

Smac Mimetic Bypasses Apoptosis Resistance in FADD- or Caspase-8–Deficient Cells by Priming for Tumor Necrosis Factor α –Induced Necroptosis^{1,2}

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Abstract

Searching for new strategies to bypass apoptosis resistance, we investigated the potential of the Smac mimetic BV6 in Jurkat leukemia cells deficient in key molecules of the death receptor pathway. Here, we demonstrate for the first time that Smac mimetic primes apoptosis-resistant, FADD- or caspase-8–deficient leukemia cells for TNF α -induced necroptosis in a synergistic manner. In contrast to TNF α , Smac mimetic significantly enhances CD95-induced apoptosis in wild-type but not in FADD-deficient cells. Interestingly, Smac mimetic- and TNF α -mediated cell death occurs without characteristic features of apoptosis (i.e., caspase activation, DNA fragmentation) in FADD-deficient cells. By comparison, Smac mimetic and TNF α trigger activation of caspase-8, -9, and -3 and DNA fragmentation in wild-type cells. Consistently, the caspase inhibitor zVAD.fmk fails to block Smac mimetic- and TNF α -triggered cell death in FADD- or caspase-8–deficient cells, while it confers protection in wild-type cells. By comparison, necrostatin-1, an RIP1 kinase inhibitor, abolishes Smac mimetic- and TNF α -induced cell death in FADD- or caspase-8–deficient. Thus, Smac mimetic enhances TNF α -induced cell death in leukemia cells via two distinct pathways in a context-dependent manner: it primes apoptosis-resistant cells lacking FADD or caspase-8 to TNF α -induced, RIP1-dependent and caspase-independent necroptosis, whereas it sensitizes apoptosis-proficient cells to TNF α -mediated, caspase-dependent apoptosis. These findings have important implications for the therapeutic exploitation of necroptosis as an alternative cell death program to overcome apoptosis resistance.

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Abbreviations: BIR, baculovirus IAP repeat; cIAP1, cellular inhibitor of apoptosis 1; DIABLO, direct IAP binding protein with low pI; IAP, inhibitor of apoptosis; Smac, second mitochondria-derived activator of caspase; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; XIAP, X-linked inhibitor of apoptosis; zVAD.fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

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Introduction

Apoptosis is a form of programmed cell death that typically leads to caspase activation as a common effector mechanism and may proceed via two major routes, namely, the death receptor (extrinsic) and the mitochondrial (intrinsic) pathways [1]. Stimulation of death receptors of the tumor necrosis factor (TNF) receptor superfamily on the cell surface, including CD95 (APO-1/Fas), TNF-related apoptosis-inducing ligand (TRAIL) receptors, or TNF receptor 1 (TNFR1), triggers caspase-8 activation in a multimeric complex including the adaptor protein FADD, resulting in subsequent cleavage of downstream effector caspases such as caspase-3 [2]. In the mitochondrial pathway, cytochrome *c* and second mitochondria-derived activator of caspase (Smac)/direct IAP binding protein with low *pI* (DIABLO) are released from mitochondria into the cytosol, which in turn triggers caspase-3 activation via the apoptosome complex and via binding to X-linked inhibitor of apoptosis (XIAP), respectively [3].

While necrosis has previously been viewed as an uncontrolled, accidental mode of cell death, it is now well appreciated that necroptosis (programmed necrosis) is a regulated, caspase-independent form of cell death that occurs when caspase activation is inhibited or absent [4]. The serine/threonine kinase RIP1 has been identified as a critical mediator of TNF α -initiated necroptosis that becomes phosphorylated on the induction of necroptosis and interacts with RIP3 to form the necrosome complex [5]. In addition, RIP1 is involved in the regulation of apoptosis after death receptor ligation [6,7], implying that apoptotic and necrotic pathways share some common components.

Inhibitor of apoptosis (IAP) proteins are a family of eight proteins, which, per definition, all possess a baculovirus IAP repeat (BIR) domain that mediates the binding and inhibition of caspases [8]. By comparison, only some IAP proteins, namely, XIAP, cellular inhibitor of apoptosis 1 and 2 (cIAP1 and cIAP2), also harbor a RING domain with E3 ubiquitin ligase activity that mediates (auto)ubiquitination and proteasomal degradation [8]. XIAP is well characterized for its anti-apoptotic activity through binding to and inhibiting caspase-9 and -3/-7 via its BIR3 domain and the linker region preceding BIR2 domain, respectively [9]. Recently, cIAP1 and cIAP2 were identified as E3 ubiquitin ligases for the serine/threonine kinase RIP1 that polyubiquitinate RIP1 via K63-linked chains [10,11]. Depending on its ubiquitination status, RIP1 either promotes survival by stimulating nuclear factor κ B activation once it is ubiquitinated or contributes to cell death in its deubiquitinated form, which allows its interaction with key components of death receptor signaling such as FADD and caspase-8 [5]. Smac mimetics have been shown to trigger autoubiquitination and proteasomal degradation of IAP proteins with a RING domain including cIAP1 and cIAP2 [12–14] and, thus, can indirectly favor deubiquitination of RIP1 [10].

Resistance to apoptosis represents a characteristic feature of human cancers and represents a major unsolved obstacle in clinical oncology [15]. IAP proteins are expressed at high levels in many malignancies including leukemia and contribute to evasion of apoptosis [16]. We previously reported that IAP antagonists sensitize cancer cells to apoptosis and overcome Bcl-2-imposed resistance to apoptosis by switching type II cells that depend on the mitochondrial contribution to TRAIL-induced apoptosis into type I cells, which signal to apoptosis irrespective of high Bcl-2 levels [17–19]. Searching for novel strategies to bypass cancer cell resistance to apoptosis, we investigated in the present study whether Smac mimetics can also overcome defects in the death receptor pathway of apoptosis.

Materials and Methods

Cell Culture

Human wild-type (WT) Jurkat T-ALL, FADD-deficient, caspase-8-deficient, or caspase-8-deficient and Bcl-2-overexpressing variants of human Jurkat clones deficient in FADD, caspase-8 or caspase-8-deficient, and Bcl-2-overexpressing cells were kind gifts from Dr J. Blenis or Dr S. Nagata [20–22]. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 1 mM L-glutamine, 25 mM HEPES buffer, 50 U/ml penicillin, and 50 μ g/ml streptomycin. Leukemia blasts were derived from children treated for ALL at the Ludwig Maximilians University's Children's Hospital after informed consent was obtained in accordance with the Declaration of Helsinki. The study was approved by the local ethical committee. Samples were obtained by bone marrow puncture at initial diagnosis before the onset of therapy, isolated using Ficoll Isopaque (Amersham Bioscience, Freiburg, Germany) and stimulated directly after isolation. Smac mimetic BV6 is a bivalent IAP antagonist compound [13]. Caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk) was obtained from Bachem (Heidelberg, Germany) and TNF α and necrostatin 1 (Nec-1) were purchased from Biomol (Hamburg, Germany). Enbrel was kindly provided by Pfizer. All chemicals were purchased by Sigma (Steinheim, Germany) unless indicated otherwise.

