The Journal of Immunology

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J Immunol 2004; 172:1907-1915; ;
doi: 10.4049/jimmunol.172.3.1907
http://www.jimmunol.org/content/172/3/1907

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

TNF-α-Induced Apoptosis of Macrophages Following Inhibition of NF-κB: A Central Role for Disruption of Mitochondria[1]

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Previously, we established that suppressing the constitutive activation of NF-κB in in vitro matured human macrophages resulted in apoptosis initiated by a decrease of the Bcl-2 family member, A1, and the loss of mitochondrial transmembrane potential ($\Delta\psi_m$). This study was performed to characterize the mechanism of TNF-α-induced apoptosis in macrophages following the inhibition of NF-κB. The addition of TNF-α markedly enhanced the loss of $\Delta\psi_m$ and the induction of apoptotic cell death. Although caspase 8 was activated and contributed to DNA fragmentation, it was not necessary for the TNF-α-induced loss of $\Delta\psi_m$. The inhibition of NF-κB alone resulted in the release of cytochrome *c* from the mitochondria, while both cytochrome *c* and second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI were released following the addition of TNF-α. Furthermore, c-Jun N-terminal kinase activation, which was sustained following treatment with TNF-α when NF-κB was inhibited, contributed to DNA fragmentation. These observations demonstrate that cytochrome *c* and second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI may be differentially released from the mitochondria, and that the sustained activation of c-Jun N-terminal kinase modulated the DNA fragmentation independent of the loss of $\Delta\psi_m$. *The Journal of Immunology,* 2004, 172: 1907–1915.

Tumor necrosis factor-α is a pleiotropic cytokine that regulates multiple cellular responses, including inflammation and cell survival (1–3). Ligation of TNF receptors (TNFR1 and TNFR2) by TNF-α activates NF-κB and the c-Jun N-terminal kinase (JNK)[5]/stress-activated protein kinase pathway, both of which mediate cellular activation, gene transcription, and survival (4, 5). Macrophage-generated TNF-α is critical to the pathogenesis of chronic inflammatory conditions, such as rheumatoid arthritis (reviewed in Ref. 6). However, TNFR ligation may also promote a death signal mediated by the recruitment of TNFR-associated death domain protein, Fas-associated death domain protein, and the initiator caspase 8. Activated caspase 8 may cleave caspase 3 directly or may cleave the Bcl-2 family protein Bid (7, 8), which targets the mitochondria, inducing the loss of mitochondrial transmembrane potential ($\Delta\psi_m$), and release of proteins that initiate an additional death signal through the activation of caspase 9 (9, 10). Either pathway may result in the activation of the effector caspases, including caspases 3 and 7 (11). The release of second mitochondria-derived activator of caspase/direct inhibitor of apoptosis (IAP)-binding protein with low pI (Smac/DIABLO) from mitochondria facilitates caspase 3 activation by inhibiting the X-linked IAP-like protein (XIAP)-mediated suppression of caspase 3 (12).

Cells resistant to TNF-α-induced apoptosis may become sensitive following the inhibition of NF-κB, although the mechanisms for the induction and execution of cell death have yet to be fully elucidated, and appear cell type specific (13–17). Furthermore, multiple cell types from mice deficient in mediators of TNF-α-induced NF-κB activation, including RelA (p65), I-κB kinase β, and I-κB kinase γ/NF-κB essential modulation, are highly sensitive to TNF-α-mediated apoptosis (18–22). NF-κB regulation of a number of molecules, including Flip, IAPs, A1, Bcl-x_L, and Gadd45β, may be critical for protecting cells from TNF-α-induced apoptosis (reviewed in Ref. 23). Therefore, NF-κB activation mediates inhibition of a death-inducing signal following treatment with TNF-α; however, the mechanisms involved remain to be clarified, particularly in macrophages.

Human monocyte-differentiated macrophages express constitutively activated NF-κB and are resistant to many apoptotic stimuli, including Fas and TNFRs (24–27). As the primary producers of TNF-α, macrophages have evolved to survive the cytotoxic effects of TNF-α and, therefore, provide an excellent model for elucidating the mechanism(s) of NF-κB-mediated protection from TNF-α-induced apoptosis (28). We previously demonstrated that the constitutive activation of NF-κB in macrophages was essential for maintaining mitochondrial integrity and viability, mediated by the

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Received for publication June 6, 2003. Accepted for publication November 20, 2003.

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[1] This work was supported by awards from the National Institutes of Health (AR049217), the Veterans Administration Research Service (Merit Review), and the National Arthritis Foundation (to R.M.P.).

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[5] Abbreviations used in this paper: JNK, c-Jun N-terminal kinase; $\Delta\Psi_m$, mitochondrial transmembrane potential; IAP, inhibitor of apoptosis; Ac, acetyl; Ad, adenoviral; AFC, aminomethyl coumarine; DEVD, Asp-Glu-Val-Asp; IETD, Ile-Glu-Thr-Asp; moi, multiplicity of infection; PDTC, pyrrolidine dithiocarbamate; PI, propidium iodide; Rh123, rhodamine 123; Smac/DIABLO, second mitochondria-derived activator of caspase/direct IAP-binding protein with low pI; XIAP, X-linked IAP-like protein.

0022-1767/04/$02.00

expression of the Bcl-2 family member A1, even in the absence of an exogenous death-inducing signal (29). The data presented in this work demonstrate that treatment of macrophages with TNF-α, in conjunction with NF-κB inhibition, potentiated mitochondrial damage and DNA fragmentation. Unexpectedly, TNF-α-mediated mitochondrial dysfunction did not require caspase 8 activation. Furthermore, the sustained activation of JNK induced in TNF-α-treated macrophages following NF-κB inhibition contributed to DNA fragmentation independent of the loss of $\Delta\psi_m$. Further studies will be required to characterize this pathway, which may provide insights to new approaches to suppress chronic inflammation mediated by macrophages.

Materials and Methods

Materials

Pyrrolidine dithiocarbamate (PDTC), LY 294002, and polymyxin B sulfate were obtained from Sigma-Aldrich (St. Louis, MO). Human TNF-α was from R&D Systems (Minneapolis, MN). JNK inhibitor peptide (JNKI1) was purchased from Alexis Biochemicals (San Diego, CA). Smac-N7 peptide was purchased from Calbiochem (San Diego, CA). RPMI 1640, DMEM, FBS, PBS, L-glutamine, penicillin, and streptomycin were obtained from Life Technologies (Gaithersburg, MD). Propidium iodide (PI) was from Roche Molecular Biochemicals (Indianapolis, IN), and rhodamine 123 (Rh123) was from Molecular Probes (Eugene, OR).

