

The c-FLIP-NH₂ terminus (p22-FLIP) induces NF- κ B activation

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c-FLIP proteins (isoforms: c-FLIP_L, c-FLIP_S, and c-FLIP_R) play an essential role in the regulation of death receptor-induced apoptosis. Here, we demonstrate that the cytoplasmic NH₂-terminal procaspase-8 cleavage product of c-FLIP (p22-FLIP) found in nonapoptotic malignant cells, primary T and B cells, and mature dendritic cells (DCs) strongly induces nuclear factor κ B (NF- κ B) activity by interacting with the I κ B kinase (IKK) complex via the IKK γ subunit. Thus, in addition to inhibiting apoptosis by binding to the death-inducing signaling complex, our data demonstrate a novel mechanism by which c-FLIP controls NF- κ B activation and life/death decisions in lymphocytes and DCs.

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Abbreviations used: CHX, cycloheximide; DED, death effector domain; DISC, death-inducing signaling complex; EMSA, electrophoretic mobility shift assay; IKK, I κ B kinase complex; siRNA, small interfering RNA.

c-FLIP is a well-described inhibitor of death receptor-mediated apoptosis (1). At the mRNA level, it can be found in multiple splice variants, whereas at the protein level only three isoforms, c-FLIP_L, c-FLIP_S, and c-FLIP_R, have been detected so far (1–4). All three c-FLIP isoforms contain two death effector domains (DEDs), which are structurally similar to the NH₂-terminal part of procaspase-8. c-FLIP_L also contains catalytically inactive caspase-like domains (p20 and p12).

c-FLIP proteins are recruited to the death-inducing signaling complex (DISC) by DED interactions (3–5). Both short c-FLIP isoforms, c-FLIP_S and c-FLIP_R, block death receptor-induced apoptosis by inhibiting procaspase-8 activation at the DISC (2, 3). The role of c-FLIP_L at the DISC is still a matter of controversy (6, 7). Some reports describe c-FLIP_L as an antiapoptotic molecule, functioning in a way analogous to c-FLIP_S, whereas others describe c-FLIP_L as a proapoptotic molecule, facilitating the activation of procaspase-8 at the DISC. This proapoptotic role may explain the phenotype of c-FLIP-deficient mice characterized by heart failure and death at embryonic day 10.5. The same phenotype has been reported for caspase-8- and FADD-deficient mice (8–11).

In addition to its antiapoptotic role in death receptor-induced apoptosis, c-FLIP proteins were invoked to play a prominent role in NF- κ B signaling (12–14). The transcription factor NF- κ B family regulates the expression of genes

crucial for innate and adaptive immune responses, cell growth, and apoptosis (15). In mammalian cells, the NF- κ B family is composed of five members: RelA, RelB, c-Rel, p50/NF- κ B1, and p52/NF- κ B2 (16). In most cells, the NF- κ B dimer is sequestered in the cytosol by inhibitors of the κ B protein (I κ B), and its nuclear translocation can be induced by a wide variety of stimuli (16). These stimuli trigger activation of the I κ B kinase (IKK) complex, which consists of two catalytic subunits, IKK α and IKK β , as well as a regulatory subunit, IKK γ /NEMO. When the IKK complex is activated, I κ B is phosphorylated, and the I κ Bs are degraded in a ubiquitin-dependent manner. The NF- κ B dimers can then be translocated into the nucleus, where target gene transcription is induced.

Recently, it has been demonstrated that overexpression of c-FLIP_L activates NF- κ B (13, 17). In another study, upon overexpression, c-FLIP_L was shown to interact with established components of the TNFR-mediated NF- κ B activation pathway, TRAF1, TRAF2, and RIP (12). In addition, it has been reported that c-FLIP_L-mediated NF- κ B activation requires cleavage to p43-FLIP, also demonstrated to interact with TRAF2 (18). In TNFR-mediated NF- κ B activation, TRAF2 and RIP were described to act upstream of the IKK complex (19, 20).

Here, we show that in nonapoptotic cells, c-FLIP forms heterodimers with procaspase-8 resulting in a novel NH₂-terminal fragment of c-FLIP (p22-FLIP). p22-FLIP turned out to be the key mediator of NF- κ B activation by

The online version of this article contains supplemental material.

direct binding to the IKK complex. These findings provide a new mechanism of c-FLIP-mediated NF- κ B activation and shed light on the regulation of life/death decisions made in lymphocytes.

RESULTS

A new form of c-FLIP can be detected in malignant B and T cells

In addition to the three previously described c-FLIP protein isoforms, c-FLIP_L, c-FLIP_R, and c-FLIP_S (2, 3, 21), we have detected a new prominent protein band with the anti-FLIP mAb NF6 directed against the DED region of c-FLIP (Fig. 1 A). The molecular mass of this protein is \sim 22 kD. The p22 protein was observed in total cellular lysates (Fig. 1 A) and in immunoprecipitates (Fig. 1 B) from B lymphoblastoid cell lines Boe^R and Raji and the T cell lines HUT78 and Jurkat A3, but not in CEM and SKW6.4 cells. The viability of the cells used for analysis was verified by negative prop-

idium iodide and annexin V staining (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20051556/DC1>). P22 protein was the most prominent in Boe^R cells (Fig. 1, A and B). We call this protein p22-FLIP.

The detection of p22-FLIP with the anti-FLIP mAb NF6 indicated the presence of DEDs in p22-FLIP because the antibody was raised against the NH₂ terminus of c-FLIP. Furthermore, p22-FLIP disappeared upon the addition of zVAD-fmk (Fig. 1 C). This suggests that p22-FLIP is likely a caspase-dependent cleavage product of c-FLIP. We then analyzed the primary structure of c-FLIP_{L/S} and found an aspartate residue at position 198 (Fig. 1 D). Cleavage at Asp¹⁹⁸ could result in the formation of an NH₂-terminal DED-containing cleavage product with a molecular mass of \sim 22 kD, corresponding to p22-FLIP.

To test this hypothesis, we generated a cDNA corresponding to the c-FLIP NH₂-terminal fragment resulting from cleavage at Asp¹⁹⁸. Subsequently, p22-FLIP was translated in vitro and added to cell lysates of Boe^R cells, followed by immunoprecipitation with the anti-FLIP mAb NF6. The products of immunoprecipitation as well as the corresponding lysates were analyzed by Western blot (Fig. 1 E). After adding the in vitro-translated c-FLIP NH₂-terminal fragment, the band corresponding to p22-FLIP increased considerably. Thus, we conclude that the molecular mass of the in vitro-translated product was indeed identical to endogenous p22-FLIP. These data provide the first evidence that p22-FLIP is an NH₂-terminal cleavage product of c-FLIP generated by cleavage at Asp¹⁹⁸.