Western Blot Analysis and Immunoprecipitation

Western blot analysis was performed as described previously [23] using the following antibodies: mouse anti-caspase-8 (1:1000) from Alexis Biochemicals (Eggenstein, Switzerland); rabbit anti-caspase-3 (1:1000) from Cell Signaling (Beverly, MA); rabbit anti-caspase-9 (1:1000), mouse anti-XIAP, and mouse anti-RIP1 (1:1000) from BD Biosciences (Heidelberg, Germany); goat anti-cIAP1 (1:1000) and rabbit anti-survivin (1:1000) from R&D Systems, Inc (Wiesbaden, Germany); and rabbit anti-cIAP2 (1:1000) from Epitomics (Burlingame, CA) or mouse anti- β -actin as loading control (1:5000) from Sigma followed by goat-antimouse, goat-antirabbit, or donkey-antigoat IgG conjugated to horseradish peroxidase (1:5000) from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence was used for detection (Amersham Bioscience). Immunoprecipitation was performed as described previously [10].

Determination of Cell Death

Apoptosis was determined by fluorescence-activated cell sorting analysis (FACSscan; BD Biosciences) of DNA fragmentation of propidium iodide-stained nuclei or by forward side scatter analysis as described previously [23]. Briefly, cells were harvested, washed with PBS, and resuspended in hypotonic buffer containing 50 μ g/ml propidium iodide, 0.1% sodium citrate, and 0.4% Triton X-100. The amount of hypodiploid DNA (sub-G₁ fraction) was determined by FACS analysis. For microscopy of apoptotic cells, cells were collected by centrifugation (5 minutes at 600 rpm) and stained with 0.02% diamidine phenylindole dihydrochloride (Roche Diagnostics, Mannheim, Germany) in methanol for 5 minutes to stain nuclear DNA. Pictures were taken using an Olympus AX70 "Provis" microscope (Hamburg, Germany). Necrotic cell death was determined by measuring loss of plasma membrane integrity by propidium iodide-emitted fluorescence and flow cytometry. Briefly, cells were harvested, washed with

PBS, and resuspended in propidium iodide-containing PBS (1 μ g/ml propidium iodide).

Statistical Analysis

Statistical significance was assessed by Student's *t* test (two-tailed distribution, two-sample, unequal variance). The interaction between Smac mimetic and TNF α was analyzed by the combination index (CI) method using CalcuSyn software (Biosoft, Cambridge, UK). Combina-

tion index less than 0.9 indicates synergism; 0.9 to 1.1, additivity; and greater than 1.1, antagonism.

Results

On the basis of our recent findings that IAP inhibitors bypass Bcl-2-conferred resistance to TRAIL-induced apoptosis [17,18], we investigated in the present study whether the Smac mimetic BV6 that neutralizes XIAP, cIAP1, and cIAP2 can overcome apoptosis resistance owing to defects in the death receptor pathway.

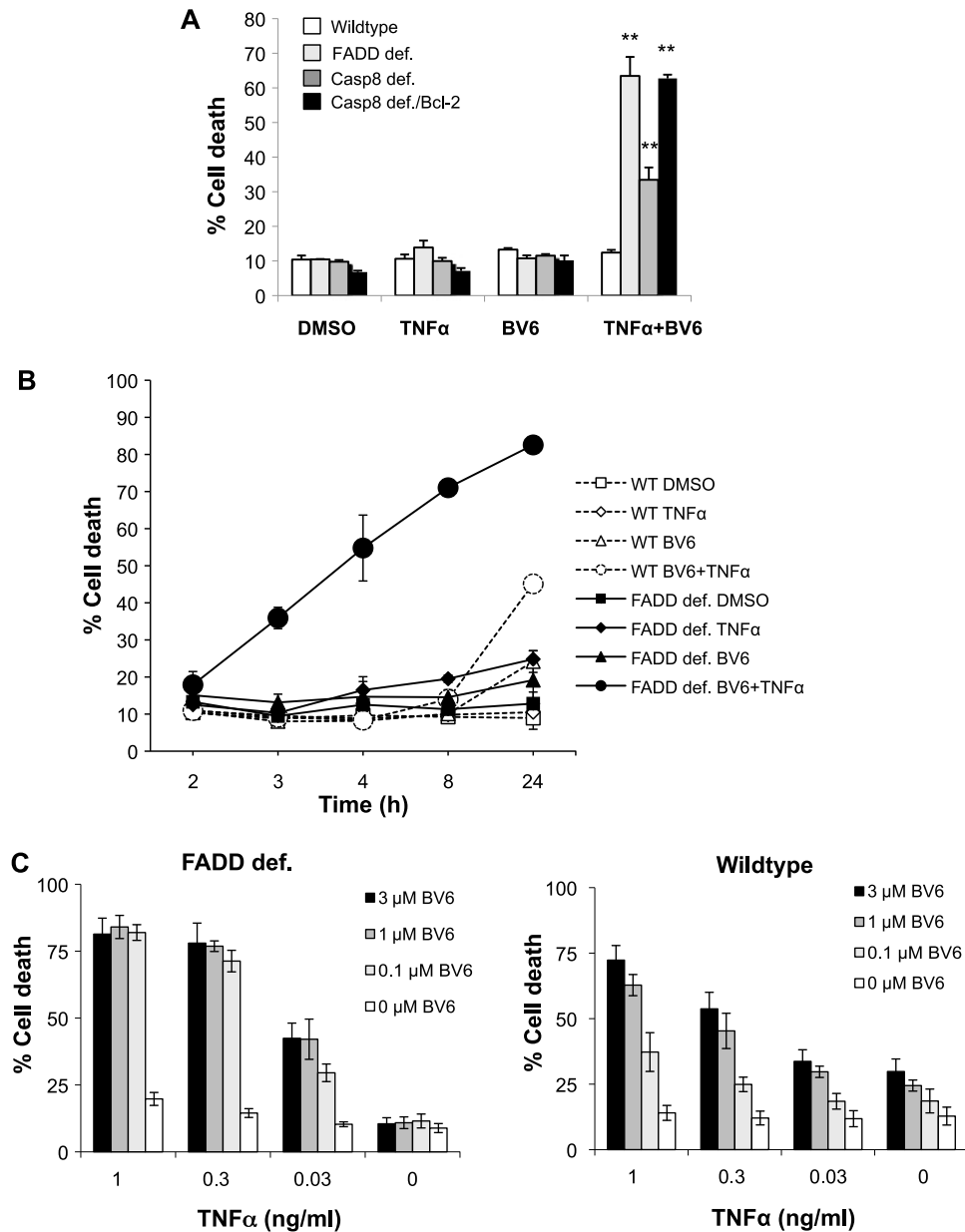


Figure 1. Smac mimetic sensitizes FADD- or caspase-8-deficient cells for TNF α -induced cell death. (A) WT (white bars), FADD-deficient (light gray bars), caspase-8-deficient (dark gray bars), or caspase-8-deficient and Bcl-2-overexpressing (black bars) Jurkat cells were pretreated with BV6 (1 μ M, 2 hours) before being stimulated with 1 ng/ml TNF α for 4 hours. (B) WT (closed symbols) or FADD-deficient (open symbols) Jurkat cells were pretreated with BV6 (1 μ M, 2 hours) before being stimulated with 1 ng/ml TNF α for indicated times. (C) FADD-deficient (left panel) or WT (right panel) Jurkat cells were pretreated for 2 hours with indicated concentrations of BV6 (white bars indicate 0 μ M; light gray bars, 0.1 μ M; dark gray bars, 1 μ M; black bars, 3 μ M) before adding the indicated concentrations of TNF α for 24 hours. (D) Primary leukemic blasts from three different children with ALL before the onset of chemotherapy were treated with 100 ng/ml TNF α and/or 100 nM BV6 or dimethyl sulfoxide (DMSO). In A to C, cell death was analyzed by PI staining; and in D, by forward side scatter analysis. In A to C, data are the mean and SD of at least three independent experiments performed in triplicate. ****P** < .001 comparing cells treated with BV6 and TNF α compared with DMSO-treated cells. In D, data are the mean of one experiment performed in duplicate.

