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Intrinsic cleavage of receptor-interacting protein kinase-1 by caspase-6

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Necroptosis is a form of programmed cell death that occurs in the absence of caspase activation and depends on the activity of the receptor-interacting protein kinases. Inactivation of these kinases by caspase-mediated cleavage has been shown to be essential for successful embryonic development, survival and activation of certain cell types. The initiator of extrinsic apoptosis, caspase-8, which has a pro-death as well as a pro-life function, has been assigned this role. In the present study we demonstrate that caspase-6, an executioner caspase, performs this role during apoptosis induced through the intrinsic pathway. In addition, we demonstrate that in the absence of caspase activity, intrinsic triggers of apoptosis induce the receptor-interacting-kinase-1-dependent production of pro-inflammatory cytokines. We show that ubiquitously expressed caspase-6 has a supporting role in apoptosis by cleaving this kinase, thus preventing production of inflammatory cytokines as well as inhibiting the necroptotic pathway. These findings shed new light on the regulation of necroptosis as well as cell death in an inflammatory environment wherein cells receive both intrinsic and extrinsic death signals.
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The main form of programmed cell death in metazoans is apoptosis, a process that is crucially dependent on the sequential activation of a family of aspartate-specific proteases, the caspases.[1,2] Apoptosis is a clean form of cell death, wherein the DNA is degraded and the cell contents are wrapped in apoptotic bodies to be taken up by neighboring cells or resident macrophages. As such, apoptosis is non-inflammatory. Necrosis has always been considered an accidental form of non-programmed cell death resulting from extensive cell damage and elicits an inflammatory response. A programmed form of necrosis, generally referred to as 'necroptosis', has recently emerged.[3–5] The program of necroptosis is executed through the sequential activation of the receptor-interacting protein kinases (RIPKs)-1 and -3 and downstream effectors in the absence of caspase activation upon ligation of a variety of both cell surface and intracellular receptors.[6–9]

Caspase activity is essential to prevent necroptotic cell death, either through direct cleavage of the RIPKs[10,11] or through cleavage of other proteins such as the deubiquitinating enzyme (DUB) CYLD,[12] which enhances the pro-necroptotic potential of RIPK1 by removing K63-linked ubiquitin chains.[13,14]

In addition, RIPK1 has been shown to be activated upon genotoxic stress induced by DNA double-stranded breaks (DSBs) and downstream of Toll-like receptor signaling in a novel signaling platform dubbed the 'RIPoptosome'.[15–17] In this platform, the role of RIPK1 is not so much to initiate necroptosis, but either to initiate signaling,[15] resulting in the transcription of pro-survival and pro-inflammatory genes, or to activate caspase-8 in a FADD-dependent manner.[16,17]

Thus, caspase-8 could indirectly participate in DSB-induced apoptosis. However, caspase-8 has a survival role as well as an apoptotic role. Like all caspases, caspase-8 only gains activity upon dimerization. Caspase-8 is not only activated by homodimerization, but also by heterodimerization with the inactive caspase-8 homolog cellular FLICE-like inhibitory protein ($cFLIP_L$).[2] The homodimer readily cleaves and activates the executioner caspase-3as well as the pro-apoptotic Bcl-2 family member Bid, but the more readily assembled heterodimer has restricted substrate specificity.[18–20] The homodimer is therefore thought to execute the pro-apoptotic functions of the caspase whereas the heterodimer, wherein caspase-8 remains unprocessed, is thought to execute the non-apoptotic functions of this caspase. As such, expression of $cFLIP_L$ is essential for restricting the pro-necroptotic activities of RIPK1.[21–23]

Traditionally, DSB-induced apoptosis is considered intrinsic apoptosis: initiated by caspase-9, following cytochrome *c* release from the mitochondria, as opposed to extrinsic caspase-8-dependent apoptosis, which is initiated by death receptor ligation.[24] As caspase-8 is not normally activated in the intrinsic pathway of apoptosis, or is at best activated late in the RIPoptosome, we decided to investigate whether caspase-8 activation is required for the control of RIPK1 activity during DSB-induced cell death.

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Abbreviations: AV, Annexin V; bEVD-AOMK, biotinyl-GluValAsp-acyloxymethyl ketone; cFLIP, cellular FLICE-like inhibitory protein; DSBs, double strand breaks; FKBP, FK506 binding protein; Nec1, necrostatin-1; RIPK, receptor-interacting protein kinase; SytR, Sytox Red; zVAD, carbobenzoxy-ValAlaAsp-fluoromethyl ketone

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Results

FADD is dispensable for RIPK1 cleavage during DSB-induced apoptosis. In the absence of the adaptor protein, FADD, essential for the activation of the initiator caspases-8 and -10, cells become susceptible to TNFα-induced necroptosis, while gaining resistance to TRAIL and FAS-induced apoptosis.[25] To determine the nature of DSB-induced cell death in the absence of caspase-8/10 activity, Jurkat T cells deficient in FADD ($FADD^{DEF}$) or retrovirally reconstituted with FADD ($FADD^{REC}$; Figure 1a) were treated with increasing doses of the topoisomerase-I inhibitor etoposide in the presence or absence of the broad-spectrum caspase inhibitor carbobenzoxy-ValAlaAsp-fluoromethyl ketone (zVAD) and/or the RIPK1 inhibitor necrostatin-1 (Nec1). Cell death was determined after AV/Sytox Red (AV/SytR) double staining by flow cytometry. Both cell types cells were equally susceptible to etoposide-induced death and cell death was completely inhibitable by zVAD in both cell types but not by Nec1 (Figure 1b). Thus, etoposide does not induce the necroptotic propensity of RIPK1. Furthermore, this result suggests that caspase-8/10 activation is not required to initiate, prevent or enhance etoposide-induced death (Figures 1b and c). Finally, cell lysates were analyzed on western blots probed for RIPK1 and RIPK3 as well as cleaved PARP, an indicator of executioner caspase activity and thus apoptosis (Figure 1d). RIPK1 was found to be cleaved in both cell types upon etoposide stimulation, correlating with the amount of PARP cleavage. RIPK3 cleavage was not observed. This suggests that RIPK1 cleavage is an apoptotic event and independent of caspase-8/10 activation.

RIPK1 cleavage was equally observed following treatment with several alternative inducers of apoptotic cell death (Figures 1e and f). Cell death was additionally confirmed by determining loss of viability with Cell Titer Blue and release of lactate dehydrogenase (Supplemental Figure S1a). $FADD^{DEF}$ cells appeared to be slightly more susceptible to DSB-induced

