

A20 Inhibits Tumor Necrosis Factor (TNF) Alpha-Induced Apoptosis by Disrupting Recruitment of TRADD and RIP to the TNF Receptor 1 Complex in Jurkat T Cells

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Tumor necrosis factor receptor 1 (TNFR1) can trigger distinct signaling pathways leading to either the activation of NF- κ B transcription factors or apoptosis. NF- κ B activation results in the expression of antiapoptotic genes that inhibit the apoptosis pathway that is activated in parallel. However, the molecular mechanism of this inhibition remains poorly characterized. We have isolated a Jurkat T-cell mutant that exhibits enhanced sensitivity to TNF-induced apoptosis as a result of a deficiency in I- κ B kinase γ (IKK γ)/NEMO, an essential component of the IKK complex and NF- κ B pathway. We show here that the zinc finger protein A20 is an NF- κ B-inducible gene that can protect the IKK γ -deficient cells from TNF-induced apoptosis by disrupting the recruitment of the death domain signaling molecules TRADD and RIP to the receptor signaling complex. Our study, together with reports on the role of other antiapoptotic proteins such as c-FLIP and c-IAP, suggests that, in order to ensure an effective shutdown of the apoptotic pathway, TNF induces multiple NF- κ B-dependent genes that inhibit successive steps in the TNFR1 death signaling pathway.

The regulation of survival and death in lymphoid cells is a tightly regulated process, and alteration of the delicate balance between the two can lead to a variety of autoimmune and inflammatory diseases. Members of the tumor necrosis factor (TNF) superfamily are critical regulators of the balance between the two opposing processes, and binding to their cognate receptors can trigger signaling pathways leading to either survival or death (25). Indeed, in the case of TNF receptor 1 (TNFR1/CD120a), ligation of a single class of receptor can trigger either cell survival or apoptosis depending on which signaling pathway predominates in the cell type examined. Studies conducted over the past several years have identified a number of molecules that are involved in the proximal signaling mechanism of TNFR1. Upon binding of TNF alpha to TNFR1, the death domain present in the cytoplasmic tail of the receptor is able to recruit and associate with two death domain-containing signaling molecules known as TRADD (TNFR1-associated death domain) and RIP (receptor-interacting protein) (13–15, 45). However, the mechanisms by which these death domain interactions are regulated remain poorly understood. Both TRADD and RIP are required for signaling to NF- κ B, as well as the apoptosis pathway (13–15, 18, 36, 42). In turn, TRADD and RIP associate with TRAF2 and another death domain protein called FADD (FAS-associated death domain protein) (13, 14, 41, 45). It is at this juncture that the NF- κ B and apoptotic signals are thought to bifurcate: TRAF2 recruitment leads to the activation of downstream kinases and NF- κ B activation, whereas FADD recruitment leads to the activation of caspase 8 and apoptosis (6, 14, 24).

In most instances, TNF binding to TNFR1 induces an

NF- κ B response but does not result in apoptosis unless the cells are simultaneously treated with protein synthesis inhibitors. The molecular basis for this observation was subsequently explained by the demonstration that cells lacking NF- κ B transcription factors became sensitive to TNF-induced apoptosis and that this was due to the lack of expression of NF- κ B-dependent antiapoptotic genes (2, 24, 43, 47). It appears that the NF- κ B pathway triggered by TNFR1 antagonizes the apoptosis pathway that is simultaneously activated. However, the identities of the antiapoptotic genes that are regulated by NF- κ B and the mechanisms by which these molecules inhibit TNF-mediated apoptosis remain to be fully explored.

In this study, we address the above question by using a Jurkat T-cell mutant that we isolated that is defective in I- κ B kinase γ (IKK γ)/NEMO expression. Inactive NF- κ B transcription factors in the cytoplasm are bound to inhibitory I- κ B proteins, and upon stimulation, the I- κ B is phosphorylated on specific serine residues by IKK (17). This phosphorylation event targets I- κ B for ubiquitination and degradation by the proteasome, thereby allowing the free NF- κ B factors to translocate to the nucleus. IKK is a multimeric complex, and at its core, it consists of two catalytic subunits, IKK α and IKK β , and an essential regulatory subunit, IKK γ /NEMO (10, 11, 26, 37, 38, 49–51). As a result of the IKK γ deficiency, the mutant Jurkat T-cell line that we isolated lacks a functional IKK and is unable to activate NF- κ B (11, 50). In the absence of the protective effects of NF- κ B, these IKK γ -deficient cells now became highly sensitive to TNF-induced apoptosis compared to their wild-type counterpart. We sought to identify the antiapoptotic genes that are regulated by NF- κ B in our Jurkat T-cell model. Our analyses indicate that the zinc finger protein A20 is not induced in the IKK γ -deficient cells and that its ectopic expression in the mutant cells was sufficient to confer resistance to TNF-mediated cell death. Furthermore, we show here that A20 blocks the TNF apoptosis pathway at a very early

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step by inhibiting the recruitment of the death domain-containing signaling molecules TRADD and RIP to the receptor.

MATERIALS AND METHODS

Cell lines and complementation of mutant. 3T8 is a CD3⁺ Jurkat T-cell line that has been sequentially transfected with two reporter constructs containing the human CD14 and the rat Thy1 gene under the control of eight copies of the NF- κ B enhancers. Mutant 8321 is a CD3⁺ derivative of 3T8 generated by ICR191 mutagenesis and subsequent negative enrichment for cells that do not respond to phorbol myristate acetate (PMA) according to protocols described previously (42). For complementation, mutant cells were transfected by electroporation with an expression plasmid encoding either bacterial glutathione S-transferase (GST) as a negative control or human IKK γ under the control of the spleen focus-forming virus long terminal repeat. Transfectants were selected by puromycin, cloned by limiting dilution, and analyzed for IKK γ expression by immunoblotting with anti-IKK γ monoclonal antibody (MAb). Two representative IKK γ -complemented 8321 clones, 1 and 2, are shown. All Jurkat T-cell lines were cultured in Iscove's modified Dulbecco's medium (Sigma-Aldrich, St. Louis, Mo.) supplemented with 10% bovine calf serum (HyClone, Logan, Utah), 2 mM L-glutamine, 50 μ M β -mercaptoethanol, and 15 μ g of gentamicin/ml. 293 EbnaT cells were cultured in Dulbecco's modification of Eagle's medium (Gibco, Grand Island, N.Y.) with 10% bovine calf serum and 15 μ g of gentamicin/ml.

Flow cytometry analysis. For cell surface staining, cells were incubated with fluorochrome-conjugated anti-rat Thy1 (Cedarlane Laboratories, Hornby, Ontario, Canada) for 30 min on ice and then washed three times with phosphate-buffered saline. Cells were analyzed on a FACSCalibur or FACScan flow cytometer, and data were analyzed with CellQuest software (Becton Dickinson, San Jose, Calif.).

Cloning and expression of A20. The human A20 full-length gene was amplified by PCR from a phytohemagglutinin (PHA)-activated T-cell cDNA library with *Pfu* Turbo polymerase (Stratagene, La Jolla, Calif.) and subcloned into a retrovirus expression vector. Both strands of the cloned A20 were sequenced and verified by automated sequencing (PE Applied Biosystems, Foster City, Calif.). The retroviral vector used is a derivative of the Moloney murine leukemia virus vector pMMP412 (35) into which an internal ribosome entry site-puromycin resistance cassette was inserted immediately downstream of the A20 gene. As a negative control, the A20 gene was replaced by the bacterial GST gene.

Retroviral pseudotyping and transduction. Human 293 EbnaT cells were seeded at a density of 4×10^6 cells in a 10-cm-diameter dish. The next day, cells were transfected by using calcium phosphate with 2.5 μ g of plasmid pMD.G encoding vesicular stomatitis virus G protein and 7.5 μ g of plasmid pMD.OGP encoding gag-pol (33) (both kindly provided by Richard Mulligan), together with 10 μ g of the retroviral expression construct encoding either the irrelevant control GST or myc-tagged full-length A20. At 48 h posttransfection, the viral supernatant was collected, centrifuged at $800 \times g$, and used to infect Jurkat T cells. A quantity of 5×10^6 Jurkat T cells was resuspended in 10 ml of viral supernatant in the presence of 4 μ g of Polybrene/ml, aliquoted into a 24-well plate, and spun in a microtiter rotor at $800 \times g$ for 1 h at room temperature. Infected Jurkat T cells were cultured for 48 h and then selected by using 1 μ g of puromycin/ml for 10 to 14 days. The drug-resistant cells were pooled, and the bulk population of cells was analyzed for the expression of myc-A20 by immunoblotting with anti-myc MAb. All experiments were conducted with the bulk pools of drug-resistant Jurkat T cells.

Reverse transcriptase PCR. Jurkat T cells were treated as indicated and harvested for total RNA isolation by using RNA STAT-60 (Iso-Tex Diagnostics, Friendswood, Tex.). First-strand cDNA was synthesized by using Superscript II reverse transcriptase (Gibco). The cDNA reaction mixtures were subjected to serial 10-fold dilutions followed by 25 cycles of amplification of 1 min each at 94, 58, and 72°C with a final 7-min extension at 72°C. The following primers were used: A20-forward, AAGCCGGCTGCGTGATTTTG; A20-reverse, GTCTTC GGGGGCAGGCTCACC; GAPDH (glyceraldehyde-3-phosphate dehydrogenase)-forward, CTCATGACCACAGTCCATGCCATC; and GAPDH-reverse, CTGCTTCACCACTTCTTGATGTC. PCR products were analyzed on 2% agarose gels and visualized by ethidium bromide staining.

