 0.5% Nonidet-P40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM DTT, 0.5 mM PMSF, 20 mM β-glycerol phosphate, 1 mM sodium vanadate, and 1 μg/ml leupeptin). Cell lysates were fractionated by SDS-polyacrylamide gel and Western blotted. The proteins were visualized by ECL according to the manufacturer’s instruction (Amer sham Biosciences). For immunoprecipitation assays, cells were treated with TL1A as indicated in the figure legends and then collected in lysis M2 buffer. The lysates were mixed and precipitated with anti-DR3 Abs (R&D Systems) and protein G-Agarose beads by incubation at 4°C for overnight. The beads were washed five times with lysis buffer, and the bound proteins were resolved in 4–20% SDS-polyacrylamide gels and detected by Western blot analysis.

EMSA

Nuclear extracts were isolated using the Biovision Nuclear/Cytosol fractionation kit following the manufacturer’s guidelines (Biovision). Gel shifts were performed with the Promega Gel shift assay system (Promega) using 10 μg nuclear extract and following the recommendations outlined in the manufacturer’s protocol. All consensus oligos were also purchased from Promega.

Cytotoxicity assay

TL1A- and Fas-induced cell death was determined using tetrazolium dye colorimetric test (MTS test). The MTS absorbance was then read using a plate reader at 490 nm.

Results

TL1A can induce several events in T cells, including the activation of NF-κB and MAPK (7, 22). In addition, TL1A also enhances proliferation and cytokine production of T cells stimulated through the TCR (23). We therefore sought to determine whether TRADD was important for these events. CD4+ and CD8+ T cells were purified from lymph nodes of wild-type and TRADD −/− mice and labeled with CFSE. Because TL1A increases T cell proliferation better when the CD28 costimulatory signal is absent (23), we preactivated T cells with anti-CD3 only and treated with TL1A or IL-2 for 72 h. In contrast to wild-type T cells, which proliferated very well in response to TL1A (as seen by the dilution peaks on the CFSE histogram), CD4+ TRADD-deficient T cells showed very minimal proliferation (Fig. 1A). Similar differences were seen in the CD8+ T cells (Fig. 1B). However, IL-2–induced proliferation was minimally affected by the absence of TRADD in either CD4+ or CD8+ T cells, suggesting that the defect in the proliferative response was specific for TL1A treatment.

Next we sought to determine whether the lack of proliferation was due to a deficiency in the downstream signaling pathways and whether TRADD has a role in DR3-mediated MAPK signaling and NF-κB activation. We examined phosphorylation of JNK and JNK

FIGURE 1. TRADD-deficient T cells do not proliferate in response to TL1A treatment. CFSE-labeled wild-type and knockout T cells were treated with TL1A (50 μg/ml; top panels) or IL-2 (100 U/ml; bottom panels) for 72 h, then stained with anti-CD4 or anti-CD8 Abs and analyzed by flow cytometry. Histograms are gated on CD4+ (A) and CD8+ cells (B).
IkBα in CD3-activated T cells after TL1A treatment. As shown on Fig. 2A and 2B, wild-type CD4+ and CD8+ T cells demonstrated a potent activation of these two pathways. In contrast, TL1A induced only a very weak phosphorylation of JNK and minimal phosphorylation of IkBα in TRADD-deficient T cells. Similar results were obtained if T cells were preactivated with CD3 in the presence of CD28 or if they were not preactivated at all (data not shown). This was true despite similar expression levels of DR3 in wild-type and knockout cells (data not shown). The defect in signaling was specific for TL1A because the PMA-dependent activation of JNK was normal in both wild-type and TRADD knockout cells (Fig. 2C). Phosphorylation of p38 and ERK was not observed in response to TL1A stimulation, whereas both kinases were strongly activated when T cells were treated with PMA (Supplemental Fig. 1).

We also assessed NF-κB DNA-binding activity in wild-type T cells versus TRADD-deficient T cells using EMSA. TNF-α treatment was used as a positive control (Fig. 2D). Strong NF-κB DNA-binding activity was observed in wild-type T cells 30 min after TL1A treatment and was decreased by 4 h (Fig. 2D). However, we observed little NF-κB binding to DNA in TRADD-deficient T cells in the presence or absence of both stimuli, and we observed little, if any, increase at 6 (Supplemental Fig. 2) or 8 h (not shown). Thus, our data suggest that TRADD is necessary for potent DR3-mediated signaling in response to TL1A.

