Inhibition of p38 Kinase Reveals a TNF-α-Mediated, Caspase-Dependent, Apoptotic Death Pathway in a Human Myelomonocyte Cell Line

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TNF-α transduces signals of survival or death via its two receptors, R1/p55/p60 and RII/p75/p80. The role of caspases as effectors of cell death is universally accepted, although caspase inhibitors may potentiate TNF cytotoxicity in some instances. In conditions when macromolecular synthesis is blocked, caspases are part of the machinery that executes TNF-triggered apoptotic death in U937, a human myelomonocyte cell line, and in the Jurkat T cell line. However, inhibition of p38 mitogen-activated protein kinase (p38 MAPK) triggered TNF cytotoxicity in U937 cells and murine splenic macrophages, but not the Jurkat cell line. TNF induced expression of the antiapoptotic protein c-IAP2 (cytoplasmic inhibitor of apoptosis protein 2), and was blocked in the presence of a p38 MAPK inhibitor, which also induced caspase-dependent, TNF-mediated apoptosis in U937 cells. Thus, inhibition of p38 MAPK resulted in the activation of caspase 9 and cleavage of the adaptor molecule BH3 interacting domain death agonist, and blocked NF-κB-mediated transactivation, without affecting the nuclear translocation of NF-κB. Collectively, these data show that activation of p38 MAPK is critical to cell survival by TNF in U937 cells, and demonstrate lineage-specific regulation of TNF-triggered signals of activation or apoptosis. The Journal of Immunology, 2001, 166: 6570–6577.
Materials and Methods

Cells and reagents

U937, a human myelomonocytic cell line, and Jurkat, a T lymphoblastoid cell line of human origin, were used in all experiments. Murine spleen macrophages were isolated by adherence on plastic petri dishes. In each experiment, unfractionated cells (20 × 10⁶ cells/ml) from three individual spleens were incubated for 45 min in complete medium in tissue culture dishes. At the end of incubation, floating cells were removed by gentle swirling and decantation of dish contents. Plates were washed twice with complete medium using a similar procedure. After the final wash, 2 ml of cold medium was added to dishes, and adherent cells were removed using a cell scraper and used in functional assays. rTNF and Abs to TNFR1 and TNFRII were obtained from R&D Systems (Minneapolis, MN). Cycloheximide (CHX), Hoechst 33342, and propidium iodide were obtained from Sigma (St. Louis, MO). The p38 MAPK inhibitor PD 169316 was purchased from Calbiochem (San Diego, CA). The peptide inhibitors Z-Val-Ala-Asp(O-methyl)-fluoromethyl ketone (ZVAD-FMK), Boc-Asp(O-methyl)-FMK (BD-FMK), Ile-Glu-Thr-Asp-FMK (IETD-FMK), Val-Glu-Ile-Asp-FMK (VEID-FMK), Leu-Glu-His-Asp-FMK (LEHD-FMK), and Z-Phe-Ala-FMK were obtained from Enzyme Systems Products (Dublin, CA), dissolved as 20 mM stock solutions in DMSO, and stored at −70°C. The 5,5′,6,6′-tetrachloro-1,1′,3′,3′-tetraethylbenzimidazolylcarbocya-nine iodide (JC-1) and 3,3′-diethylxocarbocyanine (DiOC₂) were obtained from Molecular Probes (Eugene, OR), made up as stock solutions in DMSO, and stored at −70°C. Abs to cleaved caspase 9, phosphoisoforms of p38, and ATF-2 were from New England Biolabs (Beverly, MA); cytoplasmic inhibitor of apoptosis protein 1 (c-IAP1), c-IAP2, TNFR-associated factor-2 (TRAF-2), Bcl-x1, p38 MAPK, Fas, and NF-κB were from Santa Cruz Biotechnology (Santa Cruz, CA); and biotin- or avidin-conjugated Abs were obtained from Jackson ImmunoResearch (West Grove, PA). Ab to BH3 interacting death domain agonist (BID) was obtained from R&D Systems.

Apoptotic nuclear damage and cell lysis

Cell lysis was quantitated by flow cytometry using propidium iodide. Apoptotic nuclear morphology was assessed using Hoechst 33342, as described previously (26). All assays of nuclear morphology were scored double blind. For imaging nuclei, cells were fixed with 0.1% paraformaldehyde for 10 min at room temperature, then washed and stained with 20 nM of the dye SYTOX Green (Molecular Probes) for 10 min at ambient temperature. At the end of incubation, the cells were washed and resuspended in PBS (10⁶/ml) for analysis of nuclear morphology.

