Competitive Control of Independent Programs of Tumor Necrosis Factor Receptor-Induced Cell Death by TRADD and RIP1

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Stimulation of tumor necrosis factor receptor 1 (TNFR1) can initiate several cellular responses, including apoptosis, which relies on caspases, necrotic cell death, which depends on receptor-interacting protein kinase 1 (RIP1), and NF- κ B activation, which induces survival and inflammatory responses. The TNFR-associated death domain (TRADD) protein has been suggested to be a crucial signal adaptor that mediates all intracellular responses from TNFR1. However, cells with a genetic deficiency of TRADD are unavailable, precluding analysis with mature immune cell types. We circumvented this problem by silencing TRADD expression with small interfering RNA. We found that TRADD is required for TNFR1 to induce NF- κ B activation and caspase-8-dependent apoptosis but is dispensable for TNFR1-initiated, RIP1-dependent necrosis. Our data also show that TRADD and RIP1 compete for recruitment to the TNFR1 signaling complex and the distinct programs of cell death. Thus, TNFR1-initiated intracellular signals diverge at a very proximal level by the independent association of two death domain-containing proteins, RIP1 and TRADD. These single transducers determine cell fate by triggering NF- κ B activation, apoptosis, and nonapoptotic death signals through separate and competing signaling pathways.

Tumor necrosis factor alpha (TNF- α) is a pleiotropic cytokine that belongs to the TNF ligand superfamily. Through associations with its receptors on a variety of cell types, TNF- α participates in a broad range of biological activities, including cell differentiation, tissue development, inflammatory responses, lipid metabolism, and cellular cytotoxicity (1, 24). Programmed cell death (PCD) caused by death receptors includes both apoptosis and nonapoptotic mechanisms (13). For example, TNF- α can trigger different forms of PCD that are morphologically distinguished as apoptosis and necrosis-like death (3, 9, 25). By using either the Jurkat human T-cell leukemia line or mouse embryonic fibroblasts that are deficient in the Fas-associated death domain (FADD) protein, receptor-interacting protein kinase 1 (RIP1), and caspase-8, substantial evidence indicates that TNF receptor 1 (TNFR1) occupancy can initiate either FADD/caspase-8-dependent apoptosis or a RIP1-dependent nonapoptotic death program that results in necrosis (3, 9, 30, 36; L. Zheng and M. Lenardo, unpublished data). How the TNFR1 signal is distributed to these different pathways has not been determined.

There are two TNF- α receptors, TNFR1 and TNFR2, both of which belong to the TNFR superfamily. Similar to other members of the superfamily, TNFR1 and TNFR2 harbor four characteristic cysteine-rich domains (CRDs) in their extracellular portions. We have recently shown that the first extracellular CRD, the most membrane-distal domain of TNFRs, contributes to homotypic receptor self-assembly, presumably a prerequisite for the second and third CRDs to bind directly

with TNF- α (4). However, these two TNFRs have different intracellular domains which can initiate distinctive intracellular signals. TNFR1 has a single intracellular death domain (DD), whereas TNFR2 has a TNFR-associated factor (TRAF)-binding motif (24). The DD is an 80-amino-acid hexahelical bundle homologous to the DDs that exist in some other members of the TNFR superfamily designated the death receptor subfamily (18). The DD is an essential protein-binding domain that accounts for the ability of TNFR1 to induce apoptotic and nonapoptotic cell death and/or inflammatory signals, depending on the conditions. In this report, we wished to examine how TNFR1 transmits signals that determine different cellular outcomes.

The DD and its flanking sequences in TNFR1 provide docking sites for intracellular adaptors and proximal signal molecules (for details, see references 1 and 24). In response to TNF- α stimulation, the silencer of DD is displaced from the intracellular DD of TNFR1, which allows TNFR1 to associate with other signaling molecules, including the TNFR-associated DD (TRADD) protein (11, 14), TRAF2, TRAF1 (28), and other DD-containing adaptors, such as RIP1 (10) and the FADD protein (26, 31). The recruitment of TRAF2 to the TNFR1 signaling complex is important for NF- κ B activation (31) and, more prominently, for c-Jun N-terminal kinase (JNK) activation (19, 27).