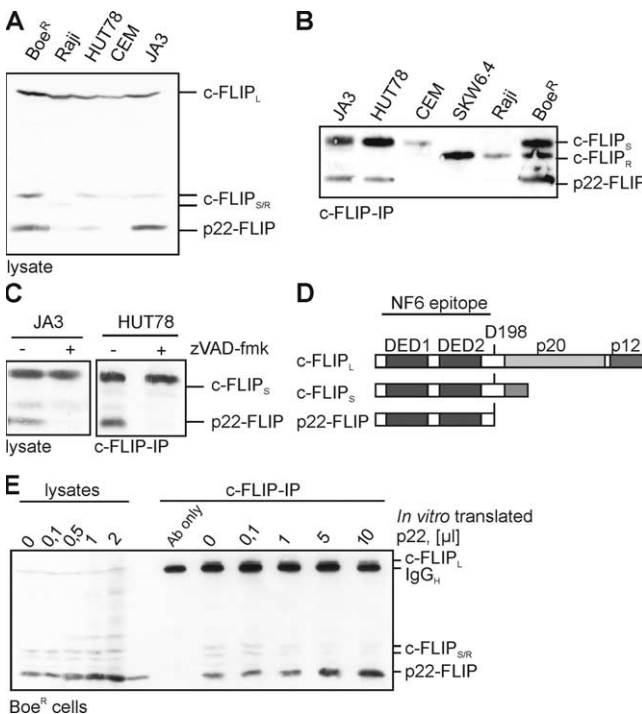


Figure 1. Caspase-dependent presence of p22-FLIP in tumor cell lines. (A) Total cellular lysates of the indicated T and B cell lines were subjected to 12% SDS-PAGE and Western blot analysis using the anti-FLIP mAb NF6. The positions of c-FLIP_L, c-FLIP_{S/R}, and p22-FLIP are indicated. (B) Western blot analysis of c-FLIP proteins after immunoprecipitation from various cell lines (5×10^7 cells each) using anti-FLIP mAb NF6. Positions of c-FLIP_{S/R} and p22-FLIP are indicated. (C) HUT78 and Jurkat A3 cells were preincubated with or without 20 μ M zVAD-fmk for 30 min. Analysis of c-FLIP proteins by Western blot was performed as in A. (D) Schematic representation of c-FLIP proteins. DEDs are depicted in black. The cleavage site for generation of p22-FLIP (D198) is shown. The epitope for anti-c-FLIP mAb NF6 is indicated. (E) The NH₂ terminus of c-FLIP encoding the amino acids 1–198 was in vitro translated, [³⁵S] labeled, and added in the indicated amounts to the lysates of Boe^R cells as well as to immunoprecipitates of c-FLIP from 5×10^7 Boe^R cells.

p22-FLIP identification as the NH₂-terminal cleavage product of c-FLIP

To study whether p22-FLIP can be generated from both c-FLIP_L and c-FLIP_S, we performed the following experiment in vitro. c-FLIP_L, c-FLIP_S, and FLAG-c-FLIP_L were translated in vitro, [³⁵S] labeled, and added to total cellular lysates of HUT78 and J16 cells (Fig. 2 A). Upon incubation, all c-FLIPs were cleaved into the NH₂-terminal fragment p22-FLIP (for c-FLIP_L and c-FLIP_S), the NH₂-terminal FLAG-p22-FLIP (for FLAG-c-FLIP_L), and the COOH-terminal fragment p33-FLIP (for c-FLIP_L and FLAG-c-FLIP_L). Consistent with the results obtained previously, p22-FLIP was not detected upon the addition of zVAD-fmk. Thus, we observed a caspase-dependent processing of c-FLIP_{L/S} into p22-FLIP in nonapoptotic cells.

To provide conclusive evidence that p22-FLIP is indeed the cleavage product of c-FLIP resulting from cleavage at Asp¹⁹⁸, we generated an uncleavable D198A mutant of FLAG-c-FLIP_L. In vitro-translated [³⁵S]-labeled D198A-FLAG-c-FLIP_L and WT-FLAG-c-FLIP_L were added to the lysates of HUT78 cells (Fig. 2 B). As anticipated, FLAG-p22-FLIP was generated in a caspase-dependent manner only from WT-FLAG-c-FLIP. In addition, Boe^R cells were transfected with WT- and D198A-FLAG-c-FLIP constructs, and consistent with the in vitro data, FLAG-p22-FLIP generation was observed only in the cells transfected with the WT

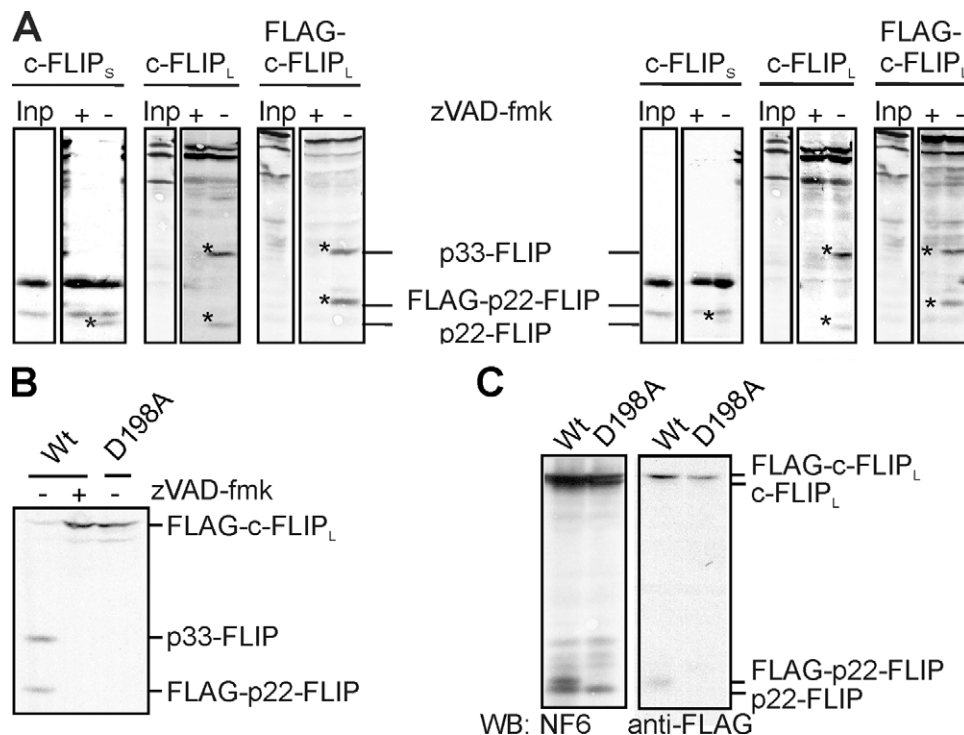


Figure 2. Identification of the p22-FLIP cleavage site. (A) In vitro-translated [³⁵S]-labeled c-FLIP_S, c-FLIP_L, or FLAG-c-FLIP_L was added to the lysates of HUT78 (left) and Jurkat A3 cells (right) and incubated overnight at 4°C in presence or absence of 50 μM zVAD-fmk. Reactions were separated on 12% SDS-PAGE gels, blotted, and subjected to autoradiography. (B) In vitro-translated [³⁵S]-labeled

construct (Fig. 2 C). Collectively, these data demonstrate that p22-FLIP is the NH₂-terminal cleavage product of c-FLIP generated by caspase cleavage at Asp¹⁹⁸.

p22-FLIP is generated by procaspase-8 activity and inhibits death receptor-induced apoptosis

To unravel the mechanism of p22-FLIP formation and find the caspase directly involved in c-FLIP processing, we investigated whether procaspase-8 might generate p22-FLIP. Procaspase-8, which, as a proform, was reported to possess catalytic activity (7, 22) and form heterodimers with c-FLIP in the cytosol by DED interactions, represented a likely candidate (6, 23). Therefore, we immunoprecipitated procaspase-8 from HUT78 cells with an anti-caspase-8 mAb and added in vitro-translated [³⁵S]-labeled c-FLIP_L (Fig. 3 A, left). Interestingly, we observed cleavage of c-FLIP_L into the NH₂-terminal fragment p22-FLIP and the COOH-terminal fragment p33-FLIP by procaspase-8. The processing was blocked by zVAD-fmk.

