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Immunity, Vol. 11, 783–793, December, 1999, Copyright ©1999 by Cell Press

Regulated Commitment of TNF Receptor Signaling: A Molecular Switch for Death or Activation

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Summary

Tumor necrosis factor receptor (TNFR) superfamily members can induce a context-dependent apoptosis or cell activation. However, the mechanisms by which these opposing programs are selected remain unclear. We show that in T cells, TNFR2 (TNFRSF1B) signaling is dramatically affected by the intracellular mediator RIP, a protein Ser/Thr kinase required for NF-κB activation by TNFR1 (TNFRSF1A). In the presence of RIP, TNFR2 triggers cell death, whereas in the absence of RIP, TNFR2 activates NF-κB. RIP is induced during IL2-driven T cell proliferation, and its inhibition reduces susceptibility to TNF-dependent apoptosis. Evidence that signaling outputs are shaped by intracellular constraints helps reconcile conflicting views of TNFR1 and TNFR2 as apoptotic mediators.

Introduction

To date, the relative roles of TNFR1 and TNFR2 in TNF-induced cell death have proven difficult to establish unambiguously. Substantial evidence supports the view that engagement of TNFR1 can trigger apoptosis in many cell types either in the absence or presence of inhibitors of RNA or protein synthesis (Engelmann et al., 1990; Tartaglia et al., 1991, 1993a). These inhibitors are now thought to unmask or potentiate cell death by preventing the de novo induction of an NF-κB-dependent antiapoptotic response (Beg and Baltimore, 1996; Liu et al., 1996; Van Antwerp et al., 1996; Wang et al., 1996). Although in many cells TNFR2 delivers signals that activate inflammation programs (Tartaglia et al., 1991; Vandenabeele et al., 1992; Rothe et al., 1994), engagement of TNFR2 in some circumstances has been found to have proapoptotic effects of varying magnitude, ranging from stimulation of TNFR1-mediated apoptosis (Grell et al., 1995; Vandenabeele et al., 1995; Weiss et al., 1997) to seemingly autonomous induction of cell death (Heller et al., 1992; Grell et al., 1993, 1995; Haridas et al., 1998). In the search for resolution of these apparent contradictions, explanations based on the different activities of membrane versus soluble TNF (Grell et al., 1995) or on the endogenous expression of membrane TNF (Grell et al., 1999) have been advanced. However, an equally consistent possibility is that the outcome of TNF signaling cannot be completely explained by fixed signal transduction properties ascribed to each receptor.

Evidence that TNF plays a dualistic role in activated T cells, which principally express TNFR2 (Ware et al., 1991; Zheng et al., 1995), is particularly relevant. On resting or submaximally stimulated naive cells, TNF promotes proliferation (Yokota et al., 1988) through a pathway involving TNFR2 (Gehr et al., 1992; Tartaglia et al., 1993b; Grell et al., 1998). However, late in activation, TNF induces apoptosis (Sarin et al., 1995), and a TNFR2-dependent cell death pathway has been described that affects activated CD8-positive cells (Zheng et al., 1995; Alexander-Miller et al., 1998; Herbein et al., 1998). Susceptibility to death is controlled by prior activation, so that T cells that have been exposed to IL-2 are prone to apoptosis, but resting cells are not (Lenardo, 1991).

A consensus signaling pathway for TNFR1-mediated apoptosis has been proposed and recently reviewed (Ashkenazi and Dixit, 1998). The intracellular portion of the receptor contains a death domain (DD) (Tartaglia et al., 1993c) and binds other DD-containing proteins upon binding of ligand. A multiprotein complex including the DD-containing signaling effectors TRADD and RIP has been shown to form at the receptor upon TNF engagement (Hsu et al., 1995, 1996a, 1996b; Stanger et al., 1995). Death domain proteins like FADD/MORT1 (Boldin et al., 1995; Chinnaiyan et al., 1995) and RAIDD (Duan and Dixit, 1997) may also be aggregated to the signaling complex and have been reported to interact through N-terminal death effector domains with caspases to activate their proteolytic activity and induce apoptosis (Thornberry and Lazebnik, 1998).

The mechanism of TNFR2-mediated cell death, however, remains understudied. Unlike TNFR1 and CD95, TNFR2 lacks a death domain, and although members of the TNF receptor–associated factor (TRAF) family of proteins (Rothe et al., 1994) have been shown to play a role in cell death induced by receptors lacking a death domain (Lee et al., 1996; Force et al., 1997), the precise effectors mediating activation of the apoptotic machinery by TNFR2 are presently unknown. We show here that in a human T cell line TNFR2-mediated death requires RIP and that the induction of RIP during human T cell activation promotes a developmental change in TNFR2 signaling from NF-κB activation to apoptosis.

Results

Jurkat (human T cell leukemia) cells express TNFR1 but not TNFR2 (Ting et al., 1996) and are largely insensitive to TNF-induced cell death (Figure 1B). To explore the apoptotic effects of TNF in this system, we created cell lines that express TNFR2 (Smith et al., 1990) or a mutated form of IκB-α (IκB.DN) that behaves as a dominant-negative regulator of NF-κB activation (Brockman et al., 1995). Figure 1A documents the expression of these molecules in the resulting transfected cell lines.

In accord with results from previous reports (Wallach, 1984; Tartaglia et al., 1991; Van Antwerp et al., 1996), preincubation of cells with cycloheximide (CHX) unmasks the apoptotic activity of TNFR1 (Figure 1B), and Jurkat cells expressing IκB.DN rapidly succumb to TNF-induced apoptosis (Figure 1C). However, expression of