Mitochondrial transmembrane potential

For DiOC₂ staining, cell pellets were incubated for 20 min in the dark at 37°C in 0.5 ml staining solution (PBS containing 40 μM DiOC₂) as described previously (26). Cells were washed in excess PBS and resuspended in 5 ml PBS containing propidium iodide to identify dead cells, and the propidium iodide-negative subset was analyzed flow cytometrically. For staining with JC-1, cell pellets were resuspended in staining solution (200 μl PBS containing 5 μg/ml JC-1) and incubated at 37°C for 10 min. At the end of incubation, cells were washed in excess PBS and resuspended in 0.3 ml PBS and analyzed flow cytometrically, according to the manufacturer’s instructions. All flow cytometric analysis was performed on a Becton Dickinson FACS (Becton Dickinson, Mountain View, CA).

Immunofluorescence analysis by flow cytometry

Cells fixed with 1% paraformaldehyde at room temperature for 20 min were washed twice in chilled permeabilization buffer (PBS containing 0.3% saponin, 0.1% sodium azide, and 1% FCS). A total of 20 μl of relevant Ab (diluted in permeabilization buffer) was added directly to the cell pellets, and the cells were incubated on ice for 30 min. Primary Ab was followed by secondary biotin-conjugated Ab, and all samples were finally stained with PE-avidin. Each step of Ab staining was followed by two washes in permeabilization buffer to remove excess Ab. At the end of the incubation with PE-avidin, cells were washed once with permeabilization buffer, followed by a wash in FACS buffer (PBS containing 1% FCS and 0.1% sodium azide), and resuspended in 0.4 ml FACS buffer for flow cytometric analysis.

Immunofluorescence analysis by microscopy

Cells were fixed in 70% ethanol for 2 h, washed twice in saponin buffer, and stained as described for flow cytometric analysis, except that biotin-conjugated Ab was followed by avidin conjugated to Alexa-546 (Molecular Probes). After the last wash, cells were resuspended in a minimal volume of PBS, mounted on slides, and photographed using a Pro-Series 3, CCD camera (Media Cybernetics).

Western blot analysis

The 10⁶ cells treated under various conditions were harvested, washed twice by centrifugation in chilled PBS, lysed by the addition of SDS sample buffer (62.5 mM Tris-HCl, 2% w/v SDS, 10% glyceral, 50 mM DTT, and 0.1% bromophenol blue), vortexed to reduce sample viscosity, denatured by boiling, and then cooled on ice. Samples were microcentrifuged for 5 min before loading on 10% SDS-PAGE gels. Proteins were transferred onto Hybond-ECL nitrocellulose membrane (Amer sham, Little Chalfont U.K.). Membranes were blocked for 1 h in blocking buffer (5% Biotto, 0.1% Tween 20 in PBS) and incubated overnight with primary Ab in blocking buffer at 4°C, with gentle rocking. Membranes were washed three times for 5 min each with 10 ml of PBS containing 0.1% Tween-20, and then incubated for 1 h at 25°C with goat anti-rabbit HRP (Pierce, Rockford, IL), diluted 1/5000 in blocking buffer. After another three washes, proteins were detected by chemiluminescence, according to the manufacturer’s instructions (Pierce).

NF-κB reporter assay

A total of 6–10 × 10⁶ U937 cells was transfected with 5 μg of thymidine kinase-6 NF-κB luciferase by electroporation at 250 mM and 960 μF capacitance. Twenty-four hours after transfection, live cells were harvested by Ficoll density centrifugation, and cells were treated with TNF (10 ng/ml), or TNF + PD, or continued in medium alone. Six to 8 h after initiation of treatment, cell lysates were assayed for luciferase activity using a substrate provided by Promega (Madison, WI), according to the manufacturer’s instructions. Background readings using a luminometer from Turner Design (Palo Alto, CA). Promoter activity is expressed as fold induction over readings obtained from mock-transfected cells cultured in medium alone.

