The death domain kinase RIP1 links the immunoregulatory CD40 receptor to apoptotic signaling in carcinomas

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D40, a tumor necrosis factor (TNF) receptor family member, is widely recognized for its prominent role in the antitumor immune response. The immunostimulatory effects of CD40 ligation on malignant cells can be switched to apoptosis upon disruption of survival signals transduced by the binding of the adaptor protein TRAF6 to CD40. Apoptosis induction requires a TRAF2-interacting CD40 motif but is initiated within a cytosolic death-inducing signaling complex after mobilization of receptor-bound TRAF2 to the cytoplasm. We demonstrate that receptor-interacting protein 1 (RIP1) is an integral component of this complex and is required for CD40 ligand-induced caspase-8 activation and tumor cell killing. Degradation of the RIP1 K63 ubiquitin ligases cIAP1/2 amplifies the CD40-mediated cytotoxic effect, whereas inhibition of CYLD, a RIP1 K63 deubiquitinating enzyme, reduces it. This two-step mechanism of apoptosis induction expands our appreciation of commonalities in apoptosis regulatory pathways across the TNF receptor superfamily and provides a telling example of how TNF family receptors usurp alternative programs to fulfill distinct cellular functions.

Introduction

Receptor-interacting protein 1 (RIP1) is a death domain–containing kinase with diverse and context-specific roles in inflammation, cell survival, and apoptosis (Festjens et al., 2007; Galluzzi et al., 2009b). Genetic evidence has demonstrated that RIP1 is required for the pro-inflammatory and antiapoptotic functions of TNF receptor 1 (TNFR1) by mediating nuclear factor of activated T cells (NF-κB) and MAPK signaling (Kelliferi et al., 1998; Vivarelli et al., 2004), whereas other studies have shown that RIP1 is an integral component of a cytoplasmic apoptosis-inducing signaling complex mediated by TNFR1 engagement (Micheau and Tschopp, 2003; Jin and El-Deiry, 2006; O’Donnell et al., 2007; Wang et al., 2008; Legarda-Addison et al., 2009). RIP1 is also required for caspase-8 activation within a Fas ligand (CD95L)-triggered death-inducing signaling complex after mobilization of receptor-bound TRAF2 to the cytoplasm. We demonstrate that receptor-interacting protein 1 (RIP1) is an integral component of this complex and is required for CD40 ligand-induced caspase-8 activation and tumor cell killing. Degradation of the RIP1 K63 ubiquitin ligases cIAP1/2 amplifies the CD40-mediated cytotoxic effect, whereas inhibition of CYLD, a RIP1 K63 deubiquitinating enzyme, reduces it. This two-step mechanism of apoptosis induction expands our appreciation of commonalities in apoptosis regulatory pathways across the TNF receptor superfamily and provides a telling example of how TNF family receptors usurp alternative programs to fulfill distinct cellular functions.

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membrane-proximal region of the receptor cytoplasmic C terminus binds TRAF6, whereas a membrane-distal domain recruits TRAF2 and TRAF3 (Fig. 1 A). To address the impact of specific CD40–TRAF interactions on apoptotic signaling, we used a panel of HeLa cell clones stably expressing wild-type or mutated CD40 sequences that were unable to directly associate with TRAF6 (CD40mT6), TRAF2/TRAF3 (CD40mT2/mT3), or all TRAFs (CD40mT2/T3/T6; Fig. 1 A; Tsukamoto et al., 1999; Jabara et al., 2002; Benson et al., 2006). We have previously used this cell system to demonstrate that the TRAF2/TRAF3-interacting domain of CD40 is primarily responsible for the engagement of NF-κB, JNK, and p38 cascades, whereas the TRAF6-binding region contributes to NF-κB signaling (Davies et al., 2005b).

