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Inhibitor of Apoptosis Protein-1 Regulates Tumor Necrosis Factor–Mediated Destruction of Intestinal Epithelial Cells

Thomas Grabinger,[1] Konstantin J. Bode,[1] Janine Demgenski,[1] Carina Seitz,[1] M. Eugenia Delgado,[1] Feodora Kostadinova,[1] Cindy Reinhold,[1] Nima Etemadi,[2,3] Sabine Wilhelm,[4] Matthias Schweinlin,[4] Kay Hänggi,[5] Janin Knop,[5] Christof Hauck,[6] Heike Walles,[4,7] John Silke,[2,3] Harald Wajant,[8] Ueli Nachbur,[2,3] Wong W. Wei-Lynn,[5] and Thomas Brunner[1]

[1]Biochemical Pharmacology, Department of Biology, University of Konstanz, Germany; [2]Cell Signaling and Cell Death Division, The Walter and Eliza Hall Institute of Medical Research, Parkville, Australia; [3]Department of Medical Biology, University of Melbourne, Melbourne, Australia; [4]Department of Tissue Engineering and Regenerative Medicine, University Hospital Würzburg, Würzburg, Germany; [5]Institute of Experimental Immunology, University of Zurich, Zurich, Switzerland; [6]Department of Biology, University of Konstanz, Konstanz, Germany; [7]Translational Center Würzburg, Würzburg branch of the Fraunhofer IGB, Würzburg, Germany; and [8]Division of Molecular Internal Medicine, Department of Internal Medicine II, University Hospital Würzburg, Germany

BACKGROUND AND AIMS: Tumor necrosis factor (TNF) is a cytokine that promotes inflammation and contributes to pathogenesis of inflammatory bowel diseases. Unlike other cells and tissues, intestinal epithelial cells undergo rapid cell death upon exposure to TNF, by unclear mechanisms. We investigated the roles of inhibitor of apoptosis proteins (IAPs) in the regulation of TNF-induced cell death in the intestinal epithelium of mice and intestinal organoids. **METHODS:** RNA from cell lines and tissues was analyzed by quantitative polymerase chain reaction, protein levels were analyzed by immunoblot assays. BIRC2 (also called cIAP1) was expressed upon induction from lentiviral vectors in young adult mouse colon (YAMC) cells. YAMC cells, the mouse colon carcinoma cell line MC38, the mouse macrophage cell line RAW 264.7, or mouse and human organoids were incubated with second mitochondrial activator of caspases (Smac)-mimetic compound LCL161 or recombinant TNF-like weak inducer of apoptosis (TNFSF12) along with TNF, and cell death was quantified. C57BL/6 mice with disruption of *Xiap, Birc2* (encodes cIAP1), *Birc3* (encodes cIAP2), *Tnfrsf1a*, or *Tnfrsf1b* (*Tnfrsf1a* and *b* encode TNF receptors) were injected with TNF or saline (control); liver and intestinal tissues were collected and analyzed for apoptosis induction by cleaved caspase 3 immunohistochemistry. We also measured levels of TNF and alanine aminotransferase in serum from mice. **RESULTS:** YAMC cells, and mouse and human intestinal organoids, died rapidly in response to TNF. YAMC and intestinal crypts expressed lower levels of XIAP, cIAP1, cIAP2, and cFLIP than liver tissue. Smac-mimetics reduced levels of cIAP1 and XIAP in MC38 and YAMC cells, and Smac-mimetics and TNF-related weak inducer of apoptosis increased TNF-induced cell death in YAMC cells and organoids—most likely by sequestering and degrading cIAP1. Injection of TNF greatly increased levels of cell death in intestinal tissue of cIAP1-null mice, compared with wild-type C57BL/6 mice, cIAP2-null mice, or XIAP-null mice. Excessive TNF-induced cell death in the intestinal epithelium was mediated TNF receptor 1. **CONCLUSIONS:** In a study of mouse and human cell lines, organoids, and tissues, we found cIAP1 to be required for regulation of TNF-induced intestinal epithelial cell death and survival. These findings have important implications for the pathogenesis of TNF-mediated enteropathies and chronic inflammatory diseases of the intestine.

Keywords: TWEAK; TNF Signaling; IBD; Mouse Model.

Tumor necrosis factor (TNF)−α is a cytokine with a broad spectrum of activities. Although predominantly expressed by activated macrophages and dendritic cells, a large variety of different cell types, including epithelial cells in different tissues, can produce TNF.[1,2] TNF is one of the first cytokines released during an immune response and generally has important regulatory functions in the control of immunologic processes due to its potent pro-inflammatory properties.[3] When TNF binds to its receptors, TNFR1 and TNFR2, it triggers a series of signaling cascades, generally leading to cellular activation, gene expression and survival.[4] These cellular responses are often initiated by the activation of mitogen-activated protein kinases and nuclear factor−κB (NFκB). Many of the TNF-induced and NFκB-regulated target genes are also cytokines with pro-inflammatory activities, including TNF itself, leading to amplification and spreading of the initial inflammatory stimulus.[5] TNF is not only critical during the onset of protective immune responses, but is also involved in the pathogenesis of numerous acute and chronic

Abbreviations used in this paper: **IAP, inhibitor of apoptosis protein; IBD, inflammatory bowel disease; IEC, intestinal epithelial cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NFκB, nuclear factor κB; RIPK1, receptor-interacting protein kinase 1; Smac, second mitochondrial activator of caspases; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; TWEAK, tumor necrosis factor related weak inducer of apoptosis; XIAP, X-linked inhibitor of apoptosis protein; YAMC, young adult colonocytes.**

inflammatory diseases, including inflammatory bowel disease (IBD)[6] and rheumatoid arthritis.[7] TNF is an important therapeutic target, and TNF-neutralizing drugs are successfully used in the treatment of chronic inflammatory diseases, such as rheumatoid arthritis and IBD.

Although TNF has pro-inflammatory properties promoting cellular activation, proliferation and survival, historically it was the first cell death (necrosis)-promoting cytokine identified due to its ability to induce necrotic cell death in transplanted tumor tissue.[8] It became the founding member of a large family of ligands with diverse functions and activities. Among these, Fas/CD95 ligand (TNFSF6) and TNF-related apoptosis-inducing ligand (TNFSF10) are particularly well known for their potent cell death–promoting activities.[9] Although Fas/CD95 ligand and TNF-related apoptosis-inducing ligand induce cell death in a large variety of cell lines and/or tissues, most cells and tissues are generally resistant to TNF-induced cell death. This resistance is explained by the fact that TNF-induced NF-κB activation promotes the expression of various survival proteins (eg, cFLIP), which prevent TNF-induced apoptosis or necroptosis.[10,11]

Although TNF alone fails to trigger cell death in most tissues and cell types, there is one important exception, the intestinal epithelium. TNF injection or induction of TNF expression in vivo (eg, after lipopolysaccharide injection and associated macrophage activation) leads to a very rapid induction of intestinal epithelial cell death, resulting in almost immediate shedding of mature epithelial cells from the villus tip and a somewhat delayed apoptosis of intestinal crypt cells.[12] TNF-induced intestinal epithelial cell death is observed during the pathogenesis of a variety of inflammatory diseases, including sepsis, graft-vs-host disease,[13] bacterial and viral infections,[14,15] and, in particular, IBD.[1,16,17] Neutralization of TNF substantially reduces intestinal epithelial cell death under these inflammatory conditions and, importantly, contributes to improved survival. The reasons for this exquisite sensitivity of intestinal epithelial cells (IECs) to TNF is presently unknown and poorly investigated, but is likely to be very important for the regulation of TNF-induced intestinal immunopathologies, such as IBD.