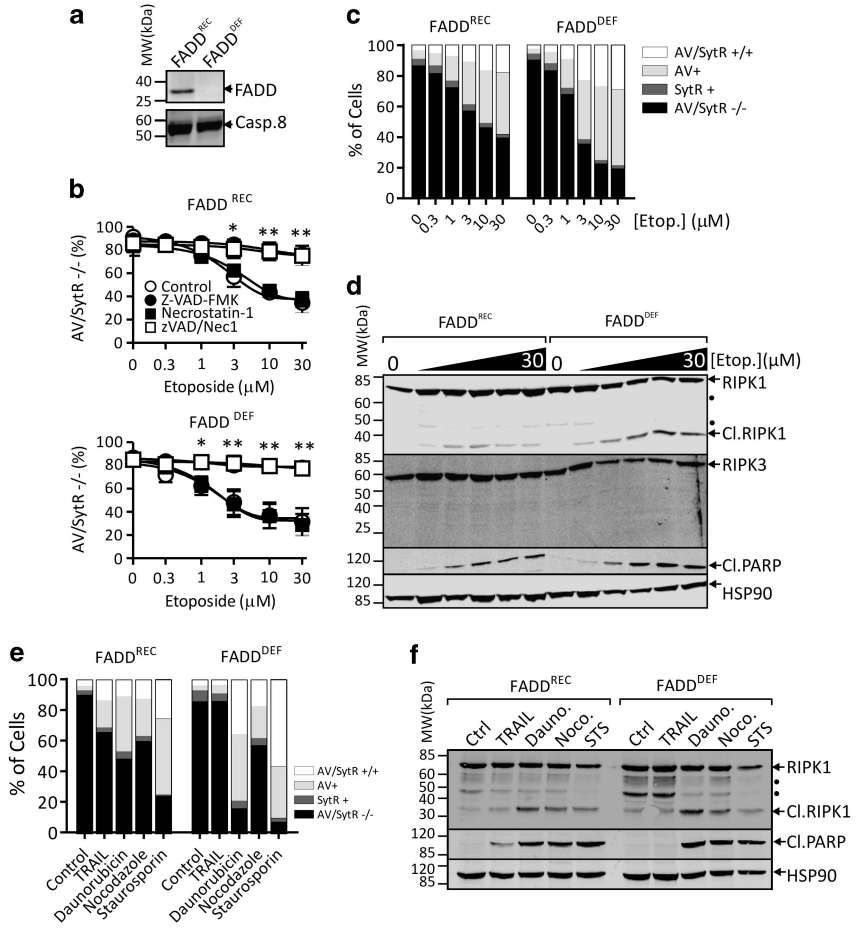

Figure 1 RIPK1 cleavage is an apoptotic event that occurs independently of FADD. Jurkat cells (clone 5C3) that were either deficient in FADD ($FADD^{DEF}$) or retrovirally reconstituted with FADD ($FADD^{REC}$), as demonstrated on western blot probed with specific antibodies against the indicated targets in (**a**), were stimulated with increasing amounts of etoposide (0.3, 1, 3, 10 or 30 μM) for 18 h, either in the absence (control; ○) in the presence of the pan-caspase inhibitor Z-VAD-FMK (●), the RIPK1 inhibitor necrostatin-1 (■) or both inhibitors (zVAD/Nec1; □). Cell death was determined after 24 h by flow cytometry after staining the cells with FITC-labeled Annexin V (AV) and Sytox Red (SytR) (**b** and **c**). Cell lysates were analyzed on western blots probed with specific antibodies against the indicated proteins (**d**) . Alternatively, cells were stimulated with either 100 ng/ml TRAIL, 1 μM Daunorubicin, 20 μg/ml Nocodazole or 100 nM Staurosporin for 18 h (**e** and **f**). Full-length and cleaved (Cl.) bands are indicated by arrows, unidentified bands on the RIPK1 blots are indicated by dots (●). Data either represent the mean ± S.E.M. of three independent experiments, analyzed by a Student's *t*-test as compared with an untreated sample (*$P<0.05$, ** $P<0.01$), or a representative graph/blot of at least three independent experiments is shown

apoptosis with etoposide or daunorubicin, but this effect was not found to be significant ($P>0.05$). RIPK1 cleavage was similar in both cell types with all death-inducing agents, except TRAIL (Figure 1f and Supplemental Figure S1b). See Figures S1c for further elucidation of the banding pattern observed with the anti-RIPK1 antibody. Thus, RIPK1 cleavage is neither etoposide-specific nor specific for DSB-induced death, but is a general phenomenon that occurs during apoptosis.

Etoposide titration on caspase-8-deficient cells corroborated that during intrinsic apoptosis, RIPK1 cleavage is caspase-8 independent (Supplemental Figures S1e–g).

Caspase-6 readily cleaves RIPK1 *in vitro*. It has been assumed that caspase-8/10 activity is required to cleave and inactivate the RIPKs.[10,11,26] Yet, we observe that RIPK1 cleavage occurs independently of FADD and caspase-8. In the caspase-8-deficient cells, RIPK1 could in principle have been cleaved by caspase-10. However, the cleavage was not noticeably reduced. Indeed, cleavage of RIPK1 appeared to be enhanced in caspase-8-deficient cells, and the cells displayed resistance to TRAIL-induced death but were equally susceptibile etoposide as the parental Jurkat cells (Supplemental Figure S1c), suggesting no compensatory expression/activation of caspase-10 in the extrinsic pathway.

Although the caspase-8 homodimer, the heterodimer with $FLIP_L$ and caspase-10 are capable of cleaving RIPK1, they do so with very low efficiency.[19,27] Furthermore, the caspase cleavage site identified in RIPK1 (^{321}LQLD/C^{325}) is not an optimal caspase-8 cleavage site.[1] Yet, during apoptosis a sizable portion of RIPK1 gets cleaved, in a caspase-dependent manner independent of FADD. Therefore, we decided to investigate the ability of the other apoptotic members of the caspase family to cleave RIPK1.

Apoptotic members of the caspase family 2, 3, 6, 7, 8, 9 and 10, were recombinantly expressed and purified from *E. coli* and the active concentration of each was determined by zVAD titration.[28] Caspases were assayed in the presence of 0.8 M NaCitrate buffer to ensure maximal activity and incubated at increasing concentrations for 1 h at 37 °C with FLAG-purified RIPK1. The reaction was stopped by boiling the samples in SDS sample buffer, and RIPK1 cleavage was analyzed on western blots. The only apoptotic caspase that cleaved more than 50% of total RIPK1 under these experimental conditions was the executioner caspase-6 (Figure 2). Caspases-8 and -10 were also able to cleave RIPK1 at higher concentrations, but the relative amount of cleavage achieved by these initiator caspases did not approach the efficiency with which the executioner caspase-6 cleaved RIPK1 (Table 1). A more

