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Abstract

Background: A frequent feature of hepatocellular carcinoma is resistance to apoptosis induced by a variety of agents, including the pleiotropic cytokine tumor necrosis factor-α (TNF). Compared with other cell types, hepatocytes and hepatoma-derived cell lines are resistant to TNF-induced apoptosis. Resistance to TNF is largely ascribed to activation of the prosurvival transcription factor NF-κB and can be overcome by co-administration of low doses of protein synthesis inhibitors or other drugs.

Aims: This study analyses the molecular mechanisms by which TNF, in combination with cycloheximide (CHX), induces apoptosis in human hepatoma-derived Huh7 cells, focusing on the role played by JNK.

Methods: Huh7 cell cultures were treated with TNF+CHX in the presence or absence of either the pancaspase inhibitor zVADfmk or the JNK inhibitor SP600125, or after suppression of JNK expression by RNAi. Apoptosis was assessed by both light microscopy and flow cytometry. JNK and caspase activation were assessed by western blotting and/or enzymatic assay.

Results: TNF+CHX-induced death of Huh7 cells was partially prevented when JNK activity or expression was suppressed. Moreover, apoptosis was significantly reduced by zVADfmk. The combination of SP600125 and zVADfmk completely abrogated cell death.

Conclusions: These results demonstrate a causal role for JNK and caspases in TNF+CHX-induced apoptosis of Huh7 human hepatoma cells. Strategies aimed at enhancing both pathways may be useful in overcoming the resistance of hepatocarcinoma cells to TNF-dependent apoptosis.

Key words: TNF, JNK, apoptosis, hepatocarcinoma, caspases

1. Introduction

Tumor necrosis factor-α (TNF) is a pleiotropic cytokine that can elicit proliferative or cytotoxic responses, depending on cell type and status [1,2]. In particular, TNF plays a pivotal role in liver homeostasis since, depending on the circumstances, it can promote either hepatocyte survival or death [3,4]. Accordingly, it can drive liver regeneration after partial hepatectomy and liver injury or trigger cell death in pathophysiological settings such as fulminant hepatic failure, ischemia-reperfusion and viral hepatitis [4-6]. Finally, TNF significantly contributes to hepatocarcinogenesis as well as to modulate the susceptibility of hepatocellular carcinoma (HCC) cells to apoptosis.

Although cells of the hepatocytic lineage generally appear poorly susceptible to TNF-induced apoptosis, their responsiveness can be enhanced by agents that cause translational or transcriptional inhibition (e.g., cycloheximide, actinomycin D, D-galactosamine), suggesting that the cytokine promotes the expression of protective factors. Importantly, TNF plays a role also in the hepatocytic death triggered Fas activation [5] and by antiblastic drugs such as 5-fluorouracil, bleomycin [7,8] or taxol (Minero et al., in preparation). Enhanced TNFR1 (TNF receptor 1) expression or secondary TNF production and release have been reported among the mechanisms involved [7].

NF-κB and JNK signaling stand among the main modulators of TNF cytotoxicity and their balance is crucial in shaping the outcome of TNF action on liver cells. Landmarks in this regard were observations showing that IKKβ mutant mice undergo embryonic lethality due to compromised NF-κB activation [9], while lethality is delayed in IKKβ/JNK1 double mutants [9]. NF-κB indeed mediates inhibition of JNK signaling in TNF-exposed hepatocytes while JNK activation enhances caspase-dependent cell death [10]. In keeping with these observations, the JNK inhibitor SP600125 attenuates TNF cytotoxicity in primary cultures of RelA-deficient hepatocytes [11]. By contrast, constitutive activation of JNK in many tumors and in as many as 75% of HCCs [12] is believed to exert a strong influence on tumor cell resistance to antineoplastic agents. The

part played by JNK activation in apoptosis of hepatocytes or hepatoma cells is still unsettled, however: although a promoting role has been most frequently observed, there are reports of an antagonistic action [13] or even of no effect at all [14].

Aim of the present study has been to investigate the relevance of JNK activity to apoptosis by TNF, particularly in Huh7 cells, a human HCC-derived line. The mechanisms underlying cell death by TNF+CHX have been studied in these cells, demonstrating that both a caspase- and a JNK-dependent pathway are critically involved in this process. These findings might suggest a new approach to strongly potentiate the susceptibility of HCC cells to TNF cytotoxicity.

2. Materials and methods

2.1 Cell cultures and treatments

Human hepatoma Huh7 cells (kindly provided by C. Traboni, actually at Okairos, Pomezia, Italy) were grown in Dulbecco's modified Eagle's medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. For the experiments, cells were plated in Petri dishes (30,000/cm^2) and grown at 37°C in a humidified 5% CO_2 atmosphere.

Forty-eight hours after plating, cells were treated with TNF+CHX (TNF 15 ng/ml; Peprotech, NJ, USA; CHX, 10 µg/ml; Sigma, St Louis, MO, USA). Where indicated, cells were pretreated for 1 hour prior to adding TNF+CHX with the JNK inhibitor SP600125 (20 µM; Calbiochem, Germany) or the polycaspase inhibitor zVADfmk (10-50 µM; Alexis Laboratories, CA, USA) both provided by Apotech Corporation (Switzerland), and cyclosporine A (2.5 and 5 µM; Sigma, St Louis, MO, USA). TNF was dissolved in DMEM, whereas all the inhibitors were dissolved in DMSO (Sigma, St Louis, MO, USA). Control experiments ruled out any effect of DMSO at the final concentration used (data not shown).

2.2 Morphological analysis

Phase contrast microscopy: Cells were seeded in chamber slides and treated as described above. At the indicated time points cells were examined by light microscopy (Axiovert 35, Zeiss) and photographed (Coolpix 4500, Nikon).

DAPI staining: Changes in nuclear morphology characteristic of apoptosis were visualized by fluorescence microscopy after DAPI-staining. Cells washed with PBS and fixed with 95% ethanol for 5 min at room temperature were incubated for 15 min with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; 1µg/ml), then washed twice with PBS. Cells were observed in a fluorescence microscope (Leitz, Dialux 20) and photographed (Coolpix 4500, Nikon).