Other studies have shown that ligation of a CD40 mutant lacking a functional TRAF6 binding site is defective in activation of ERK and Akt in lymphoid cells (Mukundan et al., 2005; Benson et al., 2006) and that ectopic expression of TRAF6 but not TRAF6 binding to CD40. CD40mT2/T3/T6 combines the aforementioned mutations and perturbs the binding of all TRAFs (Davies et al., 2005b). WT, wild type. (B) The TRAF6-interacting domain of CD40 transduces ERK and Akt signaling. Lysates from CD154-stimulated HeLa/CD40 and HeLa/CD40mT6 cells were analyzed for the expression of phosphorylated, active ERK and Akt, or the total proteins. (C) The TRAF2/TRAF3-interacting domain of CD40 mediates CD154-induced death signals. HeLa clones expressing the WT or mutated CD40 sequences described in A were stimulated with CD154 for 12 h before assessment of apoptosis. Mean values of percentage apoptotic cells from at least three independent experiments are shown with the exception of HeLa/CD40mT6 clone 10, where two determinations were performed. (D and E) The TRAF2/TRAF3-binding domain of CD40 mediates cell death via caspase-8 activation. HeLa/CD40mT6 clone 21 cells were stimulated with CD154 for the indicated time points, and lysates were analyzed for the expression of caspase-8, caspase-3, or β-actin [D]). Inhibitors of caspase-8 and -3 but not caspase-9 protect HeLa/CD40mT6 cells from CD154-induced apoptosis assessed by cell death ELISA. Data are expressed as fold increase (±SD; n = 4) in apoptosis induced by CD154 relative to untreated cultures, which was given the arbitrary value of 1. [F] RT-PCR showing up-regulation of TNF mRNA after treatment of HeLa/CD40mT6 cells with CD154. GAPDH, glyceraldehyde 3-phosphate dehydrogenase. (G) Early CD154-mediated death signals are independent of autocrine TNF production. HeLa/CD40mT6 cells were exposed to 0.5 µg/ml neutralizing anti-TNF mAb and then treated as described in C before assessment of apoptosis. Error bars indicate SD.

Results and discussion

The TRAF2/TRAF3-interacting domain of CD40 mediates death signals

CD40 signals through TNF receptor-associated factor (TRAF) proteins (Bishop, 2004, 2007; Eliopoulos, 2008). Specifically, a membrane-proximal region of the receptor cytoplasmic C terminus binds TRAF6, whereas a membrane-distal domain recruits TRAF2 and TRAF3 (Fig. 1 A). To address the impact of specific CD40–TRAF interactions on apoptotic signaling, we used a panel of HeLa cell clones stably expressing wild-type or mutated CD40 sequences that were unable to directly associate with TRAF6 (CD40mT6), TRAF2/TRAF3 (CD40mT2/mT3), or all TRAFs (CD40mT2/T3/T6; Fig. 1 A; Tsukamoto et al., 1999; Jabara et al., 2002; Benson et al., 2006). We have previously used this cell system to demonstrate that the TRAF2/TRAF3-interacting domain of CD40 is primarily responsible for the engagement of NF-κB, JNK, and p38 cascades, whereas the TRAF6-binding region contributes to NF-κB signaling (Davies et al., 2005b).

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channel CD40 signals to RIP1. We tested this hypothesis by performing coimmunoprecipitation experiments in lysates from CD40-negative 293 cells transfected with FLAG-tagged RIP1 in the presence or absence of a CD40 receptor expression vector. When overexpressed, CD40 stimulates signal activation through formation of receptor multimers and recruitment of TRAFs in a ligand-independent manner (Rothe et al., 1995; Pullen et al., 1999). As shown in Fig. 3 A, although very little endogenous TRAF2 coprecipitated with FLAG-RIP1 in the absence of CD40, this interaction dramatically increased upon transfection of increasing amounts of CD40 expression vector.
hypothesize that the absence of RIP1 in CD40-bound TRAF2 complexes is the result of the overlapping requirement of the C terminus of TRAF2 for binding to both CD40 (Rothe et al., 1995) and RIP1 (Liu et al., 1996). Once released from CD40, presumably after the CD154-mediated ubiquitination and degradation of TRAF3 (Matsuzawa et al., 2008; for review see Eliopoulos, 2008), the C terminus of TRAF2 could become accessible to RIP1, allowing the formation of a pro-apoptotic signaling complex. The difference in the kinetics of CD40–TRAF2 and RIP1–TRAF2 interactions after CD40 stimulation (Fig. 3, B and C) is compatible with this model.