The interactions of the three DD-containing proteins involved in TNFR1 proximal signals pose interesting regulatory questions. TRADD has been shown to associate with the cytoplasmic tail of TNFR1 through homotypic DD interactions. This recruitment initiates two major TNF-induced responses, namely, programmed death by apoptosis and NF- κ B activation (11). FADD has been found in the TNFR1 signaling complex

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upon stimulation (26) and is believed to mediate the TNFR1-induced activation of caspase-8 and/or caspase-10 that causes apoptosis. However, the association of FADD with TNFR1 seems to require TRADD as an adaptor (9, 12). RIP1, another DD molecule found in the TNFR1 signaling complex, induces NF- κ B (33) and a necrotic program of death involving reactive oxygen species (ROS) (9, 10, 21, 32). Even though it possesses a DD itself, RIP1 seems to still require TRADD as an adaptor to indirectly associate with TNFR1 (10). Thus, TRADD has been considered a central signaling adaptor for TNFR1 stimulation (1, 13, 15). Much evidence suggests that through homotypic DD interactions, TRADD is recruited to TNFR1 as a first step which promotes the subsequent binding of all other signal transducers. The place of formation and the components of the TNFR1 signaling complex during TNF- α stimulation have been controversial, partly due to differences in the experimental approaches used by different investigators (26, 29). Nevertheless, recent data show that upon TNF- α stimulation, signaling complexes can form at the membrane as part of a secondary internal signaling complex or by internalization of the receptor with a signal complex attached to its cytoplasmic tail. In one model, there is an early recruitment of TRADD/RIP1 to the cytoplasmic tail of TNFR1, and then the complex dissociates from the receptors on the membrane and travels into the cytoplasm, recruiting additional proteins into the complex, including FADD and caspase-8 (26). Alternatively, TNFR1 recruits adaptors and other signaling molecules residing on the membrane and then internalizes as a whole, forming the stabilized signaling complex (29). Nevertheless, as a serine/threonine protein kinase, RIP1 can deliver both survival and death signals during TNF- α stimulation. A major question posed by the complex array of signaling proteins is how these specific signaling responses from TNFR1 are triggered.

It has been well established that stimulation of TNFR1 can induce the activation of NF- κ B. In view of the known PCD pathways, it is interesting that most of the key pro-PCD components are constitutively expressed, which allows cells to promptly sense and execute death signals. In contrast, many of the anti-PCD molecules, such as the cellular FLICE-like inhibitory protein (cFLIP) (17), inhibitor of apoptosis proteins (cIAPs), TRAFs (35), and Bcl-2/Bcl-XL (6), are regulated by NF- κ B induction (for a review, see reference 15). Thus, NF- κ B may play a central role in regulating life and death during death receptor stimulation in an inducible manner.

Since TRADD-knockout animals or deficient cell lines are unavailable, it has been difficult to definitively address the physiological role of TRADD. Previous studies suggested that TRADD serves as a central signaling adaptor for both FADD and RIP1 in association with TNFR1. Here we show that silencing the expression of TRADD with small interfering RNA (siRNA) can prevent TNF- α -induced NF- κ B activation and caspase-8-dependent apoptosis but has no effect on TNFR1-induced, RIP1-dependent nonapoptotic PCD. We also address the roles of RIP1, FADD, and caspase-8 in the two PCD pathways induced by TNF- α in T-cell lines.

MATERIALS AND METHODS

Cells and reagents. Wild-type Jurkat cells (A3) were purchased from ATCC. Mutant Jurkat cell lines deficient in RIP1, caspase-8 (Jurkat I9.2), and FADD (Jurkat I2.1) were kindly provided by Brian Seed and John Blenis. All cells were

cultured in a 5% CO₂ incubator at 37°C with complete RPMI 1640 medium containing 10% fetal calf serum, 2 mM glutamine, and 100 U/ml each of penicillin and streptomycin. Recombinant human TNF- α and goat anti-human TNFR1 antibodies were purchased from R&D. Cycloheximide (CHX) and butylated hydroxyanisole (BHA) were obtained from Sigma. Antibodies against FADD, TRADD, RIP1, caspase-8, and cytochrome *c* were obtained from BD Pharmingen. Anti-NF- κ B (p65) was bought from Santa Cruz. siRNA duplex oligonucleotides were purchased from Dharmacon, and their sense-strand sequences are as follows: human RIP1, 5'-GGAGCAAACUGAAUAAUGAUU-3'; and human TRADD, 5'-GGAGGAUGCGCUGCGAAUUU-3'.

Transfection by electroporation. Jurkat cells were cultured under optimized conditions for high-efficiency transfection. Briefly, 4×10^6 to $\sim 8 \times 10^6$ cells were resuspended in 0.4 ml of complete RPMI 1640 medium. The cells were mixed with 200 pmol of siRNA duplex oligonucleotide or the indicated amount of plasmid DNA in a 0.4-cm cuvette and electroporated using an Electro Cell Manipulator 600 instrument (BTX) under the following conditions: 260 V, 720 Ω , and 1,050 μ F at room temperature. The transfected cells were cultured for approximately 1 to 4 days prior to further analysis.

