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TNF-receptor associated factor 6-deficient fibroblast is sensitive to the TNF-α-induced cell death: Involvement of reactive oxygen species

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Abstract

Tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6) has mainly been involved in signaling from CD40 and IL-1 receptor family. While TNF-α exerts various biological effects including cell death, the role of TRAF6 in the TNF-α signaling remains to be unclear. Here, we demonstrated that murine embryonic fibroblasts (MEFs) derived from TRAF6 knockout (TRAF6KO) mice have increased sensitivity to actinomycin D plus TNF-α-induced cell death compared with wild-type MEF. Reactive oxygen species (ROS) were accumulated more in TRAF6KO MEF than in wild-type MEF. An antioxidant, butylated hydroxyanisole (BHA) completely inhibited TNF-α-induced cell death and DNA fragmentation. Thus, the TNF-α-induced cell death in TRAF6KO MEF was ROS-dependent. Reconstitution of full-length TRAF6 but not N-terminal-deleted TRAF6 constructs in TRAF6KO MEF reversed TNF-α-induced cell death, ROS accumulation, and DNA fragmentation completely. Thus, we concluded that resistance against TNF-α-induced cell death is rendered by TRAF6, which regulates ROS accumulation.

Keywords: TRAF6; TNF-α; Actinomycin D; Cell death; TRAF6KO; MEF; ROS; Antioxidant; BHA

Tumor necrosis factor receptor (TNFR)-associated factors (TRAFs) belong to a family of intracellular adaptor proteins that mediate signaling downstream of various cell surface receptors including members of TNFR superfamily as well as Toll-like receptor (TLR) and the IL-1R family [1–3]. Currently, seven mammalian TRAFs, TRAF1 to 7 have been identified, which contain a C-terminal receptor-binding TRAF domain, consisting of TRAF-N and TRAF-C, except TRAF1 and TRAF7 [1–3]. Among them, TRAF6 is the only TRAF family member that participates in the signal transduction of both TNFR and IL-1R/TLR superfamily [4,5]. The role of TRAF6 has been extensively studied by the gene-targeted mice [1,3,6–10]. Namely, TRAF6-deficient mice exhibit exencephaly [1], osteopetrosis, and defective IL-1, CD40, and LPS signaling [6,7], and are essential for normal bone formation [8,9]. In addition, TRAF6 is shown to be essential for the induction of self-tolerance in thymic stroma and TRAF6 gene deficiency develops autoimmunity [10].

Biological effects of TNF-α are mediated by two distinct receptors, TNFR1 and TNFR2. Activation of death receptor TNFR1 by TNF-α leads to the recruitment of TNFR1-associated death domain protein (TRADD), which serves as a platform to form various signaling complexes. TRADD can recruit FADD and lead to caspase 8 activation and apoptosis [11,12]. However, TNF-α is not cytotoxic to most cells, because TRADD can recruit TRAF2 and receptor interacting protein (RIP) to form distinct complexes leading to the activation of NF-κB and c-Jun N-terminal kinase (JNK) [12–14]. It has been well-established that activation of NF-κB serves to protect cells against apoptotic stimuli such as TNF-α [14–16], therefore TNF-α-induced apoptosis requires NF-κB inhibition.

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Since the role of TRAF6 in the TNF-α-induced apoptosis and necrosis is still controversial, we employed embryonic fibroblast (TRAF6KO MEF) from the TRAF6 gene knockout mice in these experiments. In the TRAF6KO MEF, no apparent cell death was induced by TNF-α but the cell death of apoptotic and necrotic type was induced in the presence of actinomycin D (ActD), revealing that TRAF6 protects ActD plus TNF-induced cell death. In this type of cell death, reactive oxygen species (ROS) were critically involved. While the involvement of ROS in TNF-α-mediated apoptosis is also highly controversial [17,18], the role of ROS is discussed.

Materials and methods

Reagent and antibodies. Recombinant murine TNF-α was purchased from Peprotech (London, UK). ActD, staurosporine, butylated hydroxyanisole (3-tert-butyl-4-hyrodoxyanisole, BHA), and anti-Flag antibody (Ab) were purchased from Sigma–Aldrich (Tokyo). Anti-TRAF2 and Anti-TRAF6 and Anti-β-actin Abs were purchased form Santa Cruz Biotechnology (CA, USA). Peroxidase-coupled anti-rabbit and anti-goat IgG were obtained from Dako (Dako-Japan, Tokyo).

