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TNFR80-Dependent Enhancement of TNFR60-Induced Cell Death Is Mediated by TNFR-Associated Factor 2 and Is Specific for TNFR60

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Costimulation of TNFR80 can strongly enhance TNFR60-induced cell death. In this study, we show that this enhancement is TNFR60 selective, as neither TNF-related apoptosis-inducing ligand/Apo2 ligand-, Apo1/Fas-, ceramide-, nor daunorubicin-mediated cell death was affected by costimulation of TNFR80. We further demonstrate that TNFR-associated factor 2 (TRAF2) is critically involved in both negative and positive regulation of TNF-induced cell death. Overexpression of TRAF2 and of a TRAF2 mutant, deficient in nuclear factor-κB activation, selectively desensitized and enhanced, respectively, TNFR60-induced cell death in HeLa cells. However, upon costimulation of TNFR80, which mediates activation of nuclear factor-κB and the c-Jun amino-terminal kinase via TRAF2, TNF-induced cell death is drastically enhanced in parental and TRAF2-transfected, but not in TRAF2 (87–501)-transfected cells. These data point to a critical role of TRAF2 in the apoptotic TNFR cross talk, whereby the TNFR80-dependent enhancement of TNFR60-induced cell death is due to TNFR80-mediated negative regulation of TRAF2 function(s). An interference with TRAF2 function was confirmed independently by analysis of c-Jun amino-terminal kinase activation via TNFR60 upon prestimulation of TNFR80. We propose that the apoptotic TNFR cross talk is based on TNFR80-mediated abrogation of antiapoptotic TRAF2-dependent signaling pathways initiated by TNFR60, but not Apo1/Fas or the apoptotic TNF-related apoptosis-inducing ligand receptors. The Journal of Immunology, 1998, 161: 3136–3142.

Umor necrosis factor is a pleiotropic cytokine that is mainly produced by activated macrophages and lymphocytes. TNF initiates inflammatory, immune regulatory, and pathophysiologic responses by binding to two distinct cell surface receptors of 60 kDa (TNFR60) and 80 kDa (TNFR80) (reviewed in Ref. 1). The TNFR are the name-giving members of a receptor superfamily whose members have in common three to six extracellular copies of a canonical motif of cysteine-rich pseudo-repeats, each comprising six conserved cysteines in a stretch of about 40 amino acids. Further members of the TNFR superfamily are CD40, CD30, CD27, Apo1/Fas, OX40, 4-1BB, nerve growth factor receptor, LT-βR, and several viral gene products (reviewed in Refs. 2 and 3). The ligands of these receptors also form a complementary family of molecules, most of which are expressed primarily as biologically active type II membrane proteins, from which soluble forms are produced by proteolytic cleavage or alternative splicing (reviewed in Refs. 2 and 3).

Until recently, the molecular mechanisms of intracellular signal initiation by binding of TNF and TNF-related cytokines to their cognate receptors remained undefined. In fact, none of the TNFR superfamily members possesses sequences implying any known catalytic activity. However, during the last 4 yr, a rapidly growing number of proteins has been identified that directly or indirectly associate with the cytoplasmic domains of the TNFR superfamily members. Most of these associated factors belong to two groups of signal-transduction molecules, the so-called death domain proteins and the TNFR-associated factor (TRAF) family. Both groups are defined by distinct sequence motifs that are involved in further protein/protein interactions (reviewed in Ref. 4). The death domain has been defined originally as a part of the intracellular regions of TNFR60 and Apo1/Fas, and is responsible for the generation of the apoptotic signal by these receptors (5, 6). TRADD (TNFR1-associated death domain protein; 7), FADD/MORT1 (Fas-associated protein with death domain; 7), FADD/MORT1 (Fas-associated protein with death domain/mediator of receptor-induced toxicity; 8–10), and RIP (receptor-interacting protein; 11) are death domain-containing proteins that have been identified by yeast two-hybrid screens using the intracellular domain of TNFR60 or Apo1/Fas as baits. Overexpression of these molecules leads to activation of NF-κB and/or induction of apoptosis (7–11). FADD/MORT1 is part of the inducible Apo1/Fas death-inducing signaling complex (12), and is believed to represent the physical link to proapoptotic proteases of the caspase family (13, 14).