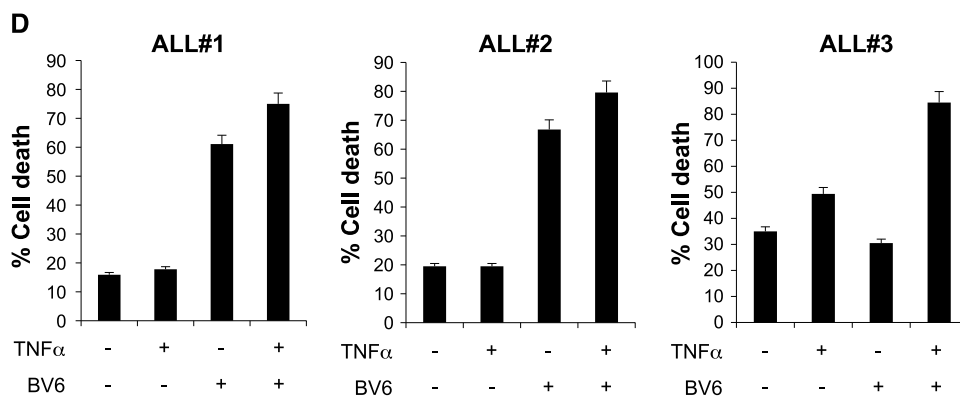


Figure 1. (continued).

Smac Mimetic Sensitizes Apoptosis-Resistant Leukemia Cells for TNF α -Induced Cell Death

As models for defective death receptor-mediated apoptosis, we used the T-ALL leukemia cell line Jurkat and several variants with deficiencies in key molecules of the death receptor pathway, namely, FADD-deficient cells, caspase-8-deficient cells and caspase-8-deficient cells that also overexpress Bcl-2.

To investigate whether the antagonism of IAP proteins by Smac mimetic sensitizes leukemia cells for cell death, we preincubated cells for 2 hours with the Smac mimetic to downregulate cIAP1/2 expression levels (Figure W1), followed by stimulation with TNF α . Interestingly, Smac mimetic significantly enhanced TNF α -induced cell death in FADD-deficient cells, caspase-8-deficient cells, as well as caspase-8-deficient and Bcl-2-overexpressing cells rapidly within 4 hours (Figure 1A). By comparison, WT cells did not respond to the combination treatment at this time point (Figure 1A). Importantly, kinetic analysis revealed that Smac mimetic- and TNF α -induced cell death occurred more rapidly in FADD-deficient cells compared with WT cells (Figure 1B). Dose titration studies showed that nanomolar concentrations of Smac mimetic sensitized FADD-deficient cells for TNF α -induced cell death (Figure 1C). Calculation of CI revealed that the interaction of Smac mimetic plus TNF α is strongly synergistic (Table 1). Of note, equimolar concentrations of Smac mimetic were even more effective in increasing TNF α -mediated cell death in FADD-deficient cells than in WT cells (Figure 1C, e.g., for 0.3 ng/ml TNF α and 0.1 μ M BV6). Also, TNF α as single agent triggered cell death at higher concentrations in FADD-deficient cells in a dose-dependent manner (Figure W2).

Table 1. Synergistic Induction of Cell Death by BV6 and TNF α .

TNF α (ng/ml)	BV6 (μ M)	CI
1	3	<0.1
1	1	<0.1
1	0.3	<0.1
0.3	3	0.1
0.3	1	<0.1
0.3	0.3	0.2

Combination index was calculated as described in Materials and Methods for cell death induced by combined treatment of Jurkat FADD $^{-/-}$ cells for 24 hours with indicated concentrations of BV6 and TNF α . CI values of 0.1 to 0.3 indicate strong synergism.

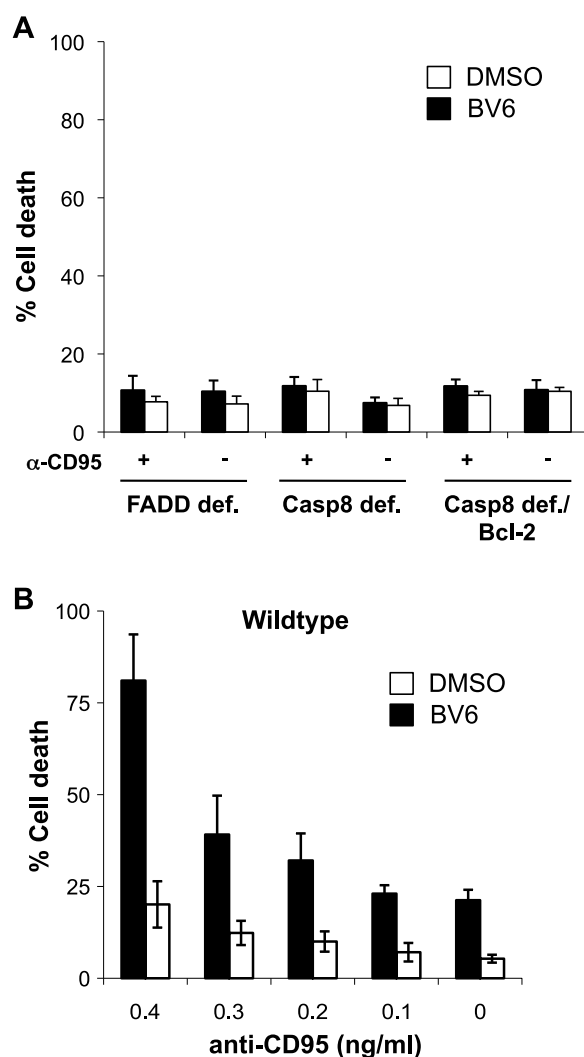


Figure 2. FADD-deficient cells remain resistant to CD95-induced cell death even in the presence of Smac mimetic. FADD-deficient, caspase-8-deficient, or caspase-8-deficient and Bcl-2-overexpressing Jurkat cells (A) or WT (B) Jurkat cells were pretreated with BV6 (1 μ M, 2 hours, black bars) before stimulating for 72 hours with 0.4 ng/ml agonistic anti-CD95 (A) or indicated concentrations of agonistic anti-CD95 (B). Cell death was analyzed by PI staining. Data are the mean and SD of three independent experiments performed in triplicate.

