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Tumor Necrosis Factor-induced Nonapoptotic Cell Death Requires Receptor-interacting Protein-mediated Cellular Reactive Oxygen Species Accumulation*

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The mechanism of tumor necrosis factor (TNF)-induced nonapoptotic cell death is largely unknown, although the mechanism of TNF-induced apoptosis has been studied extensively. In wild-type mouse embryonic fibroblast cells under a caspase-inhibited condition, TNF effectively induced cell death that morphologically resembled necrosis. In this study, we utilized gene knockout mouse embryonic fibroblasts cells and found that tumor necrosis factor receptor (TNFR) I mediates TNF-induced necrotic cell death, and that RIP, FADD, and TRAF2 are critical components of the signaling cascade of this TNF-induced necrotic cell death. Inhibitors of NF-κB facilitated TNF-induced necrotic cell death, suggesting that NF-κB suppresses the necrotic cell death pathway. JNK, p38, and ERK activation seem not to be required for this type of cell death because mitogen-activated protein kinase inhibitors did not significantly affect TNF-induced necrotic cell death. In agreement with the previous reports that the reactive oxygen species (ROS) may play an important role in this type of cell death, the ROS scavenger butylated hydroxyanisole efficiently blocked TNF-induced necrotic cell death. Interestingly, during TNF-induced necrotic cell death, the cellular ROS level was significantly elevated in wild type, but not in RIP$^{-/-}$, TRAF2$^{-/-}$, and FADD$^{-/-}$ cells. These results suggest that RIP, TRAF2, and FADD are crucial in mediating ROS accumulation in TNF-induced necrotic cell death.

Tumor necrosis factor (TNF)[1], a proinflammatory cytokine, plays an important role in diverse cellular events such as cell proliferation, differentiation, and death (1–4). TNF elicits its biological effects in responding cells by binding to the receptors, TNFRI and TNFRII. Both TNF receptors belong to the TNF/nerve growth factor receptor superfamily, which is characterized by multiple cysteine-rich domains in their extracellular regions (1–3). TNFRI, but not TNFRII, is a death receptor (1–4). Although many of the TNF-mediated processes can be regulated by either one of its receptors, TNFRI and TNFRII, apoptosis is mainly induced through TNFRI (1–4).

Tremendous progress has been made recently in our understanding of the mechanisms of the distinct pathways of TNF receptor signaling. The binding of TNF to TNFRI leads to the trimerization of TNFRI followed by recruitment of TNFRI-associated death domain protein (TRADD) (2–4). TRADD serves as a platform to recruit other adapter proteins, FAS-associated death domain protein (FADD), TNFR-associated factor 2 (TRAF2), and receptor-interacting protein (RIP, the death domain kinase) to build a receptor complex, which may be modified and released from the membrane to form different sub-complexes during the signaling process (1, 3–5). Several distinct TNFRI signaling pathways have been identified, namely, NF-κB, JNK, p38, and ERK activation and cell death, which are elaborately regulated and mutually interfering (2, 6).

RIP and TRAF2 are two key factors in TNF-induced NF-κB activation (7–9). Upon TNF ligation, TNFRI receptor complex is formed, and the IKK complex is then recruited through TRAF2 and activated (10). Although the mechanism of Iκβ kinase activation is not fully understood, studies have shown that it is mediated by RIP and may involve MEK kinase (MEKK)3 (10, 11). Activated Iκβ kinase then phosphorylates IκBs, the inhibitory proteins of NF-κB that mask NF-κB nuclear translocation signal to sequester NF-κB in the cytoplasm (6, 12). Subsequently, IκBs are rapidly polyubiquitinated and degraded, and the released NF-κB is translocated into the nucleus to activate its target genes (6, 12). It has been shown that several target genes of NF-κB, such as *cIAP-1*, *cIAP-2*, and *IEX-1L*, have anti-apoptotic properties (13, 14). RIP and TRAF2 are also crucial in JNK, p38, and ERK activation (15, 16). The cascade of these MAP kinase activation pathways is not well understood in detail, although MEKK1 and ASK1 are suggested to play a role (17, 18). The role of JNK in TNF-induced cell death is still controversial: it may be pro- or anti-apoptotic, necrotic, or irrelevant (19–23).

TNF induces both apoptotic and necrotic cell death, depending upon cell types and treatment condition (24, 25). FADD is essential for TNF-induced apoptosis as it recruits caspase-8 (26, 27). Caspase-8 is then activated, thereby initiating a caspase cascade, which results in apoptosis (28, 29). BID, a member of the Bcl2 family and a target of caspase-8, may play

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[1] The abbreviations used are: TNF, tumor necrosis factor; TNFRI/II, tumor necrosis factor receptor I/II; TRADD, TNFRI-associated death domain protein; TRAF2, TNFR-associated factor 2; FADD, FAS-associated death domain protein; RIP, receptor-interacting protein; MEKK, MEK kinase; JNK, Jun C-terminal kinase; ERK, extracellular signal-regulated kinase; NF-κB, nuclear factor κB; Z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; MEF, mouse embryonic fibroblasts; CM-H2 DCFDA, 5(6)-chloromethyl-2′,7′-dichlorodihydrofluorescence diacetate; BHA, butylated hydroxyanisole; ECL, enhanced chemiluminescence; LDH, lactate dehydrogenase; CHX, cycloheximide.

