

BNip3 is a mediator of TNF-induced necrotic cell death

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Abstract Tumor necrosis factor (TNF) is a pleiotropic cytokine involved in immune modulation, inflammatory reactions, and target cell death in many pathologic conditions. The cell death pathways triggered by TNF include the caspase-8/Bid-dependent apoptotic pathway and the caspase-independent necrosis pathway (necroptosis). While the signaling pathways activated after binding of TNF to the TNF receptor (TNFR) and subsequent insertion of Bid/Bax/Bik into the outer mitochondrial membrane are relatively well known, other cell death pathways and the participating signaling molecules remain to be clarified. BNip3 is a pro-death protein and a member of the BH3-only Bcl-2 family. When ectopically overexpressed or induced by hypoxia, BNip3 induces various types of cell death via mitochondrial or non-mitochondrial death cascades. In this study using A549 alveolar epithelial cells of the lung, we show that BNip3 is transcriptionally and translationally upregulated by TNF, and its expression level determines the sensitivity to necroptosis induced by TNF. However, BNip3 does not appear to be involved in caspase-8/Bid-dependent apoptotic cell death in these alveolar lung cells. Finally, we show that the generation of reactive oxygen species (ROS) is essential for mitochondrial insertion of BNip3, which is an important step in BNip3-induced mitochondrial catastrophe. Our results indicate that BNip3 is a candidate therapeutic target in pathologic conditions in which TNF causes tissue damage.

Keywords BNip3 · TNF · Cell death · Reactive oxygen species

Introduction

Tumor necrosis factor (TNF) is a pleiotropic cytokine that is produced by immune cells in response to a range of stimuli such as inflammation, viral infection, or neoplastic transformation. TNF induces cytotoxic effects that lead to target cell death [1, 2], and the death signals initiated by TNF propagate via diverse pathways. Cell death mechanisms include perturbation of mitochondria by the insertion of Bid/Bax/Bik [3–5], production of cathepsin B [6, 7], generation of reactive oxygen species (ROS) [8], or the activation of c-jun N-terminal kinase (JNK) [9, 10]. These result in two distinct forms of cell death: caspase-dependent classical apoptosis or caspase-independent necrosis, termed necroptosis [11, 12]. Necroptosis induced by TNF requires the production of ROS. [13]. In addition, there are several other signaling molecules suggested to play a role in necrotic cell death: RIP1 [14], RIP3 [15], and lysosomal proteases such as calpains and cathepsins [16]. Although these molecules appear to be central in the initiation of caspase-independent necrosis by death receptors including TNFR, the molecular pathways leading to necroptosis are not clearly understood. Moreover, the necrosis effector of RIP1 is unknown.

BNip3, a mammalian Bcl-2 homolog that belongs to a set of pro-apoptotic Bcl-2 family proteins, has been identified as an adenoviral E1B 19K-interacting protein. [17, 18]. Functional inactivation of BNip3 by its interaction with E1B 19K suggests that BNip3 is involved in eradication of virus-infected cells, possibly TNF-mediated target cell removal. Although BNip3 induces various types of cell

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death through different molecular pathways [18–20], sensitization of virus-infected cells to TNF-induced necrotic cell death further increases the possibility of BNip3 involvement in necrotic cell death. Based on this assumption, we hypothesized that BNip3 might play a role in TNF-induced necrotic cell death.

In this study, we investigated whether BNip3 is involved in TNF-induced cell death of respiratory epithelial cells because they are victims of the cytotoxic effect of TNF, as seen in many disorders such as acute lung injury, acute respiratory distress syndrome, and emphysema [21]. Our findings demonstrate that BNip3 is a mediator of TNF-induced caspase-independent necroptosis and a potential therapeutic target in pulmonary pathologic conditions in which TNF exerts cytotoxic effects in respiratory alveolar cells.

Materials and methods

Cell culture and reagents

A549 human lung alveolar epithelial cancer cells, LN18 glioblastoma cells, and U937 lymphoma cells were obtained from the American Type Culture Collection (Rockville, MD, USA), and cultured in RPMI medium containing 10% fetal bovine serum under 5% CO₂. TNF was purchased from Life Technology Inc. (MD, USA). IETD-CHO, z-VAD-FMK, necrostatin-1 [5-(indol-3-ylmethyl)-(2-thio-3-methyl)hydantoin], and SP600125 were purchased from Calbiochem (CA, USA). Anti-BNip3 polyclonal antibody was produced by immunizing a rabbit with keyhole limpet hemocyanin-conjugated amino acid residues 112–124 (KNSDWIWDWSSRP) of human BNip3 (GenBank Accession No., NM_004052) and purification of the antibody from the immune serum by affinity chromatography. Anti-Bid, anti-caspase-3, anti-poly(ADP-Ribose) polymerase, anti-HIF-1 α , anti-tubulin, anti-GFP, and anti-TNFR1 antibodies were purchased from Abcam (Cambridge, UK). RIP1 and RIP3 antibodies were from R&D Systems (Minneapolis, MN) and IMGENEX (San Diego, CA), respectively. Unless specified otherwise, reagents were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA).

Cell death, LDH, and caspase-3 cleavage assays

The amount of cell death induced by TNF in untransfected cells was measured by the Trypan blue exclusion method. Cell death in cells overexpressing particular proteins was evaluated by confocal microscopic (META 510, Zeiss, Germany) analysis of pyknotic, shrunken nuclei or fragmented apoptotic bodies among transfected cells after staining with propidium iodide (PI) (excited at 543 nm

using He–Ne laser and detected with 560 nm band pass filter). A minimum of 500 cells per experiment was counted by three different pathologists. Release of lactate dehydrogenase (LDH) from cells into the culture medium was measured using a LDH assay kit (Calbiochem). Caspase-3 activity was measured using a colorimetric caspase-3 assay kit (Calbiochem) according to the manufacturer's instructions.

Plasmid construction and transient transfection

DNA encoding BNip3 was amplified by polymerase chain reaction (PCR) from a human kidney cDNA library (Clontech Laboratories, Inc., CA, USA) and subcloned into pEGFP or the TA vector tagged with EGFP or myc/V5, respectively (Invitrogen Corporation, CA, USA). Transmembrane domain-deleted BNip3 (BNip3 Δ TM) was constructed by the splicing overlap extension method, as described previously [22]. Cells were transiently transfected with each plasmid using Lipofectamine 2000 transfection reagent according to the manufacturer's protocol (Invitrogen). GFP-expressing cells were observed under confocal microscope (excited at 588 nm using Argon laser and detected with 520 nm band pass filter).

Quantitative RT-PCR

BNip3 and HIF-1 α mRNA levels were quantified by real-time PCR using the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal standard. Total RNA (1 μ g) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Carlsbad, CA) with random hexamer priming. The resultant cDNA was amplified using a LightCycler (Roche Diagnostics Ltd., Lewes, UK). Real-time PCR analysis was carried out with the LightCycler-FastStart DNA Master SYBR Green I mix (Roche Diagnostics) and specific primers for GAPDH, BNip3, and HIF-1 α . The sequences of the gene-specific primers are as follows: BNip3 sense, 5'-TGC TCT GTG AAG GCA TTT AC-3', and antisense, 5'-CAG AAT TGC TTG AAC CTC TC-3'; HIF-1 α sense, 5'-ACT TCT GGA TGC TGG TGA TT-3', and antisense, 5'-GAT GAG TAA AAT CAA ACA CA-3'; GAPDH sense, 5'-TGC TCT GTG AAG GCA TTT AC-3', and antisense, 5'-CAG AAT TGC TTG AAC CTC TC-3'. The expression levels of each mRNA species and their estimated crossing points were determined relative to the standard preparation using LightCycler computer software.