Cell culture and cell viability. TRAF6KO and wild-type MEFs were established elsewhere [8–10]. Cells were maintained in Dulbecco's MEM (Nissui Seiyaku, Tokyo) supplemented with 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and 10% fetal bovine serum (Gibco-BRL, NY, USA).

Wild-type and TRAF6KO MEFs were incubated in 96-well plates (Nalge Nunc International, Tokyo) at 5×10^3 per well. Cells were pretreated with ActD, for 30 min prior to the stimulation with TNF-α (Peprotech). Cell viability was determined by the 3-(4,5-dimethylthiazol-23-yl)-2,5-diphenyltetrazolium bromide (MTT) method as described elsewhere [19]. Statistical differences between wild-type and TRAF6KO MEF groups were assessed with Students' *t*-test.

Western blot analysis. Cell lysates were prepared by suspending 2×10^6 cells in 120 μl of lysis buffer as described elsewhere, and immunoblot analysis was performed as described previously [19], with various Abs (mostly dilution at 1:1000) and horseradish peroxidase-coupled anti-rabbit or anti-goat IgG (1:1000) and visualized using an enhanced chemiluminescence's Western blot detection system (Amersham Biosciences, Piscataway, NJ, USA).

DNA fragmentation and evaluation of apoptosis/necrosis. A DNA fragmentation assay was performed as described previously [19]. Annexin V-positive cells and/or PI-positive were analyzed by Vybrant Apoptosis Assay kit #3 (Molecular Probes, OR, USA) following the manufacturer's protocol. Briefly, 8×10^5 cells were incubated with Annexin V and PI at room temperature for 15 min and then added 0.4 ml buffer. The cells were analyzed using FACS Calibur using the CELLQuest program (Becton-Dickinson, CA, USA).

Flow cytometric analysis of intracellular ROS. Intracellular ROS levels were determined by staining with 5-(and -6)-chloromethyl-2′,7′-dichlorodihydrofluorescence diacetate (CM–H_2DCFHDA; Molecular Probes, OR, USA), which is oxidized to dichlorofluorescein (DCF) by ROS. 8×10^5 cells were incubated with 1 μM CM–H_2DCFHDA for 30 min in the stimulated medium, at 37° C and 5% CO_2. The stained cells were analyzed by flow cytometry with excitation 485 nm and emission 545 nm.

Retroviral construction and transduction. Generation of stable transfectants by using retroviral vector was performed as described previously [8]. Retroviral expression vector for full-length TRAF6 and N-terminus deleted TRAF6 (amino acid 229–530, ΔNTRAF6) were constructed by inserting each cDNA into MSCV-IRES-PURO (Clontech). 293 cells were transiently transfected by calcium phosphate precipitation to generate viral supernatants. After infection, TRAF6KO MEF was selected with 2 μg/ml puromycin (Nakarai, Osaka) to isolate stable transfectants. Puromycin-resistant pools were used in these experiments.

Results

TRAF6KO MEF showed increased sensitivity to ActD plus TNF-α-induced cell death

To test the role of TRAF6 in TNF-α-induced cell death, wild-type MEF and TRAF6KO MEFs were stimulated with TNF-α, but TNF-α alone did not induce significant cell death in both MEFs at the doses tested (0.1–30 ng/ml) (data not shown). While treatment with ActD alone at the doses of 0.01–3 μg/ml did not induce significant differences between two MEFs, pretreatment of ActD (0.03 μg/ml) for 30 min following TNF-α stimulation induced marked cell death in TRAF6KO MEF but not in the wild-type MEF (Fig. 1A). TNF-α-induced cell death exhibited dose-dependent manner in the TRAF6KO MEF and low doses of TNF-α (>0.1 ng/ml) in combination of ActD- (0.03 μg/ml) induced cell death around 40% and reached 80% cell death at 10 ng/ml TNF-α. In contrast, other apoptosis-inducing agents such as staurosporine induced cell death at higher doses in both wild-type MEF and TRAF6KO MEF similarly with no differences between these MEFs (Fig. 1B). We confirmed that TRAF6KO MEF did not express TRAF6 protein, but wild-type MEF express both TRAF2 and TRAF6 normally (Fig. 1B, inlet).