Inhibitor of apoptosis proteins (IAPs) are a group of proteins related to baculovirus IAP. Mammalian cells express XIAP (X-linked IAP), cIAP1 (cellular IAP1) and 2, NAIP (neuronal IAP) and survivin.[18,19] Most IAPs are directly or indirectly involved in the regulation of cell death. While baculovirus IAP was initially described as a caspase inhibitor, characterization of mammalian IAPs (cIAP1, cIAP2, and XIAP) revealed that they can also regulate cell death via their E3 ubiquitin ligase activity (reviewed in Silke and Vucic[19]). In particular, cIAP1 and 2 are recruited to the receptor complex of various TNFR family members, and regulate the activity of other co-recruited signaling proteins by ubiquitination.[20,21] The role of IAPs has been particularly well characterized for TNFR1 signaling. cIAP1 and 2 are recruited to TNFR1 via a TRADD/TRAF2-dependent process and ubiquitinate other signaling proteins of the TNFR1 receptor complex, including RIPK1 (receptor-interacting kinase 1).[22,23] Absence of cIAP1 and 2, or their pharmacologic inhibition by second mitochondrial activator of caspases (Smac)-mimetics generally results in sensitization of cells to TNF-induced cell death due to inhibition of the induction of protective NF-κB-dependent survival signals, the formation of the pro-apoptotic TNFR1-induced complex II, or the RIPK1/RIPK3-dependent induction of necroptosis.[24–26]

Although cIAP1 and 2 have overlapping activities, their respective roles in the regulation of TNF-induced cell death in specific cell types and tissues are incompletely understood.[27] In particular, their relative contribution to the high sensitivity of IECs to TNF-induced cell death and associated enteropathy has not been explored. Here we show that IECs express only low levels of IAPs compared with liver tissue. Smac-mimetics further sensitize mouse and human IEC to TNF-induced cell death in vitro, and genetic deletion of cIAP1, but not cIAP2 or XIAP, results in massive acceleration of TNF-induced IEC cell death and enteropathy in vivo. TNF–related weak inducer of apoptosis (TWEAK), which triggers recruitment of cIAP1 and cIAP2 to the TWEAK receptor Fn14 and depletes the cytosolic pool of these proteins,[28,29] was also found to sensitize IEC to TNF-induced cell death in vitro and in vivo. Our findings show that cIAP1 expression is a limiting and nonredundant factor in the regulation of TNF-induced cell death in the intestinal epithelium. Therefore, cIAP1-regulated TNF sensitivity likely plays a critical role in TNF-mediated tissue destruction and the pathogenesis of intestinal immunopathologies, such as IBD.

Materials and Methods

Mice

C57BL/6, *Tnfrsf1a*$^{-/-}$ (TNFR1-null), *Tnfrsf1b*$^{-/-}$ (TNFR2-null), and *Xiap*$^{-/-}$[30] mice were bred and kept in individually ventilated cages at the central animal facility of the University of Konstanz; *Birc2*$^{-/-}$ (cIAP1-null), *Birc2*$^{-/-}$ *x Tnfrsf1a*$^{-/-}$ and *Birc2*$^{-/-}$ *x Tnfrsf1b*$^{-/-}$ mice at the animal of the University of Zürich (Switzerland); and *Birc3*$^{-/-}$ (cIAP2-null) mice[31] at the animal facility of the Walter and Eliza Hall Institute in Melbourne, Australia. As controls, wild-type C57BL/6 mice from the respective animal facility were used.

Cell Lines

The conditionally immortalized young adult mouse colon (YAMC) cell line (kind gift of R. Whitehead, Vanderbilt University)[32] was maintained at permissive conditions (33°C, 5% CO_2) in RPMI 1640 medium (Sigma, St Louis, MO), supplemented with 5% fetal calf serum (PAA, GE Healthcare, Freiburg, Germany), ITS solution (Gibco, Grand Island, NY), 50 μg/mL Gentamicin (Sigma), and 5 U/mL mouse interferon-γ Six hours prior to treatment, YAMC cell culture was changed to 37°C, 5% CO_2 and medium without mouse interferon-γ. The mouse colon carcinoma cell line MC38 was obtained from American Type Culture Collection (Manassas, VA) and cultured in Iscove's modified Dulbecco's medium (Sigma), 10% fetal calf serum, minimum essential medium amino-acid solution (Sigma), 4 mM L-glutamine and 50 μg/mL gentamycin (Sigma) at 37°C and 5% CO_2. The mouse macrophage cell line RAW

264.7 was obtained from American Type Culture Collection and maintained in Dulbecco's modified Eagle medium, 10% fetal calf serum, and 50 μg/mL gentamicin.

Antibodies and Reagents

Antibodies against cleaved caspase 3 (Asp175, #9661), cIAP-1 (#4952) and Bcl-x_L (#2764) were purchased from Cell Signaling Technology (Danvers, MA). Antibody against c-Flip (#ADI-AAP-440E) was purchased from Enzo Life Sciences (Farmingdale, NY), anti-tubulin antibody (#T5168) was purchased from Sigma-Aldrich, epidermal growth factor (#315-09), Noggin (#250-38), interferon-γ (#315-05), and R-Spondin-1 (#120-38) were purchased from Peprotech (Rocky Hill, NJ). The Smac mimetic compound LCL161 was kindly provided by Novartis (Basel, Switzerland). Mouse recombinant TWEAK and BV6 was produced as described previously.[29,33,34]

RNA Isolation and Quantitative Polymerase Chain Reaction

Total RNA from cell lines and tissue was isolated and reverse transcribed using the High Capacity cDNA Reverse Transcriptions Kit (Applied Biosystems, Carlsbad, CA). Quantitative polymerase chain reaction was done on a StepOnePlus Real-Time PCR system (Applied Biosystems) using SYBR green master mix (Applied Biosystems) All gene-specific primers were designed to span an exon exon junction. For $cFLIP_S$ and $cFLIP_L$ splice variant-specific primer sequences were used. Gene expression was normalized using β-actin. Primer sequences used are listed in Supplementary Table 1.

Intestinal Crypt Isolation and Organoid Culture

Isolation and culture of mouse and human intestinal crypts were done as described previously.[35,36] Mouse organoids were cultured in 96-well plates, and human organoids in 24-well plates for 3 days (37°C, 5% CO_2) before indicated treatments.

Lentiviral Transduction of Young Adult Mouse Colon Cells

YAMC cells were transduced using a 4-hydroxytamoxyfen-inducible lentiviral constructs resulting in inducible expression of cIAP1.[37] Cell were cultured with or without 60 nM 4-hydroxytamoxifen for 16 hours, exposed to increasing concentrations of TNF and cell death was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Cell Death Assay for Organoids and Cell Lines

MTT assays in mouse organoids and cell lines were done as described previously.[35] Cell death in human organoids was detected using an M30 CytoDeath ELISA (VLVbio, Nacka, Sweden) and normalized to the DNA content in the sample.

Western Blot

Cells were lysed in NP-40 lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris, pH 8.0, 1 mM EDTA). Concentration of total protein was determined using BCA assay (Pierce, Rockford, IL) following manufacturer's recommendation. Samples were boiled with sodium dodecyl sulfate sample buffer (95°C, 5 minutes), resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis gels (12%) and transferred to a polyvinylidene difluoride membrane. Immunoblot analyses were performed using specific antibodies and visualized with a biomolecular imager (ImageQuant LAS4000; Fujifilm, Tokyo, Japan).

Determination of Cell Death by Flow Cytometry

Cells and supernatant of 24-well plates were harvested, centrifuged at 1000 $\times$ g, 4°C, 5 minutes and resuspended in Annexin-V binding buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$) with 1 μg/mL fluorescein isothiocyanate labeled Annexin V. Cell suspension was analyzed using a LSFortessa cytometer (BD Biosciences, San Jose, CA).

Immunohistochemistry

Mice were injected with phosphate-buffered saline control or 4 $\mu\gamma$ murine TNF or human FcTNF for 4 hours. Serum was harvested, and tissues from small intestine, large intestine, and liver were collected, formalin-fixed, dehydrated, and paraffin-embedded. Apoptotic cells in tissue sections were detected using an anti-cleaved caspase 3 antibody as described previously.[38] Quantification of cleaved caspase 3 positive cells per 100 crypts was done in the small intestine.

Transaminase Assay

For quantitative determination of alanine aminotransferase activity in the serum of mice, alanine aminotransferase reagent colorimetric endpoint method kit (Teco Diagnostics, Anaheim, CA) was used after manufacturer's recommendation with 1:5 diluted serum.