Kill assay. Cells were seeded into 96- or 48-well plates at 10^5 /ml in the presence (exclusively for the wild-type Jurkat cells) or absence (for all other Jurkat cell lines) of 0.2 μ g/ml of CHX. One to 10 ng of TNF- α /ml was added to the cultures for up to 48 h. An aliquot of each sample was taken out, mixed with 25 nM 3,3'-diethyloxycarbocyanine (DiOC6; Molecular Probes), and then cultured for another 30 min. Cells were then stained with 10 μ g/ml of propidium iodide (PI) and analyzed with a FACScan instrument, using constant time (30 s) acquisition (4). TNF- α -induced specific death was quantified by comparing the numbers of viable cells (PI⁻ DiOC6^{high}) in the TNF- α -treated samples with those in the control samples.

Light and transmission electron microscopy. The Jurkat cell line 4E3, which stably expresses TNFR2, and RIP1- and FADD-deficient Jurkat cells were subjected to TNF- α stimulation (5 ng/ml) for 0, 2, 4, 8, and 24 h. The cells were collected, washed once with phosphate-buffered saline (PBS), and then fixed at room temperature with 2 ml of 2.5% glutaraldehyde in PBS. The fixed samples were then processed further for light and transmission electron microscopy (TEM) by routine methods.

Immunoprecipitation and Western blot assay. Fifty million cells were stimulated with 100 ng/ml of recombinant TNF- α for 10 min at 37°C. The cells were washed twice with 10 ml of cold PBS and lysed on ice with 1.5 ml of lysis buffer (20 mM Tris-Cl, pH 7.6, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, and 1 $\times$ Complete protease inhibitor cocktail [Roche]). The samples were microcentrifuged at 4°C for 10 min at top speed. The cytosol lysates were precleared by incubation with 50 μ l of protein G-Sepharose beads for 30 min and centrifugation. The precleared lysates were subjected to immunoprecipitation by using 15 μ g of anti-TNFR1 and 60 μ l of 50% protein G beads overnight. The immunoprecipitated beads were subjected to 4 to 20% Tris-glycine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the separated proteins were transferred to nitrocellulose membranes. The membranes were then blocked with 5% nonfat milk in PBS-0.1% Tween 20 and probed with anti-RIP1, anti-TRADD, anti-FADD, and anti-caspase-8 antibodies.

NF- κ B activation assay. To determine the nuclear translocation of NF- κ B, $\sim 200,000$ cells per sample were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and then Celspin mounted onto slides. The fixed cells were then permeabilized with 0.05% Triton X-100 in PBS and stained with fluorescein isothiocyanate-labeled rabbit anti-NF- κ B antibodies (Santa Cruz) for 40 min and with 1 μ g/ml of Hoechst 33342 (Sigma-Aldrich) for 5 min at room temperature. The slides were then rinsed with PBS three times and cover mounted for fluorescence microscopy. The Dual-Glo luciferase assay system (Promega) was used to measure NF- κ B activity. Jurkat cells were first cotransfected by electroporation, as described above, with the NF- κ B-driven firefly luciferase plasmid pNF- κ B.luc and the thymidine kinase-driven *Renilla* luciferase-expressing plasmid pRL-TK.luc at a 10:1 to 15:1 ratio, with 5 to 10 μ g DNA in total. The transfected cells were cultured for 24 h and then stimulated with 10 ng/ml of TNF- α for 6 to 12 h before being harvested. The assay was then carried out according to the manufacturer's manual (Promega). Dual luciferase activities of the lysates were determined by using a TD-20/20 luminometer according to the manufacturer's instructions (Turner Designs).

Cytochrome *c* release assay. Cytosolic extracts were prepared by a selective digitonin-based plasma membrane permeabilization technique, as follows. Cells (5×10^6) were washed twice in PBS and incubated with extraction buffer (50 μ g/ml digitonin, 250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, pH 7.5) supplemented with 1 $\times$ Complete protease inhibitor cocktail (Roche) for 5 min on ice. Extracts were then spun at $800 \times g$

for 15 min at 4°C, and the resulting supernatants were stored as cytosolic fractions. The protein-normalized samples were subjected to Western blot assay as described above. Anti-cytochrome *c* was used at 0.5 µg/ml for blotting.