ActD plus TNF-α-induced cell death in TRAF6KO MEF contains apoptotic and necrotic cell death

We examined whether DNA fragmentation was induced by the ActD plus TNF-α treatment, and found that while no apparent DNA fragmentation occurred in wild-type MEF at least up to 10-h incubation period, significant DNA fragmentation was observed as early as at 4 h and markedly at 8–10 h in TRAF6KO MEF (Fig. 1C).

In order to estimate how apoptotic and necrotic cell death is involved, profiles of Annexin-V and PI staining during this cell death were analyzed by flow cytometry. ActD plus TNF-α-treated TRAF6KO MEF displayed, at early 2 h, Annexin-V staining, i.e., PS translocation from inside to outside of membrane, before their loss of membrane integrity assessed by PI staining (Fig. 1D). ActD plus TNF-α-treated TRAF6KO MEF lost their membrane integrity without PS translocation at 8 h (Fig. 1D). These results indicated that ActD plus TNF-α-induced cell death in TRAF6KO MEF included both apoptotic cell death at early stimulation time (2 h) and later, necrotic type of cell death.

Accumulation of ROS in the ActD plus TNF-α-induced cell death in TRAF6KO MEF

Since ROS have been reported to be involved in various cell death, we examined whether ROS is produced during induction of cell death. Wild-type and TRAF6KO MEFs were stimulated with ActD, TNF-α or ActD plus TNF-α for 2 h, and the cells were labeled with a cell-permeable fluorescent dye, CM–H_2DCFDA,