To ensure that the mature caspase-8 heterotetramer could not process c-FLIP into p22-FLIP, we added recombinant active caspase-8 to in vitro-translated c-FLIP_L (Fig. 3 A, right). As expected, recombinant caspase-8 cleaves c-FLIP_L in a “classic apoptotic” fashion and generated p43-FLIP and p12-FLIP. Thus, we show that caspase-8 and procaspase-8 process c-FLIP in two mutually exclusive ways. The active

WT-FLAG-c-FLIP_L or D198A-FLAG-c-FLIP_L was added to the lysates of HUT78 cells and incubated overnight at 4°C in the presence or absence of 50 μM zVAD-fmk and visualized as in A. (C) Boe^R cells were transfected with WT-FLAG-c-FLIP_L or D198A-FLAG-c-FLIP_L and incubated with or without 20 μM zVAD-fmk, and c-FLIP proteins were analyzed by Western blot.

caspase-8 heterotetramer generates the well-characterized p43-FLIP and p12-FLIP cleavage products, whereas procaspase-8 activity induces formation of the novel p22-FLIP cleavage product as well as the COOH-terminal p33-FLIP. This is consistent with a report on different substrate specificities of the caspase-8 proform and active caspase-8 (7). Clearly, the present study is the first to demonstrate two different caspase-8 specificities with respect to c-FLIP cleavage.

c-FLIP proteins are well-established inhibitors of death receptor-mediated apoptosis. To examine the role of p22-FLIP in death receptor-mediated apoptosis, we generated stable BJAB cell lines overexpressing either high (p22-FLIP_{high}) or low (p22-FLIP_{low}) amounts of p22-FLIP. The amount of p22-FLIP in these cell lines was validated by Western blot (Fig. 3 B). Both p22-FLIP_{high} and p22-FLIP_{low} BJAB cells were characterized by reduced sensitivity toward CD95- and TRAIL-induced apoptosis as compared with the vector-transfected control (Fig. 3, C and D, respectively). The reduction in apoptosis was more prominent in the p22-FLIP_{high} cells. These data provide evidence that compared with other c-FLIP proteins, p22-FLIP is an even stronger inhibitor of death receptor-induced apoptosis.

c-FLIP proteins were reported to have a short half-life (24, 25). We and others have demonstrated that cycloheximide (CHX) sensitizes cells toward death receptor-induced

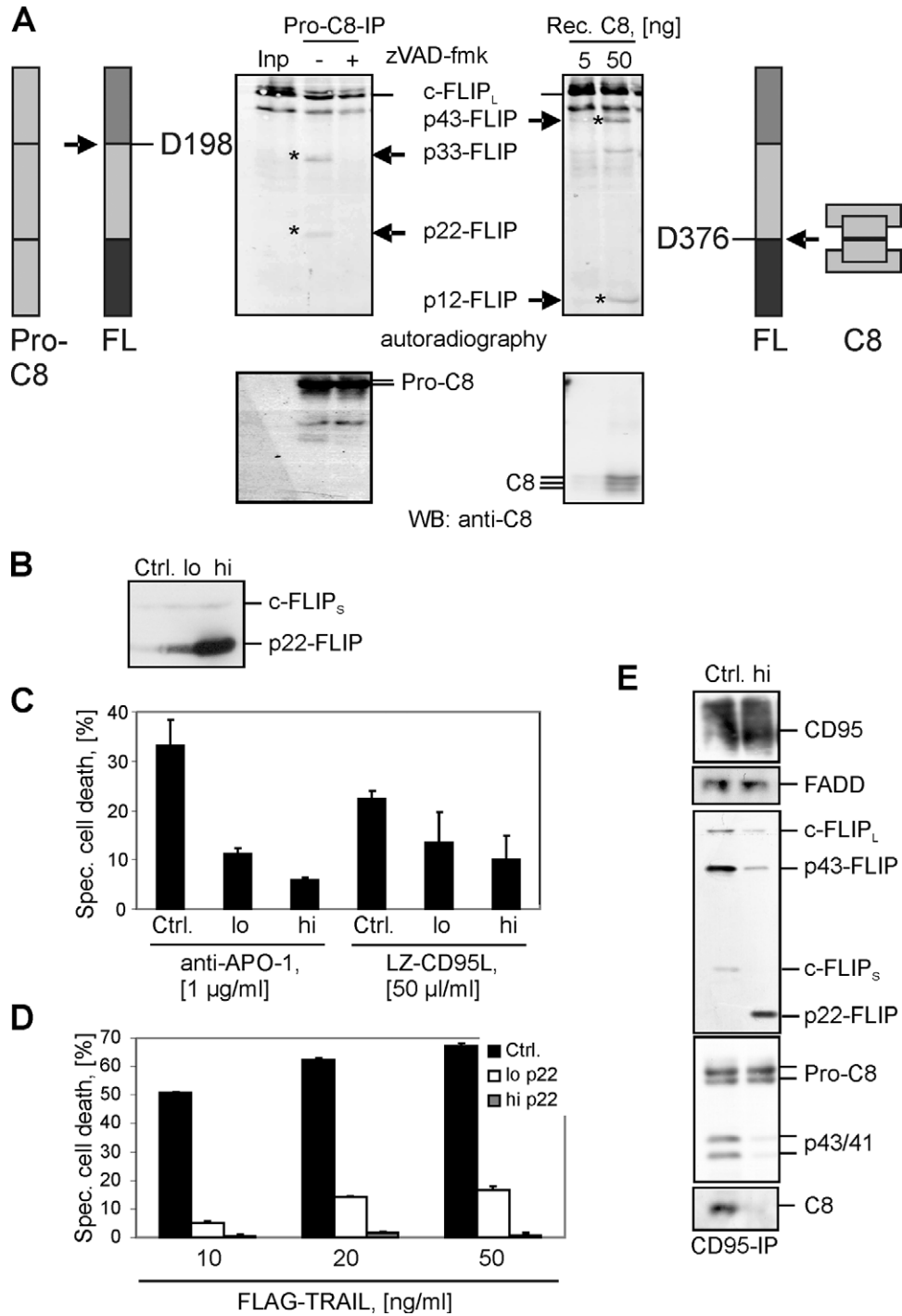


Figure 3. p22-FLIP is generated by procaspase-8 and inhibits death receptor-induced apoptosis. (A) Procaspase-8 was immunoprecipitated from HUT78 cells using anti-caspase-8 mAb C15 and then incubated for 1 h at 37°C together with in vitro-translated [³⁵S]-labeled c-FLIP_L in the presence or absence of zVAD-fmk. c-FLIP processing was analyzed by autoradiography (top left). c-FLIP cleavage products p22 and p33 are indicated. Afterward, the same membrane was subjected to Western blot analysis using anti-caspase-8 mAb C15 (bottom left). [³⁵S]-labeled c-FLIP_L was incubated with the indicated concentrations of recombinant caspase-8 for 1 h at 37°C. c-FLIP pro-

cessing was analyzed by autoradiography (top right). c-FLIP cleavage products p12 and p43 are indicated. Afterward, the same membrane was subjected to Western blot analysis using anti-caspase-8 mAb C15 (bottom right). (B) Analysis of p22-FLIP expression in BJAB cell lines stably overexpressing high or low amounts of p22-FLIP (p22-FLIP_{high} or p22-FLIP_{low}, respectively). Endogenous expression of c-FLIP_s is used as a loading control. (C) p22-FLIP_{high}, p22-FLIP_{low}, and vector-transfected BJABs (Ctrl.) were stimulated with 1 µg/ml anti-APO-1 antibodies or 50 µl/ml LZ-CD95L for 16 h. Specific cell death was calculated as described in Materials and methods. (D) p22-FLIP_{high}, p22-FLIP_{low}, and