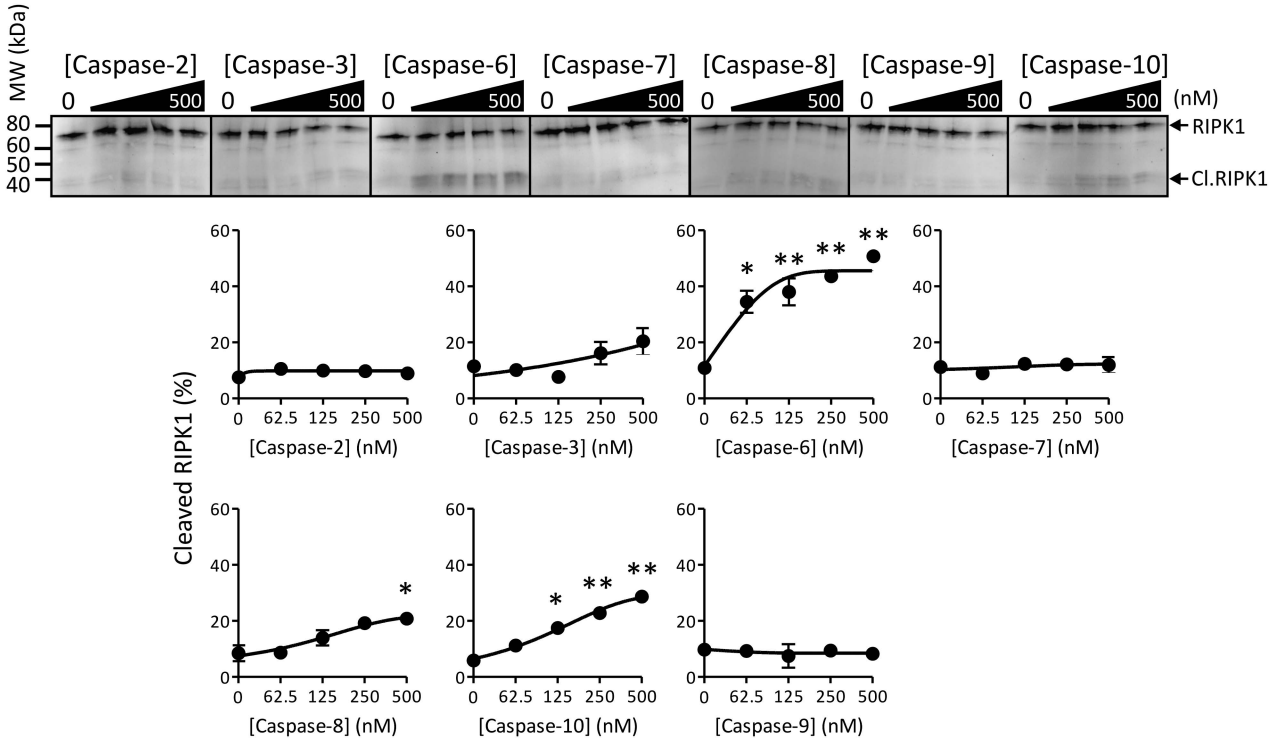

Figure 2 Recombinant RIPK1 is cleaved by caspase-6 *in vitro*. The active-site concentration of the indicated recombinant human caspases was determined by Z-VAD-FMK titration and increasing concentrations of the caspases (ranging from 0 to 500 nM) were incubated with FLAG-tagged human RIPK1, expressed and purified from HEK293 T cells, for 1 h at 37 °C in 0.8 M citrate buffer. The reaction was stopped by boiling the samples in SDS sample buffer and the samples were analyzed on western blots probed with a specific antibody against RIPK1. The bands of full-length and cleaved (Cl.) RIPK1 are indicated by arrows. Note that endogenous (non-FLAG-tagged) RIPK1 is copurified with FLAG-tagged RIPK1 and that a small percentage ($\sim$10%) of RIPK1 gets cleaved during expression. Results were analyzed by expressing the fluorescent signal of the cleaved bands as a percentage of the sum of the signals of the full-length and cleaved bands, as determined on a Li-Cor Odyssey infrared scanner. Data represent the mean $\pm$ S.E.M. of three independent experiments. The relative increase in cleavage was analyzed by an unpaired Student's *t*-test as compared to the cleavage observed in the untreated sample in the same series (*$P<0.05$, ** $P<0.01$)

Table 1 Catalytic parameters of cleavage by caspases-6 and -8 for the indicated substrates

	Caspase-6	Caspase-8
RIPK1	1.39 ± 0.40	0.19 ± 0.04
Bid	2.26 ± 0.34	20.2 ± 6.01
Caspase-8 (^{371}VETD/S^{375})	2.72 ± 0.15	n.d.
Pro-caspase-3	2.18 ± 0.45	5.69 ± 0.42

Numbers represent the apparent k_{cat}/K_M ($10^3\,M^{-1}s^{-1}$) ± S.D. as determined on western blot (RIPK1) or blue gel (all others) in at least three independent experiments. $k_{cat}/K_{M(app)}$ ($10^3\,M^{-1}s^{-1}$)

detailed comparison of caspases-6 and -8 activities on RIPK1 is shown in Supplemental Figure S2. No significant RIPK1 cleavage was observed with the other caspases tested.

Caspase-6 is required to cleave RIPK1 upon cytochrome-*c*-induced caspase activation. To test whether endogenous caspase-6 was capable of cleaving endogenous RIPK1 in the pathway of intrinsic apoptosis, hypotonic extracts from Jurkat cells were immune depleted for caspase-6 and/or -8 (Figure 3a and Supplemental Figure S3a). Upon addition of exogenous cytochrome *c*, induction of caspase-3-like activity occurred at the same rate in all extracts (Figure 3b). Samples were collected at the indicated time points and subjected to western blot analysis (Figure 3c). RIPK1 cleavage was significantly reduced in caspase-6-depleted extracts, whereas depletion of caspase-8 had no appreciable effect (Figures 3c and d). Thus, caspase-6 is absolutely required to cleave RIPK1 downstream of caspase-9 activation. Note that endogenous RIPK1 is a much better substrate for caspase-6 than recombinantly purified RIPK1 used in the experiments of Figure 2. In addition, caspase-6 activity was also essential to cleave caspase-8 in this pathway, generating a band of around 43 kDa, indicative of cleavage between the large and small subunits of caspase-8.

Alternatively, when recombinant caspases-3, 6 or 8 were added to the lysates, it became clear that caspase-6 acts downstream of caspase-3 to achieve RIPK1 cleavage. Caspase-3-dependent RIPK1 cleavage was significantly reduced in caspase-6-depleted extracts, whereas active caspase-8 alone could also cleave endogenous RIPK1 in the absence of caspase-6, albeit with lesser efficiency than caspase-6 itself (Supplemental Figures S3b–d).

Finally, we demonstrate that in murine embryonic fibroblasts from caspase-6 knockout mice RIPK1 cleavage only occurs upon reconstitution with caspase-6, but not upon transfection with an empty vector (Figures 4a and b). Although caspase-6 activates spontaneously in this system, as demonstrated by a significant increase in lamin cleavage (Figure 4c), no substantial amount of cell death was observed in these cells (data not shown).

Caspase-6 cleaves, but does not activate caspase-8. Caspase-8 itself is also a substrate for caspase-6 and cleavage by caspase-6 might potentiate this caspase, increasing its potency and thus its ability to cleave RIPK1.[19,29–31] It has been shown that cleavage of caspase-8 is neither necessary nor sufficient for activation; only dimerization is strictly required (reviewed in van Raam and Salvesen[2]). We decided to revisit this issue to further exclude a role for caspase-8 in our model and to address a few outstanding issues in the relationship between caspases-8 and -6.

To determine the efficiency of cleavage of caspase-8 by caspase-6 and the exact cleavage site, increasing concentrations of recombinant caspase-6 were titrated against full-length recombinant caspase-8, employing the catalytic mutant C360A (Figure 5a). Bands 3 and 4, as indicated on the right side of the gel, were identified by Edman degradation to be the N-terminus of our construct (GSSHH, 3) and the product of caspase-8 cleavage after the first caspase cleavage site in the inter subunit linker (SEEQP, 4), respectively. The identity of the remaining bands was deduced from their respective molecular weights relative to the identified bands. Thus, caspase-6 primarily cleaves caspase-8 at ^{371}VETD/S^{375} with a moderate catalytic rate (Table 1 and Figure 5a).

We employed a broad-spectrum biotinylated activity-based probe for caspases, biotinyl-GluValAsp-acyloxymethyl ketone (bEVD-AOMK),[32] to test whether cleavage by caspase-6 was sufficient for caspase-8 activation and to demonstrate that caspase-6 is really activated and not just cleaved in our system. Caspase-9 was activated in cytosolic extracts by the addition of cytochrome *c* and the activated downstream caspases were labeled with bEVD-AOMK at the indicated time points. Caspase were captured from the lysates with Neutravidin beads and both input and beads were analyzed on western blots (Figure 5b). Caspases-3 and -6 were captured from the lysates as early as 30 min after addition of cytochrome *c*, while no active caspase-8 could be detected.

To demonstrate that caspase-8 can be labeled if it gains activity and that activation of this species is dimerization dependent, we employed a recombinant caspase-8 fusion protein (Figure 5c). In this caspase-8 species, the N-terminal DED domains had been replaced an artificial dimerization domain derived from the sequence of the FK506 binding protein (FKBP) and the three Asp residues in the linker between the FKBP and the large subunit of the caspase had been mutated to Ala, to prevent auto-processing during expression in *E. coli* (FKBP-Casp.8D$_3$A).[19] Labeling of this caspase-8 species increased with increasing concentrations of the FKBP-specific dimerizing compound, AP20187 (Figure 5d). Addition of zVAD prevented labeling altogether. A small amount of FKBP-Casp.8D$_3$A was labeled in the absence of the dimerizing agent, as self-dimerization during expression and purification of the caspase could not be prevented. This experiment demonstrates that active caspase-8 can be labeled with bEVD-AOMK, labeling is activation dependent and that activation of caspase-8 depends on dimerization.