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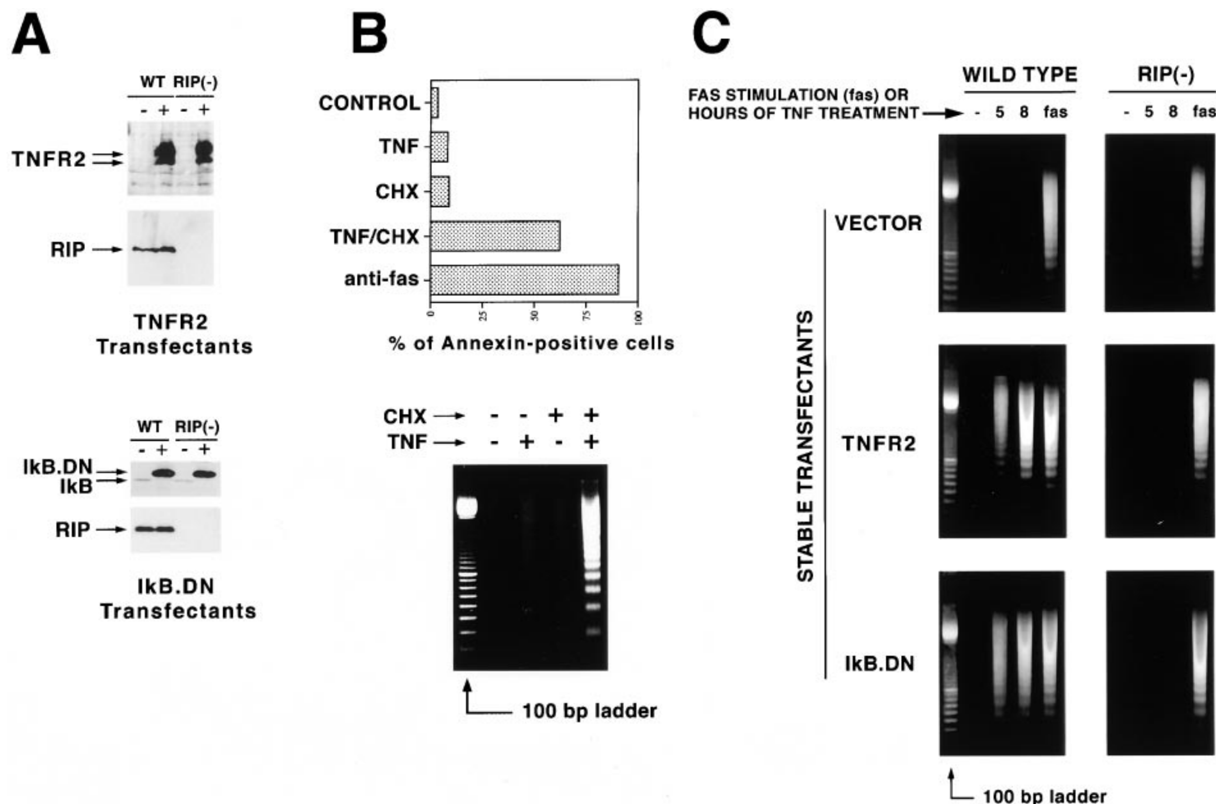


Figure 1. TNF-Mediated Apoptosis of Jurkat Transfectants Expressing TNFR2 or IκB.DN

(A) Immunoblot analysis of transfectant cell lines. Wild-type (WT) and RIP^- Jurkat cells were stably transfected with vectors either without insert (−) or expressing TNFR2 or a dominant-negative form (IκB.DN) of IκB-α (IκB-α S32A, S36A) (+). The presence of two TNFR2 bands is likely due to glycosylation. Both endogenous and transfected IκB-α were detected by the antibody used. The DN form has a peptide tag at the amino terminus that gives it a slightly higher molecular mass. IκB.DN expression blocked NF-κB activation induced by a wide variety of stimuli (data not shown). The blots were reprobed with an anti-RIP antibody to show that levels of RIP were unaffected by transfection.

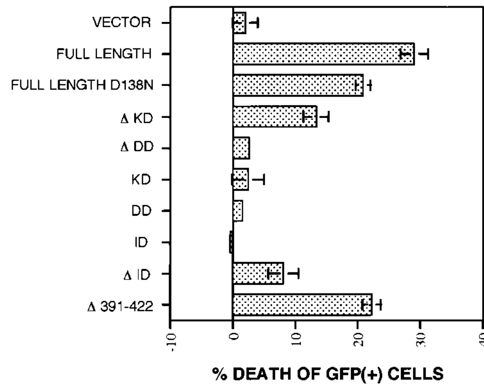
(B) Cycloheximide (CHX) unmasks TNFR1-mediated cell death in Jurkat cells. When indicated, cells were preincubated for 1 hr in 10 μg/ml of cycloheximide, and, after that, TNF (20 ng/ml) or anti-fas/CD95 (100 ng/ml) were added for 6 hr. Upper panel: cells were stained with Annexin-V-FITC, and the percentage of Annexin-V-positive cells was measured by flow cytometry. Lower panel: cells were lysed, and low molecular weight DNA was fractionated on a 1% agarose gel.

(C) Cells expressing TNFR2 or IκB.DN undergo RIP-dependent apoptosis. Cells were cultured for the indicated times in the absence or presence of 20 ng/ml of TNF or for 5 hr in the presence of 100 ng/ml of anti-CD95 antibody. Low molecular weight DNA was isolated and analyzed as in (B). Annexin-V stainings confirmed these results and showed that cell death was always greater than 40% in those experimental points showing DNA ladder formation (data not shown). Cell death always reached more than 80% when treatments were done overnight (data not shown). Similar results were obtained when CHX was used instead of IκB.DN as a sensitizing agent (data not shown).

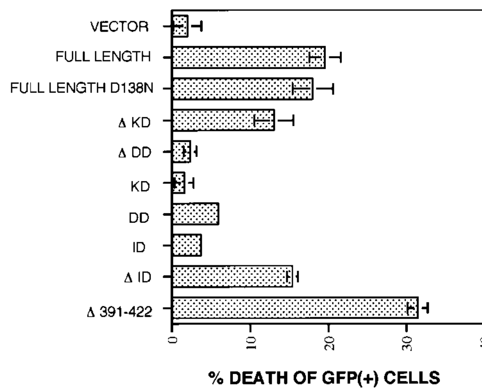
TNFR2 in Jurkat cells renders them sensitive to TNF-dependent cell death in the absence of cycloheximide or IκB.DN (Figure 1C), suggesting the existence of a death pathway resistant to NF-κB activation. An alternative explanation might be that the presence of TNFR2 compromises the efficacy of the antiapoptotic program that prevents TNFR1 from inducing cell death, for instance by blocking the protective pathway itself or by inhibiting NF-κB activation.

While the Ser/Thr kinase RIP (Stanger et al., 1995) is required for TNFR1-mediated NF-κB induction, it has not been found necessary for CD95-mediated cell death (Ting et al., 1996; Kelliher et al., 1998), and any potential role it might play in TNF-dependent death in Jurkat cells might be masked by the strong protective effect of NF-κB activation (Beg and Baltimore, 1996; Liu et al., 1996; Van Antwerp et al., 1996; Wang et al., 1996). Although the parental cells bearing IκB.DN were highly susceptible, RIP-deficient cells (Ting et al., 1996) expressing IκB.DN were found to be highly resistant to TNF-induced cell death (Figure 1C), indicating that RIP is required for TNFR1-dependent apoptosis in this setting. RIP-deficient Jurkat cells expressing TNFR2 also did not die when exposed to TNF but retained the capacity to undergo apoptosis when triggered by CD95 (Figure 1C). Thus, cell death induced by TNF seems to require RIP both in IκB.DN- and TNFR2-expressing Jurkat cells.

Although RIP-null cells do not undergo apoptosis, it remains possible that the mutant cell line harbors other mutations that are responsible for the death-resistance phenotype. To show that RIP itself is sufficient to correct the phenotypic defect, we reintroduced it into RIP-deficient cells by transfection. As shown in Figure 2, transient transfection of wild-type RIP into RIP^- cells expressing TNFR2 or IκB.DN resulted in cell death after TNF treatment. Various mutated forms of RIP (Ting et al., 1996) were transfected into the RIP^- cell lines to establish which elements of RIP are required for apoptosis.