Annexin V staining. Apoptosis was analyzed by annexin V staining based on the translocation of phosphatidylserine from the inner to the outer layer of the plasma membrane as previously described (42). Briefly, cells were treated with TNF (Peprotech, Rocky Hill, N.J.) or anti-FAS (clone CH11; Upstate Biotechnology, Charlottesville, Va.) for the indicated time, washed with phosphate-buffered saline, and resuspended in binding buffer (10 mM HEPES [pH 7.4], 140 mM NaCl, 2.5 mM CaCl₂). In experiments examining the loss of RIP expression, cells were pretreated with 1 μ M geldanamycin (Biomol, Plymouth Meeting, Pa.)

for 14 h before stimulation with TNF or anti-FAS. Phycoerythrin-labeled annexin V (Pharmingen, San Diego, Calif.) was added to the cell suspension and incubated at room temperature for 15 min. Samples were then diluted in binding buffer and analyzed immediately by flow cytometry.

DNA content. Cells were pelleted and resuspended in hypotonic solution (0.1% sodium citrate, 0.1% Triton) containing 50 μ g of propidium iodide/ml. DNA content was subjected to flow cytometric analysis. Cells with a normal DNA content ($\geq 2N$) were scored as viable whereas cells with a hypodiploid DNA content ($\leq 2N$, termed A^o) were scored as apoptotic.

Cell viability assays. Cellular viability after TNF treatment was measured with the Cell Titer 96 Aqueous One Solution cell proliferation assay (Promega, Madison, Wis.) according to the manufacturer's instructions. Briefly, cells were plated in 96-well microtiter plates and treated in triplicate with control medium or TNF for the indicated times. Viability was assessed through an MTS conversion assay, and A_{490} was determined for each well. After the value of background absorbance was subtracted from each measurement, the viability of cells in response to treatment with TNF was calculated as the ratio (A_{490} TNF)/(A_{490} no treatment).

Coimmunoprecipitation and immunoblotting. The TNFR1 coimmunoprecipitation experiments were performed as previously described (9). Jurkat T cells (5×10^7 /sample) that were either untreated or treated with TNF were centrifuged and resuspended in lysis buffer (0.1% NP-40, 20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 30 mM NaF, 2 mM pyrophosphate) freshly supplemented with protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 μ g of aprotinin/ml, 1 μ g of leupeptin/ml, and 1 μ g of pepstatin/ml). After incubation on ice for 15 min, detergent-insoluble material was removed by centrifugation at $20,800 \times g$ for 15 min. The supernatants were collected and incubated overnight with goat anti-TNFR1 antibody. The immune complexes were then precipitated with protein A-agarose beads for 2 h and washed extensively with lysis buffer. Immunoprecipitated proteins were eluted with 2 \times loading buffer, separated on a sodium dodecyl sulfate (SDS)-10% polyacrylamide gel, and transferred to nitrocellulose membranes. Membranes were probed with anti-RIP or anti-TRADD MAb, followed by horseradish peroxidase-labeled secondary antibody, and visualized with chemiluminescence (NEN, Boston, Mass.). For analyses of total cell extracts, cells were lysed as described above and 25 μ g of the whole-cell lysates was separated by SDS-10% polyacrylamide gel electrophoresis (PAGE) and subjected to immunoblotting with the indicated antibodies. Antibodies used were from the indicated sources: myc, Roche, Indianapolis, Ind.; TNFR1, R & D Systems, Minneapolis, Minn.; RIP, Pharmingen; IKK γ , Imgenex, San Diego, Calif.; IKK α , Santa Cruz Biotechnology, Santa Cruz, Calif.; caspase 8, caspase 3, and poly(ADP-ribose) polymerase (PARP), Cell Signaling Technology, Beverly, Mass.; and TRADD, Transduction Laboratories, Lexington, Ky.

RESULTS

IKK γ -deficient Jurkat T cells cannot activate NF- κ B in response to TNF and are highly sensitive to TNF-induced apoptosis. As part of our efforts to dissect TNFR1 signaling, we had generated a series of Jurkat T-cell signaling mutants as described previously (42). Briefly, the parental Jurkat T-cell line 3T8, which expresses TNFR1 but not TNFR2 (42), was created by sequential stable transfection of two reporter genes consisting of NF- κ B enhancer elements upstream of either CD14 or Thy1. Stimulation of this cell line with NF- κ B activators such as TNF or the phorbol ester PMA results in the surface expression of CD14 and Thy1. In order to obtain mutants with defects further downstream in the signaling cascade, the parental cell line was chemically mutagenized with ICR191 and cells that failed to express CD14 or Thy1 in response to PMA were repeatedly enriched by using a cell sorter. Clonal cell lines from this negatively enriched pool were obtained by limiting dilution. Initial characterization of one of these mutants revealed that clone 8321, in contrast to parental cells, failed to express Thy1 in response to a number of stimuli including TNF (Fig. 1a), PMA, and PHA (data not shown). That observation suggested that the genetic defect in clone 8321 affected a component of the NF- κ B signaling pathway shared by multiple stimuli and may lie in the IKK complex.

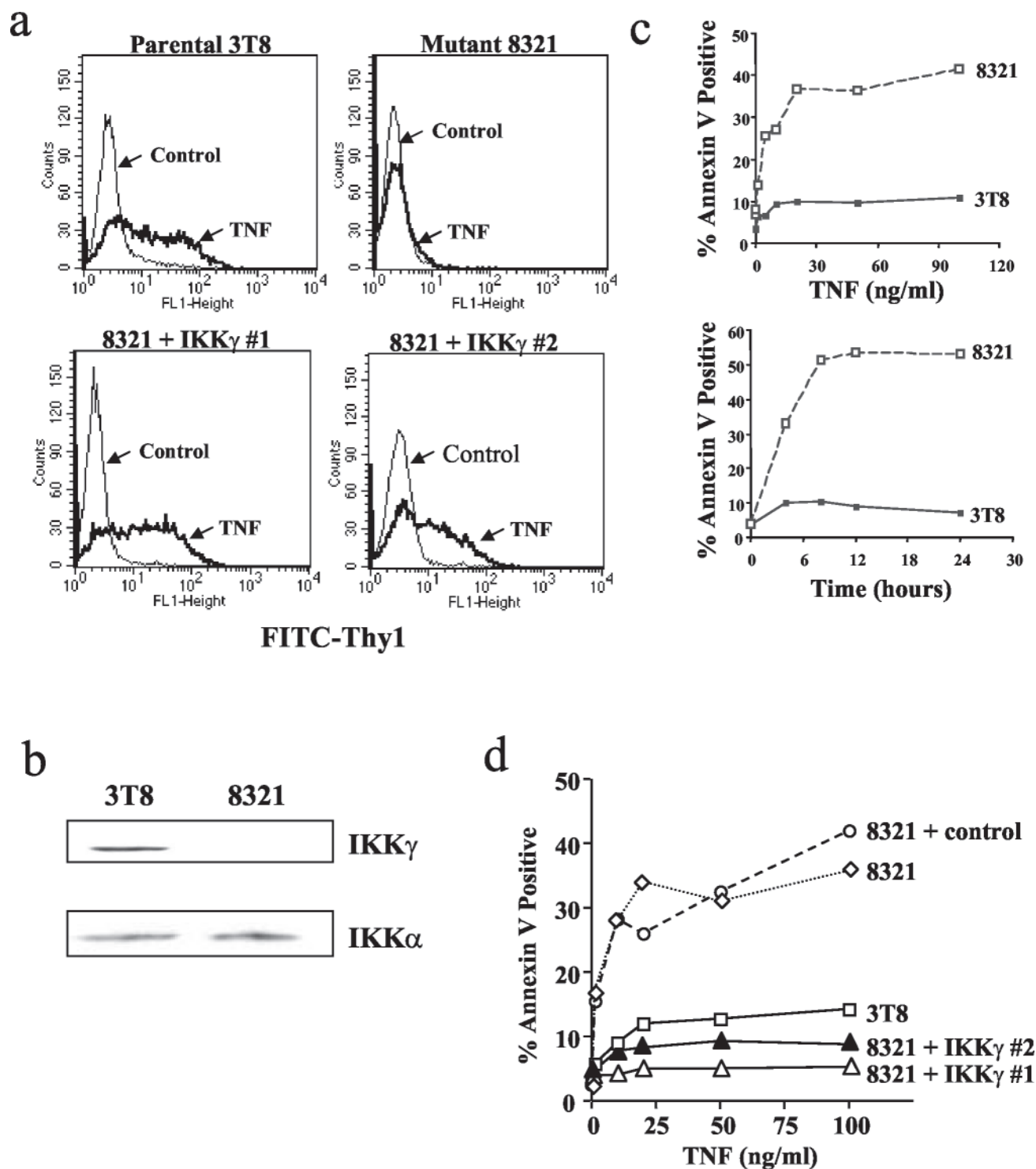


FIG. 1. IKK γ -deficient Jurkat T cells do not activate NF- κ B in response to TNF but are highly sensitive to TNF-induced apoptosis. (a) The parental 3T8 Jurkat T-cell line has NF- κ B-CD14 and NF- κ B-Thy1 reporter constructs. Parental 3T8 (top left panel), mutant 8321 (top right panel), and two 8321 clones complemented with an IKK γ expression plasmid (bottom two panels) were left unstimulated (thin line) or stimulated with 20 ng of TNF/ml for 8 h (thick line). Surface expression of Thy1 was analyzed by flow cytometry with fluorescein isothiocyanate-conjugated anti-Thy1. For complementation, 8321 cells were stably transfected with an IKK γ expression plasmid, and the responses of the complemented clones 1 and 2 are shown. Clones obtained from 8321 cells transfected with an irrelevant control vector were also similarly analyzed, and their responses were identical to untransfected 8321 cells (data not shown). (b) Detergent-soluble lysates from 3T8 and 8321 cells were immunoblotted with either IKK γ MAb (upper panel) or IKK α MAb (lower panel) to show equal loading. (c) 3T8 and 8321 cells were treated with the indicated concentrations of TNF for 4 h (upper panel) or with 20 ng of TNF/ml for the indicated times (lower panel). Apoptosis was analyzed by flow cytometry of cells stained with fluorochrome-conjugated annexin V. (d) Indicated cell lines were treated with various concentrations of TNF for 4 h and stained with fluorochrome-conjugated annexin V.