2.3 Flow cytometry

DNA distribution analysis was performed as described elsewhere [15]. Briefly, Huh7 cells were trypsinized, washed with PBS, fixed in ice-cold 70% ethanol for 30 min, incubated at room temperature in PBS containing DNase-free RNase (Type II-A) and propidium iodide, at final concentrations of 0.4 and 0.18 mg/ml, respectively. Cells were then analysed with a FACScan flow cytometer (Becton & Dickinson, Mountain View, CA, USA) equipped with a 488 nm argon laser and three filters, transmitting respectively at 530 nm (FL1), 585 nm (FL2) and above 620 nm (FL3). Data were recorded using the CellQuest software (Becton & Dickinson, Mountain View, CA, USA). The percentage of apoptotic cells was assessed by evaluating the accumulation of hypodiploid cells, characterized by a subG1 DNA fluorescence [16].

2.4 siRNA transfection

Huh7 cells were seeded ($10^4/cm^2$) in 6 cm Petri dishes in a total volume of 3 ml DMEM supplemented with 10% fetal bovine serum. Twenty-four hours after plating cells were transfected using 10 µl Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA), 300 µl OptiMEM (Invitrogen) and 100 pmol siRNA (control and JNK specific; Invitrogen). The target sequence for JNK siRNA was 5'-AAAAAGAATGTCCTACCTTCT-3', which is specific for both human and mouse JNK [17]. Treatments with TNF+CHX were performed 72 hours after transfection.

2.5 Total cell extracts and subcellular fractions for western blotting

Purified cytoplasmic and nuclear fractions were prepared from cells resuspended in lysis buffer (10 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 5 mM KCl, 0.01 mM EDTA, pH 8.0, 0.1% Triton X-100, 1 mM DTT, 0.1 mM PMSF, 2 µg/ml aprotinin, 2 µg/ml leupeptin). By centrifugation (5600 rpm, 5 min, 4°C; Biofuge 17 RS, Heraeus Sepatech) was obtained the cytoplasmic fraction (supernatant), that was stored at -80°C. To isolate the nuclear fraction, pellets were resuspended in a second lysis buffer (20 mM HEPES, pH 7.9, 25% glycerol, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM

EDTA, pH 8.0, 0.5 mM DTT, 0.2 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin), incubated for 30 min on ice and centrifuged as described above. The resulting nuclear fraction (supernatant) was stored at -80°C. p-c-Jun, PARP, NF-κB and GAPDH were quantified in the nuclear fraction, while p-JNK, c-FLIP and β-actin, were quantified in the cytoplasmic fraction. GADD45β and total NF-κB were analysed in total cell extracts prepared by homogenization in PBS containing 1% Nonidet P40, 0.5 % sodium deoxycholate, 0.1% SDS, 0.1 mM PMSF, 2 μg/ml aprotinin and 100 mM sodium orthovanadate), kept on ice 30 minutes, sonicated and centrifuged at 5600 rpm for 5 min at 4°C.

Protein concentration was determined with a modified Bradford technique using the Bio-Rad protein assay kit (BioRad, CA, USA) according to the manufacturer instructions. Aliquots of cytosolic extracts or nuclear fractions (50 μg protein) were resolved by SDS-polyacrilamide gel electrophoresis and transferred to nitrocellulose membranes (BioRad, CA, USA). Nonspecific binding was blocked with 5% nonfat dry milk in TBS-Tween 0.05% for 1 hour. Blots were washed with TBS-Tween 0.05% and then incubated overnight with polyclonal rabbit IgG specific for p-SAPK/JNK (1:1000; Cell Signaling Technology, MA, USA), JNK (1:200, Santa Cruz, CA, USA), p-c-Jun (1:1000; Cell Signaling Technology, MA, USA), NF-κB p65 (1:100; Santa Cruz, CA, USA), c-FLIP$_{S/L}$ antibody (1:1000; Sigma, St Louis, MO, USA), GADD45β (1:200; Santa Cruz, CA, USA), PARP (1:200; Santa Cruz, CA, USA) and monoclonal rabbit IgG anti-c-Jun (1:500; Cell Signaling Technology, MA, USA). Secondary antibodies were peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG diluted 1:3,000 or 1:10,000, respectively, in TBS-Tween-milk 2.5%. Immunocomplexes were revealed by the ECL detection system (Santa Cruz, CA, USA). Filters were then stripped by incubation in 62.5 mM Tris–HCl, pH 6.7, containing 100 mM 2-mercaptoethanol and 2% SDS for 30 min at 50°C, and reprobed with a mouse polyclonal antibody directed against β-actin (Sigma, St Louis, MO, USA) or against GAPDH (Santa Cruz, CA, USA) to normalize sample loading.

2.6 Caspase 3 activity

Huh7 cells were washed with PBS, resuspended in 20 mM HEPES-KOH, pH 7.5, containing 10 mM KCl, 1 mM Na-EDTA, 1 mM DTT, 1 mM PMSF and 10 μg/ml leupeptin), incubated on ice for 15 min, sonicated, centrifuged (13,000 rpm, 15 min, 4°C; Biofuge 17 RS, Heraeus Sepatech) and the supernatant was collected. Aliquots corresponding to 30 μg protein were incubated with fluorogenic substrates for caspase 3 (Ac-DEVD-AMC; Biomol, Philadelphia, PA, USA) at 20 μM concentration for 1 hour at 37° C in reaction buffer (25 mM HEPES, pH 7.5, 0.1% CHAPS, 10% sucrose, 0.1 mg/ml BSA and 10 mM DTT). Reaction was blocked with 0.01% ice-cold trichloroacetic acid and fluorescence read with a LS 55 (Perkin-Elmer) spectrofluorometer (excitation: 380 nm, emission: 460 nm). Free AMC (amino-methyl-coumarin) was used as the working standard. Results are expressed as specific activity.

2.7 Data presentation and analysis

Most experiments were performed in triplicate and repeated more than three times. Quantification of western blots was performed by densitometric analysis and normalized *vs* β-actin or GAPDH levels: bands were analyzed using the TotalLab software (NonLinear Dynamics, Newcastle upon Tyne, UK). All results are expressed as mean ± SD. Significance of the differences was evaluated by one-way ANOVA.