Tumor Necrosis Factor Enzyme Linked Immunosorbent Assay

TNF in the serum was detected using a commercially available enzyme-linked immunosorbent assay (BioLegends, San Diego, CA).

Statistics

Graph Pad Prism software (version 5.01, GraphPad Software, La Jolla, CA) was used for statistical analysis. All experiments were performed in triplicate and repeated at least 3 times. All values are reported as mean $\pm$ SD. Where indicated, statistical analysis was evaluated with unpaired 2-tailed Student t tests, with a P value $<.05$ considered statistically significant. When multiple groups were compared, 1-way analysis of variance was performed.

Study Approval

All animal experiments complied with animal experimentation regulations of Germany, Switzerland, and Australia, and were approved by the Ethics Review Committee of the respective regional council. Experiments using human tissue samples were approved by the Institutional Ethics Committee on Human Research of the Julius-Maximilians-University Würzburg (study approval number 182/10).

Results

Tumor Necrosis Factor Induces Cell Death in Intestinal Epithelial Cells in vivo and in vitro

TNF contributes to the pathogenesis of different inflammatory diseases of the intestine, at least in part via the direct induction of cell death in IECs and associated disruption of the epithelial barrier function.[39] The underlying reason why IECs are so sensitive to TNF is not understood. In order to study the mechanisms of TNF-induced cell death in IECs, we first aimed at establishing an in vitro/ex vivo model system recapitulating TNF-induced IEC apoptosis in vivo. Mice were injected with a sublethal dose of soluble mouse TNF, stimulating TNFR1 in particular.[40] After 6 hours, mice were euthanized and tissue sections were analyzed for apoptosis induction by staining for cleaved caspase 3, indicative of ongoing apoptosis in the tissue.[38] While very few cleaved caspase 3−positive cells were detected in the small and large intestine of control treated mice, a high frequency of apoptotic cells was detected after TNF injection, in both small and large intestine (Figure 1*A*). Cell death detection was particularly prominent in the intestinal crypts. In contrast, TNF injection (in the absence of a tissue-specific sensitizer, such as D-galactosamine) failed to promote detectable cleaved caspase 3−positive cells in the liver (Figure 1*A*).

Because apoptosis was predominantly detected in intestinal crypts, TNF-induced cell death was further studied in ex vivo cultured intestinal organoids.[35,41] Stimulation of these organoids with TNF resulted in a rapid loss of organoid integrity and the appearance of cells with altered morphology, which were propidium iodide−positive (Figure 1*B*).[35] The rapid induction of organoid death was further confirmed using a quantitative viability staining method (Figure 1*C*).

We further analyzed which TNF receptor mediates cell death in isolated mouse intestinal organoids. In agreement with the in vivo findings,[42] we observed an almost complete abrogation of TNF-induced cell death in *Tnfrsf1a*-deficient (TNFR1-null) organoids, while organoids from wild-type and *Tnfrsf1b*-deficient (TNFR2-null) mice showed a dose-dependent response, though organoids from TNFR2-null mice exhibited a somewhat reduced cell death (Figure 1*C*). This indicates that both TNF receptors mediate cell death in IECs, but predominantly TNFR1. TNF sensitivity was also confirmed in human organoids (Figure 1*D*)

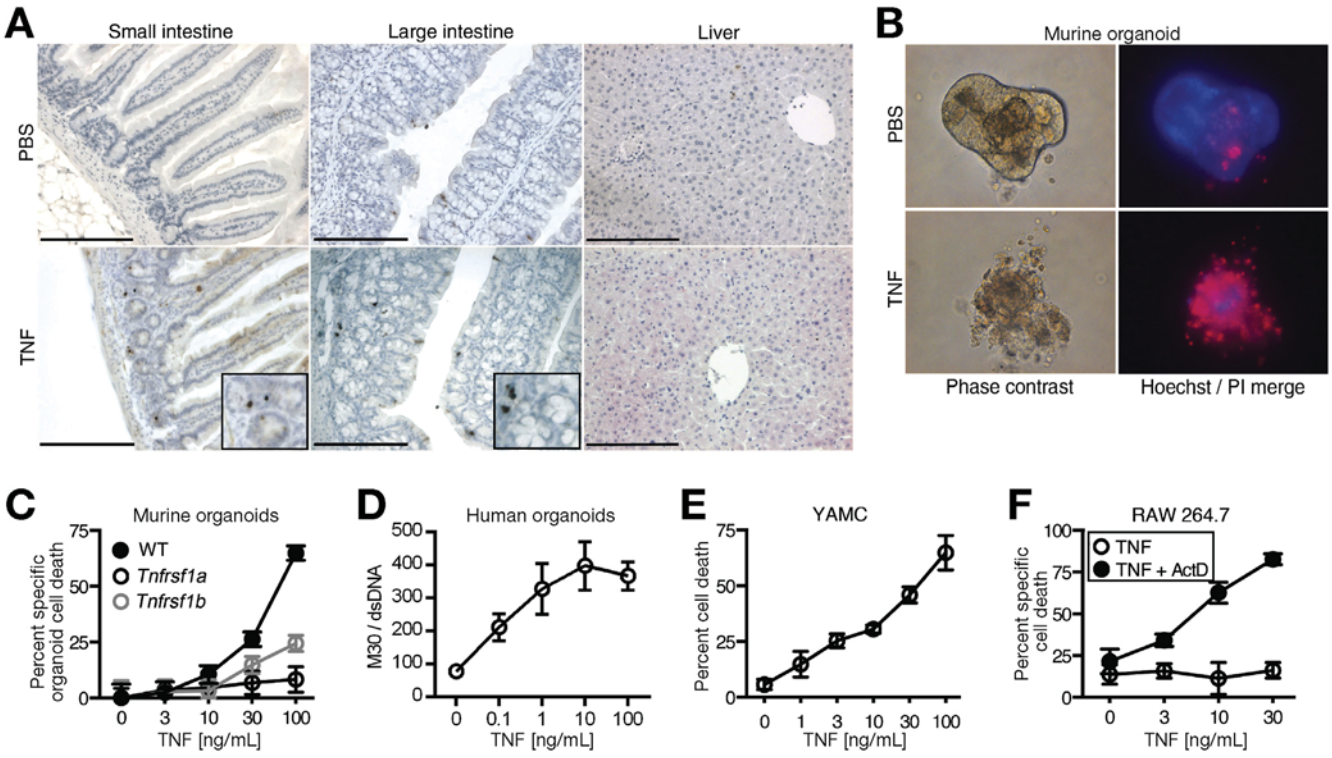
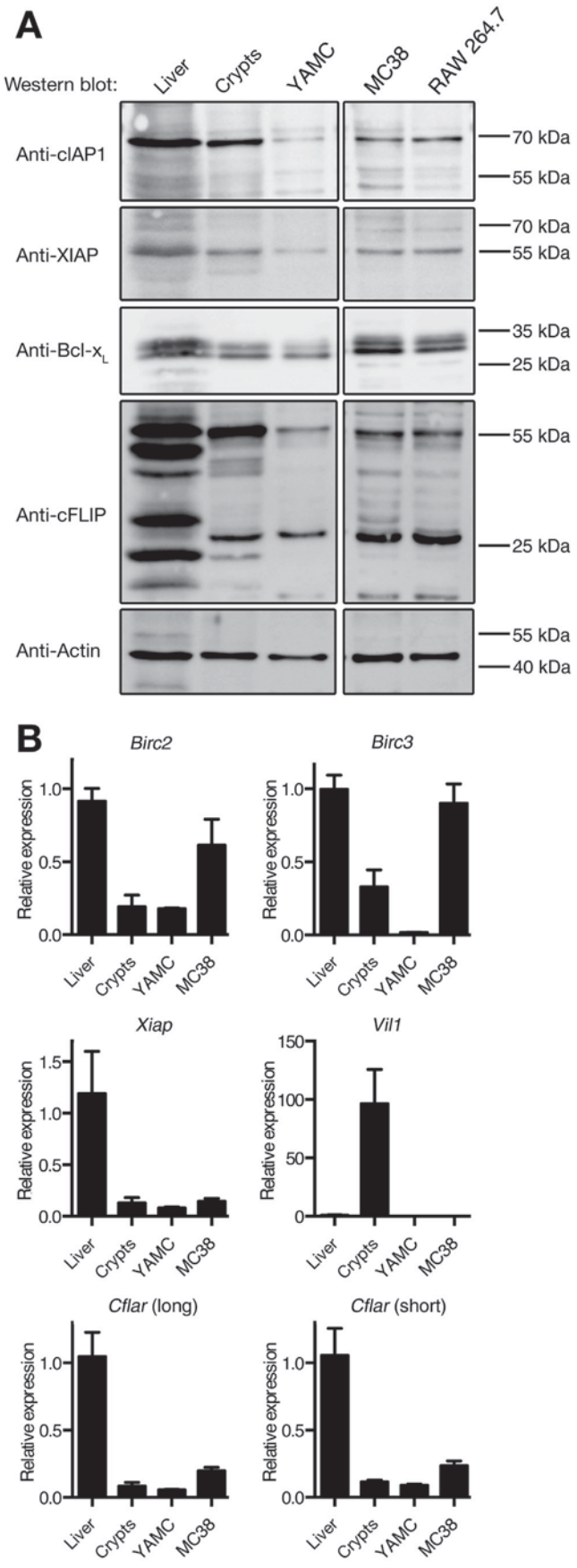


