The Journal of Immunology

Apoptosis Induced by the Toll-Like Receptor Adaptor TRIF Is Dependent on Its Receptor Interacting Protein Homotypic Interaction Motif

William J. Kaiser and Margaret K. Offermann

J Immunol 2005; 174:4942-4952; ;
doi: 10.4049/jimmunol.174.8.4942
http://www.jimmunol.org/content/174/8/4942

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Apoptosis Induced by the Toll-Like Receptor Adaptor TRIF Is Dependent on Its Receptor Interacting Protein Homotypic Interaction Motif[1]

William J. Kaiser and Margaret K. Offermann[2]

TLRs detect specific molecular features of microorganisms and subsequently engage distinct signaling networks through the differential use of Toll/IL-1R (TIR)-domain-containing adapter proteins. In this study, we investigated the control of apoptosis by the TIR domain-containing adapter proteins MyD88, TIR-domain containing adapter protein (TIRAP), TIR-domain-containing adapter-inducing IFN-β (TRIF), TRIF-related adapter molecule (TRAM), and sterile α motifs and β-catenin/armadillo repeats (SARM). Upon overexpression, TRIF was the sole TIR-adapter to potently engage mammalian cell death signaling pathways. TRIF-induced cell death required caspase activity initiated by the Fas/Apo-1-associated DD protein-caspase-8 axis and was unaffected by inhibitors of the intrinsic apoptotic machinery. The proapoptotic potential of TRIF mapped to the C-terminal region that was found to harbor a receptor interacting protein (RIP) homotypic interaction motif (RHIM). TRIF physically interacted with the RHIM-containing proteins RIP1 and RIP3, and deletion and mutational analyses revealed that the RHIM in TRIF was essential for TRIF-induced apoptosis and contributed to TRIF-induced NF-κB activation. The domain that was required for induction of apoptosis could activate NF-κB but not IFN regulatory factor-3, yet the activation of NF-κB could be blocked by superrepressor IκBα without blocking apoptosis. Thus, the ability of TRIF to induce apoptosis was not dependent on its ability to activate either IFN regulatory factor-3 or NF-κB but was dependent on the presence of an intact RHIM. TRIF serves as an adaptor for both TLR3 and TLR4, receptors that are activated by dsRNA and LPS, respectively. These molecular motifs are encountered during viral and bacterial infection, and the apoptosis that occurs when TRIF is engaged represents an important host defense to limit the spread of infection. *The Journal of Immunology,* **2005, 174: 4942–4952.**

Toll-like receptors are sentinels of the mammalian innate immune system that detect invariant molecular components of bacteria, viruses, yeast, and fungi during pathogen invasion (1–3). The human genome encodes at least 10 TLRs (4–9), and the pathogen-associated molecules recognized by some TLRs have been identified such as peptidoglycan (TLR2) (10–12), dsRNA (TLR3) (13), LPS (TLR4) (14–16), flagellin (TLR5) (17), ssRNA (TLR7/8) (18–20), and unmethylated CpG DNA (TLR9) (21). TLRs are characterized by an ectodomain composed of leucine-rich repeats and a cytoplasmic signaling domain called a Toll/IL-1R (TIR)[3] domain, which upon receptor activation, recruits one or more intracellular TIR domain-containing adapter proteins. The four mammalian TLR-adapter proteins, MyD88, TIR domain-containing adapter protein (TIRAP; MyD88 adapter-like protein, Mal), TIR-domain-containing adapter-inducing IFN-β (TRIF; TIR-containing adapter molecule-1), and TRIF-related adapter molecule (TRAM; TIR-containing adapter molecule-2/TIR-containing protein), bridge TLR activation to distinct signaling networks that regulate the host defense against infection (22–30). A fifth mammalian intracellular TIR-domain containing adapter was recently identified called sterile α motifs (SAM) and β-catenin/armadillo repeats (SARM), and sequences similar to SARM are also present in nematode, insect, and fish (31–35). Though the function of SARM remains unidentified, the nematode ortholog of SARM, TIR-1, is necessary for the cellular response to fungal infection and signals independently of the single TLR expressed in *Caenorhabditis elegans*, suggesting a potential role for SARM in innate immunity (36, 37).

MyD88 serves as an adapter for most members of the TLR and IL-1R superfamily (38, 39). In addition to a TIR domain, MyD88 contains an N-terminal death domain (DD) that interacts with the DD of the serine/threonine kinase IL-1R-associated kinase (IRAK)4 to promote IRAK4 autophosphorylation and subsequent phosphorylation of IRAK1 (40, 41). Phosphorylated IRAK1 binds the adaptor TRAF6, ultimately leading to IκB kinase (IKK) complex activation of NF-κB (42, 43). MyD88 functions independently of other TIR-adapter proteins for signaling by most TLRs, but it cooperates with a second adaptor protein, TIRAP, for responsiveness to activated TLR2 and TLR4 (44, 45). Both TLR3 and TLR4 can transduce signals independent of MyD88 through the adaptor TRIF (46, 47). Most, if not all, signaling by TLR3 occurs through TRIF without involvement of MyD88, whereas TLR4 can signal through either MyD88 or through TRIF. TRIF functions independent of other adaptors when activated by TLR3, whereas TLR4-induced signaling through TRIF occurs in cooperation with a fourth TIR adapter protein called TRAM (23, 25, 27).

Winship Cancer Institute, Emory University, Atlanta, GA 30322

Received for publication August 13, 2004. Accepted for publication January 28, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Public Health Service Grant RO-1 CA79402.

[2] Address correspondence and reprint requests to Dr. Margaret K. Offermann, Winship Cancer Institute, Emory University, 1365-B Clifton Road NE, Atlanta, GA 30322. E-mail address: mofferm@emory.edu

[3] Abbreviations used in this paper: TIR, Toll/IL-1R; DD, death domain; DED, death effector domain; DN, dominant negative; EGFP, enhanced GFP; FADD, Fas/Apo-1-associated DD protein; cFLIP$_s$, cellular FLIP, short isoform; IKK, IκB kinase; IRAK, IL-1R-associated kinase; IRF, IFN regulatory factor; ISRE, IFN-stimulated response element; RIP, receptor interacting protein; PRD, positive regulatory domain; RAIDD, RIP-associated ICH-1 homologous protein with death domain; RHIM, RIP homotypic interaction motif; SAM, sterile α motif; SARM, SAM and β-catenin/armadillo repeat; SR, super repressor; TBK, TANK-binding kinase; TIRAP, TIR-domain containing adapter protein; TRAM, TRIF-related adapter molecule; TRIF, TIR-domain-containing adapter-inducing IFN-β; TRADD, TNFR-associated DD protein.

0022-1767/05/$02.00

TRIF is the TLR adaptor that characteristically induces IFN-β transcription (22, 29, 47), a process that requires the coordinate activation of both IFN regulatory factor (IRF)-3 and NF-κB (48). The activation of IRF-3 is dependent on phosphorylation of specific serine residues by the two noncanonical IKKs, IKK-ϵ and TANK-binding kinase (TBK)1 (47, 49–52). Phosphorylated IRF-3 then translocates to the nucleus where it regulates the expression of numerous host defense genes including type I IFNs (53–55). The activation of NF-κB by TRIF involves direct binding of the adaptor TRAF6 that recruits the IKK complex (56, 57). The TRAF6 binding site is N-terminal to the TIR, whereas a distinct region that is C-terminal to the TIR domain binds the kinase receptor interacting protein (RIP)-1 and can activate NF-κB in the absence of the TRAF6 binding domains (22, 56, 57).

Apoptosis is a host defense against pathogen invasion that can be triggered by TLR ligands such as lipoproteins, dsRNA, and LPS (58–62). Apoptosis occurs through activation of members of the caspase family of cysteine proteases (63). Protein-protein interactions resulting from DD are often involved in caspase activation. For example, the DD of Fas can complex with the DD of the adaptor protein Fas/Apo-1-associated DD protein (FADD) that in turn recruits caspase-8, leading to its activation (64). MyD88 is the only TLR adaptor protein that has a DD. Although MyD88 can bind FADD and activate caspase-8, overexpression of MyD88 does not generally induce high levels of apoptosis (60, 61).

In this study, we examined the proapoptotic potential of the five human TIR domain-containing adapter proteins and show that TRIF was the only one that efficiently induced apoptosis when overexpressed in 293T cells. TRIF-induced apoptosis occurred through activation of the FADD-caspase-8 axis and could proceed without engagement of the intrinsic pathway. A RIP homotypic interaction motif (RHIM) present in the C terminus of TRIF formed complexes with both RIP1 and RIP3, and disruption of these interactions by mutating the RHIM eliminated the ability of TRIF to induce apoptosis. The domain that was required for induction of apoptosis could activate NF-κB but not IRF-3, but its ability to induce apoptosis was not directly linked to its ability to activate NF-κB.

Materials and Methods

Cell culture and transfections

Human embryonic kidney 293T cells were maintained in DMEM containing 4.5 g/ml glucose, 10% FCS (Invitrogen Life Technologies), 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin (Invitrogen Life Technologies). Transient transfections were performed using Lipofectamine2000 according to the manufacturer's protocol (Invitrogen Life Technologies).

Expression vectors and mutagenesis

Plasmids that express MyD88, TIRAP, TRAM, TRIF, and SARM as C-terminal FLAG epitope-tagged or enhanced GFP (EGFP) fusion proteins were constructed in the plasmids p3XFLAG14 (Sigma-Aldrich) and pEGFP-n1 (BD Biosciences Clontech). The open reading frames for MyD88, TRAM, and TRIF were PCR amplified from IMAGE cDNA clones obtained from American Type Culture Collection, the SARM cDNA was obtained from Open Biosystems, and TIRAP (β isoform) was PCR amplified from HUVEC cDNA (26). To generate a SARM expression vector, a 707-aa protein open reading frame was PCR amplified from an IMAGE cDNA (accession number BC040429). The TRIF cDNA (accession number BC035331) contained a frame-shift that was corrected by site-directed mutagenesis to restore the full-length 712-aa open reading frame and was cloned into p3XFLAG14 and pEGFP-n1 and pcDNA3.1/myc/His (22, 46). Site-directed mutagenesis was performed using the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene) and confirmed by sequence analysis. A series of vectors that express truncated forms of TRIF were generated by cloning PCR amplified sequences into p3XFLAG14 or pEGFP-n1, except for the ΔB3 mutant that was generated by deletion of sequence downstream of the PshAI site in TRIF. To mutate the RHIM domain of TRIF, the amino acids VQLG, which correspond to residues 687–690 in full-length TRIF, were replaced with four alanines by site-directed mutagenesis. Mutation of P434H was also achieved by site-directed mutagenesis.