3. Results

3.1 TNF+CHX-induced apoptosis in Huh7 cells

A 18 hours treatment of Huh7 cells with TNF in combination with CHX resulted in extensive apoptotic cell death, in agreement with a previous report [18]. Nuclear morphology in DAPI-stained cells is illustrated in Fig. 1A. By flow-cytometry, the proportion of hypodiploid cells, exhibiting a subG1 DNA fluorescence [16], amounted to 8-12% in control cultures and ranged around 90% after 18 hours on TNF+CHX. Rare apoptotic figures observed microscopically (not shown) and low percentages of subG1 cells assessed by flow cytometry (8 and 12%, respectively) in cultures treated with TNF or CHX separately were comparable to control values (Fig. 1B).

Apoptosis was largely yet only partially prevented in Huh7 cells exposed to TNF+CHX in the presence of the polycaspase inhibitor zVADfmk at 10, 20 and 50 μM concentrations (Fig. 1A-B), by themselves not cytotoxic (Fig. 1B and data not shown). These observations point to a process at least partly dependent on caspases, but the canonical initiator caspases did not appear involved. In fact, no early activation of caspase 8 and just a slight activation of caspase 9 after 6 h treatment could be detected in Huh7 cultures exposed to TNF+CHX (see below and Fig. S1). The activation of caspase 9 likely depends on caspase 3 activity, that increased already after 2 h treatment (data not shown), and persisted after 6 h on TNF+CHX (Figure 4D-E). Moreover, the absence of any mitochondrial membrane depolarization detectable by flow-cytometry over the first 6 hours of treatment (Fig. S2A) or of any protection exerted by 2.5-5 μM cyclosporine A (Fig. S2B), a well known inhibitor of mitochondrial permeability transition [19], ruled out a role for the intrinsic pathway in this apoptotic process; this was consistent with the lack of early caspase 9 activation.

3.2 Role of JNK activation

Various inhibitors were used to shed light on the mechanisms underlying apoptosis by TNF+CHX in Huh7 cells. Cell death was not reduced at all in cultures cotreated with antioxidants (butylated hydroxyanisole, vitamin E; not shown) or the iron chelator deferoxamine

(desferrioxamine mesylate; not shown), ruling out a role for oxidative stress. Interestingly, these same agents were found to partially protect rat hepatoma HTC cells from TNF+CHX [20]. No effect was exerted by SB203580, a selective inhibitor of the p38 MAPK (% of hypodiploid cells: TNF+CHX = 86 ± 1.8, TNF+CHX+SB203580 = 84.5 ± 2.0). Finally, a significant though partial protection was detected both morphologically (Fig. 2A) and flow-cytometrically (Fig. 2B) in cells cotreated with the JNK inhibitor SP600125. A similar pattern, though less pronounced, was observed in HepG2 cells, a human hepatoblastoma line (Fig. S3A-B).

The protection afforded by SP600125 suggested an involvement of JNK signaling in TNF+CHX-induced apoptosis. Therefore, we next established by western blotting the kinetics of JNK activation from the levels of phosphorylated (p) JNK and c-Jun, one of its main substrates. While unchanged with respect to controls in CHX-treated cells (Figure S4A), the two p-JNK bands, as well as p-c-Jun levels markedly increased in cultures exposed to TNF+CHX (Fig. 2C), already after 15 min treatment (Figure S4B). The accumulation of both p-JNK forms was substantially reduced in the presence of SP600125 (Fig. 2C), denoting its specific dependence on JNK activation.

As an independent approach to assess the relevance of JNK activation to cell death, Huh7 cells were transfected with a specific siRNA that recognizes a sequence shared by human and mouse JNK1-JNK2. This manipulation resulted in marked JNK protein depletion at 72 hours (Fig. 3A) and, in cells subsequently exposed to TNF+CHX for 18 hours, in a 40% decrease of subG1 cells (Fig. 3B). Therefore, apoptosis was prevented to a similar extent by depletion or pharmacological inhibition of JNK. In siRNA-transfected cells exposed to TNF+CHX the inhibition of apoptosis was a bit further increased by SP600125 (Fig. 3B), likely reflecting a tighter JNK inhibition.

Finally, Huh7 cells were examined to evaluate whether and how JNK inhibition by SP600125 and caspase inhibition by zVADfmk interacted in antagonizing TNF+CHX-induced death. As Fig. 4A illustrates, death was totally suppressed by the two inhibitors combined.

Interestingly, while zVADfmk did no alter p-JNK levels in TNF+CHX-treated cultures (Fig. 4B-C), SP600125 reduced caspase 3 activation and cleavage of its endogenous substrate poly-ADP ribose polymerase (PARP; Fig. 4D-E).

Several reports suggest that, due to their antagonistic role in cell death, the proapoptotic action of JNK on hepatocytes and HCC cell lines can only be deployed when NF-κB activation and related induction of antiapoptotic proteins are impaired (see Discussion). In the present study, NF-κB nuclear levels were markedly increased in Huh7 cells exposed to TNF+CHX for 2 to 6 hours, but much less so in cultures cotreated with SP600125 (Fig. 5A-B). Total NF-κB levels being unchanged (Fig. 5A), these effects should likely be ascribed to changes in nuclear translocation of this transcriptional regulator. Regardless of the amount of NF-κB translocated into the nucleus, however, the NF-κB-dependent production of protective factors in TNF+CHX-exposed Huh7 cells was presumably blocked by CHX. Two findings in Fig. 5A and 5C indicate that this was indeed the case. The manifold protective effects of NF-κB include upregulation of GADD45β, which negatively modulates the MKK4/MKK7-JNK axis [reviewed in 21], and of the caspase 8 inhibitor c-Flip. Expression of the former was unchanged during the experimental period considered (Fig. 5A), that of the latter rapidly decreased (Fig. 5C-D).

4. Discussion

This study demonstrates that JNK and caspases play a major and distinct role in apoptosis induced by TNF+CHX in Huh7 cells. Inhibitors of JNK or caspases resulted in partial prevention of apoptosis, that was fully suppressed by their combination.