RIP (-) CELLS EXPRESSING TNFR2



RIP(-) CELLS EXPRESSING IkB.DN

Figure 2. Transfected RIP Restores TNF-Induced Death in RIP$^-$ Cells Expressing Either TNFR2 or IκB.DN

RIP$^-$ cells expressing either TNFR2 or IκB.DN were electroporated with a mixture of 2 μg of a plasmid encoding GFP and 10 μg of control vector or vector expressing either wild-type RIP or the indicated RIP mutants (KD, kinase domain; ID, intermediate domain; DD, death domain). After 24 hr, cells were split, incubated overnight in the presence or absence of 20 ng/ml of TNF, and analyzed for cell death using flow cytometry. Results shown are the average of triplicate measurements and are representative of no fewer than three independent experiments showing similar results.

Deletion of either the death or the intermediate domain substantially impaired the ability of RIP to restore TNF-induced apoptosis, whereas deletion of the kinase domain had an incomplete effect (Figure 2). A mutated version of RIP lacking amino acids 391 to 422 efficiently restored TNF-induced cell death (Figure 2), despite being unable to mediate NF-κB activation by TNFR1 (Ting et al., 1996). The dependence of cell death on the presence or absence of the different domains was similar in the two systems (Figure 2), suggesting that a common function of RIP was exploited by the two death pathways.

To weigh the possibility that TNF-induced apoptosis in Jurkat cells expressing TNFR2 is trivially dependent on a TNFR1-restricted process, for example by transfer of ligand to TNFR1, we explored the functional anatomy of the death response. Support for the nontrivial involvement of TNFR2 comes from studies using agonistic and antagonistic anti-TNFR1 antibodies. Specific stimulation of TNFR1 with agonistic antibodies in Jurkat cells expressing TNFR2 fails to induce apoptosis (Figure 3A, upper panel), whereas those antibodies are able to efficiently induce cell death in cells expressing IκB.DN (Figure 3A, upper panel). In addition, ligand-blocking antibodies against TNFR1 substantially inhibit TNF-induced cell death in Jurkat cells expressing IκB.DN, but not in those expressing TNFR2 (Figure 3A, lower panel). Transfection of C terminally deleted versions of TNFR2 into Jurkat cells showed that the intracellular domain and, in particular, a fragment between amino acids 415 and 435 known to contain the TRAF2 binding site (Rothe et al., 1994), are essential for efficient TNF-induced apoptosis in this system (Figure 3B). This finding is in accord with results in other cell types (Weiss et al., 1997; Declercq et al., 1998) and suggests that TNFR2 stimulates the cell death pathway through a process dependent on intracellular factors. Although these findings point to a predominant role of TNFR2 in transmitting the apoptotic signal in Jurkat cells expressing TNFR2, some requirement for TNFR1 costimulation cannot be excluded. Since Jurkat cells expressing TNFR2 show a blockade in the TNFR1 pathway to apoptosis (Figure 3A, upper panel), cell death induced by TNFR2 appears insensitive to the degree of NF-κB activation that suffices to inhibit apoptosis through the TNFR1 pathway (see below).

To further explore the mechanism by which TNF provokes apoptosis in Jurkat cells, plasmids expressing antiapoptotic proteins, including dominant-negative FADD/MORT1 (FADD.DN), Bcl-X_L, and CrmA (Boise et al., 1993; Miura et al., 1995; Chinnaiyan et al., 1996), were transfected into wild-type cells expressing either TNFR2 or IκB.DN. As shown in Figure 3C, all three proteins tested were able to inhibit cell death mediated by TNFR2 and TNFR1 to a similar extent. This suggests

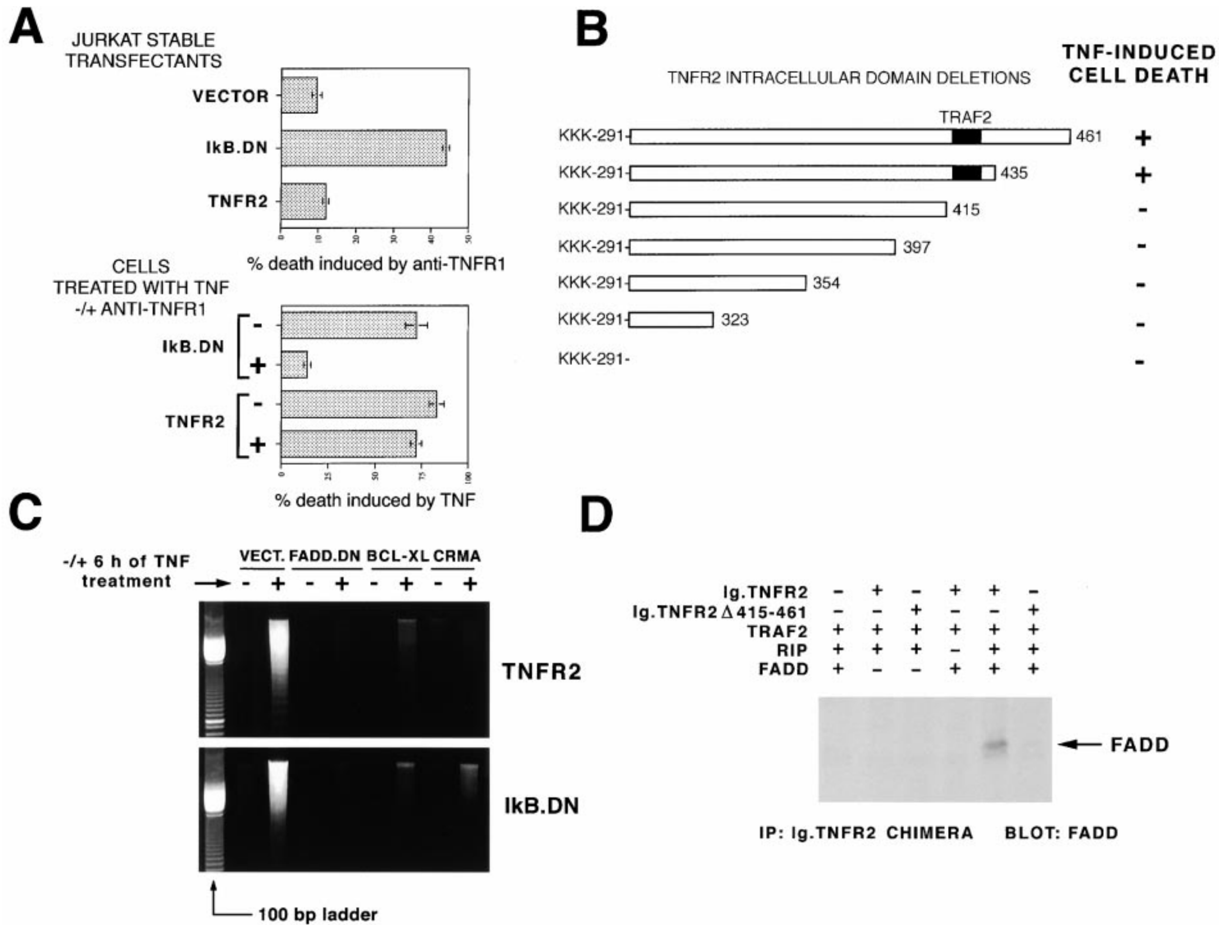


Figure 3. The TNFR2 Apoptosis Pathway in T Cells

(A) Agonistic and antagonistic anti-TNFR1 antibodies define a predominant role for TNFR2 in TNF-induced apoptosis in Jurkat cells expressing TNFR2. Jurkat cells expressing either TNFR2 or IκB.DN were incubated overnight in the presence or absence of 10 μg/ml of an agonistic anti-TNFR1 antibody (upper panel) or with TNF (20 ng/ml) for 24 hr in the absence or presence of 50 μg/ml of a neutralizing anti-TNFR1 antibody (lower panel). The percent death was measured using flow cytometry.