In addition, we demonstrate in Supplemental Figure S4a that recombinant caspase-6 alone readily releases cytochrome *c* from isolated mitochondria in the absence of caspase-8 (see Figure 3a). Finally, we show that caspase-6 can process both Bid and caspase-3 directly, with reasonable kinetics (Supplemental Figures S4b and c and Table 1). These findings explain the discrepancy between the study of Cowling *et al.*,[29] who suggested that caspase-6-dependent

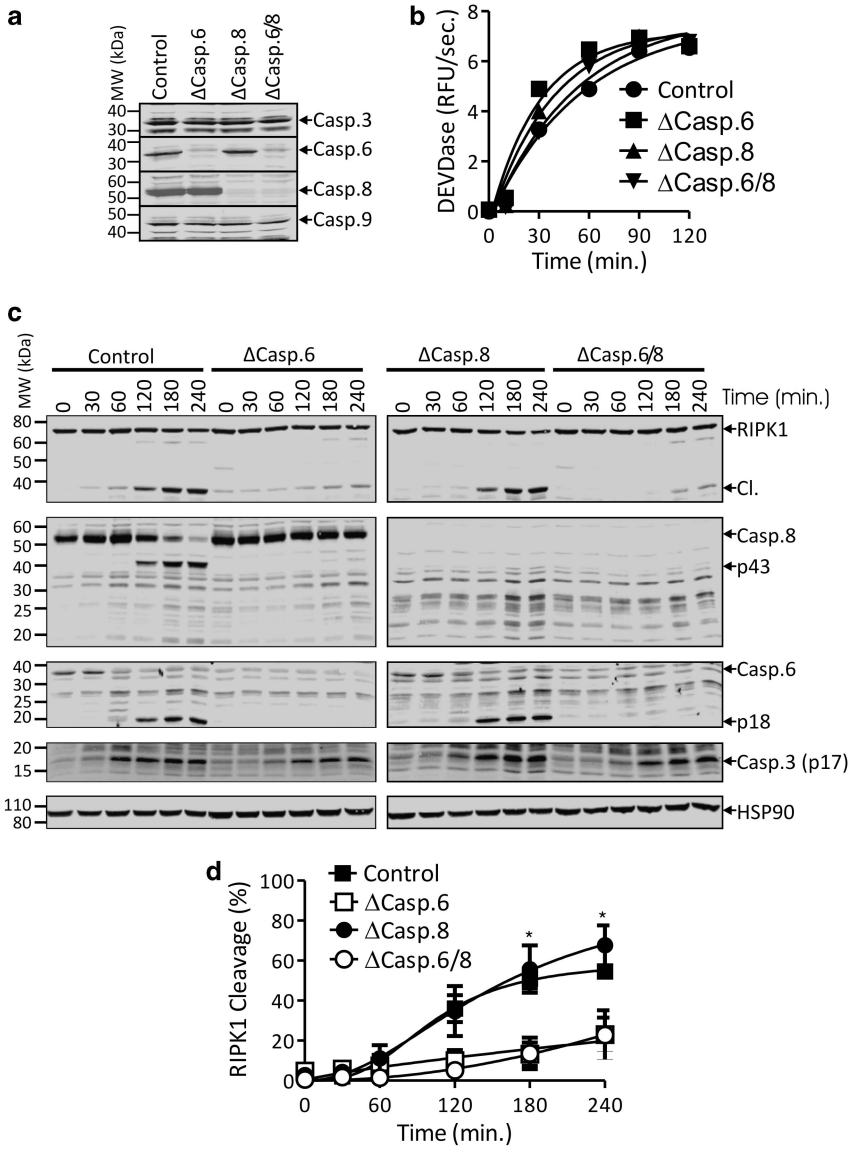

Figure 3 Caspase-6 is essential for RIPK1 cleavage following cytochrome-*c*-induced caspase activation. Jurkat cytosolic extracts were immune depleted for either caspase-6 (ΔCasp.6), -8 (ΔCasp.8) or both (ΔCasp.6/8), as indicated. Samples of the depleted lysates were subjected to western blot analysis to determine the amount of depletion achieved, relative to caspases-3 and -9 (**a**). Extracts were then activated by the addition of cytochrome *c* and ATP and the degree of activation was determined by monitoring caspase-3-like DEVDase activity over time (**b**). No significant difference was observed between the different extracts. Samples were taken at the indicated time points and subjected to western blot analysis, probed with specific antibodies against the indicated target proteins, to determine cleavage of these target proteins (**c**). HSP90 is shown as a loading control. Results were analyzed by expressing the fluorescent signal of the cleaved bands as a percentage of the sum of the signals of the full-length and cleaved bands, as determined on a Li-Cor Odyssey infrared scanner (**d**). Data represent the mean ± S.E.M. of three independent experiments. The relative increase in RIPK1 cleavage between the control and ΔCasp.6 extracts was analyzed by an unpaired Student's *t*-test (*$P<0.05$)

cleavage could activate caspase-8 as they observed Bid-dependent cytochrome *c* release upon addition of recombinant caspase-6 to isolated mitochondria, and later studies that demonstrated that cleavage alone is insufficient to activate caspase-8 (reviewed in van Raam and Salvesen[2]). Thus, caspase-6 can have a supporting, but limited, role in apoptosis; either by increasing the potency of preformed caspase-8 dimers or by directly processing Bid and caspase-3.

Caspase activity prevents RIPK1-dependent cytokine production during DSB-induced apoptosis. One context wherein intrinsic RIPK1 cleavage has been shown to be important is DSB-induced apoptosis, as it has been suggested that late-phase signaling of RIPK1 during DSB-induced apoptosis can lead to the production of pro-inflammatory cytokines, such as TNFα and IL6, in the absence of caspase activity.[15] In theory, such cytokine production can lead to feed-forward signaling and trigger secondary necroptosis, triggered by TNFα rather than by DSBs.[15,17]

To demonstrate this monocytic U937 cells, known for their ability to produce TNFα, were preincubated for 30 min with or without zVAD and/or Nec1 and subsequently stimulated for

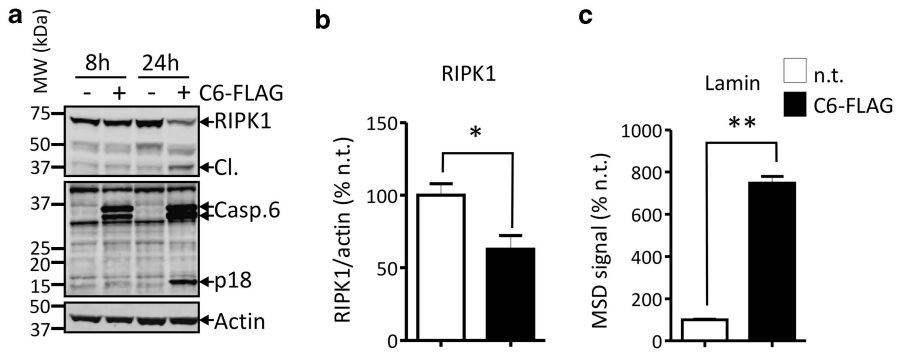

Figure 4 RIPK1 is cleaved in caspase-6$^{-/-}$ cells reconstituted with caspase-6. Mouse embryonic fibroblasts from caspase-6$^{-/-}$ mice were reconstituted with full-length caspase-6 fused to a C-terminal FLAG tag or transfected with empty vector. Samples were taken after 8 and 24 h and analyzed by western blot for RIPK1 cleavage, expression and cleavage of caspase-6-FLAG and actin as a loading control (**a**). Specific bands from full-length and cleaved proteins are indicated by arrows. A quantification of the RIPK1 blot is shown in (**b**). Caspase-6 activity was determined with a cleaved lamin ELISA, shown in (**c**). Data represent the mean ± S.E.M. of at least three independent experiments. The relative decrease of full-length RIPK1 or increase of cleaved lamin between the control (n.t., white bars) and caspase-6 transfected samples (C6-FLAG, black bars) was analyzed by an unpaired Student's *t*-test (*$P<0.05$, **$P<0.01$)