Results

Caspase inhibitors block TNF + CHX-induced apoptosis in U937 and Jurkat cells

U937 cells were cultured with 5 ng/ml TNF in the presence or absence of a broad spectrum caspase inhibitor peptide, ZVAD-FMK or CHX. Cells were scored for lysis using propidium iodide after 18 h in culture. As seen in Fig. 1A, U937 cultured with TNF alone showed minimal levels of apoptosis (<10% apoptotic nuclei at 18 h), but the presence of 200 ng/ml CHX resulted in a sharp increase in apoptotic nuclear damage in these cells (Fig. 1, A and B).
B). As previously reported (25), the caspase inhibitor ZVAD-FMK also potentiated TNF-induced death (Fig. 1A), but a large proportion of the dead (propidium iodide-positive) cells in the TNF + ZVAD preparations had a distorted, condensed morphology (Fig. 1, A and C), and did not show distinct nuclear fragmentation.

To explore the mechanism by which TNF + CHX induced apoptotic death, we used cell-permeable, peptide inhibitors to investigate the role of caspases in this pathway. Maximal levels of apoptotic nuclear damage were induced in 6–8 h by TNF (5 ng/ml) + CHX (500 ng/ml) in U937 cells (Fig. 2A). Under these conditions, pretreatment with BD-FMK, a broad spectrum caspase inhibitor; VEID-FMK, an inhibitor of caspase 6; or IETD-FMK, an inhibitor of caspase 8, blocked TNF + CHX-induced apoptotic nuclear damage in a dose-dependent manner (Fig. 2A). In the same experiment, BD-FMK and VEID-FMK only partially inhibited the loss of mitochondrial transmembrane potential (Fig. 2B), while IETD-FMK had no effect, even at concentrations that block nuclear damage. Concentrations of IETD-FMK higher than 50 μM have not been tested, as the resultant toxicity masked the protective effects, if any, of this inhibitor. In contrast, LEHD-FMK, an inhibitor of caspase 9, did not block either nuclear or mitochondrial damage in these cells (Fig. 2, A and B). The partial inhibition of mitochondrial damage by the caspase inhibitors (Fig. 2B) suggests a role for other proapoptotic molecules such as reactive oxygen species in this pathway (22, 25). ZVAD-FMK expectedly did not block either nuclear (Fig. 2A) or mitochondrial damage (Fig. 2B) triggered by TNF + CHX. The peptides used as caspase-specific inhibitors are based on optimal peptide recognition motifs that have been described for the different caspases (27). These have been used to design caspase-specific substrates as well (28). Although both BD-FMK and ZVAD-FMK are broad spectrum inhibitors of caspases, differences in their ability to inhibit death have been observed in other systems as well (29). A recent study has suggested that ZVAD-FMK acts at a very early step in apoptotic cascades to block caspasas or a hitherto undefined, but related activity in cells (28).

Similar assays performed in the Jurkat T cell line showed that Jurkat cells are relatively resistant to TNF + CHX compared with U937, in that maximal levels of apoptotic nuclear damage are only observed 16–18 h after culture. Jurkat cells were pretreated with 25 μM of the various peptides for 45 min and then cultured with TNF + CHX for 16 h. All the peptides tested, including LEHD-FMK, blocked nuclear damage (Fig. 2C), although ZVAD-FMK expectedly was most potent in inhibiting apoptotic death at concentrations as low as 5 μM (data not shown) in these cells. To address the possibility of nonspecific effects of the peptides, we also tested an inhibitor of cathespin, Z-Phe-Ala-FMK, which did not block TNF + CHX-mediated death in these cells (data not shown).