C-terminal V5 epitope-tagged expression vectors were generated for multiple DD-containing proteins. Truncations containing DD of FADD (aa 96–208), RIP-associated ICH-1 homologous protein with death domain (RAIDD) (aa 99–199), IRAK1 (aa 1–211), IRAK2 (aa 1–96), IRAK4 (aa 1–186), and MyD88 (aa 1–114) were PCR amplified from IMAGE cDNAs or from HUVEC cDNA and then TOPO cloned into pcDNA3.1D/V5/His (Invitrogen Life Technologies), except IRAK4 (1–186) which was cloned into pcDNA3.1/myc/His. TNFR-associated DD protein (TRADD), Bax, cellular FLIP, short isoform (cFLIP$_s$), caspase-2, caspase-8-dominant negative (DN), and caspase-9-DN were all also TOPO cloned into pcDNA3.1D/V5/His (Invitrogen Life Technologies). The plasmids used as template for amplifying caspase-8-DN and caspase-9-DN were previously described (65). TRADD and caspase-2 were PCR amplified from IMAGE clones, and cFLIP$_s$ and Bax were amplified from cDNA provided by S.-Y. Sun (Winship Cancer Institute, Emory University, Atlanta, GA). Site-directed mutagenesis was performed to change the active-site cysteine to alanine in caspase-2 to generate a caspase-2-DN expression vector. All constructs TOPO cloned into pcDNA3.1D/V5/His expressed C-terminal V5-epitope tagged proteins except for Bax, which was cloned to retain a stop codon.

Vectors that express N-terminal myc-tagged Bcl2, Bcl-X$_L$, TBK1, IRF-3, IRF-3-ΔN (aa 56–452), RIP1, RIP2, murine RIP3, and RIP4 were generated by inserting into pcDNA3–6myc (N-terminal 6Xmyc tag) sequences amplified from human cDNA, except for RIP3 which was from murine cDNA. RIP1 cDNA was provided by L. R. Gooding (Department of Microbiology, Emory University), and all others were IMAGE expressed sequence tags. RIP1, RIP2, murine RIP3, and RIP4 were also cloned into p3XFLAG10 (N-terminal 3XFLAG tag). The RIP1 intermediate domain (RIP1-ID, aa 301- 488), was PCR amplified and cloned into the expression vectors pcDNA3–6myc and p3XFLAG10. A kinase dead TBK1 was generated by site-directed mutagenesis of the catalytic lysine 38 (K38) to alanine. The baculoviral caspase inhibitor, p35, from pUSE-p35 (Upstate), and the cowpox caspase inhibitor CrmA from plasmid provided by L. R. Gooding (Department of Microbiology, Emory University) were subcloned into the expression vector pcDNA3.1$^+$ (Invitrogen Life Technologies). Plasmid expressing a IκBα-super repressor (SR) (S32A/S36A) was obtained from BD Biosciences Clontech.

Cell death assays

Cells were cotransfected with 250 ng of pEGFPn1 (BD Biosciences Clontech) and 1.25 μg of the indicated expression vector(s). The concentration of transfected DNA was balanced with the appropriately matched empty vector. Twenty-four hours posttransfection, a minimum of 300 EGFP-positive 293T cells from three independent samples were scored as viable or apoptotic based on cellular and nuclear morphology. The morphologic criteria for apoptosis included fragmentation of DNA based on staining of DNA with Hoescht 33258 (Molecular Probes). We have previously compared these morphologic criteria of apoptosis to TUNEL and annexin staining and have found very similar results.

Western blot analysis

Cells were pelleted at 500 $\times$ g and then lysed at 4°C with agitation for 30 min in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, and protease inhibitor mixture (BD Pharmingen). For western blot analysis, cellular extracts or immunoprecipitates were combined with 3$\times$ SDS loading buffer (187.5 mM Tris-HCl, pH 6.8, 6% SDS, 30% glycerol, 0.03% bromophenol blue, 125 mM DTT). Proteins were resolved by SDS-PAGE using Criterion gels (BioRad), and then electrotransferred to Immobilon-P membranes (Millipore). Nonspecific binding sites were blocked by incubating membranes in TBS containing 0.05% Tween 20 and 5% (w/v) dry nonfat milk. Membranes were immunoblotted with one of the following primary Abs: cleaved caspase-3 D175 (Cell Signaling Technology), DFF45 (BD Pharmingen), c-Myc 9E10 (BD Pharmingen), or FLAG M2 (Sigma-Aldrich). Anti-mouse Ig G-HRP (BioRad) or anti-rabbit IgG-HRP (BioRad) were used as secondary Abs, and the immunoblots were visualized with the ECL system (Amersham Bioscience).

Immunoprecipitations

293T cells were seeded on 60-mm dishes and transfected the following day with 2.5 μg of DNA for each of the indicated constructs. To maintain cell viability, cells were also cotransfected with 2.5 μg of plasmid encoding the baculoviral caspase inhibitor p35. Twenty-four hours posttransfection,

cells were washed with PBS and then lysed in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) containing protease inhibitor mixture (Sigma-Aldrich) for 30 min with agitation and then pelleted at 12,000 × g for 10 min. Ten percent of the soluble fraction was combined with 3× SDS loading buffer and reserved for analysis of expressed proteins. The remainder of the soluble fraction was added to 20 μl of anti-FLAG M2-Agarose Affinity Gel (Sigma-Aldrich) and incubated on a roller shaker for 4–6 h at 4°C. The resin was washed five times with 0.5 ml wash buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl). After the final wash, the supernatant was removed with a 28-gauge insulin syringe, and the proteins bound to the resin were eluted in 100 μl of elution buffer (0.1 M glycine, pH 3.5) for 5 min at room temperature with agitation. The resin was centrifuged for 15 s at 10,600 × g; the supernatant was collected and then combined with 10 μl of 10× wash buffer (0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl). Immunoprecipitates were resolved by SDS-PAGE and analyzed by Western Blot as described.

Luciferase assays

The ISG54 IRSE and NF-κB luciferase reporter plasmids were obtained from Stratagene. To generate pGL3-IFN-β, the sequence corresponding to (−110 to +20) from −110IFN-β-CAT (48) was PCR amplified and cloned into pGL3-Basic (Promega). pGL3-PRD(II)$_2$ was cloned by inserting annealed complimentary oligos encoding two tandem positive regulatory domain II (PRDII) sites from the IFN-β promoter into pGL3-Basic (49, 66). pGL3-PRD(III-I)$_5$ was cloned by inserting annealed complimentary oligos encoding a total of five PRD(III-I) sites from the IFN-β promoter into pGL3-Basic (49, 66). Twenty-four-well plates were seeded with 293T cells and transfected 18–24 h later with 100 ng of the indicated firefly luciferase reporter plasmid, 500 ng of the indicated experimental plasmid(s), and 2.5 ng of phRL-TK that encodes *Renilla* luciferase under the control of the constitutively active herpes simplex thymidine kinase promoter (Promega). The amount of total DNA transfected was balanced with the appropriately matched empty vector backbone, and each experimental condition was performed in triplicate. Firefly and *Renilla* luciferase activities were determined using the Promega Dual Luciferase System, with firefly luciferase activity normalized to *Renilla* luciferase activity.

Results

Differences in apoptosis induction by adapter molecules

A schematic of the domain architecture of the five TIR domain containing adapter is shown in Fig. 1*A*. MyD88 is the only adaptor that contains a DD. Both TRIF and SARM are considerably longer than the other TIR domain containing adapter proteins. TRIF has 400 aa N-terminal to the TIR domain, but unlike the other four adapter molecules that contain the TIR at their C terminus, its TIR domain is flanked by a C-terminal extension. SARM is composed of an N-terminal series of β-catenin/armadillo motifs and two tandem SAM before a C-terminal TIR domain (31, 37). TIRAP and TRAM are the smallest of the five adapters and possess short N-terminal extensions with no known homology to other protein domains.

