TL1A-induced NF-κB Activation and c-IAP2 Production Prevent DR3-mediated Apoptosis in TF-1 Cells*

We recently identified TL1A, an endothelium-derived T cell costimulator and a ligand for tumor necrosis factor receptor superfamily members DR3 and decoy receptor 3 (1). To elucidate the signaling events triggered by TL1A-DR3 interaction and to understand the molecular mechanisms regulating DR3-mediated apoptosis, we have studied the effect of TL1A and an agonistic DR3 monoclonal antibody in human erythroleukemic TF-1 cells, which express DR3 endogenously. TL1A induced the formation of a DR3 signaling complex containing TRADD, TRAF2, and RIP and activated the NF-κB and the ERK, JNK, and p38 mitogen-activated protein kinase pathways. However, TL1A or an agonistic DR3 monoclonal antibody did not induce apoptosis in these cells nor were there detectable levels of FADD or procaspase-8 seen in the signaling complex. Interestingly, DR3-mediated apoptosis was induced in TF-1 cells in the presence of a NF-κB pathway-specific inhibitor but not in the presence of mitogen-activated protein kinase inhibitors, either alone or in combination, suggesting that DR3-induced NF-κB activation was responsible for resistance to apoptosis in these cells. Consistent with this, we found that TL1A significantly increased the production of c-IAP2, a known NF-κB-dependent anti-apoptotic protein, and that the NF-κB inhibitor or cycloheximide prevented its synthesis. Furthermore, inhibition of c-IAP2 production by RNA interference significantly sensitized TF-1 cells to TL1A-induced apoptosis. Our study identifies a molecular mechanism by which TL1A and DR3 regulate cell fate in TF-1 cells.

Death receptors of the tumor necrosis factor receptor (TNFR) superfamily induce programmed cell death or apoptosis that plays crucial roles in immune modulation, organ development, and tissue homeostasis (2–5). Although TRAIL-induced apoptosis through activation of DR4 or DR5 is important in eliminating cancer and virus-infected cells, FasL-mediated Fas activation leads to activation of effector caspases, including caspase-3, -6, and -7, and ultimately cell death. On the other hand, signal transduction induced by TNF binding to TNFR1 first recruits intracellular TNFR-associated death domain (TRADD). As a platform adaptor, TRADD then recruits the adaptor protein FADD, TNFR-associated factor-2 (TRAF2), and receptor interactive protein (RIP) to stimulate apoptosis, mitogen-activated protein kinase (MAPK) activation, and NF-κB activation, respectively. Consistent with their ability to activate different signaling pathways, death receptors such as Fas, DR4, and DR5 preferentially induce apoptosis, whereas TNFR1 mediates cell growth, survival, differentiation, or death depending on the cellular context. From the study of TNF-induced apoptosis, it became apparent that NF-κB is a key regulator of a the life or death decision of a cell because it plays a major role in inhibition of apoptosis by activation of gene expression (9–14). Among the many NF-κB target genes, members of the cellular inhibitor of apoptosis proteins (c-IAP), such as c-IAP1 and c-IAP2, have been shown to prevent cell death by directly inhibiting effector caspases (15–19) and/or inducing degradation of apoptosis inducer Smac/DIABLO (20).