Figure 1. TNF-induced cell death in IECs in vivo and in vitro. (*A*) Wild-type mice were injected with phosphate-buffered saline (PBS) or TNF for 4 hours, and apoptosis in small and large intestine and liver was detected using cleaved caspase 3 immunohistochemistry (n = 12 mice per group) Scale bar = 150 μm. (*B*) Representative microscopic (phase contrast and Hoechst/propidium iodide staining) images of PBS- and TNF-treated mouse intestinal organoids. Scale bar = 150 μm. (*C*) Organoids from wild-type (WT), *Tnfrsf1a*$^{-/-}$ (TNFR1-null) and *Tnfrsf1b*$^{-/-}$ (TNFR2-null) mice were treated with TNF and cell death was measured by MTT reduction (mean ± SD of triplicates, n = 3). (*D*) Human organoids were treated with increasing doses of TNF and cell death was analyzed by M30 enzyme linked immunosorbent assay (mean ± SD of triplicates, n = 3). (*E*) YAMC cells were treated with indicated doses of TNF and cell death was assessed by MTT. Mean ± SD of triplicate of representative experiments are shown (n = 4). (*F*) RAW 264.7 macrophage cells were either pretreated with actinomycin D (60 ng/mL) or PBS, and TNF-induced cell death was measured by MTT reduction (mean ± SD of triplicates, n = 2).



We next set out to identify an intestinal cell line, which shows similar responses to TNF as primary IECs. When the intestinal epithelial cell line YAMC[43] was exposed to increasing concentrations of TNF, a dose-dependent induction of cell death was detected (Figure 1*G*). In contrast, the macrophage cell line RAW 264.7 failed to die in response to TNF alone, but required sensitization via actinomycin D (Figure 1*H*).

Intestinal Epithelial Cells Express Low Levels of Inhibitors of Apoptosis

In order to understand the basis of the high sensitivity of IECs to TNF-induced cell death, we investigated the relative expression of apoptosis-regulating proteins in primary IEC and YAMC cells in comparison to liver, RAW264.7 and the mouse colorectal tumor cell lines MC38, that are insensitive to TNF-induced cell death. High protein levels of XIAP and cIAP1 were detected in liver extracts, whereas crypts expressed relatively high levels of cIAP1 but low levels of XIAP. YAMC cells were expressing low levels of both proteins (Figure 2*A*). A similar finding was made for cFLIP$_S$ and cFLIP$_L$. In comparison, relatively high expression levels of Bcl-x$_L$ were found in all cells and tissues. These findings on a protein level were confirmed by quantitative reverse transcription polymerase chain reaction, where, compared with the liver, intestinal crypts and YAMC cells expressed much lower levels of *Xiap*, *Birc2* (cIAP1), *Birc3* (cIAP2), and *cFlip* (Figure 2*B*).

Smac Mimetics Further Sensitize Intestinal Epithelial Cells to Tumor Necrosis Factor Induced Cell Death

We next assessed the effect of the IAP inhibitor and Smac-mimetics on TNF-induced cell death. As reported previously in other cell types,[44] we observed that LCL161 caused a dose-dependent decrease in cIAP1 and XIAP levels in MC38 cells, and confirmed the degradation of the proteins in primary mouse intestinal crypts (Figure 3*A*). Treatment of YAMC cells with LCL161 resulted in a profound sensitization to TNF-induced cell death (Figure 3*B*), but not chemotherapeutic drug-induced cell death (Figure 3*C* and *D*). This sensitizing effect of IAP inhibition was further confirmed in primary IECs in mouse and human intestinal organoid cultures. Cell death induction by TNF alone was further enhanced by pretreating organoids with LCL161, respectively BV6, while Smac-mimetic alone resulted in only a minimal increase over basal cell death (Figure 3*E* and *F*). Although Smac-mimetics have been shown previously to

Figure 2. IAP expression in IECs. Protein (*A*) and messenger RNA (*B*) expression of the indicated genes in liver, isolated crypts, and the cell lines YAMC, MC38, and RAW 264.7. Representative immunoblots of 3 independent experiments are shown. Mean values ± SD of samples from 3 individual mice are shown for quantitative polymerase chain reaction (n = 2). *Birc2*, cIAP1; *Birc3*, cIAP2; *Cflar* (short), cFLIP$_S$; *Cflar* (long), cFLIP$_L$; Vil-1, Villin.