Early activation of the effector caspase-3 was detected in Huh7 cells, yet only a partial protection from TNF+CHX was granted by the polycaspase inhibitor zVADfmk, used at a concentration (10 μM) that does not affect other proteases [22]. These findings indicate an involvement of the caspase system in the cell death process. However, caspase 8 plays no role in TNF+CHX-induced apoptosis of Huh7 cells, while the involvement of the intrinsic apoptotic pathway is ruled out by the lack of MMPT, of caspase 9 hyperactivation, and of protection by cyclosporine A. Despite the very similar experimental model used, the present findings are in disagreement with those of Liedtke et al. [13] as to caspase 9 activation and MMPT, though not on caspase 3 activation (see below).

Clearcut evidence for a proapoptotic role of JNK was provided by Chang and coworkers [9]: (i) *jnk1*$^{-/-}$ mice are protected against concanavalin A-induced fulminant hepatitis. Such protection is associated with decreased caspase-8 and 3 activation and c-FLIP degradation; (ii) JNK accelerates c-FLIP proteasomal degradation by activating the ubiquitin-ligase Itch. Several studies support a proapoptotic role of JNK activation, even in response to TNF [23], yet a general consensus on this issue is lacking [12,24,25]. In fact, varying JNK activation patterns have been observed: sustained JNK phosphorylation appears to be proapoptotic, but its transient activation favors survival [26]. In the present work, JNK activation in TNF+CHX-treated Huh7 cells was detectable after 15 min treatment and persisted over 6 hours at least. JNK inhibition, pharmacological (SP600125) or by RNAi, partially abrogated apoptosis. Similarly, SP600125 was reported to inhibit apoptosis in TNF+actinomycin D-treated primary hepatocytes [27, 28] or in human hepatoma HepG2 cells treated with H_2O_2, staurosporine or the flavonoid luteolin [29, 30].

NF-κB relays signals that compete with those of JNK with respect to cell death. Extensive hepatocyte death and embryonic lethality was reported in mice unable to activate NF-κB [31]. Indeed, NF-κB protects both hepatocytes and HCC cells from apoptosis by inducing several antiapoptotic factors [21]. The caspase-8 inhibitor c-FLIP, in particular, plays a pivotal role: while NF-κB promotes c-FLIP production, JNK-dependent Itch phosphorylation leads to its decay (see above). HCC cell resistance to apoptosis by death receptor ligands may also depend on constitutive c-FLIP overexpression [32, 33] or NF-κB activation [34], whereas c-FLIP downregulation markedly sensitizes cells to apoptosis [34]. In the present study, c-FLIP was markedly reduced in both TNF+CHX- and CHX-treated cells. Such drop, however, was not contrasted by SP600125, suggesting a causal role of protein synthesis blockade by CHX. Besides c-FLIP, a role in the NF-κB *vs* JNK antagonism is also played by Bcl-2 and Bcl-x_L, whose overexpression, constitutive [35] or inducible [36], defines a prosurvival mechanism. By contrast, Bcl-2 and Bcl-x_L antiapoptotic activity is blocked by JNK-dependent phosphorylation in vinblastine-treated KB-3 carcinoma cells [37-40], while constitutively active JNK results in Bax activation [38, 39]. Noteworthy, in cells not expressing Bcl-2, such as hepatocytes or most HCC cells, survival depends on Bcl-x_L, as shown by the spontaneous apoptosis occurring in rat HTC cells after Bcl-x_L depletion [41]. Not only Bcl-x_L phosphorylation is critical for mitochondria protection, but it also renders beclin-1 availabile for autophagy induction [42]. However, p-Bcl-x_L levels do not change in Huh7 cells exposed to TNF-CHX (data not shown).

Some of the effects of NF-κB on TNF hepatotoxicity are due to suppression of JNK activation [43]. Notable, in this regard, is NF-κB-dependent GADD45β upregulation, that suppresses JNK phosphorylation via MKK7 inhibition [44, 45]. By contrast, NF-κB blockade by *RelA* or *IKKβ* ablation promotes TNF-induced JNK signaling [43, 45], marked and sustained JNK activation by concanavalin A [46], prolonged JNK activation and hepatocyte apoptosis by TNF. Consistently, SP600125 inhibits TNF-induced death in primary hepatocytes isolated from

IKKβΔHep- or *RelA*-deficient mice [11, 46]. In the present study, apoptosis of Huh7 cells by TNF+CHX partly depends on JNK activation, but is also associated with increased nuclear translocation of NF-κB, although GADD45β levels remain unaltered, likely due to CHX-induced protein synthesis inhibition.

Apparently in conflict with the present and other findings, inhibition of JNK activation by TAK1 dominant-negative, a MAP3K family member,was reported to enhance TNF+CHX-induced apoptosis in Huh7 cells [13]. However, while RNA interference or SP600125 [47] specifically inhibit JNK, TAK1 downregulation could affect JNK or alternative pathways. As an example, TAK1 activates NF-κB via IKK [48, 49].

Taking a more general point of view, a large share (75%) of human HCCs show constitutive JNK activation [40] that probably confers them a selective advantage. Along this line, a proapoptotic action of JNK inhibition was also shown in TRAIL-treated HCC cells, though not in normal hepatocytes [12]. Moreover, a recent study [40] shows that the susceptibility of HCC-derived cell lines to paclitaxel-induced death mainly reflects the extent of JNK activation: marked in 'death-prone' cell lines, but marginal in 'death-reluctant' cells. The former are protected against apoptosis by SP600125, the latter significantly less or not at all. The results shown in the present study seem to conform to this pattern. Indeed, Huh7 cells have low basal levels of active JNK that increase upon TNF+CHX exposure and are substantially protected from apoptosis by SP600125. By contrast, HepG2 cells, characterized by constitutively high levels of active JNK (Figure S2; [40]), are less sensitive than Huh7 cultures to TNF+CHX-induced apoptosis and less protected by SP600125 (see Figure S2). In this view HCCs should be regarded as a heterogeneous family of cancers, where, from a therapeutic point of view, JNK inhibition might selectively be either beneficial or detrimental.

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Figure legends

Figure 1. Effect of TNF and CHX, alone or in combination, on Huh7 cells.