Suppression of BNip3 expression by siRNA

Pairs of 21-nucleotide sense and antisense RNA oligomers were chemically synthesized separately and annealed by

Bioneer Corp. (Daejeon, Korea). The oligonucleotides for BNip3 were as follows: sense, 5'-AAG GAA CAC GAG CGU CAU GAA-3', and antisense, 5'-UUC AUG ACG CUC GUG UUC CUU-3', which corresponds to coding nucleotides 436–456 of human BNip3. The selected sequence was subjected to a BLAST search to ensure that no other sequences in the human genome sequence were targeted. siRNA was introduced into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Mitochondrial preparation and alkali extraction

Mitochondria were freshly isolated from cells by differential centrifugation as described previously [23]. For alkaline extraction, the mitochondrial pellets were resuspended at 1 mg protein/ml in freshly prepared 100 mM Na₂CO₃, pH 11.5 and incubated for 20 min on ice [24]. The membranes were then pelleted by centrifugation at 300,000×g for 30 min at 4°C. Mitochondrial membrane pellets corresponding to 100 µg of protein (the alkali-resistant fraction) and the corresponding volume of supernatant (the alkali-sensitive fraction) were separated by 12% SDS-PAGE and subjected to immunoblot analysis using an anti-BNip3 polyclonal antibody.

Immunoblotting and densitometric analysis

Cells were harvested and suspended in lysis buffer containing 10 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 1.1 mM Na₃VO₄, and 10 mM NaF. Cell fractionation for nuclear HIF-1 was performed as described [25]. Extracted proteins were separated by SDS-PAGE on 12% polyacrylamide gels and electrophoretically transferred to nylon membranes. The membranes were probed with primary antibody, followed by incubation with horseradish peroxidase (HRP)-coupled secondary antibody. Detection was performed with a chemiluminescence system (Amersham Pharmacia Biotech., NJ, USA). Densitometric analysis (Perkin-Elmer, USA) was performed for quantitative evaluation of the amount of protein produced.

Flow cytometric measurements of ROS

To quantify the production of ROS, cells were suspended in PBS containing 5 µM dichlorofluorescein diacetate (DCFH-DA), followed by incubation at 37°C for 30 min. After washing with PBS, cells were analyzed by cytomics FC500 using CXP software (Beckman Coulter, CA, USA).

Statistical analysis

All data are presented as mean ± SD from three or more independent experiments. Statistical significance was assessed with one-way ANOVA, followed by Dunnett's multiple-range test. Differences with a *p* value less than 0.05 were considered statistically significant.

Results

TNF induces necrotic cell death in A549 adenocarcinoma cells of the lung

Since TNF exerts its cytotoxic effects via specific binding to the receptor TNFR1, we initially determined the expression of TNFR1 in A549 cells. As shown in Fig. 1a, both TNFR1 mRNA (upper panel) and protein (lower panel) are expressed in these cells. Binding of TNF to TNFR1 can trigger one of two distinct forms of cell death: classical caspase-dependent apoptosis or caspase-independent necrotic cell death [11]. To determine the cytotoxic effects and the type of cell death facilitated by TNF in A549 cells, we treated cells with TNF and analyzed cell death based on morphological and biochemical assays. The death rate of A549 cells increased with increasing concentration of TNF (Fig. 1b), and cell death was characterized by cytoplasmic condensation and pyknotic nuclei without apoptotic body formation (Fig. 1c). Dose-dependent release of LDH into the culture medium was also noted (Fig. 1d); this is frequently encountered after necrotic cell death due to rupture of the cell membrane. However, we did not observe any significant increase in caspase activity or evidence of Bid cleavage to tBid, compared with etoposide-treated positive control cells or TNF-treated LN18 cells (Fig. 1e, f). Consistent with these findings, necrostatin-1 (Nec-1), an inhibitor of necroptosis [26], significantly decreased TNF-induced cell death and LDH release, but the pan-caspase inhibitor zVAD-FMK did not (Fig. 1b–d). Thus, TNF triggers necrotic rather than apoptotic cell death in A549 cells.

TNF increases the expression of BNip3 in A549 cells

Next, to determine whether TNF affects the expression levels of BNip3, we performed real-time RT-PCR and Western blot analysis using mRNA and cell lysates from TNF-treated A549 cells. As shown in the left panel of Fig. 2a, we observed a time-dependent increase in BNip3 transcript levels (up to approximately 4-fold) in response to TNF treatment. Representative RT-PCR results are shown in the right panel. Consistent with the RT-PCR data, protein levels increased approximately 4.5-fold (dimer) and