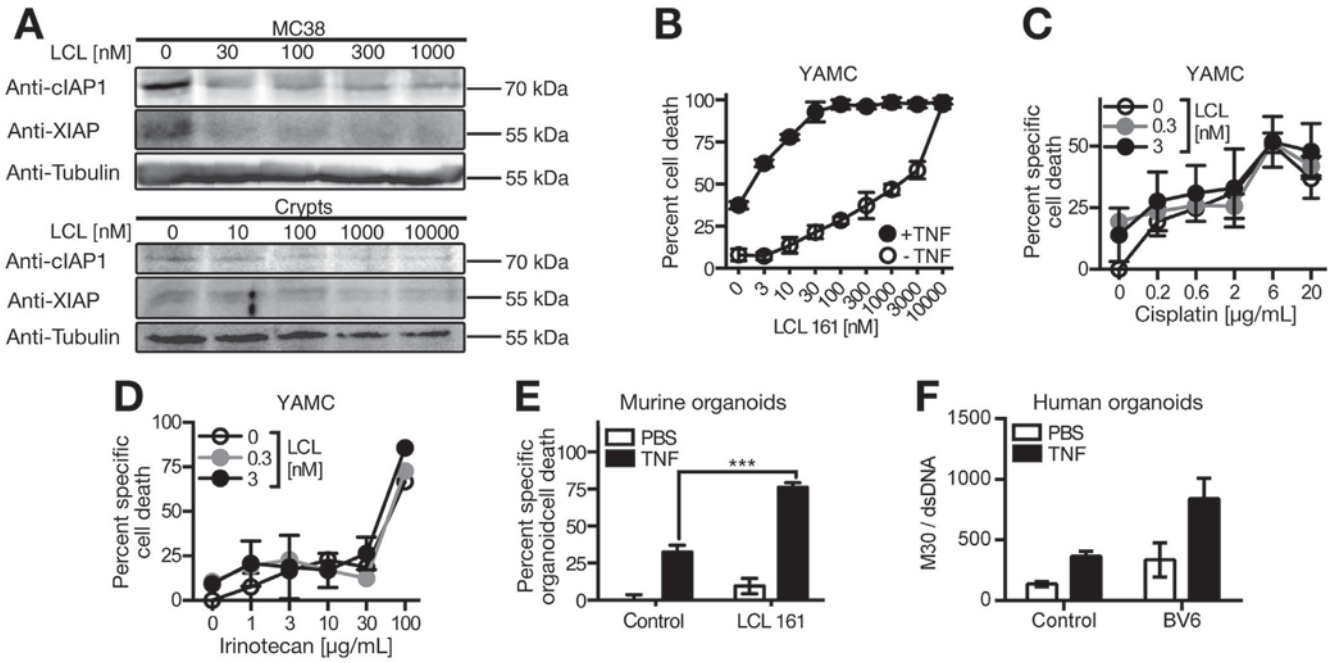


Figure 3. The Smac-mimetic LCL161 sensitizes IECs to TNF-induced cell death. (*A*) MC38 and isolated crypts were treated with the indicated concentrations of LCL161 for 2 hours, and protein levels of cIAP1 and XIAP were detected by immunoblotting (n = 3). (*B*) YAMC cells were pretreated with indicated concentrations of LCL161 for 2 hours, and then stimulated with buffer control or TNF (30 ng/mL). (C, *D*) YAMC cells were pretreated with indicated concentrations of LCL161, and stimulated with the chemotherapeutic agents cisplatin (*C*) or irinotecan (*D*) (mean ± SD values of triplicates, n = 2). (*E*) Mouse organoids were pretreated with LCL161 (30 nM), followed by stimulation with TNF (30 ng/mL). Organoid cell death was measured by MTT reduction. ***$P < .001$. Mean ± SD values of triplicates, n = 3. (*F*) Human organoids were pretreated with BV6, then stimulated with TNF (1 ng/mL), and cell death was analyzed by M30 enzyme-linked immunosorbent assay. Mean ± SD values of triplicates, n = 3.

promote cell death via the activation of the NF-κB pathway, induction of TNF expression and TNF-mediated cell death,[25] we failed to detect Smac-mimetic-induced TNF in the supernatant of YAMC cells (Supplementary Figure 1).

Tumor Necrosis Factor Related Weak Inducer of Apoptosis Sensitizes Intestinal Epithelial Cells to Tumor Necrosis Factor Induced Cell Death

TWEAK (TNFSF12) is a member of the TNF family, which has been found to regulate IEC cell death during T helper 2 (interleukin 13)–mediated experimental colitis.[45,46] Furthermore, TWEAK has been shown to sensitize cells to Fas/CD95 ligand- and TNF-induced cell death by promoting the sequestration and degradation of TRAF2 and cIAP1.[28,29,47] We set out to test whether TWEAK-induced cIAP1 degradation could also sensitize IEC to TNF-induced cell death. While TWEAK alone only weakly induced cell death in YAMC cells, it potently enhanced TNF-induced cell death (Figure 4*A*). This observation was also confirmed in primary mouse intestinal organoids (Figure 4*C*) and a similar observation was made in human organoids (Figure 4*D*). In contrast, no sensitization to cisplatin-induced cell death was observed (Figure 4*B*). To test the relevance of this finding in vivo, wild-type mice were either injected with TWEAK or TNF alone, or the combination of both. While TWEAK alone failed to induce significant apoptosis induction in intestinal crypts, it significantly enhanced TNF-induced crypt cell apoptosis, as measured by the detection of caspase 3–positive cells (Figure 4*D*). These findings were confirmed by detection of increased cleaved lamin A–positive crypt cells upon co-treatment with TNF and TWEAK, though only a trend toward significant differences was seen (Supplementary Figure 2)

X Linked Inhibitor of Apoptosis Protein Is Not Involved in the Regulation of Tumor Necrosis Factor Induced Intestinal Epithelial Cell Death

Because Smac released from the mitochondria also inhibits XIAP and thereby enhances the mitochondrial apoptosis pathway, we tested the role of XIAP in the regulation of TNF-induced intestinal crypt cell apoptosis. However, TNF-induced apoptosis, as analyzed by the detection of cleaved caspase 3–positive cells, was comparable in both wild-type and XIAP-null mice (Figure 5*A* and *B*), indicating that regulation of TNF-induced apoptosis by XIAP is not relevant in intestinal crypts. Because XIAP has been implicated in the regulation of death receptor-induced apoptosis of type II cells,[30] we also analyzed TNF-induced cell death induction in the liver. Yet, TNF did not promote a significant increase in alanine transaminase in the serum or apoptotic cells in liver tissue (Supplementary Figure 3), indicative of liver damage, either in wild-type or in XIAP-null mice (Figure 5*C*). In comparison, injection of lipopolysaccharide

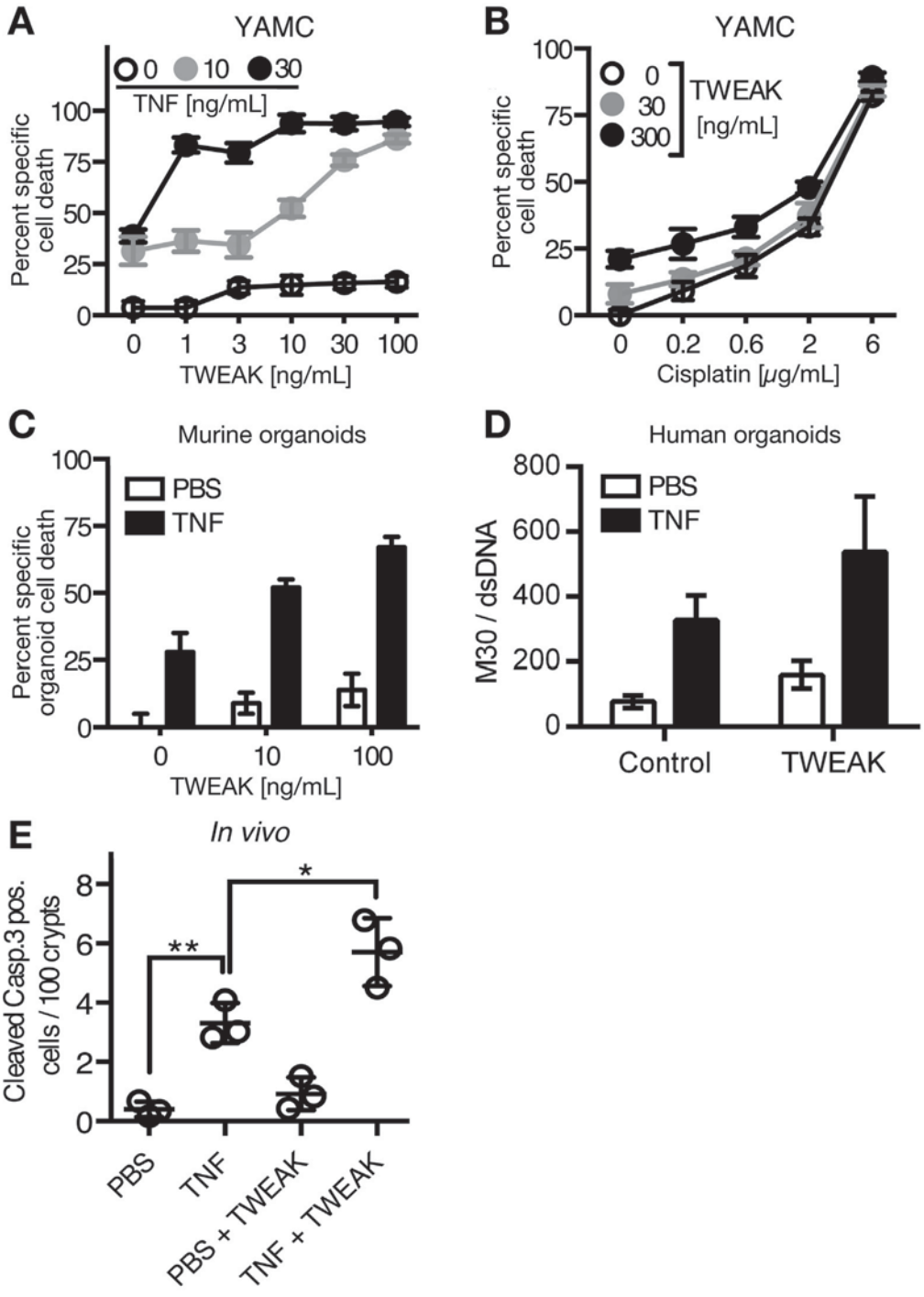


Figure 4. TWEAK sensitizes IECs to TNF-induced cell death. YAMC cells were pretreated with indicated concentrations of TWEAK, and then treated with either TNF (*A*) or the chemotherapeutic agent cisplatin (*B*). Cell death was measured by MTT reduction. Mean ± SD values of triplicates are shown (n = 3). Mouse organoids (*C*) and human organoids (*D*) were pretreated with TWEAK, and stimulated with phosphate-buffered saline (PBS) or TNF. Mean ± SD values of triplicates are shown (n = 2). (*E*) Mice were injected with PBS or TWEAK (200 μg), and PBS or 10 μg TNF (n = 3 mice per group). The number of cleaved caspase 3 positive cells per 100 crypts was quantified. **P* < .05; ***P* < .01.