(A) DAPI staining of Huh7 cells treated for 18 hours with TNF and CHX and effect of 50 μM zVADfmk. a, controls; b, TNF+CHX; c, zVADfmk+TNF+CHX. Arrows indicate apoptotic-like figures. Scale bars correspond to 20 μm. (B) Flow-cytometric evaluation of apoptotic cells (hypodiploid DNA content). Cells pretreated with 10, 20 or 50 μM zVADfmk 1 hour before TNF+CHX. Data are means ± SD of 4 experiments. Significance of the differences: ***$p < 0.001$ *vs* controls [C]: °°° $p < 0.001$ *vs* TNF+CHX.

Figure 2. JNK involvement in TNF-induced death of Huh7 cells.

(A) Phase contrast images of cultures treated for 18 hours: a, controls; b, SP600125; c, TNF+CHX; d, TNF+CHX+SP600125. Arrows indicate apoptotic-like figures. The scale bar equals 40 μm. (B) Effect of JNK inhibition on apoptosis levels after 18 hour treatment. Cells were given 20 μM SP600125 (SP) 1 hour before TNF+CHX. Significance of the differences: *** $p < 0.001$ *vs* t_0; °°° $p < 0.001$ *vs* TNF+CHX. (C) Effect of SP600125 on JNK and c-Jun phosphorylation (activation) 2, 4 and 6 hours after TNF+CHXtreatment (***** indicates the band corresponding to JunD).

Figure 3. JNK inhibition protects Huh7 cells from death.

(A) Western blot analysis of JNK levels in control cultures and in cells treated with JNK-specific siRNA for 24, 48 and 72 hours. (B) Effect of JNK-siRNA on TNF+CHX-induced apoptosis. Cells transfected with siJNK for 72 hours, then exposed to TNF+CHX for further 18 hours, with or without SP600125. Histograms represent the percentages of cells with subG1 DNA content. Significance of the differences: *** $p < 0.001$ *vs* C; °°° $p < 0.001$ *vs* TNF+CHX; $^{§§§}p < 0.001$ *vs* TNF+CHX+siJNK; $^{\#}p < 0.05$ *vs* TNF+CHX+SP.

Figure 4. The inhibition of JNK and caspases results in differential protection of Huh7 cells from death.

(A) Death suppression by zVADfmk and SP600125: cells pretreated for 1 hour with SP600125 (20 μM) and zVADfmk (50 μM), separately or in combination, then exposed to TNF+CHX for additional 18 hours. Histograms represent the percentages of apoptotic cells. Significance of the differences: ***$p < 0.001$ *vs* C; °°°$p < 0.001$ *vs* TNF+CHX; $^{\dagger}p < 0.05$ *vs* TNF+CHX+zVADfmk and $^{\#\#\#}p < 0.001$ *vs* TNF+CHX+SP. (B) Representative western blots for JNK and pJNK and (C) densitometric analysis of the latter in cultures pretreated as in C and exposed to TNF+CHX for additional 2 hours. (D) Effect of SP600125 on caspase 3 activation and PARP cleavage (E) in Huh7 cells after 6 hours of TNF+CHX treatment. Caspase 3 enzymatic activity assayed as indicated in Materials and Methods. Data (means ± SD) expressed as nkatal × 10^{-5}/mg protein. Significance of the differences: *** $p<0.001$ *vs* C; °°° $p<0.001$ *vs* TNF+CHX. (E) Representative Western blot showing appearance of the 85 kDa PARP fragment during TNF+CHX treatment. Cells were pretreated for 1 hour with SP600125 (20 μM).

Figure 5. Effect of SP600125 on NF-κB, GADD45β and cFLIP levels.

(A) Representative western blots of GADD45β and total and nuclear NF-κB; (B) densitometric analysis of nuclear NF-κB. Cells pretreated for 1 hour with SP600125, then exposed to TNF+CHX for 2, 4 and 6 hours; (C) Representative western blots and densitometric analysis of c-FLIP in Huh7 cultures treated with TNF+CHX for 2, 4 and 6 hours or (D) with CHX alone for 2 and 4 hours. Significance of the differences: ***$p < 0.001$ *vs* C; °°°$p < 0.001$ *vs* TNF+CHX.

Legends to supplemental figures

Figure S1. Caspase 8 and 9 enzymatic activity in Huh7 cells treated with TNF+CHX.

Caspase 8 and caspase 9 enzymatic activity assayed as indicated in Supplemental materials and methods. Data (means ± SD) expressed as nkatal × 10^{-5}/mg protein. Significance of the differences: * $p<0.05$ *vs* C; ° $p<0.05$ *vs* TNF+CHX.

Figure S2. The mitochondrial pathway to apoptosis.

(A) Evaluation of the mitochondrial membrane potential ($\Delta\psi_m$) by the flow-cytometric JC-1 test on Huh7 cells exposed to TNF+CHX for 2, 4 and 6 hours. Histograms depict the percentage of cells with depolarized mitochondria at each time point (Lysis software, Becton-Dickinson). (B) Effect of cyclosporine A (CsA, 2.5 or 5 μM) supplied to cultures 1 hour prior to 18 hour exposure to TNF+CHX. Histograms illustrate the percentage of hypodiploid cells. Significance of the differences: *** $p< 0.001$ *vs* C.

Figure S3. JNK involvement in TNF+CHX-induced apoptosis in HepG2 cells and primary hepatocytes.

(A) Levels of apoptotic cells after JNK inhibition. Cells pretreated for 1 hour with 20 μM SP600125 before treatment with TNF+CHX for 18 hours. Significance of the differences: *** $p<0.001$ *vs* t0; °°° $p< 0.001$ *vs* TNF+CHX. (B) Effect of SP600125 on JNK phosphorylation after 2 hours of TNF+CHX-treatment.

Figure S4. JNK phosphorylation after treatment with CHX (A) or TNF+CHX (B).

(A) Representative western blot of phosphorylated JNK in Huh7 cells exposed to CHX, in the presence or in the absence of SP600125. (B) Representative western blot of phosphorylated and total JNK in Huh7 cells exposed to TNF+CHX for 15, 30, or 60 min.