(B) The TRAF2 binding region of TNFR2 is required for TNF-mediated cell death. Jurkat cells were electroporated with a mixture of 2 μg of a plasmid expressing GFP and 10 μg of a plasmid encoding the indicated C-terminal deletions of TNFR2. After 24 hr, cells were split and incubated overnight in the absence or presence of 20 ng/ml of TNF. Cells were subjected to flow cytometry, and the percentage of live transfected cells was evaluated. +, greater than 45% death; −, less than 3% death.

(C) TNF-induced death in cells expressing TNFR2 or IκB.DN is blocked by dominant-negative FADD/MORT1 (FADD.DN), Bcl-X_L, and CrmA. Cells expressing TNFR2 or IκB.DN were electroporated with a mixture of 2 μg of CD14 expression plasmid and 10 μg of either vector alone (VECT.) or vector bearing FADD.DN (amino acids 80–205), Bcl-X_L, or CrmA. After 36 hr, cells expressing CD14 were selected using anti-CD14 magnetic beads. Equal numbers of cells were then either treated for 6 hr with 20 ng/ml of TNF or left untreated, and the extent of apoptosis was measured by low molecular weight DNA formation.

(D) FADD accumulates in a TNFR2 signaling complex in the presence of TRAF2 and RIP. 293EBNA/T cells were transfected as indicated with DNA mixtures containing equal amounts of plasmids expressing TRAF2, RIP, FADD.DN, and a chimeric membrane protein having an extracellular domain comprising the human IgG1 constant region, a CD7 transmembrane domain, and either the complete intracellular domain of TNFR2 or a deleted version lacking residues 415 to 465 (spanning the TRAF2 binding site). An antiapoptotic plasmid expressing CrmA and Bcl-X_L was cotransfected in each case. Cells were lysed 36 hr after transfection, and the Ig.TNFR2 chimera were immunoprecipitated using protein A–Sepharose. The presence of FADD.DN in the precipitates was detected by blot analysis with an antibody that recognizes the death domain of FADD.

that TNFR2, like TNFR1, mediates apoptosis through a FADD/MORT1-dependent caspase pathway that can be inhibited by CrmA and Bcl-X_L. Because the apoptotic signals mediated by TNFR2 require the TRAF2 binding site, we looked for a mechanism coupling TRAF2 to apoptosis. TRAF2 has been previously shown to bind RIP (Hsu et al., 1996b), and RIP and FADD/MORT1 are known to interact (Varfolomeev et al., 1996), although the precise geometry of these associations is not understood.

We performed coimmunoprecipitation studies to determine whether RIP can bridge TNFR2-bound TRAF2 to FADD by simultaneously interacting with both molecules. Cells were cotransfected with combinations of plasmids expressing TRAF2, RIP, the death domain of FADD, and a transmembrane immunoglobulin chimera bearing a TNFR2 intracellular domain (Figure 3D). Following immunoprecipitation of the TNFR2 component, FADD was detected only in the presence of RIP and only when the TNFR2 intracellular domain retains an

intact TRAF2-binding site (Figure 3D). Thus, a probable logic for the pathway is that TNFR2 engagement leads to the recruitment of TRAF2, association of RIP, and binding of FADD/MORT1.

NF-κB activation initiates a response that blunts the apoptotic action of several agonists of cell death, including TNFR1 (Beg and Baltimore, 1996; Liu et al., 1996; Van Antwerp et al., 1996; Wang et al., 1996). Thus, a diminished NF-κB activation might facilitate apoptosis through TNFR2. However, Jurkat cells expressing TNFR2 triggered a strong NF-κB response upon TNF treatment (Figure 4A), indicating that the NF-κB-activated protective response fails to protect against TNFR2-induced apoptosis.

Activation of NF-κB by TNFR1 has been shown to require RIP (Ting et al., 1996; Kelliher et al., 1998), and the RIP-null cells used here show an absolute block in TNFR1-mediated NF-κB induction (Ting et al., 1996). Unexpectedly, TNF was able to activate NF-κB in RIP$^-$ cells expressing TNFR2 (Figure 4B), indicating that TNFR2 is capable of activating NF-κB by a pathway that intersects the TNFR1-initiated pathway somewhere downstream of RIP. As a result, apoptosis and NF-κB activation induced by TNFR2 are uncoupled at the level of RIP.

These findings imply that, in T cells, RIP may be able to determine the outcome of TNF signaling. In the absence of RIP, only TNFR2 would be functional, triggering NF-κB activation, and both apoptosis and the induction of NF-κB through TNFR1 would be blocked. In the presence of RIP, TNF-induced apoptosis should proceed efficiently through a TNFR2-dependent pathway, despite induction of NF-κB activity through both receptors. This differential signaling mimics naturally occurring events in T cell activation. Activated human and murine T cells principally express TNFR2 (Ware et al., 1991; Zheng et al., 1995), and TNFR2 has been found to mediate TNF-induced proliferation in the initial phase of the T cell activation response (Gehr et al., 1992; Tartaglia et al., 1993b; Grell et al., 1998). Later in the process, T cells become sensitive to apoptosis induced by TNF (Sarin et al., 1995), and when activated murine CD8-positive cells are restimulated through the T cell receptor, TNFR2 mediates activation-induced cell death triggered by the autocrine secretion of TNF (Zheng et al., 1995).

To determine whether developmental regulation of RIP controls susceptibility to cell death in vivo, we studied TNF sensitivity and RIP expression in activated T cells from multiple human donors. Both CD4- and CD8-positive cells are resistant to TNF-induced apoptosis 2 days after phytohemagglutinin (PHA) stimulation (Figure 5A). At this time, NF-κB activation, measured as nuclear translocation of the p65 (RelA) subunit of NF-κB, is intact (Figure 5B), and cells express TNFR2 (Figure 5C). However, after the cells are cultured 4–10 more days in the presence of IL-2, they become susceptible to TNF-induced cell death (Figure 5A), while retaining unchanged their ability to activate NF-κB (Figure 5B). The transition to a death-sensitive state occurs without significant variation in the expression of TNFR2 (Figure 5C) or FADD (Figure 5D), and TNFR1 expression remains undetectable throughout (data not shown). Consistent with previous reports (Sarin et al., 1995), the presence of IL-2 during TNF treatment inhibits cell death (Figure 5A).