Detection of ROS. Intracellular ROS were detected with dichlorodihydrofluorescein diacetate (DCFDA; Molecular Probes) according to the manufacturer's instructions. Briefly, Jurkat cells were treated with 10 µM DCFDA (in PBS) at 37°C in the dark for 30 min before being harvested, and the cells were then washed once with PBS. Cellular staining was measured by a flow cytometer (FACSCalibur; Becton Dickinson). The percentage of DCFDA-positive cells was analyzed using Flowjo software, with the cells gated on forward scatter plots.

RESULTS

TNF-α causes two forms of PCD in Jurkat cells. It has recently been shown that both CD95 and TNFR1 can initiate caspase-dependent and caspase-independent cell death programs (3, 9, 15). We found that in the absence of the protein synthesis inhibitor CHX, wild-type Jurkat cells were not sensitive to death induced by TNF-α (up to 100 ng/ml) (Fig. 1A and data not shown). However, Jurkat cells became sensitive to TNF-α stimulation in presence of 0.1 to 0.3 µg/ml of CHX. In contrast to the wild-type cells, FADD^{-/-} and RIP1^{-/-} Jurkat cells died in response to TNF-α stimulation without CHX. However, TNF-α-induced death in FADD^{-/-} cells was totally caspase independent, whereas it was mainly caspase dependent in the RIP1^{-/-} cells, as reflected in their differential sensitivity to the pan-caspase inhibitor zVAD-FMK (Fig. 1A). Another interesting observation was that the caspase-independent death of FADD^{-/-} cells manifested a different morphology in cells examined by microscopy. We observed dramatic nuclear condensation but no obvious chromatin segregation or apoptotic body formation compared to wild-type or RIP1^{-/-} cells stimulated by TNF-α. In contrast, when FADD was intact, TNF-α caused typical apoptosis (Fig. 1B, upper panels). When examining cells by TEM, we found that nonapoptotic death manifested massive vesicle formation, cell membrane damage, and damaged nuclear chromatin (Fig. 1B, lower panels). These data establish that TNF-α can kill lymphoid cells by two distinct mechanisms.

TRADD is not essential for TNF-α-induced death in FADD^{-/-} Jurkat cells. Most of the proximal molecules in TNFR1 signaling pathways have been examined in detail by using systems that have corresponding gene deficiencies (9). However, TRADD has been an exception due to the unavailability of genetic knockout animals or genetically deficient cell lines. Nevertheless, substantial evidence suggests that TRADD is an essential adaptor for TNFR1 in recruiting both FADD/caspase-8 and RIP1 to form the TNFR1 signaling complexes (10, 12). To definitively examine the role of TRADD during TNF-α signaling, we used siRNA against TRADD in Jurkat 42.3 cells, which constitutively express TNFR2 in a background of FADD deficiency. These cells are very sensitive to TNF-α-induced nonapoptotic death without a requirement for using CHX (15). Surprisingly, we observed that silencing the expression of TRADD not only failed to protect Jurkat cells but actually enhanced TNF-α-induced death (Fig. 2A). Thus, TRADD is clearly not necessary for TNF-α-induced death.

In contrast, we found that when RIP1 expression was knocked down, FADD-deficient Jurkat cells became completely resistant to TNF-α-induced death (Fig. 2A). Similar results were observed using caspase-8 deficient Jurkat (I9.2) cells (Fig. 2B). These data suggest that in the absence of FADD or caspase-8 function, where apoptosis is blocked,

FIG. 1. TNF-α induces two types of programmed death in human Jurkat leukemia T cells. (A) TNF-α (10 ng/ml) induced death in wild-type (WT), FADD^{-/-}, and RIP1^{-/-} Jurkat cells in the presence of medium (open bars), 20 µM zVAD-FMK (shaded bars), 0.1 µg/ml CHX (hatched bars), or both zVAD-FMK and CHX (filled bars). The percentage of TNF-α-specific death was calculated by the following formula: % death = (1 - live counts of TNF-α-treated cells/live counts of control cells) × 100. The means and standard deviations of the percentages of cell death are presented. Data were derived from two experiments with triplicate samples in each. The significance of the paired samples was examined by *t* tests and denoted as follows for comparison with the respective controls: *, *P* < 0.05; **, *P* < 0.01. (B) Photomicrographs of cell preparations stained with hematoxylin and eosin revealing the morphology of TNF-α-induced death. The upper two rows show light microscopic images of Jurkat 4E3, RIP1^{-/-}, and FADD^{-/-} cells subjected to mock or TNF-α (10 ng/ml) stimulation for 8 h. Green arrowheads indicate cells undergoing apoptosis with typical apoptotic bodies, and red arrowheads indicate cells dying through nonapoptotic death. The lower two panels are TEM images that correlate with the above light microscopic samples.