Figure 1

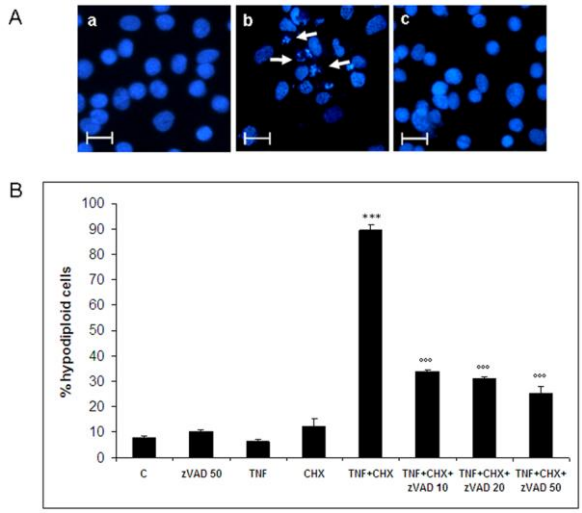


Figure 2

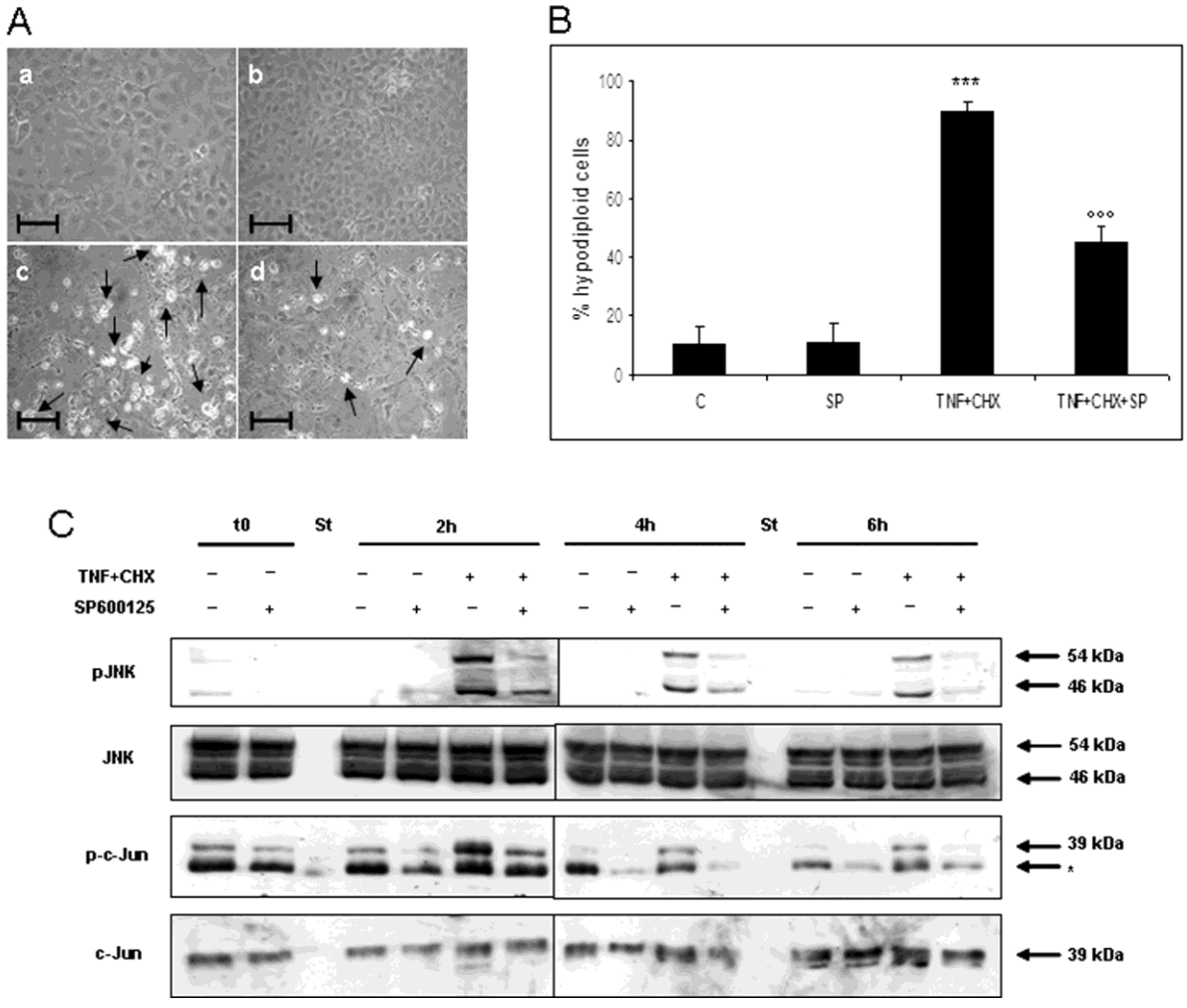


Figure 3