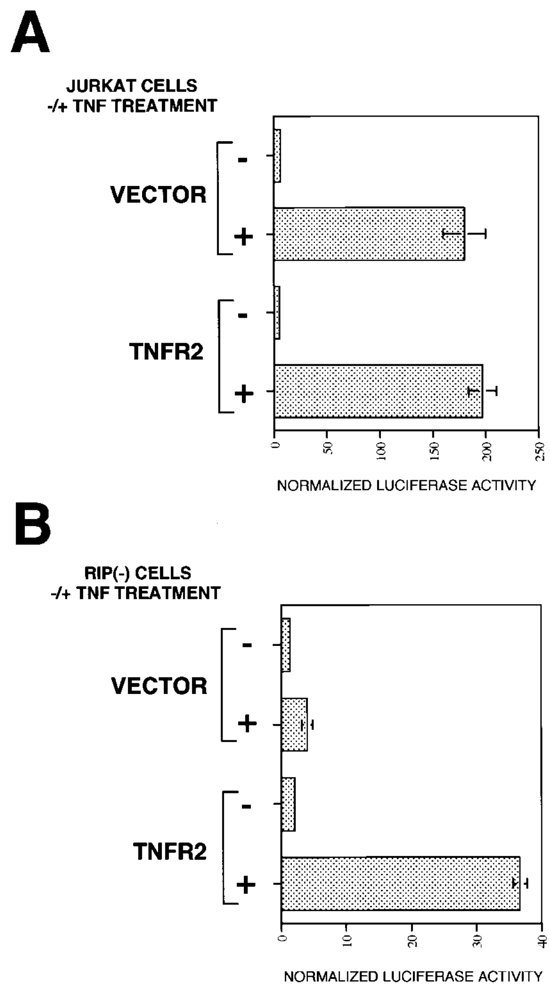


Figure 4. NF-κB Activation Induced by TNF in Wild-Type and RIP$^-$ Cells Expressing TNFR2

(A) TNF strongly activates NF-κB in Jurkat cells expressing TNFR2. Wild-type cells bearing either vector or TNFR2 expression plasmid were electroporated with a mixture of DNA containing 4 μg of an NF-κB-luciferase reporter and 12 μg of a vector expressing both CrmA and Bcl-X_L. After 36 hr, cells were split into three wells and treated for 6 hr with 20 ng/ml of TNF, 10 ng/ml of phorbol-12-myristate-13-acetate (PMA) plus 1 μM of ionomycin, or left untreated. They were then lysed, and the luciferase activity was measured. Values shown are averages of triplicate measurements, normalized as the fraction of luciferase activity induced by treatment with PMA/ionomycin.

(B) RIP is not required for TNFR2-mediated activation of NF-κB. RIP$^-$ cells transfected with either vector or TNFR2 expression plasmid were electroporated with an NF-κB-luciferase reporter plasmid (4 μg). After 16 hr, cultures were exposed for 6 hr to either 20 ng/ml of TNF, 10 ng/ml of PMA plus 1 μM of ionomycin, or no agent. Luciferase assay and normalization were conducted as described in (A).

RIP expression is strongly induced in human T cells between day 2 and day 10 of activation (Figure 5D) with good expression apparent by day 5 (data not shown). To evaluate whether RIP expression may be necessary for apoptosis in this context, we developed a system to interfere with RIP expression and measure its impact on TNF-induced cell death. Since the RIP-deficient mice have a perinatal lethal phenotype (Kelliher et al., 1998) and it has not been possible to create T cell lines from

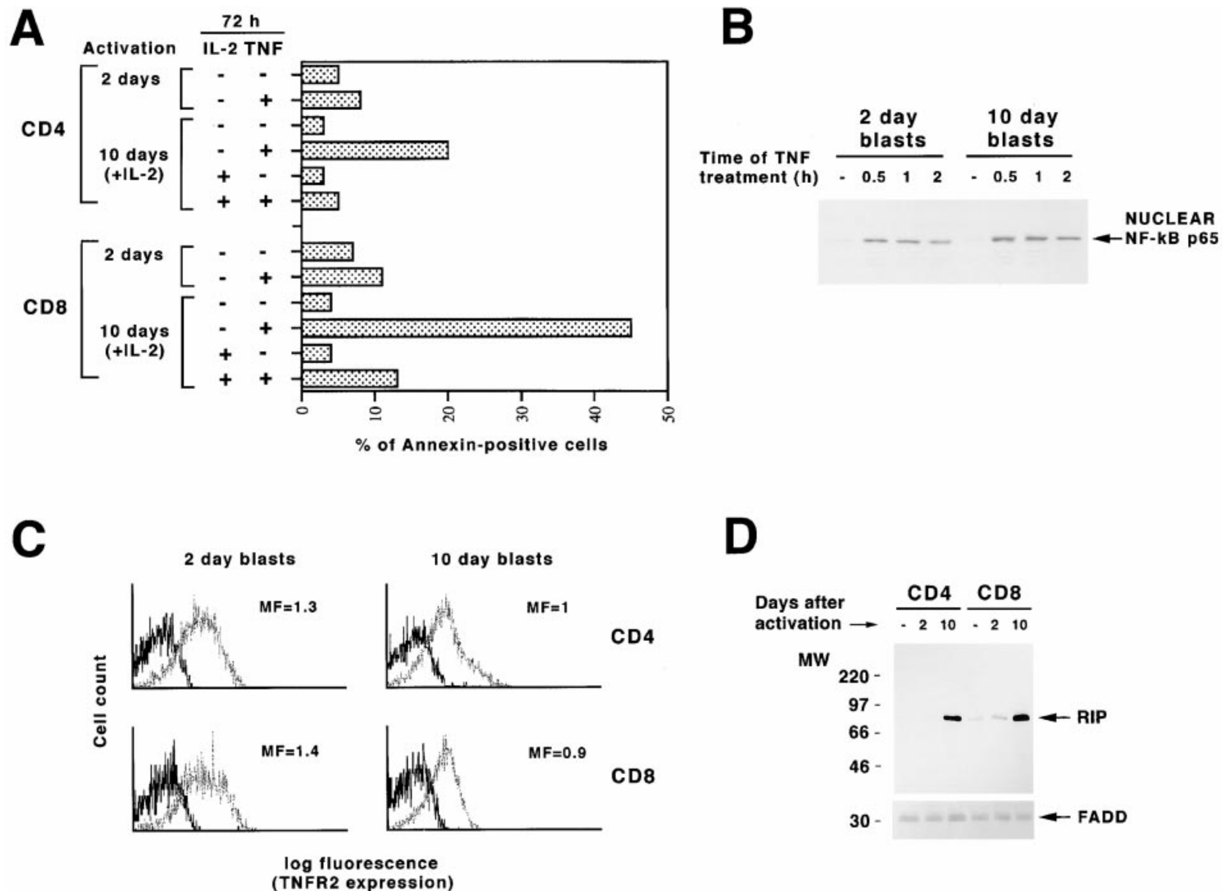


Figure 5. Analysis of TNF-Induced Cell Death and NF-κB Activation during Human T Cell Activation

(A) Activated T cells acquire sensitivity to TNF-induced cell death after IL2 exposure. Peripheral blood mononuclear cells (PBMCs) were purified and activated with 2 μg/ml of PHA for 2 days. CD4 and CD8 cells were purified with magnetic beads and subsequently incubated for 8 days in the presence of 30 U/ml of IL-2. At the indicated times, cells were harvested, washed, and incubated for 72 hr in the absence or presence of 50 ng/ml TNF. Where indicated, 30 U/ml of IL-2 was present during TNF treatment. Cell death was evaluated by Annexin-V-FITC staining.