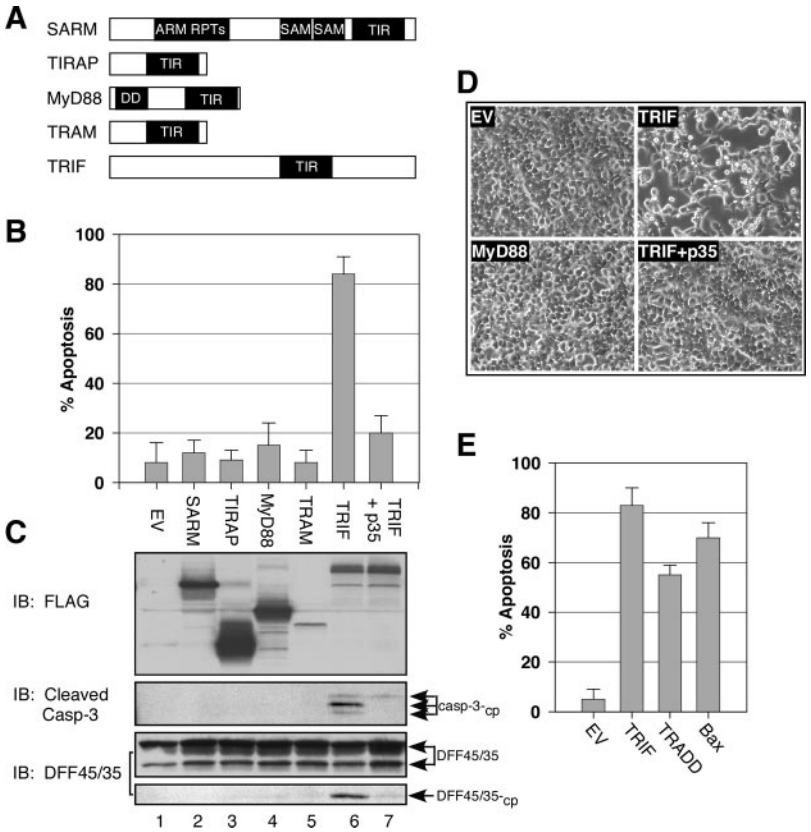

FIGURE 1. Induction of apoptosis by TIR domain-containing adapter proteins. *A,* A schematic representation of the domain architecture of the five TIR-adapter proteins is shown. The TIR domain is located at the C terminus of each adapter except TRIF where it more centrally located. MyD88 contains an N-terminal DD. SARM contains a series of N terminus proximal armadillo repeats (ARM RPTs) and two tandem SAM adjacent to the TIR domain. *B,* 293T cells were transfected in 12-well plates with 250 ng of pEGFPn1, 250 ng of plasmid expressing a C-terminal 3XFLAG epitope-tagged TIR-adapter protein, or 250 ng of empty vector (EV), p3XFLAG14-EV. Where indicated, cells were also transfected with 1 μg of a baculoviral p35 expression vector. Plasmid DNA concentrations were balanced with pcDNA3.1$^+$. Twenty-four hours posttransfection, the percentages (mean ± SD) of EGFP-positive cells ($n > 300$) scored as apoptotic were calculated. *C,* Twenty-four hours after transfection of 293T cells with FLAG epitope-tagged TIR adapter protein expression vectors, cell lysates were size-fractionated by SDS-PAGE and then immunoblotted with the Ab indicated at the *left* of each blot. Immunoreactive proteins are labeled. A 30-s exposure is shown for the DFF45/35 and a 5-min exposure of the same immunoblot is shown for the 10-kDa DFF45/35 caspase cleavage product (cp). *D,* 293T cell morphology 24 h posttransfection with empty vector (EV), TRIF, MyD88, or TRIF and baculoviral p35 (TRIF + p35). *E,* The percentages of transfected cells undergoing apoptosis (mean ± SD) were determined 24 h after transfection with 250 ng of pEGFP-n1, 1 μg of the empty vector pcDNA3.1$^+$, and 250 ng of pcDNA3.1-TRIF-myc-His, pcDNA3.1D-Bax, pcDNA3.1D-TRADD-V5-His, or pcDNA3.1$^+$ empty vector.

We transiently transfected 293T cells with FLAG epitope-tagged versions of each of the five adapter molecules, and a GFP expression vector was cotransfected with each TIR adaptor for the identification of transfected cells. Analysis of GFP expression demonstrated that the percentage of transfected cells was comparable for all adaptors (data not shown). Twenty-four hours post-transfection, we calculated the percentage of GFP-expressing cells with an apoptotic morphology that was characterized by cell shrinkage, membrane blebbing, and nuclear fragmentation. TRIF induced apoptosis in the majority of transfected cells, but there was no increase in cell death in response to the other four TIR adapters (Fig. 1, *B* and *D*). Western blot analysis using Ab to the FLAG epitope indicated that the level of expression of all of the adaptors except TRAM was higher than the expression of TRIF, so that their inability to induce apoptosis was not due to inadequate protein expression (Fig. 1*C*). Additional cell lines including HeLa and MCF-7 also responded to TRIF but not the other adaptors with an increase in apoptosis after transfection of epitope tagged or untagged constructs (data not shown).

We used a biochemical approach to confirm that TRIF-induced cell death was due to apoptosis. Apoptosis involves a proteolytic cascade that leads to the proteolytic activation of caspase-3, an effector caspase that serves as a point of convergence of intrinsic and extrinsic apoptotic pathways (67, 68). Activated caspase-3 then cleaves multiple cellular substrates, including the nuclease inhibitors DFF45 and its isoform DFF35, generating characteristic cleavage products (DFF45/35-$_{cp}$) indicative of apoptosis (69, 70). The cleavage of DFF45/35 releases the nuclease that causes irreversible DNA fragmentation during apoptosis. Western blot analysis demonstrated that the cells that were transfected with TRIF contained processed caspase-3 and cleaved DFF45/35, whereas cells transfected with the other adaptors did not (Fig. 1*C*). This indicates that the cell death induced by TRIF was due to apoptosis. Furthermore, the cell death that resulted from TRIF could be prevented by the broad-spectrum caspase inhibitor from baculovirus, p35 (71). Expression of p35 prevented TRIF-induced activation of caspase-3 and proteolysis of DFF45/35 (Fig. 1*C*), thereby restoring cell viability to control levels (Fig. 1, *B* and *D*). The amount of cell death resulting from TRIF was as high or higher than the amount induced by TRADD or Bax (Fig. 1*E*), proapoptotic molecules that activate extrinsic and intrinsic apoptotic pathways, respectively. Thus, TRIF is unique among the TIR-adapters in being a potent inducer of apoptosis.

TRIF-induces apoptosis through the FADD/caspase-8 axis

Proapoptotic signals first activate large prodomain caspases that subsequently target effector caspases such as caspase-3. For example, extrinsic death signals such as the cytokines Fas ligand or TRAIL promote caspase-8 and caspase-10 activation, genotoxic stress stimulates caspase-2, and apoptotic signals radiating from mitochondria activate caspase-9 (72–76). To better define the mechanism by which TRIF initiates caspase activation, we examined the ability of TRIF to induce apoptosis in the presence of DN forms of the initiator caspases-2, -8, or -9, each containing an inactivating mutation of the catalytic site. Of the three mutant caspases tested, only caspase-8-DN suppressed TRIF-induced apoptosis (Fig. 2*A*). cFLIP$_s$ and CrmA, which affect caspase-8 but not caspase-9 activity (77, 78), also blocked TRIF-induced apoptosis. In contrast, overexpression of the mitochondrial arbiters of cell death, Bcl2 and Bcl-X$_L$ (79, 80), did not affect the level of TRIF-induced cell death. Thus, apoptosis induced by TRIF overexpression likely involves caspase-8 activation and does not require mitochondrial amplification of the death signal.

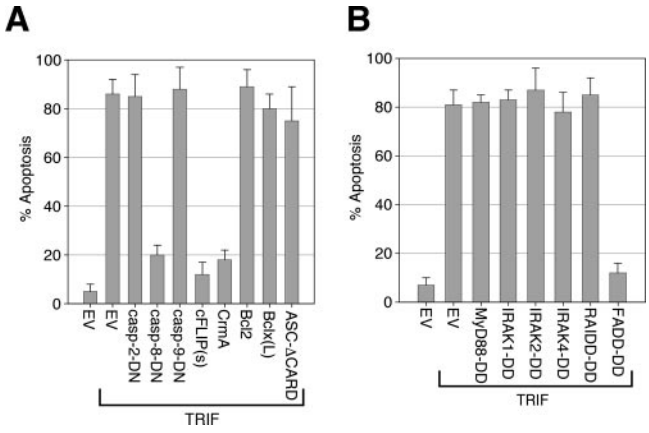

FIGURE 2. Identification of signaling pathways involved in TRIF-induced apoptosis. 293T cells were cotransfected with an EGFP expression vector, p3XFLAG14-TRIF, or the empty vector p3XFLAG14, and the indicated expression vector at a ratio of 1:1:4. All DNA concentrations were balanced with the empty vector pcDNA3.1$^+$. Twenty-four hours posttransfection, the percentage of apoptosis was calculated by directly scoring EGFP-positive cells ($n > 300$) as apoptotic based on cell morphology. Data represent the mean ± SD. EV, Empty vector.

Although TRIF lacks a DD, a DD-containing truncation of IRAK1 (aa 1–211) can suppress TRIF-induced activation of NF-κB (29), suggesting that DD-containing proteins might function downstream of TRIF. DD-containing truncations of MyD88, IRAK-1, IRAK-2, and IRAK-4 had no effect on TRIF-induced apoptosis (Fig. 2*B*) indicating that these DD-containing proteins involved in TLR-signaling do not relay proapoptotic signals from TRIF to caspase-8. The adapter protein RAIDD links caspase-2 to the DD-containing protein Pidd (75). Consistent with the inability of caspase-2-DN to block TRIF-induced apoptosis (Fig. 2*A*), the DD of RAIDD also failed to block apoptosis induced by TRIF (Fig. 2*B*). The only DD tested that blocked TRIF-induced apoptosis was the DD of FADD. FADD contains both a DD and a death effector domain (DED) and couples the ligation of death receptors to caspase-8 or -10 (81). This occurs through DD-DD interactions with either death receptors (e.g., Fas) or adapters (e.g., TRADD) and DED-DED interactions with caspase-8 or -10. Overexpression of a FADD-DD truncation, which contained the DD and lacked the DED domain, suppressed TRIF-induced apoptosis. This provides additional evidence that TRIF-induced apoptosis occurs through activation of the extrinsic apoptotic pathway, as indicated by the inhibition that occurred with caspase-8-DN, c-FLIPs and CrmA.