DR3 is another member of the death receptors in the TNFR superfamily, and its expression is mostly restricted to lymphocytes (21–26). Overexpression of DR3 in cell lines induced NF-κB activation and triggered apoptosis (21, 22, 24). DR3 appeared to bind to downstream signal transducers TRAF2, RIP, FADD, and caspase-8 through the association with TRADD (21). Similar to TNFR1, DR3-induced apoptosis is FADD- and caspase-8-dependent in embryonic fibroblasts (27, 28). However, these results were obtained in transient systems where DR3 and/or the signaling molecules were overexpressed. The physiological signal transducers activated by DR3 through engaging its cognate ligand have not been studied. Recently, we have reported the identification and initial characterization of TL1A, a new member of the TNF superfamily of cytokines and a long sought ligand for DR3 (1). Interestingly, we found that 24 h of TL1A treatment of the TF-1 human erythroleukemic line or PHA-activated T cells, which both naturally express DR3, did not result in significant apoptosis. Programmed cell death and lymphocyte homeostasis. Identification of the signaling molecules and signal transduction pathways activated by the death receptors has greatly enhanced our understanding of this death receptor-dependent extrinsic apoptosis pathway as well as the physiological and pathological functions of the death receptors (Refs. 6–8 and references within). Among them, TNFR1, Fas, DR4, and DR5 are the best studied. Binding of FasL or TRAIL to cell surface Fas or DR4 (or DR5), respectively, triggers the association of the intracellular adaptor protein Fas-associated death domain (FADD) to the death domain of the receptor and the activation of caspase-8 and/or caspase-10. These initiator caspases induce a cascade of caspase activation that leads to the activation of effector caspases, including caspase-3, -6, and -7, and ultimately cell death.

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The abbreviations used are: TNF, tumor necrosis factor; TNFR, TNF receptor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; FADD, Fas-associated death domain; TRADD, TNFR-associated death domain; TRAF2, TNFR-associated factor-2; RIP, receptor interactive protein; c-IAP, cellular inhibitor of apoptosis protein; DR, death receptor(s); DR3Δ DD, DR3 death-domain deleted; c-FLIP, FLICE inhibitory protein; XIAP, X chromosome-linked IAP; Bel-2, B-cell lymphoma 2; mAb, monoclonal antibody; CHX, cycloheximide; FBS, fetal bovine serum; SEAP, secreted alkaline phosphatase; siRNA, small interfering RNA; SRE, serum response element.

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death was induced in TF-1 cells when TL1A was used together with a protein synthesis inhibitor, cycloheximide (CHX) (1). Furthermore, the physiological role of DR3 in thymocyte apoptosis was suggested by DR3 knockout studies, in which targeted disruption of DR3 expression in mice led to reduced removal of autoreactive T cells in the thymus (29). To understand the molecular basis for this cell context-dependent apoptosis in TF-1 cells, we investigated the pathways and signaling pathways activated by TL1A-DR3 interaction and studied the roles of these pathways in DR3 apoptosis induction in TF-1 cells. TF-1 cells are CD34− myeloid progenitor cells, and understanding the role of TL1A and DR3 in the growth regulation of these cells could have physiological importance and clinical relevance. Our study demonstrated that in these cells, TL1A binding to endogenous DR3 initiates the formation of a signaling complex containing TRADD, TRAF2, and RIP but no detectable amount of FADD, procaspase-8, or c-IAP1. Although both the NF-κB and all three MAPK pathways were activated by either TL1A or a DR3 agonistic monoclonal antibody (mAb), we found that only DR3-induced NF-κB activation played a major role in cell fate determination in these cells, and it is mediated at least in part through c-IAP2, a known NF-κB-induced anti-apoptosis factor.

**EXPERIMENTAL PROCEDURES**

**Constructs, Recombinant Proteins, and Stable Cell Lines—** Constructs for human DR3 ΔΔ DD, rhTL1A, and DR3-Fc, as well as stable cell lines expressing pcDNA3.1 vector control and DR3 ΔΔ DD, have been described previously (1). Death domain-truncated mouse DR3 (mDR3 DD) or stable cell lines expressing human DR3 (DD or mouse DR3 DD) were maintained in RPMI 1640 medium containing 1% FBS and RPMI 1640 medium containing 1% FBS and 1% nonidet P-40, 150 mM NaCl, 1 mM EDTA, plus 50 mM HEPES, pH 7.5, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, plus 50 mM HEPES, pH 7.5, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, plus 10 mM Na3VO4, and 10 mM NaF) at 4 °C for 30 min. The cell pellet was taken at each time point, spun down, and lysed in 1 ml of lysis buffer (50 mM HEPES, pH 7.5, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, plus 50 mM HEPES, pH 7.5, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, plus 10 mM Na3VO4, and 10 mM NaF) at 4 °C for 30 min. The cell pellets were removed by centrifugation at 14,000 rpm for 10 min. The cell lysates were incubated with 50 μg of F05 mAb or control mIgG1 and 40 μl of protein A/G-agarose beads (Santa Cruz Biotechnology, CA) at 4 °C for 5 h with gentle rotation. The beads were then washed twice with lysis buffer containing 1 mM NaCl and twice again with lysis buffer before boiling in 1× SDS sample buffer for SDS-PAGE and Western blotting.