plus D-galactosamine caused strong liver damage (Figure 5*C* and Supplementary Figure 3).

cIAP1 is a Critical Regulator of Tumor Necrosis Factor Induced Intestinal Epithelial Cell Apoptosis

To test the role of cellular IAPs in the regulation of TNF-induced IEC cell death, we analyzed TNF-induced cell death in the absence of cIAP1. Injection of cIAP1-null mice with TNF resulted in a massive increase of IEC apoptosis, as demonstrated by the high frequency of cleaved caspase 3−positive cells in the small and large intestine of cIAP1-null mice in comparison to wild-type mice (Figure 6*A* and *B*). Histologic analysis showed that 5 hours post TNF injection, massive crypt cell death was observed, and large numbers of mature IECs were shed into the gut lumen, leading to excessive epithelial erosion (Supplementary Figure 4*A* and *B*). Whereas wild-type mice survived 24 hours and longer, despite the significant induction of IEC cell death, cIAP1-null mice had to

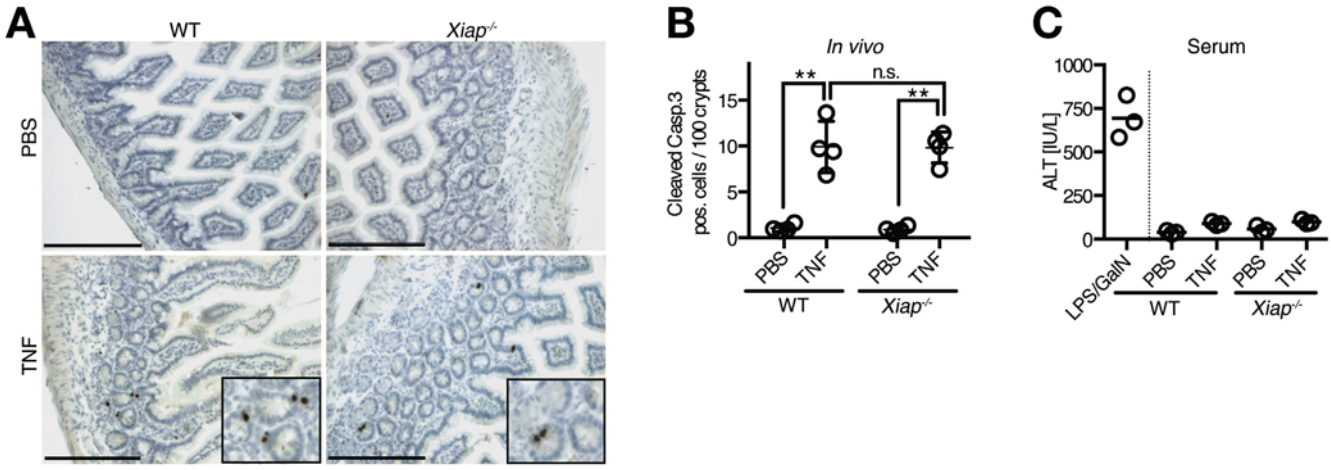


Figure 5. XIAP is not involved in the regulation of TNF-induced IEC cell death. (*A*) Wild-type (WT) and *Xiap*$^{-/-}$ mice were injected with phosphate-buffered saline (PBS) or TNF (4 μg) and cleaved caspase 3 positive cells in the small intestine was analyzed (n = 4 per group; scale bar = 150 μm). *Inserts* show magnification. (*B*) Quantification of cleaved caspase 3 positive cells in intestinal crypts. $^{**}P < .01$; NS, not significant. (*C*) Serum transaminase (alanine aminotransferase [ALT]) activity of TNF-treated WT and *Xiap*$^{-/-}$ mice (n = 3 per group). Serum from lipopolysaccharide plus D-galactosamine-treated mice served as positive control. Individual data points and mean values are shown.

be euthanized after 5 hours post TNF injection due to moribundity. Although cIAP1-null mice were exquisitely sensitive to TNF, no signs of increased spontaneous apoptosis were evident in control treated mice (Figure 6*A* and *B*). Of interest, the mortality of cIAP1-null mice appeared to be predominantly mediated by the massive induction of IEC cell death and associated enteropathy, as few signs of liver damage were seen in either wild-type or cIAP1-null mice (Figure 6*C* and Supplementary Figure 3). The increased sensitivity of cIAP1-null intestinal epithelial cells was also confirmed in mouse intestinal organoid cultures, which showed a comparable TNF sensitivity as wild-type organoids treated with LCL161 (Figure 6*D*). In contrast, when cIAP1 was inducibly expressed in YAMC cells, increased resistance to TNF was observed (Supplementary Figure 5). An increased endogenous production of TNF due to the activation of the non-canonical NFκB pathway[48] could be excluded as a cause for increased IEC cell death, as serum TNF levels as well as intestinal expression levels of TNF messenger RNA in cIAP1-null mice were comparable to that in wild-type and Xiap-deficient mice (Supplementary Figure 6). To further analyze the respective role of TNF receptors in this massive induction of apoptosis in cIAP1-null mice, intestinal tissue damage was analyzed in *Birc2*$^{-/-}$ *x Tnfrsf1a*$^{-/-}$ (cIAP1 x TNFR1-null) and *Birc2*$^{-/-}$ *x Tnfrsf1b*$^{-/-}$ (cIAP1 x TNFR2-null) double-deficient mice. Whereas deletion of *Tnfrsf1b* had no impact on TNF-induced tissue damage, complete protection was seen in *Tnfrsf1a*-deficient mice (Figure 6*E* and *F*), supporting again the idea that TNFR1 is the critical cell death–promoting TNF receptor.

cIAP2 Is Not Involved in the Regulation of Tumor Necrosis Factor Induced Intestinal Epithelial Cell Apoptosis

cIAP1 and 2 have often, but not always, overlapping activities.[31,48] However, when cIAP2-null mice were injected with TNF, cell death induction in the intestinal crypts was found to be comparable to that observed in wild-type animals (Figure 7*A* and *B*), indicating that cIAP2 has no crucial role in regulating TNF-induced cell death in IECs. In comparison, an approximately 10-fold higher frequency in TNF-induced crypt cell death was observed in cIAP1-null mice treated at the same time or in a separate experiment (Figure 6*B* and 7*B*). This finding was confirmed in mouse intestinal organoid cultures (Figure 7*C*).