TIR-adapter proteins differ in their ability to transactivate NF-κB and IRF-dependent reporter constructs

Distinct gene expression profiles are activated by different TLRs, in part through the differential use of TIR-adapter proteins. Because differences in gene induction may account for the different levels of apoptosis by the TIR-adapter proteins, we compared the ability of the adapters to activate NF-κB and/or IRF-dependent reporter gene expression. We used two different NF-κB-dependent reporter genes: one containing two tandem copies of the PRDII element from the IFN-β promoter (labeled PRD II) (82) and the other containing five κB sites from the Ig κ L chain (labeled NF-κB). TRIF, MyD88, TIRAP, and TRAM activated NF-κB reporter gene expression, but the magnitude of the induction differed between the different adaptors (Fig. 3, *A* and *B*). SARM was the only TIR adaptor that did not induce an increase in NF-κB-dependent reporter activity. TIRAP induced less than half of the NF-κB reporter activity induced by either MyD88 or TRIF, and the induction by TRAM was only ~10% of the activity resulting from either

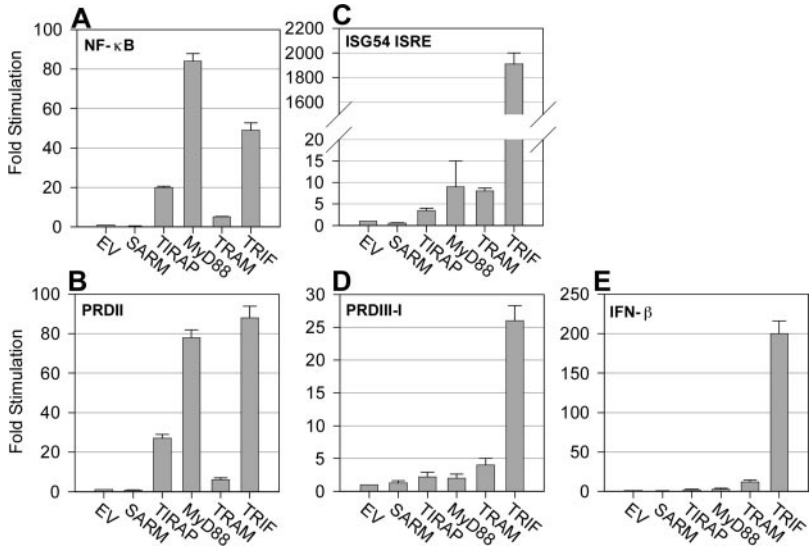

FIGURE 3. TIR-containing adapter protein activation of NF-κB and IRF-dependent reporters. 293T cells were transfected with 500 ng of TIR-adapter protein expression vector or empty vector, 100 ng of the indicated firefly luciferase reporter plasmid, and 5 ng of the *Renilla* luciferase expression vector, phRL-TK. 24 h post-transfection, firefly and *Renilla* luciferase activities were assayed. Firefly luciferase activity was divided by *Renilla* luciferase activity to normalize for transfection efficiency. The level of luciferase activity was then directly compared with the constitutive level present in cells transfected with empty vector. Data are expressed as the mean relative stimulation ± SD ($n = 3$) from a representative experiment.

MyD88 or TRIF. MyD88 induced higher levels of the κ L chain-derived NF-κB reporter than TRIF, whereas TRIF induced higher levels of the PRD II NF-κB reporter than did MyD88, but the differences in efficacy were relatively minor. In addition, both NF-κB-dependent reporters yielded a similar pattern of activation by the other TIR domain-containing proteins. We thus used only the κ-chain derived NF-κB reporter for subsequent studies.

More striking differences were observed when we tested the ability of the TIR-containing adapter proteins to transactivate IRF-dependent reporter gene expression. TRIF induced an 1800-fold increase in reporter expression using a reporter that contained five copies of the IFN-stimulated response element (ISRE) from the gene encoding ISG54 (ISG54 ISRE) (Fig. 3*C*). Induction by TIRAP, MyD88, and TRAM ranged between 5- and 10-fold above baseline levels, whereas there was no increase above baseline in response to SARM. IRF-3 binds adjacent PRDIII and PRDI sites in the IFN-β promoter (48), and a reporter gene containing five PRDIII-I sites (PRDIII-I) was stimulated >25-fold above basal levels by TRIF and slightly <5-fold by TRAM (Fig. 3*D*). The other adapter molecules did not significantly stimulate PRDIII-I reporter gene expression. Although the magnitude of the induction differed, the patterns of induction of the ISG54 ISRE and PRD III-I reporters were similar with the different adaptors. Thus, subsequent studies used the ISG54 ISRE to assess IRF-dependent transcriptional responses.

IFN-β induction involves the coordinate activation of both IRF-3 and NF-κB transcription factors (48). TRIF was the only adapter that induced high levels of activity from a reporter driven by the IFN-β promoter (Fig. 3*E*). This was expected because TRIF strongly activated both IRF-3 and NF-κB-dependent reporters. TRAM stimulated a several-fold increase in IFN-β reporter activity; however, compared with TRIF, this level of induction was minor. None of the other adapter molecules induced IFN-β reporter gene expression.

The C terminus of TRIF is responsible for induction of apoptosis through a mechanism that is not dependent on TRIF-induced activation of NF-κB or IRF-3

To map the proapoptotic region(s) of TRIF and determine whether apoptosis was linked to the transcriptional responses induced by TRIF, we generated a series of constructs that expressed truncations and/or mutants of TRIF (Fig. 4*A*). Each of these expression vectors contained a FLAG tag at the C terminus, and expression of each of these was confirmed by western blot analysis (data not shown). The expression vectors could be broadly classified as those that contained deletions or mutations that affected the TIR, deletion of sequences that were N-terminal to TIR or deletion of sequences that were C-terminal to TIR. The TIR-domain is important for TRIF-binding to TLR3 and TLR4 and binds the transcription factor IRF-3 (22, 56, 83, 84). The binding of TRAF6 occurs at a site that is N-terminal to TIR (56), whereas RIP-1 binds to sequences that are C-terminal to TIR (83, 85). We compared the ability of each construct to induce transcription of either the NF-κB or ISRE reporter and to induce apoptosis to identify domains associated with each of these functions. The majority of constructs induced at least one of these functions, whereas those that failed to induce any of these responses functioned as trans-dominant negatives. Thus, functional studies confirmed expression of each of the vectors, and their relative activities in the three different assays were used to define domains involved in the distinct responses.

Sequences in the TIR were more important for ISRE activation than for NF-κB activation or for induction of apoptosis (Fig. 4). The TIR domain in isolation did not induce apoptosis or activate NF-κB or ISRE reporters, whereas mutation of the TIR within full length TRIF (TRIF-FL/M) reduced ISRE activation by 40% without affecting NF-κB activation or apoptosis (Fig. 4*B*). The domain required for apoptosis was located C-terminal to the TIR. A C-terminal fragment (TRIF-C) lacking the TIR was sufficient to activate NF-κB and induce apoptosis, but it did not activate ISRE. The levels of NF-κB activation resulting from TRIF-C was at 116% the level of TRIF-FL, and apoptosis was at 60% of the level induced by TRIF-FL. When the C-terminal 67 aa of TRIF were deleted (TRIF ΔC2), increased apoptosis no longer occurred, whereas there continued to be some activation of both NF-κB and ISRE reporters. Thus, the NF-κB-dependent reporter could be activated by two distinct portion of TRIF: the C-terminal fragment (TRIF-C) and a construct lacking the C-terminal fragment (TRIF ΔC2). The ISRE reporter was most effectively activated by TRIF constructs containing the TIR and sequences N-terminal to the TIR (FL, ΔC, and ΔC2). When TRIF sequences N-terminal to TIR were deleted but the TIR and the C terminus were intact (TRIF ΔN), there was a 75% reduction in ISG54 ISRE reporter activity and a 20% reduction in NF-κB-dependent reporter activity, but it continued to induce apoptosis. When a single amino acid was mutated in the TIR of the construct lacking sequences N-terminal to