**SEAP Reporter Assay—** TF-1 cell lines stably expressing the SEAP reporter under the control of NF-κB or SRE enhancer were described previously (31). The cells in log phase growth were washed and grown in RPMI 1640 medium containing 0.2% FBS overnight before being treated with different concentrations of rhTL1A or F05 in the presence or absence of SN50 or SN50M. The supernatants were collected 8–24 h later, and SEAP activity was measured using a Phospha-Light BP300 system (Applied Biosystems).

**Western Blot Analyses of rhTL1A- and F05-induced Activation of Various Signaling Pathways—** TF-1 cells in log phase growth, at 1× 106 cells/ml, were kept in RPMI 1640 medium containing 1% FBS for overnight. The cells were then treated with various amounts of rhTL1A, F05, or control proteins for different time points. At the end of each time point, 1 ml of cells were removed and spun at 5000 rpm for 1 min before being boiled in 100 μl of SDS sample buffer. The signaling molecules were separated on 12% SDS-PAGE, transferred to nitrocellulose membranes, and blotted with primary antibodies (1 μg/ml) followed by secondary antibodies (1:1000 dilution) and detected with ECL kit (Amersham Biosciences). Each membrane was stripped and probed for up to five times.

**Western Blot Detection of Anti-apoptotic Proteins in TF-1 Cells—** TF-1 cells in log phase growth were washed and suspended in RPMI 1640 medium containing 1% FBS and granulocyte-macrophage colony-stimulating factor (2 ng/ml) at 1× 106 cells/ml. The cells were treated with buffer or rhTL1A (500 ng/ml) for different time points or for 3 h with or without inhibitor CHX (10 μg/ml), SN50 (100 μg/ml), or SN50M (100 μg/ml). Aliquots of cells were removed at each time point and lysed with RIPA buffer (50 mM HEPES, pH 7.5, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1× protease inhibitor mixture, 10 mM Na3VO4, and 10 mM NaF) at 4 × 106 cells/ml. The lysates were then removed of cell debris and analyzed by SDS-PAGE and Western blotting.

**c-IAP2 siRNA Generation and Electroporation—** Twenty-one-nucleotide double-stranded siRNAs corresponding to nucleotides 387–407 (siRNA-1, 5′-aagcagatactgtggg3′) and 997–1017 (siRNA-2, 5′-aagtcaggcttcgctctc3′) of the human c-IAP2 coding region and non-silencing siRNA control (5′-aaccgttgcaagtggctc3′) were purchased from Qiagen. 1.5 nmol of siRNA was transfected into 1.5 × 106 cells in 0.5 ml of cold RPMI-1640 medium by electroporation using a Gene Pulser Electroporator II (Bio-Rad) at 250 V and 400 microfarads.