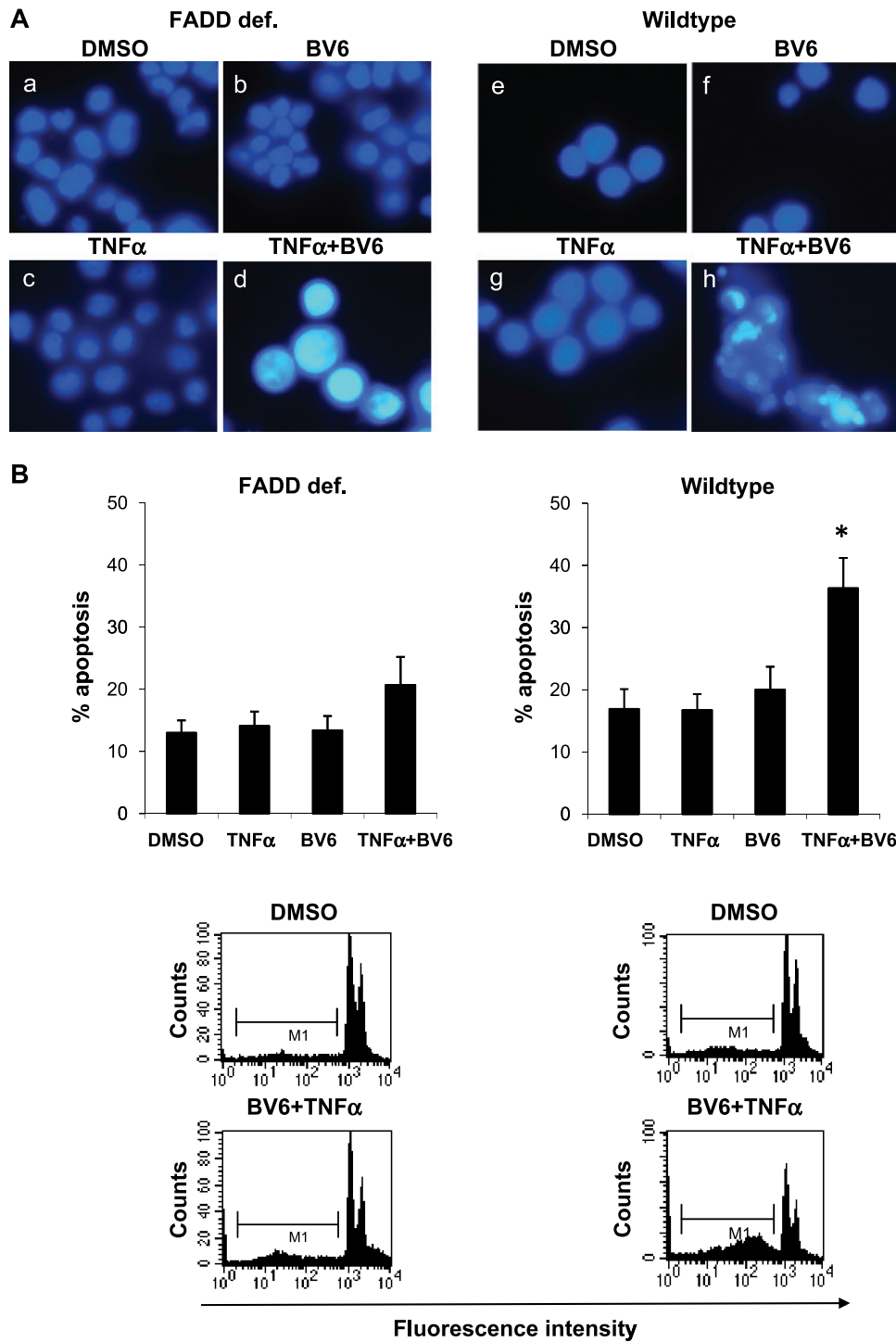


Figure 3. Lack of DNA fragmentation in Smac mimetic- and TNF α -induced cell death in FADD-deficient cells. (A) Nuclear morphology was assessed by diamidine phenylindole dihydrochloride staining and fluorescence microscopy. FADD-deficient cells (a–d): (a) DMSO, (b) treatment with 1 μ M BV6 for 8 hours, (c) treatment with 1 ng/ml TNF α for 8 hours, and (d) pretreatment with 1 μ M BV6 for 2 hours before 8 hours of stimulation with 1 ng/ml TNF α for 8 hours. WT cells (e–h): (e) DMSO, (f) treatment with 1 μ M BV6 for 8 hours, (g) treatment with 1 ng/ml TNF α for 8 hours, and (h) pretreatment with 1 μ M BV6 for 2 hours before 8 hours of stimulation with 1 ng/ml TNF α for 8 hours. Representative pictures are shown; magnification, $\times 60$. (B) FADD-deficient and WT Jurkat cells were pretreated with BV6 (1 μ M, 2 hours) before being stimulated with 1 ng/ml TNF α for 4 hours. DNA fragmentation was analyzed by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei. Quantitative analysis of three independent experiments performed in triplicate with mean and SD (upper panel) and representative histograms of flow cytometric analysis (lower panel) are shown.

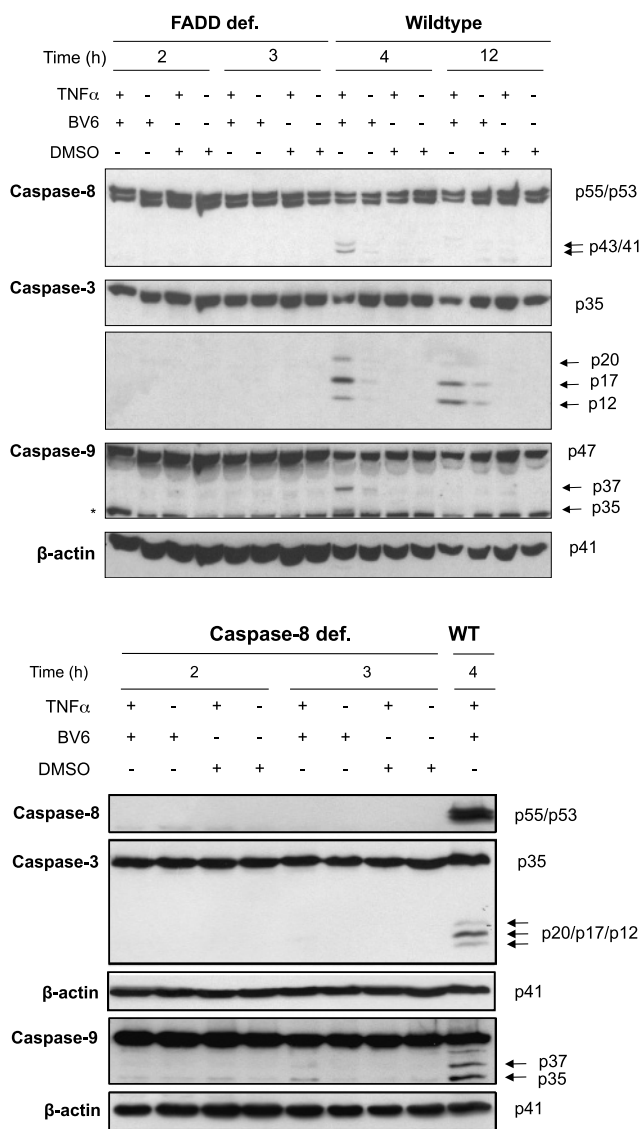


Figure 4. Lack of caspase activation in Smac mimetic- and TNF α -induced cell death in FADD- or caspase-8-deficient cells. FADD- or caspase-8-deficient and WT Jurkat cells were pretreated with BV6 (1 μ M, 2 hours) before being stimulated with 1 ng/ml TNF α for indicated time points. Caspase activation was analyzed by Western blot. Cleavage fragments are indicated by arrows. β -Actin served as loading control. Asterisk indicates an unspecific band. A representative experiment of two is shown.