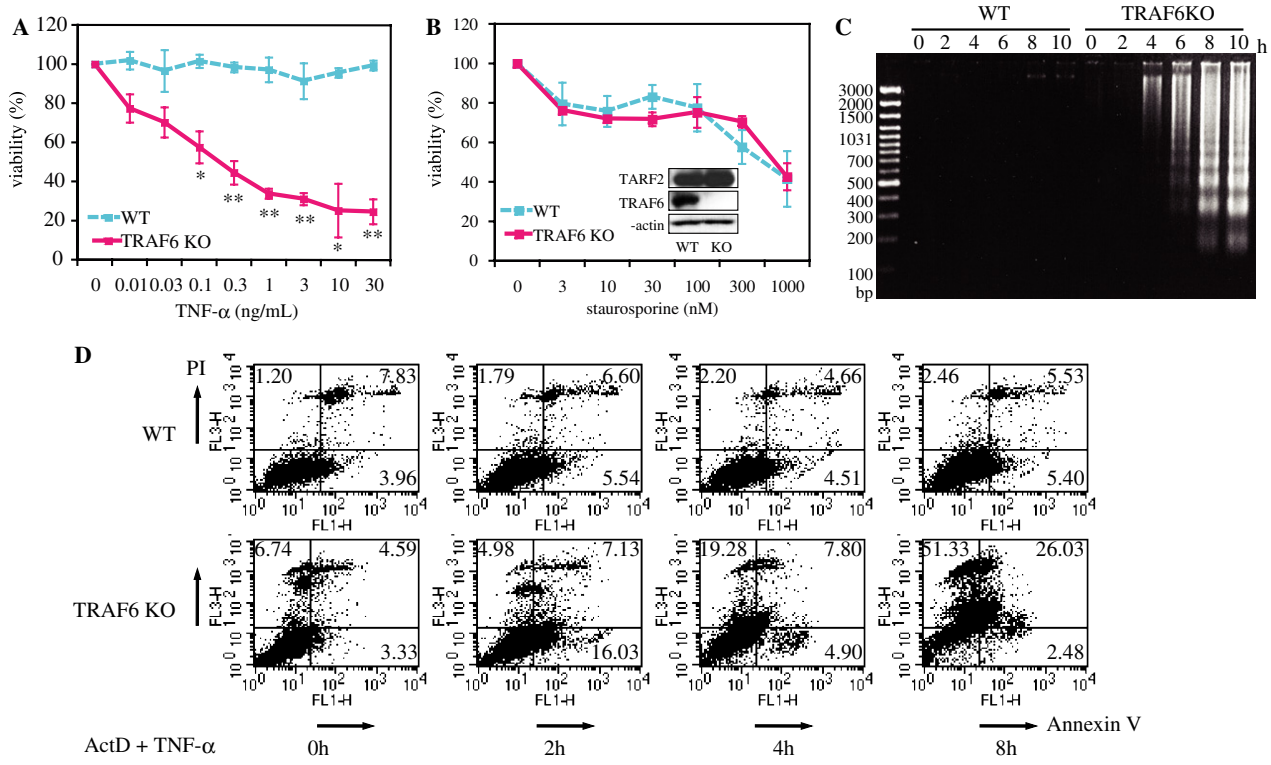

Fig. 1. TRAF6KO MEFs shows increased sensitivity to ActD plus TNF-α stimulation. (A) Wild-type and TRAF6KO MEFs were pretreated with ActD (0.03 μg/ml) for 30 min and then stimulated with TNF-α at the indicated doses for 16 h. (B) Wild-type and TRAF6KO MEFs were treated with staurosporine at the indicated doses for 16 h. Cell survival was determined with MTT assay. Data are expressed as means ± SD ($n = 3$), $^{*}p < 0.05$, and $^{**}p < 0.005$ between TRAF6KO and wild-type MEF. (inlet) Western blot analysis using anti-TRAF2, TRAF6, and β-actin antibodies, respectively. (C,D) Wild-type and TRAF6KO MEF were pretreated with ActD (0.03 μg/ml) for 30 min and then stimulated with TNF-α (1 ng/ml) for the indicated periods. (C) DNA was subjected to a 2% agarose gel electrophoresis. (D) The cells stained by Annexin V and PI were analyzed by FACS. Figures indicate the % of cells in each compartment.

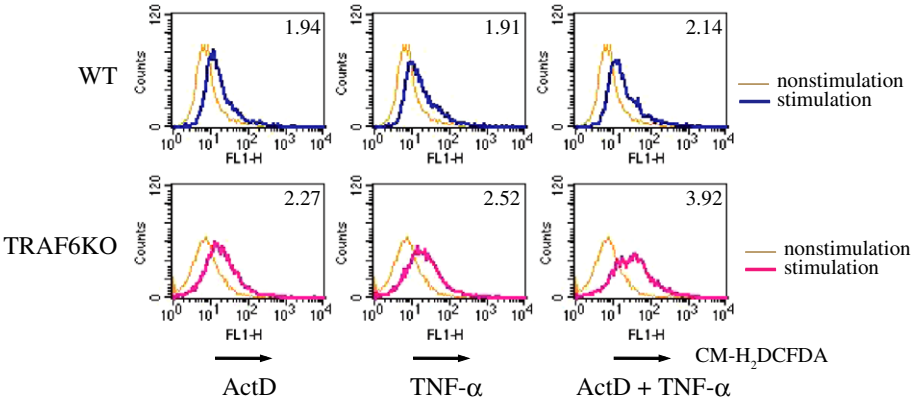

Fig. 2. ActD plus TNF-α-induced cell death in TRAF6KO MEF was mediated by ROS. Wild-type and TRAF6KO MEFs were pretreated with or without ActD (0.03 μg/ml) for 30 min and then stimulated with TNF-α (1 ng/ml) for 2 h. ROS levels were determined by staining with CM–H_2DCFDA, which is oxidized to dichlorofluorescein (DCF) by ROS. Stained cells were analyzed by flow cytometry. Figures indicate the ratio of mean fluorescence intensity by the "stimulation" with ActD plus TNF-α vs that by the "nonstimulation".

which is oxidized by ROS in the cells and was analyzed by flow cytometry. As shown in Fig. 2, TRAF6KO MEF produced significant level of ROS by the stimulation with ActD plus TNF-α (3.92-fold), but only low levels either with ActD (2.27) or TNF-α (2.52) alone. No significant ROS accumulation was observed in the wild-type MEF.

An antioxidant, BHA suppressed ActD plus TNF-α-induced ROS accumulation and cell death in TRAF6KO MEF

We examined further whether an antioxidant, butylated hydroxyanisole (BHA), could suppress the ActD plus TNF-α-induced cell death and ROS accumulation in TRAF6KO MEF. BHA inhibited cell death in dose-dependent manner

with almost complete suppression at 100–300 μM (Fig. 3A). BHA at 300 μM reversed ROS accumulation completely (Fig. 3B), DNA fragmentation (Fig. 3C) as well as the appearance of Annexin V and PI double positive cells in TRAF6KO MEF (Fig. 3D). Thus, we assumed that ROS produced by the ActD plus TNF-α-stimulation was responsible for the cell death in TRAF6KO MEF.