RIP1 is required for CD40-induced death signaling in tumor cells

We next explored the functional role of RIP1 in CD40 death signaling by using geldanamycin, a compound which induces the degradation of RIP1 by disrupting the function of the RIP1-associating chaperone protein HSP90 (Lewis et al., 2000). Treatment of different tumor lines with geldanamycin induced a time-dependent decrease in RIP1 expression (Fig. S1 A) and protected them from CD154-induced apoptosis (Fig. S1 B). These results demonstrate a correlation between RIP1 protein levels, modulated by geldanamycin, and sensitivity to CD154-induced apoptosis but do not exclude the possibility that geldanamycin impacts on CD40-induced cell death through molecules other than RIP1. Therefore, we used RNAi to explore more specifically the role of RIP1 in death signaling. The knockdown of RIP1 in both EJ and HeLa/CD40mT6 (Fig. 4 A) dramatically reduced CD154-induced cytotoxicity, as determined by morphological changes (Fig. 4 B), nuclear condensation, and degradation detected by propidium iodide staining (Fig. 4, C and E), oligo-nucleosomal enrichment (Fig. 4, D and F), crystal violet staining (Fig. 5 F), or Annexin V staining and flow cytometry (not depicted). In contrast, diminished RIP1 did not affect CD95L-induced apoptosis (Fig. 5 F; Jin and El-Deiry, 2006).

To confirm that RIP1 is a bona fide regulator of CD40 death signaling, we assessed the effect of RIP1 knockdown on apoptosis induced by CD154 transgene expression. Adenovirus-mediated delivery of the CD154 gene to carcinoma cells elicits pro-apoptotic effects through membrane-bound CD154 (Gomes et al., 2009; Vardouli et al., 2009), which is likely to mimic CD154 expressed on activated T lymphocytes. EJ cells transfected with control or RIP1 siRNA were transduced with recombinant adenovirus (RAd) expressing CD154 or, as a control, the lacZ gene, then analyzed for transgene expression (Fig. S1 C) or exposed to CHX before assessment of apoptosis (Fig. S1 D). It was found that RIP1 knockdown provided protection from the cytotoxic effect of RAd-CD154 and CHX treatment (Fig. S1 D). Collectively, these data provide compelling evidence that RIP1 has an essential role in the regulation of CD40-mediated death signaling in tumor cells.

RIP1 is critically involved in TNF-induced JNK, ERK, and NF-κB signaling in most (Kelliher et al., 1998; Devin et al., 2003; Lee et al., 2003) but not all settings (Wong et al., 2010). We have found that the knockdown of RIP1 did not significantly influence the CD154-mediated degradation of IkBα, a hallmark

The TRAF2-RIP1 link was further explored in physiological conditions under which endogenous proteins were analyzed. To this end, lysates were prepared from HeLa/CD40mT6 cells before and after stimulation with CD154 and sequentially immunoprecipitated with anti-CD40 and RIP1 antibodies. In anti-CD40 immunoprecipitates, no detectable association of endogenous CD40 and TRAF2 was observed in the absence of stimulation, but this interaction was rapidly induced after CD40 ligation, as described previously (Rothe et al., 1995; Matsuzawa et al., 2008). RIP1 did not coprecipitate with CD40 before or after stimulation (Fig. 3 B). Interestingly, TRAF2 was readily detected in anti-RIP1 immunoprecipitates from CD154-stimulated cultures (Fig. 3 C).

These data suggest that RIP1 is not a component of the CD40-bound TRAF2 signaling complex but forms a separate, cytoplasmic association with TRAF2 after CD40 ligation.
of canonical NF-κB signaling, ERK or JNK phosphorylation, or the processing of p100 NF-κB2 to p52 (Fig. S2).

**CYLD and cellular inhibitors of apoptosis (cIAPs) are involved in CD40-mediated cell death**

Ubiquitination and phosphorylation are two RIP1 posttranslational modifications that influence RIP1-mediated cell death. cIAP1/2 are required for TNF-induced K63-linked ubiquitination of RIP1 (Bertrand et al., 2008), which functions to inhibit TNF-induced apoptosis (O’Donnell et al., 2007), whereas RIP1 deubiquitination by CYLD facilitates its direct interaction with caspase-8 and initiation of cell death (Wang et al., 2008). Because cIAP1/2 are involved in CD40-mediated MAPK signaling (Matsuzawa et al., 2008), we hypothesized that they may also function in the CD40 death pathway. In line with this prediction, knockdown of cIAP1/2 increased EJ cell killing by CD154 and CHX treatment (Fig. 4. G and I), whereas the knockdown of CYLD inhibited it (Fig. 4. H and I).