Cell isolation and culture

Buffy coats (Lifesource, Glenview, IL) were obtained from healthy donors. Mononuclear cells, isolated by Histopaque (Sigma-Aldrich) gradient centrifugation, were separated by countercurrent centrifugal elutriation (JE-6B; Beckman Coulter, Palo Alto, CA) in the presence of 10 μg/ml polymyxin B sulfate, as previously described (29, 30). Isolated monocytes were ≥90% pure, as determined by morphology, nonspecific esterase staining, and CD14 (BD Biosciences, Franklin Lakes, NJ) expression examined by flow cytometry (data not shown). Monocytes were adhered to plates (Costar, Cambridge, MA) for 1 h in RPMI 1640 and 1 μg/ml polymyxin B sulfate. Following adherence, human blood-isolated monocytes were differentiated in vitro for 7 days in RPMI 1640 containing 20% heat-inactivated FBS, 1 μg/ml polymyxin B sulfate, 0.35 mg/ml L-glutamine, 120 U/ml penicillin, and streptomycin (20% FBS/RPMI 1640) (26). Seven-day differentiated macrophages strongly expressed maturation markers including CD71 and the integrin $\alpha_v\beta_5$ (31).

Determination of subdiploid DNA content

At the indicated time points, cells were harvested, fixed in 70% ethanol, and stained with PI (50 μg/ml), as previously described (32). The apoptotic profile was determined by flow cytometry using a Beckman Coulter EpicsXL flow cytometer and system 2 software. The subdiploid DNA peak (<2 N DNA), immediately adjacent to the G_0/G_1 peak (2 N DNA), represents apoptotic cells and was quantified by histogram analyses. Objects with minimal light scatter representing debris were excluded, as previously described (33), so that quantitation of the subdiploid population would not be inappropriately skewed.

Analysis of $\Delta\psi_m$

Mitochondrial dysfunction was assessed using the cationic lipophilic green fluorochrome Rh123, as previously described (34). Disruption of $\Delta\psi_m$ is associated with a lack of Rh123 retention and a decrease in fluorescence. Cultures were incubated with Rh123 (0.1 μg/ml) for 30 min, harvested, and analyzed by flow cytometry. Mean fluorescence was recorded for each sample, and control cultures at each respective time point were designated as 100% fluorescence. For histogram analysis, objects with minimal light scatter representing debris were gated out. Where indicated, Rh123 samples were washed, fixed in 70% ethanol, and analyzed for subdiploid DNA content.

Western blot analysis

Whole cell extracts were prepared from in vitro matured, 7-day differentiated macrophages that were treated, as indicated. Extracts were electrophoresed on SDS-PAGE 12.5% polyacrylamide gels and transferred to Immobilon-P (Millipore, Bedford, MA) by semidry blotting. Membranes were blocked for 1 h at room temperature in PBS/0.2% Tween 20/5% nonfat dry milk (PBS/Tween/milk). The membranes were then incubated overnight at 4°C in PBS/Tween/milk with the indicated Abs: anti-caspase 9 (Calbiochem), anti-cytochrome *c* (BD PharMingen, San Diego, CA), anti-Smac/DIABLO (kindly provided by X. Wang, University of Texas Southwestern Medical Center, Dallas, TX), mouse anti-Bax (Sigma-Aldrich), mouse anti-cytochrome oxidase subunit IV (COX IV) (Molecular Probes), anti-XIAP (R&D Systems), mouse anti-caspase 8 (kindly provided by M. Peter, University of Chicago), or anti-tubulin (Calbiochem). Membranes were washed in PBS/Tween/milk and incubated with either donkey anti-rabbit or anti-mouse secondary Ab conjugated to HRP (1/2000 dilution; Amersham Pharmacia Biotech, Piscataway, NJ). Visualization of the protein bands was performed using the ECL Plus kit, as recommended by the manufacturer (Amersham Pharmacia Biotech).

Caspase activity assay

Seven-day differentiated human macrophages (0.6×10^6) were treated and harvested. Cell lysates were prepared, as instructed by the manufacturer. The lysates were incubated for 1 h at 37°C with either the fluorogenic caspase-3 substrate, acetyl-Asp-Glu-Val-Asp-aminomethylcoumarine (Ac-DEVD-AFC) or fluorogenic caspase-8 substrate, acetyl-Ile-Glu-Thr-Asp-aminomethyl coumarine (Ac-IETD-AFC) synthetic, fluorogenic substrate, both purchased from Enzyme Systems Products (Livermore, CA). Samples were read on a fluorometer at 400 nm excitation and 505 nm emission.

Caspase inhibition

Macrophages were pretreated for 1 h with 50 μM of the general caspase inhibitor zVAD.fmk (Enzyme System Products) or vehicle control, DMSO, followed by culture with 200 μM of PDTC and 10 ng/ml TNF-α for an additional 12 h. Alternatively, macrophages were infected with control or I-κBα-expressing adenoviral vectors for 24 h. The cells were then pretreated with zVAD.fmk or the caspase 8 inhibitor IETD.fmk for 1 h before the addition of TNF-α. At the indicated time points, the cells were harvested and the cells or lysates were examined, as described in *Results*.

Confocal microscopy

Monocytes were plated on glass slides and in vitro matured to macrophages for 7 days. The macrophages were treated with PDTC or PDTC plus TNF-α for 16 h, then incubated with Mitotracker Red (25 nM, CMXRos; Molecular Probes) for 30 min, and subsequently stained with anti-Bax, as previously described (35, 36). Briefly, macrophages were fixed in 2% formaldehyde in PBS, permeabilized with 0.1% Triton X-100, and incubated with mouse anti-Bax Ab, followed by FITC-labeled anti-mouse H and L chain. Confocal microscopy was performed with 510 laser-scanning/confocal microscope.

Kinase assay

In vitro matured macrophages were treated as indicated, harvested in lysis buffer, and clarified by centrifugation. JNK was immunoprecipitated from 60–120 μg total protein extract by GST-c-Jun beads from Cell Signaling Technology (Beverly, MA). The kinase activity, determined according to the manufacturer's directions, was assayed at 30°C for 30 min in 50 μl of kinase buffer in the presence of 10 μM of ATP and 50 μCi of [γ-^{32}P]ATP. The proteins were resolved by 12% SDS-PAGE, followed by autoradiography.

Statistical analysis

Significance was determined by Student's *t* test.