**TNF + CHX triggers the activation of caspase 9 in Jurkat cells**

The differential induction of caspase 9 in Jurkat cells, suggested in the previous experiments, was confirmed by assaying for the expression of cleaved caspase 9 in intact, treated cells. Furthermore, to aid comparison and normalize the kinetics of the apoptotic response in the two cells, we treated Jurkat cells with TNF in the presence of a high dose of CHX (5 μg/ml) so as to induce TNF + CHX-mediated apoptosis with kinetics similar to U937 cells. Using a polyclonal Ab that specifically recognized the cleaved form of the endogenous protease, Jurkat cells treated with the higher dose of CHX and TNF were stained after 2 or 4 h for expression of caspase 9. A small shift indicative of cleaved caspase 9 was detected at 2 h (data not shown); however, by 4 h all the cells stained positive with this Ab (filled histogram, Fig. 2D). Specific fluorescence was not detected in control, unstimulated cells, or in cells treated with Ab isotype controls (dotted and shaded line open histograms, Fig. 2D). Under these conditions, TNF + CHX induced 60.5% apoptotic nuclear damage by 6 h, which was reduced to 20.6% in LEHD-FMK-treated cells. As expected from the functional assays, cleaved caspase 9 could not be detected in U937 cells (Fig. 2E) at either early or later time points in the death pathway, although U937 treated with TNF + CHX (0.5 μg/ml) showed a measurable increase in nuclear damage by 2 h. Thus, TNF + CHX induced 73% apoptotic damage compared with 2.3% apoptotic nuclei scored in control, untreated cells 6 h after initiation of the assay. These data indicate a role for caspase 9 in TNF-mediated apoptosis in Jurkat cells, but not U937 cells, in conditions when TNF cytotoxicity is induced by CHX.

Although CHX has been used routinely in many studies to analyze TNF-mediated cytotoxicity, it can affect multiple events in cells. Notwithstanding, the experiments with CHX in this study...
suggest the contextual regulation of TNF-initiated signaling events in the two cell types under study. In U937 cells, TNF induced necrosis in the presence of a caspase inhibitor (Fig. 1 and Ref. 25), and activates proapoptotic caspases in the presence of CHX (Fig. 1, B and D, and Fig. 2). Thus, it was of interest to try and identify the nature of the input signals that can regulate caspase activation in TNF-treated U937 cells.

**Inhibition of p38 MAPK potentiates TNF cytotoxicity in U937 cells**

TNF signaling leads to the activation of kinase signaling cascades, and these can regulate caspase activation in various cell death pathways. To further characterize the regulation of TNF-mediated signaling in U937 cells, we tested for the effect of an inhibitor of p38 MAPK, PD169316 (30–32). As shown in Fig. 3A, TNF, in the presence of PD169316 (TNF + PD), induced apoptotic nuclear damage at concentrations of PD that were nontoxic (Fig. 3A, open squares) to the cells. Agonist Ab to TNFR1 (Fig. 3B) triggered apoptotic death in the presence of 20 μM PD in a dose-dependent manner, but there was no induction of cytotoxicity by an Ab to TNFRII (Fig. 3B). These results are comparable with the effects of TNFR1 and TNFRII Abs in the presence of CHX (Fig. 3C) in these cells. PD169316 did not potentiate TNF-mediated apoptotic death in Jurkat cells (Fig. 3D), suggesting a specific role for p38 MAPK in TNF-mediated survival in U937 cells.

The immunoblot in Fig. 3F shows an experiment that demonstrates the induction of phosphorylated p38 MAPK in cells treated with 10 ng/ml TNF for 30 min (lane 2) as compared with untreated cells (lane 1). This experiment shows that pretreatment of cells with PD169316 before the addition of TNF blocks the phosphorylation of p38 MAPK (lane 3). Lane 4 served as a positive control, and shows the induction of phosphorylated p38 MAPK within 30 min in U937 cells treated with hydrogen peroxide (19). Interestingly, in keeping with the differences in TNF-triggered signaling observed in U937 and Jurkat cells, a role for p38 MAPK as a possible effector of Fas-mediated death has been recently suggested in Jurkat cells (33). In subsequent experiments, we have further characterized the TNF + PD-mediated death pathway in U937 cells.

**Inhibition of p38 MAPK triggers a caspase-dependent death pathway in U937 cells**

Because PD169316 triggered TNF-mediated apoptosis, we tested the effect of caspase inhibitors on TNF + PD-mediated apoptosis in the U937 cell line. In the experiment shown in Fig. 3E, 25 μM of the caspase inhibitors BD-FMK, VEID-FMK, or IETD-FMK blocked TNF + PD-mediated apoptosis, whereas ZVAD-FMK was unable to block apoptotic nuclear damage (Fig. 3E). Unexpectedly, LEHD-FMK, the inhibitor of caspase 9, also blocked nuclear damage in TNF + PD-treated cells.