Importantly, Smac mimetic also cooperated with TNF α to trigger cell death in primary leukemic blasts (Figure 1D), highlighting the potential clinical relevance.

To explore whether the Smac mimetic primes FADD- or caspase-8-deficient cells also to other death receptor ligands besides TNF α , we extended these experiments to agonistic anti-CD95 antibodies. In contrast to the synergism of Smac mimetic and TNF α , FADD- or caspase-8-deficient cells remained completely resistant to CD95-induced cell death even in the presence of Smac mimetic (Figure 2A). By comparison, Smac mimetic substantially enhanced CD95-induced cell death in WT Jurkat cells (Figure 2B).

Together, this set of experiments demonstrates that the Smac mimetic BV6 synergizes with TNF α to rapidly and efficiently trigger cell death

in apoptosis-resistant leukemia cells that lack essential components of the death receptor pathway such as FADD or caspase-8.

Smac Mimetic and TNF α Induce Nonapoptotic Cell Death in Apoptosis-Resistant Cells

Because we observed differences in the kinetic and the efficacy of cell death induction by Smac mimetic and TNF α between WT and FADD-deficient cells, we examined in more detail the molecular mechanisms of cell death. The analysis of nuclear morphology revealed that Smac mimetic and TNF α trigger nuclear condensation without signs of fragmentation in FADD-deficient cells, whereas fragmented nuclei, a characteristic feature for apoptotic cells, were detected in WT cells (Figure 3A). Consistently, the assessment of DNA fragmentation by FACS analysis of propidium iodide-stained nuclei in permeabilized cells showed that Smac mimetic and TNF α caused little DNA fragmentation in FADD-deficient cells compared with a marked increase in fragmented DNA in WT cells (Figure 3B). Furthermore, we determined activation of caspases as another biochemical hallmark of apoptosis. To this end, different time points were chosen for FADD-deficient and WT cells because of the more rapid induction of cell death in FADD-deficient cells (Figure 1B). Interestingly, no caspase cleavage fragments were detected on treatment with Smac mimetic and TNF α in FADD- or caspase-8-deficient cells (Figure 4). In contrast, Smac mimetic cooperated with TNF α to induce cleavage of caspase-8, -3, and -9 in WT cells (Figure 4). The slight increase in caspase-3 cleavage on treatment with BV6 alone in WT cells (Figure 4) is consistent with the slight induction of cell death by BV6 in these cells (Figure 1C). Together, this set of experiments demonstrates that Smac mimetic and TNF α cooperate to trigger typical apoptotic events such as caspase activation and DNA fragmentation in WT cells, whereas all these characteristic features of apoptosis are lacking in FADD-deficient cells. This points to a nonapoptotic mode of cell death in FADD-deficient cells on exposure to Smac mimetic and TNF α .

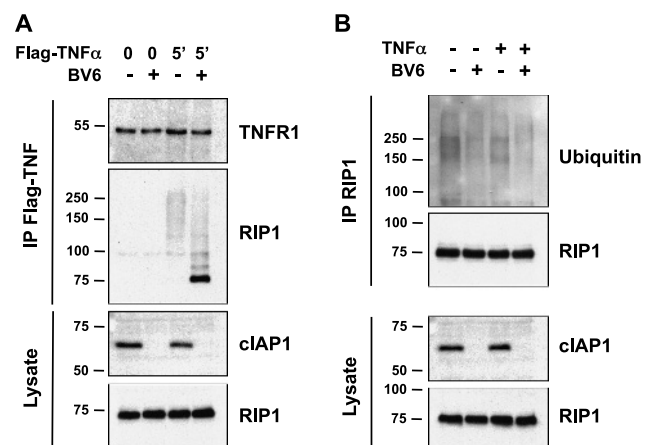


Figure 5. Constitutive and TNF α -induced RIP1 ubiquitination is reduced by Smac mimetic exposure. Jurkat cells were pretreated with BV6 (1 μ M, 2 hours) before stimulated for 5 minutes with 2 μ g/ml Flag-tagged TNF α (A) or 50 ng/ml TNF α (B). RIP1 ubiquitination was analyzed by immunoprecipitating TNFR1 using anti-Flag antibody (A) or by immunoprecipitating RIP1 followed by Western blot analysis.