**Gene Pulser Electroporator II (Bio-Rad) at 250 V and 400 microfarads.**
TL1A-induced NF-κB Activation and c-IAP2 Production

Escherichia coli-produced rhTL1A (amino acids 72–251), anti-DR3 mAb F05 potently induced caspase activation (Fig. 1B) and apoptosis (Fig. 1C) in TF-1 cells in the presence of 10 μg/ml of CHX, whereas an isotype control mouse IgG1 (mIgG1) or the heat-inactivated rhTL1A (TL1A HI), mAb F05, or mIgG1 in the presence of CHX (10 μg/ml) for 6 h. Caspase activity was measured as described under “Experimental Procedures.” The results are expressed as averages of triplicate reactions plus standard errors. The data were analyzed and graphed using GraphPad Prism 3.0 software. C, effect of TL1A and anti-DR3 mAb F05 on apoptosis in TF-1 cells. TF-1 cells (1 × 10^6 cells/ml) were treated with TL1A, F05, or mIgG1 (0.2 μg/ml each) in the presence or absence of CHX (10 μg/ml) for 5 h before staining with annexin V and propidium iodide (Molecular Probes). The relative percentage of annexin V-only positive cells is shown.

TL1A-DR3 Signaling Complex Formation—Although DR3 has been shown to interact with TRAF2, RIP, FADD, and caspase-8 through TRADD in transient systems (21), DR3-induced signaling complexes have not been studied in cells that naturally express DR3. To identify the physiological signal transducers recruited to DR3 immediately after TL1A binding, we treated TF-1 cells with rhTL1A for various time periods (0, 5, 15, 30, and 60 min), and the DR3 signaling complex was immunoprecipitated from total cell lysates with antibody F05 or control mIgG1. After analysis of the precipitated protein content by Western blotting, we consistently found that TRADD, the platform adaptor, and signal transducers TRAF2 and RIP were recruited to DR3 (Fig. 2 and data not shown). This DR3 signaling complex can be detected as early as 5 min after TL1A stimulation and remains intact for at least 60 min (Fig. 2). Interestingly, more RIP proteins appear to be recruited to the complex at the 60-min time point than any of the earlier time points. Very little, if any, FADD, procaspase-8, or c-IAP1 protein was detected in the same complex, whereas significant amounts of these molecules were present in TF-1 cells (Fig. 2, compare Input lane with others). We also noted that untreated TF-1 cells lack a detectable amount of the c-IAP2 protein (see Figs. 6 and 7). Our finding indicates that a physiological DR3 signaling complex in TF-1 cells contains TRADD, TRAP2, and RIP but not significant amounts of FADD, procaspase-8, or c-IAP1.

TL1A and Anti-DR3 Activate NF-κB and MAPK (ERK, JNK, and p38) Pathways in TF-1 Cells—It is known that TRADD, RIP, and TRAF2 are key effectors transducing signals from TNFR1 to downstream mediators that lead to the activation of NF-κB and MAPK, particularly JNK (c-Jun N-terminal kinase, also known as stress-activated protein kinase) (Refs. 6–8 and references within). To explore the DR3 signal transduction pathways in cells naturally expressing this receptor and to compare the effect of rhTL1A with DR3 mAb F05, we analyzed the phosphorylation of pathway-specific kinases or substrates in TF-1 cells. As expected, granulocyte-macrophage colony-stimulating factor, a positive control for the experiment, showed strong activation of ERK1,2 in TF-1 cells, whereas TNF, a known pleiotropic cytokine, induced activation of all pathways tested (Fig. 3A). Similar to TNF, rhTL1A activated not only the NF-κB pathway, indicated by IkBα degradation, but also JNK, p38, and ERK1,2 MAPK (Fig. 3A). All four signaling pathways were activated in a dose- and time-dependent manner, because 1 μg/ml of rhTL1A induced stronger phosphorylation than the 0.1 μg/ml dose. Different kinases/substrates were activated at different time points. Noticeably, activation of NF-κB and ERK1 and ERK2 were evident at 30 min and lasted till at least 90 min post-treatment, whereas JNK was activated later at the 60- or 90-min time point. Activation of the p38 MAPK appeared to be relatively weak and
transient (peaked at 10 min and disappeared at 30-min time point). Remarkably, signal transduction induced by the DR3 agonistic antibody F05 gave results very similar to those induced by rhTL1A, strongly suggesting that TL1A signals mainly through the DR3 receptor in TF-1 cells.