These experiments suggested a role for p38 MAPK in the modulation of caspase 9 activation. Alternatively, it is also possible that caspase 9 activation or activity is directly inhibited by CHX, which may explain the lack of effect of the peptide LEHD-FMK on TNF + CHX-mediated death (Fig. 2). Therefore, we tested for inhibition of caspase 9 by CHX. As shown in Fig. 4A, TNF + PD-induced expression of activated caspase 9 (Fig. 4B, solid line) was not blocked by 0.5 μg/ml CHX (filled histogram in Fig. 4, A or B) or 50 ng/ml CHX (data not shown). Caspase 9 expression in control, untreated cells is indicated by the solid line histogram in Fig. 4A. In functional assays for caspase 9 activity, we tested whether 0.5 μg/ml (CHX) or lower concentrations of CHX (50 ng/ml, CHX10) would block the inhibitory effect of LEHD-FMK on TNF + PD-mediated death. As shown in Fig. 4C, PD and CHX have additive effects on TNF-mediated death. Apoptotic death cells treated with LEHD-FMK + TNF + PD + CHX were comparable with that of LEHD-FMK + TNF + CHX for each concentration of CHX. These data are consistent with the possibility...
that LEHD-FMK blocks the TNF + PD-triggered, caspase 9 component of the death pathway and does not affect TNF + CHX-mediated cell death. Additionally, these experiments suggest that caspase 9 activation is not regulated directly by CHX.

In preliminary experiments, shown in the immunoblot in Fig. 4D, there is a loss of expression or immunoreactivity of the death-adaptor molecule BID, as early as 2 h following treatment with TNF + PD (lanes 3 and 5) as compared with cells treated with TNF (lane 2) or left untreated (lane 1). These conditions lead to induction of apoptotic damage by 6 h. Furthermore, cells treated with IETD-FMK (lane 3) at concentrations that block caspase 8-mediated cleavage of BID show restoration of BID expression as compared with cells treated with TNF + PD (lane 2). All blots were reprobed for expression of Fas, which showed that changes in BID immunoreactivity were not owing to alterations in cell number. Thus caspase 8-mediated cleavage of BID can lead to the activation of caspase 9 in TNF + PD-treated cells. As shown in Fig. 4E–G, IETD-FMK partially blocks the activation of caspase 9 (G) induced by TNF + PD (F), suggesting that caspase 9 activation may, in part, be dependent on caspase 8-mediated cleavage of BID in U937 cells treated with TNF + PD.

**Inhibition of p38 MAPK blocks antiapoptotic gene expression**

To assess whether the effect of p38 MAPK on TNF signaling was limited to U937 cells alone, we also tested for the effect of PD on TNF-induced cytotoxicity in freshly isolated murine splenic macrophages. As shown in Fig. 5A, adherent macrophages isolated from murine spleens when cultured overnight showed minimal induction of apoptotic damage. Cells treated with TNF for 18 h also showed minimal cytotoxicity as compared with control, untreated cells. However, as was seen with U937 cells, PD169316 induced a striking increase in TNF-mediated apoptotic nuclear damage (solid bars) and cell lysis (hatched bars) as well.