Based on the data obtained earlier from the overexpression experiments (2, 4), it is believed that, similar to TNFR1, DR3 interacts with TRADD, and then TRADD recruits RIP and TRAF proteins to the signaling complex. To observe the formation of the physiological DR3 signaling complex, we performed immunoprecipitation experiments with DR3-specific Abs. In agreement with the previous results, TRADD, RIP, and TRAF2 were recruited to DR3 after 5 min of TL1A treatment in wild-type T cells (Fig. 3). Similar to TNFR1-associated RIP, DR3-associated RIP was presumed to be ubiquitinated based on the pattern seen in Western blotting with an anti-RIP Ab. In contrast, in TRADD-deficient T cells, we observed neither RIP1 nor TRAF2 recruitment to DR3, indicating that TRADD is required for the recruitment of these two critical signaling molecules to the DR3 signaling complex in T cells.

It has been shown previously that overexpression of DR3 induces apoptosis in cell lines (2–5), and the human leukemia cell line TF-1, which has high endogenous levels of DR3, undergoes apoptosis when treated with TL1A in the presence of cycloheximide (7, 22). However, consistent with a previous study (7), we confirmed that primary T cells from lymph nodes were resistant to cell death under these conditions (data not shown). We also tested whether thymocytes can be sensitized to cell death. T cells from wild-type and TRADD knockout mice were treated with cycloheximide and TL1A or anti-Fas Abs for 16 h. Apoptosis induced by Fas cross-linking was the same in wild-type and TRADD-

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**FIGURE 2.** TRADD is required for MAPK and NF-κB activation. Western blot analysis of lysates of wild-type and TRADD knockout CD4+ (A), CD8+ (B) T cells, and pan T cells (C) treated with TL1A (50 μg/ml) (A, B) for the indicated times or PMA (100 nM) (C). D, Nuclear extracts of wild-type and knockout T cells treated with TNF-α (30 ng/ml) and TL1A (50 μg/ml) for indicated times were analyzed by EMSA.

**FIGURE 3.** TRADD is required for formation of the functional DR3 signaling complex. Cell extracts from wild-type and TRADD knockout T cells treated with TL1A (50 μg/ml) for 5 min were immunoprecipitated with anti-DR3 (ip:DR3) and analyzed by Western blotting. Input, 2% of extract before immunoprecipitation; Ub-, ubiquitinated.

**FIGURE 4.** TL1A does not induce apoptosis in primary T cells. MTS assay of the viability of wild-type and TRADD knockout T cells treated for 24 h with cycloheximide (C) alone (10 μg/ml), cycloheximide and TL1A (50 μg/ml), or cycloheximide and anti-FAS (10 ng/ml) (A) and SMAC mimetic (SM) alone (10 nM), SMAC mimetic and TL1A (50 μg/ml), or SMAC mimetic and anti-FAS (10 ng/ml) (B). Error bars shown represent SEM.
deficient thymocytes (Fig. 4A, Supplemental Fig. 3). No cell death was observed after TL1A treatment in the presence or absence of cycloheximide. Additionally, cross-linking of the flag-tagged TL1A with the flag-specific M2 Abs did not result in apoptosis of the T cells (data not shown). Furthermore, when the SMAC mimetic SM-164 was used instead of cycloheximide, it again sensitized thymocytes to Fas- but did not result in TL1A-induced apoptosis (Fig. 4B).

Discussion

Due to the lack of knockout mice, the involvement of adapter protein TRADD in TNFR1 signaling had been a controversial issue for a long time. Recently, our laboratory and others have generated TRADD knockout mice and have demonstrated that TRADD is indispensable for MAPK and NF-kB activation as well as apoptotic and necrotic cell death in TNFR1 signaling (18–20). Interestingly, TRADD turned out to be not solely a TNFR1 adaptor molecule, but also an adaptor in several other signaling pathways. In the absence of TRADD, TRIF-dependent signaling events downstream of TLR3 and TLR4 are attenuated, suggesting that TRADD functions outside of DR signaling (18–20). TRADD has also been proposed to serve as a negative regulator of IFN-γ signaling through the formation of a complex with STAT1α (24). The antiviral pathways mediated through RIG-like helicases is also dependent on TRADD, and TRADD is a necessary adaptor to mediate the downstream Cardif-dependent immune responses, such as the activation of the NF-κB and IRF3 transcription factors and the production of IFN-β (25). A role for TRADD in other DR signaling pathways has also been suggested. The p75TNFR receptor was shown to interact with TRADD in breast cancer cells after stimulation with nerve growth factor (26). Most other studies in relationship to other DR have relied on overexpression studies. For example, both DR4 and DR5 can interact with TRADD when overexpressed in cell lines (27). Likewise, the implication of the involvement of TRADD in the DR3 signaling pathway has also largely been based on overexpression data.