Results

TNF-α potentiates macrophage apoptosis induced by NF-κB inhibition

To delineate the role of TNF-α-induced NF-κB activation in the protection against TNF-α-mediated apoptosis of in vitro matured human macrophages, an adenoviral vector expressing a superrepressor I-κBα (AdI-κBα), with serine to alanine mutations that retard its degradation, was used (29). Compared with the inhibition of NF-κB alone by superrepressor I-κBα, the addition of TNF-α greatly enhanced the loss of macrophage viability, defined as the inability to exclude PI (Fig. 1*A*). The mode of TNF-α-induced cell death, when NF-κB was inhibited, was apoptotic determined by DNA fragmentation (Fig. 1*B*). The apoptotic cell death was associated with the loss of mitochondrial transmembrane potential ($\Delta\psi_m$), defined as the loss of mitochondrial retention of Rh123

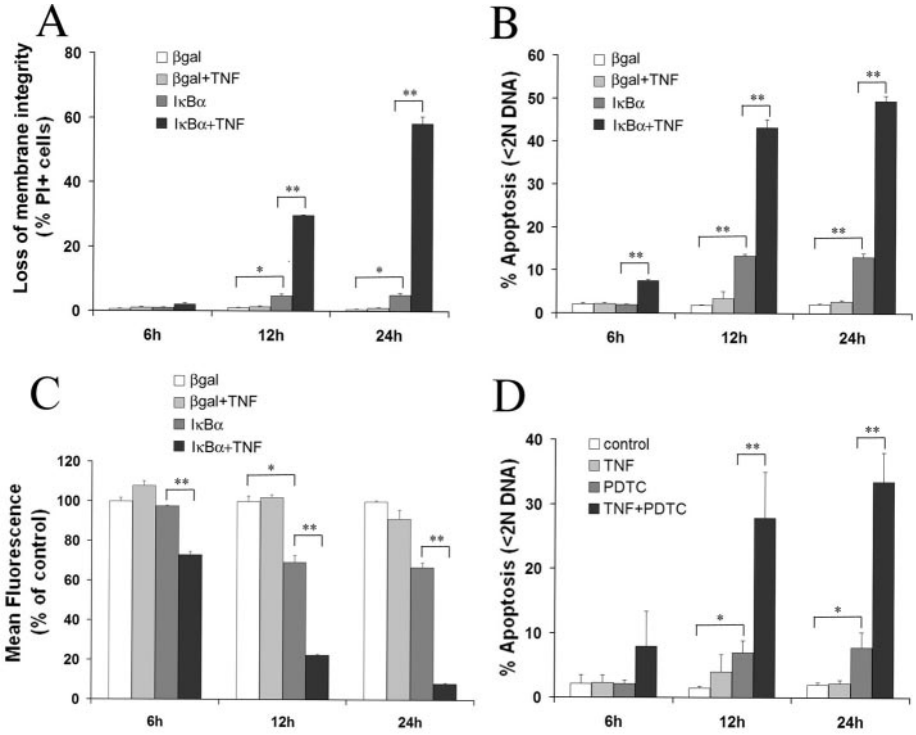

FIGURE 1. TNF-α potentiates macrophage apoptosis induced by NF-κB inhibition. *A–C*, TNF-α increases apoptosis induced by inhibition of NF-κB by ectopic expression of superrepressor I-κBα. In vitro differentiated macrophages were infected with AdI-κBα or control (Adβgal) at 100 multiplicity of infection (moi) for 2 h. The infected macrophages were then treated with 10 ng/ml TNF-α for 6–24 h, as indicated. The cells were harvested and analyzed for loss of membrane integrity, determined by the inability to exclude PI (*A*, % cell death, PI^+ cells), for DNA fragmentation, measured by analysis of subdiploid DNA (*B*, % Apoptosis), and for loss of $\Delta\psi_m$, determined by Rh123 retention (*C*, Mean Fluorescence). *D*, Addition of TNF-α increases macrophage apoptosis when NF-κB is inhibited by PDTC. In vitro differentiated macrophages were treated with 200 μM of PDTC, 10 ng/ml TNF-α alone, or TNF-α plus PDTC simultaneously, as indicated. Cells were harvested at 6, 12, and 24 h for analysis. Apoptosis was determined by analysis of subdiploid DNA, using flow cytometry, as previously described (29, 30). The results in each panel represent the mean $\pm$ 1 SE of at least three independent experiments, each performed in triplicate. $*$, Indicates $p < 0.05$; $**$, represents $p < 0.01$ between the different treatment groups.

(Fig. 1*C*) (29). To confirm that the effects observed were due to the inhibition of NF-κB, a second method of suppressing NF-κB activation, the addition of PDTC was used (29). PDTC also resulted in a marked increase of TNF-α-induced apoptosis (Fig. 1*D*), which was associated with the loss of $\Delta\psi_m$ and the inability to exclude PI (data not shown).

TNF-α treatment plus NF-κB inhibition resulted in the activation of caspases 9 and 3

We previously demonstrated that macrophage apoptosis induced by the inhibition NF-κB alone did not result in caspase 3 activation, even though caspase 9 was cleaved (29). Therefore, we examined the effect of TNF-α on caspase 3-like activity, determined by cleavage of a DEVD fluorogenic substrate containing a caspase 3-specific cleavage site. Treatment of macrophages with PDTC alone did not result in caspase 3-like activity at 12 h (Fig. 2*A*) or 24 h (Fig. 2*B*). In contrast, macrophages treated with TNF-α plus PDTC displayed a significant increase in caspase 3-like activity at both 12 h (Fig. 2*A*) and 24 h (Fig. 2*B*), which was comparable to that observed following the inhibition of the phosphatidylinositol-3 kinase/Akt-1 pathway in macrophages or Fas ligation in monocytes, as previously described (30, 37).

To clarify the mechanism of caspase 3 activation, caspase 9 activation was assessed using Western blot analysis. Seven-day differentiated macrophages treated with PDTC, with or without TNF-α, demonstrated no change in procaspase 9 levels at 6 h (Fig. 2*C*). However, at 24 h, parallel cultures revealed that procaspase 9 was diminished in cells treated with PDTC, with or without TNF-α (Fig. 2*C*). Treatment with TNF-α alone did not affect caspase 9 activation assessed by Western blot. These data demonstrate that differences in procaspase 9 cleavage were not responsible for the increased caspase 3-like activity observed following the addition of TNF-α.

Selective release of Smac/DIABLO by TNF-α and the role of the mitochondria

To determine the mechanism of caspase 3 activation by TNF-α, cytosolic extracts were examined by Western blot for the release of cytochrome *c* and Smac/DIABLO from the mitochondria. Following the inhibition of NF-κB by PDTC, in the absence or presence of TNF-α, cytochrome *c* was released into the cytosol (Fig. 3*A*). No cytochrome *c* oxidase subunit IV was detected in the same cytosolic extracts (data not shown). Because caspase 3 was activated only when TNF-α was added, the cytosolic extracts were also examined for Smac/DIABLO, which binds to and inhibits XIAP from suppressing caspase 9 activity and caspase 3 activation. The release of Smac/DIABLO from the mitochondria was not detected when NF-κB activation was inhibited, in the absence of TNF-α (Fig. 3*A*). In contrast, when TNF-α was added with PDTC, Smac/DIABLO was detected in the cytosolic extracts. Because the expression of XIAP may be regulated by NF-κB activation, the macrophages were also examined for XIAP. XIAP was readily detected in differentiated macrophages, and neither the inhibition of NF-κB alone, nor with TNF-α, suppressed the expression of XIAP (Fig. 3*B*).