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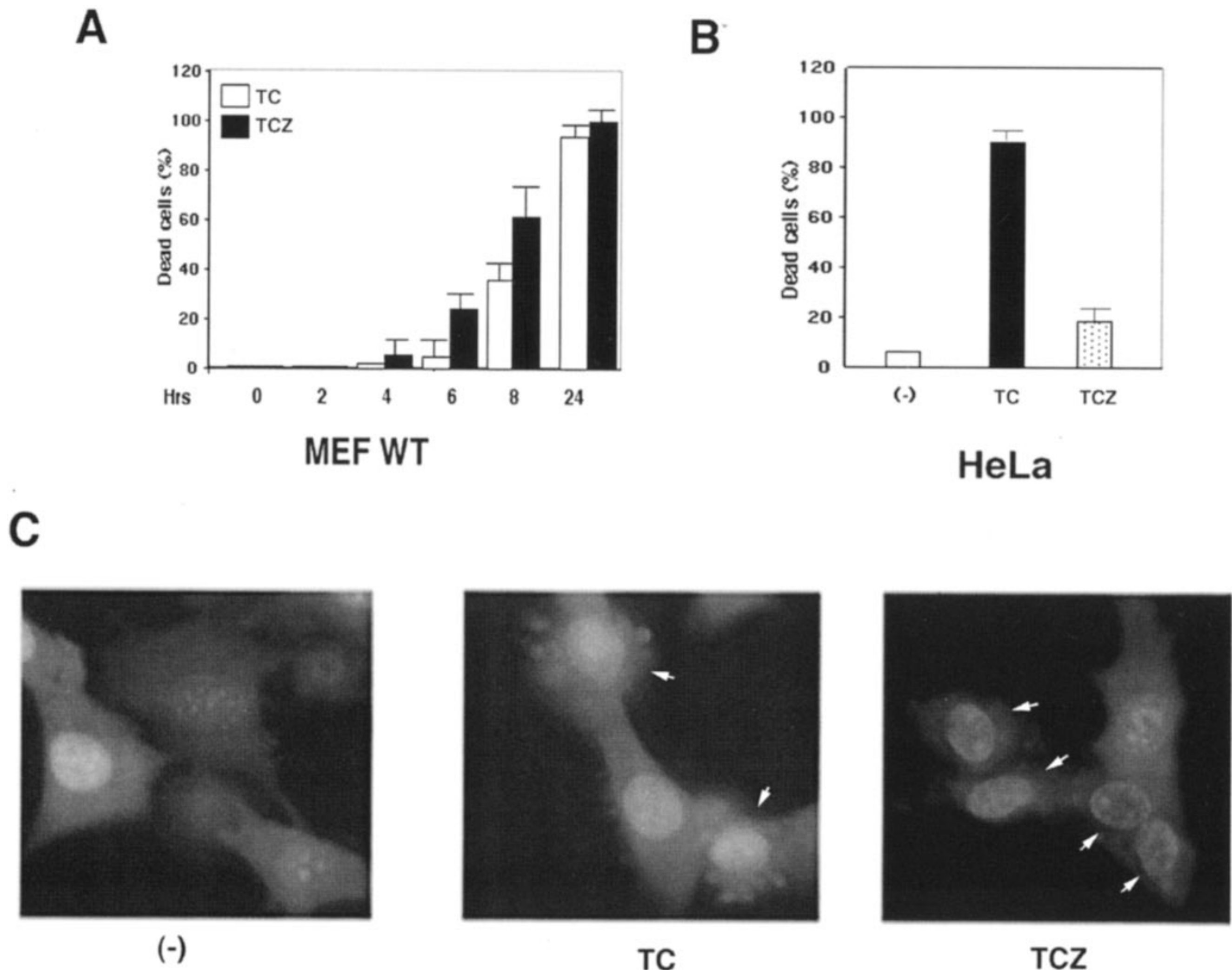

FIG. 1. **TNF-induces necrotic cell death in wild-type MEF cells.** *A*, wild-type MEF cells were pretreated with Z-VAD-fmk (20 μM) for 30 min or remained untreated and then treated with recombinant mouse TNF (30 ng/ml) plus CHX (10 μg/ml) for the indicated time periods. Cell death was quantified by measuring the percentage of LDH leakage. Data shown is the average of the results of three independent experiments. *B*, HeLa cells were pretreated with Z-VAD-fmk (20 μM) for 30 min or remained untreated and then treated with human TNF (30 ng/ml) plus CHX (10 μg/ml) for 24 h. Cell death was quantified by measuring the percentage of LDH leakage. Nontreated cells served as a control. *C*, wild-type MEF cells cultured on cover slides were treated as described in *A* for 8 h or remained untreated and then stained with ethidium bromide and acridine orange; they were immediately observed and photographed under a fluorescence microscope. *Arrowheads* indicate dead cells. *TC*, TNF and CHX; *TCZ*, TNF, CHX, and Z-VAD-fmk.

a critical role in caspase-8 activation and amplification of apoptosis signals through the mitochondria (20, 30, 31).

Despite the rapid progress in elucidation of the molecular mechanisms of TNF signaling to apoptosis, the signaling pathway to necrotic cell death in response to TNF is not well studied. RIP was found to be essential for Fas, TNF-related apoptosis-inducing ligand (TRAIL), and TNF-induced necrosis in lymphocytes (32). ROS was found to be required for necrotic cell death in L929 cells, a unique murine fibroblast cell line that is defective in TNF-induced apoptosis (33–35), as well as in NF-κB subunit p65- or TRAF2/TRAF5-null mouse embryonic fibroblast (MEF) cells (19). Activation of poly(ADP-ribose) polymerase (PARP) and depletion of cellular ATP is also suggested to be involved in TNF-induced necrosis (36). However, the roles of each component of the TNFRI signaling complex in TNF-induced necrotic cell death have not been well addressed. Moreover, the signaling mechanism that leads to TNF-induced necrotic cell death has remained elusive. In this study, by utilizing gene knockout cells, we systematically delineated the necrotic signaling pathway induced by TNF. Here we established that the RIP-, TRAF2-, and FADD-mediated ROS accumulation plays a pivotal role in TNF-induced necrotic cell death.

EXPERIMENTAL PROCEDURES

Reagents and Plasmids—Anti-RIP and JNK1 antibodies were purchased from PharMingen. Anti-FLAG (M2) and actin antibodies were obtained from Sigma. Anti-TRAF2 was obtained from Santa Cruz Biotechnology. Anti-FADD was a gift from Dr. Jianke Zhang. The mammalian expression plasmids for TRAF2, RIP, and FADD have been described previously (23, 37). Z-VAD-fmk, BAY-11, MG132, p38 inhibitor SB203580, JNK inhibitor SP600125, and MEK inhibitor U0126 were purchased from Calbiochem. 5(6)-chloromethyl-2′,7′-dichlorodihydrofluorescence diacetate (CM-H2 DCFDA) was obtained from Molecular Probes. Butylated hydroxyanisole (BHA) was obtained from Sigma.