Reconstitution of TRAF6 reversed ActD plus TNF-α-induced cell death in TRAF6KO MEF

Finally, whether the ActD plus TNF-α-induced cell death, ROS accumulation, and DNA fragmentation could be reversed by the ectopic expression of TRAF6 was tested. Full-length TRAF6 (FLTRAF6) and N-terminus deleted TRAF6 (ΔNTRAF6) constructed in the MSCV-IRES-PURO were stably transfected in the TRAF6KO MEF, and the expression of the transfected genes was verified by Western blotting with anti-Flag Ab (Fig. 4A). Reconstitution of FLTRAF6 but not ΔNTRAF6 completely reversed ActD plus TNF-α-induced cell death, ROS accumulation, and DNA fragmentation (Fig. 4B–D). These results indicated that TRAF6 plays critical role in regulating accumulation of ROS in TRAF6KO MEF which leads to the cell death.

Discussion

We demonstrated here that TRAF6KO MEF have increased sensitivity to TNF-α-induced cell death in the presence of ActD. The cell death did not occur in the wild-type MEF, indicating that TRAF6 is critical to the protection from cell death. Interesting observation is that the cell death is totally dependent on the accumulation of ROS, which is totally rescued by a phenolic antioxidant reagent, BHA.

While BHA as well as a butylated hydroxytoluene (BHT) exerts cytotoxicity and induces apoptosis in some leukemic cells, including HL-60 [20] or U937 cell lines [21], these phenolic antioxidants, which also work as lipid peroxide quenchers, suppress TNF cytotoxicity [22], possibly dependent on the sensitivity of the cells to the type and amounts of radicals. Similarly, TNF-α-induced programmed cell death can also be suppressed by BHA [23,24]. It has been well-recognized that if NF-κB activation is prevented, TNF-α can induce either apoptotic or necrotic cell death [25]; in addition, ROS is involved in the TNF-α-induced programmed cell death [25]. Recently,