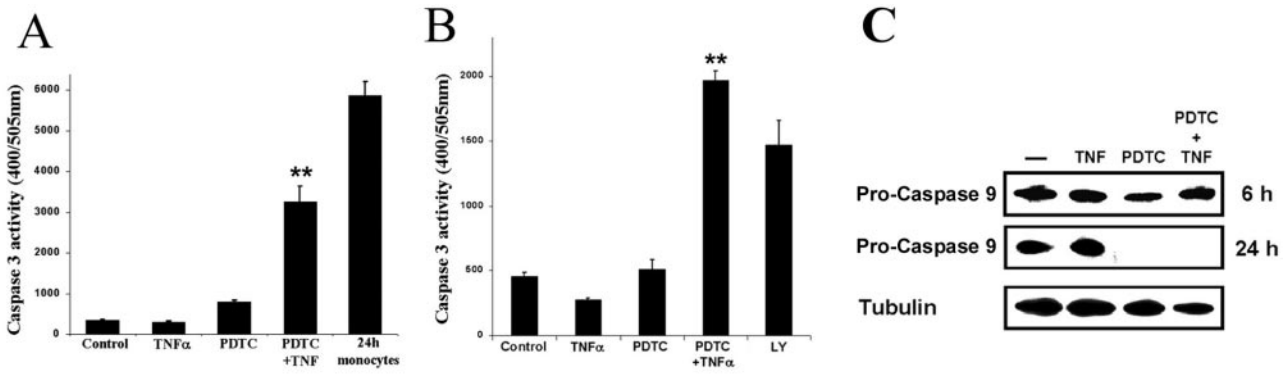

FIGURE 2. TNF-α is necessary to activate caspase 3, but not caspase 9, following inhibition of NF-κB. Macrophages were incubated with 10 ng/ml TNF-α, 200 μM of PDTC, or both TNF-α plus PDTC, as indicated. Cells were harvested at 12 (*A*) or 24 (*B*) h, lysed, and incubated with Ac-DEVD-AFC at 37°C for 1 h to detect caspase 3-like activity, as described in *Materials and Methods*. Macrophages incubated with 50 μM LY294002 (LY, *B*) or primary monocytes cultured for 24 h (*A*) were used as positive controls for caspase 3 activity, as previously described by us (29, 37). The results are presented as the mean ± 1 SE of an experiment, performed in triplicate, which is representative of three independent experiments. **, Indicates $p < 0.01$ determined by Student's *t* test compared with control. *C*, Caspase 9 is activated in TNF-α-treated macrophages following NF-κB inhibition by PDTC. Seven-day macrophages were treated, as described above, and harvested at 6 and 24 h. Cleavage of procaspase 9 was detected by Western blot analysis. Whole cell extracts (30 μg) were subjected to SDS-PAGE on 12.5% polyacrylamide gels, then transferred to Immobilon P and probed with Abs to caspase 9 and tubulin. The data are representative of three experiments.

To determine whether the Smac/DIABLO released may be responsible for the enhanced apoptosis induced by TNF-α when NF-κB was inhibited, a cell-permeable Smac/DIABLO peptide, Smac-N7 peptide (Calbiochem), was used. Previous data have demonstrated that this peptide binds and inactivates XIAP, and thus promotes the activation of procaspase 3 and the catalytic activity of caspase 9 (38–41). Smac-N7 significantly increased the apoptosis induced by the superrepressor I-κBα-mediated inhibition of NF-κB, while Samc-N7 alone did not induce apoptosis in human macrophages (Fig. 3*C*). The presence of Smac-N7 also enhanced caspase 3 activity following inhibition of NF-κB by AdI-κBα infection (Fig. 3*D*). Together, these observations suggest that following the inhibition of NF-κB alone, cytochrome *c* was released from the mitochondria, resulting in the cleavage of caspase 9, and that the XIAP expressed was sufficient to prevent the activation of caspase 3. However, when TNF-α was added, Smac/DIABLO was also released from the mitochondria, potentially inhibiting the function of XIAP, thereby permitting the activation of caspase 3.

These observations suggest that mitochondrial dysfunction may play an essential role in the apoptosis induced by TNF-α when NF-κB is inhibited. The translocation of Bax to mitochondria may be important in apoptosis, mediated through release of cytochrome *c* (42–44) and Smac/DIABLO (12, 45). To determine whether Bax translocation might contribute to the mitochondrial dysfunction observed in response to TNF-α when NF-κB is inhibited, macrophages were treated with TNF-α and PDTC. The membrane fraction, which includes mitochondria, was harvested and used for the detection of Bax by Western blot analysis. Little Bax was detected in the membrane fraction, when the macrophages were cultured in medium alone. In contrast, Bax was readily detected in the membrane faction when the macrophages were treated with TNF-α and PDTC for 18 h (Fig. 4*A*). To confirm the translocation of Bax to the mitochondria, confocal microscopy was used. In the cells cultured in medium alone, Bax (green) was diffusely distributed, with little colocalization with mitochondria (red) (Fig. 4*B*). After treatment with TNF-α and PDTC, Bax colocalized with the mito-