To determine whether TL1A-mediated signal transduction can transduce signals to the nucleus and induce pathway-specific gene expression, we examined the effect of rhTL1A and F05 on reporter gene activation in stably transfected TF-1 cells, in which the SEAP reporter gene is regulated either by the NF-κB enhancer or the serum response element (SRE) (31). Because SRE-dependent transcription is regulated by ERK, JNK, and p38 MAPK (32, 33), the SRE-SEAP reporter can be used to measure the combined effect of these three MAPK. When TF-1/NF-κB-SEAP cells were used, both rhTL1A and F05 induced SEAP production similarly (up to 5-fold at 100 ng/ml) in a dose-dependent manner, whereas no activation was seen with heat-inactivated rhTL1A or isotype control antibody (Fig. 3B). Similar results were seen when TF-1/SRE-SEAP cells were used. A 5–6-fold induction of SEAP activity was observed with 100 ng/ml of either rhTL1A or F05 (Fig. 3B). These results demonstrated that TL1A-DR3 interaction activated the NF-κB and MAPK pathways and led to pathway-specific gene expression.

Fig. 3. DR3 signal transduction in TF-1 cells. A, signaling pathways activated by rhTL1A and F05. TF-1 cells (1 × 10⁶ cells/ml) were treated with buffer alone, rhTL1A (0.1 and 1 μg/ml), anti-DR3 mAbs F05 (0.1 and 1 μg/ml), or control antibody (mIgG1, 1 μg/ml) for 10, 30, 60, or 90 min, and the signaling molecules were detected by Western blotting with specific antibodies. Granulocyte-macrophage colony-stimulating factor (5 ng/ml) and TNF (20 ng/ml) were used as positive controls in the same experiment. The results were from three blots stripped and then probed with each of the antibodies shown. One of two experiments with similar results is shown. B, TL1A-DR3 interaction induces pathway-specific gene expression. Stable TF-1/NF-κB-SEAP and TF-1-SRE-SEAP cells were incubated with 0–1000 ng/ml of rhTL1A, heat-inactivated rhTL1A (TL1A HI), anti-DR3 mAb F05, or control mIgG1 for 24 h. SEAP reporter activity was then measured from culture supernatant. The results are shown as the averages of triplicate reactions with standard errors.

Effect of NF-κB and MAPK Inhibitors on DR3-induced Apoptosis in TF-1 Cells—To understand the specific role of each of the signaling pathways activated by DR3 in the life and death decision making of the cell, we examined the apoptotic effect of pathway-specific inhibitors in the presence or absence of the DR3 agonistic antibody F05 in TF-1 cells. A time course (3, 6, 12, and 18 h) analysis was carried out to monitor the progression of DR3-mediated apoptosis. The NF-κB pathway-specific peptide inhibitor SN50 (34) was chosen for this study because it specifically inhibited TL1A-mediated NF-κB activation in TF-1/NF-κB-SEAP cells, whereas the SN50 control peptide SN50M had no effect (Fig. 4A). As shown in Fig. 4 (B and C), F05 (open bars), but not the control antibody mIgG1 (shaded bars), potently induced caspase activation (up to 15-fold) when
SN50 or CHX was present for 3–6 h. No significant caspase activation was observed in the presence of the other inhibitors, including SN50M, MEK1 inhibitor PD98059 (35), MEK1 and 2 inhibitor U0126 (36), JNK inhibitor SP600125 (37), and p38 MAPK inhibitor SB203580 (38), alone or in combination, when compared with untreated cell controls (Fig. 4, B and C). A similar result was seen for the 12-h time point, although the background caspase activity of untreated cells had increased and the nonspecific toxic effect of some of the inhibitors (SP and SN50) became evident (Fig. 4D). At the 18-h time point, only samples treated with CHX or a combination of SN50, PD98059, SP600125, and SB203580 (SN50 + 3mix) gave DR3-specific caspase activation, whereas any other conditions showed caspase activation independent of F05 (Fig. 4E). Interestingly, we consistently observed that the presence of the three MAPK inhibitors significantly reduced the toxic effect of SN50 to the TF-1 cells (compare shaded bars of SN50-treated with SN50 + 3mix-treated samples in all four time points). Similar results