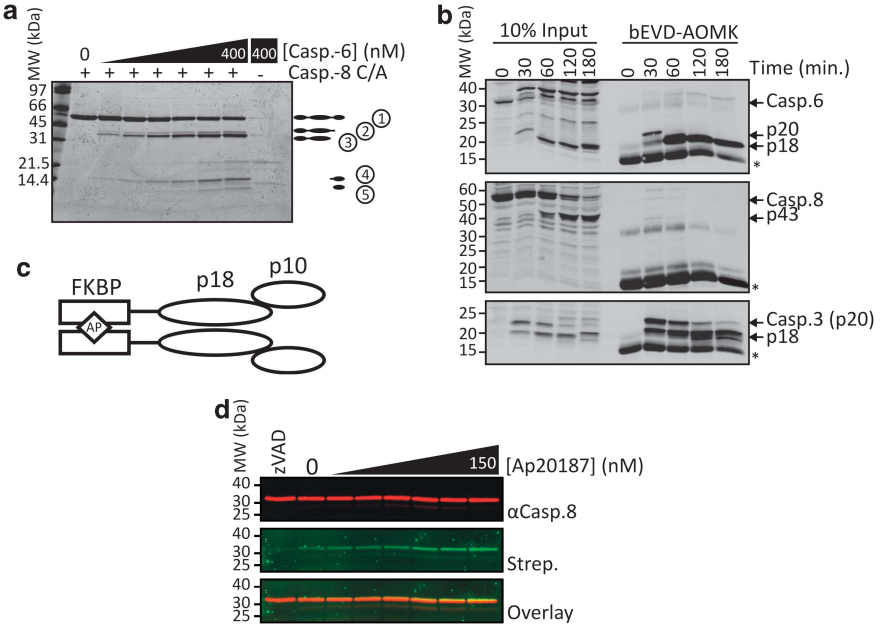

Figure 5 Caspase-6 cleaves, but does not activate, caspase-8.To determine cleavage of caspase-8 by caspase-6, recombinant caspase-6 was titrated on recombinant full-length caspase-8 at the indicated concentrations and incubated for 1 h at 37 °C in caspase buffer. Samples were analyzed by SDS-PAGE and a blue gel is shown of a typical experiment (**a**). The identity of bands and was confirmed by N-terminal sequencing (data not shown). The identity of the remaining bands was deduced from their respective molecular weights relative to the identified bands, as depicted on the right side of the gel as a cartoon of caspase-8. To establish whether cleavage by caspase-6 was sufficient to activate caspase-8, caspase-9 activation was induced in Jurkat cytosolic extracts by the addition of cytochrome *c* and dATP (**b**), resulting in the activation of downstream caspases. Samples were taken at the indicated time points; active caspases were labeled with bEVD-AOMK, pulled down from the extracts and analyzed on western blots probed with specific antibodies against the indicated proteins. Ten percent of the input before neutravidin pull down was loaded on the left sides of the gels. The caspase-3 blot was only probed for cleaved caspase-3. Full-length and cleaved caspases are indicated by arrows. *indicates a non-specific neutravidin band. To demonstrate that dimerization activates caspase-8 and that active caspase-8 is labeled with the activity-based probe bEVD-AOMK, a caspase-8 construct wherein the N-terminal DED domains were replaced with an inducible heterodimerization domain (FKBP) was used (**c**). This construct undergoes auto-processing between the large and small subunits, but has been rendered non-cleavable between the FKBP domain and the large subunit. 100 nM recombinant protein was incubated for 30 min with increasing amounts of the dimerizing compound AP20187 or zVAD, followed by an additional 10 min of incubation with 200 nM bEVD-AOMK (**d**). Samples were analyzed on western blots probed for caspase-8 and a fluorescently labeled secondary antibody (top panel, red) and with fluorescently labeled streptavidin (Strep., middle panel, green). The lower panel shows an overlay of the two signals, as detected on a Li-Cor Odyssey infrared scanner

18 h with increasing doses of etoposide. Cell death was determined by flow cytometry after Annexin-V-FITC/Sytox Red double staining (Figure 6a). TNFα concentrations were determined in the cell supernatant with an L929 sensitivity assay, as explained in the methods section, demonstrating directly that endogenous TNFα production can lead to tissue damage. As shown in Figure 6b, etoposide induces the production of significant amounts of TNFα, when caspase activation was blocked. This TNFα production was almost completely prevented by preincubation with Nec1, suggesting