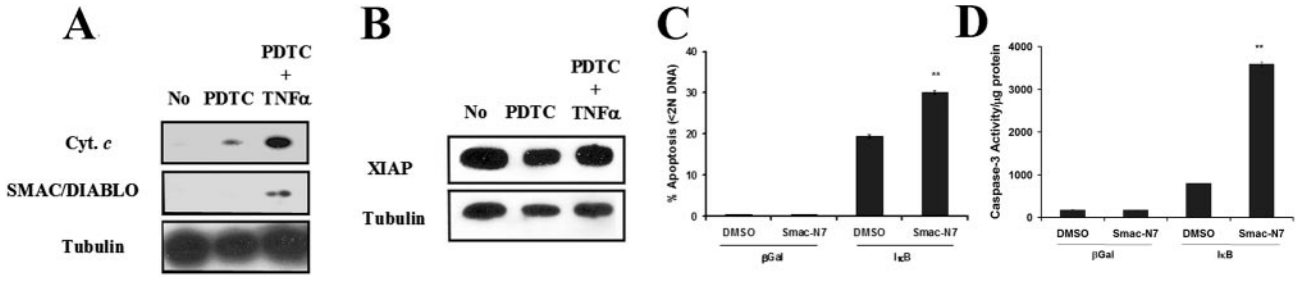

FIGURE 3. The TNF-α-induced release of Smac/DIABLO contributes to apoptosis in macrophage. *A*, Inhibition of NF-κB alone by PDTC or in the presence of TNF-α differentially releases cytochrome *c* and Smac/DIABLO from mitochondria. Monocyte-differentiated macrophages were treated with PDTC alone (200 μM) or with TNF-α (10 ng/ml) for 24 h. The cytosolic protein was isolated by methods previously described by us (37). Western blots (15 μg) were probed with Abs to cytochrome *c*, Smac/DIABLO, and tubulin, as described in *Materials and Methods*. *B*, Inhibition of NF-κB by PDTC does not affect XIAP. Macrophages were treated and harvested, as described above. Whole cell lysates (30 μg) were used for Western blot analysis and probed with Abs to XIAP or tubulin. Each panel is representative of two or three experiments. *C*, Smac peptide enhances apoptosis. In vitro matured macrophages were infected with superrepressor AdI-κBα at 100 moi or control (Adβgal) for 24 h. The infected macrophages were then treated with 90 μMol of Smac-N7 cell-permeable peptides (Calbiochem, San Diego, CA) or control DMSO for 20 h, as indicated. The cells were harvested and analyzed for the apoptotic phenotype, measured by analysis of subdiploid DNA (% Apoptosis). The results in each panel represent the mean ± 1 SE of two independent experiments, each performed in triplicate. **, Represents $p < 0.01$ between the different treatment groups. *D*, Smac peptide enhances caspase 3 activation. Cells treated as described in *C* were harvested, lysed, and incubated with Ac-DEVD-AFC at 37°C for 1 h to detect caspase 3-like activity.

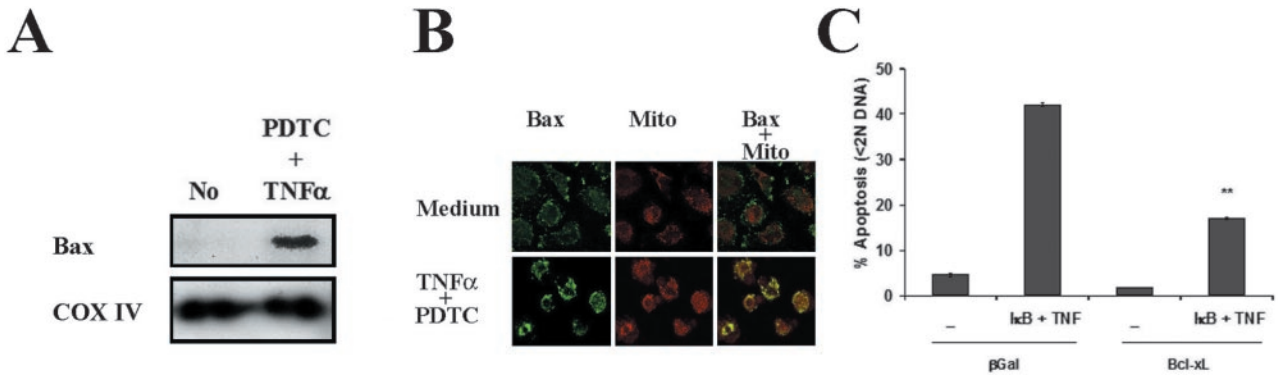

FIGURE 4. The TNF-α-induced localization of Bax to the mitochondria contributes to apoptosis. *A*, Bax translocation, following TNF-α plus PDTC. Differentiated macrophages were treated with vehicle control alone (No) or with PDTC alone (200 μM) plus TNF-α (10 ng/ml) for 24 h. The membrane faction was isolated and probed with Abs to Bax and cytochrome oxidase IV (COX IV). *B*, Bax colocalizes to the mitochondria. Macrophages cultured on glass slides were treated with PDTC alone (200 μM) or with TNF-α (10 ng/ml) for 16 h, then were incubated with Mitotracker Red (25 nM, CMXRos; Molecular Probes) for 30 min to identify the mitochondria. The cells were fixed, permeabilized, and stained with Bax and examined by confocal microscopy, as described in *Materials and Methods*. *C*, Bcl-x_L suppresses apoptosis. In vitro matured macrophages were infected with 6 moi of AdBcl-x_L plus superrepressor AdI-κBα at 100 moi or control (Adβgal plus AdI-κBα) for 24 h. The infected macrophages were then treated with 10 ng/ml TNF-α or medium for 20 h, as indicated. The cells were harvested and analyzed for the apoptotic phenotype, measured by analysis of subdiploid DNA (% Apoptosis). The results in each panel represent the mean $\pm$ 1 SE of two independent experiments, each performed in triplicate. **, Represents $p < 0.01$ between the different treatment groups.

chondria (yellow) (Fig. 4*B*). The important role of mitochondrial dysfunction in the apoptosis induced by TNF-α when NF-κB activation is inhibited was further supported by the ectopic expression of Bcl-x_L, a Bcl-2 family antiapoptotic molecule, which may protect the mitochondria from Bax-mediated apoptosis. Supporting the potential importance of Bax, the ectopic expression of Bcl-x_L significantly reduced the apoptosis induced by TNF-α, when NF-κB was inhibited with the superrepressor I-κBα (Fig. 4*C*).