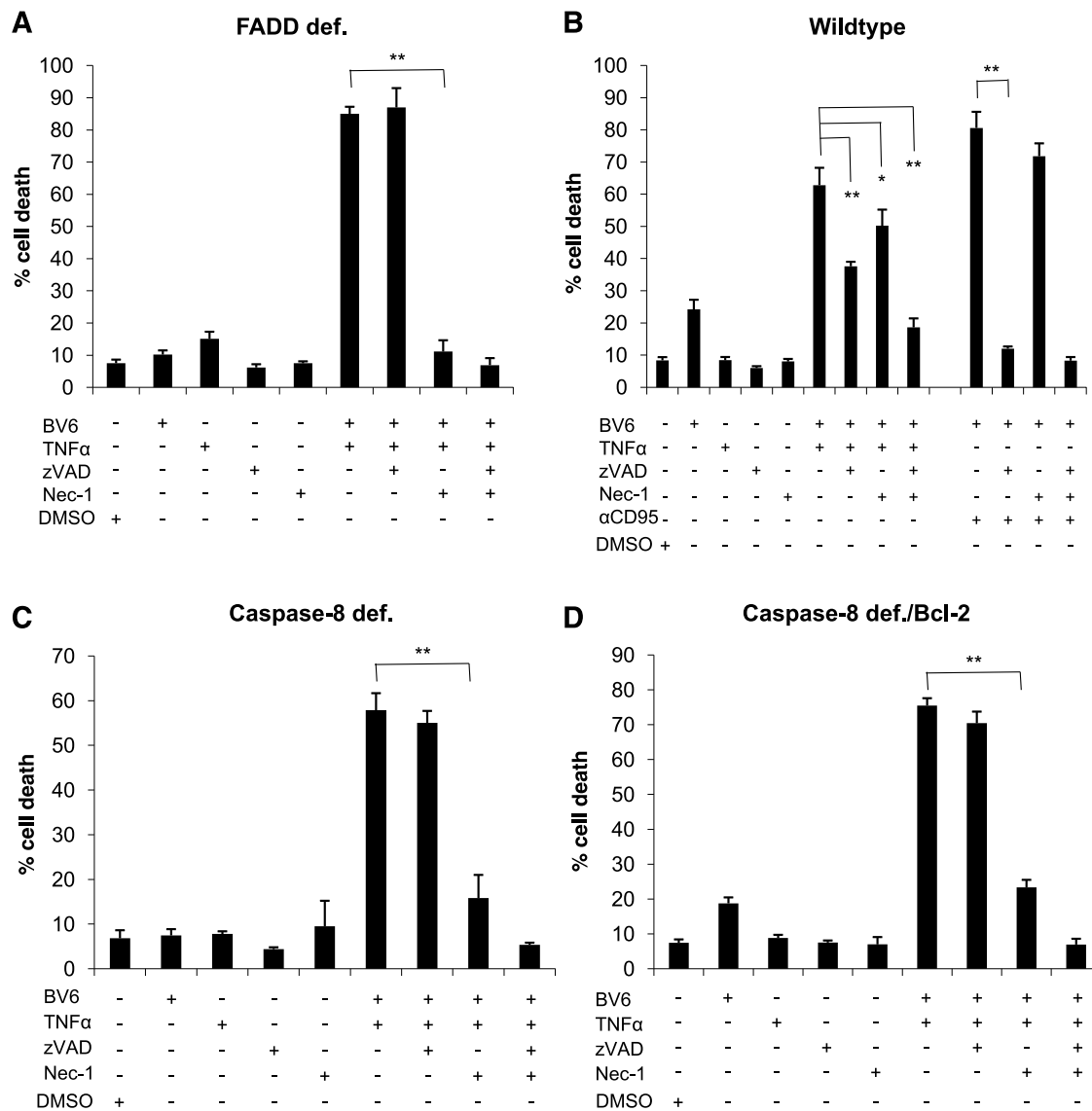


Figure 6. Requirement of RIP1 for Smac mimetic- and TNF α -induced cell death in FADD- and caspase-8-deficient cells. FADD-deficient (A) and WT (B) caspase-8-deficient (C) or caspase-8-deficient and Bcl-2-overexpressing (D) Jurkat cells were pretreated with BV6 (1 μ M, 2 hours) before being stimulated with 1 ng/ml TNF α for 24 hours in the presence or absence of 20 μ M zVAD.fmk or 30 μ M necrostatin 1. Treatment with 1 ng/ml agonistic anti-CD95 for 24 hours was used as a positive control for apoptosis. Cell death was analyzed by PI staining. Data are the mean and SD of three independent experiments performed in triplicate. * $P < .05$. ** $P < .001$.

Smac Mimetic and TNF α Act in Concert to Trigger RIP1-Dependent Necroptosis

Because cIAP1 and cIAP2 were recently identified as E3 ubiquitin ligases of RIP1 [10,11], we investigated whether pretreatment with Smac mimetic results in reduced ubiquitination of RIP1 by immunoprecipitating either TNFR1 or RIP1. Stimulation of cells with Flag-tagged TNF α followed by immunoprecipitation of TNFR1 revealed that exposure to Smac mimetic impaired the TNF α -induced ubiquitination of RIP1 (Figure 5A). Similarly, immunoprecipitation of RIP1 showed that Smac mimetic markedly reduced constitutive as well as TNF α -stimulated RIP1 ubiquitination (Figure 5B). Together, these experiments demonstrate that Smac mimetic reduces ubiquitination of RIP1.

Next, we asked whether down-regulation of cIAP1 and cIAP2 by Smac mimetic may facilitate RIP1-dependent cell death. To address this question, we used necrostatin 1, a specific small-molecule inhib-

itor of RIP1 [24,25]. Strikingly, Smac mimetic- and TNF α -triggered cell death was completely blocked by the addition of necrostatin 1 in FADD-deficient cells (Figure 6A). In contrast, necrostatin 1 caused only a minor reduction in Smac mimetic- and TNF α -induced cell death in WT cells (Figure 6B). To investigate the functional requirement of caspases for cell death induction, we used the broad-range caspase inhibitor zVAD.fmk. Notably, the addition of zVAD.fmk failed to confer protection against Smac mimetic- and TNF α -triggered cell death in FADD-deficient cells, whereas zVAD.fmk significantly reduced cell death in WT cells (Figure 6, A and B). Interestingly, this protective effect of zVAD.fmk in WT cells was further enhanced by the combined use of zVAD.fmk and necrostatin 1 (Figure 6B). This indicates that RIP1-dependent cell death is initiated in WT cells under conditions where caspase activation is inhibited. Similar to FADD-deficient cells, the addition of necrostatin 1 profoundly reduced the Smac mimetic- and TNF α -induced cell death also in caspase-8-deficient cells as well as in

caspase-8–deficient and Bcl-2–overexpressing cells, whereas zVAD.fmk failed to block cell death induction in these cells (Figure 6, C and D). Together, these findings demonstrate that Smac mimetic and TNF α trigger two distinct cell death programs in Jurkat cells in a context-dependent manner: In FADD- or caspase-8–deficient Jurkat cells, Smac mimetic and TNF α induce RIP1-dependent and caspase-independent necroptosis, whereas they trigger caspase-dependent apoptotic cell death in WT Jurkat cells.

Discussion

In the present study, we identify a novel mechanism of how Smac mimetics can bypass apoptosis resistance of leukemia cells by demonstrating that the Smac mimetic BV6 promotes TNF α -induced necroptosis as an alternative cell death program in apoptosis-resistant cells that lack essential molecules of the death receptor pathway such as FADD or caspase-8. Thus, Smac mimetic can prime leukemia cells to either apoptotic or necroptotic cell death after exposure to TNF α , depending on the cellular context and the presence and functionality of key signaling molecules.

In the presence of FADD and caspase-8 (i.e., in WT Jurkat cells), Smac mimetic enhances TNF α -induced apoptosis. Apoptotic cell death is demonstrated by several characteristic features, including caspase activation, DNA fragmentation, and inhibition of cell death by the caspase inhibitor zVAD.fmk. In contrast, in the absence of either FADD or caspase-8, Smac mimetic primes cells for TNF α -initiated necroptosis that critically depends on RIP1 and lacks prototypic features of apoptosis. Thus, in apoptosis-resistant cells, which are devoid of signaling molecules that are critically required for death receptor–induced apoptosis such as FADD or caspase-8, Smac mimetic in combination with TNF α activates RIP1-dependent necroptosis as an alternative cell death program to ensure the demise of the cell. Necroptotic cell death is confirmed by pharmacological inhibition of RIP1, a critical mediator of necroptosis [4], by biochemical features (i.e., lack of caspase activation, insensitivity to the caspase inhibitor zVAD.fmk) as well as by morphologic characteristics (i.e., nuclear condensation without fragmentation). Of note, Smac mimetic– and TNF α -induced necroptosis occurs in a highly synergistic manner, as calculated by CI. Consistently, we recently reported in a murine prototypic model of necrosis that depletion of cIAP1 by Smac mimetic sensitizes L929 cells for TNF α -mediated necroptosis that critically depends on RIP1 [26].