We then tested whether p38 MAPK mediated cell survival by modulating expression of various endogenous antiapoptotic proteins of the Bcl-2 family, including Bcl-2 and Bcl-xL, or the inhibitors of apoptosis proteins, c-IAP1 and c-IAP2. The c-IAP family are key players in the TNF-mediated antiapoptotic response (2, 6). These proteins are transcriptionally regulated and block death by inhibiting the activation of caspases. Earlier experiments have shown that the expression of inhibitor of apoptosis protein is among the early responses triggered by TNF via NF-κB (6), and is therefore sensitive to inhibition by agents such as CHX. U937 cells were cultured with TNF for 2 h in the presence or absence of 20 μM PD169316, and analyzed for the cytoplasmic levels of c-IAP1, c-IAP2, Bcl-xL, Bcl-2, and TRAF-2. U937 cells express very low levels of c-IAP1 and c-IAP2 (data not shown). TNF-induced expression of c-IAP2 (Fig. 5C, solid line) was markedly reduced by PD169316 (Fig. 5C, filled histogram); expression of c-IAP1 (Fig. 5B, solid line) was partially blocked (Fig. 5B, filled histogram), but the high levels of constitutively expressed TRAF-2 (Fig. 5D, shaded histogram) were unchanged by this inhibitor (Fig. 5D).
harvested, lysed, and assayed for NF-κB line histogram, respectively, in each figure. U937 cells were stained for expression of BclxL (are shown in D for both TNF and TNF + PD-treated cells. The isotype controls for TNF or TNF + PD-treated cells are indicated by the fine or dotted line histogram, respectively, in each figure. U937 cells were stained for expression of BclxL (E) either in untreated cells (filled histogram, med) or after treatment with 5 ng/ml TNF (gray thick line, TNF) for 3 h. The dotted line and dark line represent isotype controls for untreated and TNF-treated cells, respectively. F–H, U937 cells were incubated for 20 min in the presence (G and H) or absence (F) of 5 ng/ml TNF. Cells in H were preincubated with 20 μM PD169316 for 30 min before the addition of TNF. Cells were harvested, fixed, and stained with an Ab that recognizes the p65 subunit of NF-κB, as described in Materials and Methods. Cells were visualized using appropriate filters on an Olympus microscope and imaged using a CCD camera at ×40 magnification. I, Luciferase assay for NF-κB promoter activity. U937 cells were transfected with the thymidine kinase-6 NF-κB luciferase (luc) reporter plasmid or mock transfected, and 24 h later the transfection groups were treated with TNF or TNF + PD. Eight hours after addition of TNF, cells were harvested, lysed, and assayed for NF-κB-driven luciferase activity. Luciferase activity has been shown as fold induction over mock-transfected cells cultured in medium alone. J, U937 cells treated with TNF, TNF + PD, and 0.2 mM hydrogen peroxide, or left untreated were analyzed for expression of the phosphoisoform of p38 MAPK (p-p38), and the same blot was stripped and reprobed for expression of the phosphoisoform of ATF-2 (p-ATF2) and total p38 MAPK.

U937 cells do not express Bcl-2 (data not shown). BclxL is expressed in U937 cells at very low levels (Fig. 5E, filled histogram); its expression is not enhanced further by TNF (Fig. 5E, heavy gray line), nor altered in TNF + PD-treated cells. The role of p38 MAPK in TNF-mediated up-regulation of c-IAP2 in U937 cells appears similar to its role in CD40-mediated signaling in dendritic cells (34).

p38 MAPK does not affect the nuclear translocation of NF-κB
Activation of the transcription factor NF-κB is central to the survival pathway triggered by TNF in many systems. p38 MAPK appeared to function as a TNF-triggered survival signal in U937 cells, and because the effect of p38 kinase on NF-κB-induced gene expression has been shown in other systems (13, 14), we tested whether p38 MAPK can also modulate NF-κB activation. NF-κB is normally trapped in the cytoplasm by its inhibitor I-κB, but the association is regulated by phosphorylation of I-κB by members of the MAPK kinase family. We wished to test whether p38 MAPK blocked the nuclear translocation of NF-κB. Thus, cells treated with TNF show an early nuclear translocation (within 20 min of addition of TNF) of NF-κB, which can be visualized by staining with an Ab that recognizes the p65 subunit of NF-κB, as shown in Fig. 5F, F–H. As shown in Fig. 5F, the fluorescent signal with NF-κB Ab in control, untreated cells is confined principally to the cytoplasmic region of the cells. The nucleus can be seen as a central, sparsely stained region that is largely devoid of fluorescence in these cells. Exposure to TNF for 20 min results in the translocation of NF-κB to the nucleus, which can be visualized as the bright fluorescence in the nucleus (Fig. 5G). The fluorescence is sustained for at least 45 min after addition of TNF (data not shown). The translocation of NF-κB is not inhibited in TNF + PD-treated cells, as these cells also show bright nuclear localized fluorescence (Fig. 5H), comparable with that seen in cells treated with TNF alone.

To examine the effect of PD on TNF-induced NF-κB-dependent gene transcription, we transfected a NF-κB reporter construct in U937 cells. As shown in Fig. 5I, U937 cells treated with TNF for