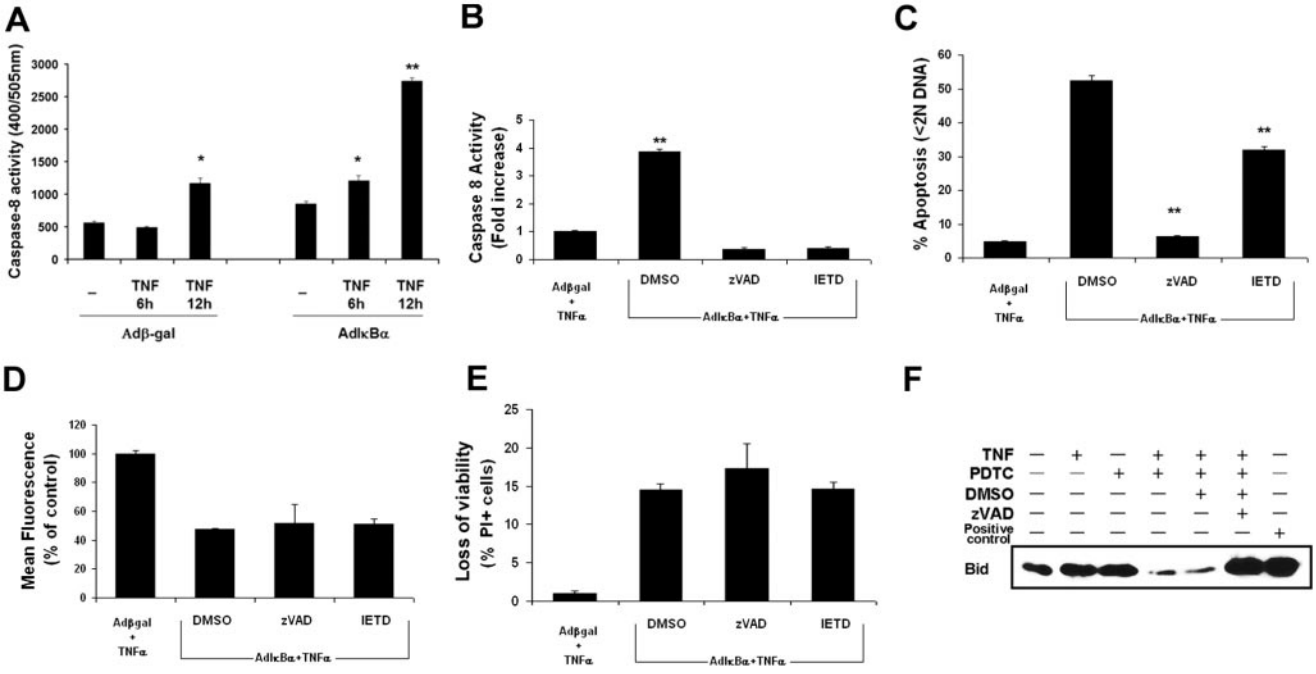

FIGURE 5. Activation of caspase 8 in macrophages following inhibition of NF-κB and treatment with TNF-α. *A*, In vitro differentiated macrophages were infected with AdI-κBα or the control Adβgal at 100 moi for 2 h. The macrophages were then treated with 10 ng/ml TNF-α for 6 or 12 h, as indicated. Cells were harvested, and IETD or caspase 8-like activity was determined using Ac-IETD-AFC. The results presented represent the mean $\pm$ 1 SE of an experiment performed in triplicate, which is representative of three independent experiments. *B*, In vitro differentiated macrophages were infected with AdI-κBα or the control Adβgal at 100 moi for 2 h, then treated with zVAD-fmk (50 μM) or zIETD.fmk (20 μM), or DMSO control, as indicated, then with 10 ng/ml TNF-α for 16 h. The cells were harvested, and lysates were prepared to detect caspase 8-like activity. *C–E*, Macrophages were treated as described in *B* and harvested and analyzed for loss of viability (*C*, % Cell death), DNA fragmentation, defined as subdiploid DNA (*D*, % Apoptosis), and the loss of $\Delta\psi_m$, determined by Rh123 retention (*E*, Mean Fluorescence). The results are presented as the mean $\pm$ 1 SE of an experiment, performed in triplicate, which is representative of two independent experiments. *, Indicates $p < 0.05$; **, indicates $p < 0.01$ determined by Student's *t* test compared with medium control or adenoviral vector control Adβgal. *F*, Bid cleavage requires caspase activation. Seven-day differentiated macrophages were treated with 10 ng/ml TNF-α, 200 μM of PDTC, both TNF-α plus PDTC, or pretreated with TNF-α for 2 h before addition of PDTC. Additional cultures were pretreated with control vehicle (DMSO) alone or zVAD.fmk (50 μM) for 1 h before addition of both TNF-α plus PDTC. Cells were harvested at 12 h, and whole cell extracts (50 μg) were analyzed by Western blot analysis using anti-Bid Abs. The results are representative of three experiments.

Caspase 8 activation is not necessary for mitochondrial dysfunction induced by TNF-α treatment following NF-κB inhibition

To characterize the mechanism(s) responsible for the TNF-α-induced apoptosis of macrophages, the paradigm that TNF-α-induced apoptosis is initiated through the activation of caspase 8 was examined. When superrepressor I-κBα was used to inhibit NF-κB activation, the addition of TNF-α for 12 h resulted in 2- to 3-fold increase of caspase 8-like activity, using an IETD fluorogenic substrate (Fig. 5*A*). To determine whether caspase 8 plays an essential role in TNF-α-induced macrophage apoptosis, the effect of caspase inhibition was examined. The addition of the broad-based caspase inhibitor zVAD.fmk, or the caspase 8 inhibitor, IETD.fmk, suppressed the TNF-α-induced caspase 8-like activity in macrophages expressing the superrepressor I-κBα, even below the baseline observed for control cells infected with the Adβgal (Fig. 5*B*). Although addition of zVAD.fmk, which is also capable of inhibiting caspases 9 and 3, completely prevented DNA fragmentation, the caspase 8-specific inhibitor IETD.fmk resulted in only a partial, but significant ($p < 0.01$), reduction of DNA fragmentation (Fig. 5*C*). Despite their ability to inhibit caspase activation and DNA fragmentation, neither zVAD.fmk nor IETD.fmk protected against the loss of $\Delta\psi_m$ (Fig. 5*D*) or of the ability to exclude PI (Fig. 5*E*) following the addition of TNF-α in macrophages ectopically expressing I-κBα. Similarly, when NF-κB was inhibited with PDTC, zVAD.fmk failed to protect macrophages against the TNF-α-induced loss of $\Delta\psi_m$ or of the ability to exclude PI, although zVAD-.fmk was effective at preventing DNA fragmentation (data not shown). Furthermore, following inhibition of NF-κB by PDTC, Bid was cleaved only when TNF-α was added; however, Bid activation was prevented by caspase inhibition with zVAD.fmk (Fig. 5*F*). These observations suggest that, in macrophages, although Bid cleavage was caspase dependent, caspase activation and Bid cleavage were not necessary for the loss of $\Delta\psi_m$, or for the loss of membrane integrity, induced by TNF-α when NF-κB was inhibited.

TNF-α-mediated JNK activation is prolonged when NF-κB is inhibited

Because TNF-α did not require caspase 8 activation to induce mitochondrial dysfunction and apoptotic cell death, an alternate apoptotic pathway was considered. TNF-α signaling also activates the mitogen-activated protein kinase pathway, which may contribute to the regulation of apoptosis (46–49). Therefore, the TNF-α-induced activation of JNK was assessed in in vitro matured macrophages, in the absence or presence of NF-κB inhibition. JNK kinase activity, as determined by c-Jun phosphorylation using an in vitro kinase assay, was present 10 min after treatment with TNF-α alone or TNF-α plus PDTC (Fig. 6*A*). After 30 (data not shown) and 60 (Fig. 6*A*) min, JNK activity in macrophages treated with TNF-α alone abated. In contrast, at 30 (data not shown) and 60 (Fig. 6*A*) min, JNK remained active in cells treated with TNF-α plus PDTC. Remarkably, JNK activity was sustained even at 12 and at 24 h (data not shown), when macrophages were treated with both TNF-α plus PDTC, but not when treated with TNF-α or PDTC alone. The persistent TNF-α-induced activation of JNK was also observed at 18 h when the superrepressor I-κBα was used (Fig. 6*B*). Thus, in macrophages, TNF-α alone induced a transient activation of JNK, which was sustained when NF-κB was inhibited.