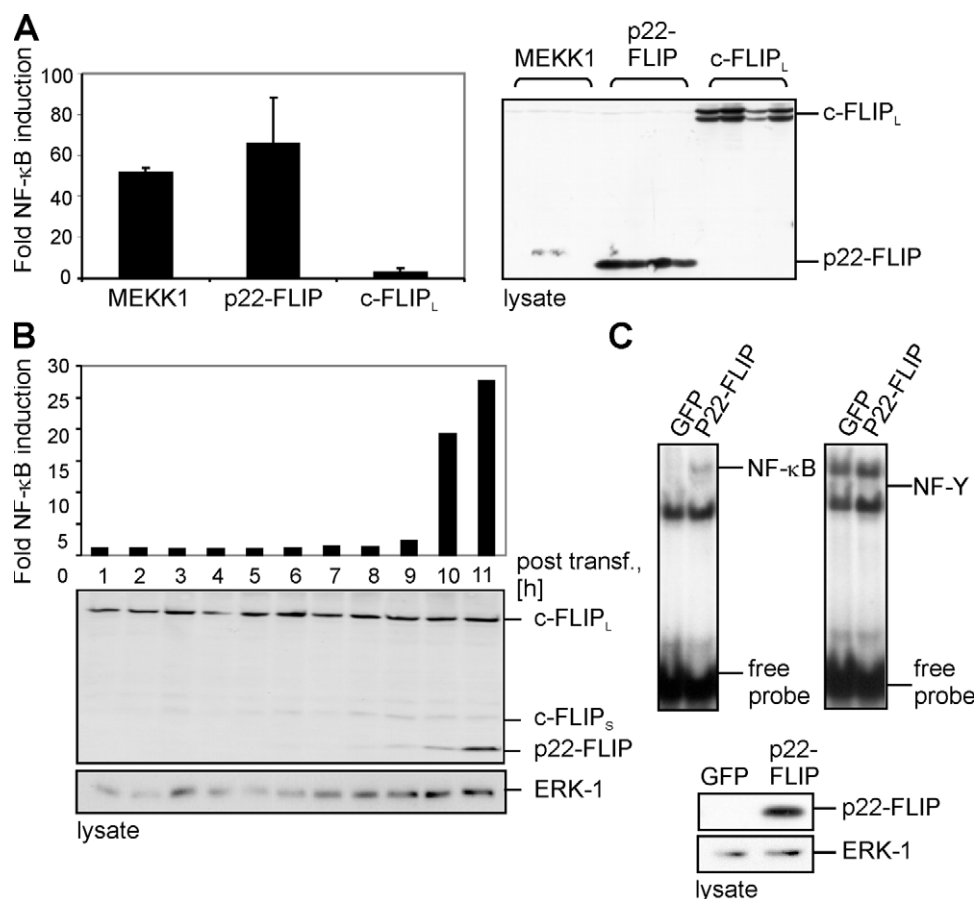


Figure 4. p22-FLIP is a strong inducer of NF-κB. (A) 293T cells were cotransfected with MEKK1, p22-FLIP, c-FLIP_L, and luciferase reporter plasmid. GFP transfections were performed to control the transfection efficiency. Western blot analysis using anti-FLIP mAb NF6 was performed to control equal protein expression (right). (B) 293T cells were cotransfected with p22-FLIP and the luciferase reporter plasmid. After the indicated periods of time, cells were lysed and NF-κB

luciferase activity was determined (top). Western blot analysis using anti-FLIP mAb NF6 was performed to determine the expression level of p22-FLIP. (C) Nuclear extracts, which were prepared from 293T cells transfected with p22-FLIP or GFP, were subjected to EMSAs using ³²P-labeled oligonucleotides containing an NF-κB (left) or an NF-γ (right) binding site. p22-FLIP expression was verified by Western blot.

apoptosis, which correlates with a decrease of c-FLIP levels (24, 25). To understand whether sensitization also involves a decrease of the p22-FLIP level, we studied Boe^R cells that contain high levels of p22-FLIP. Treatment of Boe^R cells with CHX resulted in a substantial decrease of the p22-FLIP level within 4 h (Fig. S2 A, available at <http://www.jem.org/cgi/content/full/jem.20051556/DC1>). This decrease correlated with an enhanced sensitivity toward CD95- and TRAIL-induced apoptosis (Fig. S2 B). These data provide additional evidence that the p22-FLIP level in Boe^R cells correlates with the sensitivity toward death receptor-induced apoptosis, pointing toward the inhibitory role of p22-FLIP.

The antiapoptotic action of c-FLIP in death receptor-mediated apoptosis involves inhibition of caspase-8 activation

at the DISC (2, 3, 24). To investigate whether p22-FLIP is also recruited to the DISC, we immunoprecipitated the CD95 DISC from p22-FLIP_{high} cells. Indeed, p22-FLIP was recruited to the DISC (Fig. 3 E). As expected, the activation of procaspase-8 at the DISC of p22-FLIP_{high} cells was lower than in vector-transfected cells. The amount of caspase-8 cleavage products in the DISC, p43/p41, and p18 was markedly reduced in p22-FLIP_{high} cells, whereas the amounts of FADD and CD95 were similar in both p22-FLIP_{high} and vector-transfected cells. This observation is consistent with the reduced sensitivity of p22-FLIP_{high} cells toward CD95-induced apoptosis and shows that p22-FLIP effectively blocks caspase-8 activation at the DISC, thereby inhibiting CD95-induced apoptosis.

vector-transfected BJABs (Ctrl.) were stimulated with the indicated concentrations of FLAG-TRAIL for 16 h. (E) CD95 DISCs were immunoprecipitated from 5×10^7 cells of p22-FLIP_{high} and vector-transfected

BJABs (Ctrl.) and analyzed by Western blot with anti-caspase-8 mAb C15, anti-FLIP mAb NF6, anti-CD95 polyclonal antibody C20, and anti-FADD mAb.

p22-FLIP is a strong inducer of NF-κB

We established an inhibitory role of p22-FLIP in death receptor-induced apoptosis; however, the questions of why p22-FLIP is present in nonapoptotic malignant cells and which functional role p22-FLIP might play in the nonapoptotic scenario were not answered. Using NF-κB luciferase activation assays with p22-FLIP and c-FLIP_L, we observed that p22-FLIP is a strong inducer of NF-κB (Fig. 4 A). Moreover, p22-FLIP-mediated NF-κB activation was much stronger than that observed with c-FLIP_L, even though expression levels of p22-FLIP and c-FLIP_L were similar (Fig. 4 A).

To clarify whether NF-κB activation correlates with the expression level of p22-FLIP, we performed transient transfections of 293T cells with p22-FLIP (Fig. 4 B). The maximum of NF-κB activity at 10 h paralleled the increase of p22-FLIP expression. Moreover, the observed expression level of p22-FLIP in 293T cells (Fig. 4 B) compared with c-FLIP_L was still lower as compared with the ratio p22-FLIP/c-FLIP_L in Boe^R cells (Fig. 1 A). Thus, we demonstrate that the induction of NF-κB is specific for p22-FLIP and does not depend on a high expression level of p22-FLIP.