FIGURE 5. PD169316 potentiates TNF cytotoxicity in splenic macrophages and inhibits the expression of TNF-triggered antiapoptotic proteins in U937 cells, but does not inhibit NF-κB nuclear translocation. A, Murine splenic macrophages were cultured overnight in the different conditions shown in the figure and analyzed for the induction of apoptotic nuclear damage and cell lysis. B–E, Expression of c-IAP1 (B) and c-IAP2 (C) in U937 cells treated with TNF (solid line, open histogram) or TNF + PD (filled histogram), 2.5 h after addition of 5 ng/ml TNF. Cells stained with TRAF-2 (filled histogram) are shown in D for both TNF and TNF + PD-treated cells. The isotype controls for TNF or TNF + PD-treated cells are indicated by the fine or dotted line histogram, respectively, in each figure. U937 cells were stained for expression of BclxL (E) either in untreated cells (filled histogram, med) or after treatment with 5 ng/ml TNF (gray thick line, TNF) for 3 h. The dotted line and dark line represent isotype controls for untreated and TNF-treated cells, respectively. F–H, U937 cells were incubated for 20 min in the presence (G and H) or absence (F) of 5 ng/ml TNF. Cells in H were preincubated with 20 μM PD169316 for 30 min before the addition of TNF. Cells were harvested, fixed, and stained with an Ab that recognizes the p65 subunit of NF-κB, as described in Materials and Methods. Cells were visualized using appropriate filters on an Olympus microscope and imaged using a CCD camera at ×40 magnification. I, Luciferase assay for NF-κB promoter activity. U937 cells were transfected with the thymidine kinase-6 NF-κB luciferase (luc) reporter plasmid or mock transfected, and 24 h later the transfection groups were treated with TNF or TNF + PD. Eight hours after addition of TNF, cells were harvested, lysed, and assayed for NF-κB-driven luciferase activity. Luciferase activity has been shown as fold induction over mock-transfected cells cultured in medium alone. J, U937 cells treated with TNF, TNF + PD, and 0.2 mM hydrogen peroxide, or left untreated were analyzed for expression of the phosphoisoform of p38 MAPK (p-p38), and the same blot was stripped and reprobed for expression of the phosphoisoform of ATF-2 (p-ATF2) and total p38 MAPK.
6–8 h in three experiments showed a 10- to 15-fold increase in NF-κB reporter activity relative to control, untreated cells. This induction was markedly blocked in cells treated with TNF + PD, but complete inhibition was not consistently observed in these assays. As shown in the immunoblots in panel 1, concentration of PD that results in a reduction in levels of phospho-p38 kinase in TNF + PD-treated cells also blocked induction of expression of the downstream transcription factor phospho-ATF-2 in these cells. Hydrogen peroxide (H2O2)-treated cells were used as a positive control in these experiments, as described for Fig. 3.

**Discussion**

TNF triggers the expression of a largely overlapping battery of genes in diverse cell types (2, 6), although it elicits widely varying responses of survival, death, or differentiation. This suggests that additional cell-specific factors, not necessarily transcriptionally regulated, modify the outcome of TNF signaling, and this study identifies p38 MAPK as one such candidate in U937 cells. Although other signaling pathways have not been examined in this study, we find that p38 MAPK is a critical component of the TNF survival pathway in U937, a myelomonocyte cell line, and in murine splenic macrophage preparations as well. In T cells, p38 MAPK is believed to contribute to cell death triggered by Fas (33), although a more recent study has shown that TNF-triggered activation of p38 MAPK in Jurkat cells may not contribute to the apoptotic response in these cells (35). Thus, p38 kinase activity may be a critical determinant for TNF-triggered survival in some cells particularly of the myeloid lineage, including dendritic cells and eosinophils (12, 18), but may also contribute to death in other cells (16, 17).

p38 MAPK has been implicated as a mediator of apoptosis (16, 17), but recent reports have also described a protective role for this kinase in TNF-mediated signaling (3, 19). A recent report has indicated a protective role for p38 MAPK in response to oxidative stress in U937 (19), although the role of antiapoptotic proteins, if any, in this system was not discussed. TNF-mediated death is blocked by cytokines in T cells (36), and because p38 MAPK regulates expression of cytokines in different cells (10, 12, 13), an analysis of cytokines produced in response to TNF signaling in U937 cells may reveal additional antiapoptotic signals generated via this pathway. The up-regulation of antiapoptotic proteins, such as c-IAP2, is one way by which p38 MAPK contributes to TNF-mediated survival, and thereby the determination of cell fate.