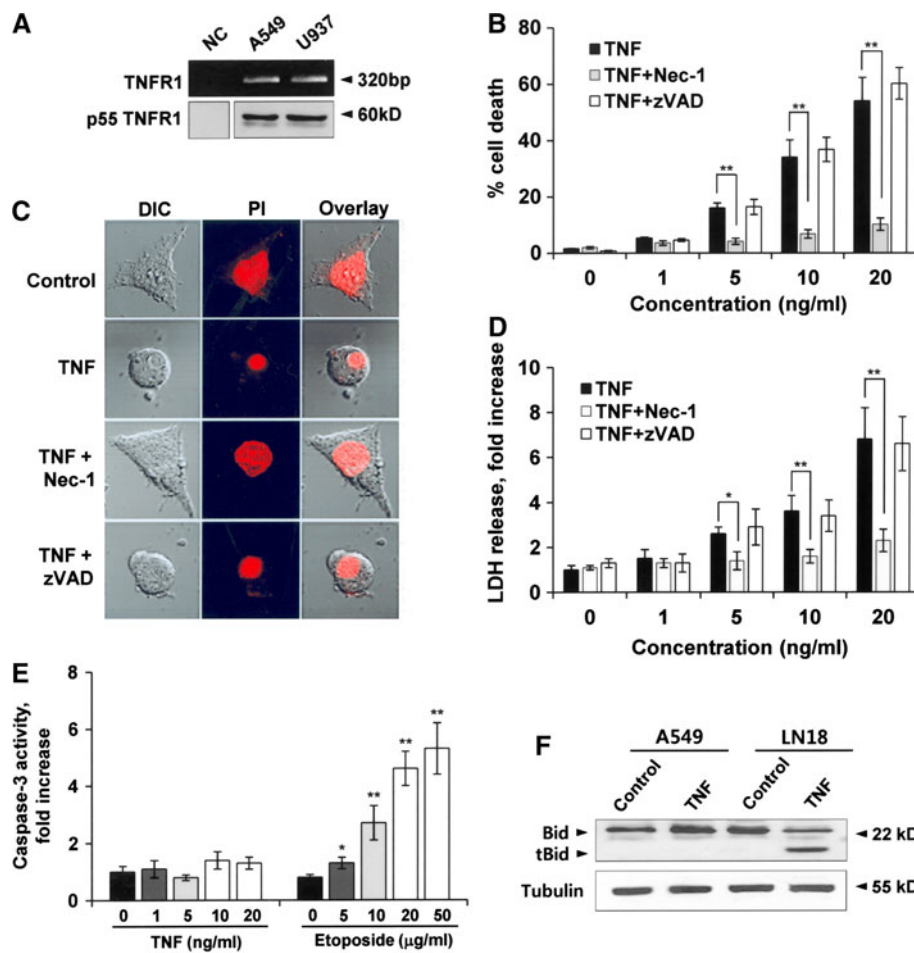


Fig. 1 TNF induces necroptosis in A549 cells. **a** Expression of p55 TNFR1 mRNA and protein. mRNA and protein extracted from the indicated cells was subjected to RT-PCR (*upper panel*) or Western blot (*lower panel*), respectively. As a negative control (lane NC), PCR or Western blot was performed without cDNA or primary antibody, respectively. U937 cells were used as a positive control for TNFR1. **b** Cell death triggered by TNF was significantly inhibited by necrostatin (Nec)-1. A549 cells were treated with the indicated concentration of TNF in the presence or absence of 20 μM necrostatin-1 or 50 μg/ml zVAD-FMK (zVAD) for 48 h, and cell death was assessed by trypan blue exclusion. **c** Cells were treated with 10 ng/ml TNF for 48 h in the presence or absence of 20 μM necrostatin-1 or 50 μg/ml zVAD-FMK, and viewed with a Nomarski differential interference contrast (DIC)-equipped inverted confocal

microscope after nuclear staining with PI. The fluorescence was excited at 543 nm and detected with 560 nm band pass filter using a cooled CCD camera. **d** Cells were treated as in (c) and LDH release into the culture medium was measured. Results are shown as the fold increase compared to TNF-untreated control cells. **e** Cells were treated with the indicated concentration of TNF or etoposide for 48 h, and caspase-3 activity was determined as described in the “Materials and methods” section. Etoposide was used as a positive control of apoptosis. **f** Lysates from A549 and LN18 cells, untreated or treated with 10 ng/ml of TNF for 24 h, were subjected to Western blot analysis to detect cleaved Bid. Tubulin was used as a loading control. All experiments were performed at least three times, and the means ± SD or representative images are shown. * $P < 0.05$, ** $P < 0.01$

2.7-fold (monomer) compared with levels observed in control cells after 48 h of TNF treatment (Fig. 2b, left panel). Representative blots are shown in the right panel. However, neither mRNA nor protein levels of HIF-1α, a key transcriptional regulator of BNip3 in response to hypoxia, were significantly altered (Fig. 2c). Taken together, our data indicate that binding of TNF to TNFR1 in A549 cells results in transcriptional upregulation of BNip3 independent of HIF-1α.

BNip3 overexpression induces caspase-independent necrosis-like cell death in A549 cells

BNip3 induces apoptotic, necrotic, or autophagic cell death via diverse molecular pathways depending on the cell type or experimental conditions [18–20]. We therefore investigated the characteristics of BNip3-induced cell death in A549 cells. Cells were transfected with GFP-tagged BNip3 (pEGFP-BNip3)- or Bax-expressing plasmid (pcDNA-Bax),

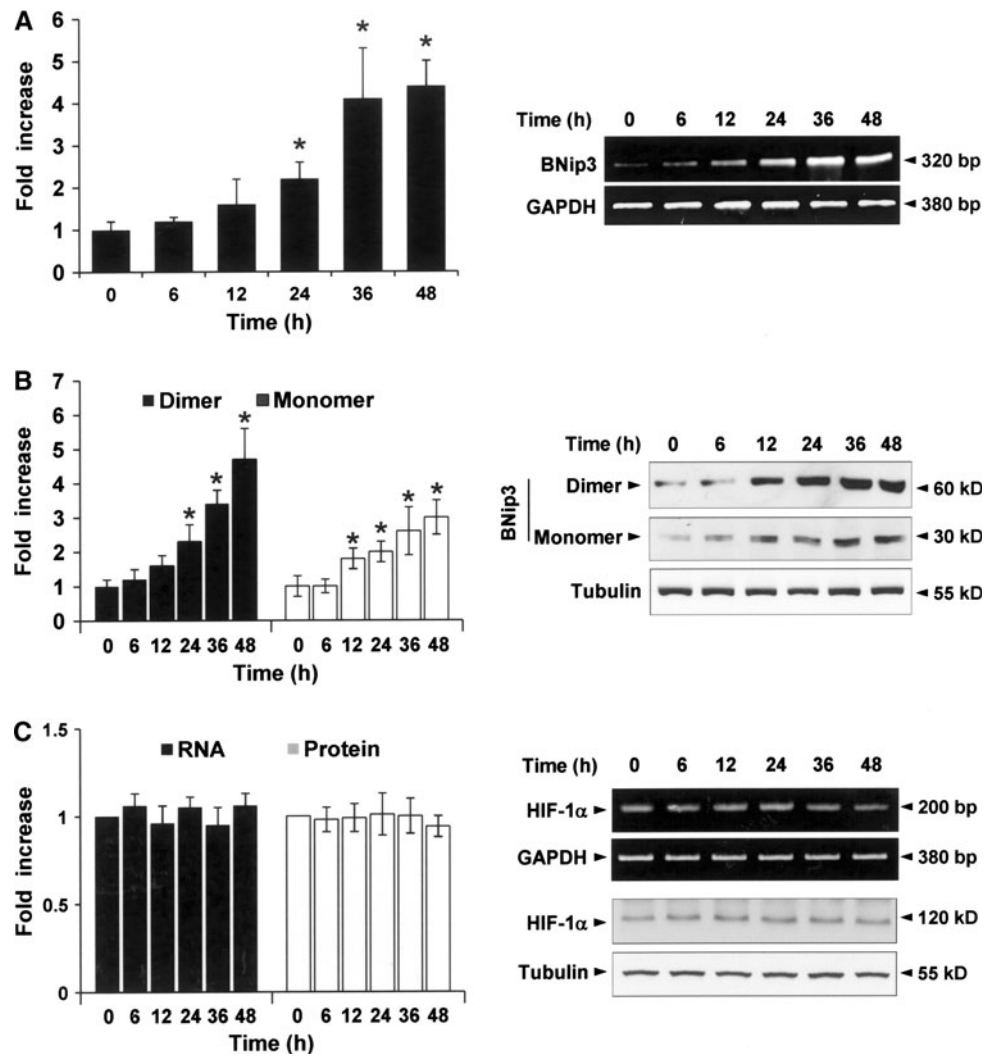


Fig. 2 TNF upregulates mRNA and protein expression of BNip3. **a** mRNA from A549 cells treated with 10 ng/ml TNF for the indicated times was quantitated using real-time RT-PCR as described in the “Materials and methods” section (*left panel*). Data are shown as the fold increase compared to control cells. Representative RT-PCR results are shown in the *right panel*. **b** Lysates extracted from A549 cells treated with 10 ng/ml TNF for the indicated times were subjected to Western blotting and densitometric quantification was performed (*left panel*) after normalization to tubulin. Representative Western blot results are shown in the *right panel*. **c** mRNA of (a) or