Subsequent immunoblotting analysis with antibodies against candidate molecules revealed that clone 8321 has a specific defect in the expression of IKK γ . IKK γ was readily detectable with an anti-IKK γ MAb in the parental 3T8 Jurkat cells but not in mutant 8321 cells, whereas the IKK α catalytic subunit was present in equivalent amounts in the two cell lines (Fig. 1b). Transfection of an expression vector encoding human IKK γ , but not an irrelevant control, restores TNF-induced NF- κ B activation and Thy1 expression in mutant 8321, demonstrating that the absence of IKK γ was the basis for the mutant phenotype (Fig. 1a).

Wild-type Jurkat T cells are largely resistant to TNF-induced cell death, and we therefore tested to see if the IKK γ -deficient Jurkat line exhibits any alteration in its apoptosis response to TNF. Consistent with published reports (2, 27, 43, 47), the defect in IKK γ expression and, consequently, the absence of TNF-induced NF- κ B activation resulted in enhanced susceptibility to TNF-mediated apoptosis in mutant 8321 (Fig. 1c). Stimulation of mutant 8321 cells with TNF for 4 h resulted in significant dose-dependent cell death as measured by annexin V reactivity, whereas parental 3T8 Jurkat cells remained largely viable (Fig. 1c, upper panel). Likewise, a time course study showed that mutant 8321 cells stimulated with 20 ng of TNF/ml rapidly succumbed to TNF-induced apoptosis with maximal apoptosis observed by 8 h, but this was not observed in the parental cells (Fig. 1c, bottom panel). Restoration of the TNF-induced NF- κ B response in the mutant cells with an IKK γ expression vector (Fig. 1a) returned the TNF sensitivity of the mutant cells to that observed in wild-type Jurkat cells (Fig. 1d). Taken together, these results confirm that the IKK γ -deficient cells possess enhanced sensitivity to TNF-induced apoptosis as a result of the lack of NF- κ B activation.

TNF-induced A20 gene expression in Jurkat T cells is NF- κ B dependent. The data presented above suggest that, in wild-type Jurkat T cells, TNF induces the expression of NF- κ B-dependent genes that then antagonize the apoptosis pathway. Therefore, the IKK γ -deficient Jurkat cells would be a good model system to identify these antiapoptotic genes and to study the mechanisms that they utilize to inhibit apoptosis. To do so, we began to screen for candidate genes that are induced by TNF in the parental Jurkat cells but not in the IKK γ -deficient mutant by reverse transcriptase PCR. One candidate gene that we tested was the zinc finger protein A20 gene which was originally identified as an immediate-early gene induced by TNF and has been reported elsewhere to have antiapoptotic effects (31, 32, 39). More recently, A20 has been shown elsewhere to associate with signaling molecules of the TNF pathway (40, 52). Although a low level of A20 expression was detectable in nonstimulated parental 3T8 Jurkat cells, A20 gene transcripts were induced approximately ninefold after 2 h of TNF stimulation (Fig. 2). In contrast to parental Jurkat cells, TNF stimulation did not induce the expression of A20 in the IKK γ -deficient Jurkat cells (Fig. 2). Therefore, the lack of IKK γ expression resulted in the inability of TNF to induce A20 gene expression, and this result is consistent with the fact that there are two NF- κ B sites in the A20 gene promoter (21). As A20 has been implied to be antiapoptotic in some systems and A20 $^{-/-}$ mice exhibit increased TNF-mediated cytotoxicity (22, 32, 39), the experiment in Fig. 2 suggests that the lack of A20

induction in the IKK γ -deficient cell line may contribute to its enhanced sensitivity to TNF-induced apoptosis.

A20 expression protects IKK γ -deficient cells against TNF-induced apoptosis. To examine whether the lack of A20 induction played a role in the increased susceptibility to TNF in the IKK γ -deficient cells, we tested to see if A20 expression in the mutant cells could inhibit TNF-induced cell death. Mutant 8321 cells were transduced with retrovirus encoding either an irrelevant control protein or a myc-tagged full-length human A20, upstream of the gene coding for puromycin resistance in a bicistronic configuration. 8321 cells transduced with the retroviruses were selected with puromycin, and the bulk pools of drug-resistant cells were used for all subsequent experiments. The exogenous myc-A20 was readily detected in the A20-transduced cells but not in the control-transduced cells by immunoblotting with anti-myc MAb (Fig. 3a).

The two groups of cells were next analyzed for their sensitivity to TNF-mediated killing. We used three different methods corresponding to different stages in cell death to quantify the level of apoptosis: annexin V reactivity to analyze the early changes in plasma membrane lipid composition, propidium iodide staining to measure DNA fragmentation, and MTS conversion assays to measure cell viability. Similar to untransduced IKK γ -deficient 8321 cells (Fig. 1c), TNF induces an apoptotic response in the 8321 cells expressing the irrelevant control protein in a time- and dose-dependent manner as assayed by annexin V staining (Fig. 3b). In contrast, 8321 cells expressing A20 were effectively protected from TNF killing (Fig. 3b). Similarly, the percentage of control-expressing 8321 cells with subdiploid DNA content increased from 6.3 to 58.6% upon stimulation with 20 ng of TNF/ml for 20 h, indicating increased DNA fragmentation. On the other hand, only a slight increase in subdiploid DNA content from 5.4 to 7.6% was observed in the A20-expressing 8321 cells (Fig. 3c). Finally, cell viability was assessed by MTS conversion assays after a 24-h treatment with various doses of TNF (0 to 100 ng/ml). The viability of control-expressing 8321 cells was dramatically reduced in the presence of increasing amounts of TNF whereas a significant proportion of the A20-expressing cells that were correspondingly treated remained viable (Fig. 3d). As an additional control in this experiment, parental 3T8 Jurkat cells transduced with the control virus were also analyzed in a similar fashion. Indeed, a comparison of the responses of the A20-expressing 8321 cells and the control parental 3T8 cells indicated that the expression of A20 was sufficient to return the TNF sensitivity of the IKK γ -deficient cells to that seen in the control parental 3T8 cells (Fig. 3d). Since the A20-expressing cells that we used in our experiments are from a bulk puromycin-resistant population, it is highly unlikely that the effect that we observed in the A20-expressing cells was due to clonal variation. In addition, the levels of surface expression of TNFR1 on the control and on A20-expressing cells were equivalent as assayed by the use of biotinylated TNF and phycoerythrin-conjugated streptavidin (data not shown).

A20 has been shown elsewhere to associate with IKK γ upon TNFR1 ligation, and further, IKK γ can be recruited to the activated receptor (9, 52). Therefore, we had initially speculated that the inhibition of apoptosis by A20 was dependent on IKK γ , as the IKK γ -mediated recruitment of A20 to the receptor-signaling complex could then enable A20 to inhibit apo-