We also assayed p22-induced NF-κB activity by electrophoretic mobility shift assay (EMSA; Fig. 4 C), which independently confirmed that p22-FLIP induces NF-κB. Thus, we demonstrated that p22-FLIP is a strong inducer of NF-κB.

p22-FLIP induces NF-κB by direct interaction with the IKK complex

To get more insight into the mechanism of p22-mediated NF-κB induction, we coexpressed p22-FLIP with the inhibitors of NF-κB (IκBα and IκBβ) and with components of the IKK complex (IKKα, IKKβ, DN-IKKα, and DN-IKKβ; Fig. 5 A). Cotransfections of p22-FLIP with increasing amounts of IκBα and IκBβ inhibited NF-κB activation. Looking at more upstream events, we observed that cotransfection with DN-IKKα and DN-IKKβ also led to suppression of NF-κB activation. Collectively, the results indicated that p22-FLIP is a strong inducer of NF-κB acting via the canonical NF-κB pathway.

We further examined whether p22-FLIP directly interacts with the IKK complex. FLAG-tagged IKKα, IKKβ, and IKKγ were transiently cotransfected with p22-FLIP into 293T cells, and then immunoprecipitated with anti-FLAG and anti-FLIP antibodies (Fig. 5 B). We did not observe any interaction between p22-FLIP and IKKα or IKKβ. However, p22-FLIP was coimmunoprecipitated with FLAG-IKKγ and vice versa. Thus, we showed that p22-FLIP interacts with the IKK complex via IKKγ.

In addition, we compared NF-κB induction by p22-FLIP, c-FLIP_L, and p43-FLIP upon transient transfections in 293T cells (Fig. 5 C). Interestingly, the addition of zVAD-fmk resulted in a decrease in NF-κB activation for p43-FLIP and c-FLIP_L, but did not affect p22-FLIP-mediated NF-κB induction. These results strongly indicate that p43-FLIP and

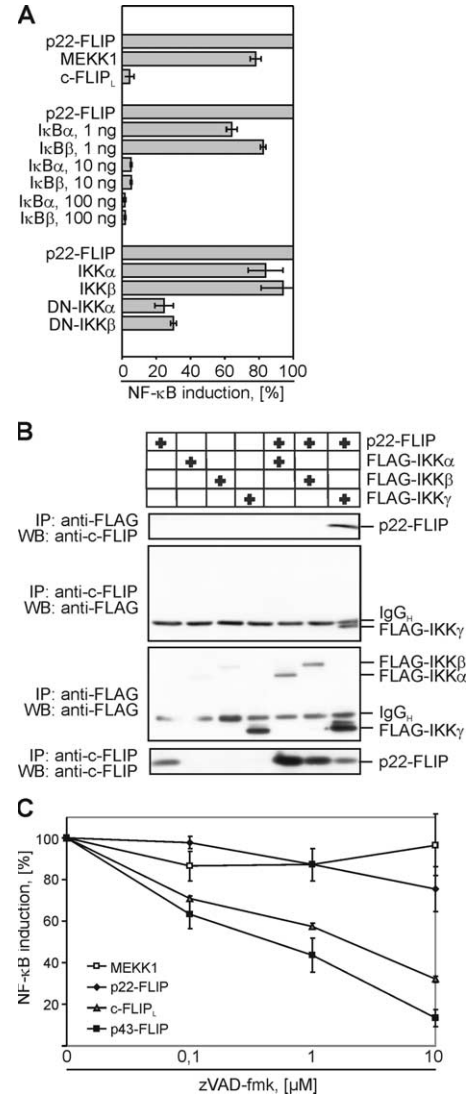


Figure 5. p22-FLIP induces NF-κB by direct interaction with the IKK complex. (A) 293T cells were cotransfected with luciferase reporter plasmid and either MEKK1, p22-FLIP, or c-FLIP_L (top part of the diagram). 293T cells were cotransfected with p22-FLIP, the luciferase reporter plasmid, and any one of the constructs IκBα, IκBβ, WT-IKKα, WT-IKKβ, mutated IKKα, or IKKβ (bottom part of the diagram). Transfection efficiency was examined using GFP transfections. NF-κB luciferase activity was determined as described in Materials and methods. (B) FLAG or FLIP immunoprecipitations were performed from 293T cells that were transfected with p22-FLIP and any one of the constructs FLAG-IKKα, FLAG-IKKβ, or FLAG-IKKγ. Immunoprecipitated products were subjected to 12% SDS-PAGE gels and analyzed by Western blot using anti-FLIP mAb NF6 and anti-FLAG mAb. (C) 293T cells were cotransfected with MEKK1, p22-FLIP, c-FLIP_L, p43-FLIP, and the luciferase reporter plasmid. Transfected cells were incubated for 16 h in the presence of the indicated concentrations of zVAD-fmk and lysed, and NF-κB luciferase activity was determined.

c-FLIP_L require further cleavage to induce NF-κB activity, whereas p22-FLIP does not. Thus, we show that for the induction of NF-κB activity, both p43-FLIP and c-FLIP_L have to be processed into p22-FLIP.

p22-FLIP induces NF- κ B during activation of primary lymphocytes and maturation of primary DCs

Next, we examined primary human T and B cells for the presence of p22-FLIP. Interestingly, p22-FLIP was absent in freshly prepared cells, but was generated upon activation of these cells with PHA (Fig. 6 A). The same phenomenon was observed in DCs. p22-FLIP was generated upon LPS stimulation, indicating that p22-FLIP is present during maturation of DCs (Fig. 6 A). Also, the increase of p22-FLIP levels in primary cells correlated with the increase of c-FLIP levels and, correspondingly, with the increase of the ratio of c-FLIP to procaspase-8. This observation provides additional evidence for the proposed mechanism of procaspase-8-mediated c-FLIP cleavage to p22-FLIP (Fig. 3).

To find out whether p22-FLIP also directly interacts with the IKK complex in primary human T cells, we performed immunoprecipitations using an anti-IKK γ antibody (Fig. 6 B). We observed p22-FLIP in the IKK complex. Thus, we conclude that p22-mediated NF- κ B induction occurs via the same mechanism in primary cells.