lysate of the nuclear fraction of (b) was subjected to real-time RT-PCR or Western blot analysis to detect HIF-1 α transcripts or translates, respectively. Real-time PCR results and densitometric quantification of Western blots are shown as fold increase compared with control (*left panel*). Representative RT-PCR and Western blot results are shown in the *right panel*. GAPDH and tubulin were used as loading controls for RT-PCR and Western blot experiments, respectively. All experiments were performed at least three times, and the means \pm SD or representative images are shown. * $P < 0.01$

or empty GFP plasmid (pEGFP), and morphologic and biochemical assessments were performed. Cells that overexpressed BNip3 began to die 24 h after transfection, and approximately 60% of cells overexpressing BNip3 were dead after 72 h (Fig. 3a). Dying cells were shrunken, and their nuclei were small and condensed without typical apoptotic body formation (Fig. 3b). Release of LDH into the culture medium implying rupture of cell membrane was detected (Fig. 3c). However, we did not detect caspase

cleavage activity (data not shown). In line with these findings, necrostatin-1 effectively suppressed BNip3-induced cell death and LDH release while the pan-caspase inhibitor zVAD-FMK did not (Fig. 3a). At this concentration of caspase inhibitor, cell death induced by Bax overexpression was suppressed (Fig. 3a). These results suggest that BNip3 induces caspase-independent necrosis-like cell death similar to that encountered after treating cells with TNF.

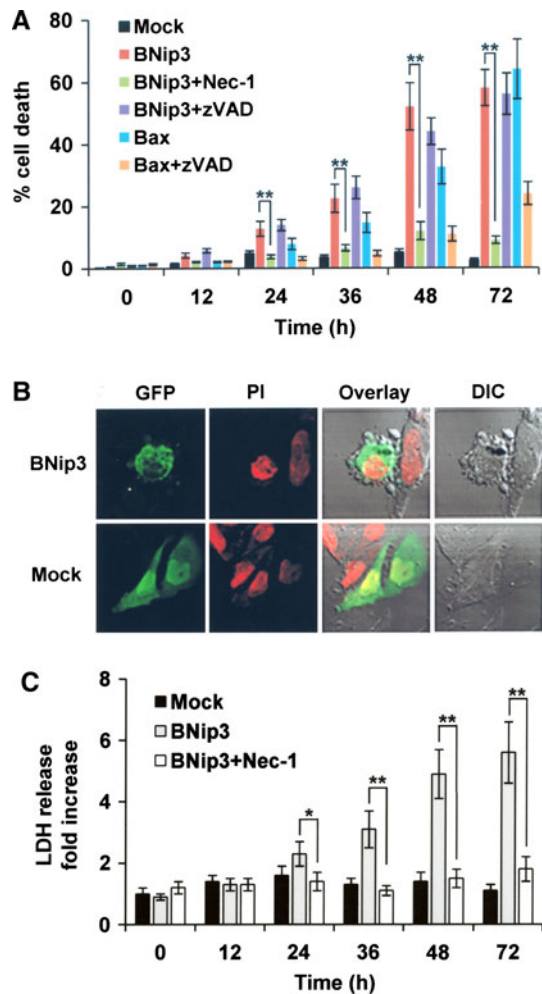


Fig. 3 BNip3 induces caspase-independent necrotic cell death. Cells were transfected with empty pEGFP (mock), pEGFP-BNip3 (BNip3), or pcDNA-Bax (Bax) expression plasmid ($1 \mu\text{g}/10^6$ cells) in the presence or absence of necrostatin-1 ($20 \mu\text{M}$) or zVAD-FMK ($50 \mu\text{g}/\text{ml}$). **a** After the indicated times, dead cells with shrunken pyknotic nuclei were counted among at least 500 transfected cells and the rate of cell death was calculated. **b** Forty-eight hours after transfection, GFP-expressing cells were viewed (excited at 588 nm using Argon laser and detected with 520 nm band pass filter) with a DIC-equipped inverted confocal microscope after nuclear staining with PI (excited at 543 nm using He-Ne laser and detected with 560 nm band pass filter). **c** Cells were treated as in (a) and LDH release into the culture medium was measured after the indicated times. Results are shown as the fold increase compared to untransfected control cells. All data are shown as means \pm SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$

The expression level of BNip3 determines the sensitivity of A549 cells to TNF

Because the BNip3-induced death of A549 cells had many features in common with that induced by TNF, we hypothesized that BNip3 may participate in cell death pathways activated by TNF. To this end, we evaluated the

effects of different expression levels of BNip3 on cellular susceptibility to TNF. A549 cells were transfected with increasing amounts of empty pEGFP or pEGFP-BNip3 plasmid. Twelve hours after transfection, cells were treated with 1 ng/ml TNF, a concentration at which cell viability did not decrease significantly within our experimental time frame (Fig. 1b). Twenty-four hours after TNF treatment, cell death rates were determined by counting the number of dead cells among BNip3-overexpressing cells. As shown in Fig. 4a, cell death rates increased in proportion to the level of ectopic BNip3 expression to approximately 40% with transfection of $2 \mu\text{g}$ pEGFP-BNip3. In contrast, without TNF treatment, only 21% of BNip3-overexpressing cells were dead after 24 h (Fig. 4a). The levels of ectopically expressed BNip3 are shown in Fig. 4b. We also performed Western blotting for RIP1 and RIP3 to show that sensitivity to TNF was not affected by these proteins (Fig. 4b, lower panel). These results suggest that BNip3 sensitizes cells to TNF. This was confirmed by functional inactivation of BNip3 and a knockdown approach using a dominant negative (DN) transmembrane domain (TM)-truncated form of BNip3 (pEGFP-BNip3 ΔTM) [27, 28] and small interference RNA (siRNA), respectively. Cells were transfected with increasing amounts of pEGFP-BNip3 ΔTM for 24 h, treated with 20 ng/ml TNF for an additional 48 h, and then the number of dead cells among the transfected cells was counted. As shown in Fig. 4c, expression of ectopic BNip3 ΔTM decreased the sensitivity of A549 cells to TNF. The levels of ectopically expressed BNip3 ΔTM , RIP1, and RIP3 are shown in Fig. 4d. Furthermore, we transfected cells with siRNA targeting BNip3 (siBNip3) or scrambled siRNA (siCon) to downregulate endogenous BNip3 expression, and then determined the effects of TNF on cells. Consistent with the functional inactivation assay using BNip3 ΔTM , downregulation of BNip3 expression by siBNip3 transfection also decreased the sensitivity of A549 cells to TNF (Fig. 4e). The expression levels of endogenous BNip3, RIP1, and RIP3 are shown in Fig. 4f. Our data suggest that BNip3 expression level is an important determinant of TNF-induced cytotoxicity in A549 cells.