(B) TNF-induced NF-κB activation does not change during T cell activation and proliferation. Cells were activated, purified using a mixture of CD4- and CD8-specific magnetic beads, and cultured as above. Day 2 or day 10 cells were collected, washed, and incubated overnight to reduce spontaneous activation of NF-κB. T cells were then treated with TNF (50 ng/ml) for the indicated times and lysed, and nuclear extracts were isolated. Equal amounts of nuclear protein were subjected to SDS-PAGE, and the p65 (RelA) subunit was detected by immunoblot.

(C) TNFR2 expression does not vary significantly during IL-2-dependent human T cell expansion. Cells were activated with PHA, purified and cultured as in (A) with the exception of TNF treatment, and subjected to flow cytometry using an anti-TNFR2 or an irrelevant control antibody. Shown is the mean fluorescence intensity (MF) obtained with anti-TNFR2 antibody (light line).

(D) RIP expression is induced during IL-2-dependent T cell expansion, whereas FADD expression remains constant. Cells were activated and purified as in (C), washed, and lysed in a buffer containing 1% NP-40. Extracts were normalized for protein content, and equal amounts of protein were loaded in each well, resolved by SDS-PAGE, and subjected to immunoblotting using a specific anti-RIP antibody. Western blots were reprobed with an anti-FADD specific antibody.

such mice to date, we explored alternative approaches based on retroviral transfer of antisense constructs. Because RNAs that lack open reading frames are known to be labile, we inserted a green fluorescent protein (GFP) coding sequence upstream of antisense RIP in a murine leukemia virus–based vector (Riviere et al., 1995). An improved efficiency of the GFP-RIP antisense transcript over conventional antisense RNA was apparent in the inhibition of cell death provoked by overexpression of RIP in 293T cells (Figure 6A). Retroviral transduction of the GFP-antisense RIP transcription unit into CD8 T cell blasts from four different donors decreased susceptibility to TNF-induced cell death at day 10 after activation, as established by comparison with cells not expressing construct or cells expressing GFP only (Figure 6B). Similar constructs bearing an irrelevant antisense segment downstream of GFP lacked death-protective activity, indicating that the effect is specific to antisense RIP (Figure 6C).

Thus, the findings in primary human T cells are consistent with observations from experiments with Jurkat cells. In human T cells, RIP is needed for TNFR2 induction of death but not NF-κB activation. In resting T cells or activated T cells that have not been exposed to IL2, TNF activates NF-κB through TNFR2 and a TRAF2-dependent pathway (Rothe et al., 1995). In activated T cells that have been exposed to IL2, RIP is induced and is proposed to bridge the TNFR2–TRAF2 complex to

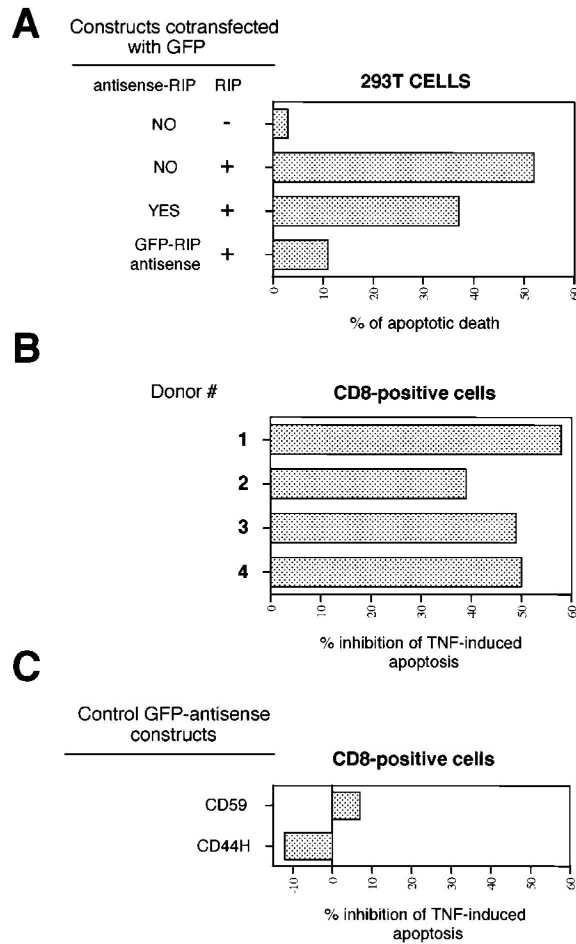


Figure 6. Retroviral Transduction of RIP Antisense Inhibits the IL2-Driven Transition of CD8 T Cells to a Death-Sensitive State

(A) Potentiation of antisense activity by an upstream open reading frame. 293T cells were transfected as indicated with 10 μg of a mixture of mammalian expression plasmids containing GFP (2 μg), RIP (2 μg), and either antisense RIP or a construct containing the GFP open reading frame upstream of antisense RIP (GFP-RIP antisense) (6 μg). The percentage of GFP-positive cells undergoing apoptotic cell death versus the total number of GFP-positive cells was scored 36 hr after transfection.

(B) GFP-RIP antisense inhibits TNF-induced apoptosis in CD8 T cell blasts. PBMCs from four independent donors were isolated and cultured in the presence of PHA as described in Figure 5. After 2 days, cells were transduced overnight with retrovirus expressing either GFP or GFP-RIP antisense. A day later, CD8-positive cells were isolated using magnetic beads and cultured for 8 days in the presence of IL2 (30 U/ml) to induce competency for TNF-triggered cell death. Apoptosis assays were performed with Annexin-V-phycoerythrin. The death-protective activity was assessed for each culture by comparison of the percent death in the transduced (green) and nontransduced (nongreen) cells. Data for the antisense constructs are presented as a percentage of the death protection (nil) provided by GFP transduction alone.

(C) Antisense CD59 or CD44H do not significantly interfere with TNF-induced apoptosis of CD8 T cell blasts. Cells were cultured and processed as in (B) with the exception that GFP and GFP-CD59 antisense or GFP and GFP-CD44H antisense constructs were used to transduce activated PBMCs. Susceptibility to TNF-induced apoptosis was calculated as in (B).