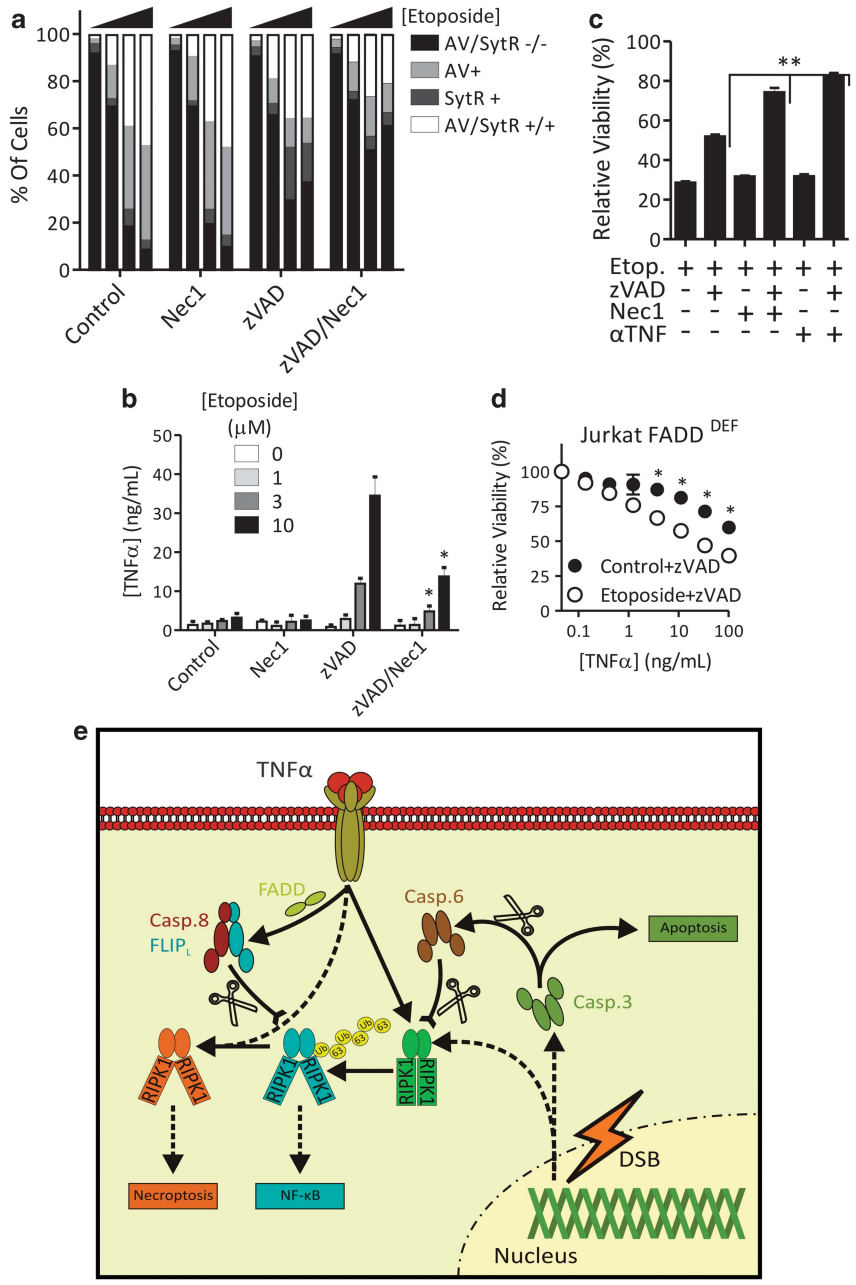

Figure 6 Lack of caspase-6 activation during etoposide-induced apoptosis leads to RIPK1-dependent TNFα production. U937 cells were treated overnight with increasing amounts of etoposide in the presence/absence of the indicated inhibitors (20 μM each). Cell death was determined by flow cytometry after Annexin V/Sytox Red double staining (**a**). TNFα concentrations were determined in the culture supernatant with an L929 sensitivity assay (**b**). The mean values ± S.E.M. of three independent experiments are shown and the relative difference in TNFα levels between zVAD- and zVAD/Nec1-treated samples was analyzed with an unpaired Student's *t*-test (*$P<0.01$). To establish that necroptotic cell death was induced by autocrine TNFα signaling in the presence of zVAD, U937 cells were incubated with etoposide and zVAD, were indicated, in the presence of Nec1 or a neutralizing anti-TNFα antibody (**c**). Where no anti-TNFα was used, an isotype control antibody was used instead. Viability was determined after 24 h with a Cell Titer Blue assay after an additional 2 h of incubation with the assay reagent. Relative viability is expressed as a percentage of the control, non-treated cells. The mean values ± S.E.M. of three independent experiments are shown. Statistical significance between etoposide/zVAD-treated and cells additionally treated with Nec1 or anti-TNFα was determined with a Student's *t*-test (**$P<0.001$). $FADD^{DEF}$ Jurkat cells were treated with increasing amounts of TNFα in the absence (●) or presence (○) of 1 μM etoposide to induce DSB as well zVAD (20 μM) (**d**). Cell viability was determined after 18 h with a Cell Titer Blue assay after an additional 2 h of incubation with the assay reagent. Relative viability is expressed as a percentage of non-TNFα-treated cells. Data represent the average ± S.E.M. of three independent experiments. Statistical significance between etoposide only and etoposide/zVAD-treated cells was determined with a Student's *t*-test (*$P<0.01$). (**e**) TNF Receptor signaling activates the caspase-8/$FLIP_L$ heterodimer in a FADD-dependent manner and RIPK1 in its NF-κB activating (K63-ubiquitinated) propensity. However, in the same signaling pathway, the transition between NF-κB activating and necroptosis-inducing RIPK1 is promoted through activation of DUBs. This transition is prevented by caspase-8 activity in a heterodimer with $FLIP_L$. Upon induction of DSB, RIPK1 is activated in its NF-κB signaling propensity, and caspase-3 and downstream caspase-6 are activated as well. DSBs do not normally induce the transition between NF-κB-activating and necroptosis-inducing RIPK1. Caspase-3 induces apoptosis, and caspase-6 silences RIPK1. In the absence of caspase-6, caspase-3 would still be activated and the cells would become apoptotic. However, under certain conditions, the balance might be shifted toward necroptosis or survival (NF-κB activation) instead

it is RIPK1 dependent. The U937 cells produced such high levels of TNFα that autocrine signaling led to necroptosis in the presence of zVAD, as indicated by the fact that only the combination of zVAD and Nec1 prevented death of these cells (Figures 6a and c). To demonstrate that etoposide/zVAD-dependent death was indeed induced by autocrine TNFα signaling, the cells were incubated in the presence of a neutralizing anti-TNFα antibody, as indicated in Figure 6c. Although zVAD alone partially rescued the cells from etoposide-induced death, Nec1 and anti-TNFα only significantly improved the viability in the presence of zVAD. These results were confirmed with the alternative broad-spectrum caspase inhibitor Q-VD-OPH (qVD), indicating that it is not a zVAD-specific effect (Supplemental Figures S5a and b). Thus, although DSBs does not induce necroptosis directly, it can result in TNFα production if the intrinsic caspase cascade is blocked and trigger necroptosis indirectly through autocrine signaling.

To test whether the induction of DSBs could sensitize cells to TNFα-induced necroptosis, $FADD^{DEF}$ Jurkat cells were incubated with increasing amounts of TNFα, in the presence or absence of etoposide and either zVAD or Nec1 alone or both zVAD and Nec1, whereupon viability was determined with a Cell Titer Blue assay (Figure 6d and Supplemental Figure S5c). Control cells only display a reduction in viability with higher concentrations of TNFα (>3 ng/ml), but cells treated with etoposide and zVAD display a reduction in viability starting at much lower concentrations of TNFα (>0.3 ng/ml). In the absence of zVAD, when caspase-6 is activated, etoposide-treated cells responded similar to TNFα as cells not treated with etoposide. Thus, upon induction of DSBs these cells become significantly more sensitive to TNFα-induced necroptosis in the absence of caspase activity. Note that the $FADD^{DEF}$ Jurkat cells used for these experiments do not produce measurable amounts of TNFα upon etoposide/zVAD treatment (data not shown).

As we have shown that etoposide induces intrinsic apoptosis, independent of caspase-8 activation, that caspase-6 cleaves RIPK1 in the intrinsic pathway of apoptosis and that the observed TNFα production is RIPK1 dependent, we suggest that a critical role of caspase-6 during apoptosis is to prevent late-phase RIPK1-dependent cytokine production by dying cells, and that caspase-6 protects the cells from necroptotic death in an inflammatory environment (Figure 6e).

Discussion

Our data indicates that caspase-6 cleaves RIPK1 downstream of caspase-3 in the intrinsic pathway of apoptosis. However, activation of RIPK1 in this pathway does not directly lead to necroptotic death in the absence of caspase activity (Figure 1b) unless the TNF receptor is also engaged (Figure 6). Thus, we hypothesize that in the presence of caspase activity, DSBs lead to complete inactivation of RIPK1 through caspase-6-mediated cleavage, with apoptosis as a net effect, whereas in the absence of caspase activity DSBs lead to signaling toward NF-κB and production of pro-inflammatory cytokines. Autocrine signaling by TNFα can feed-forward in the system and promote necroptosis if caspase-8 is not concomitantly activated (Figure 6).

As such, the executioner caspase-6 has a unique function during apoptosis – to terminate signaling. A number of cells presumably die daily as a consequence of excessive stress or damage induced by elements form the environment, such as cytotoxic agents or UV radiation. In the absence of a control mechanism, the death of these cells could trigger inflammation. This can be observed in cancer, for example, which is characterized by cells that fail to activate caspases, in spite of extensive stress or damage, localized necrosis and inflammation.[33] We propose that caspase-6 is a major part of this control mechanism that prevents the induction of inflammation by dying cells.

The role of caspase-6 in apoptosis is unclear, and the only apoptotic substrates that have thus far been identified for caspase-6, besides caspase-8, are the nuclear envelope proteins, lamin A and SATB1.[34,35] Overexpression of caspase-6 does not induce apoptosis,[36] although concomitant inhibition of the proteasome potentiates apoptosis induction by this caspase.[37] Caspase-6 knockout mice do not have a phenotype consistent with decreased apoptosis or increased inflammation[38] but in mice, and not in humans, RIPK1 can additionally be cleaved by the mitochondrial serine protease HtrA2/Omi.[39]

The only immunological phenotype that is clearly described for the caspase-6 knockout mice is a defect in the B-cell compartment. Their B cells appear to be arrested in G_1 and therefore proliferate less and differentiate more readily into antibody-producing plasma cells.[40] In the absence of RIPK1 cleavage, activating T cells undergo necroptosis, whereas B cells fail to activate and monocytes do not differentiate into macrophages (reviewed in Green *et al*.[41]). Although it is most likely that caspase-8 cleaves RIPK1 during the development of these cells, a role for caspase-6 cannot be excluded. In addition, nucleophosmin-1 was recently proposed as an endogenous inhibitor of caspases-6 and -8 in the cytosol and to contribute to acute myeloid leukemia by preventing myeloid differentiation.[42] Cleavage of RIPK1 is essential for myeloid differentiation and it could be that in this case inhibition of caspase-6 prevents this cleavage.