BNip3 is inserted into mitochondria in response to death signals triggered by TNF

BNip3 is normally loosely attached to mitochondria, but can readily integrate into the outer mitochondrial membrane following death-triggering stimuli such as hypoxia [29]. To further investigate the role of BNip3 in TNF-induced cell death pathways, mitochondria isolated from TNF-treated or -untreated A549 cells were incubated with

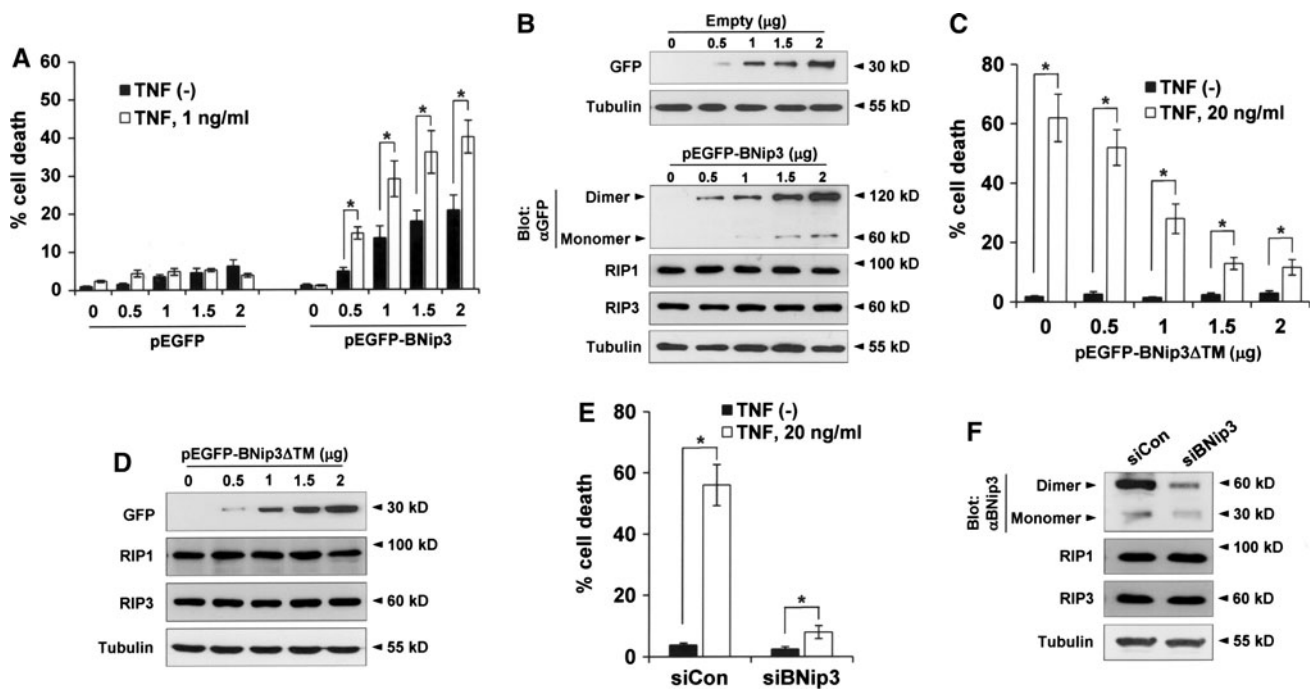


Fig. 4 The expression level of BNip3 protein in A549 cells determines sensitivity to TNF. **a** Cells were transfected with the indicated amounts of empty pEGFP or pEGFP-BNip3 expression plasmid. Twelve hours after transfection, cells were left untreated or further treated with 1 ng/ml TNF for an additional 24 h, then dead cells were counted among 500 BNip3-overexpressing cells after nuclear staining with PI. **b** Cell lysates of (**a**) were subjected to Western blot analysis using anti-EGFP antibody for the quantitative determination of GFP (*upper panel*) or GFP-BNip3 (*lower panel*) expression. Western blot for RIP1 or RIP3 was performed to determine the expression level of each protein. Tubulin was used as a loading control. **c** Cells were transfected with the indicated amounts of pEGFP-BNip3 Δ TM for 24 h, and further incubated with or without 20 ng/ml TNF for an additional 48 h. At least 500 cells overexpressing BNip3 Δ TM were counted and the rate of cell death was

determined. **d** Cell lysates of (**c**) were subjected to Western blot analysis using anti-EGFP antibody for quantitative determination of BNip3 Δ TM expression. Western blot for RIP1 or RIP3 was performed to determine the expression level of each protein. Tubulin was used as a loading control. **e** Cells were transfected with 100 nm siRNA targeted to BNip3 (siBNip3) or control siRNA (siCon) for 48 h, and further incubated with or without 20 ng/ml TNF for an additional 48 h. At least 500 cells were counted after nuclear staining with PI and the rate of cell death is shown. **f** Cell lysates of (**e**) were subjected to Western blot analysis using anti-BNip3, anti-RIP1, or anti-RIP3 antibodies to validate downregulation of endogenous BNip3 or to determine the levels of RIP1 and RIP3 after TNF treatment. Tubulin was used as a loading control. All experiments were performed at least three times and the results are shown as mean \pm SD of three or more independent experiments. * $P < 0.01$

alkaline buffer to dislodge loosely membrane-associated protein, as described previously [19], and then subjected to Western blot analysis for BNip3. As shown in the left panel of Fig. 5a, after alkali extraction BNip3 was predominantly detected in the supernatant of TNF-untreated control cells, but was readily detectable in the mitochondrial compartment of TNF-treated cells (right panel). Next, to compare this finding with the previous cell death assays, cells were treated with TNF in the presence or absence of necrostatin-1 or zVAD-FMK, and BNip3 insertion into mitochondria was determined after alkali extraction. Consistent with the cell death assay, mitochondrial insertion of BNip3 in response to TNF was inhibited by necrostatin-1 (Fig. 5b), but not by zVAD-FMK (Fig. 5c). These results indicate that TNF-induced death signaling is sufficient to induce the integration of BNip3 into the mitochondrial membrane in a necrostatin-1-dependent manner.

Generation of reactive oxygen species occurs upstream of mitochondrial insertion of BNip3 in response to TNF

TNF facilitates cell death via the activation of downstream molecules including Jun N-terminal kinase (JNK) or ROS [9, 30]. In addition, RIP1, which plays a pivotal role in receptor-induced necroptosis, exerts its effects by generation of ROS [31]. Therefore, to determine the identity of the upstream signal(s) that activates BNip3, we investigated whether stimulation of the JNK pathway or ROS production following binding of TNF to TNFR1 are required for insertion of BNip3 into mitochondria. To this end, A549 cells were treated with TNF in the presence or absence of SP600125, a specific JNK inhibitor, or the antioxidant *N*-acetylcysteine (NAC), and BNip3 insertion into mitochondria was analyzed. As shown in Fig. 6a, BNip3 was present exclusively in the mitochondrial fraction of