Cell Culture and Transfection—RIP$^{-/-}$ and TRAF2$^{-/-}$ MEF cells were described previously. TNFRI$^{-/-}$ and TNFRII$^{-/-}$ MEF cells were generated from TNFRI and TNFRII knockout mice, respectively. FADD$^{-/-}$ and FADD reconstituted FADD$^{-/-}$ MEF cells were provided by Dr. Jianke Zhang (38). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were transfected with LipofectAMINE (Invitrogen) as described previously (37, 39). Stable transfected RIP and TRAF2 cell lines were selected with

hygromycin from the TRAF2$^{-/-}$ cells transfected with each gene of interest and pHygromycin.

Western Blot Analysis—After treatment with different reagents as described in the figure legends, cells were collected and lysed in M2 buffer (20 mM Tris, pH 7, 0.5% Nonidet P-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerol phosphate, 1 mM sodium vanadate, 1 μg/ml leupeptin). Fifty micrograms of the cell lysate from each sample was fractionated by SDS-PAGE and Western blotted. The proteins were visualized by enhanced chemiluminescence (ECL), according to the manufacturer's (Amersham Biosciences) instruction (39).

Cell Death Assays—Cells cultured in 12-well plates were treated as described in the figure legends. Cell death was observed under the microscope and characterized by cell rounding up and detaching from the plates. For quantification of cell death, a cytotoxicity detection kit was used to measure the percentage of lactate dehydrogenase (LDH) leakage according to the manufacturer's instructions (Roche Applied Science). Each experiment was repeated at least three times with duplicate samples. The figures shown are averages of the results of three experiments.

For morphological study of cell death, cells were cultured on cover slides and treated with TNF, cycloheximide, and Z-VAD-fmk for 5 h. The cells were stained with 50 μg/ml of ethidium bromide and 50 μg/ml of acridine orange and immediately observed and photographed under a fluorescence microscope.

Detection of ROS Accumulation—Cells in 12-well plates were cultured in phenol red-free medium and treated with TNF, cycloheximide, and Z-VAD-fmk for the indicated time periods. CM-H2-DCFDA (1 μM/ml) was added 30 min before collecting cells. The stained cells were analyzed with a flow cytometer (FACSCalibur, BD Biosciences) and data were processed with the CellQuest program (BD Biosciences).

RESULTS

TNF Induces Necrotic Cell Death in MEF Cells When Caspases Are Blocked—To study the mechanism of TNF-induced nonapoptotic cell death, we decided to use MEF cells as a model because this enabled us to utilize the gene-knockout fibroblast cells that are null for several components of the TNF receptor signaling cascade. First, we tested whether TNF induces nonapoptotic cell death in this type of cell. We pre-treated wild-type mouse fibroblast cells with Z-VAD-fmk, a membrane-permeable pan-caspase inhibitor, followed by treatment with TNF and cycloheximide (CHX). As a control, cells without pre-treatment were treated with TNF/CHX to induce apoptosis. Although the caspases were blocked after Z-VAD-fmk pre-treatment (data not shown), cells were still sensitive to TNF-induced cell death, which was even more extensive at early time points when compared with that without Z-VAD-fmk pretreatment (Fig. 1*A*). However, Z-VAD-fmk pretreatment efficiently blocked TNF-induced apoptotic cell death in HeLa cells, which not only indicated the effectiveness of Z-VAD-fmk used in our study, but also suggested that the caspase-independent cell death is cell type-specific (Fig. 1*B*). The cell death induced by TNF under the caspase-blocked condition is morphologically distinct from the typical apoptosis because it did not exhibit the classic apoptosis features such as cell membrane blebbing, shrinkage of cytoplasm and nucleus, and chromatin condensation (Fig. 1*C*). Instead, the dead cells maintained morphologically normal nuclei and showed disrupted cytoplasmic membrane and loose cytoplasm, which resembles the morphology of necrosis. These results suggest that, under caspase-inhibited conditions, TNF induces necrotic cell death in MEF cells.

TNF Induced Necrotic Cell Death Mainly through TNFRI—To see which of the TNF receptors transduces the necrotic cell death signal, we treated the TNFRI- or TNFRII-knockout MEF cells and compared them with wild-type cells. As expected, TNF-induced apoptosis occurred through TNFRI, the same as when treated with TNF plus CHX; cell death is defective in TNFRI$^{-/-}$ cells, whereas in TNFRII$^{-/-}$ cells, it was similar to that in wild-type cells (Fig. 2). When pre-treated with Z-VAD-fmk to induce necrotic cell death, TNFRII$^{-/-}$ cells died and

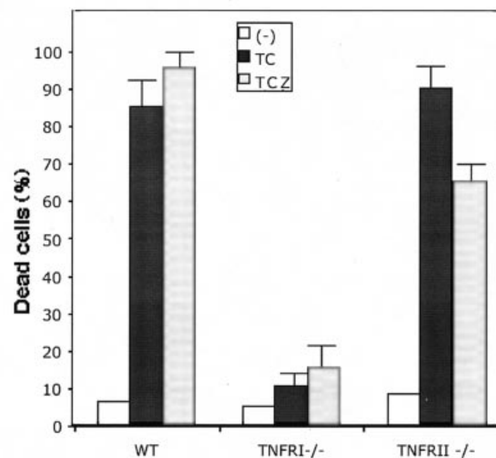

FIG. 2. **TNF induced necrotic cell death mainly through TNFRI.** TNFRI$^{-/-}$, TNFRII$^{-/-}$, and WT MEF cells were treated for 24 h as described in Fig. 1. Cell death was quantified by measuring the percentage of LDH leakage. Data shown are the average of the results of three independent experiments. *TC*, TNF and CHX; *TCZ*, TNF, CHX, and Z-VAD-fmk.

showed necrotic morphology (data not shown). However, the extent of cell death was reduced compared with wild-type cells, suggesting that, although TNFRII is not essential for TNF-induced necrotic cell death, it contributes to this process. This result is consistent with a recent report (40). In contrast, necrotic cell death was not observed in TNFRI$^{-/-}$ cells (Fig. 2), suggesting that TNF-induced necrotic cell death signal is mediated mainly through TNFRI.