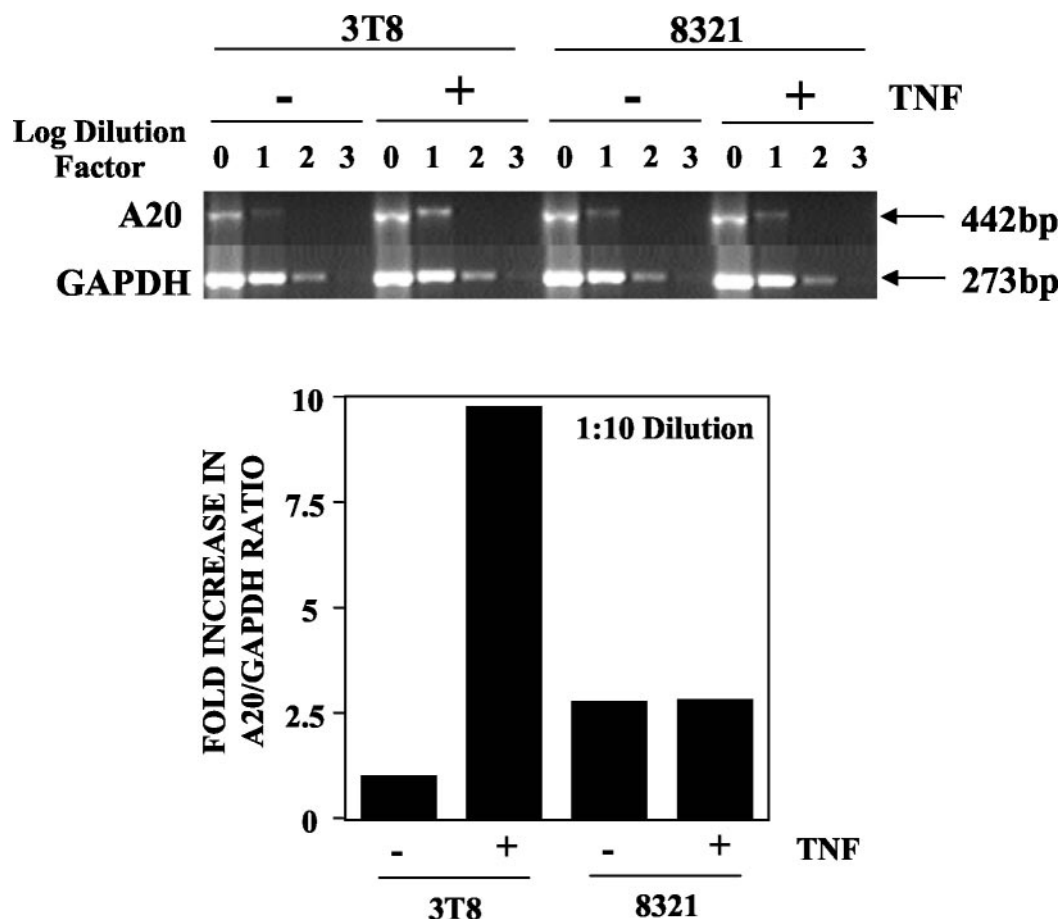


FIG. 2. TNF-induced A20 gene expression in Jurkat T cells is NF- κ B dependent. Parental 3T8 and mutant 8321 cells were treated with or without TNF (50 ng/ml) for 2 h. Total RNA isolated from each sample was reverse transcribed, and the resulting cDNA reaction mixtures were diluted 10-fold serially. A20 gene expression was analyzed by PCR of the serially diluted cDNA reaction mixtures (upper panel). The GAPDH gene was similarly analyzed to indicate equivalent RNA isolation and amplification in all samples (bottom panel). The 1:10 dilution of the cDNA reaction mixtures gave positive PCR signals that were within the linear range. The intensities of the bands were also quantified by densitometry, and the results from the 1:10 dilution are depicted in the bar graph below. For each sample, the ratio of the intensity of A20 to GAPDH was calculated and the value of the untreated parental 3T8 sample was set at 1.0. All other values shown are relative to that sample.

ptosis signaling molecules present in the complex. However, our results demonstrated that ectopically expressed A20 effectively blocked TNF-induced apoptosis in the IKK γ -deficient cells. This clearly shows that the interaction with IKK γ is not required for A20 to exert its antiapoptotic effect. Nevertheless, the induction of endogenous A20 by TNF is IKK γ dependent and requires an intact NF- κ B signaling pathway.

A20 expression inhibits TNF-induced activation of caspases. TNFR1 activation leads to the recruitment of TRADD and RIP to the receptor complex (13, 15, 45). TRADD and RIP then interact with FADD to initiate the death pathway through FADD's recruitment of caspase 8 (3, 6, 14, 24, 28). The clustering of caspase 8 results in its autoactivation and initiates the subsequent caspase proteolytic cascade (29, 30). To elucidate the underlying mechanism of A20 suppression of TNF-induced apoptosis, we examined whether the activation of the caspase cascade is affected by A20. We first determined whether A20 could block the processing of procaspase 8 induced by TNF. Control- or A20-expressing 8321 Jurkat cells were treated with 20 ng of TNF/ml for various lengths of time, lysed, and analyzed by immunoblotting with caspase 8 Mab. Treatment with

TNF resulted in the characteristic cleavage of inactive procaspase 8 to the active p43/41 form in the control-expressing 8321 cells, whereas in the A20-expressing 8321 cells, this processing of procaspase 8 was completely eliminated (Fig. 4). We also determined if the activation of caspase 3, a downstream effector caspase, is affected by A20. Control-expressing 8321 cells undergoing apoptosis induced by TNF showed an increase in the level of the catalytically active p19/17 form of caspase 3, and this response was significantly reduced in the A20-expressing 8321 cells (Fig. 4). The cleavage of downstream proteolytic substrates in response to TNF was also similarly affected by A20 expression. PARP, a substrate of caspase 3, was cleaved upon TNF stimulation in the control-expressing cells, and this cleavage was substantially decreased in the A20-expressing cells (Fig. 4). Taken together, these data demonstrate that the activation of the caspase cascade was inhibited by A20.

A20 specifically inhibits TNF- but not FAS-mediated apoptosis. The inhibitory effect of A20 on TNF-induced caspase 8 activation suggested that A20 either directly inhibits caspase 8 or inhibits some component of the TNFR1 pathway upstream

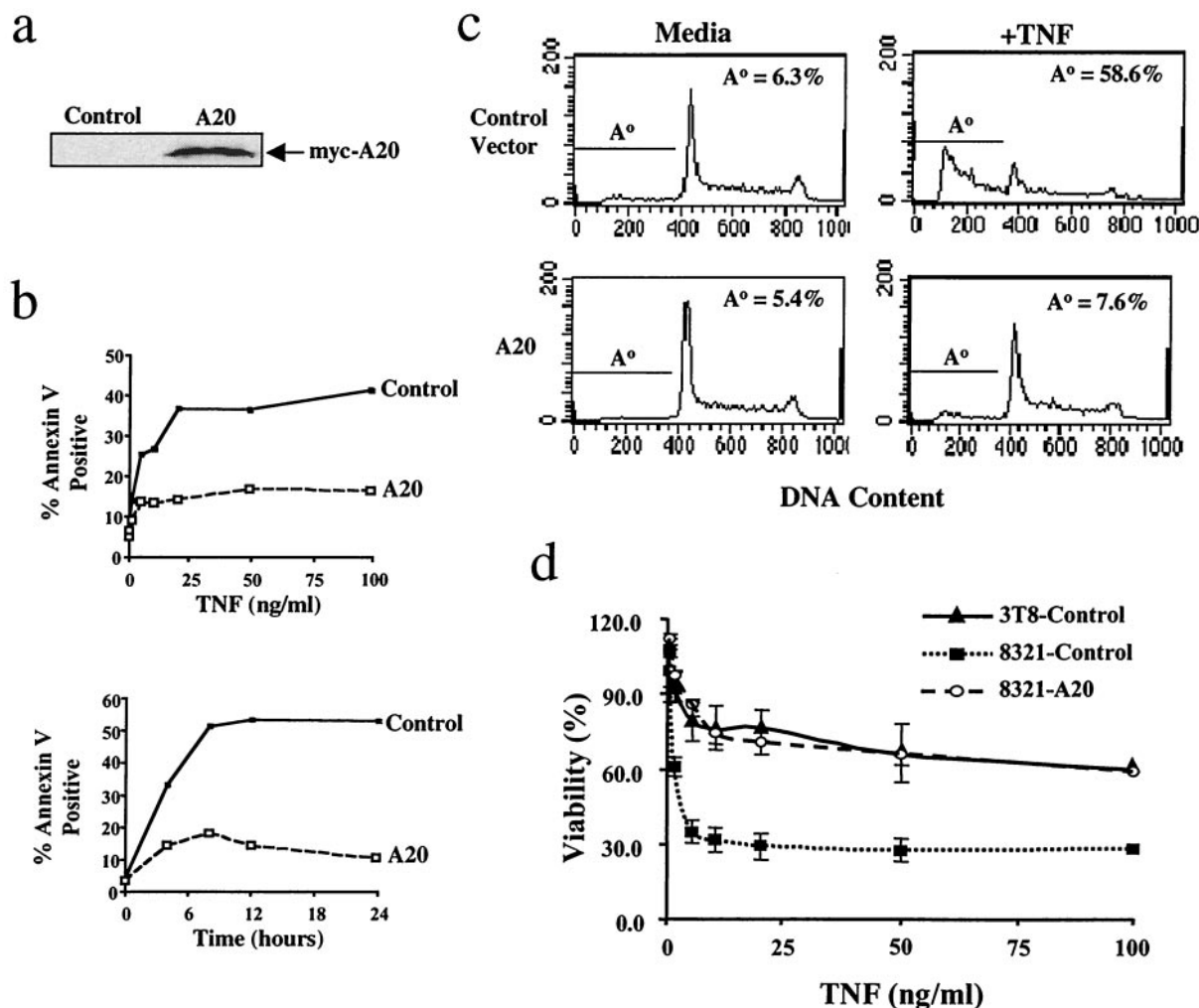


FIG. 3. A20 expression protects IKK γ -deficient Jurkat mutant 8321 against TNF-induced apoptosis. (a) 8321 cells were transduced with retroviruses encoding either irrelevant control or myc-tagged human A20 full-length gene, upstream of the puromycin resistance gene in a bicistronic configuration. Detergent-soluble lysates from puromycin-resistant cells were analyzed by anti-myc immunoblotting. (b) Control or A20-expressing 8321 cells were stimulated with the indicated concentrations of TNF for 4 h (top panel) or with 20 ng of TNF/ml for the indicated times (lower panel). Apoptotic cells were detected by annexin V reactivity as described for Fig. 1. (c) Control or A20-expressing 8321 cells were left unstimulated or stimulated with TNF (20 ng/ml) for 20 h. Cells were fixed, stained with propidium iodide, and analyzed by flow cytometry. A° indicates the percentage of cells with subdiploid DNA content ($<2N$). (d) Parental 3T8 cells expressing control protein (closed triangles) and 8321 cells expressing either the control (closed squares) or A20 protein (open circles) were treated with the indicated doses of TNF for 24 h. Cell viability was determined by MTS conversion assays in which the viability of untreated cells was normalized to 100%. Each data point represents the mean value and standard deviation from triplicate wells.

of caspase 8. To distinguish between these possibilities, we examined the effect of A20 on the related FAS/CD95 death receptor pathway. Although TNFR1 and FAS are highly homologous, the two receptors utilize different proximal signaling molecules to activate the caspase pathway. TNFR1 requires the adapters TRADD and RIP to transduce signals to FADD and the downstream caspase 8, whereas the FAS receptor does not require TRADD or RIP (1, 36, 42). Instead, FAS can interact directly with FADD, leading to the activation of caspase 8 (4, 5). If A20 inhibits the TNF apoptotic pathway at the level of FADD or caspase 8, it should then also inhibit FAS-triggered apoptosis. On the other hand, if A20 inhibits the TNF apoptotic pathway upstream of FADD or caspase 8, it would inhibit a signaling step that is unique to TNFR1 and would not inhibit FAS-triggered apoptosis.