Smac mimetic compounds induce degradation of cIAPs and thus amplify death ligand-induced cancer cell killing (Li et al., 2004; Wang et al., 2008; Geserick et al., 2009). When EJ cells were treated with the Smac mimetic LBW242 (Gaither et al., 2007), cIAP1/2 were rapidly degraded (Fig. S3 A) and the cells became susceptible to CD154-induced apoptosis (Fig. S3 B). This effect was blocked by RIP1 knockdown or the pan-caspase inhibitor zVAD-fmk, further highlighting the critical involvement of the cIAP1/2–RIP1–caspase axis in the CD40 death pathway.

Moreover, the catalytic activity of RIP1 may also contribute to the CD40-triggered death pathway, as necrostatin-1,
lysates, neither RIP1 nor TRAF2 coprecipitated with caspase-8. However, both proteins (but not CD40) were found in complex with caspase-8 after exposure to CD154 (Fig. 5 C).

RIP1 has a death domain motif, and caspase-8 has a death effector domain (DED). Fas-associated death domain protein (FADD) possesses both motifs and has been proposed to link RIP1 and caspase-8 in death receptor signaling (Chinnaiyan et al., 1995; Kischkel et al., 2000; Sprick et al., 2000). Although FADD knockdown blocked CD95L-induced killing (Fig. 5, D and F), it did not impact on CD40-mediated apoptosis (Fig. 5, F and H). FADD knockdown also fails to influence TNF-induced, RIP1-mediated death signals in tumor cells (Jin and El-Deiry, 2006; Wang et al., 2008). FAF1 is a pro-apoptotic protein that possesses a DED-interacting domain responsible for association with the DED of caspase-8 and an atypical death domain (Ryu et al., 2003). Knockdown of FAF1 in EJ cells (Fig. 5 E) was an allosteric RIP1 kinase inhibitor (Degterev et al., 2008), inhibited CD40-mediated apoptosis by 40% (Fig. 4 J).

Figure 5. RIP1 associates with caspase-8 and is required for its activation. (A and B) RIP1 knockdown suppresses CD154-induced caspase-8 cleavage in EJ (A) or HeLa/CD40mT6 (B) cells. Lysates were immunoblotted for caspase-8 or, as a control, β-actin or p50 NF-κB. (C) RIP1, TRAF2, FAF1, and caspase-8 interact upon CD154 stimulation. Cells were stimulated with CD154, and lysates were immunoprecipitated with a goat polyclonal against caspase-8. Immunoprecipitates were immunoblotted using rabbit polyclonal antibodies against RIP1, TRAF2, CD40, and FAF1 or a monoclonal anti-caspase-8, as indicated. Results are representative of three independent experiments. The Ig light chains are indicated by asterisks. (D and E) Expression of FADD (D) and FAF1 (E) before and after transfection with the respective siRNAs. (F and G) Crystal violet staining of RNAi-transfected EJ cells treated with CHX in the presence or absence of CD154 or CD95L. (H) FAF1 but not FADD knockdown protects from the pro-apoptotic effects of CD40 ligation. After knockdown, EJ cells were exposed to CD154 and CHX for 5 h before assessment of apoptosis. Error bars indicate SD. (I) Proposed model of CD40-induced death signaling in tumor cells. CD40-mediated apoptosis involves the formation of a secondary cytoplasmic complex of TRAF2, RIP1, FAF1 and caspase-8 (Casp-8). RIP1 is required for caspase-8 activation and cell death, whereas apoptosis is antagonized by survival signals predominantly mediated by the TRAF6-binding domain of CD40, which may operate at the level of the death-inducing signaling complex and/or downstream of it.

As caspase-8 activation is required for CD40 induced apoptosis (Fig. 1 E), we examined whether RIP1 functions upstream of caspase-8 in this pathway. Cells were depleted of RIP1 and treated with CD154 in the presence (EJ) or absence (HeLa/CD40mT6) of CHX before analysis of caspase-8 by immunoblotting. RIP1 knockdown was found to suppress caspase-8 activation in both cases (Fig. 5, A and B).