TNF-induced Necrotic Cell Death Requires RIP, TRAF2, and FADD—As TNF-induced necrotic cell death is mainly mediated by TNFRI, we decided to investigate which component of the TNFRI signaling complex is required for this cell death process. Therefore, we utilized gene knockout cells that have RIP, TRAF2, or FADD deletions. As shown in Fig. 3, *A* and *B*, whereas TNF-induced apoptosis in RIP$^{-/-}$ and TRAF2$^{-/-}$ cells is comparable with wild-type cells, TNF-induced necrotic cell death was defective in RIP$^{-/-}$ cells and markedly decreased in TRAF2 $^{-/-}$ cells, suggesting that RIP and TRAF2 are required for TNF-induced necrotic cell death. To rule out the possibility that flaws other than the deletion of their respective genes in the knockout cells may affect the process of necrosis, we reconstituted RIP or TRAF2 expression in RIP$^{-/-}$ or TRAF2$^{-/-}$ cells (Fig. 3*C*) and tested the sensitivity to TNF-induced necrotic cell death in the resulting cells. The reconstitution of RIP or TRAF2 expression efficiently restored TNF-induced necrotic cell death in RIP$^{-/-}$ or TRAF2$^{-/-}$ cells. These results suggested a crucial role of RIP in TNF-induced necrosis, which is consistent with a previous study (32). Additionally, our results suggest that TRAF2 also plays an important role in TNF-induced necrotic cell death.

FADD is a critical component of the TNF-induced apoptosis pathway (26, 27). To see whether TNF-induced necrotic cell death requires FADD, we compared FADD$^{-/-}$ MEF cells with wild-type MEF cells. As expected, TNF-induced apoptosis is defective in FADD$^{-/-}$ cells (Fig. 3, *A* and *B*). Surprisingly, TNF-induced necrotic cell death is also defective in this type of cell, indicating that TNF-mediated necrotic cell death also requires FADD. To confirm this result, FADD$^{-/-}$ cells with FADD expression reconstituted (Fig. 3*C*) were treated with apoptotic (TNF/CHX) and necrotic (TNF/CHX/Z-VAD-fmk) conditions. The reconstitution of FADD expression not only efficiently restored TNF-induced apoptosis (data not shown), but also partially restored TNF-induced necrotic cell death (Fig. 3*D*), suggesting that despite a crucial role for FADD in apoptosis, FADD also plays a