To obtain more insight into the role of p22-FLIP in T cell activation, we studied the proliferation of primary T cells upon silencing of c-FLIP using small interfering RNA (siRNA). The silencing of c-FLIP resulted in down-regulation of c-FLIP_{L/S/R} as well as its cleavage product, p22-FLIP (Fig. 6 C). Primary T cells were stimulated with either PHA or anti-CD3/CD28, and thymidine incorporation was measured after 3 d of additional culture (Fig. 6 C). Interestingly,

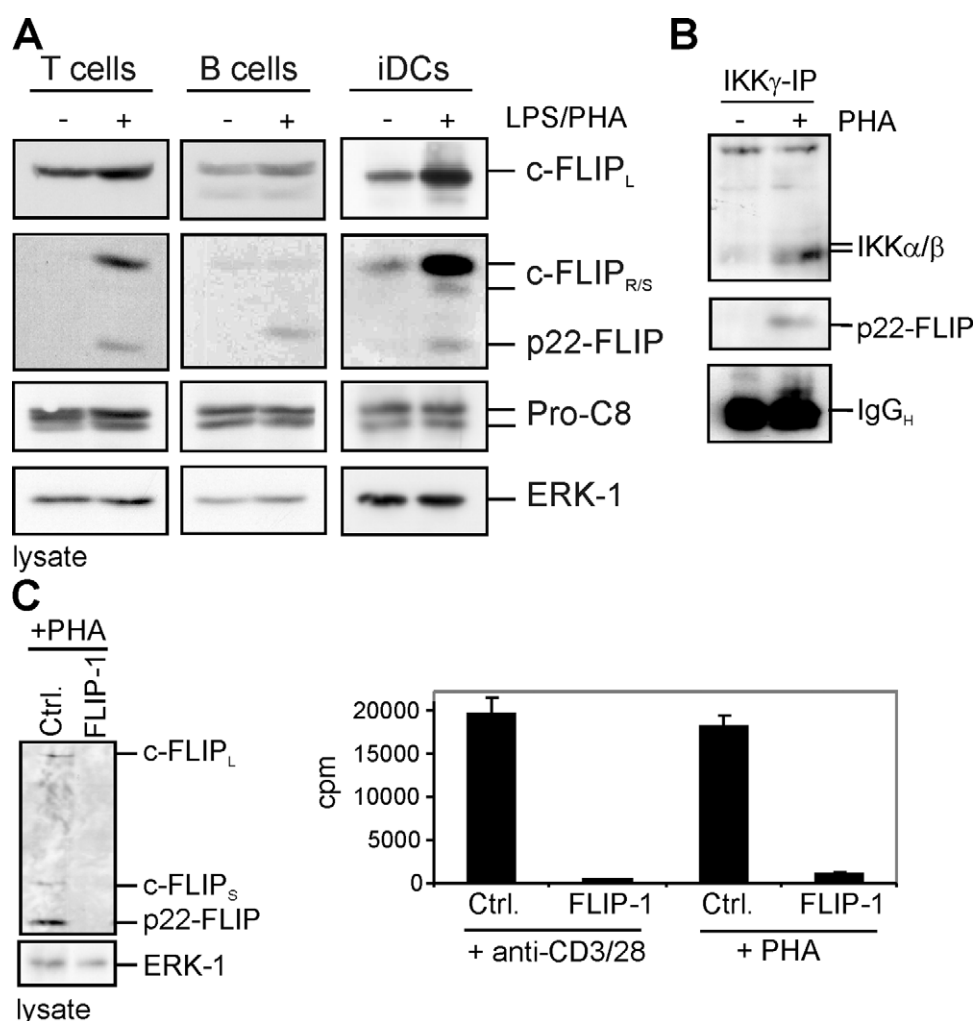


Figure 6. p22-FLIP is a key mediator of NF- κ B induction. (A) Primary human T and B cells were stimulated with 1 μ g/ml PHA. Primary immature iDCs were stimulated with 500 ng/ml LPS. Western blot analysis was performed using the anti-FLIP mAb NF6, the anti-caspase-8 mAb C15, and the anti-ERK1 mAb. (B) 10^7 primary human T cells were stimulated with 1 μ g/ml PHA for 12 h and lysed, and anti-IKK γ immunoprecipitation was performed. Western blot analysis was performed using anti-IKK α/β antibody and anti-FLIP mAb NF6. (C) Primary human T cells

were transiently transfected with double-stranded siRNA oligonucleotides comprising a FLIP-specific sequence (FLIP-1) or a nonspecific sequence (Ctrl.). 48 h after transfection, cells were stimulated with 1 μ g/ml PHA for 3 d (right) or 0.5 μ g/ml anti-CD3/28 for 24 h (left) or 3 d (right). After incubation, cells were lysed and Western blot analysis was performed using the anti-FLIP mAb NF6 (left), or the proliferation was measured after incorporation of tritiated thymidine (3 H)TdR during the last 18 h.

silencing of c-FLIP led to a complete stop of cell proliferation. Thus, we show that the absence of c-FLIP and, consequently, p22-FLIP led to severe defects in T cell proliferation.

DISCUSSION

c-FLIP proteins were demonstrated to induce NF- κ B activation (12–14). However, the exact underlying mechanism of this process has not been established yet. In this study, we identified a new mechanism of c-FLIP-mediated NF- κ B activation and showed that NF- κ B activation requires c-FLIP processing into the NH₂-terminal DED-containing fragment, p22-FLIP (Fig. 7). p22-FLIP is generated by procaspase-8 cleavage of both c-FLIP isoforms, c-FLIP_L and c-FLIP_S. Furthermore, p22-FLIP is a strong activator of NF- κ B, acting directly at the level of the IKK complex by binding to IKK γ . In addition to its role as an activator of NF- κ B, p22-FLIP can block apoptosis by directly binding to the DISC.

p22-FLIP is generated by cleavage of c-FLIP_{L/S} at Asp¹⁹⁸. The resulting NH₂-terminal fragment, p22-FLIP, contains two tandem DEDs and has a high structural homology to v-FLIP of the Kaposi's sarcoma-associated herpesvirus and other v-FLIPs (14) that are also characterized by the presence of two DEDs followed by a short COOH terminus.

We argue that p22-FLIP is the final cleavage product of c-FLIP that serves as a mediator of NF- κ B activation. This result is in contrast to previous studies, where it was suggested that p43-FLIP is a cleavage product of c-FLIP that mediates NF- κ B activation (18). We have clearly shown that upon zVAD-fmk treatment, p43-FLIP-mediated activation of NF- κ B is decreased, whereas the p22-FLIP-mediated NF- κ B response remained unaltered. These results indicate that p43-FLIP requires further processing.

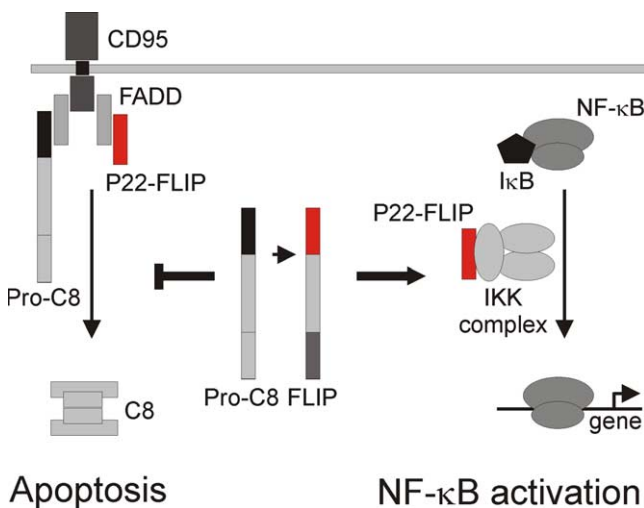


Figure 7. The dual function of p22-FLIP in the cell. p22-FLIP induces NF- κ B by interacting with IKK γ in the IKK complex (right side). In addition, p22-FLIP can block death receptor-mediated apoptosis by binding to the DISC via DED interactions and inhibiting procaspase-8 activation (left side).

Importantly, our study demonstrates a new NF- κ B-activating pathway initiated by procaspase-8. We show that independently of death receptor stimulation, procaspase-8 generates the p22-FLIP cleavage product, which leads to the induction of NF- κ B (Fig. 7). Recently, procaspase-8 was reported to play a prominent role in NF- κ B induction via its involvement in the MALT1-Bcl-10 adaptor complex that is formed upon TCR stimulation (26). Our findings further elucidate the involvement of procaspase-8 in NF- κ B induction.