There are several lines of evidence that two TNFR60-signaling cascades bifurcate at TRADD, one leading to the activation of NF-κB and the other coupling via FADD/MORT1 to the apoptotic caspase cascade (15, 16). The serine threonine kinase RIP is recruited to the TNFR60-signaling complex in a ligand-dependent manner, and might also be involved in the activation of NF-κB as

4 Abbreviations used in this paper: TRAF, TNF receptor-associated factor; Apo2L, Apo2 ligand; FADD, Fas-associated protein with death domain; IAP, inhibitors of apoptosis; JNK, c-Jun amino-terminal kinase; MORT, mediator of receptor-induced toxicity; NODD, nuclear factor-κB-dependent cell death protective factor; RIP, receptor-interacting protein; TRADD, TNF receptor 1-associated death domain protein; TRAIL, TNF-related apoptosis-inducing ligand.
well as induction of cell death (17), although the position of RIP within the signaling cascades is yet rather unclear (18).

The TRAF protein family currently comprises six members that interact with molecules of the TNFR superfamily or with the IL-1R. Rothe et al. (19) have isolated the first two members of the TRAF protein family based on their interaction with the cytoplasmic domain of TNFR80. TRAF3, also designated as CD40bp, CAP-1, LAP1, or CRAF1, was identified by its association with CD40 (20–23) and TRAF5 by binding to the LT-βR (24). TRAF4, also termed CART1, was isolated by differential screening of libraries of malignant and nonmalignant breast tissues (25) and TRAF6 by homology screening of an expressed sequence tag library (26). Overexpression studies and analysis of various deletion mutants of members of the TNFR superfamily suggested that TRAF2, 5, and 6 are critically involved in the activation of NF-κB by CD40 (27, 28), CD30 (29), LT-βR (24), TNFR80 (27), and TNFR60 (15), as well as the membrane receptor for IL-1 (26). The C-terminal TRAF domain of TRAF proteins comprises about 230 amino acid residues, and can be divided in the N- and C-TRAF domain. For most of the TRAF molecules, the capability to interact with several members of the TNFR superfamily and other TRAFs has been established (reviewed in Ref. 4). Moreover, some of the TRAF molecules bind to cytoplasmic proteins that are capable or suspected to modulate TNF-initiated apoptosis or TNF-dependent activation of NF-κB. These include the inhibitors of apoptosis (IAP; 30), the antiapoptotic molecule A20 (31), and the molecule TANK (TNF activator of NF-κB)/I-TRAF (TRAF-interacting protein) (32, 33), as well as the TRAF-interacting protein TRIP (34), respectively. In particular, Hsu et al. (15) have shown that interaction of the death domain protein TRADD with TRAF2 is critically involved in TNFR60-dependent activation of NF-κB.

The common usage of the above-described signal-transduction molecules by various members of the TNFR superfamily opens up the possibility of a complex pattern of receptor cross talk. In fact, we and others have recently described synergistic action of TNFR80, CD40, and TNFR60 in the induction of cell death (35–37). In this study, we demonstrate that TRAF2 is involved in the enhancement of TNFR60-mediated cell death by TNFR80. This apoptotic cross talk occurs upstream of FADD/MORT1 in the TNFR60-signaling pathway or at a pathway selectively used by TNFR60 because no interference with the apoptotic signaling induced by the cytokines Apo1/Fas and TRAIL/Apo2 ligand or with ceramide- and UV-induced cell death was observed.

Materials and Methods

Abs and reagents

The TNFR80-specific agonistic mAb MR2-1 was kindly provided by W. Buurman (University of Limburg, Maastricht, The Netherlands). FITC-labeled goat anti-mouse IgG plus IgM Ab was obtained from Dianova (Hamburg, Germany). All other reagents were obtained from Sigma (Deisenhofen, Germany), if not otherwise stated. A 1:1 mixture of purified recombinant Flag-tagged human TRAIL (10 μg/ml) and anti-Flag M2 Ab (20 μg/ml; Kodak International Biotechnologies, New Haven, CT) was incubated at room temperature for 10 min and diluted to the final concentrations indicated in the figure legends.