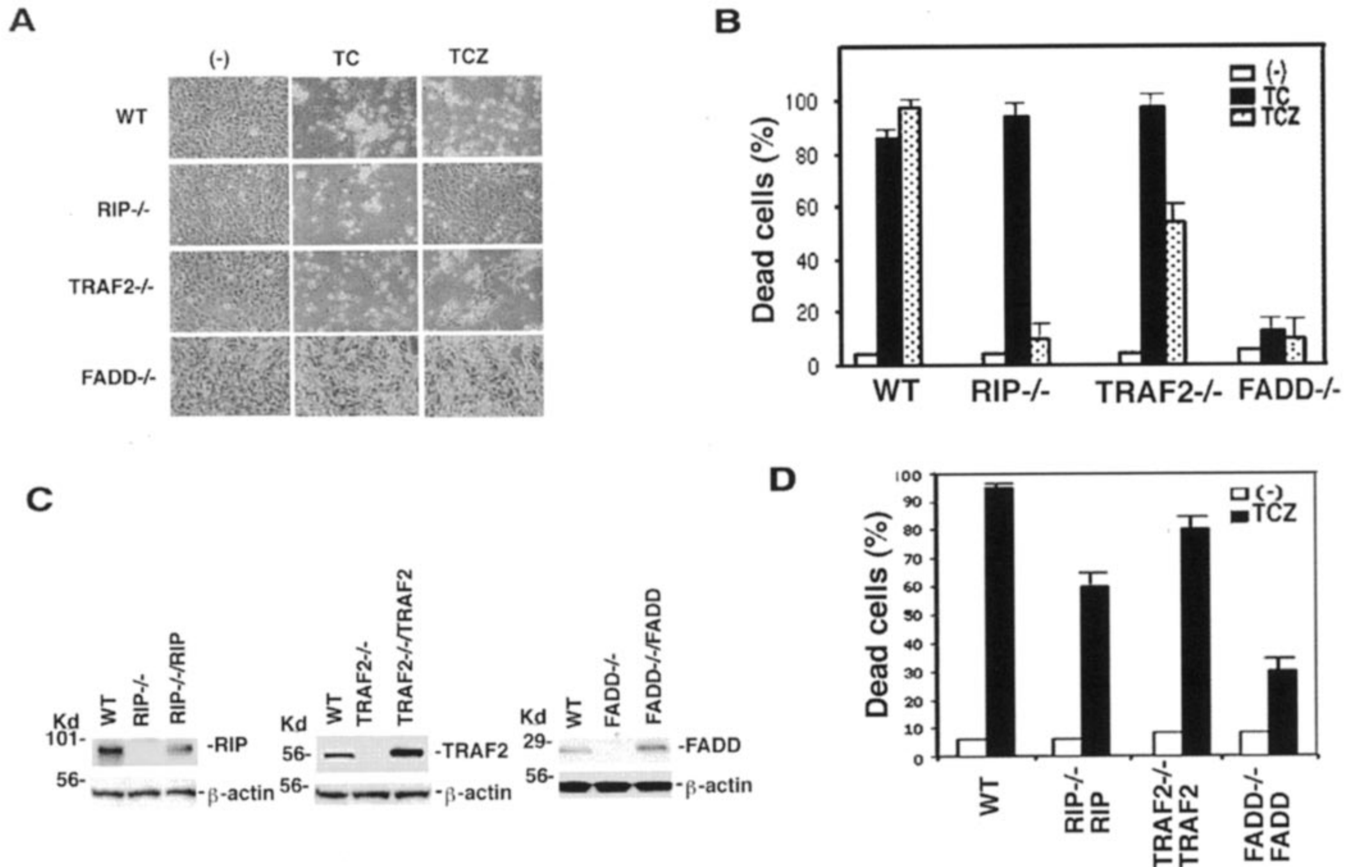

FIG. 3. **TNF-induced necrotic cell death in RIP$^{-/-}$, TRAF2$^{-/-}$, and FADD$^{-/-}$ MEF cells.** *A*, RIP$^{-/-}$, TRAF2$^{-/-}$, and FADD$^{-/-}$ MEF cells were treated for 24 h as described in Fig. 1 and photographed under a light microscope. *B*, cells were treated as in *A*; cell death was quantified by measuring the percentage of LDH leakage. Data shown are the average of three independent experiments. *C*, cell extracts of RIP$^{-/-}$, TRAF2$^{-/-}$, and FADD$^{-/-}$ MEF cells and each respective reconstituted cells with stable transfections were Western blotted for RIP, TRAF2, and FADD, respectively. WT MEF cells were used as a control. *D*, each reconstituted cell with stable transfections were treated with Z-VAD-fmk, TNF, and CHX for 24 h as described in Fig. 1. Cell death was quantified by measuring the percentage of LDH leakage. WT MEF cells were used as a control. Data shown is the average of the results of three independent experiments.

positive role in TNF-induced necrotic cell death.

NF-κB Activation Blocks TNF-induced Necrotic Cell Death—Because TNF-induced NF-κB activation is anti-apoptotic and necrotic cell death occurs when apoptosis is abrogated in MEF cells, we sought to investigate whether activation of NF-κB is required for TNF-induced necrotic cell death. NF-κB is a transcription factor that exerts its function through augmenting the expression of its target genes. The high concentration of cycloheximide (10 μg/ml), which was included to induce necrotic cell death, might block *de novo* protein synthesis and, therefore, it is unlikely that NF-κB is required for necrotic cell death. To test this possibility, we chose the NF-κB inhibitor, IκBα, as a read-out marker, because the IκBα gene is one of the NF-κB-activated targets. Normally, when cells are exposed to TNF, IκBα is first degraded and then recovers rapidly (6, 12, Fig. 4*A*, *lanes 5–7*). However, under the necrotic cell death condition that included CHX, no recovery of IκBα was observed (Fig. 4*A*, *lane 3*). These results confirmed that, under our necrotic cell death conditions, NF-κB-activated gene expression was blocked; however, necrotic cell death was still observed (Fig. 4*C*). Because no correlation between NF-κB-activated gene expression and necrotic cell death was observed, it is unlikely that NF-κB is required for TNF-induced necrotic cell death.

Next, we tested whether NF-κB had a negative role in TNF-induced necrotic cell death in MEF cells. Cells were pretreated with NF-κB inhibitor BAY-11 or proteasome inhibitor MG132, followed by TNF plus Z-VAD-fmk treatment. BAY-11 inhibits NF-κB activation through the blockage of IκB phosphorylation, whereas MG132 inhibits NF-κB activation through the blockage of IκB degradation (41). As shown in Fig. 4*B*, both Bay-11 and MG132 blocked NF-κB activation efficiently, as measured by IκBα degradation. Although there was no cell death observed when cells were treated with TNF/Z-VAD-fmk, a significant portion of cells died when pretreated (in addition to TNF/Z-VAD-fmk treatment) with Bay-11 or MG132 to block NF-κB (Fig. 4*C*). This result suggests that NF-κB negatively regulates TNF-induced necrotic cell death in MEF cells. Because CHX is required to achieve the full necrotic effect and Bay-11 or MG132 only partially facilitated TNF-induced necrotic cell death, there might be other factors in addition to NF-κB that negatively regulate the TNF-induced necrotic cell death pathway.

TNF-induced Necrotic Cell Death Does Not Require JNK, p38, and ERK Activation—To see whether the activation of MAP kinases is required for TNF-induced necrotic cell death, we pretreated the wild-type MEF cells with JNK inhibitor SP600125, p38 inhibitor SB203580, or MEK inhibitor U0126 before induction of TNF-induced necrotic cell death. U0126 blocks ERK activation through blockade of MEK, the upstream kinase of ERK. Although the inhibitors efficiently inhibited their respective MAP kinases (data not shown), there was only a marginal effect by each inhibitor on TNF-induced necrotic cell death (Fig. 5). These results suggest that neither JNK, p38, nor ERK is required for TNF-induced necrotic cell death.

Important Role of ROS in TNF-induced Necrotic Cell Death—It has been reported previously that TNF induces the accumulation of cellular ROS, and that ROS is required for