This observation prompted us to investigate the hypothesis that caspase-8 interacts with RIP1 and TRAF2. HeLa/CD40mT6 cells were stimulated with CD154, and lysates were subjected to immunoprecipitation using anti-caspase-8 antibody. In control lysates, neither RIP1 nor TRAF2 coprecipitated with caspase-8. However, both proteins (but not CD40) were found in complex with caspase-8 after exposure to CD154 (Fig. 5 C). RIP1 has a death domain motif, and caspase-8 has a death effector domain (DED). Fas-associated death domain protein (FADD) possesses both motifs and has been proposed to link RIP1 and caspase-8 in death receptor signaling (Chinnaiyan et al., 1995; Kischkel et al., 2000; Sprick et al., 2000). Although FADD knockdown blocked CD95L-induced killing (Fig. 5, D and F), it did not impact on CD40-mediated apoptosis (Fig. 5, F and H). FADD knockdown also fails to influence TNF-induced, RIP1-mediated death signals in tumor cells (Jin and El-Deiry, 2006; Wang et al., 2008). FAF1 is a pro-apoptotic protein that possesses a DED-interacting domain responsible for association with the DED of caspase-8 and an atypical death domain (Ryu et al., 2003). Knockdown of FAF1 in EJ cells (Fig. 5 E) was
found to partially reduce the cytotoxic effect of CD154 and CHX treatment (Fig. 5, G and H), and FAF1 is detected in the RIP1–caspase-8 complex (Fig. 5 C).

We have recently shown that TRAF2 is largely responsible for the CD154-mediated sequential activation of NF-kB and IRF1, which act in concert to ensure the synchronous synthesis of components of the antigen presentation machinery required for the engagement of antitumor immune responses (Moschonas et al., 2008). Results presented in this study demonstrate that TRAF2 is also required for CD40-mediated tumor cell killing (Figs. 1 and 2). Together, these observations suggest that TRAF2 is a master regulator of the antitumor functions of CD40 in malignant epithelial cells.

However, our data also show that apoptosis is not triggered at the level of the receptor, but requires the function of a cytosolic complex containing TRAF2, RIP1, FAF1, and caspase-8, which is antagonized by signals emanating from the TRAF6-binding domain of CD40 (Fig. 5 I). Considering the breadth of CD40 expression and the diversity of its roles, the identification of two signaling complexes regulating cell survival versus death could be exploited to fine-tune CD154-based anticancer strategies.

**Materials and methods**

**Cell culture, adenovirus constructs, and reagents**

The bladder carcinoma EJ, the cervical cell line HeLa, and CD40-expressing clones were maintained in RPMI medium supplemented with 10% FCS. The early passage ovarian AGE60 (Vardouli et al., 2009; and the human embryonic kidney (HEK) 293 cell line were cultured in Dulbecco’s modified Eagle medium supplemented with 10% FCS (Invitrogen). Parental 293 and HeLa cells are CD40-negative (Davies et al., 2005b). Human recombinant soluble CD40L was kindly provided by Amgen Inc., or purchased from Enzo Life Sciences, Inc. Geldanamycin, kinase, and caspase inhibitors were obtained from EMD and dissolved in dimethyl sulfoxide before use. RAds expressing CD154 and lacz have been described previously (Vardouli et al., 2009). The Smac mimetic LBW242 was provided by L. Zawel (Novartis Institutes for Biomedical Research, Basel, Switzerland).