Discussion

TNF is an important pro-inflammatory and disease-promoting cytokine involved in the pathogenesis of numerous inflammatory disorders. Its role in the pathogenesis of IBD is particularly well studied, and it is an important therapeutic target in Crohn's disease and ulcerative colitis.[49,50] It can either be directly inhibited by neutralizing antibodies or soluble receptors or targeted indirectly by glucocorticoids, which prevent NF-κB activation, TNF expression and associated TNFR-induced inflammatory processes. TNF mediates its deleterious effects via different mechanisms. On one hand, TNF promotes expression of pro-inflammatory cytokines, chemokines, and adhesion molecules, mostly via NF-κB -dependent processes, and thereby induces the recruitment of immune cells to target tissues. Associated immune cell activation and effector functions, in turn, often result in excessive tissue damage. However, TNF can also have direct destructive and cytotoxic activities on cells. TNF-induced signaling in IECs leads to activation of the myosin light-chain kinase, and associated disruption of tight junctions.[51] The increase in intestinal epithelial barrier permeability often results in penetration of bacteria and bacterial products, stimulation of the immune system, and triggering of a vicious cycle of inflammation and tissue destruction. Therefore, understanding of

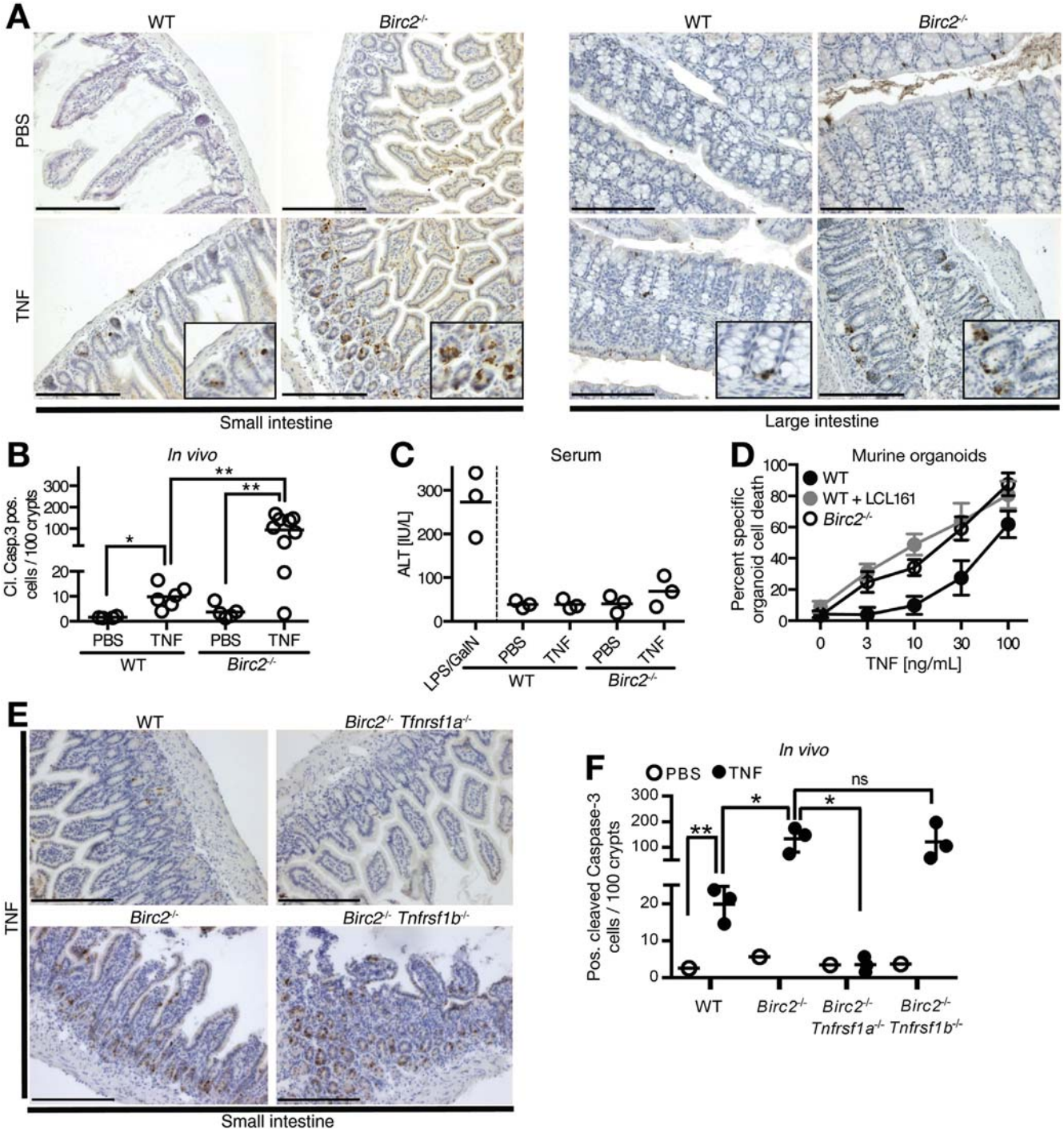


Figure 6. cIAP1 is a critical regulator of TNF-induced IEC apoptosis. (*A*) Wild-type (WT) or cIAP1-null (*Birc2*$^{-/-}$) mice were treated with PBS or TNF (4 μg), and apoptosis in small and large intestine was detected using cleaved caspase 3 immunohistochemistry (n = 6 per group; scale bar = 150 μm). *Insets* show magnifications. (*B*) Apoptotic cells within the crypt region of tissue from the same experiment were quantified. *$P < .05$; **$P < .01$. Mean values and individual data points are shown. (*C*) Serum transaminase activity of TNF-treated WT and *clap1*$^{-/-}$ mice (n = 3 per group). Alanine aminotransferase (ALT) values of lipopolysaccharide plus D-galactosamine-treated WT mice served as positive control for excessive liver damage. Mean values and individual data points are shown. (*D*) Organoids from WT or cIAP1-null (*Birc2*$^{-/-}$) mice were treated with indicated concentrations of TNF. WT organoids were also pretreated with LCL161 (60 nM). Cell death was measured by MTT reduction. Mean ± SD values of triplicates of a representative experiment are shown (n = 2). (*E*) WT, *Birc2*$^{-/-}$ (cIAP1-null), *Birc2*$^{-/-}$ *Tnfrsf1a*$^{-/-}$ (cIAP1-null TNFR1-null), and *Birc2*$^{-/-}$ *Tnfrsf1b*$^{-/-}$ (cIAP1-null TNFR2-null) mice were injected with 4 μg TNF and cell death in small intestine was detected using cleaved caspase 3 immunohistochemistry. (*F*) Quantification of experiment shown in (E) (n = 3 per TNF-treated group. *$P < .05$; **$P < .01$.