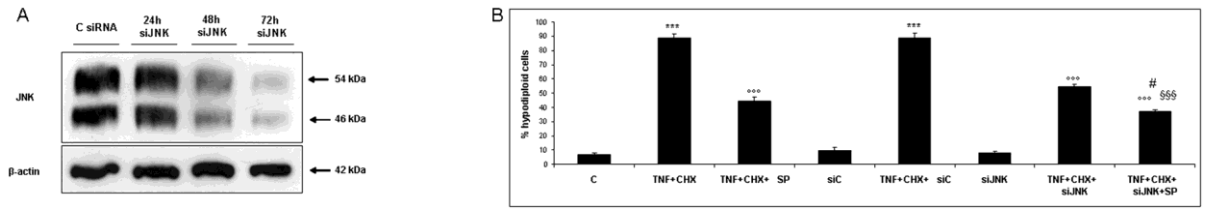


Figure 4

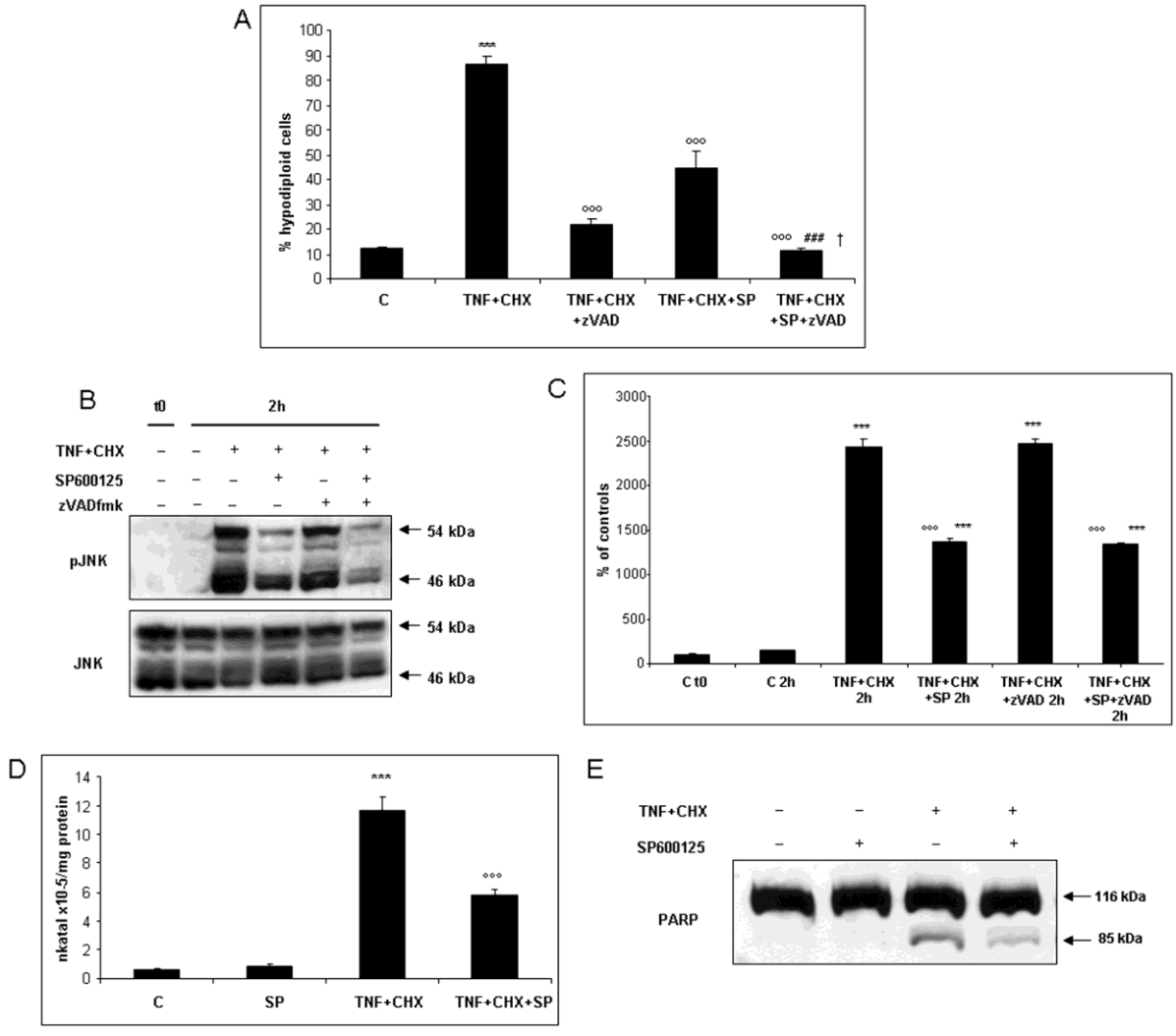
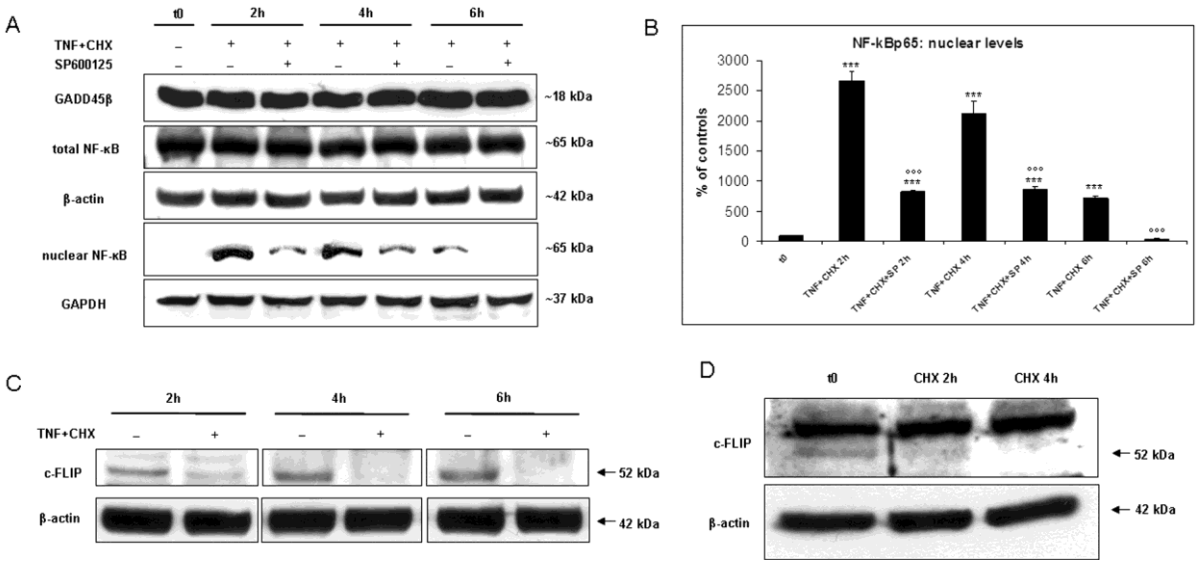