**Antibodies, immunoprecipitations, and immunoblotting**

Phospho-specific antibodies against JNK, ERK, Akt, and the corresponding antibodies that recognize both the phosphorylated and unphosphorylated forms, the FAF1, and monoclonal caspase Abs were purchased from Cell Signaling Technology and used at dilutions of 1:500–1:1000. The cIκB/MADE (C21), RIP1 (C20), TRAF2 (C20), CD40 (H120 and C20), and caspase-8 (C20) antibodies were kindly provided by L. Zawel (Novartis Institutes for Biomedical Research, Basel, Switzerland). Phospho-specific antibodies against JNK, ERK, and the corresponding antibodies that recognize both the phosphorylated and unphosphorylated forms, the FAF1, and monoclonal caspase Abs were purchased from Cell Signaling Technology and used at dilutions of 1:500–1:1000. The cIκB/MADE (C21), RIP1 (C20), TRAF2 (C20), CD40 (H120 and C20), and caspase-8 (C20) antibodies were obtained from Santa Cruz Biotechnology, Inc., the β-actin and FLAG M2 antibodies were obtained from Sigma-Aldrich, and the cIAP1/2 Ab was obtained from R&D Systems. Anti–rabbit IgG-HRP and anti–mouse IgG-HRP were obtained from Sigma-Aldrich. Phospho-specific antibodies against JNK, ERK, Akt, and the corresponding antibodies that recognize both the phosphorylated and unphosphorylated forms, the FAF1, and monoclonal caspase Abs were purchased from Cell Signaling Technology and used at dilutions of 1:500–1:1000. The cIκB/MADE (C21), RIP1 (C20), TRAF2 (C20), CD40 (H120 and C20), and caspase-8 (C20) antibodies were obtained from Santa Cruz Biotechnology, Inc., the β-actin and FLAG M2 antibodies were obtained from Sigma-Aldrich, and the cIAP1/2 Ab was obtained from R&D Systems. Anti–rabbit IgG-HRP and anti–mouse IgG-HRP were obtained from Sigma-Aldrich. Immunoblotting was performed as described previously (Eliopoulos et al., 2003; Moschonas et al., 2008). For immunoprecipitation, cells (1–2 × 10^7) were lysed in 1 ml of DISC immunoprecipitation buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM EDTA, and 1% Triton X-100) with protease inhibitor cocktail (Roche). Cell lysates (900 µl) were incubated overnight with 2–3 µg of antibody at 4°C. Complexes were precipitated by protein G–agarose (Millipore) and suspended in 50 µl of SDS sample buffer after three washes with DISC immunoprecipitation buffer. Immunoprecipitates were subjected to SDS-PAGE and Western blotting. The neutralizing anti-TNF mAb2101 was purchased from R&D Systems and used at 0.5 µg/ml.

**Quantitative measurement of apoptosis**

For the assessment of apoptosis, we used the MTT Assay (Promega) and the Annexin V Assay (BD Pharmingen) to measure cell death.

**Cytochemical staining**

To estimate apoptosis based on nuclear morphology, the fluorescent DNA staining dye propidium iodide (Sigma-Aldrich) was used. Approximately 2 × 10^6 cells in 25 µl were stained by adding 1 µl of 100 µg/ml propidium iodide. Uptake of the dye was examined by fluorescence microscopy. To estimate the apoptotic index, a minimum of 300 cells was examined by at least two independent investigators and quantified by recording the relative number of cells showing condensed or fragmented chromatin. Unless stated otherwise, data shown depict apoptosis measurements using this assay, with error bars representing SD of n independent experiments indicated in the figure legends.

**Annexin V staining**

Cells were lightly trypsinized and incubated with Annexin V FITC (BD) and propidium iodide for 15 min before assessment of fluorescence intensity on a flow cytometer (BD).

**RNAi**

For the delivery of siRNAs, 5 × 10^6 EJ or HeLa/CD40+/i cells were plated into each well of a 24-well plate (Costar), and two rounds of transfection with siRNA duplexes were performed as described previously (Davies et al., 2005b; Moschonas et al., 2008). The sequences of the siRNAs used were as follows: RIP1 siRNA, 5′-GUACUCGCCGUUUCUGUAAA-3′; TRAF2 siRNA, Dharmacon siGENOME SMARTpool M-005198 (Thermo Fischer Scientific); TRAF3 siRNA, Dharmacon siGENOME SMARTpool M-005252 (Thermo Fischer Scientific); FADD siRNA, Dharmacon siGENOME SMARTpool M-003800 (Thermo Fischer Scientific); and FAF1 siRNA, Dharmacon siGENOME SMARTpool M-009106 (Thermo Fischer Scientific). The siRNA sequences for clpA1, clpA2, and CYLD were as described previously (Wang et al., 2008). clpA1 and clpA2 were knocked down simultaneously.

**Light microscopy**

Morphological changes related to apoptosis were observed using an inverted microscope (DMIRE2; Leica) equipped with a digital camera (DFC300 FX; Leica). Camera image acquisition was controlled by IM50 software (Leica), and single images were exported as TIFF files. Individual frames were prepared for presentation using Photoshop (Adobe). Cells were seeded into 4-well, chambered coverglass units with coverslip-quality glass bottoms (Lab-Tek; Thermo Fischer Scientific) and, after treatment, were examined with a 63× dry objective lens.

**Online supplemental material**

Fig. S1 shows data supporting the involvement of RIP1 in CD40-mediated apoptosis. Fig. S2 illustrates that RIP1 is dispensable for CD154-stimulated NF-κB and MAPK signaling. Fig. S3 shows that RIP1 is required for apoptosis induced by a Smac mimetic and CD154 combination treatment. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201003087/DC1.

The authors declare no conflict of interest.

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