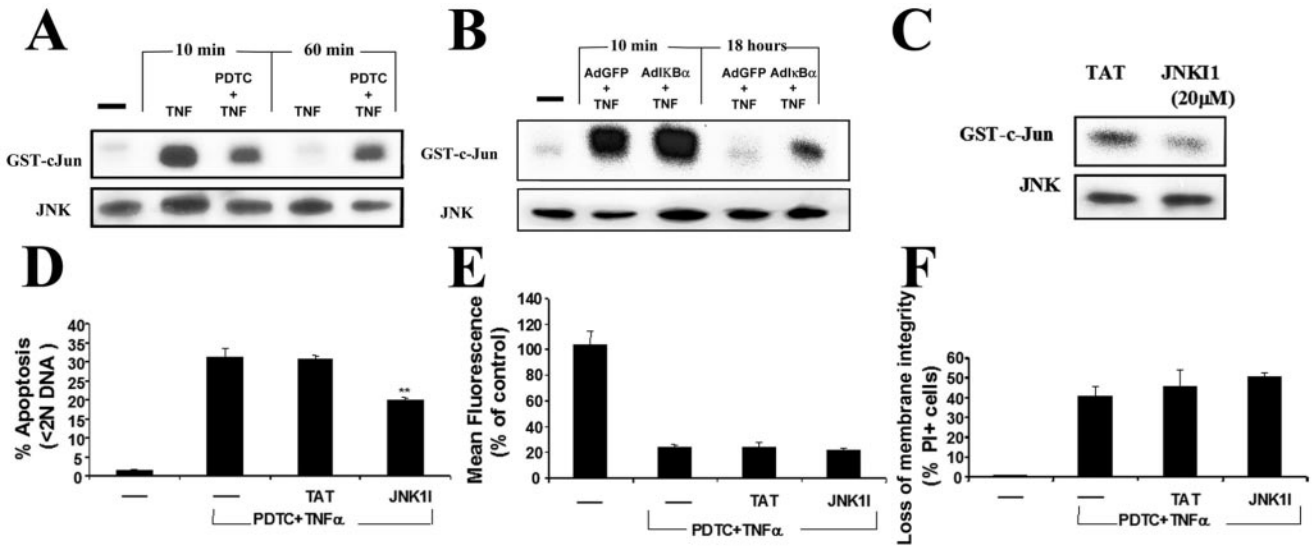

FIGURE 6. NF-κB inhibition results in sustained TNF-α-induced JNK activation in macrophages, which is not essential for mitochondrial dysfunction or cell death. In vitro matured human macrophages were incubated with 10 ng/ml TNF-α, 200 μM of PDTC, or both TNF-α plus PDTC, where indicated, for 10 and 60 min (*A*). Alternatively, macrophages were infected with AdI-κBα or the control AdGFP at 100 moi, and then treated with TNF-α for 10 min or 18 h, as indicated (*B*). At the indicated time points, the cells were harvested and lysates were prepared, as described, and used for determining JNK kinase activity, as described in *Materials and Methods*. Additionally, the lysates (30 μg) were analyzed by Western blot and probed with an anti-JNK Ab (p54 JNK presented), as indicated. The results presented in each panel are representative of two or three experiments. *C*, Inhibition of JNK activity in vivo. Macrophages were pretreated with JNKI1 (20 μM) or control TAT peptide (20 μM) for 1 h, and then PDTC plus TNF-α were added. After 24 h, the cells were harvested, and the kinase activity was performed, as described in *Materials and Methods*. The results presented in each panel are representative of three experiments. *D–F*, Differentiated macrophages were pretreated with JNKI1 (20 μM) or the TAT control peptide for 1 h. PDTC (200 μM) plus TNF-α (10 ng/ml) were then added for 24 h. The cells were harvested and analyzed for the loss of membrane integrity (*D*), DNA fragmentation (*E*), or loss of $\Delta\psi_m$ (*F*). **, Indicates $p < 0.01$ compared with the TAT control. The results presented are the mean ± 1 SE of an experiment performed in triplicate, which is representative of three experiments.

The role of persistent JNK activation in macrophage apoptosis

To determine whether the persistent activation of JNK was responsible for the TNF-α-induced apoptosis observed, a cell-permeable peptide inhibitor of JNK (JNKI1), capable of inhibiting JNK1, JNK2, and JNK3 (50), was used. The addition of the peptide inhibitor suppressed JNK activity in macrophages induced by TNF-α plus NF-κB inhibition by PDTC (Fig. 6*C*). JNKI1 also suppressed DNA fragmentation (Fig. 6*D*). In contrast, JNKI1 failed to protect against the TNF-α-induced disruption of $\Delta\psi_m$ (Fig. 6*E*) or cell death (Fig. 6*F*). These observations demonstrate that the persistent activation of JNK, although contributing to DNA fragmentation, was not responsible for the loss of $\Delta\psi_m$ following treatment with TNF-α when NF-κB was inhibited.

Discussion

As the principal generators of TNF-α, macrophages are exposed to a potentially cytotoxic milieu, but remain resistant to TNF-α-mediated apoptosis (27, 51). Our study demonstrates the presence of a novel mechanism for the induction of TNF-α-induced apoptosis, which may be cell type and context specific. According to the accepted paradigm, TNF-α-induced apoptosis occurs following ligation TNF receptors, mediated via the death domain interaction with TNFR-associated death domain protein, which binds Fas-associated death domain protein, resulting in the recruitment and activation of caspase 8 (52–54). In certain cells, inhibiting of NF-κB activation permits TNF-α-induced caspase 8 and Bid activation and apoptotic cell death (46, 55–57). However, when TNF-α-induced apoptosis was dependent upon caspase 8, zVAD-fmk protected against the development of mitochondrial dysfunction and apoptotic cell death (10). In contrast, in macrophages, although TNF-α induced caspase 8-like activity when NF-κB was inhibited, caspase 8 activation was not essential for the loss of either $\Delta\psi_m$ or viability, even though it contributed to DNA fragmentation. Because neither IETD.fmk nor zVAD.fmk protected the mitochondria, it is possible that activated caspase 8 may have affected DNA fragmentation through effects on caspase 3 activation, as observed in type I cells (58).