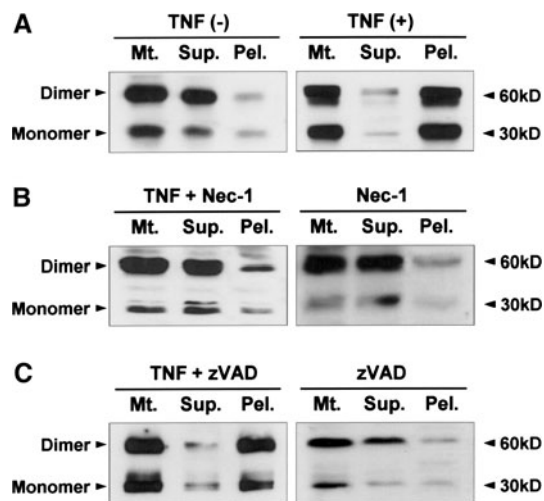


Fig. 5 Mitochondrial insertion of BNip3 in response to death signals triggered by TNF. **a** Cells were untreated (*left panel*) or treated (*right panel*) with 20 ng/ml TNF for 12 h, and mitochondria were isolated. After exposure to alkali buffer as described in the “Materials and methods” section, total proteins from the supernatant (Sup.) and mitochondrial pellets (Pel.) were subjected to Western blot analysis for BNip3. Mt., mitochondrial proteins before alkali exposure. **b** Cells were treated with 20 μ M necrostatin-1 with (*left panel*) or without (*right panel*) 20 ng/ml TNF for 12 h. Mitochondrial isolation and alkali extraction were performed as in (**a**). **c** Cells were treated with 50 μ g/ml zVAD-FMK with (*left panel*) or without (*right panel*) 20 ng/ml TNF for 12 h. Mitochondrial isolation and alkali extraction were performed as in (**a**). Representative blots of four independent experiments are shown

TNF-treated cells after alkali treatment irrespective of the presence of SP600125. In contrast, treatment of cells with NAC inhibited the movement of BNip3 from the cytosolic compartment to mitochondria (Fig. 6b). In line with the results of BNip3 mitochondrial insertion, NAC decreased the rate of TNF-induced cell death whereas SP600125 failed to suppress cell death by TNF in a time course analysis (Fig. 6c). To further show that ROS are an important executor of mitochondrial BNip3 insertion, we determined ROS levels in NAC- or necrostatin-1-treated and untreated cells. Flow cytometric analysis using DCFH-DA showed suppression of ROS generation by NAC (Fig. 6d, left panel) or necrostatin-1 (Fig. 6d, right panel). Together, our results suggest that ROS generated by TNF are required for BNip3 insertion into mitochondria.

BNip3 is not involved in the classical apoptotic pathway activated by TNF

BNip3 induces caspase-dependent apoptosis, caspase-independent necrosis-like cell death, or genetically regulated autophagic cell death [19, 20]. The data presented above demonstrate that BNip3 is involved in the necrotic death of A549 cells. To determine whether BNip3 also

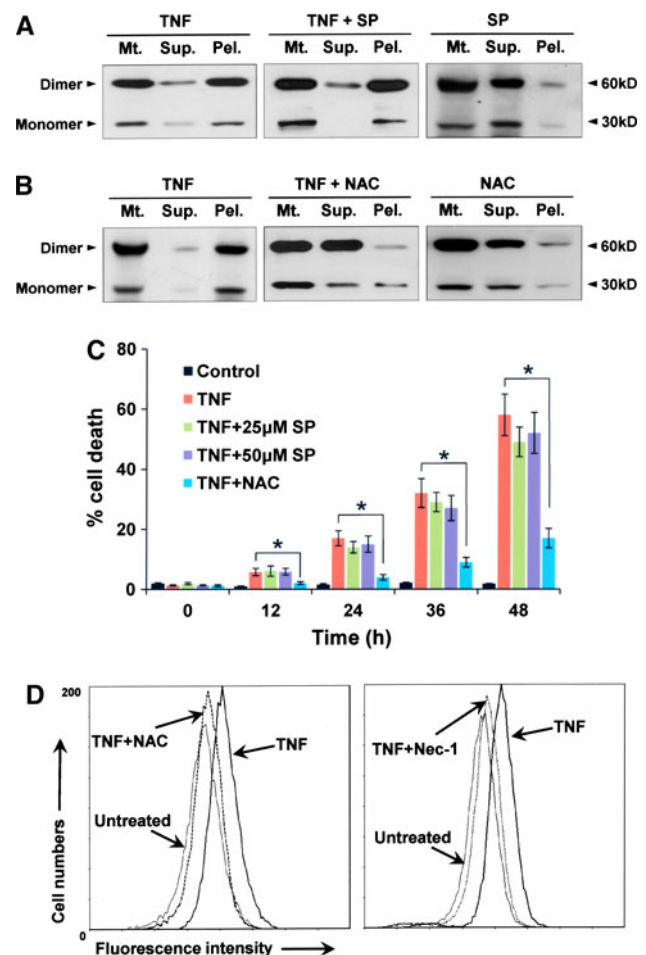


Fig. 6 TNF-induced mitochondrial insertion of BNip3 is dependent on reactive oxygen species. **a** Cells were treated with 20 ng/ml TNF in the presence (*middle panel*) or absence (*left panel*) of 50 μ M SP600125 (SP), or with SP only (*right panel*) for 12 h. Alkali extraction and Western blot analysis were performed as described in Fig. 5. **b** Cells were treated with 20 ng/ml TNF in the presence (*middle panel*) or absence (*left panel*) of 5 mM N-acetylcysteine (NAC), or with NAC only (*right panel*) for 12 h. Alkali extraction and Western blot analysis were performed as described in Fig. 5. **c** Cells were untreated or treated with 20 ng/ml TNF in the presence or absence of 25 or 50 μ M SP600125, or 5 mM NAC for the indicated time duration, and the cell death rate was determined by trypan blue exclusion. **d** Cells were treated with 20 ng/ml TNF in the presence or absence of 5 mM NAC (*left panel*) or 20 μ M necrostatin-1 (*right panel*) for 12 h, and ROS levels were measured by flow cytometry using DCFH-DA fluorescent dye. A representative histogram plot of three independent experiments is presented. Means \pm SD or representative data of three independent experiments are shown. * $P < 0.01$

plays an important role in apoptotic cell death, we treated LN18 glioblastoma cells with TNF and characterized the cell death. Morphologically, cell death was characterized by fragmentation of nuclei and apoptotic body formation (Fig. 7a). In addition, activation of caspase-3 and cleavage of Bid and PARP were noted (Fig. 7b, c). In line with these findings, cell death was suppressed by the caspase inhibitor