Caspase-6 has primarily been studied in the context of a variety of neurological disorders.[43] In the context of neuronal development and aging, caspase-6 has been suggested to activate in a non-caspase-3-dependent manner through autoactivation.[36,44] In neurons, such activation has been suggested to occur downstream of death receptor 6, but the exact mechanism of activation remains unclear.[45] We recently suggested that inactive caspase-6 could exist as a tetramer in the cell and that activation primarily requires the dissociation of the tetramer into active dimers.[46] Such a mode of activation would be permissive for caspase-6 activation outside apoptosis.

Caspase-6 cleaves caspase-8, and although cleavage does not activate caspase-8, it does appear to be required to stabilize the caspase-8 dimer and to generate a fully active species.[2] As such, by targeting the two signaling components of the RIPoptosome, caspase-6 inhibits the pro-survival/pro-necroptotic functions of this complex while favoring apoptosis.

In conclusion, we identify RIPK1 as a substrate of caspase-6 in the apoptotic cascade. Cleavage of RIPK1 appears to be required to terminate signaling in a cell marked for death and

prevent the production of pro-inflammatory cytokines. Thus, caspase-6 could have a non-apoptotic role in the regulation of inflammation and/or cellular differentiation. This function, as well as the precise mechanism of caspase-6 activation in a cellular context, requires further investigation.

Materials and Methods

Reagents. All chemical reagents were obtained from Sigma-Aldrich (St. Louis, MS, USA), unless otherwise indicated. Caspase substrates were obtained from Enzo Lifesciences (Farmingdale, NY, USA). Carboxybenzyl-Val-Ala-DL-Asp(OMe)-fluoromethylketone (zVAD), which was obtained from Bachem (Bubendorf, Switzerland). Biotylated-Val-Glu-Asp-acetyloxymethylketone (bVED-AOMK) was a kind gift from Dr. Matt Bogyo of Stanford University, CA, USA. Human TNFα was obtained from Peprotech (Rocky Hill, NJ, USA), Etoposide was obtained from Enzo Lifesciences, Killer TRAIL from Alexis (Lausanne, Switserland).

Antibodies. Rabbit anti-RIP1 (D94C12, #3493), -cleaved PARP (#9541) and cleaved caspase-3 (#9661) were all obtained from Cell Signaling Technology (Beverly, MA, USA). Rabbit anti-caspase-6 (Mch2; #06–691), used for detection of full-length caspase-6 and immune precipitation, was obtained from Upstate Biotechnology (Lake Placid, NY, USA). Mouse anti-HSP90 (#610418) was obtained from BD Transduction Laboratories (Franklin Lakes, NJ, USA). Rabbit anti-caspase-6 large subunit (AB10512) was obtained from Millipore (Billerica, MA, USA). Rabbit anti-RIP3 (IMG-5846A) was obtained from Imgenex (San Diego, CA, USA). All of these antibodies were used at a 1 : 1000 dilution in Tris-buffered saline, 0.002% Tween20 (TBST) supplemented with 2% (*w/v*) bovine serum albumin, fraction V. The mouse monoclonal anti-caspase-8 (C15) antibody was a kind gift from Dr. Markus Peter of Northwestern University, Chicago, IL, USA. This hybrydoma supernatant was used at a 1 : 200 dilution. Secondary antibodies, donkey anti-mouse IRDye 680LT and donkey anti-rabbit IRDye 800 CW, were both obtained from Li-Cor (Lincoln, NE, USA) and used at a 1 : 5000 dilution. All primary antibodies were incubated with the blot overnight at 4 °C; secondary antibodies were incubated for 1 h at room temperature. The blots were extensively washed with TBST in between. The neutralizing anti-TNF antibody (clone 104C) was a kind gift from Dr. Carl Ware (SBMRI) and was used at a concentration of 5 μg/ml.

Flow cytometry. To determine cell death, cells were pelleted and resuspended in AV binding buffer (BD Biosciences, San Jose, CA, USA) containing fluorescein isothiocyanide (FITC)-labeled AV (1 : 250 *v/v*; BD Biosciences). Cell were labeled for 30 min on ice, pelleted again, resuspended in AV binding buffer containing Sytox Red (1 : 1000 *v/v*; Invitrogen) and immediately analyzed on a FACSCanto II flow cytometer (BD Biosciences, Carlsbad, CA, USA) equipped with FACSDiva software. Ten thousand events were collected per sample in a gate set for intact, single cells by forward and side scatter. Markers were set for negative (untreated) and positive (maximum concentration etoposide) samples.

Plasmids. Human Caspase-2 (ΔCARD), -3, -6, -7, -8 (ΔDED), -9 (ΔCARD) and -10 (ΔDED) were cloned in pET23b *E. coli* expression vectors, as described.[47] FKBP-D3A-Caspase-8 was cloned in a pET29b expression vector and purified from *E. coli* as described.[19] Caspases used as substrates were inactivated by mutating the active-site cysteine (285,bythecaspase–1nomenclature) to alanine, by overlapping PCR. The construct for murine Bid with an N-terminal 6xHis tag in pET21 was a kind gift from Dr. John Reed of the Sanford-Burnham Medical Research Institute. Human RIPK1 with an N-terminal FLAG tag in pCDNA3.1 was a kind gift from Dr. Jürg Tschopp, formerly of the University of Lausanne, Lausanne, Switzerland.

Expression and purification of proteins. Recombinant caspases with a C-terminal 6xHis tag were expressed and purified from *E. coli* Bl21 DE3, as described.[47] Human RIPK1 with an N-terminal FLAG tag was expressed in and purified from HEK293T cells as follows: cells were grown to $\sim$60% confluence in 10-cm tissue culture dishes and transfected with 5-μg plasmid DNA/dish using Nanojuice (Novagen/EMD Chemicals, Gibbstown, NJ, USA), according to the manufacturer's instructions. To prevent premature cleavage of RIPK1, cells were preincubated with 20 μM zVAD. After transfection, cells were grown overnight, harvested, pooled washed and lysed in 1 ml cell lysis buffer (50 mM HEPES, 150 mM KCl, 0.1% CHAPS, 1% NP40, pH 7.4) supplemented with protease inhibitors (E64, MG132, 3,4-dichloroisocoumarin 3,4-DCI), phenylmethanesulfonyl fluoride aprotinin (all at 10 μM) and 100 μM EDTA, for 20 min on ice. The cleared lysate was collected after centrifugation and incubated overnight with 20 μl washed M2 anti-FLAG beads. Protein was eluted from the beads after washing away the unbound material with 40 μl 3 $\times$ FLAG peptide at a concentration of 5 mg/ml in PBS for 20 min on ice.

Determination of absolute caspase activity. Active-site concentrations of recombinant caspases expressed and purified from *E. coli* were determined by zVAD titration, as described in Pop *et al*.,[48] using the optimal AFC-labeled peptide substrate for each caspase. The substrates were Ac-VDVAD-AFC (caspase-2), Ac-DEVD-AFC (-3 and -7), Ac-VEID-AFC (-6), Ac-IETD-AFC (-8 and -10) and Ac-LEHD-AFC (-9). Assays were performed in citrate buffer (0.8 M NaCitrate, 20 mM PIPES, 100 mM NaCl, pH7.2, 5 mM dithiothreitol (DTT) added fresh).

SDS-PAGE and western blot. Cells were lysed by dissolving the washed pellet in cell lysis buffer supplemented with protease inhibitors (see above), usually at a concentration of 50 $\times$ 10^6/ml, for 20 min on ice. The samples were then spun at maximum speed at 4 °C, the supernatant was collected, boiled in SDS sample buffer and separated on 8–18% SDS-PAGE gels. Protein was transferred to Amersham Hybond nitrocellulose membrane (GE Healthcare, Little Chalfont, UK) blocked with TBST 2% BSA and probed with specific antibodies as indicated. Blots were scanned on an Odyssey infrared scanner (Li-Cor).