Cell death and survival pathways share some common central components, for example, the serine/threonine kinase RIP1 [4]. Whereas stimulation with TNF α in the absence of Smac mimetic causes little cell death in WT and FADD-deficient Jurkat cells, consistent with TNF α triggering ubiquitination of RIP1 and nuclear factor κ B activation in the presence of cIAP proteins [7], Smac mimetic–mediated degradation of cIAP proteins switches TNF α -stimulated survival toward cell death (Figure 6). Cell death proceeds either via the apoptotic pathway, if FADD or caspase-8 are present (i.e., in WT Jurkat cells), or alternatively via the necroptotic pathway in the absence of FADD or caspase-8 (i.e., in FADD- or caspase-8–deficient Jurkat cells) (Figure 6). In WT Jurkat cells, RIP1 may be involved in mediating either apoptosis or necroptosis, depending on the cellular context. When caspase activation occurs, RIP1 may contribute to some extent to the induction of apoptosis on treatment with Smac mimetic and TNF α via the formation of a cytosolic complex containing RIP1/FADD/caspase-8. This conclusion is supported by our data showing that the RIP1 inhibitor necrostatin 1 slightly reduces Smac mimetic–

and TNF α -induced cell death also in WT Jurkat cells in the absence of the caspase inhibitor zVAD.fmk. When caspase activation is blocked in WT Jurkat cells, Smac mimetic– and TNF α -induced cell death may proceed via the necroptotic route. In line with this notion, we found that the partial protection against Smac mimetic– and TNF α -induced cell death by the caspase inhibitor zVAD.fmk in WT Jurkat cells is further enhanced by RIP1 inhibition. This could be explained by a switch from Smac mimetic– and TNF α -induced apoptosis toward necroptosis on caspase inhibition by zVAD.fmk, which is inhibited by necrostatin 1. Induction of necrosis on caspase inhibition has previously been shown for the CD95 pathway [27,28]. However, the absence of Smac mimetic–mediated sensitization to CD95-triggered apoptosis in FADD-deficient leukemia cells in our study may suggest that other E3 ubiquitin ligases than cIAP proteins are involved in ubiquitination of RIP1 possibly in a stimulus-dependent manner, thereby preventing RIP1 to signal to necroptosis. Support for this hypothesis comes from a recent report showing that the E3 ubiquitin ligase Peli1 is required for RIP1 ubiquitination during Toll-like receptor 3 signaling [29].

Together, our results demonstrate that apoptotic and necroptotic cell death pathways are more closely interlinked than previously thought. The novelty and relevance of our findings is underscored by recent evidence of a close crosstalk between different cell death pathways. Accordingly, key components of the extrinsic apoptosis pathway including FADD and caspase-8 were shown to exert also nonapoptotic functions by preventing programmed necrosis [30–32]. Interestingly, we found that Smac mimetic– and TNF α -induced necroptosis occurs even more rapidly in FADD- or caspase-8–deficient cells compared with Smac mimetic– and TNF α -induced apoptosis in WT cells at equimolar concentrations, consistent with a more rapid kinetic of necroptotic cell death. In addition, Smac mimetic and TNF α induce necroptosis more rapidly in caspase-8–deficient cells with Bcl-2 overexpression compared with caspase-8–deficient cells without Bcl-2 overexpression, consistent with the notion that inhibition of apoptosis may favor necroptosis.

Our findings have several important implications. By demonstrating that Smac mimetic sensitizes cells that lack essential molecules of the death receptor pathway such as FADD or caspase-8 for TNF α -induced necroptosis as an alternative cell death program, these data provide first evidence that Smac mimetic can overcome resistance to TNF α -induced apoptosis. This mechanism might be relevant in cancers with local autocrine or paracrine production of TNF α , for example, in inflammatory cancers, because Smac mimetic–mediated down-regulation of cIAPs might switch the TNF α response from survival toward cell death. Against the background of our previous reports showing that small-molecule inhibitors of IAP proteins prime childhood ALL cells for TRAIL- or CD95-induced apoptosis [18,33], the current study identifies a novel molecular mechanisms of inhibitors of IAP proteins for bypassing apoptosis resistance in pediatric acute leukemia. Moreover, there is recent evidence in childhood ALL that induction of necroptosis can bypass resistance to glucocorticoids, one of the key drugs used in the clinic for childhood leukemia [34]. This underscores the potential clinical relevance of necroptosis as a new therapeutic strategy in refractory pediatric ALL. Taken together, Smac mimetics represent a promising novel approach to promote necroptosis as an alternative cell death program in apoptosis-resistant cancers, which warrants further investigation.