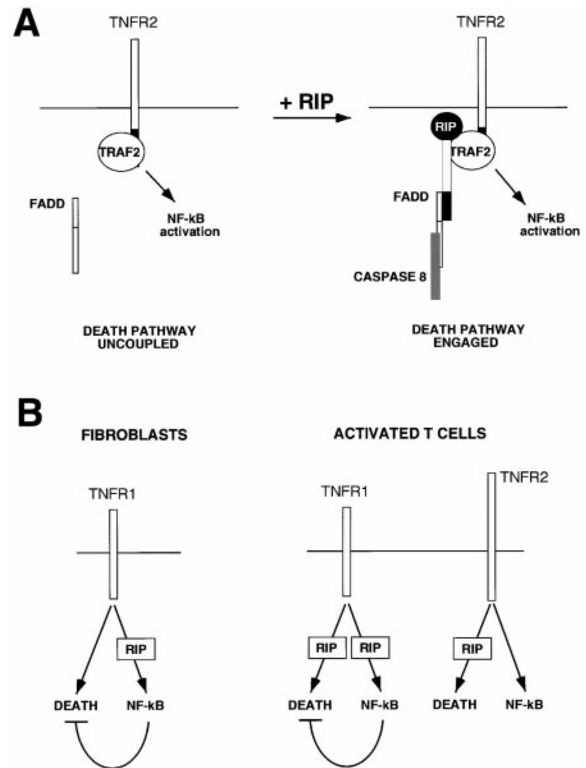


Figure 7. Context-Dependent TNF Signal Transduction

(A) The role of RIP in T cells. Early in T cell activation, the expression of RIP is low and TNFR2 induces NF-κB activation. Following exposure to IL2, RIP expression increases and, through TRAF2 or related TRAF-like factors, bridges TNFR2 to FADD/MORT1 and the apoptotic machinery.

(B) Differences in signaling in fibroblasts and T cells. In fibroblasts, RIP is essential for TNFR1-mediated NF-κB activation, but not death. In T cells, RIP is required for TNFR1-dependent NF-κB activation and death and for TNFR2-mediated death, but not NF-κB activation. TNFR2-dependent death is unaffected by NF-κB activation in this setting.

FADD/MORT1 and the apoptosis machinery (Figure 7A). Although in human T cells, RIP is needed for TNFR1-mediated induction of both death (this work) and NF-κB activation (Ting et al., 1996), its action downstream of TNFR1 clearly depends on cell type. Mouse fibroblasts are normally resistant to TNFR1-induced apoptosis but are sensitive when RIP is absent (Kelliher et al., 1998), suggesting that in these cells RIP is required for the NF-κB protective response, but not for TNFR1-mediated cell death (Figure 7B). Thus, there are likely lineage-specific differences in the TNFR1 signaling pathway as well.

Discussion

An implicit assumption in many signal transduction studies has been that a single consensus sequence of events suffices to account for the majority of changes in cellular state that follow exposure to an extracellular mediator. Indeed, for the signaling induced by G protein–coupled receptors, there appears to be a reasonable likelihood that this model is valid and that the varying

responses of cells to different ligands can largely be predicted on the basis of the varying complement of cell surface receptors exhibited by the responding cells. However, it is unclear how general this phenomenon may be, and there is reasonable evidence to suggest that in cell death pathways, and particularly in the immune system, the model has limited power to explain the observed phenomena.

Cell populations in the immune system undergo expansion and contraction in the absence of the cell–cell and cell–matrix contacts that normally regulate cell number in solid organs. Hence, regulatory mechanisms that function in the absence of specific organ context cues are required, and in this context the apposite control of apoptotic cell death (Winoto, 1997) is an important theme. In theory, population expansion and contraction can be controlled by coupling proliferative signals with an invariant death program, and indeed, at one extreme, neutrophilic granulocytes, the most vigorously proliferating cells in the immune system, are committed by default to constitutive proliferation and an apoptotic fate (Savill, 1997).

But more sophisticated controls appear necessary to manage clonal populations that must increase and decrease in response to specific stimuli. Studies in mice have shown that IL-2, an important cytokine for clonal expansion, also programs T cells for eventual death (Lenardo, 1991). The significance of this observation has been underscored by findings that mice deficient in either the IL-2 gene or the genes for the β or γ chains of the IL-2 receptor show lymphoproliferative disorders (Sadlack et al., 1993; Suzuki et al., 1995; Nakajima and Leonard, 1997). These reports conflict with the simple view that the sole function of IL-2 is expansion of activated populations and point to an important role for IL-2 signaling in population contraction in the murine immune system. Our results suggest that in humans IL-2 may have a similar role, promoting T cell proliferation as well as the transition to a death-susceptible state. TNF enters the picture in a dualistic role, acting as an activating cytokine early in immune arousal and as an apoptotic cytokine following IL-2 removal.

In T cells, antigen receptor activation and proliferation are different programs. Antigen receptor engagement does not lead directly to cell cycle transition but induces competency for proliferation by activation of both cytokine and cytokine receptor promoters. The data presented here indicate that the death susceptibility program is associated with proliferation instead of activation, as RIP induction occurs during the IL-2-driven proliferation phase, not the early activation response. Because RIP is required for both the death and NF-κB induction activities mediated by TNFR1 in T cells, TNFR1, if present, is unlikely to contribute significantly to TNF signaling prior to RIP induction.

The dual capacity to induce activation and apoptosis appears to be a common property of ligands of the TNFR superfamily. For example, CD95 aggregation triggers efficient cell death (Itoh et al., 1991) but also proliferation (Alderson et al., 1993) and NF-κB activation (Ponton et al., 1996). CD27 engagement can stimulate T cell proliferation (Goodwin et al., 1993) but can also mediate apoptosis (Prasad et al., 1997). CD30 ligand is able to induce both proliferation and death (Smith et al., 1993); and the B cell differentiation and survival (Banchereau et al., 1991) receptor CD40 also mediates apoptosis (Hess and Engelmann, 1996). The receptor for lymphotoxin-β induces NF-κB activation (Mackay et al., 1996) but triggers cell death in the presence of γ interferon (Browning et al., 1996). In the central nervous system, the low affinity nerve growth factor receptor has been implicated both in induction of cell death by default (absence of ligand) (Rabizadeh et al., 1993) and in ligand-dependent survival and NF-κB activation (Rabizadeh et al., 1993; Carter et al., 1996). Thus, throughout the TNF receptor superfamily one finds examples that death and activation represent alternative programs conditioned on receptor context and cell state.

As these examples show, it cannot be assumed that a consensus pathway applies to all cells and under all conditions, and hence it is clearly necessary to appreciate signaling pathways in a cell-specific context. Unfortunately, this considerably complicates any discussion of signaling and creates a burden to establish the basic constituents of the signaling pathway in each cell type and under each set of experimental circumstances. On the other hand, considering the breadth of expression of these receptors and the diversity of roles they subserve, the elucidation of the cell-specific features of their signal transduction is likely to shed new light on their functions in normal and deranged physiology.

Experimental Procedures

Cells and Transfections

Jurkat cells and the electroporation methods used in this study have been described in detail elsewhere (Ting et al., 1996). Adherent cells were transfected by using the calcium phosphate precipitation method as previously described (Ausubel et al., 1987).

Flag-tagged IκB-α S32A, S36A (Brockman et al., 1995), and TNFR2 (Smith et al., 1990) cDNAs were subcloned into a mammalian episomal expression plasmid that contains a puromycin resistance. To create stable transfectants, cells were electroporated with the indicated plasmids and subjected to puromycin (Sigma, St. Louis, MO) selection (1 μg/ml) for 10 days. Cells were further purified by limiting dilution to obtain clonal populations.