**Fig. 4.** Time course study of effect of pathway-specific inhibitors on DR3-specific caspase activation in TF-1 cells. A, SN50 inhibits TL1A-mediated NF-kB activation in TF-1/NF-kB-SEAP stable cell line. SN50 or SN50M (0–150 μg/ml) was incubated with or without rhTL1A (0.2 μg/ml) for 8 h, and the SEAP activity was measured from the supernatant. B–E, TF-1 cells were treated with anti-DR3 mAb F05 (0.5 μg/ml, open bars) or control antibody (mIgG1, 0.5 μg/ml, shaded bars) in the presence or absence of CHX (10 μg/ml), PD98059 (PD, 20 μM), SP600125 (SP, 20 μM), SB203580 (SB, 20 μM), U0126 (U0, 20 μM), SN50 (150 μg/ml), SN50M (150 μg/ml), or different combinations of these inhibitors for different time points. A, 3 h; B, 6 h; C, 12 h; D, 18 h. 3Mix, PD + SB + SP. Total caspase activity was then measured. The results are shown as the averages of triplicate reactions with standard errors.
were seen when rhTL1A protein was used instead of mAb F05 (data not shown). These experiments demonstrated that NF-κB is the major protective pathway activated by DR3 that protects cells from undergoing apoptosis.

**Induction of Anti-apoptotic Factor c-IAP2 by rhTL1A through NF-κB Activation**—To understand the molecular mechanism by which NF-κB protects cells from DR3-induced apoptosis, we analyzed NF-κB target gene products that have previously been shown to play a role in inhibiting apoptosis in TL1A- or F05-treated TF-1 cells (14). Among the many anti-apoptotic factors tested, including c-IAP1, c-IAP2, c-FLIP (FLICE-inhibitory protein), XIAP (X-chromosome-linked IAP), Bcl-2 (B-cell lymphoma 2), Bcl-XL, and TRAF2, we discovered that only c-IAP2, which was undetectable in untreated TF-1 cells, was significantly induced by TL1A or F05 (Fig. 5 and data not shown). c-IAP2 was detected as early as 2 h after TL1A treatment and continued to accumulate till the last time point tested (8 h). We also monitored the level of SODD (silencer of death domain), a negative regulator implicated in the control of TNFR1-induced spontaneous apoptosis (39, 40), but failed to detect any dramatic change when compared with actin internal control (Fig. 5). c-IAP2 was shown previously as a direct target gene of NF-κB pathway (41). Consistent with this, we found that both the NF-κB pathway inhibitor SN50 and a general protein synthesis inhibitor, CHX, blocked the induction of c-IAP2 protein in TL1A-treated TF-1 cells, whereas the control peptide SN50M had no effect (Fig. 6). The disappearance of the c-IAP2 protein coincided with the cleavage of initiator caspase-8 and effector caspase-3 (only the cleaved caspases are shown) and, hence, induction of apoptosis in these cells (Fig. 6). Interestingly, we also found that the level of XIAP was reduced in SN50- or CHX-treated samples in the presence of TL1A versus in the absence of TL1A.

**Inhibition of c-IAP2 Production by Small Interfering RNA Induces Caspase Activation in DR3-activated TF-1 Cells**—To analyze the direct role of c-IAP2 in TL1A and DR3 controlled cell fate determination, two c-IAP2-specific 21-nucleotide siRNAs were generated and introduced into TF-1 cells by electroporation (see “Experimental Procedures”). Both siRNA-1 (corresponding to nucleotides 387–407 of the c-IAP2 coding sequence) and siRNA-2 (nucleotides 997–1017 of c-IAP2 cDNA) inhibited c-IAP2 production in TF-1 cells induced by rhTL1A but had no effect on the actin control (Fig. 7A). In contrast, a control siRNA, siRNA-C, did not inhibit c-IAP2 production in these cells, demonstrating the specific effect of the c-IAP2 siRNAs. When transfected into TF-1 cells in the presence of rhTL1A or DR3 agonistic antibody F05, c-IAP2-specific siRNA-1 and to a lesser extent, c-IAP2-specific siRNA-2, significantly induced the caspase activity of the transfected cells but were inert in the absence of these proteins (Fig. 7B). These results demonstrated that inhibition of c-IAP2 synthesis alone greatly sensitized TF-1 cells to TL1A and DR3-induced cell death.