To test which of the above two possibilities is correct, we examined the effect of A20 expression on FAS-induced apoptosis in the 8321 cells. Control or A20-expressing 8321 cells were treated with either 20 ng of TNF/ml or 500 ng of anti-FAS MAb/ml for different lengths of time. Apoptosis was subsequently assayed by annexin V staining. In the same experiment and with the same cells, A20 expression clearly inhibited TNF-induced apoptosis but had no effect on FAS-induced apoptosis (Fig. 5a). We also examined whether FAS-induced caspase-8 activation was affected by A20. 8321 cells expressing either control protein or A20 were stimulated with various concentrations of anti-FAS MAb, and total cell lysates were immunoblotted with caspase 8 MAb. Whereas A20 was able to inhibit the processing of procaspase 8 induced by TNF (Fig. 4), it did not have any effect on caspase 8 activation induced by

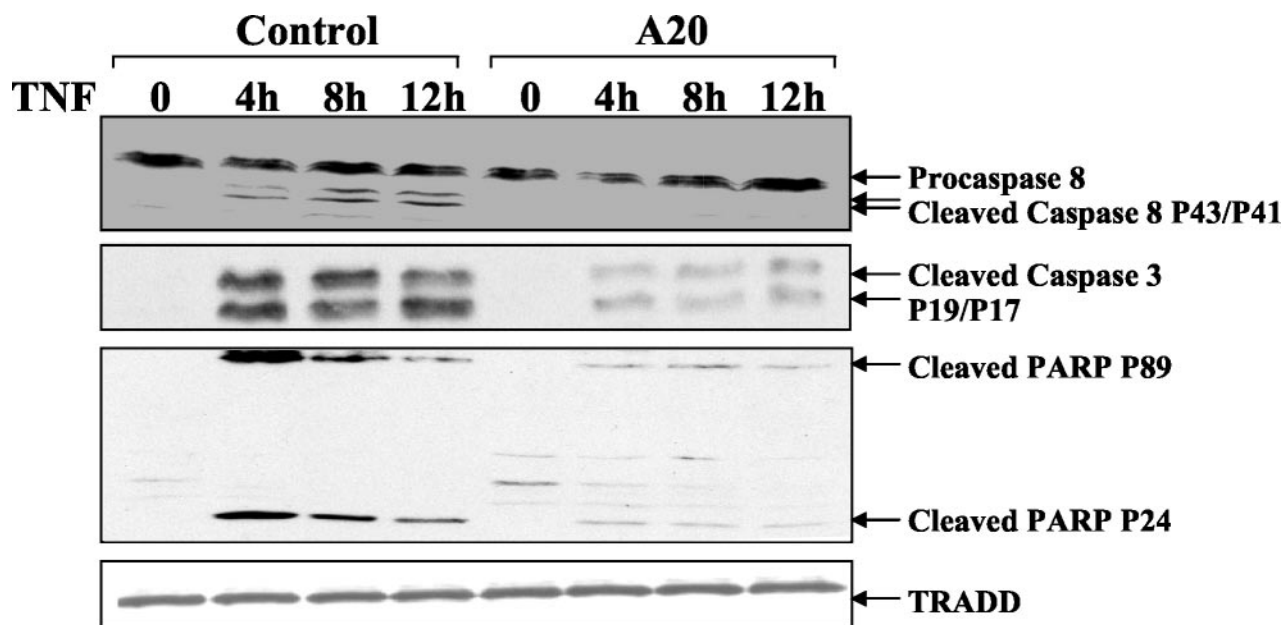


FIG. 4. A20 expression inhibits TNF-induced proteolytic cleavage of caspase 8, caspase 3, and PARP. Control or A20-expressing 8321 cells were lysed after treatment with TNF (20 ng/ml) for the indicated times. Detergent-soluble proteins were resolved by SDS-PAGE and immunoblotted with antibodies specific for caspase 8, caspase 3, and PARP. The caspase 3 and PARP antibodies used recognize the processed forms of the respective proteins. The membrane was also probed with TRADD MAb to determine equivalent loading.

FAS (Fig. 5b). This latter observation is consistent with the inability of A20 to inhibit FAS-induced apoptosis (Fig. 5a). The results in Fig. 5 suggest that A20 does not directly inhibit caspase 8; rather, it appears to target a signaling step prior to caspase 8 activation that is peculiar to the TNFR1 pathway.

A20 inhibits TNF-dependent recruitment of death domain signaling molecules to the TNFR1 complex. The experiments presented in Fig. 4 and 5 suggest that a proximal signaling component that is specific to the TNFR1 pathway is inhibited by A20. One such candidate molecule is the death domain kinase RIP. We had previously isolated a Jurkat T-cell mutant that is deficient in the expression of RIP. This genetic defect resulted in the inability of TNFR1 to induce apoptosis in this cell line, whereas FAS-induced apoptosis was unaffected (36, 42). Those analyses indicated that RIP has a unique role in apoptosis signaling triggered by TNFR1 but not by FAS. Since our previous experiment suggested that A20 inhibits a TNFR1-specific signaling component, we hypothesize that A20 may be inhibiting the proper functioning of RIP. One of the earliest biochemical signals detected upon TNFR1 ligation is the recruitment of RIP to the receptor complex (13). To test the hypothesis that A20 inhibits the functioning of RIP, we examined whether A20 has any effect on this recruitment event. Control or A20-expressing 8321 cells were left untreated or treated with 100 ng of TNF/ml for various lengths of time. Cells were then lysed in NP-40-containing buffer, TNFR1 was immunoprecipitated, and associated RIP protein was detected by immunoblotting. In control 8321 cells, stimulation with TNF resulted in the association of RIP with TNFR1 (Fig. 6a). However, this ligand-dependent association of RIP with TNFR1 was significantly reduced in the A20-expressing cells (Fig. 6a). In addition to RIP, the other apoptosis signaling adapter that is recruited to the ligated receptor is TRADD (14). Similar to

that observed with RIP, the recruitment of TRADD to the TNFR1 complex is also inhibited by A20 expression (Fig. 6a). These observations suggest that one mechanism by which A20 can disrupt apoptotic signaling from TNFR1 in T cells is by inhibiting the recruitment of the proximal signaling molecules TRADD and RIP to the receptor complex.

To test the functional effects of inhibiting RIP on the TNFR1 pathway, we examined the effect of the pharmacological agent geldanamycin, which has been shown previously to result in the loss of RIP expression (23). Consistent with that report (23), only the expression of RIP was specifically reduced in Jurkat 8321 cells treated with geldanamycin, whereas other signaling molecules were unaffected (Fig. 6b). This loss of RIP expression significantly inhibited TNF-induced apoptosis whereas FAS-induced apoptosis was not inhibited (Fig. 6b). The results obtained with geldanamycin parallel genetic studies that we had previously conducted with a Jurkat T-cell mutant deficient in RIP expression. In RIP-deficient Jurkat cells, TNFR1-induced apoptosis was impaired whereas FAS-induced apoptosis was not affected (36, 42). Therefore, both genetic and pharmacological approaches suggest that RIP function is crucial for inducing apoptosis, specifically in the TNFR1 pathway. Likewise, the inhibitory effect of A20 on the recruitment of RIP to TNFR1 is also likely to lead to the specific inhibition of TNF-induced, but not FAS-induced, apoptosis that we had observed.

DISCUSSION

In order to understand the molecular basis of the interplay between the NF- κ B transcriptional and apoptosis responses triggered by TNFR1, we have analyzed the effect of IKK γ deficiency on these two pathways in Jurkat T cells. We have