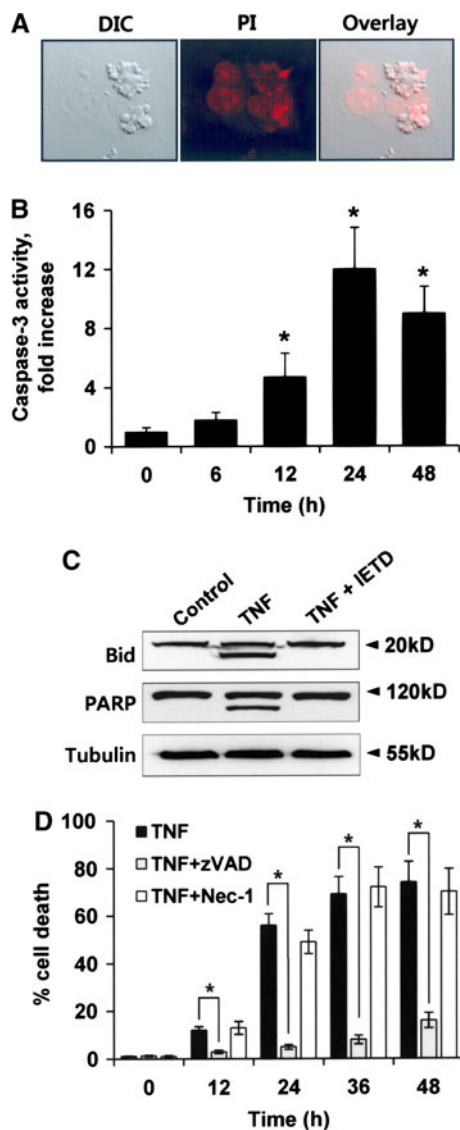


Fig. 7 TNF induces apoptotic cell death in LN18 glioblastoma cells. **a** LN18 cells were treated with 10 ng/ml TNF for 24 h and viewed with a DIC-equipped inverted confocal microscope after nuclear staining with PI. **b** Cells were treated with 10 ng/ml TNF and caspase-3 activity was determined as described in the “Materials and methods” section at the indicated time points. Results are shown as the fold increase of three independent experiments compared to control cells. **c** Lysates extracted from cells treated with 10 ng/ml TNF in the presence or absence of the caspase-8 inhibitor IETD-CHO (IETD, 30 μ g/ml) for 24 h were subjected to Western blot analysis for Bid and PARP cleavage. Tubulin was used as a loading control. Representative blots of at least three experiments are shown. **d** Cells were treated with 10 ng/ml TNF in the presence or absence of zVAD-FMK or 20 μ M necrostatin-1, and the cell death rate was determined by trypan blue exclusion at the indicated time points. Data are mean \pm SD of three or more independent experiments

zVAD-FMK, but not by necrostatin-1 (Fig. 7d), indicating that TNF induces apoptosis in LN18 cells. We next determined whether BNip3 is induced by TNF and involved in classical apoptosis, as in A549 cells. As shown

in Fig. 8a, TNF transcriptionally upregulated BNip3 expression in LN18 cells. However, ectopic overexpression of BNip3 Δ TM or downregulation of endogenous BNip3 using siBNip3 failed to inhibit TNF-induced cell death (Fig. 8b, c). Moreover, mitochondrial insertion of BNip3 was not found, even when cells were treated with TNF (Fig. 8d). These results indicate that BNip3 is involved in necrotic, but not apoptotic, TNF-triggered cell death.

Discussion

TNF is a pleiotropic pro-inflammatory cytokine secreted mainly by activated macrophages that induces multiple targets, including pro-apoptotic Bcl-2 family proteins, ROS, JNK, cathepsin B, and several types of protease, to initiate the death of target cells [6, 8, 30, 32]. Among the different types of cell death induced by TNF, caspase-dependent apoptosis is thought to be the predominant pathway. Recently, however, it has been reported that the inhibition of caspase activation converts death signals to programmed necrosis signals—so-called ‘necroptosis’—and several upstream molecules initiating necroptosis have been identified [15, 33]. Nevertheless, little is known about the signaling proteins involved in progression or execution of necroptosis. Our data indicate that BNip3 is involved in TNF-induced necroptosis.

Initially, we showed that TNF induced necrotic cell death in our experimental system and that TNF-induced cytotoxicity was not altered by inhibiting caspase activity, suggesting that caspase was not involved in TNF-induced necrotic cell death. However, a previous report by Los et al. [34] showed that suppression of caspase activation potentiates necrotic death induced by TNF in L929 mouse fibrosarcoma cells. This implies that the role of caspase in TNF-induced necrotic cell death might be different according to the intracellular signaling pathway involved.

BNip3 is stabilized or transcriptionally upregulated under hypoxic/anoxic conditions, in which HIF-1 α , a master transactivator of hypoxia, binds directly to an HRE consensus sequence in the BNip3 promoter, thereby activating expression of BNip3 [35]. Thus, HIF-1 α is thought to be a key regulator of BNip3 induction. In support of this, Giatromanolaki et al. reported that BNip3 expression is linked with hypoxia-regulated expression of proteins such as HIF-1 α and carbonic anhydrase 9 in non-small cell lung cancer [36]. However, in contrast to previous reports, we found that induction of BNip3 by TNF is independent of HIF-1 α as shown in Fig. 2, suggesting that BNip3 can be induced by HIF-1 α -independent mechanisms. Our hypothesis is supported by several published reports showing upregulation of BNip3 expression in cell

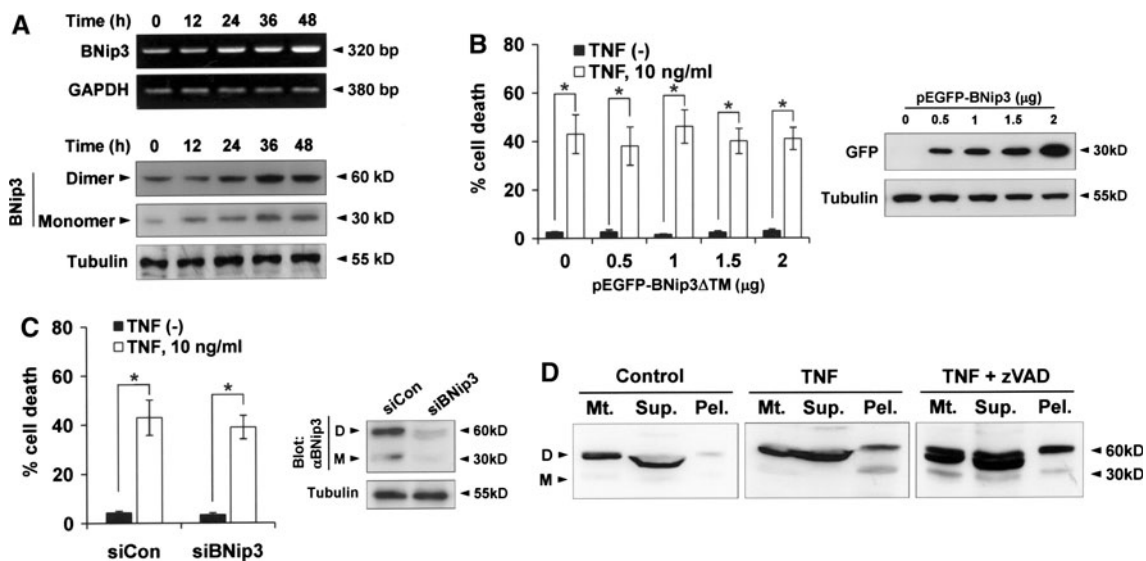


Fig. 8 BNip3 is not involved in the classical apoptotic pathway induced by TNF. **a** mRNA or protein extracted from LN18 cells treated with 10 ng/ml TNF for the indicated times was subjected to RT-PCR (*upper panel*) or Western blotting (*lower panel*) for BNip3, respectively. GAPDH or tubulin was used as a loading control. **b** Cells were transfected with the indicated amounts of pEGFP-BNip3ΔTM for 48 h, and then treated with 10 ng/ml TNF. After an additional 24 h, the number of dead cells among the BNip3ΔTM-overexpressing cells was counted (*left panel*). The expression level of BNip3ΔTM is shown in the *right panel*. Data are means \pm SD of three independent experiments. **c** Cells were transfected with siCon or

siBNip3 for 48 h, and then untreated or treated with 10 ng/ml TNF. After an additional 24 h, at least 500 cells were counted and the number of dead cells was determined (*left panel*). To validate the knockdown of BNip3, Western blot analysis using anti-BNip3 antibody was performed (*right panel*). D, dimer; M, monomer. **d** Cells were treated with 10 ng/ml TNF in the presence or absence of 40 μ M zVAD-FMK for 24 h. Mitochondrial isolation, alkali extraction, and Western blotting were performed as described in Fig. 5. Mt., mitochondrial pellet before alkali exposure; Sup, supernatant; Pel, mitochondrial pellet. * $P < 0.01$

death processes facilitated by nitric oxide [37, 38] or manganese [39].