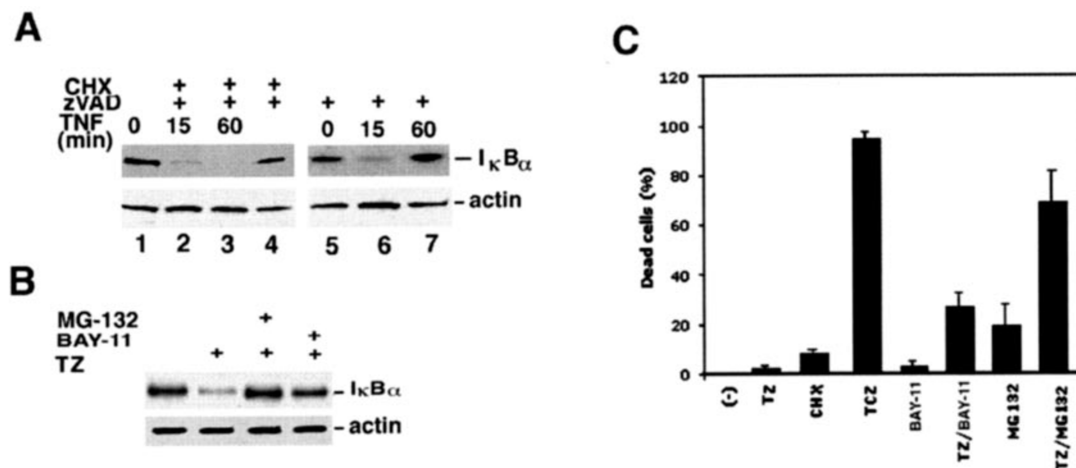

FIG. 4. **NF-κB activation blocks TNF-induced necrotic cell death.** *A*, wild-type MEF cells were pretreated with Z-VAD-fmk (20 μM) for 30 min and then treated with recombinant mouse TNF (30 ng/ml) or mouse TNF (30 ng/ml) plus cycloheximide (CHX, 10 μg/ml) for the indicated time periods. Cell extracts were Western blotted for IκBα. Actin was probed as an input control. *B*, wild-type MEF cells were pretreated with Z-VAD-fmk (20 μM) and BAY-11 (5 μM) or MG132 (10 μM) for 30 min and then treated with recombinant mouse TNF (30 ng/ml) for 15 min. Cell extracts were Western blotted for IκBα. Actin was probed as an input control. *C*, wild-type MEF cells were treated as described in *B* for 24 h, and cell death was quantified by measuring the percentage of LDH leakage. Data shown are the average of the results of three independent experiments. *TCZ*, TNF, CHX, and Z-VAD-fmk; *TZ*, TNF, and Z-VAD-fmk.

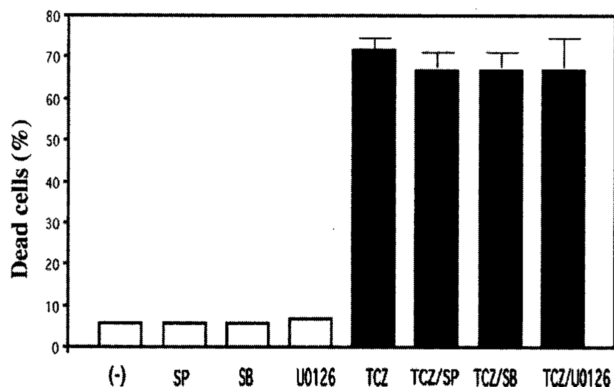

FIG. 5. **TNF-induced necrotic cell death does not require JNK, p38, and ERK activation.** Duplicated wild-type MEF cells were pretreated with Z-VAD-fmk (20 μM) and p38 inhibitor SB203580 (*SB*), JNK inhibitor SP600125 (*SP*), or MEK inhibitor U0126, and then one-half of the cells were treated with mouse TNF (30 ng/ml) plus CHX (10 μg/ml) for 24 h and the other half remained untreated. Cell death was quantified by measuring the percentage of LDH leakage. Data shown are the average of the results of three independent experiments. *TCZ*, TNF, CHX and Z-VAD-fmk.

necrotic cell death in L929 cells as well as in NF-κB subunit p65- or TRAF2/TRAF5-null MEF cells (19, 33–35, 42). To see whether ROS in MEF cells is increased under our necrotic cell death conditions, we stained the cells with CM-H2 DCFDA, a cell-permeable fluorescence dye that reacts to a broad spectrum of ROS at different time points after the induction of TNF-induced necrotic cell death. As shown in Fig. 6*A*, elevated ROS levels were detectable 4 h after TNF/CHX/Z-VAD-fmk treatment and gradually increased. The starting time point of ROS increase (4 h) correlated well with the onset of cell death (Fig. 1*A*). Then, we tested whether ROS accumulation is required for TNF-induced necrotic cell death by pretreatment of the cells with a ROS scavenger BHA. BHA dramatically inhibited ROS accumulation (Fig. 6*B*) and efficiently suppressed TNF-induced necrotic cell death (Fig. 6*C*), suggesting that, as in L929 cells, ROS plays an important role in TNF-induced necrotic cell death in MEF cells.

ROS Accumulation Is Dependent upon RIP, TRAF2, and FADD—Next we examined whether ROS accumulation under our necrotic cell death conditions requires the presence of RIP, TRAF2, and FADD. As again, in wild-type MEF cells, ROS was significantly increased 8 h after challenging the cells with TNF/CHX/Z-VAD-fmk (Fig. 7*A*). In contrast, there was no detectable ROS increase in $RIP^{-/-}$ and $FADD^{-/-}$ cells. In $TRAF2^{-/-}$ cells, the ROS level was only slightly elevated. These results suggest that RIP, FADD, and TRAF2 are components critical to transducing the signal to induce ROS accumulation in MEF cells.

DISCUSSION

In this report, we have systematically delineated the TNF-induced necrotic cell death pathway with gene knockout MEF cells. The results suggest that TNFRI mediates, and TNFRII may facilitate, necrotic cell death. The components of the TNFRI signaling complex, RIP, TRAF2, and FADD, were required for TNF-induced necrotic cell death. Although NF-κB suppressed TNF-induced necrotic cell death, MAP kinases, JNK, p38, and ERK, seemed to be dispensable. Interestingly, RIP-, TRAF2-, and FADD-mediated ROS accumulation was found to be crucial in TNF-induced necrotic cell death.

Although TNF-induced necrotic cell death had been observed as early as 1993 (43), the study of its mechanism was retarded because most of the attention and effort was focused on the mechanism of TNF-induced apoptosis. Most studies on TNF-induced necrotic cell death either used L929, a unique mouse fibroblast cell line that is defective in TNF-induced apoptosis (34, 35), or lymphocytes (32). Thus the generality of these findings should be further addressed. Additionally, the signaling cascade of the TNF-induced necrotic cell death pathway has not been clearly characterized. In this study, we found that TNFRI is essential, and TNFRII as well has some positive role in TNF-induced necrotic cell death. This result is in agreement with a recent report showing that TNFRII facilitates TNFRI-mediated programmed necrosis in Jurkat cells (40). However, the question of how TNFRII facilitates TNFRI-mediated necrotic signaling remains to be addressed. Further, as RIP and TRAF2 are critical for TNF-induced necrotic cell death but dispensable for TNF-induced apoptosis, it seems that the distinct death signals may bifurcate at the level of RIP and TRAF2 (Fig. 7*B*).