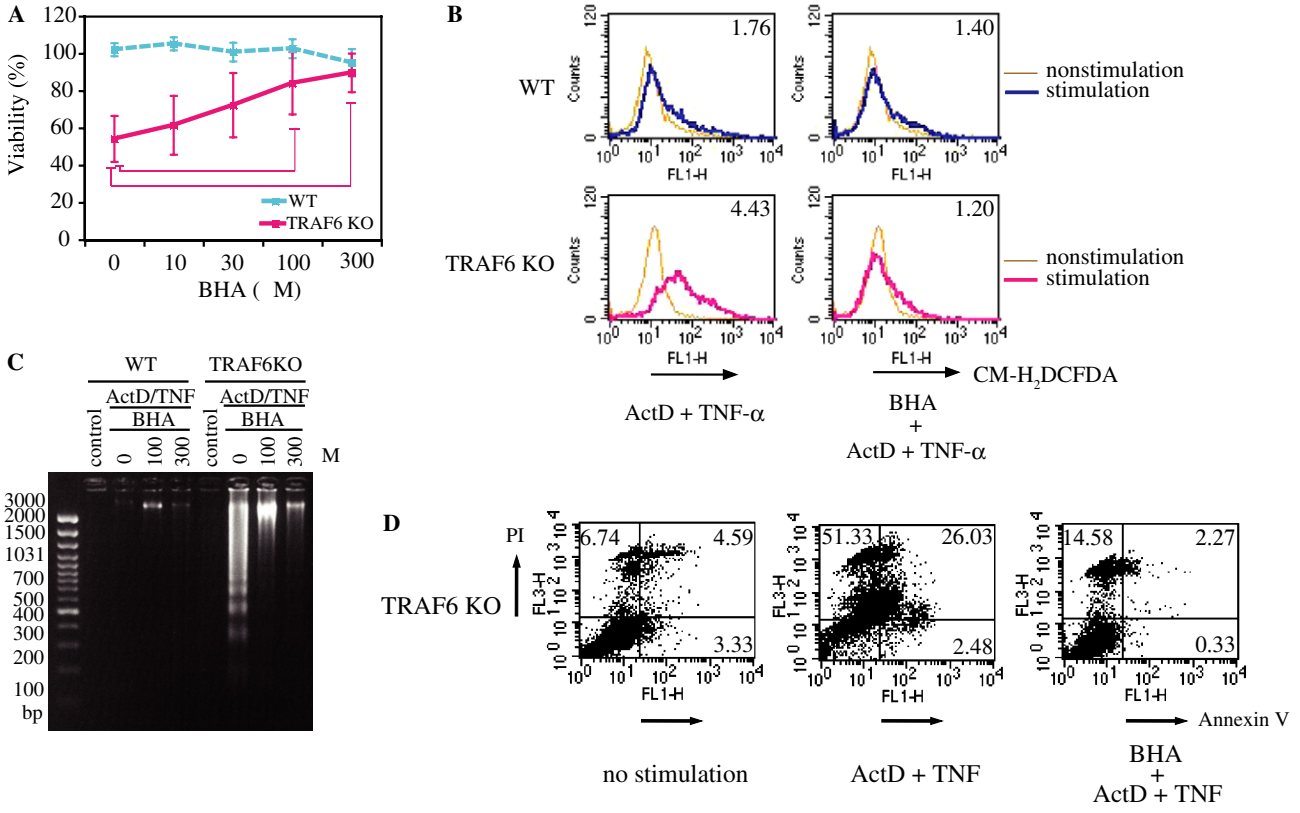

Fig. 3. An antioxidant, BHA suppressed ActD plus TNF-α-induced cell death in TRAF6KO MEF. Wild-type and TRAF6KO MEFs were pretreated with BHA (300 μM or indicated doses) for 30 min, and ActD for 30 min, followed by the stimulation with TNF-α (1 ng/ml). (A) Cell viability was determined at 16 h, as a function of doses of BHA. Cell survival was determined with MTT assay and the data were expressed as means ± SD ($n = 3$). (B) ROS accumulation was determined at 2 h in the absence or presence of 300 μM BHA. (C) DNA fragmentation. (D) Annexin V and PI staining in the absence or presence of 300 μM BHA, was determined at 8 h.