Previously, we demonstrated in macrophages that NF-κB inhibition alone, in the absence of TNF-α, resulted in the loss of $\Delta\psi_m$ and caspase 9 cleavage, while caspase 3 was not activated, and that this process was mediated by the reduction of the Bcl-2 family member A1 (29). The present study extends these observations demonstrating that the inhibition of NF-κB alone resulted in the release of cytochrome *c*, while the release of Smac/DIABLO from the mitochondria was not detected by the methods used. The addition of TNF-α, in the presence of NF-κB inhibition, induced the release of both cytochrome *c* and Smac/DABILO and the activation of caspase 3, suggesting that TNF-α activated an additional pathway that also targets the mitochondria. The difference in the effects on caspase 3 activation supports the relevance of the differential release of cytochrome *c* and Smac/DIABLO demonstrated by Western blot analysis. These observations suggest that the cytochrome *c* released following the inhibition of NF-κB alone was sufficient to promote the cleavage of caspase 9, but not caspase 3 activation. Consistent with our observations, cytochrome *c* and Smac/DIABLO were differentially released in response to TNF-related apoptosis-inducing ligand from the mitochondria of mouse embryonic fibroblasts that were deficient in *bid*, *bax*, or *bak*, compared with wild-type controls (59). These observations indicate that under certain conditions, cytochrome *c* and Smac/DIABLO may be differentially released from mitochondria.

XIAP is capable of binding caspase 3 (60) and preventing its activation by either of the apical caspases, 8 or 9 (12, 61, 62). Therefore, it is possible that the release of Smac/DIABLO from the mitochondria, induced by NF-κB inhibition plus TNF-α, but not NF-κB inhibition alone, inhibited the ability of XIAP to protect against the caspase 8-mediated activation of caspase 3 (12, 62). In addition, XIAP may bind cleaved caspase 9, preventing it from activating caspase 3 (60). Consistent with this scenario in macrophages, caspase 9 was cleaved following the inhibition of NF-κB activation, in the presence or absence of TNF-α, while caspase 3 activity was detected only in the presence of TNF-α, when the release of Smac/DIABLO from the mitochondria was detected. The apparent enhanced release of cytochrome *c* observed with TNF-α when NF-κB was inhibited was not likely responsible for the observed activation of caspase 3 following the addition of TNF-α, because all the procaspase 9 detected by Western blot was cleaved even in the absence of TNF-α. The functional significance of Smac/DIABLO release from the mitochondria was supported by the enhanced activation of caspase 3 and DNA fragmentation observed following the addition of the Smac-N7 peptide, when NF-κB was inhibited, in the absence of TNF-α. It is possible that other mitochondrial proteins such as Omi/HtrA2 may also have been involved (63). These observations are consistent with the interpretation that, in macrophages, XIAP was capable of suppressing caspase 9 activity or the activation of caspase 3 (12, 61, 62). Together these observations suggest that the release of Smac/DIABLO, following the addition of TNF-α, when NF-κB was inhibited, contributed to DNA fragmentation, due to the activation of caspase 3 by caspase 9, and possibly caspase 8.

The TNF-α-induced sustained activation of JNK, observed when NF-κB activation was prevented, may contribute to apoptosis (46, 47, 64). The sustained activation of other molecules by TNF-α, such as cytosolic phospholipase A_2, may also contribute to apoptosis (64). In macrophages, inhibition of the sustained activation of JNK suppressed the apoptotic phenotype, DNA fragmentation; however, it had no effect on the loss either of $\Delta\psi_m$ or of viability. These observations suggest that the effect of the sustained activation of JNK might be mediated by an event upstream of DNA fragmentation, which was not necessary for the loss of mitochondrial integrity. XIAP has been shown to positively or negatively regulate the persistent activation of JNK, suggesting functional intracellular interactions between these two molecules (47, 65). Although not directly examined in macrophages, where XIAP is highly expressed, it is possible that the sustained activation of JNK might, directly or indirectly, promote DNA fragmentation through interactions that suppress the antiapoptotic effects of XIAP on caspase 3 activation. Consistent with an effect of JNK mediated through XIAP, the persistent activation of JNK may induce the release of Smac/DIABLO from the mitochondria (66, 67), by a mechanism independent of caspase 8 activation, that does not promote cell death (67). Consistent with these observations, in macrophages, inhibition of JNK had no effect on the loss of $\Delta\psi_m$ nor did it protect against cell death. These observations demonstrate that, in macrophages, both caspase 8 activation and the sustained activation of JNK contribute to DNA fragmentation, but they are not essential for the loss of mitochondrial integrity or for cell death.

Ligation of TNFRs may result in apoptosis mediated by the activation of caspase 8, which subsequently cleaves Bid, which results in the loss of $\Delta\psi_m$ due to Bax/Bak (10, 42, 68, 69). In macrophages, the effects of TNF-α on the mitochondria were also critical. However, the loss of mitochondrial integrity and cell death occurred even in the presence of IETD.fmk or zVAD.fmk, suggesting a caspase-independent mechanism. Consistent with our findings, recently published papers demonstrated that TNF-α treatment may also result in necrotic, rather than apoptotic, cell death

when caspase activation is inhibited (70–72). Furthermore, although Bid activation was detected, Bid cleavage was prevented by caspase inhibition, suggesting that Bid activation was not essential for the loss of $\Delta\psi_m$ or the cell death of in vitro matured human macrophages. Data from *bid*$^{-/-}$ mice demonstrated that TNF-α was capable of inducing Bax translocation to the mitochondria (73). Although caspase 8 mediates Bax translocation through Bid (69, 74, 75), these observations in *bid*$^{-/-}$ mice suggest that the effects of TNF-α on Bax may occur in the absence of caspase 8-mediated Bid activation. Our earlier study demonstrated that, in macrophages, the inhibition of NF-κB alone resulted in the suppression of A1, which was responsible for the apoptosis observed (29). It is possible that with reduced A1, the TNF-α-induced translocation of Bax to the mitochondria was sufficient to induce further disruption of the mitochondrial integrity, resulting in enhanced apoptosis, which was suppressed by the ectopic expression of Bcl-x_L. However, because apoptosis was only partially suppressed by the Bcl-x_L, it is likely that the caspase-independent loss of $\Delta\psi_m$ and cell death observed with macrophages may be mediated by additional mechanisms, which might include the kinase domain of receptor interacting protein, reactive oxygen species, the disruption of lysosomes or the endoplasmic reticulum, neutral or acid sphingomyelinase and ceramide, arachidonic acid, or cytosolic phospholipase A_2 (64, 76–78).

In summary, these observations demonstrate that following the inhibition of NF-κB, TNF-α-induced caspase 8 activation and the sustained activation of JNK each promoted DNA fragmentation, but neither was essential for the induction of an apoptosis-like programmed cell death. Furthermore, caspase activation was not essential for the loss of $\Delta\psi_m$ and cell death, and caspase inhibition converted apoptosis-like programmed cell death to a necrosis-like programmed cell death (78), revealing the integration of caspase-dependent and -independent pathways that lead to TNF-α-induced macrophage death.

Acknowledgments

We thank Shawn Cole for assistance in virus preparation and for the assistance of the Robert H. Lurie Comprehensive Cancer Center, Flow Cytometry Core Facility of the Northwestern University Feinberg School of Medicine.

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