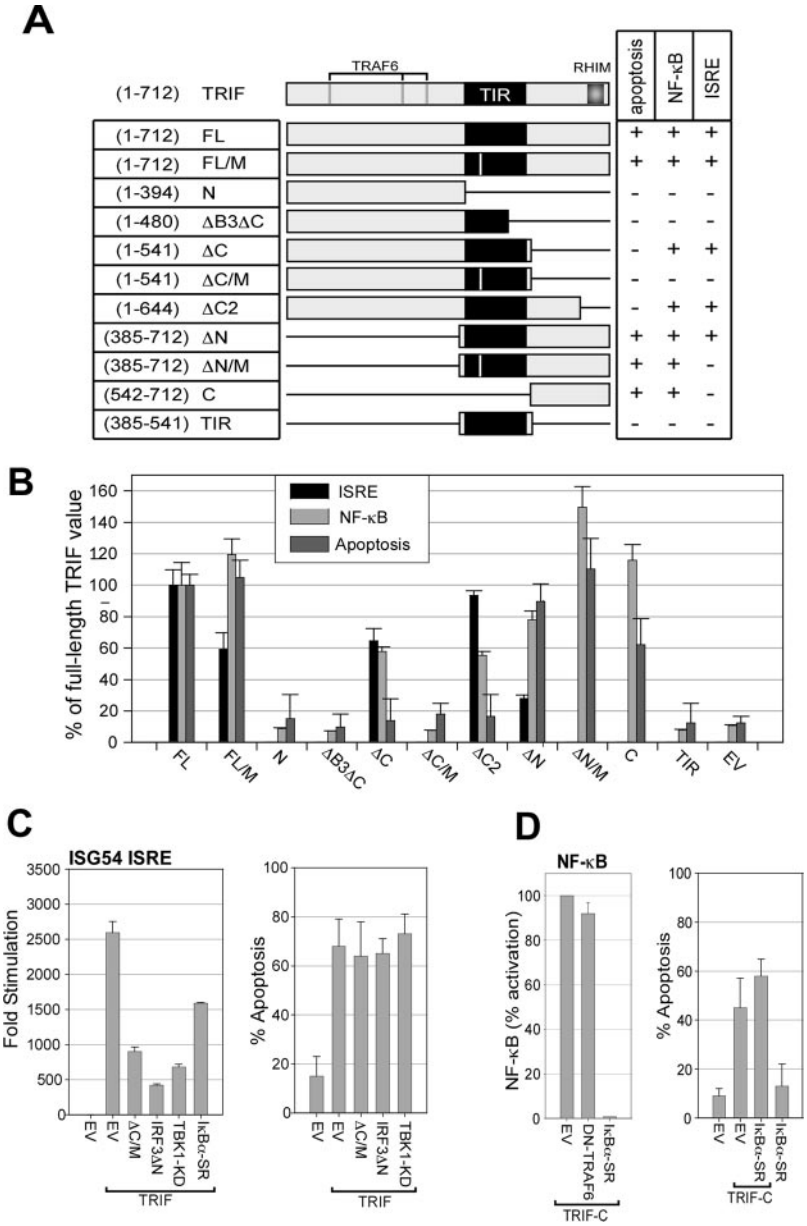

FIGURE 4. Mapping the regions of TRIF necessary for apoptosis induction. *A,* A schematic representation is shown of a series of constructs that express truncated forms of TRIF. All TRIF truncations are C-terminally 3XFLAG epitope-tagged. The amino acids of TRIF encoded by an expression vector are listed in the table on the *left* and are represented schematically with a shaded box noting the position of the TIR domain. The horizontal line indicates portions that were deleted from the TRIF. The truncations TRIF/M, ΔN/M, and ΔC/M contain the point mutation P434H in the TIR domain that is noted schematically by a solid vertical line in the shaded (TIR domain) box. The table on the *right* summarizes the ability of the various constructs to induce apoptosis, activate the NF-κB reporter, and or activate the ISG54 ISRE reporter based on the data shown in *B*. *B,* For assessment of transcriptional activity, 293T cells were transfected with 100 ng of the indicated TRIF expression vector or empty vector, 100 ng of either the NF-κB or ISG54 ISRE luciferase reporter vector, and 5 ng of the *Renilla* luciferase expression plasmid phRL-TK. Transcriptional activity that was standardized to TK was determined in triplicate for each condition. For determination of apoptosis, 293T cells were cotransfected with 250 ng of the indicated expression vector and 250 ng of an EGFP expression vector, and the percentages of EGFP-positive cells scored as apoptotic were determined 24 h posttransfection. The level of transcriptional activity and of apoptosis for each of the truncations and/or mutant constructs was compared with the level of activity generated by TRIF-FL and is plotted. *C,* The role of IRF-3 in TRIF-induced apoptosis was assessed by cotransfecting 250 ng of TRIF, 250 ng of EGFP expression vector, and 1 μg of plasmid encoding either TRIFΔC/M, IRF-3(ΔN), TBK1-K38A, or empty vector. The percentages of EGFP-positive cells scored as apoptotic were determined 24 h posttransfection. The disruption of ISG54 ISRE transcriptional activity was assessed by cotransfecting 400 ng of plasmid encoding either TRIFΔC/M, IRF-3(ΔN), TBK1-K38A, or empty vector along with 100 ng of TRIF, 100 ng of ISG54 ISRE luciferase reporter, and 5 ng of the *Renilla* luciferase expression plasmid phRL-TK. *D,* The role of NF-κB in the ability of TRIF-C to induce apoptosis was assessed by cotransfecting IκBα-SR with TRIF-C using a protocol analogous to that described in *C*, using the NF-κB-luciferase reporter to demonstrate efficacy of the IκBα -SR at blocking NF-κB activation by comparing percent activation to that seen with TRIF-C alone.

TIR (TRIF ΔN/M), its ability to transactivate ISRE was completely lost, whereas NF-κB activation was enhanced and apoptosis continued to occur. Deletion of sequences in the C terminus caused a relatively minor reduction in transactivation of the ISRE reporter. Both TRIF ΔC and TRIF ΔC2 activated ISG54-ISRE at 60–95% of the level of full length TRIF and NF-κB at ~50–60%

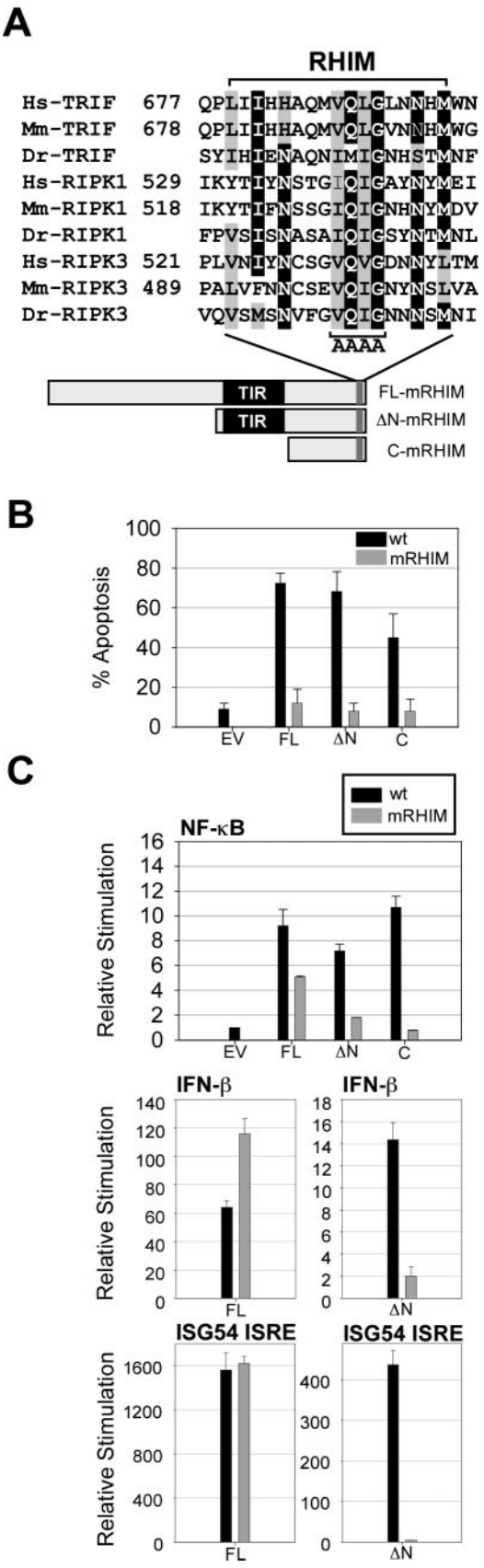

FIGURE 5. The role of the RHIM in TRIF-induced apoptosis. *A,* Sequence alignment of the RHIM present in TRIF, RIP, and RIP3 from human (Hs), mouse (Mm), and zebra fish (Dr) is shown. The RHIM alignment was shaded with the BLOSUM62 matrix at a 66% identity threshold. The number adjacent to each sequence in the alignment indicates the position of the amino acids shown within the full-length protein. To disrupt the RHIM in TRIF, the residues 687–690 TRIF were mutated to alanine and are noted by the position of the "AAAA" below the aligned sequences. TRIF constructs are represented schematically with a box indicating the C-terminal position of the RHIM in TRIF. *B,* 293T cells were cotransfected with 1.25 μg of either empty vector or the indicated TRIF-expression vector containing either wild-type or mutant RHIM and 250 ng of an EGFP expression vector. The percentage of EGFP-positive cells that were apoptotic were determined 24 h posttransfection. *C,* 293T cells were transfected with 100 ng of either empty vector or the indicated TRIF plasmid containing either wild-type or mutant RHIM and with 100 ng of the indicated luciferase reporter plasmid and 5 ng of the humanized TK *Renilla* luciferase reporter. Luciferase activity was measured 24 h posttransfection, normalized to *Renilla* luciferase activity, and then compared with the reporter activity present in empty vector-transfected cells. Data are reported as the mean relative stimulation ± SD (n = 3) from a representative experiment.

the level of the full length TRIF as long as the TIR was intact, but mutation of the TIR in a construct lacking the C terminus (TRIF ΔC/M) eliminated its transcriptional ability for both NF-κB and ISG54-ISRE.

Cotransfection of expression vectors that disrupted IRF-3 activation provided additional data that TRIF-induced apoptosis was not linked to IRF-3 activation. TRIF ΔC/M, which lacked the residues C-terminal to the TIR and contained a mutation in the TIR-domain, functioned as a DN to block TRIF-induced activation of ISG54 ISRE reporter without disrupting TRIF-induced apoptosis (Fig. 4*C*). Similarly, IRF-3 ΔN, which lacked the transactivation domain of IRF-3, and TBK-1-kinase dead (TBK-1-KD), which prevented the phosphorylation of IRF-3, both blocked TRIF-induced transaction of the ISG54 ISRE reporter without affecting TRIF-induced apoptosis. Thus, multiple mechanisms for disrupting IRF-3 activation did not prevent TRIF-induced apoptosis.

The portion of TRIF that was required for apoptosis could activate NF-κB (e.g., TRIF-C), but a distinct portion of TRIF also activated NF-κB without inducing apoptosis (e.g., TRIF ΔC) (Fig. 4*B*). This suggested that the induction of apoptosis by TRIF was not dependent on the ability of the proapoptotic fragment to activate NF-κB. When NF-κB activation was prevented by coexpressing SR IκBα (IκBα-SR), NF-κB activation by TRIF-C was fully blocked, but apoptosis was not affected (Fig. 4*D*). Thus, the ability of TRIF and its truncations to induce apoptosis could be dissociated from the activation of NF-κB.

TRIF contains a RHIM

The C terminus of TRIF that was sufficient to activate NF-κB and was necessary to induce apoptosis possessed sequence similarity to the RHIM previously identified in the kinases RIP1 and RIP3 (86). Alignment of human, mouse, and zebrafish homologues of TRIF, RIP1 and RIP3 supports the presence of a RHIM at the C terminus of TRIF that is conserved in vertebrates (Fig. 5*A*). To investigate the role of the RHIM in TRIF-induced apoptosis and NF-κB activation, we disrupted the RHIM domain by replacing the conserved residues VQLG (aa 687–690) with four alanines. All of the TRIF constructs with wild-type RHIM were proapoptotic, whereas mutation of the RHIM in the TRIF constructs eliminated the ability of TRIF to induce apoptosis (Fig. 5*B*). Mutation of the RHIM in TRIF-FL reduced NF-κB activation by 50% without reducing the magnitude of induction of the ISG54 ISRE or IFN-β reporters (Fig. 5*C*). When the TRAF 6 binding sites were absent, an intact RHIM was required for NF-κB activation (e.g., TRIF(ΔN) and TRIF(C) (Fig. 5*C*)). Thus, the RHIM in TRIF contributes to both NF-κB activation and apoptotic signaling pathways but is not important for IRF-dependent gene expression. The RHIM nonetheless contributed to IRF-dependent transactivation when sequences

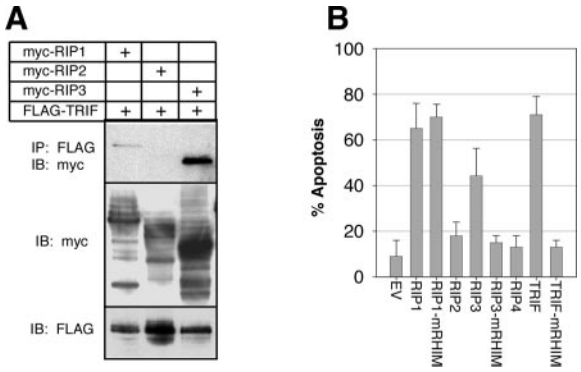

FIGURE 6. Association of RIP1 and RIP3 with TRIF. *A,* 293T cells were cotransfected with expression vectors for Myc epitope-tagged RIP 1, 2, or 3 and 3XFLAG epitope-tagged TRIF. Plasmid expressing baculoviral p35 was also cotransfected to maintain cell viability until cells were harvested 24 h posttransfection. Cellular lysates were immunoprecipitated (IP) with anti-FLAG M2 beads and analyzed by immunoblot with anti-Myc Ab. Immunoblot analysis of total cell lysate confirmed the expression Myc- and FLAG-tagged proteins. *B,* Cells were cotransfected with 250 ng of an EGFP expression vector and 250 ng of empty vector or vector encoding the indicted protein with either wild-type or mutant RHIM. The percentage of EGFP-positive cells that were apoptotic was determined 24 h posttransfection.