We have attempted to address the possible interaction of TNF-triggered p38 MAPK and NF-κB in this system. In cardiac myocytes, p38 MAPK regulates the expression of IL-6, via interactions between MKK6 (MAPK kinase), which lies directly upstream of p38, and IκB kinase, which results in NF-κB activation (13) in TNF-treated cells. This model of a direct impact of p38 MAPK on NF-κB activation is not supported by our data, which are more consistent with the effect of p38 MAPK on the TNF-triggered transcription-activating potential of NF-κB. TNF-induced nuclear translocation of NF-κB (Fig. 5G) was not modulated by PD169316 (Fig. 5G), but affected NF-κB transactivation (Fig. 5J) and expression of the antiapoptotic gene inhibitor of apoptosis protein-2 (Fig. 5C) in these cells. A similar requirement for the p38 MAPK pathway in NF-κB function, independent of its DNA binding, has been reported in response to CD40 and TNF in other systems (14, 37, 38). The mechanism by which p38 MAPK modulates NF-κB transcription is not known. One possibility consistent with the experimental results in this study and others is that p38 MAPK or a downstream kinase phosphorylates a transcriptional coactivator such as CBP/p300, which may modulate NF-κB-driven gene activation (39). We also find that p38 MAPK-mediated phosphorylation of the transcription factor ATF-2 is inhibited by PD169316 (Fig. 5J), c-IAP2, which has been functionally implicated in the inhibition of TNF-mediated death, is transcriptionally regulated by NF-κB (40). Transcription factors such as ATF-2 provide an additional layer of regulation wherein p38 MAPK may modulate NF-κB-mediated transcription of genes in response to TNF.

An earlier study has shown that inhibition of p38 MAPK does not potentiate TNF cytotoxicity in L929 cells (38). We attribute a role in cell survival to p38 MAPK, because inhibition of p38 MAPK triggered death in U937 and splenic adherent cells (Figs. 3 and 5). This study shows that inhibition of p38 MAPK resulted in activation of caspase 9 and the loss of full-length BID. Additionally, caspase 8-dependent cleavage of the adaptor molecule BID and partial inhibition of caspase 9 activation by an inhibitor of caspase 8 suggest that caspase 9 may, in part, be activated by a caspase 8-dependent signaling event.

Caspase 9 is recruited to an apoptosome complex that comprises several Apaf-1 molecules, the oligomerization of which is dependent on cytochrome c and dATP. We do not detect an early release of cytochrome c in these cells (data not shown). Heat-shock protein (Hsp) 27, a small molecular mass protein that is a pleiotropic inhibitor of cell death, is another negative regulator of caspase 9 activation that binds cytochrome c and inhibits formation of the apoptosome complex in cells (41). Hsp27 can also bind Akt kinase/protein kinase B, which also has the capability of phosphorylating and inactivating caspase 9 (42). Hsp27 is phosphorylated by p38 MAPK (43, 44), and it is possible that in the TNF-mediated survival pathway, activated p38 MAPK phosphorylates Hsp27, which inactivates the apoptosome and contributes to the inhibition of caspase 9 activation in U937 cells.

Based on biochemical and functional assays, this study provides three lines of evidence that p38 MAPK is a component of the survival response triggered by TNF in U937, a myelomonocyte cell line. Inhibition of p38 MAPK triggers TNF cytotoxicity in functional assays of death in two experimental systems, the U937 cell line and splenic macrophages. Inhibition of p38 MAPK inhibits NF-κB-mediated gene activation in reporter assays, although nuclear translocation of NF-κB is not blocked (Fig. 5). NF-κB is a well-defined mediator of the TNF-triggered survival response, and among other proteins regulates expression of the antiapoptotic protein c-IAP2 (6, 39). The TNF-induced up-regulation of c-IAP2 is also blocked by the inhibitor of p38 MAPK (Fig. 5). Thus, the experiments show that p38 MAPK can regulate NF-κB-mediated gene transcription and also the expression of the antiapoptotic protein c-IAP2, although it remains to be established whether these two events are related in the present system.

Cells use several mechanisms to regulate the activity of the caspase family of proteases. Activation of caspase-9 may be regulated by both transcription-dependent (c-IAP-mediated) (2, 6), and transcription-independent (phosphorylation of regulatory proteins) (41, 42) events. Although p38 MAPK may principally act via a phosphorylation-dependent event, in this study we present evidence that this kinase can also intersect a key transcription-dependent pathway and regulate TNF-mediated death in cells. Thus, these experiments provide additional evidence of multiple protective mechanisms influenced by this kinase. Collectively, the data indicate that the p38 MAPK pathway is critical to the TNF-triggered antiapoptotic response and functions as a key cell fate determinant in U937 cells.

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