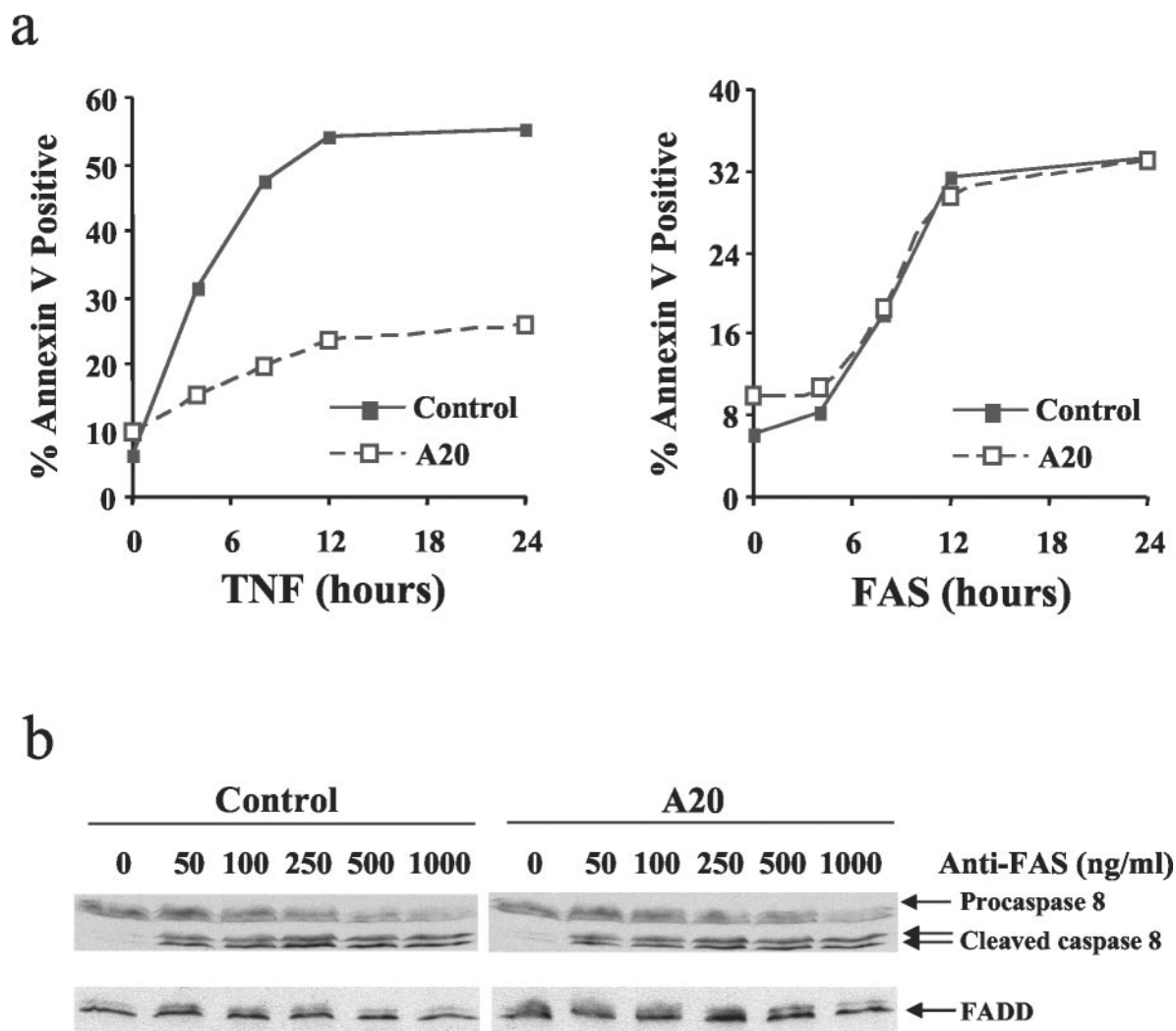


FIG. 5. A20 expression inhibits TNF- but not FAS-induced cell death and caspase activation. (a) Control or A20-expressing 8321 cells were stimulated with 20 ng of TNF/ml (left panel) or 500 ng of anti-FAS MAb/ml (right panel) for the indicated times. Cells were stained with fluorochrome-conjugated annexin V and analyzed by flow cytometry. (b) Control or A20-expressing 8321 cells were stimulated with the indicated concentrations of anti-FAS MAb for 4 h. Total cell lysates were immunoblotted with caspase 8 MAb or with FADD MAb to determine equivalent loading.

previously used somatic cell genetics to dissect TNF signaling in Jurkat T cells, and one of the mutants that we isolated in the course of these studies was deficient in the expression of IKK γ . We report here that IKK γ deficiency and the consequent lack of NF- κ B activation rendered Jurkat T cells highly sensitive to TNF-induced apoptosis. The fact that IKK γ -deficient Jurkat cells, in contrast to their wild-type counterparts, are highly sensitive to TNF-induced cell death is consistent with the notion that TNF induces the expression of NF- κ B-dependent genes that antagonize the cell death process. We subsequently analyzed candidate genes to examine which are induced by TNF in the wild-type cells but not in the IKK γ -deficient cells. One such NF- κ B-dependent gene encodes the zinc finger protein A20. To demonstrate that the lack of TNF-induced A20 expression contributed to the TNF cytotoxicity in the IKK γ -deficient cells, we showed that ectopic expression of A20 reversed the TNF sensitivity of the IKK γ -deficient cells to that

observed in wild-type cells. Therefore, A20 can inhibit the TNFR1 signaling pathway leading to apoptosis. Although IKK γ and A20 have been reported elsewhere to interact with each other (52), the fact that the antiapoptotic effect of A20 can be observed in the absence of IKK γ demonstrates that the IKK γ -A20 interaction is not required for A20 to exert its antiapoptotic effects.

How does A20 block apoptosis? The most proximal caspase that is activated during TNF-induced apoptosis is caspase 8 (3), and we tested to see if A20 affected the activation of caspase 8. The expression of A20 potentially inhibited the characteristic cleavage of the procaspase 8 zymogen to the catalytically active subunits induced by TNF. Furthermore, the processing and activation of the downstream effector caspase 3 as well as substrates were blocked by A20. The effect of A20 on caspase 8 led us to ask whether A20 is a direct inhibitor of caspase 8 or if A20 inhibits a signaling step in the TNFR1

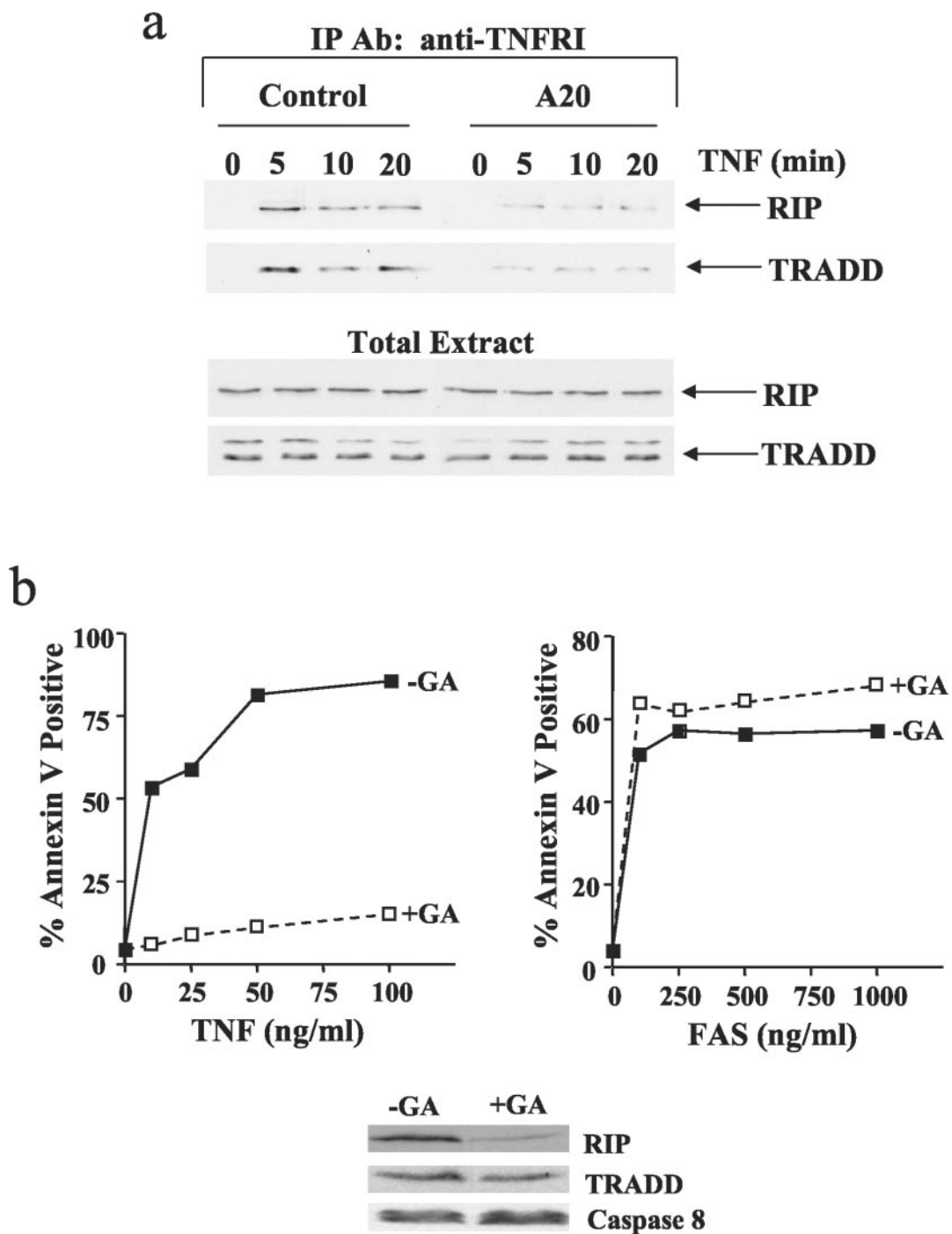


FIG. 6. A20 expression disrupts TNF-induced recruitment of TRADD and RIP to TNFR1. (a) Control or A20-expressing 8321 Jurkat cells were stimulated with TNF (100 ng/ml) for the indicated times. Cells were then lysed and immunoprecipitated with polyclonal goat anti-TNFR1. Immune complex proteins were resolved by SDS-PAGE and detected by immunoblotting with anti-RIP MAb or anti-TRADD MAb (upper panel). One percent of the total detergent extracts was similarly analyzed by immunoblotting (lower panel). (b) 8321 cells were treated or not with 1 μ M geldanamycin (GA) for 14 h. Cells were then stimulated with various concentrations of either TNF (left panel) or anti-FAS MAb (right panel) for an additional 4 h. Apoptosis was measured by annexin V staining. In the bottom panel, lysates from cells that were treated or not with geldanamycin were immunoblotted with the indicated antibodies to detect their expression levels. Among the signaling molecules examined, only the level of RIP was reduced by geldanamycin treatment.

pathway prior to caspase 8 activation. To answer that question, we examined whether A20 expression has any effect on caspase 8 activation and apoptosis triggered by FAS, a receptor that is highly related to TNFR1 (3, 46). Our results showed that A20

inhibits only the caspase 8 activation and apoptosis induced by TNFR1 and not by FAS. This argues that A20 does not directly inhibit caspase 8 activity but rather is targeting a component of the signaling pathway upstream of caspase 8 that is peculiar to

TNFR1. This observation also suggests that the level of retrovirus-mediated A20 expression in our cell lines did not result in nonspecific inhibition of cell death.