N-terminal to the TIR were lacking. The low levels of transactivation of ISG54 ISRE and IFN-β reporters that occurred with TRIF(ΔN) were abolished by mutation of the RHIM in TRIF(ΔN).

The activation of apoptosis by TRIF is dependent on an intact RHIM and is not dependent on activation of NF-κB

The RHIM that is found in both RIP1 and RIP3 is essential for heterodimerization of these proteins, whereas the related serine-threonine kinases RIP2 and RIP4 lack RHIMs. The presence of a RHIM in TRIF suggested a physical association between TRIF and RIP1 and/or RIP3 but not the RIPs lacking a RHIM, and this was confirmed using cells that were cotransfected with FLAG-tagged TRIF and myc-tagged RIP1, 2, or 3. Immunoprecipitation demonstrated that FLAG-TRIF coprecipitated the RIP1 and RIP3 but not with RIP2 (Fig. 6*A*).

Transfection studies compared the ability of TRIF and the RIPs to induce apoptosis. Transfection of either TRIF or RIP1 induced apoptosis in >60% of transfected cells, whereas RIP3 induced apoptosis in ~40% of cells (Fig. 6*B*). In comparison, overexpression of RIP2 and RIP4 had little or no effect on apoptosis compared with empty vector. When the RHIM of either TRIF or RIP-3 was mutated, enhanced apoptosis no longer occurred, whereas mutation of the RHIM of RIP-1 had no effect on its ability to induce apoptosis. This suggests that the ability of TRIF and RIP-3 to form complexes through their RHIMs was important for the induction of apoptosis, whereas RIP-1 could induce apoptosis without forming RHIM-dependent complexes.

Discussion

Of the five TIR-containing adaptors, TRIF was the only one that induced apoptosis in a high percentage of 293T cells when expressed by transfection. TRIF was also the only adaptor molecule that induced high levels of IRF-dependent transcriptional activation, whereas all of the adaptors except SARM effectively activated NF-κB responsive reporters. TRIF-induced cell death could be reversed when activation of caspase-3 was blocked with p35, demonstrating that cell death was due to apoptosis. The apoptosis induced by TRIF occurred primarily through the extrinsic apoptotic pathway, and the intrinsic pathway was not required. This was shown by the inhibition of TRIF-induced apoptosis by antagonists of the extrinsic apoptotic pathway such as FADD-DD and a catalytically inactive caspase-8, whereas inhibitors of the intrinsic pathway had no effect.

Although activation of IRF-3 can contribute to apoptosis in some cells (87), it was not involved in TRIF-induced apoptosis. Deletion of the N-terminal region of TRIF inhibited transactivation of IRF-dependent reports without inhibiting TRIF-induced apoptosis. In addition, dominant-negative forms of IRF-3 and TBK1 reduced TRIF-induced transactivation of IRF-dependent reporter constructs but had no effect on TRIF-induced apoptosis. Truncations of TRIF that were unable to activate IRF-dependent reporters were nearly as effective as full length TRIF at inducing apoptosis. Conversely, high levels of activation of IRF-dependent reporters occurred when the C-terminal fragment of TRIF that induced apoptosis was deleted. Activation of IRF-dependent reporters by TRIF was dependent on sequences within the TIR, but the TIR was not important for induction of apoptosis. A single amino acid change within the TIR of TRIF-FL was sufficient to eliminate >40% of TRIF-induced activation of IRF-dependent reporters without inhibiting NF-κB activation or apoptosis. The importance of the TIR in IRF-dependent activation probably reflects the role of the TIR in the binding of IRF-3 and IRF-7 to TRIF (83). Sequences N-terminal to TIR were also important in IRF-reporter activation and are thought to contain the binding sites for TBK1 and IKKε, proteins that are critical for the activation of IRF-3 (49, 50, 57, 83). Surprisingly, low levels of activation of IRF-3-dependent reporters occurred when sequences N-terminal to the TIR were absent as long as the TIR and C-terminal sequences were intact.

Induction of apoptosis by TRIF could also be dissociated from the activation of NF-κB. Two nonoverlapping portions of TRIF were capable of activating NF-κB: the N-terminal region that contained TRAF-6 binding sites (56, 57) and the C-terminal fragment that contained the RHIM. The domain of TRIF that was required for inducing apoptosis was localized to the RHIM found in the C terminus. NF-κB activation occurred at ~50% of the level of wild-type TRIF when the RHIM was either mutated or deleted as long as the TIR and sequences N-terminal to the TIR were intact, but mutation or deletion of the RHIM fully blocked any increase in apoptosis. When the RHIM of TRIF was intact, IκBα-SR fully blocked NF-κB activation without preventing apoptosis, indicating that activation of NF-κB was not required for TRIF-induced apoptosis. We did not see any activation of NF-κB by the N-terminal fragment unless the TIR was intact even though binding of TRAF6 to TRIF occurs at aa 250–255 and does not require the TIR (57). The C-terminal domain of TRIF activated NF-κB when the TIR and TRAF 6 binding sites were absent as long as the RHIM was intact. The involvement of both TRAF6 and RIP1 in TRIF-induced activation of NF-κB resembles the TNFR1 that uses the TRAF2 and RIP1 for the recruitment and activation of the IKK complex (88).

Mutation of the RHIM in TRIF eliminated TRIF-induced apoptosis and eliminated complex formation with RIP1 and RIP3, suggesting that the physical association between TRIF and one or both of these serine/threonine kinases was important in the induction of apoptosis. RIP-1, but not RIP-3, contains a DD that can complex with FADD and activate the extrinsic apoptotic pathway (89, 90). This process is more likely to occur when high levels of RIP-1 are expressed by transfection than in response to the constitutive levels of RIP-1 that contribute to NF-κB activation but are not associated with excessive apoptosis. The ability of DD-containing proteins to induce apoptosis is affected by protein conformation and by the relative affinity for FADD compared with other

DD-containing proteins. The DD-containing protein MyD88 can complex with FADD (60), but it did not induce apoptosis when it was expressed at levels that were as high as TRIF, indicating that the mere presence of a DD was not sufficient to induce apoptosis. Furthermore, the DD of FADD blocked TRIF-induced apoptosis, whereas the DDs of IRAKs or RAIDD had no effect, providing evidence for a specific interaction with the DD of FADD in TRIF-induced apoptosis. Mutation of the RHIM of either TRIF or RIP3 eliminated their ability to induce apoptosis, indicating that TRIF and RIP-3 needed to associate with other proteins through their RHIMs to induce apoptosis, whereas transfected RIP1 induced apoptosis without associating with RHIM-containing proteins. RIP-1 binds to and activates the IKK complex (88, 91), leading to NF-κB activation, whereas RIP-3 suppresses the ability of RIP-1 to activate NF-κB and enhances apoptosis (86). RIP-1 is important for NF-κB activation by both TNF-α and by TRIF because cells that are null for RIP-1 do not undergo NF-κB activation in response to TNF-α and have a markedly attenuated response to TRIF (85, 91). Although inhibition of NF-κB activation can often sensitize cells to proapoptotic stimuli (91, 92), the amount of apoptosis that occurred in response to TRIF was similar when NF-κB activation was intact and when NF-κB activation was inhibited by IκBα-SR. Complex formation with TRIF is not a static process, with some complexes dissociating as others form (56, 93). Thus, early activation of NF-κB that is dependent on RIP-1 might be followed by apoptosis when NF-κB activation is no longer occurring. Neither TRIF nor RIP3 have a DD, yet our studies provide evidence that recruitment of FADD through its DD is important for TRIF-induced apoptosis. The RHIM in TRIF can bind to the RHIM in either RIP1 or RIP3. In addition, the RHIMs in RIP1 and RIP3 can also bind to each other (86, 94). This raises the possibility that TRIF-induced apoptosis occurs when all three proteins are complexed, with RIP3 blocking RIP1-induced NF-κB activation and the DD of RIP1 recruiting FADD and activating the extrinsic apoptotic pathway. Further studies are required to determine whether this or a distinct mechanism leads to activation of the extrinsic apoptotic pathway by a process that is dependent on the RHIM of TRIF.

TLR3 and TLR4 use TRIF as an adaptor molecule to transmit changes induced by dsRNA and LPS, molecular motifs encountered during viral infection and Gram-negative sepsis, respectively (95–99). We demonstrate that the apoptosis induced by TRIF is dependent on an intact RHIM in TRIF and occurs through a mechanism that is not dependent on the activation of either IRF-3 or NF-κB. The expression of type I IFN and other cytokines from TRIF-induced activation of NF-κB and IRF-3 should induce changes in surrounding cells that could enhance both innate and acquired responses to the infectious agents. In addition, the apoptosis resulting from TRIF could help limit the spread of infection. The induction of apoptosis would be especially beneficial in the control of viral infection because apoptosis could prevent the production of progeny virus, a process that is dependent on use of cellular machinery.

Disclosures

The authors have no financial conflict of interest.