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References

- [1] Lockshin RA and Zakeri Z (2007). Cell death in health and disease. *J Cell Mol Med* **11**, 1214–1224.
- [2] Ashkenazi A (2008). Targeting the extrinsic apoptosis pathway in cancer. *Cytokine Growth Factor Rev* **19**, 325–331.
- [3] Kroemer G, Galluzzi L, and Brenner C (2007). Mitochondrial membrane permeabilization in cell death. *Physiol Rev* **87**, 99–163.
- [4] Vandenabeele P, Galluzzi L, Vanden Berghe T, and Kroemer G (2010). Molecular mechanisms of necroptosis: an ordered cellular explosion. *Nat Rev Mol Cell Biol* **11**, 700–714.
- [5] Festjens N, Vanden Berghe T, Cornelis S, and Vandenabeele P (2007). RIP1, a kinase on the crossroads of a cell's decision to live or die. *Cell Death Differ* **14**, 400–410.
- [6] Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, Bodmer JL, Schneider P, Seed B, and Tschopp J (2000). Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. *Nat Immunol* **1**, 489–495.
- [7] Wang L, Du F, and Wang X (2008). TNF- α induces two distinct caspase-8 activation pathways. *Cell* **133**, 693–703.
- [8] High LM, Szymanska B, Wilczynska-Kalak U, Barber N, O'Brien R, Khaw SL, Vikstrom IB, Roberts AW, and Lock RB (2010). The Bcl-2 homology domain 3 mimetic ABT-737 targets the apoptotic machinery in acute lymphoblastic leukemia resulting in synergistic *in vitro* and *in vivo* interactions with established drugs. *Mol Pharmacol* **77**, 483–494.
- [9] LaCasse EC, Mahoney DJ, Cheung HH, Plenchette S, Baird S, and Korneluk RG (2008). IAP-targeted therapies for cancer. *Oncogene* **27**, 6252–6275.
- [10] Bertrand MJ, Milutinovic S, Dickson KM, Ho WC, Boudreaux A, Durkin J, Gillard JW, Jaquith JB, Morris SJ, and Barker PA (2008). cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination. *Mol Cell* **30**, 689–700.
- [11] Varfolomeev E, Goncharov T, Fedorova AV, Dynek JN, Zobel K, Deshayes K, Fairbrother WJ, and Vucic D (2008). c-IAP1 and c-IAP2 are critical mediators of tumor necrosis factor α (TNF α)-induced NF- κ B activation. *J Biol Chem* **283**, 24295–24299.
- [12] Vince JE, Wong WW, Khan N, Feltham R, Chau D, Ahmed AU, Benetatos CA, Chunduru SK, Condon SM, McKinlay M, et al. (2007). IAP antagonists target cIAP1 to induce TNF α -dependent apoptosis. *Cell* **131**, 682–693.
- [13] Varfolomeev E, Blankenship JW, Wayson SM, Fedorova AV, Kayagaki N, Garg P, Zobel K, Dynek JN, Elliott LO, Wallweber HJ, et al. (2007). IAP antagonists induce autoubiquitination of c-IAPs, NF- κ B activation, and TNF α -dependent apoptosis. *Cell* **131**, 669–681.
- [14] Petersen SL, Wang L, Yalcin-Chin A, Li L, Peyton M, Minna J, Harran P, and Wang X (2007). Autocrine TNF α signaling renders human cancer cells susceptible to Smac-mimetic-induced apoptosis. *Cancer Cell* **12**, 445–456.
- [15] Fulda S (2009). Tumor resistance to apoptosis. *Int J Cancer* **124**, 511–515.
- [16] Fulda S (2009). Inhibitor of apoptosis proteins in hematological malignancies. *Leukemia* **23**, 467–476.
- [17] Vogler M, Walczak H, Stadel D, Haas TL, Genze F, Jovanovic M, Gschwend JE, Simmet T, Debatin KM, and Fulda S (2008). Targeting XIAP bypasses Bcl-2-mediated resistance to TRAIL and cooperates with TRAIL to suppress pancreatic cancer growth *in vitro* and *in vivo*. *Cancer Res* **68**, 7956–7965.
- [18] Fakler M, Loeder S, Vogler M, Schneider K, Jeremias I, Debatin KM, and Fulda S (2009). Small molecule XIAP inhibitors cooperate with TRAIL to induce apoptosis in childhood acute leukemia cells and overcome Bcl-2-mediated resistance. *Blood* **113**, 1710–1722.
- [19] Vellanki SH, Grabrucker A, Liebau S, Proepper C, Eramo A, Braun V, Boeckers T, Debatin KM, and Fulda S (2009). Small-molecule XIAP inhibitors enhance γ -irradiation-induced apoptosis in glioblastoma. *Neoplasia* **11**, 743–752.
- [20] Juo P, Woo MS, Kuo CJ, Signorelli P, Biemann HP, Hannun YA, and Blenis J (1999). FADD is required for multiple signaling events downstream of the receptor Fas. *Cell Growth Differ* **10**, 797–804.
- [21] Juo P, Kuo CJ, Yuan J, and Blenis J (1998). Essential requirement for caspase-8/FLICE in the initiation of the Fas-induced apoptotic cascade. *Curr Biol* **8**, 1001–1008.
- [22] Kawahara A, Ohsawa Y, Matsumura H, Uchiyama Y, and Nagata S (1998). Caspase-independent cell killing by Fas-associated protein with death domain. *J Cell Biol* **143**, 1353–1360.
- [23] Fulda S, Strauss G, Meyer E, and Debatin KM (2000). Functional CD95 ligand and CD95 death-inducing signaling complex in activation-induced cell death and doxorubicin-induced apoptosis in leukemic T cells. *Blood* **95**, 301–308.
- [24] Degterev A, Huang Z, Boyce M, Li Y, Jagtap P, Mizushima N, Cuny GD, Mitchison TJ, Moskowitz MA, and Yuan J (2005). Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. *Nat Chem Biol* **1**, 112–119.
- [25] Degterev A, Hitomi J, Germscheid M, Ch'en IL, Korkina O, Teng X, Abbott D, Cuny GD, Yuan C, Wagner G, et al. (2008). Identification of RIP1 kinase as a specific cellular target of necrostatins. *Nat Chem Biol* **4**, 313–321.
- [26] Vanlangenakker N, Vanden Berghe T, Bogaert P, Laukens B, Zobel K, Deshayes K, Vucic D, Fulda S, Vandenabeele P, and Bertrand MJ (2011). cIAP1 and TAK1 protect cells from TNF-induced necrosis by preventing RIP1/RIP3-dependent reactive oxygen species production. *Cell Death Differ* **18**, 656–665.
- [27] Vercammen D, Brouckaert G, Denecker G, Van de Craen M, Declercq W, Fiers W, and Vandenabeele P (1998). Dual signaling of the Fas receptor: initiation of both apoptotic and necrotic cell death pathways. *J Exp Med* **188**, 919–930.
- [28] Geserick P, Hupe M, Moulin M, Wong WW, Feoktistova M, Kellert B, Gollnick H, Silke J, and Leverkus M (2009). Cellular IAPs inhibit a cryptic CD95-induced cell death by limiting RIP1 kinase recruitment. *J Cell Biol* **187**, 1037–1054.
- [29] Chang M, Jin W, and Sun SC (2009). Peli1 facilitates TRIF-dependent Toll-like receptor signaling and proinflammatory cytokine production. *Nat Immunol* **10**, 1089–1095.
- [30] Oberst A, Dillon CP, Weinlich R, McCormick LL, Fitzgerald P, Pop C, Hakem R, Salvesen GS, and Green DR (2011). Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. *Nature* **471**, 363–367.
- [31] Zhang H, Zhou X, McQuade T, Li J, Chan FK, and Zhang J (2011). Functional complementation between FADD and RIP1 in embryos and lymphocytes. *Nature* **471**, 373–376.
- [32] Kaiser WJ, Upton JW, Long AB, Livingston-Rosanoff D, Daley-Bauer LP, Hakem R, Casparly T, and Mocarski ES (2011). RIP3 mediates the embryonic lethality of caspase-8-deficient mice. *Nature* **471**, 368–372.
- [33] Loeder S, Drensek A, Jeremias I, Debatin KM, and Fulda S (2010). Small molecule XIAP inhibitors sensitize childhood acute leukemia cells for CD95-induced apoptosis. *Int J Cancer* **126**, 2216–2228.
- [34] Bonapace L, Bornhauser BC, Schmitz M, Cario G, Ziegler U, Niggli FK, Schafer BW, Schrappe M, Stanulla M, and Bourquin JP (2010). Induction of autophagy-dependent necroptosis is required for childhood acute lymphoblastic leukemia cells to overcome glucocorticoid resistance. *J Clin Invest* **120**, 1310–1323.

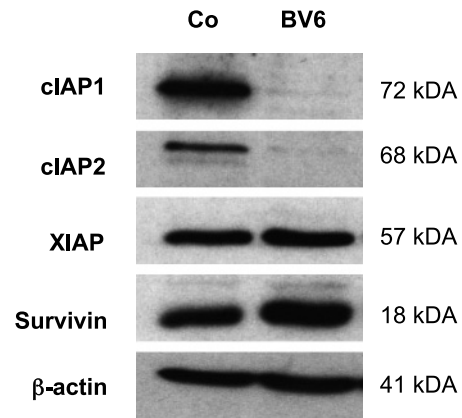


Figure W1. Down-regulation of IAP proteins by Smac mimetic. WT Jurkat cells were treated with 1 μ M BV6 for 2 hours. Protein expressions of cIAP1, cIAP2, XIAP, and survivin were analyzed by Western blot. β -Actin served as loading control.

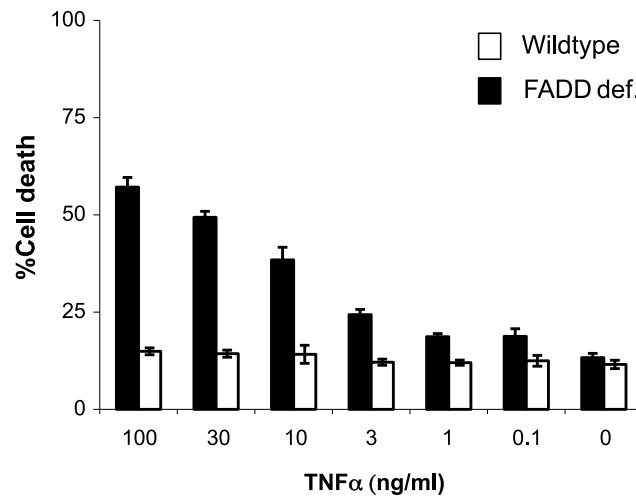


Figure W2. Dose-response of TNF α -induced cell death. WT (white bars) or FADD-deficient (black bars) Jurkat cells were treated with the indicated concentrations of TNF α for 24 hours. Cell death was analyzed by PI staining. Data are the mean and SD of three independent experiments performed in triplicate.