Cells

HeLa cells and transfectants derived thereof were grown at 37°C in a humidified 5% CO2 incubator in Click RPMI 1640 medium supplemented with 5% heat-inactivated FCS, 2 mM l-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Additionally, transfectants were permanently cultured in the presence of the appropriate selection drug.

DNA transfection

Cells were transfected by a standard electroporation procedure. In brief, 5 × 106 cells were incubated in HEBS buffer (135 mM NaCl, 5 mM KCl, 0.75 mM Na2HPO4, 5 mM dextrose, and 20 mM HEPES, pH 7.05) with 10 to 20 μg of vector DNA and, when necessary, 1 μg of a selection plasmid carrying the Escherichia coli neomycin resistance gene (pMAMneo; Clontech, Palo Alto, CA) or the Zeocin resistance gene (pZeosV; Invitrogen, San Diego, CA). Electroporation was performed by a prepulse of 320 V, 3 μF capacitance, and 800 Ω; incubation for 5 min on ice; and a final pulse of 220 V, 960 μF, and 400 Ω (Gene Pulsar; Bio-Rad, München, Germany). Transfected cells were selected in culture medium containing 600 μg/ml G418 (Life Technologies, Eggenheim, Germany), 500 μg/ml Zeocin (Invitrogen, San Diego, CA), 200 μg/ml hygromycin B (Boehringer Mannheim, Mannheim, Germany), or a combination thereof. After 2 to 3 wk of selection, individual drug-resistant colonies were isolated. Transfectants of receptor constructs were pooled and enriched for high expressors by cell sorting using a FACStar™ Plus (Becton Dickinson, San Jose, CA). Transfectants overexpressing intracellular proteins were individually expanded and characterized.

Cytotoxicity assay

HeLa cells and transfectants derived thereof were plated at a density of 1.5 × 103 cells/well in triplicates in 96-well microtiter plates in 100 μl of Click RPMI 1640 overnight at 37°C. On the next day, the reagents of interest were added in the presence of 0.5 μg/ml cycloheximide. The plates were incubated for additional 12- to 24-h culture, and cell viability was determined using crystal violet staining. Briefly, supernatants were discarded and the cells were washed once with PBS, followed by crystal violet staining (20% methanol, 0.5% crystal violet) for 15 min. The wells were washed with H2O and air dried. Residual dye was diluted with methanol for 15 min, and OD at 550 nm was measured with a SpectraMax ELISA plate reader (Dynatech, Gymersey, U.K.).

IL-6 assay

Cells were plated at a density of 1.5 × 104 cells/well in triplicates in 96-well microtiter plates in 100 μl of Click RPMI 1640 overnight at 37°C. On the next day, the cells were treated with the reagents of interest for additional 12 to 24 h. Finally, supernatants were removed and cleared by centrifugation at 15,000 rpm for 10 min, and IL-6 concentration was determined using a commercially available ELISA kit, according to the manufacturer’s recommendations (PharMingen, Hamburg, Germany).

Transfections, luciferase assays, and protein kinase assay

HeLa cells (0.8 × 107) were seeded in 24-well tissue culture plates. On the following day, the cells were transfected using the SuperFect reagent (Qiagen, Hilden, Germany) with a 3 × NF-κB luciferase reporter plasmid and a SV40 promoter-driven β-galactosidase expression plasmid to normalize the transfection efficiency. After additional 24 h, the cells were harvested in PBS, lysed in luciferase lysis buffer (Promega, Mannheim, Germany), and assayed for luciferase and β-galactosidase activities using a LUMAT 9501 Luminometer (Berthold, Bad Wildbad, Germany). JNK activity was measured by immunocomplex kinase assay with glutathione-S-transferase c-jun (1–79) as substrate, as described elsewhere (38).