References

1. Takeda, K., T. Kaisho, and S. Akira. 2003. Toll-like receptors. *Annu. Rev. Immunol. 21:335*.
2. Janeway, C. A., Jr., and R. Medzhitov. 2002. Innate immune recognition. *Annu. Rev. Immunol. 20:197*.
3. Medzhitov, R., and C. A. Janeway, Jr. 1997. Innate immunity: the virtues of a nonclonal system of recognition. *Cell 91:295*.
4. Medzhitov, R., P. Preston-Hurlburt, and C. A. Janeway, Jr. 1997. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature 388:394*.
5. Rock, F. L., G. Hardiman, J. C. Timans, R. A. Kastelein, and J. F. Bazan. 1998. A family of human receptors structurally related to *Drosophila* Toll. *Proc. Natl. Acad. Sci. USA 95:588*.
6. Takeuchi, O., T. Kawai, H. Sanjo, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, K. Takeda, and S. Akira. 1999. TLR6: A novel member of an expanding Toll-like receptor family. *Gene. 231:59*.
7. Chuang, T. H., and R. J. Ulevitch. 2000. Cloning and characterization of a subfamily of human Toll-like receptors: hTLR7, hTLR8 and hTLR9. *Eur. Cytokine Netw. 11:372*.
8. Chuang, T., and R. J. Ulevitch. 2001. Identification of hTLR10: a novel human Toll-like receptor preferentially expressed in immune cells. *Biochim. Biophys. Acta 1518:157*.
9. Du, X., A. Poltorak, Y. Wei, and B. Beutler. 2000. Three novel mammalian Toll-like receptors: gene structure, expression, and evolution. *Eur. Cytokine. Netw. 11:362*.
10. Schwandner, R., R. Dziarski, H. Wesche, M. Rothe, and C. J. Kirschning. 1999. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by Toll-like receptor 2. *J. Biol. Chem. 274:17406*.
11. Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, and S. Akira. 1999. Differential roles of TLR2 and TLR4 in recognition of Gram-negative and Gram-positive bacterial cell wall components. *Immunity 11:443*.
12. Yoshimura, A., E. Lien, R. R. Ingalls, E. Tuomanen, R. Dziarski, and D. Golenbock. 1999. Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. *J. Immunol. 163:1*.
13. Alexopoulou, L., A. C. Holt, R. Medzhitov, and R. A. Flavell. 2001. Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. *Nature 413:732*.
14. Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, et al. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science 282:2085*.
15. Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda, and S. Akira. 1999. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J. Immunol. 162:3749*.
16. Qureshi, S. T., L. Lariviere, G. Leveque, S. Clermont, K. J. Moore, P. Gros, and D. Malo. 1999. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). *J. Exp. Med. 189:615*.
17. Hayashi, F., K. D. Smith, A. Ozinsky, T. R. Hawn, E. C. Yi, D. R. Goodlett, J. K. Eng, S. Akira, D. M. Underhill, and A. Aderem. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature 410:1099*.
18. Diebold, S. S., T. Kaisho, H. Hemmi, S. Akira, and C. Reis e Sousa. 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science 303:1529*.
19. Heil, F., H. Hemmi, H. Hochrein, F. Ampenberger, C. Kirschning, S. Akira, G. Lipford, H. Wagner, and S. Bauer. 2004. Species-specific recognition of single-stranded RNA via Toll-like receptor 7 and 8. *Science 303:1526*.
20. Lund, J. M., L. Alexopoulou, A. Sato, M. Karow, N. C. Adams, N. W. Gale, A. Iwasaki, and R. A. Flavell. 2004. Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proc. Natl. Acad. Sci. USA 101:5598.*
21. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature 408:740*.
22. Yamamoto, M., S. Sato, K. Mori, K. Hoshino, O. Takeuchi, K. Takeda, and S. Akira. 2002. Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-β promoter in the Toll-like receptor signaling. *J. Immunol. 169:6668*.
23. Oshiumi, H., M. Sasai, K. Shida, T. Fujita, M. Matsumoto, and T. Seya. 2003. TIR-containing adapter molecule (TICAM)-2, a bridging adapter recruiting to Toll-like receptor 4 TICAM-1 that induces interferon-β. *J. Biol. Chem. 278:49751*.
24. Bin, L. H., L. G. Xu, and H. B. Shu. 2003. TIRP, a novel Toll/interleukin-1 receptor (TIR) domain-containing adapter protein involved in TIR signaling. *J. Biol. Chem. 278:24526*.
25. Yamamoto, M., S. Sato, H. Hemmi, S. Uematsu, K. Hoshino, T. Kaisho, O. Takeuchi, K. Takeda, and S. Akira. 2003. TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nat. Immunol. 4:1144*.
26. Horng, T., G. M. Barton, and R. Medzhitov. 2001. TIRAP: an adapter molecule in the Toll signaling pathway. *Nat. Immunol. 2:835*.
27. Fitzgerald, K. A., D. C. Rowe, B. J. Barnes, D. R. Caffrey, A. Visintin, E. Latz, B. Monks, P. M. Pitha, and D. T. Golenbock. 2003. LPS-TLR4 signaling to IRF-3/7 and NF-κB involves the Toll adapters TRAM and TRIF. *J. Exp. Med. 198:1043*.
28. Medzhitov, R., P. Preston-Hurlburt, E. Kopp, A. Stadlen, C. Chen, S. Ghosh, and C. A. Janeway, Jr. 1998. MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. *Mol. Cell 2:253*.
29. Oshiumi, H., M. Matsumoto, K. Funami, T. Akazawa, and T. Seya. 2003. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-β induction. *Nat. Immunol. 4:161*.
30. Fitzgerald, K. A., E. M. Palsson-McDermott, A. G. Bowie, C. A. Jefferies, A. S. Mansell, G. Brady, E. Brint, A. Dunne, P. Gray, M. T. Harte, et al. 2001.

Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. *Nature 413:78*.
31. O'Neill, L. A., K. A. Fitzgerald, and A. G. Bowie. 2003. The Toll-IL-1 receptor adaptor family grows to five members. *Trends Immunol. 24:286*.
32. Aravind, L., V. M. Dixit, and E. V. Koonin. 2001. Apoptotic molecular machinery: vastly increased complexity in vertebrates revealed by genome comparisons. *Science 291:1279*.
33. Jault, C., L. Pichon, and J. Chluba. 2004. Toll-like receptor gene family and TIR-domain adapters in *Danio rerio*. *Mol. Immunol. 40:759*.
34. Meijer, A. H., S. F. Gabby Krens, I. A. Medina Rodriguez, S. He, W. Bitter, B. Ewa Snaar-Jagalska, and H. P. Spaink. 2004. Expression analysis of the Toll-like receptor and TIR domain adaptor families of zebrafish. *Mol. Immunol. 40:773*.
35. Jebanathirajah, J. A., S. Peri, and A. Pandey. 2002. Toll and interleukin-1 receptor (TIR) domain-containing proteins in plants: a genomic perspective. *Trends Plant Sci. 7:388*.
36. Couillault, C., N. Pujol, J. Reboul, L. Sabatier, J. F. Guichou, Y. Kohara, and J. J. Ewbank. 2004. TLR-independent control of innate immunity in *Caenorhabditis elegans* by the TIR domain adaptor protein TIR-1, an ortholog of human SARM. *Nat. Immunol. 5:488*
37. Mink, M., B. Fogelgren, K. Olszewski, P. Maroy, and K. Csiszar. 2001. A novel human gene (SARM) at chromosome 17q11 encodes a protein with a SAM motif and structural similarity to Armadillo/β-catenin that is conserved in mouse, *Drosophila*, and *Caenorhabditis elegans*. *Genomics 74:234*.
38. Takeuchi, O., and S. Akira. 2002. MyD88 as a bottle neck in Toll/IL-1 signaling. *Curr. Top. Microbiol. Immunol. 270:155*.
39. Janssens, S., and R. Beyaert. 2002. A universal role for MyD88 in TLR/IL-1R-mediated signaling. *Trends Biochem. Sci. 27:474*.
40. Suzuki, N., S. Suzuki, G. S. Duncan, D. G. Millar, T. Wada, C. Mirtsos, H. Takada, A. Wakeham, A. Itie, S. Li, et al. 2002. Severe impairment of interleukin-1 and Toll-like receptor signalling in mice lacking IRAK-4. *Nature 416:750*.
41. Li, S., A. Strelow, E. J. Fontana, and H. Wesche. 2002. IRAK-4: a novel member of the IRAK family with the properties of an IRAK-kinase. *Proc. Natl. Acad. Sci. USA 99:5567*.
42. Cao, Z., J. Xiong, M. Takeuchi, T. Kurama, and D. V. Goeddel. 1996. TRAF6 is a signal transducer for interleukin-1. *Nature 383:443*.
43. Cao, Z., W. J. Henzel, and X. Gao. 1996. IRAK: a kinase associated with the interleukin-1 receptor. *Science 271:1128*.
44. Yamamoto, M., S. Sato, H. Hemmi, H. Sanjo, S. Uematsu, T. Kaisho, K. Hoshino, O. Takeuchi, M. Kobayashi, T. Fujita, et al. 2002. Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. *Nature 420:324*.
45. Horng, T., G. M. Barton, R. A. Flavell, and R. Medzhitov. 2002. The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. *Nature 420:329*.
46. Hoebe, K., X. Du, P. Georgel, E. Janssen, K. Tabeta, S. O. Kim, J. Goode, P. Lin, N. Mann, S. Mudd, et al. 2003. Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. *Nature 424:743*.
47. Yamamoto, M., S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Sanjo, O. Takeuchi, M. Sugiyama, M. Okabe, K. Takeda, and S. Akira. 2003. Role of adaptor TRIF in the MyD88-independent Toll-like receptor signaling pathway. *Science 301:640*.
48. Wathelet, M. G., C. H. Lin, B. S. Parekh, L. V. Ronco, P. M. Howley, and T. Maniatis. 1998. Virus infection induces the assembly of coordinately activated transcription factors on the IFN-β enhancer in vivo. *Mol. Cell 1:507*.
49. Fitzgerald, K. A., S. M. McWhirter, K. L. Faia, D. C. Rowe, E. Latz, D. T. Golenbock, A. J. Coyle, S. M. Liao, and T. Maniatis. 2003. IKKε and TBK1 are essential components of the IRF3 signaling pathway. *Nat. Immunol. 4:491*.
50. McWhirter, S. M., K. A. Fitzgerald, J. Rosains, D. C. Rowe, D. T. Golenbock, and T. Maniatis. 2004. IFN-regulatory factor 3-dependent gene expression is defective in Tbk1-deficient mouse embryonic fibroblasts. *Proc. Natl. Acad. Sci. USA 101:233*.
51. Sharma, S., B. R. tenOever, N. Grandvaux, G. P. Zhou, R. Lin, and J. Hiscott. 2003. Triggering the interferon antiviral response through an IKK-related pathway. *Science 300:1148*.
52. Kawai, T., O. Takeuchi, T. Fujita, J. Inoue, P. F. Muhlradt, S. Sato, K. Hoshino, and S. Akira. 2001. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J. Immunol. 167:5887*.
53. Taniguchi, T., K. Ogasawara, A. Takaoka, and N. Tanaka. 2001. IRF family of transcription factors as regulators of host defense. *Annu. Rev. Immunol. 19:623*.
54. Lin, R., Y. Mamane, and J. Hiscott. 1999. Structural and functional analysis of interferon regulatory factor 3: localization of the transactivation and autoinhibitory domains. *Mol. Cell Biol. 19:2465*.
55. Lin, R., C. Heylbroeck, P. M. Pitha, and J. Hiscott. 1998. Virus-dependent phosphorylation of the IRF-3 transcription factor regulates nuclear translocation, transactivation potential, and proteasome-mediated degradation. *Mol. Cell. Biol. 18:2986*.
56. Jiang, Z., T. W. Mak, G. Sen, and X. Li. 2004. Toll-like receptor 3-mediated activation of NF-κB and IRF3 diverges at Toll-IL-1 receptor domain-containing adapter inducing IFN-β. *Proc. Natl. Acad. Sci. USA 101:3533*.
57. Sato, S., M. Sugiyama, M. Yamamoto, Y. Watanabe, T. Kawai, K. Takeda, and S. Akira. 2003. Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-κB and IFN-regulatory factor-3, in the Toll-like receptor signaling. *J. Immunol. 171:4304*.
58. Hsu, L. C., J. M. Park, K. Zhang, J. L. Luo, S. Maeda, R. J. Kaufman, L. Eckmann, D. G. Guiney, and M. Karin. 2004. The protein kinase PKR is required for macrophage apoptosis after activation of Toll-like receptor 4. *Nature 428:341*.
59. Aliprantis, A. O., R. B. Yang, M. R. Mark, S. Suggett, B. Devaux, J. D. Radolf, G. R. Klimpel, P. Godowski, and A. Zychlinsky. 1999. Cell activation and apoptosis by bacterial lipoproteins through Toll-like receptor-2. *Science 285:736*.
60. Aliprantis, A. O., R. B. Yang, D. S. Weiss, P. Godowski, and A. Zychlinsky. 2000. The apoptotic signaling pathway activated by Toll-like receptor-2. *EMBO J. 19:3325*.
61. Bannerman, D. D., J. C. Tupper, J. D. Kelly, R. K. Winn, and J. M. Harlan. 2002. The Fas-associated death domain protein suppresses activation of NF-κB by LPS and IL-1β. *J. Clin. Invest. 109:419*.
62. Barber, G. N. 2001. Host defense, viruses and apoptosis. *Cell Death Differ. 8:113*.
63. Creagh, E. M., H. Conroy, and S. J. Martin. 2003. Caspase-activation pathways in apoptosis and immunity. *Immunol. Rev. 193:10*.
64. Thorburn, A. 2004. Death receptor-induced cell killing. *Cell Signal 16:139*.
65. McConnell, B. B., and P. M. Vertino. 2000. Activation of a caspase-9-mediated apoptotic pathway by subcellular redistribution of the novel caspase recruitment domain protein TMS1. *Cancer Res. 60:6243*.
66. Lee, F. S., R. T. Peters, L. C. Dang, and T. Maniatis. 1998. MEKK1 activates both IκB kinase α and IκB kinase β. *Proc. Natl. Acad. Sci. USA 95:9319*.
67. Strasser, A., L. O'Connor, and V. M. Dixit. 2000. Apoptosis signaling. *Annu. Rev. Biochem. 69:217*.
68. Hengartner, M. O. 2000. The biochemistry of apoptosis. *Nature 407:770*.
69. Gu, J., R. P. Dong, C. Zhang, D. F. McLaughlin, M. X. Wu, and S. F. Schlossman. 1999. Functional interaction of DFF35 and DFF45 with caspase-activated DNA fragmentation nuclease DFF40. *J. Biol. Chem. 274:20759*.
70. Liu, X., H. Zou, C. Slaughter, and X. Wang. 1997. DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell 89:175*.
71. dela Cruz, W. P., P. D. Friesen, and A. J. Fisher. 2001. Crystal structure of baculovirus P35 reveals a novel conformational change in the reactive site loop after caspase cleavage. *J. Biol. Chem. 276:32933*.
72. Danial, N. N., and S. J. Korsmeyer. 2004. Cell death: critical control points. *Cell 116:205*.
73. Bergeron, L., G. I. Perez, G. Macdonald, L. Shi, Y. Sun, A. Jurisicova, S. Varmuza, K. E. Latham, J. A. Flaws, J. C. Salter, et al. 1998. Defects in regulation of apoptosis in caspase-2-deficient mice. *Genes Dev. 12:1304*.
74. Lassus, P., X. Opitz-Araya, and Y. Lazebnik. 2002. Requirement for caspase-2 in stress-induced apoptosis before mitochondrial permeabilization. *Science 297:1352*.
75. Tinel, A., and J. Tschopp. 2004. The PIDDosome, a protein complex implicated in activation of caspase-2 in response to genotoxic stress. *Science 304:843*.
76. Martinon, F., and J. Tschopp. 2004. Inflammatory caspases; linking an intracellular innate immune system to autoinflammatory diseases. *Cell 117:561*.
77. Krueger, A., I. Schmitz, S. Baumann, P. H. Krammer, and S. Kirchhoff. 2001. Cellular FLICE-inhibitory protein splice variants inhibit different steps of caspase-8 activation at the CD95 death-inducing signaling complex. *J. Biol. Chem. 276:20633*.
78. Zhou, Q., and G. S. Salvesen. 2000. Viral caspase inhibitors CrmA and p35. *Methods Enzymol. 322:143*.
79. Cheng, E. H., M. C. Wei, S. Weiler, R. A. Flavell, T. W. Mak, T. Lindsten, and S. J. Korsmeyer. 2001. BCL-2, BCL-X_L sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Mol. Cell 8:705*.
80. Sun, X. M., S. B. Bratton, M. Butterworth, M. MacFarlane, and G. M. Cohen. 2002. Bcl-2 and Bcl-xL inhibit CD95-mediated apoptosis by preventing mitochondrial release of Smac/DIABLO and subsequent inactivation of X- linked inhibitor-of-apoptosis protein. *J. Biol. Chem. 277:11345*.
81. Shi, Y. 2002. Mechanism of caspase activation and inhibition during apoptosis. *Molecular Cell 9:459*.
82. Lenardo, M. J., C. M. Fan, T. Maniatis, and B. D. 1989. The involvement of NF-κB in β-interferon gene regulation reveals its role as widely inducible mediator of signal transduction. *Cell 57:287*.
83. Han, K. J., X. Su, L. G. Xu, L. H. Bin, J. Zhang, and H. B. Shu. 2004. Mechanisms of the TRIF-induced interferon-stimulated response element and NF-κB activation and apoptosis pathways. *J. Biol. Chem. 279:15652*.
84. Takeda, K., and S. Akira. 2004. TLR signaling pathways. *Semin. Immunol. 16:3*.
85. Meylan, E., K. Burns, K. Hofmann, V. Blancheteau, F. Martinon, M. Kelliher, and J. Tschopp. 2004. RIP1 is an essential mediator of Toll-like receptor 3-induced NF-κB activation. *Nat. Immunol. 5:503*.
86. Sun, X., J. Yin, M. A. Starovasnik, W. J. Fairbrother, and V. M. Dixit. 2002. Identification of a novel homotypic interaction motif required for the phosphorylation of receptor-interacting protein (RIP) by RIP3. *J. Biol. Chem. 277:9505*.
87. Heylbroeck, C., S. Balachandran, M. J. Servant, C. DeLuca, G. N. Barber, R. Lin, and J. Hiscott. 2000. The IRF-3 transcription factor mediates Sendai virus-induced apoptosis. *J. Virol. 74:3781*.

88. 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*.
89. Sun, X., J. Lee, T. Navas, D. T. Baldwin, T. A. Stewart, and V. M. Dixit. 1999. RIP3, a novel apoptosis-inducing kinase. *J. Biol. Chem. 274:16871*.
90. Grimm, S., B. Z. Stanger, and P. Leder. 1996. RIP and FADD: two "death domain"-containing proteins can induce apoptosis by convergent, but dissociable, pathways. *Proc. Natl. Acad. Sci. USA 93:10923*.
91. 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-κB signal. *Immunity 8:297*.
92. 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-κB. *Science 274:787*.
93. Jiang, Z., M. Zamanian-Daryoush, H. Nie, A. M. Silva, B. R. Williams, and X. Li. 2003. Poly(I-C)-induced Toll-like receptor 3 (TLR3)-mediated activation of NFκB and MAP kinase is through an interleukin-1 receptor-associated kinase (IRAK)-independent pathway employing the signaling components TLR3-TRAF6-TAK1-TAB2-PKR. *J. Biol. Chem. 278:16713*.
94. Yu, P. W., B. C. Huang, M. Shen, J. Quast, E. Chan, X. Xu, G. P. Nolan, D. G. Payan, and Y. Luo. 1999. Identification of RIP3, a RIP-like kinase that activates apoptosis and NFκB. *Curr. Biol. 9:539*.
95. Kaisho, T., and S. Akira. 2000. Critical roles of Toll-like receptors in host defense. *Crit. Rev. Immunol. 20:393*.
96. Kopp, E., and R. Medzhitov. 2003. Recognition of microbial infection by Toll-like receptors. *Curr. Opin. Immunol. 15:396*.
97. Barton, G. M., and R. Medzhitov. 2002. Toll-like receptors and their ligands. *Curr. Top Microbiol. Immunol. 270:81*.
98. Beutler, B., K. Hoebe, X. Du, and R. J. Ulevitch. 2003. How we detect microbes and respond to them: the Toll-like receptors and their transducers. *J. Leukocyte. Biol. 74:479*.
99. Akira, S., and H. Hemmi. 2003. Recognition of pathogen-associated molecular patterns by TLR family. *Immunol. Lett. 85:85*.