Interestingly, we could also show that the active mature caspase-8 heterotetramer p10₂-p18₂ cleaves c-FLIP_L in vitro to p43-FLIP and p12-FLIP, but not to p22-FLIP. Thus, we observe that procaspase-8 and caspase-8 heterotetramer cleave c-FLIP in two different ways. This is consistent with reports upon different substrate specificities of the caspase-8 proform and active caspase-8 (7). Of note, our study is the first one demonstrating two different caspase-8 specificities with respect to c-FLIP cleavage.

In addition, we have observed in primary cells that upon increase of the ratio of c-FLIP to procaspase-8, the amount of p22-FLIP was substantially increased. It is likely that procaspase-8 constitutively cleaves c-FLIP to p22-FLIP, forming dimers with c-FLIP. The formation of such dimers between procaspase-8 and c-FLIP in the cytosol was described previously (6, 23). Thus, the ratio of procaspase-8 to c-FLIP in a particular cell type would be the crucial factor defining the amount of generated p22-FLIP and, correspondingly, the potential to induce NF- κ B.

Our findings provide a molecular mechanism for how c-FLIP and procaspase-8 contribute to the activation and proliferation of primary lymphocytes. It was reported that caspase activity is essential for T cell activation, as it was shown in experiments with caspase inhibitors (27, 28). Analysis of caspase-8 and c-FLIP conditional knockout mice has demonstrated that those mice show severe defects in T cell activation and proliferation (29). Caspase-8 was reported to be essential for antigen-induced NF- κ B activation in T, B, and NK cells (26, 30, 31). We show that upon silencing of c-FLIP in primary cells, the proliferation of these cells is impaired. This demonstrates the importance of our new p22-FLIP-mediated NF- κ B-activating pathway in primary lymphocytes.

In conclusion, we described a new NF- κ B-activating pathway, which is mediated by two well-described apoptotic DED-containing proteins: procaspase-8 and c-FLIP via their cleavage product, p22-FLIP. The balance between DED-containing proteins may provide sensitive signaling check points that cells use for signaling cross-talk and switching between apoptosis-resistant and -sensitive phenotypes and, thus, between life and death.

MATERIALS AND METHODS

Cell lines. The T cell lines HUT78, CEM, H9, Jurkat (clone A3), and Jurkat (clone J16); the B lymphoblastoid cell lines SKW6.4, Raji, and BJAB; and the pre-B cell line Boe^h were maintained in RPMI 1640, 10 mM Hepes, 50 μ g/ml gentamycin, and 10% fetal calf serum (all from Life Technologies) in 5% CO₂.

Antibodies and reagents. Anti-FADD mAb (IgG1) was purchased from Transduction Laboratories. Anti-FLAG mAb was purchased from Sigma-Aldrich. Anti-CD95 polyclonal antibody C20 was purchased from Santa Cruz Biotechnology, Inc. Anti-caspase-8 mAb C15 (mouse IgG2b) recognizes the p18 subunit of caspase-8 (32). Anti-IKK α / β and anti-IKK γ antibodies were purchased from Santa Cruz Biotechnology, Inc., and anti-ERK1 mAb was purchased from BD Biosciences. Anti-APO-1 is an agonistic mAb (IgG3, κ) recognizing an epitope on the extracellular part of CD95 (APO-1/Fas; reference 33). FLAG-TRAIL was obtained from H. Walczak (DKFZ, Heidelberg, Germany). Horseradish peroxidase-conjugated goat anti-mouse IgG1, IgG2a, and IgG2b were from SouthernBiotech. All chemicals used were of analytical grade and purchased from Merck or Sigma-Aldrich. Plasmids encoding c-FLIP_L and c-FLIP_S have been described previously (2). The plasmid encoding FLAG-c-FLIP_L has also been described (3). NF- κ B reporter plasmid was provided by M. Li-Weber (DKFZ, Heidelberg, Germany). Constructs of FLAG-IKK α , FLAG-IKK β , DN-FLAG-IKK α (K44A), and DN-FLAG-IKK β (K44A) were provided by H. Nakano (Jun-endo University, Tokyo, Japan).

EMSA. Soluble nuclear proteins were prepared and used for EMSA as described previously (34). For each reaction, 10–20 fmol of ³²P-labeled oligonucleotides comprising an NF- κ B binding site (5'-TCAGAGGGAC-TTCCGAGAGGCG-3') or NF-Y binding site (5'-CACCTTTTAACC-AATCAGAAAAT-3') were used.

Cloning of p22-FLIP and D198A-FLAG-c-FLIP_L. p22-FLIP was cloned into the pEF4 expression vector (Invitrogen) using the PCR and the following primers: sense (encoding the KpnI restriction site): 5'-ggggtaccct-ATGTCTGCTGAAGTCATCC-3' and antisense (encoding the XbaI restriction site): 5'-gctctagacctaATCCTTGAGACTCTTTTGG-3'.

The D198A-FLAG-c-FLIP_L mutant was cloned via overlap-PCR into the pEF4 expression vector (Invitrogen). The NH₂-terminal part was amplified using the following: forward primer 1: 5'-ggggtaccctATGGACTACA-AGGACGACGACAAGGGATGTCTGCTGAAGTCATCCATCAGG-3'; reverse primer 1: 5'-CCTGAAAGTTATTTGAAGGTGCCTTGAGACTCTTTTGG-3'. The COOH-terminal part was amplified using the following: reverse primer 2: 5'-gctctagacCTATGTGTAGGAGAGGATAAG-3'; forward primer 2: 5'-CCAAAAGAGTCTCAAGGCACCTTCAAATAA-CTTCAGG-3'. The overlap-PCR was performed using the forward and reverse primer 2.

CD95 DISC analysis. Composition of the CD95 DISC was determined as follows. 5×10^7 cells were treated either with 1 μ g/ml anti-APO-1 (IgG3) for 5 min at 37°C or left untreated, washed twice in $1 \times$ PBS, and lysed in lysis buffer (30 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [Sigma-Aldrich], protease inhibitor cocktail [Roche], 1% Triton X-100 [Serva], and 10% glycerol). If pretreated with zVAD-fmk, cells were preincubated for 30 min at 37°C with the indicated concentrations of zVAD-fmk before stimulation. CD95 was immunoprecipitated with anti-APO-1 and protein A-Sepharose beads (Sigma-Aldrich) overnight at 4°C. Beads were washed five times with 20 volumes of lysis buffer. Immunoprecipitates were used for in vitro assay or analyzed using SDS-PAGE gels (35). Gels were transferred to Hybond nitrocellulose membrane (GE Healthcare), blocked with 5% nonfat dry milk in PBS/Tween (PBS plus 0.05% Tween 20) for 1 h, washed with PBS/Tween, and incubated with the primary antibody in PBS/Tween overnight at 4°C. Blots were developed with a chemoluminescence method according to the manufacturer's protocol (PerkinElmer).

Immunoprecipitations. For c-FLIP immunoprecipitation, 5×10^7 cells were lysed in a volume of 1 ml for 30 min at 0°C, followed by the addition of 100 μ l NF6 hybridoma supernatant together with 30 μ l protein A-Sepharose. For FLAG immunoprecipitation, 2×10^6 cells were transfected using the calcium phosphate method 1 d before lysis. Immunoprecipitation was performed by using 4 μ g anti-FLAG mAb together with 30 μ l protein

A-Sepharose. For IKK γ immunoprecipitation, 10^7 primary human T cells were lysed with or without stimulation, and the immunoprecipitation was performed by using 2 μ g anti-IKK γ mAb together with 30 μ l protein A-Sepharose. Immunoprecipitations were performed for 1 h at room temperature or overnight at 4°C. Beads were then washed five times with 20 volumes of lysis buffer and subjected to Western blot analysis as described above.