Figure 5



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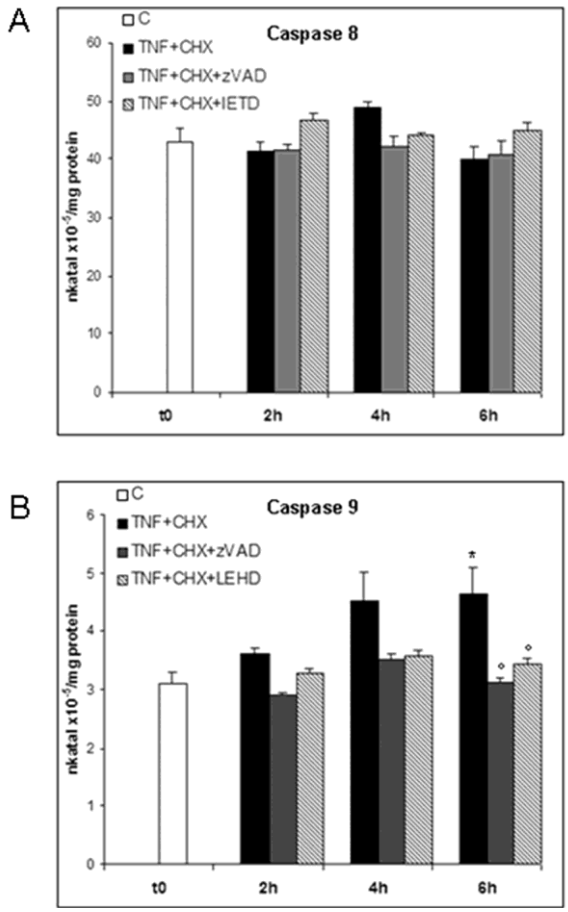

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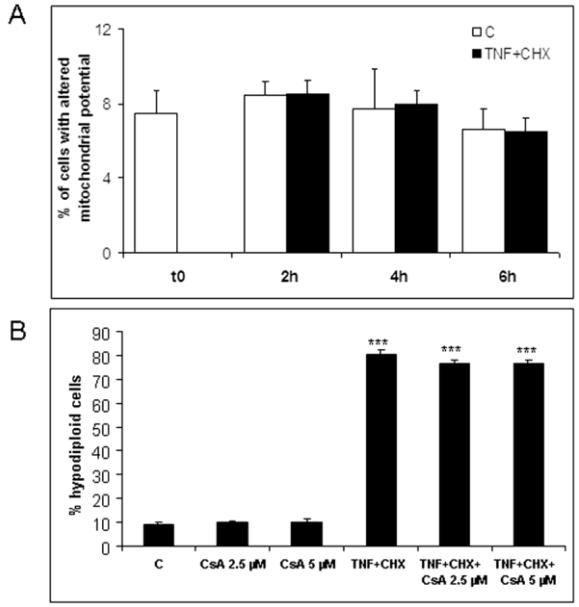

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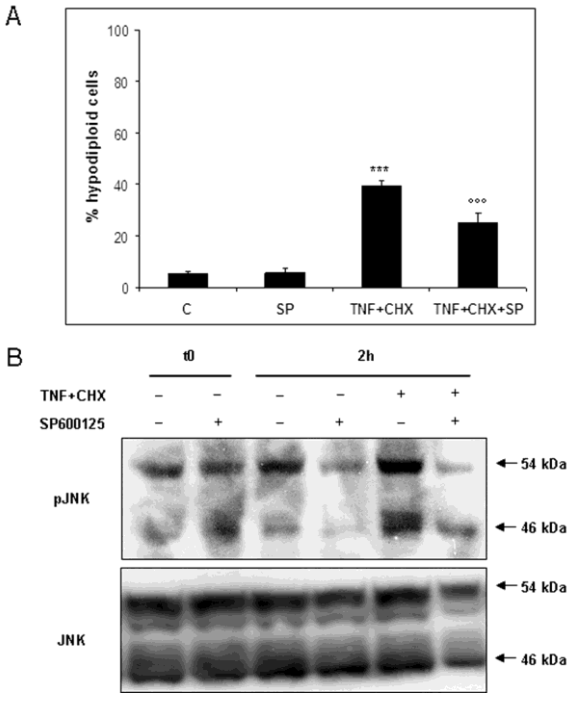

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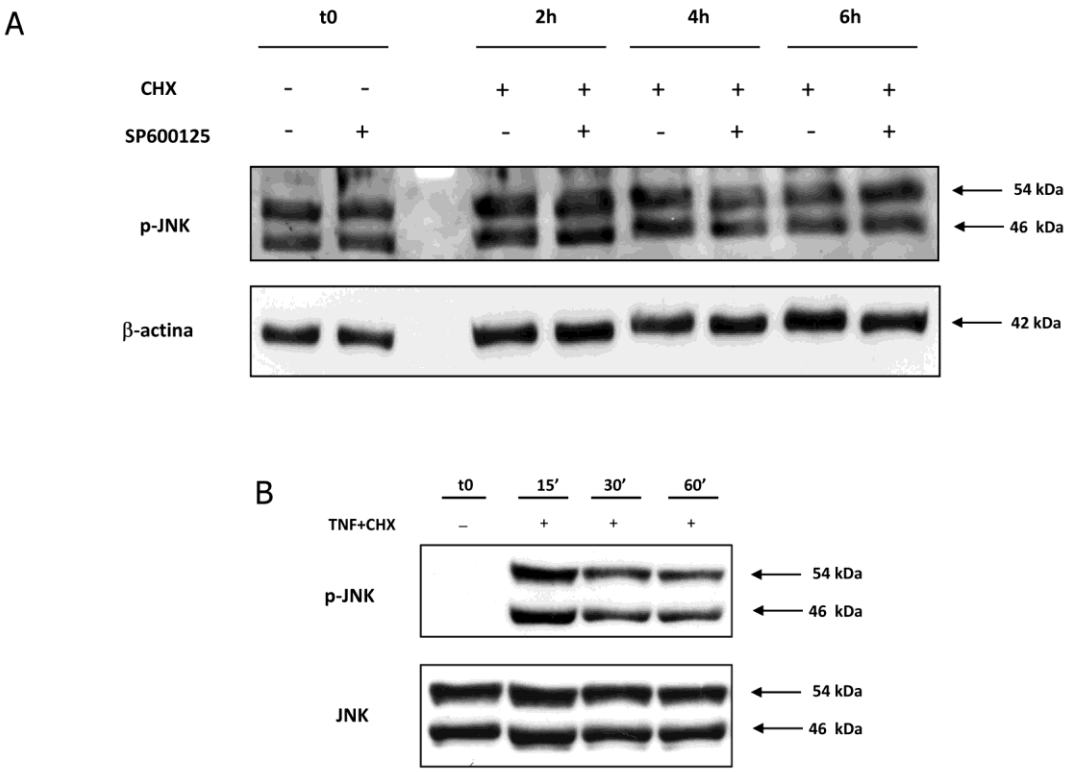
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TNF+CHX
p-JNK
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