Results

Stimulation of TNFR80 enhances TNFR60-induced cell death, but not TRAIL/Apo2L-, Apo1/Fas-, and ceramide/daunorubicin-mediated cell death

We have demonstrated recently a synergistic enhancement of TNF/TNFR60 signaling by costimulation of TNFR80 by the membrane form of TNF- or TNFR80-specific agonistic Abs (35, 37). To obtain insight into the molecular mechanisms of this TNFR cooperation, we asked whether similar cooperative mechanisms exist between TNFR80 and other death-inducing members of the TNFR family. Whereas costimulation of TNFR80 in HeLa cells transfected with TNFR80 (HeLa-TNFR80) resulted in a strong enhancement of TNF-induced cell death (Fig. 1A; Ref. 37), no enhancement of the cytotoxic effects induced by TRAIL/Apo2 ligand (TRAIL/Apo2L), a member of the TNF ligand family (39, 40), or stimulation of the Apo1/Fas Ag could be observed (Fig. 1B and C). This is remarkable because it has been shown that TNF, TRAIL/Apo2L, and stimulation of Apo1/Fas can induce cell death using similar pathways, including the molecule FADD and different members of the caspase family (41, 42). Accordingly, in our cell systems, cytotoxicity by the aforementioned stimuli could be inhibited completely by the caspase inhibitor peptide z-VAD-fmk.
The viable cells were quantified by staining with crystal violet. In Figure 1D, the open circles show the effect of dihydroceramide as a control.

Metabolites of the sphingomyelin pathways have been implicated in the induction of apoptosis, and several studies have presented evidence that the second messenger ceramide is also involved in signaling the cytotoxic effects of TNFR60 and Apo1/Fas (43–46). Therefore, we analyzed whether TNFR80 stimulation would modulate apoptosis induced by exogenously added ceramide. As shown in Figure 1E, ceramide-induced cell death was not enhanced by costimulation of TNFR80. Likewise, the cytotoxic effects of daunorubicin, a drug that has been suggested to induce apoptosis by elevating the intracellular ceramide level (47, 48), were not modulated by TNFR80 stimulation (Fig. 1F). Moreover, neither ceramide- nor daunorubicin-induced cell death was affected by the addition of z-VAD-fmk (Fig. 1, E and F), suggesting that ceramide acts downstream of the caspase cascade or on a parallel pathway.

**TRAF2-mediated protection from TNFR60-induced cell death is antagonized in the context of TNFR80 coactivation**

In a recent study, we have shown that the TRAF2 binding domain of TNFR80 is essential for the TNFR80-mediated enhancement of TNFR60-induced cytotoxicity (37). In fact, overexpression of wild-type TRAF2 in HeLa-TNFR80 cells resulted in a reduced sensitivity to the cytotoxic effects of TNF (Fig. 2A). Moreover, introduction of a deletion mutant of TRAF2 (TRAF2 (87–501)) that is still capable of associating with TNFR, but is deficient in the activation of NF-κB and JNK due to the lack of the RING finger domain (27), resulted in a dramatic enhancement of TNFR60-mediated cytotoxicity (Fig. 2A), with no change in the sensitivity toward TRAIL/Apo2L-, UV-, ceramide-, and daunorubicin-induced cell death (data not shown). Hence, upon stimulation of TNFR60, both apoptotic and antiapoptotic pathways are activated simultaneously, with TRAF2 being critically involved in the transduction of protective signals. To evaluate the potential role of TRAF2 in the TNFR80-mediated enhancement of TNFR60-induced apoptosis, the double transfectants HeLa-TNFR80-TRAF2 and HeLa-TNFR80-TRAF2 (87–501) were investigated under conditions of specific coactivation of both TNFRs. Interestingly, HeLa-TNFR80-TRAF2 double transfectants, although less sensitive toward TNFR60, Apo1/Fas, and the receptors for TRAIL/Apo2L.

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(Fig. 1D). Together, these data indicate that the TNFR80-dependent enhancement of TNFR60-induced cytotoxicity modulates a signaling pathway selectively used by TNFR60 or that the synergistic action of TNFR80 occurs upstream of FADD/MORT1 and the apoptotic caspases engaged by TNFR60, Apo1/Fas, and the receptors for TRAIL/Apo2L.**
sensitivity of TNF in wild-type cells after enhancement by costimulation of TNFR80. Therefore, it appears that the TRAF2-dependent protective response induced concomitantly with the apoptotic pathway is overruled in the context of TNFR80 stimulation. It is important to note that TNF-induced apoptosis only occurs in the presence of the protein synthesis inhibitor cycloheximide in all of our HeLa transfectants, a fact that, at the first view, is in conflict with NF-κB-dependent induction of antiapoptotic proteins. However, in our experiments, we used only 2.5 μg/ml cycloheximide, a concentration at which protein synthesis is only reduced, but not prevented. We have confirmed this by determination of IL-6 production and synthesis of a NF-κB-dependent reporter protein, which were reduced under these conditions only for 5–40% and 10%, respectively (Fig. 3).