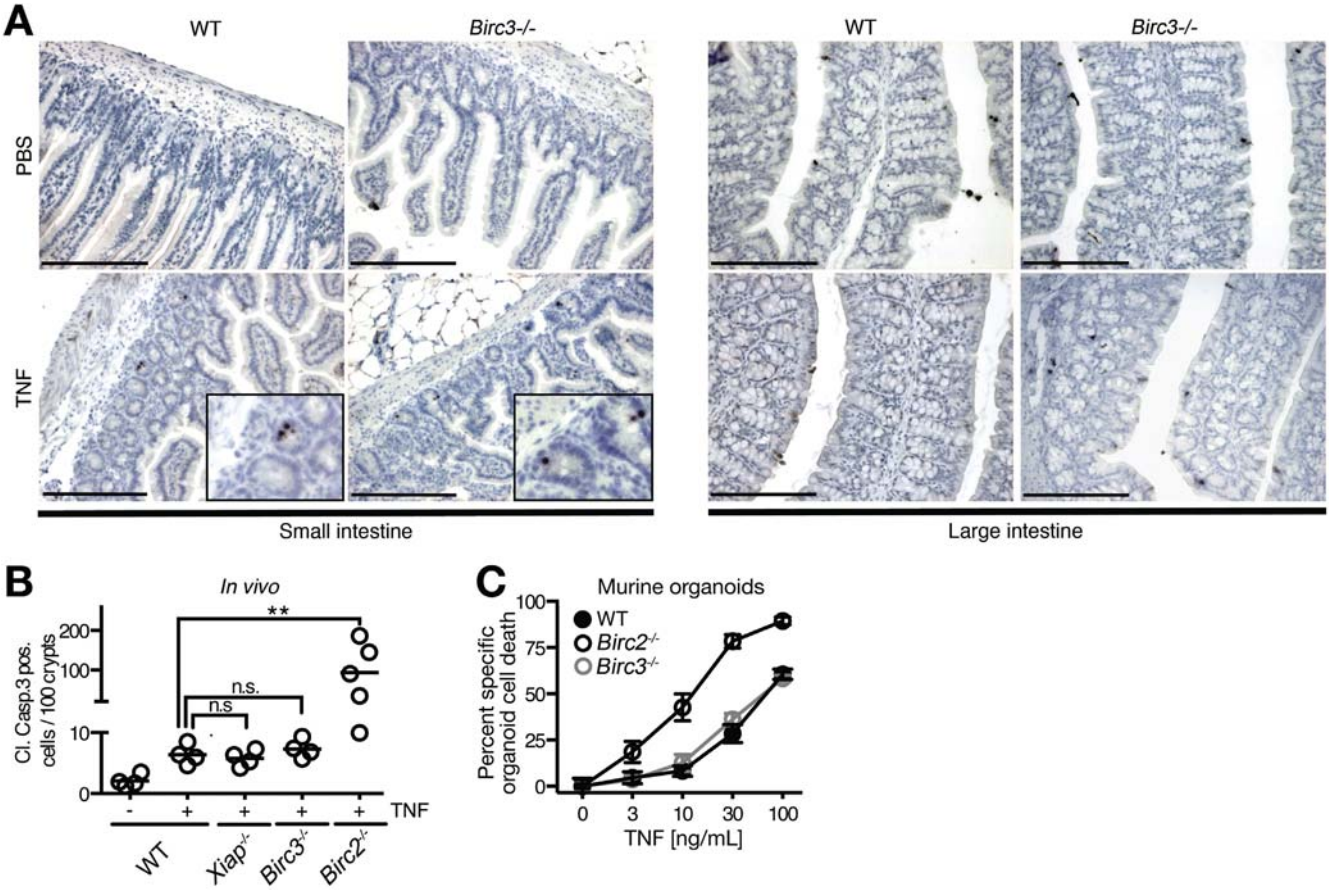


Figure 7. cIAP2 is not critical for TNF-mediated IEC cell death. (*A*) Cleaved caspase 3 immunohistochemistry of small and large intestinal tissue sections from TNF (4 μg) or PBS-injected wild-type (WT) and *Birc3*$^{-/-}$ (cIAP2-null) mice (n = 4 per group; scale bar = 150 μm). (*B*) Quantification of apoptotic crypt cells in small intestinal tissue sections of TNF-treated WT, *Xiap*$^{-/-}$, *Birc3*$^{-/-}$, and *Birc2*$^{-/-}$ mice (n = 3 5 mice per group). Individual data points and mean values are shown. $^{**}P < .01$, NS = not significant. (*C*) Intestinal organoids of WT, *Birc2*$^{-/-}$ and *Birc3*$^{-/-}$ mice were treated with indicated concentrations of TNF. Cell death was determined by MTT reduction. Mean ± SD values of triplicates of a representative experiment (n = 2) are shown.

signaling processes leading to TNF-mediated IEC cell death are very important in order to develop specific therapies.

Here, we have specifically investigated the mechanism of epithelial damage upon exogenous administration of TNF. In contrast to the liver, the intestinal epithelium is exquisitely sensitive to the pro-apoptotic action of TNF. Confirming previous reports,[42,52] we found that TNFR1 rather than TNFR2 is responsible for transducing the pro-apoptotic signals in IEC. We further provide evidence that cIAP1 contributes to the regulation of TNF sensitivity of the intestinal epithelium. Although the intestinal epithelium in wild-type mice shows a pronounced sensitivity to TNF-induced cell death, this is drastically enhanced in the absence of cIAP1. Confirming the critical role of IAPs in regulating TNF sensitivity, we found that treatment of IECs with Smac-mimetics also resulted in increased sensitivity to TNF. Similarly, TWEAK, causing cIAP1 sequestration and degradation,[28,29] strongly enhanced TNF-induced cell death in vitro, ex vivo, and in vivo.

A remarkable observation of this study is that absence of cIAP1, cIAP2, or XIAP alone does not result in excessive spontaneous cell death induction, epithelium destruction, or inflammation, in particular because in humans, mutation of XIAP has been strongly linked to IBD.[27] Although our results show a critical role for cIAP1 in controlling TNF sensitivity, absence of any IAP on its own does not cause chronic stimulation of the NF-κB pathway, associated TNF production, induction of apoptosis or necroptosis, and/or lethality, as seen in RIPK1-, caspase 8-, or FADD-deficient mice.[53–55] However, combined loss of cIAP1 and cIAP2, or cIAP1 and XIAP results in embryonic lethality.[31] Similarly, intestinal epithelium-specific deletion of RIPK1 does not cause the neonatal lethality observed in the complete RIPK1 knockout, but nevertheless results in massive intestinal inflammation and IEC apoptosis, and death within few weeks of life. This massive IEC apoptosis appears to be due to hyper-responsiveness of the intestinal epithelium to TNF/TNFR1 signaling because combined deletion of TNFR1 prevented the intestinal pathology in both *Ripk1*$^{-/-}$ and *Ripk1*$^{IEC\text{-}KO}$ mice.[56,57] In this regard, it is interesting that RIPK1 is ubiquitinated by cIAP1 and 2 in the TNFR complex, which contributes to resistance to TNF-induced cell death.[19] Alternatively, RIPK1 deficiency could also lead to reduced cIAP1 expression levels, as observed by

Dannappel et al[57] and Takahashi et al.[58] This could explain, at least in part, the similarly increased sensitivity of cIAP1- and RIPK1-deficient IECs to TNF-induced cell death. It does not, however, explain why systemic absence of RIPK1 results in neonatal lethality and IEC-specific deletion of *Ripk1* in massive spontaneous intestinal inflammation, whereas cIAP1 deficiency does not cause death or spontaneous TNF-dependent colitis. RIPK1 induces stabilization of TRAF2 and cIAP1, which prevent NF-κB-inducing kinase stabilization and activation of the non-canonical NF-κB pathway, potentially leading to TNF production and TNF-induced cell death.[59] Upon loss of *Ripk1*, IECs produce excessive TNF, which promotes inflammation and apoptosis. Of interest, neither an increase in constitutive TNF expression levels nor spontaneous intestinal inflammation was seen in cIAP1-null mice. Thus, deletion of cIAP1 or any IAP is likely not sufficient to allow spontaneous NF-κB-inducing kinase activation and TNF production and associated IEC cell death.

While we identified cIAP1 as a critical regulator of TNF-induced IEC cell death, it is surprising to note that *Xiap*, but not *Birc2* (cIAP1), gene mutations have been associated with Crohn's disease.[60] Although TNF and associated signaling pathways have been shown to be critically involved in the pathogenesis of IBD, and neutralization of TNF has a significant beneficial effect in Crohn's and colitis patients,[50] we did not observe an increased susceptibility of *Xiap*-deficient mice to TNF-induced IEC apoptosis. More likely, XIAP is involved in innate immune signaling. In this respect, it is interesting that monocytes from patients with *Xiap* mutations within the RING domain respond in a reduced manner to muramyldipeptide, in agreement with a recently described role of XIAP in NOD2 signaling,[61] Thus, the role of XIAP in the regulation of IBD may be more related to NOD2 signaling, rather than control of TNF signaling and associated IEC death. This is highlighted by the fact that mutations in *Nod2* are among the most frequent disease-associated mutations in IBD patients.[62] Although all IAPs (XIAP, cIAP1, and 2) are involved in NOD signaling,[61,63,64] only cIAP1 appears to be critical for TNFR1-induced IEC cell death, respectively survival.

In summary, here we show that cIAP1 is critically involved in the regulation of TNF-induced IEC cell death. Processes that regulate expression and stability of cIAP1 can also regulate the pathogenesis of TNF-mediated enteropathies. Our finding demonstrates a unique and limiting role of cIAP1 in a given signaling pathway, which is not redundant with cIAP2. Of interest is our observation that TWEAK sensitizes IEC to TNF-induced apoptosis, likely in a cIAP1-dependent manner, whereas inhibition of TWEAK signaling in vivo improves experimental colitis,[65] providing further evidence for the proposed mechanism. Because Smac-mimetics likely also sensitize IEC by limiting cIAP1-mediated protection, the potential use of Smac-mimetics in the clinics, for example, for sensitizing cancer cells to apoptosis, may also have potential effects on the integrity of the intestinal epithelium, particularly in the context of simultaneous bacterial infections with high TNF production.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dxdoi.org/10.1053/j.gastro.2016.11.019.

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Author names in bold designate shared co-first authorship.