**DISCUSSION**

The life or death decision of a cell is controlled by various anti- and pro-apoptotic factors in response to different biological, chemical, and physical stimuli. Interaction of the TNF family of ligands with their cognate receptors lead to cell growth, differentiation, survival, or death depending on the cellular context and the nature of the ligand and receptor interaction (4). Recent studies on the signaling mechanisms of the death receptors have revealed that members of the NF-κB and caspase families are key regulators of this life and death decision making. The balance between these positive and negative regulators ultimately determines the cell fate (5, 7, 14). Activation of Fas and DR4 (or DR5) triggers efficient apoptosis but not cell survival because these death receptors recruit mostly FADD and caspase-8, key pro-apoptotic mediators. Fas does not engage mediators like TRADD, RIP, or TRAF2 and therefore is a poor activator of NF-κB and AP-1. TNFRI, on the other hand, is a strong activator of NF-κB and AP-1 because of its ability to assemble TRADD-RIP and TRADD-TRAF2 signaling complexes efficiently. Consequently, TNF is a weak inducer of apoptosis in normal cells, and programmed cell death occurs usually when the NF-κB pathway or general protein synthesis is inhibited (5, 7, 14).

In this paper, we studied death receptor DR3 to reveal its physiological signal transducers and signaling events as well as to understand the molecular mechanisms controlling its cell fate determination. We chose human erythroleukemic cell line TF-1 as our model system because these cells express DR3 naturally and share similar responses (NF-κB activation and lack of apoptosis without CHX) to TL1A as human activated T cells (1). Although normal human T cells express DR3, none of the human T cell lines, including H9, Jurkat, Molt-3, Molt-4, and Sup-T1 have detectable levels of DR3 (25).2 Our study demonstrated that TL1A-DR3 signaling in TF-1 cells resulted in the formation of signaling complexes containing TRADD,
TRAF2, and RIP and the activation of the NF-κB and MAPK pathways. Because RIP and TRAF2 are essential effectors in TNF-induced NF-κB and JNK activation, respectively, it is reasonable to believe that they exert similar functions in TL1A-induced NF-κB and MAPK activation. The signaling molecules downstream of TRAF2 and RIP that mediate the activation of these pathways, in particular the ERK and p38 MAPK pathways, remain to be elucidated.

In contrast to previous findings using transient systems, we did not detect significant amount of FADD or procaspase-8 association with the DR3 signaling complexes in TF-1 cells. One explanation could be that in these cells under the conditions used, DR3 engaged TRADD proteins form a more stable complex with TRAF2 and RIP than with FADD or procaspase-8. However, changing the immunoprecipitation condition by eliminating the high salt washing step did not affect the outcome nor did FADD association observed at later time points (2–3 h after TL1A treatment). Alternatively, mechanisms to inhibit FADD and procaspase-8 binding to TRADD may exist, either by regulatory molecules physically blocking this association or preferentially enhancing the TRAF2 and RIP binding to TRADD. The fact that the presence of a NF-κB inhibitor or a general protein synthesis inhibitor quickly induced apoptosis suggests that these regulatory molecules have short half-lives and that their degradation allows the association of FADD and caspase-8. Because both FADD and caspase-8 are required for DR3-induced apoptosis (27, 28), the lack of efficient recruitment of these factors to DR3 may provide an early but important step toward cell survival in TF-1 cells. It also infers that DR3-mediated NF-κB and MAPK activation occurs independently of apoptosis induction in these cells. Because NF-κB and MAPK activation generally results in gene activation and protein synthesis, these signaling events further shift the balance toward cell survival instead of death in TF-1 cells. It remains to be seen whether these mechanisms apply to human peripheral T cells and developing thymocytes; both express DR3 but respond differently to targeted inactivation of DR3 (29). Although DR3 knockout mice show impaired negative selection in the thymus, demonstrating a crucial role of DR3 in thymocyte apoptosis, the peripheral lymphocyte populations are not affected. It is likely that developing thymocytes utilize different molecular mechanisms from peripheral T cells in responding to DR3 activation.