TNF-α can still efficiently program Jurkat cells to die via a pathway that depends entirely on RIP1 (9, 15). Our data also suggest that the removal of TRADD clearly exacerbates nonapoptotic death through the RIP1 pathway. Thus, TRADD is not required for TNFR1 signaling in the nonapoptotic death program, and its presence may, in fact, antagonize this death pathway.

RIP1 is dispensable for TNF-α-induced cell death. The previous data raised the question of whether RIP1 is indispensable for TNFR-induced death programs. We therefore examined the death-inducing functions of TRADD and RIP1 in wild-

FIG. 2. TRADD mediates apoptosis, whereas RIP1 mediates nonapoptotic death induced by TNF- α . Jurkat cell lines were transfected with 200 pmol of the nonsilencing (NS), TRADD, or RIP1 (RIP) siRNA, as indicated. The cells were subjected to 3 ng/ml of TNF- α stimulation for 16 h, and the percentage of cells undergoing TNF- α -induced specific death was measured by counting viable cells by flow cytometry as described in the legend to Fig. 1. The means and corresponding standard deviations are presented. Data were generated from experiments using the indicated Jurkat cell lines. (A) FADD $^{-/-}$ cells (42.3 line; three experiments); (B) caspase-8 $^{-/-}$ cells (19.2 line; seven experiments); (C) wild-type cells (A3 line; six experiments); (E) RIP1 $^{-/-}$ cells (five experiments). (D) Representative Western blot showing silenced expression of the indicated genes in wild-type Jurkat cells correlating with those in experiment C.

type (A3) Jurkat cells by siRNA silencing and found that decreasing the expression of RIP1 or TRADD alone failed to diminish TNF- α -induced death, indicating that neither was necessary to warrant a death outcome. Again, we observed enhanced TNF- α -induced death by individually silencing RIP1 or TRADD. However, when both RIP1 and TRADD were silenced, TNF- α -induced death was significantly reduced (Fig. 2C and D). An examination of the TRADD and RIP1 proteins showed a strong suppression of their expression. The small amount of residual cell death may be attributable to the rest of the protein that was not knocked down. Hence, RIP1 and TRADD apparently mediate separate pathways of TNF- α -induced death.

Although our results were consistent with a model in which TNF- α mediates two death responses, the data can also be explained by a loss of caspase-dependent apoptosis when TRADD is silenced. However, we found that in RIP1-deficient Jurkat cells,

silencing TRADD protected the cells from TNF- α -induced death. The protection was as efficient as that using the pan-caspase inhibitor zVAD-FMK in RIP1-deficient Jurkat cells (compare Fig. 1A and 2E). The addition of zVAD-FMK to the TRADD siRNA conferred no additional protection against TNF- α . Thus, RIP1 and TRADD can independently mediate separate pathways of TNF- α -induced death. TRADD seems to be essential for the FADD/caspase-8-dependent program of apoptosis, whereas RIP1 is necessary for the caspase-independent, nonapoptotic death program.

RIP1 and TRADD are both essential for TNF- α -induced NF- κ B activation. It was previously shown that both RIP1 and TRADD are involved in TNF- α -induced NF- κ B activation (10, 11, 33), which in turn up-regulates gene expression that counteracts the apoptotic death programs (2, 16, 24). Since our results showed that RIP1 and TRADD mediate separate death pathways, it was interesting to examine the effects of silencing TRADD and RIP1 on NF- κ B activation. In control Jurkat cells, a 30-min TNF- α stimulation caused significant activation and nuclear translocation of NF- κ B in the presence of non-specific oligoribonucleotides. In contrast, in Jurkat cells with RIP1 or TRADD expression silenced, the TNF- α -induced nuclear NF- κ B was greatly diminished (Fig. 3A and B). We also found that the activity of an NF- κ B-driven luciferase reporter gene was greatly decreased when the expression of RIP1, TRADD, or both was knocked down (Fig. 3C). These data suggest that unlike the death pathways where RIP1 and TRADD can operate independently, TNF- α -induced NF- κ B activation requires the cooperative actions of both.