Peripheral blood mononuclear cells (PBMCs) were purified by centrifugation onto a Ficoll/diatrizoate cushion (Organon Teknika, Durham, NC) and activated with 2 μg/ml of PHA (Sigma). Purification of CD4- and CD8-positive cells was performed using Dynabeads and the Detachabead system (Dynal, Oslo, Norway). Purity, activation, and TNFR expression were determined by flow cytometry (Coulter Epics XL), using anti-CD3, -CD4, -CD8, -CD25, -CD69 (PharMingen, San Diego, CA), or anti-TNFR2 (R and D Systems, Minneapolis, MN) antibodies. Purity was always greater than 95%. After purification, as indicated, cells were further cultured in the presence of 30 U/ml IL-2 (Sigma).

Immunoblotting and Coimmunoprecipitation Studies

To measure TNFR2, IκB.DN, or RIP expression in the Jurkat stable transfectants, equal numbers of cells were harvested and directly lysed in 2× reducing SDS sample buffer. To measure RIP and FADD expression levels in human primary T cells, cells were lysed on ice for 20 min in a buffer containing 1% NP-40 (Sigma). To evaluate NF-κB activation in primary T cells, nuclear extracts were obtained after TNF treatment, basically as previously described (Schreiber et al., 1989). Protein concentrations were measured by the bicinchoninic acid method (Pierce, Rockford, IL). For the coimmunoprecipitation studies, 293EBNA/T cells were transfected with the indicated DNAs and lysed 36 hr later in a buffer containing 0.1% NP40 as described previously (Hsu et al., 1995). The Ig.TNFR2 intracellular domain chimera were immunoprecipitated with protein A–Sepharose (Sigma) for 3 hr at 4°C. Precipitates were washed twice with lysis

buffer, twice with lysis buffer containing 0.8 M NaCl, and twice more with lysis buffer. In all cases, equivalent amounts of protein were resolved by SDS-PAGE, electrophoretically transferred to a PVDF membrane (Immobilon, Millipore, Bedford, MA), and probed with specific antibodies against TNFR2 (R and D Systems), IκB-α (Santa Cruz, Santa Cruz, CA), RIP, FADD/MORT1, or p65 NF-κB (Transduction Laboratories, Lexington, KY).

Apoptosis Assays

Annexin-V stainings were carried out following the instructions provided by the manufacturer (R and D Systems). DNA fragmentation assays were performed on 5×10^6 cells per experimental point, essentially as described previously (Herrmann et al., 1994). Low molecular weight DNA was resolved on a 1% agarose gel. Cycloheximide and TNF were purchased from Sigma and the anti-Fas antibody (IgM clone CH-11) from UBI (Lake Placid, NY).

Cell death was analyzed by flow cytometry using a previously optimized program to measure the percentage of cells present in a Forward Scatter/Side Scatter (FS/SS) gate including only propidium iodide impermeant cells. A goat anti-TNFR1 polyclonal serum and an anti-TNFR1 mouse monoclonal (R and D Systems) were used as TNFR1 agonistic and antagonistic reagents, respectively. In the case of GFP cotransfection, 1.5×10^7 cells were electroporated with a mixture of DNA containing the indicated amounts of a plasmid expressing GFP and a stoichiometric excess of plasmids expressing the indicated versions of RIP (Figure 2) or TNFR2 (Figure 3B). After 24 hr, cells were cultured overnight as described in the legend of Figures 2 and 3B and directly subjected to flow cytometry. We used the previously described program to measure the percentage of live cells that are GFP positive in each experimental condition.

For CD14 selection after transfection, three points of 2×10^7 cells each (a total of 6×10^7 cells per experimental point) were electroporated with a mixture of DNA containing a plasmid expressing CD14 and a stoichiometric excess of the indicated plasmids (Figure 3C legend). CD14-expressing cells were purified 36 hr after transfection using magnetic beads directly conjugated to an antibody against CD14 (Dynal). The percentage of CD14-positive cells was measured by flow cytometry and used to normalize cell numbers in the purified populations. Cells (5×10^6 per experimental point) were cultured and processed to detect DNA fragmentation.

Luciferase Assays

Cells (1.5×10^7) were electroporated with an NF-κB-luciferase reporter previously described (Ting et al., 1996), either alone or in combination with a stoichiometric excess of a plasmid simultaneously expressing the antiapoptotic proteins CrmA and Bcl-X_L. After the indicated culture time, cells were split into three wells and treated with TNF, PMA (Sigma) plus ionomycin (Calbiochem, San Diego, CA), or left untreated. Then, cells were harvested and lysed according to the manufacturer's instructions, using the assay system purchased from Promega (Madison, WI). Luciferase activity was quantitated with a Monolight 2010 Luminometer.

RIP Antisense Experiments

Mammalian expression vectors were constructed to express a RIP antisense RNA or antisense RIP downstream of the GFP open reading frame (GFP-RIP antisense), which was created by inserting antisense RIP downstream of the stop codon of GFP. The ability of both antisense constructs to inhibit cell death triggered by RIP overexpression was compared in 293T cells. Subconfluent cells were transfected with a mixture of plasmids containing a 1:1:3 molar ratio of GFP:RIP:antisense RIP (or GFP-RIP antisense), or irrelevant vector as indicated. After 36 hr, the percentage of green cells showing signs of apoptotic cell death versus the total number of green cells was calculated.

Retroviral constructs containing GFP, GFP-RIP antisense, GFP-CD59 antisense, or GFP-CD44H antisense were created into the pMMP vector (a generous gift from Dr. Richard Mulligan), a derivative version of the pMFG vector (Riviere et al., 1995), and transfected into subconfluent Phoenix-amphotropic packaging cells (a generous gift from Dr. Garry Nolan) using the calcium phosphate precipitation method (Ausubel et al., 1987). PBMCs from different donors were activated for 48 hr with 2 μg/ml of PHA. Virus-enriched supernatants were collected 48 hr posttransfection and diluted 1:1 in the PHA conditioned media (total volume of 3 ml). Cells (2×10^7 per experimental point) were resuspended in this mixture and added onto a 6 cm plate that was previously coated with a recombinant fibronectin fragment (RetroNectin, Takara Biomedicals, Shiga, Japan), following the instructions of the manufacturer. Transductions were done overnight. The next day, cells were harvested and CD8-positive cells purified by using magnetic beads (Dynal). Transduction efficiencies ranged from 15% to 25% in the case of GFP and from 5% to 15% in the case of the GFP-RIP, CD59, or CD44H antisense constructs. The transduced CD8 cells were cultured for 8 more days in the presence of 30 U/ml of IL2, washed and incubated for 72 hr in the absence or presence of 50 ng/ml of TNF, and stained with Annexin-V-phycoerythrin (PharMingen).

Acknowledgments

We thank Dean Ballard for the IκB S32,36A plasmid, David Goeddel for antibody reagents and comments on the manuscript, Immunex Corporation for the TNFR2 cDNA, Richard Mulligan for the retroviral pMMP plasmid, Garry Nolan for the Phoenix-amphotropic packaging cell line, and Nils Jacobson, Felix Randow, Winfried Pickl, and Adrian Ting for suggestions and critical feedback. F. X. P. was the recipient of a postdoctoral fellowship from the Lady Tata Memorial Trust. Portions of this work were supported by NIH grants AI27849 and DK43039.

Received October 25, 1999.

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