Stimulation of TNFR80 abrogates TNF- but not IL-1-induced activation of the amino-terminal c-Jun kinase

A recent study has shown that transient cotransfection of TNFR80 and TRAF2 results in a rapid depletion of TRAF2 (49). It is tempting to speculate that TNFR80-induced degradation/inactivation of TRAF2 and possibly other associated proteins contributes to the apoptotic TNFR complex investigated in our study. To test this hypothesis, we analyzed the effect of TRAF2 costimulation on TNF-mediated JNK activation since the TNF-induced JNK activation is indicative for TRAF2 action (50, 51). As shown in Figure 4, prestimulation of TNFR80 strongly interferes with JNK activation by TNF. TNF-induced JNK activation was reduced by approximately 50% after pretreatment of TNFR80 for 0.5 h, and was almost completely abrogated after 6-h pretreatment of TNFR80. In contrast, IL-1-induced activation of JNK, which is independent of TRAF2, remained largely unaffected upon TNFR80 pretreatment (Fig. 4).

Discussion

TNFR80-dependent enhancement of TNFR60-mediated cell death occurs upstream of FADD/MORT1 or on a separate TNFR60-induced pathway

As shown in Figure 1, TNFR80 triggering enhances TNFR60-mediated, but neither Apo1/Fas-mediated nor TRAIL/Apo2L-induced cell death. FADD/MORT1 is a common component of the apoptotic pathways utilized by TNFR60 and Apo1/Fas (15, 16, 52, 53). Hence, it is most likely that the TNFR60-TNFR80 cross talk occurs upstream of FADD/MORT1 or on a separate TNFR60-induced pathway, being distinct from, but coinitiated with the FADD/MORT1-dependent apoptotic pathway. The existence of mechanisms able to selectively modulate TNFR60- or Apo1/Fas-induced cell death at a point at which both pathways have not merged, is also indicated by the fact that a number of cellular systems have been described in which TNF-sensitive cells are Apo1/Fas resistant and vice versa (54–57). One possible branching point is the cytoplasmic domain of TNFR60. It has been shown recently that a nine-amino-acid binding motif outside the death
domain is required to couple the TNFR60 to neutral sphingomyelinase (58, 59), whereas acidic sphingomyelinase, NF-κB activation, and induction of apoptosis have been assigned to the death domain of the receptor (6, 60). Hsu et al. (15) have described an additional bifurcation point downstream of the death domain of TNFR60, namely the TNFR60-associated death domain protein TRADD. This molecule directly associates in a ligand-dependent manner with TNFR60 (15) and is critically involved in both NF-κB and JNK activation as well as induction of apoptosis (7, 61). In particular, amino acid residues 1–169 of TRADD are sufficient to associate with TRAF2, which is involved in NF-κB activation by TNFR60, TNFR80, and CD40 (27, 15). The N-terminal death domain of TRADD (amino acid residues 195–312) links to FADD/MORT1, which transduces the apoptotic signal to the caspase cascade (13, 14).

**TRAF2 is involved both in positive and negative regulation of TNF-induced apoptosis**

In the HeLa-TNFR80 transfectants used in this study, no further TNFR80-dependent enhancement of TNFR60-mediated cytotoxicity can be detected in cells, in which a negative regulator of NF-κB activation (TRAF2 (87–501)) is expressed. These transfectants already display a high susceptibility toward TNF in the absence of TNFR80 stimulation (Fig. 2). Furthermore, we have shown recently that TNFR cross talk is dependent on the TRAF2 binding site of TNFR80 (37). These data suggest that TNFR80 exerts its proapoptotic capability by a TRAF2-dependent interaction with a NF-κB-dependent gene product. The interference of a NF-κB-dependent gene product with TNF-induced cytotoxicity is in good accordance with recent results from several groups, demonstrating that inhibition of NF-κB activation increases the sensitivity toward TNF-induced apoptosis (61–65). The fact that overexpression of TRAF2 (87–501) resulted in a dramatic enhancement of TNFR60-mediated cytotoxicity (Fig. 2A), thus resembling the TNFR80-mediated enhancement of TNF-induced cytotoxicity in cells overexpressing TRAF2 or only endogenous TRAF2 (Fig. 2B), can be explained by the following hypothesis (Fig. 5).