TRADD mediates apoptosis, while RIP1 mediates nonapoptotic death. Our data suggest an interesting model in which TRADD and RIP1 cooperate for NF- κ B induction but compete against each other to promote the apoptotic or nonapoptotic mode of cell death, respectively. To validate this model, we first examined mitochondrial cytochrome *c* release, one of the hallmarks of caspase-dependent apoptosis (22). We detected TNF- α -induced cytochrome *c* release in wild-type Jurkat cells, peaking at 6 h. Silencing TRADD completely eliminated this process (Fig. 4A). In contrast, silencing RIP1 dramatically enhanced cytochrome *c* release, which was detectable in as soon as 2 h, indicating that RIP1 suppresses apoptosis. The elimination of both RIP1 and TRADD largely abrogated cytochrome *c* release, suggesting that TRADD was essential for the increased release caused by knocking down RIP1. We also observed that TNF- α -induced cytochrome *c* release was decreased but not eliminated in FADD $^{-/-}$ cells, caspase-8 $^{-/-}$ cells, and zVAD-FMK-treated wild-type Jurkat cells (Fig. 4B and C). The residual release may have been due to cellular degeneration. Furthermore, we found that blocking caspase activity was able to protect RIP1 $^{-/-}$ or RIP1-silenced cells from TNF- α -induced death but had no protection for TRADD-silenced cells (Fig. 2B). Taken together, these data suggest that TRADD but not RIP1 is required for TNF- α -induced, caspase-dependent apoptosis in Jurkat cells. RIP1 appears to antagonize this effect.

To characterize the biochemistry of the nonapoptotic, necrotic form of TNF- α -induced cell death, we examined the role of ROS. ROS accumulation has been suggested to be one of the key factors by which TNF- α causes cell death (21). We observed a substantial increase in ROS accumulation but no

FIG. 3. Both RIP1 and TRADD are essential for TNF- α -induced NF- κ B activation. (A) Confocal microscopic images of Jurkat cells transfected with nonsilencing (NS), TRADD, and RIP1 siRNAs and stimulated with 10 ng/ml of TNF- α for 30 min. The fixed cells were stained with fluorescein isothiocyanate-anti-NF- κ B (green) and the nuclear dye Hoechst 33342 (red). Nuclear translocation of NF- κ B can be seen in NS siRNA- but not in RIP1 or TRADD siRNA-transfected cells. Decreased expression by the siRNAs was confirmed by Western blotting (see Fig. 2). (B) Percentages of transfected cells undergoing NF- κ B nuclear translocation after 5, 10, and 30 min of TNF- α stimulation. The data represent three independent experiments. (C) Dual-luciferase reporter assays of wild-type Jurkat cells transfected with the indicated siRNAs. All samples except for the last (cotransfected with the pE-selectin and pRL-TK constructs) were cotransfected with the pNF- κ B-luc and pRL-TK plasmids, and the results are shown as means of relative luminescence units (RLU) normalized by comparing TNF- α -stimulated, NF- κ B-driven luciferase activity to TNF- α -stimulated, thymidine kinase-driven *Renilla* luciferase activity. The data represent three independent experiments.

significant difference in wild type and TRADD- or RIP1-silenced Jurkat cells after 6 h of TNF- α stimulation. Nevertheless, double silencing of TRADD and RIP1 seemed to inhibit TNF- α -induced ROS accumulation (Fig. 4D). However, when testing the role of ROS accumulation in causing cell death, we found that BHA, an ROS scavenger, can block as much as 50% of TNF- α -induced death in FADD^{-/-} and caspase-8^{-/-} Jurkat cells. In contrast, BHA did not block TNF- α lethality in RIP1-deficient or RIP1-silenced Jurkat cells, in which death is apoptotic (Fig. 4E and data not shown). Thus, the TNF- α -induced RIP1 pathway can cause death, at least partly, by ROS accumulation and damage, whereas caspase-dependent apoptosis does not require ROS for its execution. We also tested the role of mitogen-activated protein kinase inhibitors and found that they had little effect (data not shown). Taken together, our data indicate that TRADD/FADD/caspase-8 and RIP1/ROS mediate separate biochemical death programs following TNF- α stimulation.