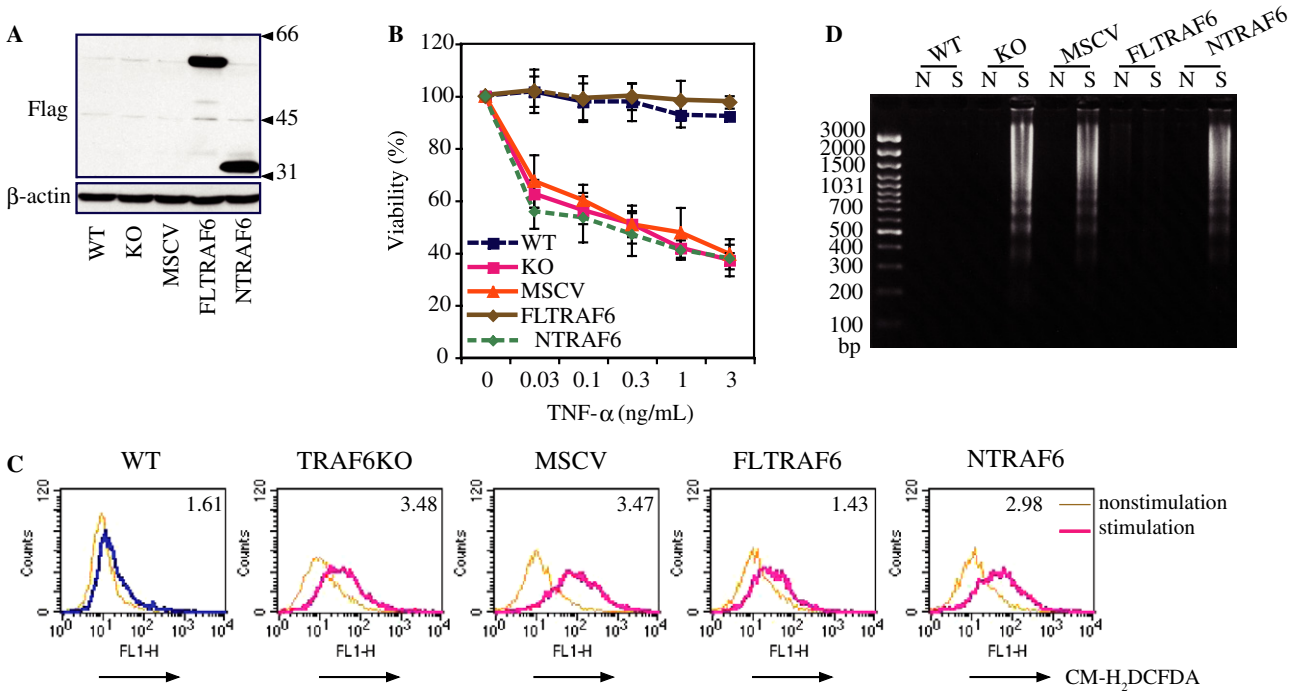

Fig. 4. Ectopic expression of TRAF6 reversed ActD plus TNF-α-induced cell death in TRAF6KO MEF. Retrovirus MSCV-IRES-PURO vector carrying each cDNA for wild-type or mutant TRAF6 was transfected in the TRAF6KO MEF. (A) Total cell lysates were blotted with anti-flag Ab. (B,C) Wild-type (WT), TRAF6KO, MSCV vector control (MSCV), full-length TRAF6 (FLTRAF6), and ΔNTRAF6 were pretreated with or without ActD for 30 min and then stimulated with TNF-α at the indicated doses. (B) Cell survival, determined at 16 h. (C) ROS accumulation at 2 h. (D) DNA fragmentation, determined at 8 h.

it has also been suggested that TNF-α-induced ROS are responsible for sustained JNK activation [26]. Concomitant to these notions, Kamata et al. [17] demonstrated that the inhibition of ROS accumulation preserves MAPK phosphatase activity, blocks sustained JNK activation, and prevents ConA-induced liver destruction. Our observation indicated that the TNF-α-induced cell death in TRAF6KO MEF is totally ROS-dependent. While typical DNA fragmentation was observed in the cell death induced by the TNF-α in the presence of ActD, this type of cell death was presumed to contain both apoptotic and necrotic types as was shown in the PI and Annexin V staining patterns (Fig. 1C and D). TNF-α can induce either apoptotic or necrotic cell death, which can be blocked by BHA in our experiments, although BHA was shown to mainly suppress TNF-α-induced necrosis [26]. Concomitant to our notion, it has been also reported that BHA suppressed TNF-α-induced both apoptosis and necrosis of IKKβKO fibroblasts [17].

Activation of death receptor TNFR1 by TNF-α leads to the recruitment of TRADD, which serves as a platform to form various signaling complexes [11]. For instance, TRADD can recruit FADD and lead to caspase 8 activation and apoptosis [12]. However, TNF-α is not cytotoxic to most cells, because TRADD can recruit TRAF2 and RIP to form distinct complexes leading to the activation of NF-κB and JNK [12,13]. It has been well-established that activation of NF-κB serves as a primary mechanism to protect cells against apoptotic stimuli such as TNF-α [15,16]. Therefore, TNF-α-induced apoptosis requires NF-κB inhibition. Alternate choice for the enhanced sensitivity to TNF-α is the use of metabolic inhibitors. On the role of metabolic inhibitors on the TNF-α-induced cytotoxicity, many studies have accumulated but the report by Sanchez-Alcazar et al. [27] should be of interest in that compared to TNF or ActD alone, combination of TNF/ActD or TNF/cycloheximide (CHX) induced a marked increase OF ROS generation as well as intracellular ATP content by L929. However, we should remind that ActD concentration used in this experiment was 0.03 μg/ml, which is far lower dose of 1 μg/ml usually used. Nevertheless, it should be noted that TRAF6 KO MEF is so sensitive to produce ROS by TNF-α in the presence of as low as 0.03 μg/ml ActD, compared with wild-type MEF. How TRAF6 is related to the ROS generation remains largely unknown and should be further explored.

In our study, reconstitution of full-length TRAF6 constructs could reverse TNF-α-induced cell death, ROS accumulation, and DNA fragmentation completely in TRAF6KO MEF (Fig. 4), while neither N-terminal-deleted TRAF6 (ΔNTRAF6) (Fig. 4B–D) nor C-terminal-deleted TRAF6 (ΔCTRAF6) (data not shown) could reverse TNF-α-induced cell death (data not shown), suggesting that full-length TRAF6 is required for protecting from TNF-α-inducing cell death.

The involvement of JNK in TNF-α-mediated cell death appeared to be minimal, since no clear activation of JNK was observed in our system (data not shown), mainly because of the weak stimulating conditions. While the

involvement of JNK in TNF-α-mediated apoptosis is highly controversial [28–30], induction of NF-κB has been shown to inhibit TNF-α-mediated JNK activation and blocking NF-κB results in sustained activation of JNK, which may directly promote TNF-α-mediated apoptosis. Despite the fact that sustained activation of JNK promotes cell death, the molecular basis on how JNK contributes to TNF-α-mediated apoptosis remains to be addressed.

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