We had previously isolated a Jurkat T-cell mutant that is deficient in the expression of the death domain kinase RIP, and analysis of this mutant showed that RIP was required for TNFR1-induced apoptosis (36) but not for FAS-induced cell death (42). Based on the genetic analysis of the RIP-deficient mutant and the specific effect of A20 on TNFR1, we postulated that A20 might be affecting the signal transduction function of RIP. Following ligation of TNFR1, RIP is recruited to the receptor as part of a multiprotein complex (13), and so we investigated whether this crucial signaling event was affected by A20. Our analysis showed that this recruitment event was impaired by A20. In addition to RIP, the other death domain-containing adapter, TRADD, is also recruited to TNFR1 following receptor ligation (14). Our results showed that the recruitment of TRADD is also similarly inhibited by A20. Although the biological properties of A20 have been studied for some time, its biochemical mechanism of action has remained unclear to date. Our studies now show that, in Jurkat T cells, A20 inhibits the recruitment of the death domain signaling adapters TRADD and RIP to the receptor, thus providing a mechanistic explanation for the observed effect of A20 on the TNFR1 apoptosis pathway. While our present study examines the function of A20 with the Jurkat T-cell model, whether A20 has a similar effect in other cell types remains to be determined.

The biological effect of inhibiting these recruitment events by A20 is likely to be functionally analogous to a deficiency in RIP or TRADD expression. We have previously explored the consequences of RIP deficiency on the TNFR1 and FAS apoptosis pathways by using a Jurkat T-cell mutant with a genetic defect in RIP expression (36, 42). In this study, we have further addressed this by making use of the pharmacological agent geldanamycin, which selectively downregulates RIP expression. Consistent with our previous genetic studies, the decreased RIP expression induced by geldanamycin also resulted in impaired TNF-induced but not FAS-induced apoptosis. Thus, by both genetic and pharmacological approaches, the loss of RIP expression resulted in the specific impairment of the TNFR1 apoptosis pathway. By inhibiting the recruitment of this essential signaling molecule to the receptor complex, the effect of A20 expression is likely to be similar to that of a loss of function in RIP. The effect of a loss of function in TRADD on TNFR1 apoptosis signaling is less clear. Neither cell lines deficient in TRADD expression nor pharmacological drugs that selectively reduce TRADD levels have been described. However, a dominant-negative mutant of TRADD has been shown elsewhere to inhibit TNFR1 signaling (34), suggesting a crucial role for TRADD in this pathway. Hence, by inhibiting the recruitment of the key proximal signaling elements in the TNFR1 pathway, i.e., TRADD and RIP, A20 is then able to inhibit the downstream signaling events that lead to apoptosis.

In addition to the antiapoptotic effects of A20 that we and others (32, 39) have described, A20 is also known to potently inhibit NF- κ B activation induced by TNFR1 (7, 16, 40). Mouse embryonic fibroblasts derived from A20-deficient mice have sustained TNF-induced NF- κ B responses as well as increased

sensitivity to TNF-induced cell death (22). Since TRADD and RIP are also essential for TNFR1-induced NF- κ B activation (34, 42), the inhibitory effect of A20 on the recruitment of these signaling molecules may also account for the effect of A20 on TNF-induced NF- κ B activation. Deletional analysis of A20 in the NF- κ B context has shown that the carboxyl-terminal domain of A20 containing the seven zinc fingers was sufficient to block TNF-induced NF- κ B activation (19, 40). Preliminary analysis suggests that the zinc finger domain of A20 is also sufficient to inhibit TNF-induced apoptosis (data not shown). Whether the effect of A20 on TRADD and RIP also results in the inhibition of TNFR1-triggered NF- κ B activation awaits further investigation.

The mechanism by which A20 exerts its inhibitory effect on the recruitment of TRADD and RIP remains unclear at this point. Since TRADD binds directly to TNFR1 and is recruited rapidly to the receptor complex (14), our observation that A20 inhibits this earliest known signaling event suggests that A20 may be acting at the level of the receptor itself or at a regulatory step involved in the localization of the death domain signaling molecules. Our present speculation is that this does not involve a direct interaction between A20 and the death domain signaling molecules or between A20 and the receptor. Others have reported that A20 does not interact directly with either TRADD or RIP by the yeast two-hybrid analysis (52), and we have also been unable to detect an interaction between A20 and RIP in our IKK γ -deficient Jurkat T cells (data not shown). Furthermore, under conditions in which we can detect TNFR1-RIP interaction, we have not been able to detect TNFR1-A20 interaction (data not shown). Therefore, it is likely that A20 exerts its effect on TRADD and RIP through some intermediary protein. A number of proteins have been reported elsewhere to interact with A20, and these include TRAF2 (40), ABIN-1 (12), ABIN-2 (44), and TXBP151 (8). TRAF2 binds to the amino-terminal portion of A20, and this domain is not required for A20-mediated inhibition of NF- κ B (19, 40). Preliminary deletional analysis of A20 indicates that this TRAF2-A20 interaction is similarly redundant for the inhibitory effect of A20 on apoptosis (data not shown). ABIN-1, ABIN-2, and TXBP151 all have been reported elsewhere to bind to the zinc finger domain of A20 (8, 12, 44). As the biochemical functions of these novel proteins are not well understood, their roles in mediating the inhibitory effect of A20 on apoptosis also await further clarification.

While the manner by which A20 inhibits the recruitment of TRADD and RIP to the ligated receptor remains to be fully understood, what is becoming clear is that nature has devised a multitier system to block TNF-induced apoptosis at multiple steps in order to effectively prevent cell death. In addition to A20, TNF induces the expression of several other NF- κ B-dependent genes that are antiapoptotic. Wang et al. reported that TRAF1 and TRAF2 cooperate with c-IAPs (cellular inhibitor of apoptosis proteins) to inhibit TNF-induced cell death (48). TRAF proteins can recruit the c-IAPs to the receptor so that the c-IAPs may be able to interact directly with caspases to inhibit their catalytic function. Therefore, this mode of inhibition occurs at the level of the caspases. Another TNF-inducible antiapoptosis protein is c-FLIP, which interacts with FADD and, in a dominant-negative manner, disrupts the association of FADD with caspase 8 (20, 27). In this case,

c-FLIP's inhibition occurs at the level of FADD prior to the autoactivation of caspase 8. As reported here, A20 exerts its inhibitory effect further upstream prior to the recruitment of the death domain signaling molecules. Hence, it appears that each one of these proteins inhibits a successive and discrete step in the TNFR1 apoptosis pathway. The combined effect of all of these proteins is likely to be a synergistic shutdown of the TNF apoptosis program.