TRADD and RIP1 can independently and competitively associate with TNFR1. We further explored the biochemical implications of the fact that RIP1 and TRADD cooperate in

NF- κ B activation but compete for transducing distinct death pathways. Since RIP1 and TRADD each contain a DD, they can be adaptors for one another in a linear manner or work independent of each other in a parallel fashion. We therefore evaluated the association of each with TNFR1. By silencing the expression of either RIP1 or TRADD, we surprisingly found that this actually improved the recruitment of the other into the TNFR1 signaling complex after TNF- α stimulation (Fig. 5A and B). This suggests that neither RIP1 nor TRADD depends on the other to bind TNFR1; they appear to be competitors rather than coadaptors in TNFR1 signaling. In other studies, we found that the TNFR1 recruitment of FADD/caspase-8 depends on TRADD but not on RIP1 (data not shown). Hence, FADD, although it has a DD itself, may not directly bind to TNFR1. As previously proposed, TRADD is likely to be an essential adaptor for the FADD/caspase-8 apoptosis complex (12). Nevertheless, RIP1-mediated death has no such TRADD requirement. Interestingly, we observed that down-modulating RIP1 expression significantly enhanced FADD association with TNFR1, even before TNF- α stimulation, whereas silencing TRADD abrogated TNFR1 from re-

FIG. 4. Cytochrome *c* release and ROS damage. Western blot assays show TNF- α -induced cytochrome *c* release in the cytosol of wild-type Jurkat cells transfected with 200 pmol of nonsilencing (NS), TRADD, RIP1, or both TRADD and RIP1 siRNAs, as indicated (A), in wild-type (A3), FADD-deficient (I2.1), and caspase-8-deficient (I9.2) Jurkat cells (B), and in zVAD-FMK-treated wild-type Jurkat cells (C). An anti- β -actin blot was used as a loading control. L, whole-cell lysates, which served as a positive control. (D) TNF- α -induced ROS accumulation in Jurkat cells silenced with the indicated siRNAs. Percentages of ROS accumulated (DCFDA⁺) in cells are shown. The data represent three independent experiments. (E) Partial inhibition of TNF- α -induced, RIP1-dependent death in FADD^{-/-} but not in RIP1^{-/-} Jurkat cells by BHA at the indicated doses. Percentages of cell loss are shown, and the data represent two independent experiments.

cruciating FADD (Fig. 5D). These data indicate that FADD requires TRADD as an essential adaptor for binding TNFR1. Another interesting observation was that upon immunoprecipitation (IP) of TNFR1, neither TRADD nor RIP1 was constitutively associated with the receptor. Following TNF- α stimulation, both TRADD and RIP1 were recruited to the receptor, and each exhibited higher-molecular-weight receptor-associated forms, likely representing ubiquitinated species for RIP1 and an unidentified modification for TRADD (Fig. 5A, B, and E and data not shown). This suggests that modification of RIP1 and TRADD within the TNFR1 signaling complex may be related to signal regulation. Nevertheless, these results agree with our observation shown in Fig. 2 that knocking down the expression of either RIP1 or TRADD can actually enhance TNF- α -induced death. However, this occurs by two different molecular mechanisms that involve two distinct mediators in separate death pathways.

TRAF2 is recruited to TNFR1 through TRADD. TRAF2 is a key component of TNFR signaling for NF- κ B and JNK activation, and it modulates sensitivity to programmed cell death (5, 20, 34). TRAF2 associates with the intermediate domain of RIP1 and the TRAF2 association domain of TRADD (12, 23). Therefore, the TNF- α -induced recruitment of TRAF2 to the

TNFR signaling complex might depend on both proteins. Surprisingly, as seen in Fig. 5C, we found that TRAF2 association with TNFR1 was dependent on TRADD but not RIP1. Therefore, despite the prominent role of RIP1, the previously reported association between RIP1 and TRAF2 may be indirect (23). However, TRAF2 might integrate signals for TNFR1.

DISCUSSION

It was previously proposed that more than one signaling pathway can mediate TNFR1-induced PCD (7, 9). Our data strongly support this model, and we further verified that TNF- α can cause death by both an apoptotic and a necrotic mechanism. The necrotic, but not apoptotic, pathway can be blocked partly by BHA and therefore is attributable to a certain extent to ROS. We examined the roles of proximal molecules involved in TNFR1 signaling and observed an interesting interplay that reveals how the modes of death are selected. We found that for TNFR1-induced apoptosis, which depends on caspase-8 activation, both FADD and TRADD are essential, but RIP1 is dispensable (Fig. 1 and 2). In contrast, RIP1 and its kinase activity seem to be required for caspase-independent necrotic death (Fig. 1 and 2 and data not shown) (3,