RIP is essential for TNF-induced NF-κB activation but dispensable for apoptosis (7, 8). Cleavage of RIP by caspase 8 triggers the apoptosis process (44). In Jurkat cells, RIP kinase activity was found to be required for TNF-, Fas-, and TRAIL-

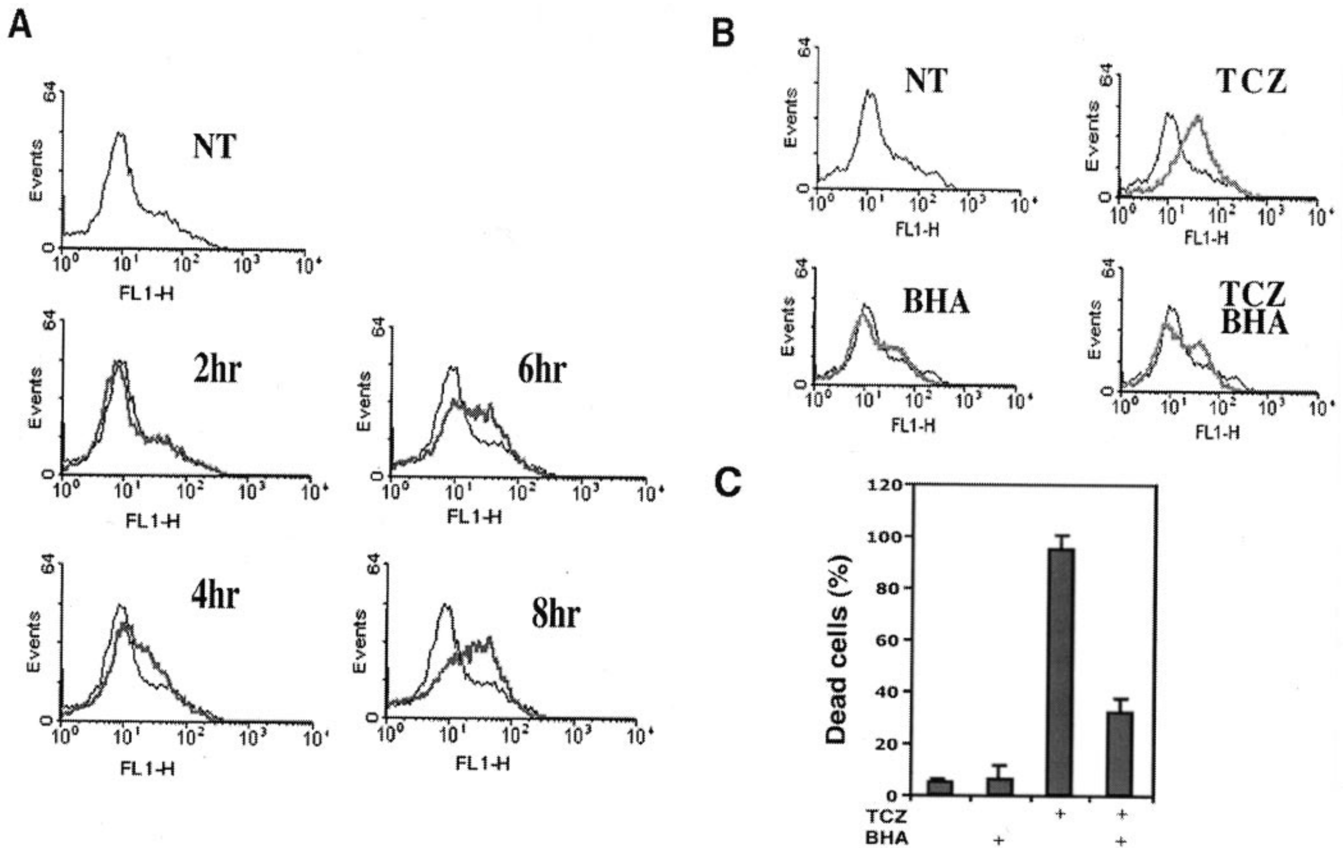

FIG. 6. **Critical role of ROS accumulation in TNF-induced necrotic cell death.** *A*, wild-type MEF cells were treated with Z-VAD-fmk, TNF, and CHX as described in Fig. 1 for the indicated time periods. 30 min before the end of treatment, CM-H2 DCFDA (1 μM) was added. ROS was detected with a flow cytometer (FACSCalibur, BD Biosciences), and data were processed with the CellQuest program (BD Biosciences). *B*, nonpretreated or BHA (100 M) pretreated (30 min) wild-type MEF cells were treated with Z-VAD-fmk, TNF, and CHX for 8 h as described in Fig. 1. 30 min before the end of treatment, CM-H2 DCFDA (1 μM) was added. ROS was detected and analyzed as in *A*. Cells treated with BHA alone were included as a control. *C*, wild-type MEF cells were treated as in *B* for 24 h; cell death was quantified by measuring the percentage of LDH leakage. Data shown are the average of the results of three independent experiments.