***In vitro* substrate cleavage.** Substrates were diluted to 1 μM final concentration and incubated with various concentrations of active caspase for 1 h at 37 °C in either 0.8 M citrate buffer or caspase buffer (20 mM PIPES, 100 mM NaCl, 10% sucrose, 5 mM DTT, 0.05% CHAPS, pH 7.4), as indicated. The reaction was stopped by boiling the samples in SDS loading buffer. Cleavage was subsequently assessed on 8–18% SDS-PAGE gels, either by staining the gels with Gel Code Blue reagent (Thermo Scientific, Waltham, MA, USA) or, in the case of FLAG-RIPK1, by western blot analysis. Gels and blots were scanned on an Odyssey infrared scanner (Li-Cor) and the fluorescent signal (the coomassie blue signal was detected in the 800 nm channel of the scanner) of full-length protein and the cleaved product was used to determine $E_{1/2}$, representing the concentration of protease that cleaves 50% of the full-length substrate in time (t). The apparent value for k_{cat}/K_M was then determined with the half-life equation[28]:

$$\frac{k_{cat}}{K_M} = \frac{\ln 2}{tE_{1/2}}$$

N-terminal sequencing. To determine the cleavage site in a substrate $\sim$100 fmol of the cleaved and untreated substrate were separated side by side by SDS-PAGE and transferred to PVDF membrane. The membrane was then dyed with coomassie blue solution (40% MeOH (*v/v*), 0.001% coomassie blue (*w/v*)) and destained with destaining solution (40% MeOH (*v/v*), 7.5% HAc (*v/v*)) to visualize the protein bands. Visible bands were then cut out and analyzed on a 492 Protein Sequencer (Applied Biosystems, Foster City, CA, USA).

Preparation of cytosolic extracts. To prepare cytosolic extracts from either Jurkat or HEK293 cells approximately 500 $\times$ 10^6 cells (Jurkat) or pooled cells from sixteen 15-cm cell culture dishes grown to 80% confluence (293) were harvested, washed with ice-cold phosphate-buffered saline (PBS; Cellgro/Mediatech inc., Manassas, VA, USA), resuspended in an equal volume of cell swelling buffer (20 mM PIPES, 10 mM KCl, 5 mM EDTA, 2 mM $MgCl_2$, pH 7.4, 4 mM DTT added fresh) and left on ice for 30 min. Swollen cells were then passed 10 times through a 20-G needle, followed by 10 passages through a 25-G and a 27-G needle. Cell lysates were cleared by centrifugation for 30 min at maximum speed and the supernatant (i.e. cytosolic extract) was harvested and stored in small aliquots at $-$80 °C until use.

Caspase depletion from cytosolic extracts. To deplete caspase-6 and/or -8 from cytosolic extracts, 40 μg antibody, either specific for caspase-6/8 or isotype control, was bound to protein A/G beads in IgG Binding Buffer (Pierce/Thermo Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Beads were then applied to 200 μl cytosolic extracts and incubated overnight at 4 °C in a rotator to deplete selected caspases. Finally, the beads with

the bound caspases were precipitated by centrifugation and the depleted extracts used for further experiments. Caspases were subsequently activated in the extracts by the addition of ATP/cytochrome *c* (see below) or the addition of active recombinant caspases at various concentrations, as indicated.

Cleaved LAMIN ELISA. The ELISA method for the detection of cleaved LAMIN in cell lysates was performed as described.[49]

Pull down of active caspases. To pull down active caspases from cytosolic extracts, the extracts were supplemented with 60 μM ATP and 10 μM MG132 to prevent proteasome activity. Extracts were then split in equal portions and caspases were either activated by the addition of cytochrome *c* (5 μM), recombinant caspase-6 (50 nM) or recombinant ΔDED-caspase-8 (50 nM). An equal volume of buffer was added to the negative control. Samples were taken at the indicated time points. Samples of 5 μl were taken at regular intervals, transferred to a white 96-well plate and 45 μl Ac-DEVD-AFC (100 μM) in caspase buffer was added to monitor caspase activation by release of free AFC on an fMax fluorescent plate reader (Molecular Devices, Sunnyvale, CA, USA). A sample of 45 μl was added directly to 2 μl bEVD-AOMK (100 μM, final concentration), left for 10 min at room temperature to label all active caspases and finally put on ice. Five microliter of these samples was taken as an input control. Ten microliter washed high capacity Neutravidin agarose (Thermo Fisher Scientific, Waltham, MA, USA) was added to the remainder of each sample at the end of the experiment and the samples were incubated overnight at 4 °C in a rotator. The next day, beads were precipitated by centrifugation and washed three times with 1 ml of ice-cold PBS. Finally, the washed beads were resuspended in SDS sample buffer, boiled for 5 min and analyzed by SDS-PAGE and western blot.

L929 sensitivity assay. To determine the production of toxic inflammatory cytokines, primarily TNFα, cells of the TNFα-sensitive murine fibroblastoma cell line L929 grown to $\sim$80% confluence in a 96-well plate were incubated overnight with supernatant samples (diluted 20 times) of Jurkat and U937 cells in the presence of 10 μM cycloheximide (Calbiochem, Billerica, MA, USA), a standard curve of human TNFα, ranging from 5000 to 40 pg/ml, or with the same concentrations of etoposide as might be left over in the medium of the Jurkat/U937 cells. The next day, cell survival was determined with an MTT assay. Briefly, Thiazolyl Blue Tetrazolium Bromide (MTT), dissolved in PBS was added to the cells to a final concentration of 1 mg/ml and incubated for 2 h at 37 °C. Afterwards, the culture medium was gently removed and the formazan crystals resulting from the metabolism of MTT by respiring, living cells were dissolved in MTT lysis buffer (4 mM HCl, 0.1% Nonidet P-40 (NP40), all in isopropanol). The absorbance of the samples was then read at 590 nm with a reference filter of 620 nm in a plate reader.

Cell lines and cell culture. Jurkat cells deficient in FADD, clone 5C3, and retrovirally reconstituted with FADD were a kind gift from Dr. Craig Walsh of UC Irvine, Irvine, CA, USA, and were generated as described.[50] Caspase-8-deficient Jurkats, clone I9.2, and parental Jurkats, clone A3, U937, L929 and HEK293T, cells were obtained from ATCC (Manassas, VA, USA). Jurkats and U937s were cultured in RPMI1640 medium (Cellgro) supplemented with 10% heat-inactivated fetal calf serum (Atlas Biologicals, Fort Collins, CO, USA), 2 mM L-Glutamine and pen/strep (Omega Scientific, Aiea, HI, USA). HEK293A cells and murine L929 cells were grown in DMEM medium with the same supplements. Embryonic fibroblasts from caspase-6 knockout mice were generated and transfected with full-length caspase-6 with a C-terminal FLAG-tag as described.[48] All cells were cultured at 37 °C with 10% CO_2 in a humidified incubator.

Statistics and data analysis. Sequence data were analyzed with Genious software v5.1.6 (Biomatters, Auckland, New Zealand), western blots and blue gels were analyzed with Odyssey software v3.0 (Li-Cor), FACS data were analyzed with FACSDiva software (BD, Franklin Lakes, NJ, USA). Statistical analysis was performed with Prism v5.00 (GraphPad Software, San Diego, CA, USA) and an unpaired Student's *t*-test with two-tailed *P*-values was used for all statistical analysis were indicated. A *P*-value below 0.05 was considered significant. Figures were prepared with CorelDRAW v11.633 (Corel Corporation, Ottawa, Ontario, Canada).

Conflict of Interest

The authors declare no conflict of interest.

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Author Contributions

BJvR performed and BJvR and GSS designed experiments, analyzed data and wrote the manuscript. DEE performed and DEE and MRH designed the experiments and analyzed the data shown in Figure 4.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)