BNip3 mediates various cytotoxic signals such as hypoxia/anoxia [20] and cytotoxic chemicals [27, 28] to induce apoptosis, necrosis, or autophagic cell death. Despite the diversity of cell death pathways mediated by BNip3, mitochondrial dysfunction caused by induction of the membrane permeability transition (MPT) plays a pivotal role in BNip3-induced cell death pathways. The recent finding that the intracellular pathogen *Shigella* induces necrotic cell death by BNip3-dependent disruption of mitochondrial membrane potential [40] further supports the importance of MPT in BNip3-induced cell death. The upstream signals that activate BNip3 may induce a conformational change in BNip3, allowing it to insert into the outer mitochondrial membrane via its C-terminal transmembrane domain, resulting in MPT. Our alkali extraction assay data from isolated mitochondria show that binding of TNF to TNFR1 triggers mitochondrial BNip3 insertion via a caspase-independent and necrostatin-1-dependent pathway. These data suggest that the BNip3/mitochondrial axis is one of the pathways activated in necroptotic cell death induced by TNF, but not in classical apoptotic cell death of LN18 cells (Figs. 7, 8). There are several explanations for the diverse functions of BNip3 in TNF-induced cell death:

(1) the BNip3 pathway leading to necroptosis may be an alternative pathway activated when the apoptotic pathway is suppressed; (2) the activation of necroptosis by BNip3 or apoptosis by caspase/Bid/Bax may be due to genetically different mechanisms that are specific to different cell types; or (3) the extent of cytoplasmic acidification in response to TNF may differ. The last possibility is supported by a previous study demonstrating that cell death occurs only with coincident cytoplasmic acidosis although BNip3 accumulates under hypoxia at neutral and acidic pH [41, 42]. While the suppression of BNip3 in LN18 cells failed to inhibit TNF-induced cell death, we found that BNip3 was upregulated by TNF (Fig. 8a). This suggests that BNip3 induction by TNF occurs through a common pathway irrespective of which type of cell death is triggered. It further shows that a simple increase in BNip3 expression is not sufficient to trigger BNip3-induced cell death, and additional molecular events may be required for the mitochondrial insertion of BNip3. This idea is reinforced by a previous study by Papandreou et al., which showed that transcriptional induction of BNip3 together with additional events triggered by anoxia are necessary for hypoxic cytotoxicity [43].

The involvement of BNip3 in the TNF signaling pathway raises further questions about the upstream signals that

activate BNip3. Because BNip3 is loosely attached to mitochondria and induces mitochondrial catastrophe by membrane insertion when activated [18, 22], it is believed that there is a signaling pathway linking TNFR1 and BNip3. We focused on two putative signaling molecules: JNK and ROS. Although the role of JNK in mediating the output of TNF-induced signaling pathways is controversial, there is strong evidence that JNK augments TNF-induced cell death [44, 45] and it has been reported that inhibition of JNK activity by a specific inhibitor prevents the onset of MPT upstream of mitochondrial disruption [46]. However, JNK-mediated mitochondrial dysfunction requires the generation of a specific truncated form of Bid, tBid, which releases mitochondrial smac/DIABLO [10, 32]; therefore, the JNK signaling pathway is unlikely to be involved in Bid-independent mitochondrial perturbation based on the results shown in Fig. 1f. Furthermore, the JNK inhibitor SP600125 did not inhibit the TNF-induced mitochondrial insertion of BNip3 (Fig. 6).

We next focused on ROS as signaling molecules because ROS produced by TNF carry out diverse roles in cells and are strong candidate molecules for Bid/caspase-independent cell death [47, 48]. Previously, Zhang et al. [49] and Aucello et al. [50] reported that oxidative stress caused induction and activation of BNip3 in cyanide-induced cell death and autophagy, respectively. Our findings directly demonstrate that ROS are key molecules responsible for mitochondrial insertion of BNip3 after TNF treatment.

In contrast to the events upstream of BNip3 activation, mitochondrial downstream pathways of BNip3-induced cell death are more diverse and complicated according to cell type or experimental conditions. Possible cell death mediators include cytochrome c, apoptosis-inducing factor (AIF), smac/DIABLO, ROS, and cathepsin [51]. As a serine protease, cathepsin is known to induce cellular autolysis and damage of neighboring cells during necrosis [52]. However, many lines of evidence show that lysosomal cathepsin plays an additional role in cell death pathways activated by various stimuli, including BNip3 [7, 53]. While it is clear that lysosomes play a significant role in cell death caused by BNip3 overexpression, the target protein responsible for the propagation of cell death is not clearly identified.

Ghavami et al. recently reported that BNip3 is involved in TNF-induced cell death of the murine fibroblast cell line L929 [53]. While their results are consistent with our data in that BNip3 was induced by TNF in a HIF-1 α -independent manner and TNF-triggered cell death was suppressed by BNip3 Δ TM overexpression, their findings differ from our observations in several regards. First, the cell death induced by TNF in L929 cells was apoptotic with PARP-1 cleavage, compared with the caspase-independent necrotic

death without PARP-1 cleavage observed in our experimental system. Furthermore, we showed that BNip3 is not involved in apoptotic death of LN18 cells. Although it is not clear why BNip3 participates in TNF-induced apoptotic death of L929 cells and necroptosis of A549 cells, it can be partly explained by different intracellular signaling pathways or participating effector molecules between L929, A549, and LN18 cells. The results of previous studies by Los et al. [34] and Frazier et al. [42] support this idea. Second, data by Ghavami et al. indicate that the role of ROS in TNF signaling is downstream of mitochondrial catastrophe induced by BNip3, i.e., lysosomal release of cathepsins. In contrast, our results show that ROS are crucial for mitochondrial insertion of BNip3 (Fig. 6a). This may suggest that ROS generated by TNF/TNFR interaction activate diverse intracellular signaling pathways upstream or downstream of mitochondrial catastrophe, depending on cell type specificity.

In summary, we demonstrated that BNip3 is a candidate protein involved in the TNF-triggered necroptosis of pulmonary alveolar epithelial cells. Both BNip3 and TNF participate in many pathological conditions of the lung including acute lung injury, acute respiratory distress syndrome, and emphysema [21]. Our results indicate that BNip3 is a candidate therapeutic target in pulmonary pathologic conditions in which TNF plays a pivotal role.

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