FIG. 5. TNF- α -initiated associations of RIP1, TRADD, TRAF2, and FADD with TNFR1. Jurkat cells were silenced for RIP1 and TRADD expression by siRNA transfection, as indicated. The cells were stimulated with 100 ng/ml TNF- α for 10 min, and the lysates were immunoprecipitated (IP) with anti-TNFR1. The electrophoretically separated IP products and lysates were probed with (A) anti-RIP1, (B) anti-TRADD, and (C) anti-TRAF2 sequentially without stripping in between. NS, nonspecific bands. The multiple arrows point to either ubiquitinated RIP1 or posttranslationally modified TRADD. The data represent five independent experiments. (D) IP with TNFR1 and probing for FADD under the indicated conditions. (E) Blot of anti-ubiquitin reactivities. Anti-ubiquitin reactivity correlated with RIP1 size in an immunoprecipitation assay with the TNFR1-associated complex before and after TNF stimulation (top) as well as with total cellular lysates (bottom) from the same cells. Ubiquitinated species are shown.

9). Thus, two distinctive PCD signals can be initiated through TNFR1. In contrast to previous assumptions, our data show that TRADD is required only for the apoptosis pathway. In support of this model, we first showed that RIP1 can associate with TNFR1 even in the absence of TRADD or FADD. Secondly, our data suggest that TRADD and RIP1 actually compete for binding with TNFR1, since silencing RIP1 substantially enhanced the TNFR1-TRADD association and vice versa (Fig. 5). This competition appears to be functionally important, since apoptotic death is increased when RIP1 is removed and necrotic death is increased when FADD or TRADD is removed (Fig. 1 and 2). Hence, it is clear that there are distinct sets of proximal molecules involved in two separate pathways of TNFR1-induced PCD that actually counter-regulate each other (Fig. 6).

It is interesting that silencing the expression of TRADD or RIP1 alone can sensitize Jurkat cells to TNF- α -induced death. Thus, cross talk between RIP1 and TRADD might initiate survival signals through NF- κ B which, in turn, down-regulate the magnitudes of death signals from each other. It is possible that RIP1 can directly bind to TNFR1 to deliver nonapoptotic signals but also interact with TRADD to coordinate NF- κ B activation. Missing this negative feedback control may cause unbalanced TNF- α signaling that leads to cell death. Further-

more, since we found that decreasing the expression of RIP1 or TRADD can abrogate TNF- α -induced NF- κ B activation (Fig. 3), a lack of either of the two DD-containing proteins seems sufficient to skew the equilibrium of the TNFR signals from survival toward death. Thus, an equilibrium between the proximal molecules in TNFR signaling is required in order for cells to live; blocking the functions of either one of these early signaling molecules can augment cell death.

It is well known that protein ubiquitination can lead to protein degradation, and under certain circumstances, it can also mediate cellular signaling (8). Interestingly, we found that in TNF- α -stimulated Jurkat T cells, RIP1 was ubiquitinated (Fig. 5E). TRADD was also modified (Fig. 5B), although we did not find evidence for ubiquitination. This modification took place mainly within the TNFR1 signaling complex, as we observed more dramatic increases in the modified RIP1 and TRADD ladders in TNFR1 coimmunoprecipitates than in corresponding cell lysates (Fig. 5). Thus, modification of these proteins is apparently a consequence of signaling; however, its mechanisms need to be further studied.

It is clear that stimulation of TNFR1 can induce multiple signals, including apoptotic, nonapoptotic, and anti-PCD pathways. However, the molecular details that underlie these pathways are still unclear and even confusing to some extent.

FIG. 6. Model of TNFR1 proximal signaling pathways. TRADD and RIP1 compete with each other for binding to TNFR1 through DD interactions and mediate separate events for programmed cell death. Recruitment of TRADD/FADD/caspase-8/caspase-10 triggers the execution of apoptosis, whereas the receptor association of RIP1 initiates a serine/threonine kinase-dependent necrosis-like death program that involves ROS accumulation. In contrast, TRADD and RIP1 seem to cooperate for NF- κ B activation, which delivers survival signals that potentially counteract either apoptosis or necrosis-like programmed cell death. Depicted are the intermediate domain (ID) and the kinase domain (KD) of RIP1.

Our data show that the most proximal molecules involved in TNFR1 signaling have powerful nonredundant roles in determining the final readout. The elucidation of the requirement of TRADD, FADD, caspase-8, RIP1, and TRAF2 for TNFR1-induced apoptosis, nonapoptotic PCD, and NF- κ B activation provides a stage for further studies on the physiological regulation of each pathway. We have found that some viruses produce caspase inhibitory proteins but, nevertheless, fail to protect the infected cells from TNF- α -induced death (3). This implies that the existence of alternative PCD pathways benefits the host in confronting different viral pathogens.

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