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REFERENCES

- Baud, V., and M. Karin. 2001. Signal transduction by tumor necrosis factor and its relatives. *Trends Cell Biol.* **11**:372–377.
- Beg, A. A., and D. Baltimore. 1996. An essential role for NF- κ B in preventing TNF- α -induced cell death. *Science* **274**:782–784.
- Boldin, M. P., T. M. Goncharov, Y. V. Goltsev, and D. Wallach. 1996. Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. *Cell* **85**:803–815.
- Boldin, M. P., E. E. Varfolomeev, Z. Pancer, I. L. Mett, J. H. Camonis, and D. Wallach. 1995. A novel protein that interacts with the death domain of Fas/APO1 contains a sequence motif related to the death domain. *J. Biol. Chem.* **270**:7795–7798.
- Chinnaiyan, A. M., K. O'Rourke, M. Tewari, and V. M. Dixit. 1995. FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell* **81**:505–512.
- Chinnaiyan, A. M., C. G. Tepper, M. F. Seldin, K. O'Rourke, F. C. Kischkel, S. Hellbardt, P. H. Kramer, M. E. Peter, and V. M. Dixit. 1996. FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor-induced apoptosis. *J. Biol. Chem.* **271**:4961–4965.
- Cooper, J. T., D. M. Stroka, C. Brostjan, A. Palmethofer, F. H. Bach, and C. Ferran. 1996. A20 blocks endothelial cell activation through a NF- κ B-dependent mechanism. *J. Biol. Chem.* **271**:18068–18073.
- De Valck, D., D. Y. Jin, K. Heyninck, M. Van de Craen, R. Contreras, W. Fiers, K. T. Jeang, and R. Beyaert. 1999. The zinc finger protein A20 interacts with a novel anti-apoptotic protein which is cleaved by specific caspases. *Oncogene* **18**:4182–4190.
- Devin, A., A. Cook, Y. Lin, Y. Rodriguez, M. Kelliher, and Z. Liu. 2000. The distinct roles of TRAF2 and RIP in IKK activation by TNF-R1: TRAF2 recruits IKK to TNF-R1 while RIP mediates IKK activation. *Immunity* **12**:419–429.
- DiDonato, J. A., M. Hayakawa, D. M. Rothwarf, E. Zandi, and M. Karin. 1997. A cytokine-responsive I κ B kinase that activates the transcription factor NF- κ B. *Nature* **388**:548–554.
- Harhaj, E. W., L. Good, G. Xiao, M. Uhlik, M. E. Cvijic, I. Rivera-Walsh, and S. C. Sun. 2000. Somatic mutagenesis studies of NF- κ B signaling in human T cells: evidence for an essential role of IKK γ in NF- κ B activation by T-cell costimulatory signals and HTLV-I Tax protein. *Oncogene* **19**:1448–1456.
- Heyninck, K., D. De Valck, W. Vanden Berghe, W. Van Crielinge, R. Contreras, W. Fiers, G. Haegeman, and R. Beyaert. 1999. The zinc finger protein A20 inhibits TNF-induced NF- κ B-dependent gene expression by interfering with an RIP- or TRAF2-mediated transactivation signal and directly binds to a novel NF- κ B-inhibiting protein ABIN. *J. Cell Biol.* **145**:1471–1482.
- Hsu, H., J. Huang, H. B. Shu, Y. Baichwal, and D. V. Goeddel. 1996. TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. *Immunity* **4**:387–396.
- Hsu, H., H. B. Shu, M. G. Pan, and D. V. Goeddel. 1996. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell* **84**:299–308.
- Hsu, H., J. Xiong, and D. V. Goeddel. 1995. The TNF receptor 1-associated protein TRADD signals cell death and NF- κ B activation. *Cell* **81**:495–504.
- Jaattela, M., H. Mouritzen, F. Elling, and L. Bastholm. 1996. A20 zinc finger protein inhibits TNF and IL-1 signaling. *J. Immunol.* **156**:1166–1173.
- Karin, M., and Y. Ben-Neriah. 2000. Phosphorylation meets ubiquitination: the control of NF- κ B activity. *Annu. Rev. Immunol.* **18**:621–663.
- Kelliher, M. A., S. Grimm, Y. Ishida, F. Kuo, B. Z. Stanger, and P. Leder. 1998. The death domain kinase RIP mediates the TNF-induced NF- κ B signal. *Immunity* **8**:297–303.
- Klinkenberg, M., S. Van Huffel, K. Heyninck, and R. Beyaert. 2001. Functional redundancy of the zinc fingers of A20 for inhibition of NF- κ B activation and protein-protein interactions. *FEBS Lett.* **498**:93–97.
- Kreuz, S., D. Siegmund, P. Scheurich, and H. Wajant. 2001. NF- κ B inducers upregulate cFLIP, a cycloheximide-sensitive inhibitor of death receptor signaling. *Mol. Cell. Biol.* **21**:3964–3973.
- Krikos, A., C. D. Laherty, and V. M. Dixit. 1992. Transcriptional activation of the tumor necrosis factor alpha-inducible zinc finger protein, A20, is mediated by kappa B elements. *J. Biol. Chem.* **267**:17971–17976.
- Lee, E. G., D. L. Boone, S. Chai, S. L. Libby, M. Chien, J. P. Lodolce, and A. Ma. 2000. Failure to regulate TNF-induced NF- κ B and cell death responses in A20-deficient mice. *Science* **289**:2350–2354.
- Lewis, J., A. Devin, A. Miller, Y. Lin, Y. Rodriguez, L. Neckers, and Z. G. Liu. 2000. Disruption of hsp90 function results in degradation of the death domain kinase, receptor-interacting protein (RIP), and blockage of tumor necrosis factor-induced nuclear factor- κ B activation. *J. Biol. Chem.* **275**:10519–10526.
- Liu, Z. G., H. Hsu, D. V. Goeddel, and M. Karin. 1996. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF- κ B activation prevents cell death. *Cell* **87**:565–576.
- Locksley, R. M., N. Killeen, and M. J. Lenardo. 2001. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* **104**:487–501.
- Mercurio, F., H. Zhu, B. W. Murray, A. Shevchenko, B. L. Bennett, J. Li, D. B. Young, M. Barbosa, M. Mann, A. Manning, and A. Rao. 1997. IKK-1 and IKK-2: cytokine-activated I κ B kinases essential for NF- κ B activation. *Science* **278**:860–866.
- Micheau, O., S. Lens, O. Gaide, K. Alevizopoulos, and J. Tschopp. 2001. NF- κ B signals induce the expression of c-FLIP. *Mol. Cell. Biol.* **21**:5299–5305.
- Muzio, M., A. M. Chinnaiyan, F. C. Kischkel, K. O'Rourke, A. Shevchenko, J. Ni, C. Scaffidi, J. D. Bretz, M. Zhang, R. Gentz, M. Mann, P. H. Kramer, M. E. Peter, and V. M. Dixit. 1996. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. *Cell* **85**:817–827.
- Muzio, M., G. S. Salvesen, and V. M. Dixit. 1997. FLICE induced apoptosis in a cell-free system. Cleavage of caspase zymogens. *J. Biol. Chem.* **272**:2952–2956.
- Muzio, M., B. R. Stockwell, H. R. Stennicke, G. S. Salvesen, and V. M. Dixit. 1998. An induced proximity model for caspase-8 activation. *J. Biol. Chem.* **273**:2926–2930.
- Opipari, A. W., Jr., M. S. Boguski, and V. M. Dixit. 1990. The A20 cDNA induced by tumor necrosis factor alpha encodes a novel type of zinc finger protein. *J. Biol. Chem.* **265**:14705–14708.
- Opipari, A. W., Jr., H. M. Hu, R. Yabkowitz, and V. M. Dixit. 1992. The A20 zinc finger protein protects cells from tumor necrosis factor cytotoxicity. *J. Biol. Chem.* **267**:12424–12427.
- Ory, D. S., B. A. Neugeboren, and R. C. Mulligan. 1996. A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. *Proc. Natl. Acad. Sci. USA* **93**:11400–11406.
- Park, A., and V. R. Baichwal. 1996. Systematic mutational analysis of the death domain of the tumor necrosis factor receptor 1-associated protein TRADD. *J. Biol. Chem.* **271**:9858–9862.
- Pickl, W. F., F. X. Pimentel-Muinos, and B. Seed. 2001. Lipid rafts and pseudotyping. *J. Virol.* **75**:7175–7183.
- Pimentel-Muinos, F. X., and B. Seed. 1999. Regulated commitment of TNF receptor signaling: a molecular switch for death or activation. *Immunity* **11**:783–793.
- Regnier, C. H., H. Y. Song, X. Gao, D. V. Goeddel, Z. Cao, and M. Rothe. 1997. Identification and characterization of an I κ B kinase. *Cell* **90**:373–383.
- Rothwarf, D. M., E. Zandi, G. Natoli, and M. Karin. 1998. IKK- γ is an essential regulatory subunit of the I κ B kinase complex. *Nature* **395**:297–300.
- Sarma, V., Z. Lin, L. Clark, B. M. Rust, M. Tewari, R. J. Noelle, and V. M. Dixit. 1995. Activation of the B-cell surface receptor CD40 induces A20, a novel zinc finger protein that inhibits apoptosis. *J. Biol. Chem.* **270**:12343–12346.
- Song, H. Y., M. Rothe, and D. V. Goeddel. 1996. The tumor necrosis factor-inducible zinc finger protein A20 interacts with TRAF1/TRAF2 and inhibits NF- κ B activation. *Proc. Natl. Acad. Sci. USA* **93**:6721–6725.
- Takeuchi, M., M. Rothe, and D. V. Goeddel. 1996. Anatomy of TRAF2. Distinct domains for nuclear factor- κ B activation and association with tumor necrosis factor signaling proteins. *J. Biol. Chem.* **271**:19935–19942.
- Ting, A. T., F. X. Pimentel-Muinos, and B. Seed. 1996. RIP mediates tumor necrosis factor receptor 1 activation of NF- κ B but not Fas/APO-1-initiated apoptosis. *EMBO J.* **15**:6189–6196.
- Van Antwerp, D. J., S. J. Martin, T. Kafri, D. R. Green, and I. M. Verma. 1996. Suppression of TNF- α -induced apoptosis by NF- κ B. *Science* **274**:787–789.
- Van Huffel, S., F. Delaei, K. Heyninck, D. De Valck, and R. Beyaert. 2001. Identification of a novel A20-binding inhibitor of nuclear factor- κ B activation termed ABIN-2. *J. Biol. Chem.* **276**:30216–30223.
- Varfolomeev, E. E., M. P. Boldin, T. M. Goncharov, and D. Wallach. 1996. A potential mechanism of "cross-talk" between the p55 tumor necrosis

- factor receptor and Fas/APO1: proteins binding to the death domains of the two receptors also bind to each other. *J. Exp. Med.* **183**:1271–1275.
46. Varfolomeev, E. E., M. Schuchmann, V. Luria, N. Chiannilkulchai, J. S. Beckmann, I. L. Mett, D. Rebrikov, V. M. Brodianski, O. C. Kemper, O. Kollet, T. Lapidot, D. Soffer, T. Sobe, K. B. Avraham, T. Goncharov, H. Holtmann, P. Lonai, and D. Wallach. 1998. Targeted disruption of the mouse caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. *Immunity* **9**:267–276.
47. Wang, C. Y., M. W. Mayo, and A. S. Baldwin, Jr. 1996. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF- κ B. *Science* **274**:784–787.
48. Wang, C. Y., M. W. Mayo, R. G. Korneluk, D. V. Goeddel, and A. S. Baldwin, Jr. 1998. NF- κ B antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. *Science* **281**:1680–1683.
49. Woronicz, J. D., X. Gao, Z. Cao, M. Rothe, and D. V. Goeddel. 1997. I κ B kinase- β : NF- κ B activation and complex formation with I κ B kinase- α and NIK. *Science* **278**:866–869.
50. Yamaoka, S., G. Courtois, C. Bessia, S. T. Whiteside, R. Weil, F. Agou, H. E. Kirk, R. J. Kay, and A. Israel. 1998. Complementation cloning of NEMO, a component of the I κ B kinase complex essential for NF- κ B activation. *Cell* **93**:1231–1240.
51. Zandi, E., D. M. Rothwarf, M. Delhase, M. Hayakawa, and M. Karin. 1997. The I κ B kinase complex (IKK) contains two kinase subunits, IKK α and IKK β , necessary for I κ B phosphorylation and NF- κ B activation. *Cell* **91**:243–252.
52. Zhang, S. Q., A. Kovalenko, G. Cantarella, and D. Wallach. 2000. Recruitment of the IKK signalosome to the p55 TNF receptor: RIP and A20 bind to NEMO (IKK γ) upon receptor stimulation. *Immunity* **12**:301–311.