In vitro c-FLIP cleavage assays. Lysates of the indicated cell lines were prepared as described above and incubated with in vitro-translated [³⁵S]-labeled c-FLIP_L, FLAG-c-FLIP_L, D198A-FLAG-c-FLIP_L, or c-FLIP_S (TNT, T7-coupled reticulocyte lysate system; Promega) overnight at 4°C (36). The procaspase-8 cleavage assay was performed as follows: 5×10^7 HUT78 cells were lysed, and procaspase-8 was immunoprecipitated using 100 μ l anti-caspase-8 C15 hybridoma together with 30 μ l protein A-Sepharose. Beads with bound procaspase-8 were incubated in reaction buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM dithiothreitol, and 10% sucrose) for 1 h at 37°C together with in vitro-translated c-FLIP_L. The recombinant caspase-8 cleavage assay was performed as follows: 5 or 50 ng/ml recombinant caspase-8 was incubated in reaction buffer for 1 h at 37°C together with in vitro-translated c-FLIP_L. Reactions were separated on 12% SDS-PAGE gels, blotted, and subjected to autoradiography.

Transfection of BJAB and Boe^R cells. Stable transfection of BJAB as well as transient transfection of Boe^R cells was performed using expression vectors or the empty vector by electroporation (950 μ F, 200 V). Selection pressure was added 48 h after transfection (100 μ g/ml zeocin) for 2 wk (BJAB cells) or 10 d (Boe^R cells). Expression was controlled by Western blot using anti-FLIP mAb NF6. The p22-FLIP-expressing BJABs as well as the empty vector-transfected control cells were subcloned.

Cytotoxicity assay. To assay apoptosis, 5×10^5 cells were incubated in 48-well plates with or without the indicated amounts of anti-APO-1, LZ-CD95L, or FLAG-TRAIL for 16 h at 37°C. Cell death was measured by FSC/SSC via flow cytometry, and specific cell death was calculated as follows: (percentage of experimental cell death – percentage of spontaneous cell death)/(100 – percentage of spontaneous cell death) \times 100.

NF- κ B activation assay. The day before transfection, 24-well titer plates were seeded with 0.5×10^5 293T cells. The cells were transfected using the calcium phosphate method with various expression vectors together with 500 ng of the NF- κ B-driven luciferase reporter plasmid. Cells were washed with PBS 16 h after transfection and lysed for 20 min at room temperature in 50 μ l lysis buffer (passive lysis buffer; Promega), followed by centrifugation (10,000 g) for 20 min to sediment insoluble materials. A total of 5 μ l of cell lysates was mixed with 50 μ l of the luciferase assay mixture (470 μ M Beetle Luciferin [Promega], 1.07 mM (MgCO₃)₄Mg(OH)₂ \times 5 H₂O, 20 mM N-Tris-(hydroxymethyl)-methylglycine, 2.67 mM MgSO₄, 100 μ M EDTA, 33.3 mM DTT, 270 μ M CoA(OAc), and 530 μ M ATP), and relative light units were measured with a Berthold duoluminomat (Bad Wildbad).

Preparation and activation of primary human lymphocytes and DCs. Human peripheral T and B cells were prepared as described previously (37). For activation, resting primary human T cells (day 0) were cultured at 2×10^6 cells/ml with 1 μ g/ml PHA for 16 h (day 1), and primary human B cells (day 0) were cultured at 2×10^6 cells/ml with 2 μ g/ml PHA. After preparing lymphocytes, the primary human monocytes were isolated using cell adhesion onto cell culture flasks. Leukocytes were resuspended in 20–30 ml RPMI 1640 with 10% FCS, and 2-ml aliquots were seeded into six-well titer plates. After incubation for 1 h, adherent cells were washed with PBS. Monocytes were differentiated into immature DCs by adding 1% human AB serum, 1% donor plasma, 1,000 U/ml GM-CSF (Schering), and 500 U/ml IL-4 (Immunotools) for 3 d. Cytokines were renewed after 3 d for an additional 3 d, and immature DCs were stimulated with 500 ng/ml LPS for 16 h.

CHX experiments. For CHX treatments, cells were incubated with 10 $\mu\text{g/ml}$ CHX for the indicated periods of time. For assaying apoptosis in a cytotoxicity assay, 10^6 cells were pretreated with 10 $\mu\text{g/ml}$ CHX for 4 h, washed, and incubated with the indicated concentrations of 1 $\mu\text{g/ml}$ anti-APO-1 or 50 ng/ml FLAG-TRAIL for 16 h at 37°C in 24-well plates. Cell death was measured by FSC/SSC via flow cytometry, and specific cell death was calculated as follows: (percentage of experimental cell death – percentage of spontaneous cell death)/(100 – percentage of spontaneous cell death) \times 100.

Annexin V and propidium iodide staining. To detect phosphatidylserine exposure by flow cytometry, the T and B cell lines were washed once with PBS, incubated for 10 min on ice in 400 μl binding buffer (2.5 mM Hepes–NaOH, pH 7.4, 35 mM NaCl, and 0.625 mM CaCl_2) with 1 μl annexin V–FITC (Qbiogene) or 10 $\mu\text{g/ml}$ propidium iodide (Invitrogen), and analyzed via flow cytometry.

siRNA-mediated knockdown of c-FLIP and proliferation assays. Primary human T cells were transfected by HiPerfect (QIAGEN) with a negative control or siRNA oligonucleotids specific for human CFLAR (Hs_CFLAR_1 HP; QIAGEN). For the lipofection, 0.2 μg (75 nM) siRNA was used, and transfected cells were rested for 48 h before further analysis. For proliferation assays, 10^5 cells were seeded into a 96-well titer plate and stimulated with either 1 $\mu\text{g/ml}$ PHA or 0.5 $\mu\text{g/ml}$ each of anti-CD3/CD28 for up to 4 d. Proliferation was measured with a scintillation counter after tritiated thymidine (^3H]TdR) incorporation during the final 15–18 h of the culture.

Online supplemental material. Fig. S1 shows the living status of tumor cell lines used, and Fig. S2 shows that the resistance of Boe^R cells toward CD95- or TRAIL-induced apoptosis is mediated by c-FLIP. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20051556/DC1>.

We thank Ralf Marienfeld for providing the FLAG-IKK α plasmid, Hiroyasu Nakano for the FLAG-IKK α and FLAG-IKK β plasmids, and Peter Angel for a MEKK1 plasmid. We thank Tobias Haas and Henning Walczak for providing FLAG-TRAIL and Min Li-Weber for advice. We thank Heiko Weyd for isolation and differentiation of primary human immature DCs. We would like to thank Sherry Sundell for critically reading the manuscript; Karsten Gülow, Dagmar Riess, and Rüdiger Arnold for their critical comments; Heidi Sauter for excellent secretarial work; and the Wilhelm Sander Stiftung, SFB 405, and Tumorzentrum Heidelberg/Mannheim for financial support.

The authors have no conflicting financial interests.

Submitted: 1 August 2005

Accepted: 6 April 2006

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