The central regulator of TNF-mediated cell death is the TRAF2 molecule, which can interfere with TNFR60-induced cytotoxicity directly at two levels: 1) induction of protective proteins via NF-κB and JNK activation, and 2) recruitment of these proteins to the TNFR60 death-signaling complex. In cells coexpressing TNFR80, however, appropriate stimulation of this receptor results in binding and degradation of TRAF2 and TRAF2-associated proteins, thereby reducing the availability of protective factors at the TNFR60 death-signaling complex. This promotes the apoptotic TNF response (Fig. 5). According to this model, in the cellular system studied in this investigation, exclusive stimulation of TNFR60 in HeLa-TNFR80 cells concomitantly induces pro- and antiapoptotic pathways. As overexpression of TRAF2 (87–501) drastically enhances TNF sensitivity, but not responsiveness to anti-Apo1 or TRAIL/Apo2L, an NF-κB-dependent cell death protective factor (NDPF) that is selective for TNF must be postulated (Fig. 5A). In addition, the efficient activation of the death proteases is counteracted by constitutively expressed, cytoplasmic-sensitive negative regulator(s), for example a cellular homologue of the CrmA gene product. This is evident from the fact that induction of cell death by TNF, anti-Apo1, and TRAIL/Apo2L in HeLa cells can occur only when protein translation is reduced. Putative candidates for this/these protective protein(s) are the IAP molecules (30), TRAF-interacting protein (TRIP) (34), TRAF1 (19), cellular FLICE-inhibitory protein/casper (66, 67), and A20 (31), because these molecules are able to form complexes with TRAF2. In fact, Speiser et al. (68) have shown recently in transgenic mice that overexpression of TRAF1 inhibits Ag-induced apoptosis by CD8+ T lymphocytes, a process in which TNF is critically involved (69, 70). Moreover, Chu et al. (71) have shown that c-IAP2 counteracts TNF-induced cell death. According to the model proposed in this work, a rather balanced cellular response with intermediate sensitivity to cell death induction will be shifted toward a vigorous apoptotic response upon costimulation of TNFR80 due to TNFR80-dependent inactivation of TRAF2: 1) TRAF2 will be recruited and/or degraded by this

**FIGURE 3.** Effect of cycloheximide on synthesis of a reporter gene product (luciferase) (A) and IL-6 production (B). A, HeLa cells were transfected with 3× NF-κB-luciferase reporter. One day later, some of the cultures were stimulated for 6 h with the indicated concentrations of TNF in the presence (filled bars) or absence (open bars) of 2.5 μg/ml cycloheximide. Then luciferase activity was determined and normalized against a cotransfected β-galactosidase standard. B, For determination of constitutive and cytokine-induced IL-6 production, cells were cultured in 96-well microtiter plates overnight at 37°C in triplicate. On the next day, cells were treated with 10 ng/ml TNF for 16 h in the presence (filled bars) or absence (open bars) of 2.5 μg/ml cycloheximide. IL-6 concentrations in supernatants were determined by an ELISA.

**FIGURE 4.** Effect of TNFR80 prestimulation on TNF- and IL-1-mediated JNK activation. HeLa-TNFR80 cells prestimulated for the indicated times with MR2-1 (1 μg/ml) in the presence of 2.5 μg/ml cycloheximide. Subsequently, cells were treated for 20 min with TNF (10 ng/ml) or IL-1β (3 ng/ml). Finally, JNK activity was measured by immunocomplex kinase assay with glutathione-S-transferase c-Jun (1–79) as a substrate.
receptor, thus reducing the production of NDPFs (e.g., TRAF1, A20, and c-IAP2); 2) NDPFs bound to TRAF2 may also be degraded; 3) still existing NDPFs cannot be recruited sufficiently to their relevant site of action, the apoptotic TNFR60-signaling complex, because of lack of the essential adapter, the TRAF2 molecule.

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