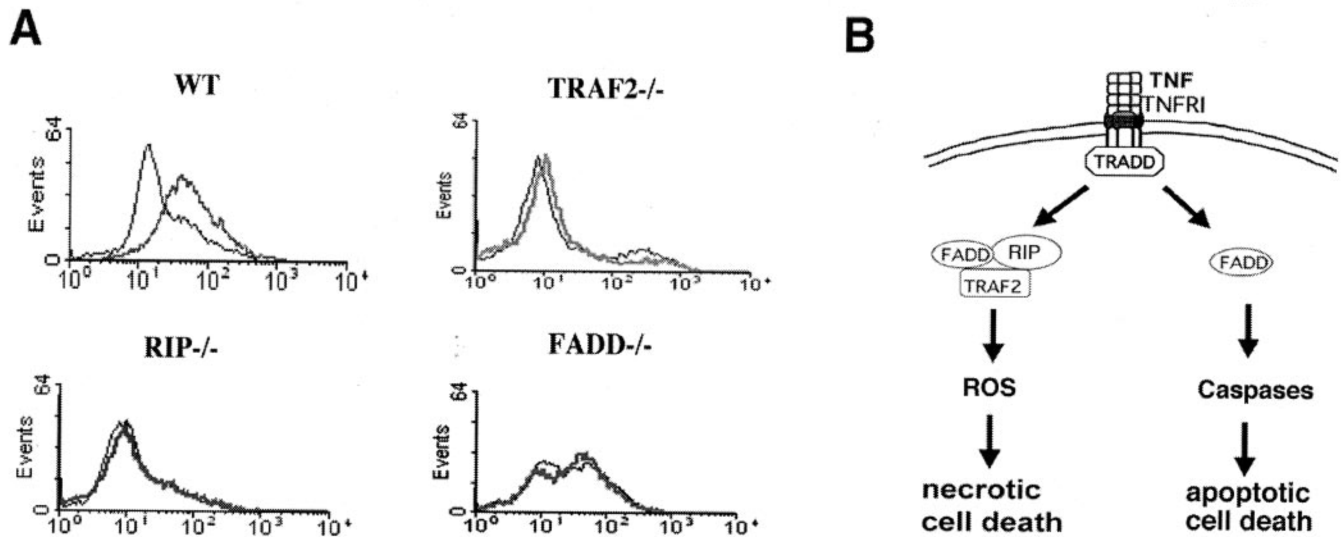

FIG. 7. **Critical role of RIP, FADD, and TRAF2 in ROS accumulation during TNF-induced necrotic cell death.** *A*, wild-type, RIP$^{-/-}$, TRAF2$^{-/-}$, and FADD$^{-/-}$ MEF cells were treated with Z-VAD-fmk, TNF, and CHX for 8 h, and ROS was detected and analyzed as described in Fig. 6. *B*, proposed model for TNF-induced necrotic cell death signaling. TNF-induced necrotic cell death was achieved by RIP-, TRAF2-, and FADD-mediated ROS accumulation, whereas TNF-induced apoptosis was achieved by FADD-mediated apoptosis.

induced necrosis (32). In this study, we confirmed that RIP is essential for TNF-induced necrotic cell death in MEF cells. However, we did not observe the requirement of RIP kinase activity for TNF-induced necrotic cell death by transient transfection of RIP$^{-/-}$ MEF cells with RIP(K45A), a kinase-deficient RIP mutant (data not shown). As our effort to establish stable lines with high level expression of RIP kinase dead mutant (RIP(K45A)) in RIP$^{-/-}$ cells has not been successful so far, the

requirement of RIP kinase activity for TNF-induced necrotic cell death in MEF cells remains to be elucidated. Because TRAF2 and FADD have also been found to be necessary for TNF-induced necrotic cell death in MEF cells, it is likely that the formation of the TNFRI signaling complex, consisting of RIP, TRAF2, and FADD, is essential for the generation of a necrotic signal when caspases are blocked. It is highly possible that TRADD, the first adaptor protein to be recruited to TNFRI, and which serves as a platform for RIP, TRAF2, and FADD interacting, is also required. Because TRADD gene knockout cells are currently not available, whether TRADD is involved in TNF-induced necrotic cell death remains for future study.

It has been reported that, in Jurkat cells, TNF-induced necrosis does not require FADD (32). However, TRAIL-induced necrosis does require FADD (32). In our study, FADD was clearly shown to be required. The reason for this discrepancy is currently unclear. It is possible that the requirement of FADD is not only death receptor-specific but also cell type-specific (24, 25, 45).

Our results demonstrate that, during TNF-induced necrotic cell death in MEF cells, ROS is accumulated. Blockage of ROS also inhibited cell death, suggesting a crucial role of ROS in this type of cell death. Consistently, the antioxidant enzyme manganese-containing superoxide dismutase has been shown to block the cytotoxicity of TNF in MCF7 cells (46). ROS accumulation is impaired in $RIP^{-/-}$, $TRAF2^{-/-}$, and $FADD^{-/-}$ cells, suggesting that these molecules are essential for transducing the signal to induce ROS, and ROS is downstream of RIP, TRAF2, and FADD for TNF-induced necrotic cell death in MEF cells. Consistent with this conclusion, it has been reported that TRAF2 mediates ROS production in HEK293 cells (47). However, because the known pathways, namely NF-κB and MAP kinase activation, are not required for TNF-induced necrotic cell death and ROS accumulation in MEF cells (data not shown), how the signal is transduced to ROS metabolic machinery is an open question. Additionally, how ROS accumulation mediated the execution of necrotic cell death is also currently unknown. It is unlikely that ROS mediates PARP activation to execute necrotic cell death, as there was no correlation between ROS accumulation, PARP activation, and ATP decrease observed in MEF cells (data not shown). Among the ROS scavengers, BHA efficiently inhibited, whereas others, such as TEMPL and NAC, only slightly or marginally inhibited TNF-induced necrotic cell death (data not shown). It is possible that the newly generated ROS in the mitochondria mediated a death signal *in situ*. This explanation is supported by the facts that BHA is the only ROS scavenger that can enter mitochondria (35).

A recent report using p65 knockout and TRAF2/TRAF5 double knockout cells showed that TNF alone induced necrosis through ROS-mediated JNK activation, which was not observed in wild-type cells (19). This finding was thought to be the result of the impaired NF-κB activation, in agreement with our finding that NF-κB suppresses TNF-induced necrotic cell death (this study). However, we found no requirement for JNK in necrosis in wild-type MEF cells. This might be due to the difference in cell lines (33, 47). Alternatively, the presence of CHX in our system may cause the bypass of JNK by blocking the anti-necrotic signals and/or facilitating the necrotic signals.

Taken together, our results revealed a critical role for RIP, TRAF2, and FADD in mediating ROS generation in TNF-induced necrotic cell death. Further study elucidating the mechanism of the signal from the TNF receptor complex to the generation of ROS and that of ROS in necrotic cell death will facilitate our understanding of cell death control and improve the value in the clinical application of TNF.

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