Studies on mice deficient in NF-κB subunits have clearly shown that this transcription factor is a major anti-apoptotic regulator in TNF-TNFRI signaling (9–14). Cells lacking NF-κB became very sensitive to TNF and underwent apoptosis, whereas wild-type cells were resistant. Our current study extended this knowledge by showing that NF-κB also plays a dominant anti-apoptotic role in TL1A-DR3 signaling. The addition of a NF-κB-specific inhibitor to TL1A- or F05-treated TF-1 cells is sufficient to induce caspase activation and programmed cell death. Clearly, continued transcription and synthesis of certain proteins are required for maintaining the state of cell survival for DR3-activated TF-1 cells. These factors could function as inhibitors for TRADD and FADD binding and/or for other death inducers including initiator and effector caspases. Blockade of this continued protein synthesis quickly tilted the balance toward apoptosis because maximal caspase activity was seen 3 h after SN50 addition (Fig. 4A). The role for MAPK in TNF-mediated apoptosis is controversial, because both pro- and anti-apoptotic activities were reported for JNK and p38 MAPK (8, 14). However, more studies favor the view that JNK activation is independent of TNF or FasL-induced apoptosis (10, 42). In line with this, our study demonstrated that none of the three MAPKs played a significant anti-apoptotic role in TL1A-mediated cell death in TF-1 cells. Nonetheless, more studies are needed to dissect the exact role of these MAPKs in TL1A-DR3 signaling.

It is known that NF-κB exerts its anti-apoptotic activity through the induction of many anti-apoptotic factors. These include c-IAP1, c-IAP2, c-FLIP, TRAF1, TRAF2, XIAP, Bcl-2, and Bcl-xL. Among them, c-IAPs are the best studied. They inhibit apoptosis induced by both the mitochondria-dependent and the death receptor-mediated death pathways through direct binding and inhibiting of death inducers (19). Interestingly, we found that in TF-1 cells, TL1A potently and specifically induces the synthesis of c-IAP2 (Fig. 5), a direct target gene product of NF-κB (41). Inhibition of c-IAP2 synthesis by RNA interference technology sensitized these cells to TL1A- and F05-induced cell death (Fig. 7), demonstrating a direct role of c-IAP2 in DR3-mediated cell fate determination in TF-1 cells. Intriguingly, c-IAP2 has been found to enhance NF-κB activation (41). This positive feedback on NF-κB signal transduction could contribute to signal amplification and ultimately cell survival. The effect of TL1A on the expression of other protective factors, including c-IAP1, c-FLIP, XIAP, TRAF2, Bcl-2, and Bcl-xL, is not apparent. However, a reduced level of XIAP was seen when TL1A was used together with SN50 or CHX (Fig. 6), suggesting that XIAP may also play a role, either independently or coordinated with c-IAP2, in the battle against apoptosis. The exact mechanisms by which c-IAP2 inhibits...
TL1A-DR3-induced apoptosis need further investigation. The evidence provided by this study clearly showed that DR3 is most analogous to TNFR1 because they employ similar signal-transduction mechanisms. DR3 appears to mediate apoptosis induced by TL1A. However, the mechanism of action of TL1A-DR3-induced apoptosis need further investigation. The manuscript and helpful discussions.

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