We took advantage of our recent generation of TRADD knockout mice and demonstrated that under more physiological conditions, TRADD plays an important role in DR3 signaling. Importantly, based on our immunoprecipitation experiments, TRADD is required for the recruitment of both RIP and TRAF2 to the DR3 complex in T cells, suggesting that TRADD is the primary adapter molecule involved in the DR3 signaling pathway, just as it is in TNFR1 signaling. This is consistent with the closer homology of the DR3 DD to the TNFR1 receptor (40% identical in mouse) than other FADD binding DRs are to TNFR1, such as the TRAIL receptor DR4/5 (28% identity) and Fas (13% identity).

Due to loss of complex formation, the TL1A-induced phosphorylation of JNK and IκBα were dramatically reduced in TRADD-deficient T cells, and no NF-κB DNA-binding activity was observed. A very small amount of residual phosphorylation of JNK and IκBα may be due to weak interaction of RIP and TRAF2 with DR3 (4). This possibility is supported by our previous findings made in our study on TNF signaling (18). The downregulation of signaling appears to have a physiologically relevant consequence in TRADD-deficient T cells, because we observed that the TL1A-induced proliferation of both CD4+ and CD8+ T cells was abrogated. Our data are consistent with recent data that also showed that TRADD-deficient CD4+ T cells failed to proliferate in response to TL1A treatment (20).

DR3 was given its name DR3 because of the DD present in the C-terminal intracellular domain of the protein. Although receptors with DDs, such as Fas and DR4/5, are clearly associated with cell death in physiological contexts, in previous studies, TL1A has only been shown to induce apoptosis in overexpression systems and in immortalized cell lines that endogenously express DR3, whereas primary T cells, the likely physiological TL1A targets, did not undergo cell death when treated with TL1A (7). Our findings are consistent with this study, as we were unable to induce cell death in primary T cells in response to TL1A. Interestingly, the presence of cycloheximide, which usually sensitizes most TNFR1-expressing cells to TNF-α–induced cell death, did not sensitize T cells to cell death. Particularly, although cellular inhibitor of apoptosis 2 (cIAP2) was found to be induced by TL1A in human TF-1 cells (22), we did not observe any induction of cIAP2 in primary T cells following the engagement of TL1A (data not shown). Therefore, the level of cIAP2 is not the main cause of the resistance of these cells to TL1A-induced apoptosis. Likewise, SMAC mimetic, although sensitizing the primary T-cells to FasL, did not sensitize the cells to TL1A. Although we did not examine this possibility, these data may suggest that DR3 is not capable of inducing the formation of a secondary complex II-type complex (14) in which FADD is recruited and then activates caspase-8. Thus, in contrast to TNFR1 signaling that can activate both survival and death pathways depending on the cellular context, the TL1A–DR3 pathway may be solely a proinflammatory and prosurvival pathway, rather than also acting as a proapoptotic pathway.

DR3 has been implicated in many inflammatory and autoimmune diseases including graft-versus-host disease (7), allergic lung inflammation (28), inflammatory bowel disease (8, 9), rheumatoid arthritis, and experimental autoimmune encephalomyelitis (23, 29). In all of the cases, TL1A plays an activating role, augmenting T cell responses and worsening the course of diseases, consistent with the fact that stimulation of DR3 does not lead to apoptosis in primary T cells. Because TNF-α also plays an important role in inflammation in many of these diseases, and TRADD is involved in both DR3 and TNFR1 signaling pathways, approaches targeting TRADD might be taken into consideration when designing new therapeutic strategies for treatment of inflammatory T cell-mediated diseases.

Disclosures

The authors have no financial conflicts of interest.

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