Full Paper

TNFα-Induced Necroptosis and Autophagy via Suppression of the p38–NF-κB Survival Pathway in L929 Cells

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Abstract. Tumor necrosis factor alpha (TNFα) has been reported to induce necroptosis and autophagy, but its mechanisms remain unclear. In this study, we found that TNFα significantly induced necroptosis and autophagy in murine fibrosarcoma L929 cells. The necroptosis inhibitor necrostatin-1 (Nec-1) completely blocked TNFα-induced necroptosis and autophagy, but inhibition of autophagy with 3-methyladenine (3MA) or Beclin 1 small interfering RNA (siRNA) promoted necroptosis, indicating that autophagy acted as a negative regulator of TNFα-induced necroptosis. The cytotoxicity of TNFα was accompanied by decreased expressions of phosphorylated p38 mitogen-activated protein kinase (p-p38) and nuclear factor-kappa B (NF-κB), and inhibition of p38 and NF-κB activation by chemical inhibitors or siRNA augmented these necroptotic and autophagic responses to TNFα in the cells. The pan-caspase inhibitor z-VAD-fmk (zVAD) exacerbated TNFα-induced necroptosis and autophagy. Combined treatment with TNFα and zVAD further decreased the expressions of p-p38 and NF-κB compared with TNFα alone treatment. Consequently, these results indicated that suppression of the p38–NF-κB survival signaling pathway promoted necroptotic and autophagic cell death in TNFα-treated L929 cells.

[Supplementary Figures: available only at http://dx.doi.org/10.1254/jphs.11105FP]

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Introduction

For a long time, apoptosis has been thought to be a predominant form of programmed cell death. However, in the absence of caspase activation, necroptosis prevails in Jurkat cells (1). Necroptosis is a newly identified type of programmed cell death initiated by activation of apoptosis induced by binding of tumor necrosis factor (TNF) or Fas to its receptor, referred to as a regulated form of necrosis, which is dependent on the serine–threonine kinase receptor-interacting protein 1 (RIP1) (2). Necroptosis can be specifically inhibited by a small molecule, necrostatin-1 (Nec-1), which targets RIP1 (3). Inhibiting RIP1 to avoid necroptosis may represent a convenient means to discriminate between necroptosis and fortuitous forms of necrosis (4). The balance of apoptosis and necroptosis regulates the normal embryonic development and T-cell proliferation; uncontrolled necroptosis would result in embryonic death (5–7). Thus, it is important to elucidate the detailed mechanisms involved in necroptosis and autophagy.

Autophagy is a physiological cellular mechanism that degrades and recycles cellular components to maintain an adequate amino acid level during nutritional starvation to protect cells from death. However, under certain environmental conditions, autophagy can also be a contributor to programmed cell death (8, 9). Three main types of autophagy have been identified: macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy (hereafter referred to as autophagy) is the major form of autophagy and is the process whereby organelles and cytosolic macromolecules are sequestered into double-membrane structures known as autophagosomes, which are subsequently de-
Tumor necrosis factor alpha (TNFα), a pleiotropic cytokine, mediates a broad range of proinflammatory activities, cell proliferation, differentiation and death (12, 13). After stimulation with TNFα, three pathways can be initiated (14 – 16). The first one is activation of transcription factor nuclear factor kappa B (NFκB) which translocates to the nucleus and mediates the transcription of a vast array of proteins involved in cell survival and proliferation. NFκB is formed through the dimerization of five subunits, including RelA (p65), c-Rel, RelB, NFκB1 (p50 and its precursor p105), and NFκB2 (p52 and its precursor p100). The RelA (p65) subunit provides the main gene regulatory function (17). Second, TNFα activates the mitogen-activated protein kinase (MAPK) pathways such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK (p38). Third, tumor necrosis factor receptor type 1–associated death domain protein (TRADD) binds to Fas-associated protein with death domain (FADD), which then recruits caspase-8. A high concentration of caspase-8 induces its autoproteolytic activation and subsequent cleaving of effector caspases, leading to apoptosis. However, recent research indicates that TNFα can induce necroptosis when caspase-8 is not activated (1) (Supplementary Fig. 1: available in the online version only). In this study, we demonstrated that TNFα induced RIP1-mediated L929 cell necroptosis and autophagy. Up-regulation of p38 and NFκB blocked TNFα-induced necroptosis and autophagy, and the pan-caspase inhibitor z-VAD-fmk (zVAD) further increased inhibition of the pro-survival p38–NFκB signaling pathway, augmenting both necroptosis and autophagy.

**Materials and Methods**

**Reagents**

Human recombination TNFα was prepared from PMAL-C2-TNF/JM109 (E. coli) in our laboratory. Crystal violet, propidium iodide (PI), acridine orange (AO), ethidium bromide (EB), 3-methyladenine (3MA), pan-caspase inhibitor zVAD, p38 inhibitor SB 203580, ERK inhibitor PD 98059, JNK inhibitor SP 600125, necroptosis inhibitor Nec-1, and NFκB inhibitor pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma Chemical (St. Louis, MO, USA). Small interfering RNA (siRNA) against mouse Beclin 1, p38, p65, control siRNA, and LipoFectamine 2000 were purchased from Invitrogen (Invitrogen, Carlsbad, CA, USA). Polyclonal antibodies to Beclin 1, light chain 3 (LC3), p38, phosphorylated p38 (p-p38), NFκB (p65), IκB, β-actin, and horseradish peroxidase–conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

L929 cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Beijing Yuanheng Shenyang Research Institution of Biotechnology, Beijing, China), 100 μg/ml streptomycin, 100 U/ml penicillin, and 0.03% L-glutamine, and maintained at 37°C with 5% CO2 at a humidified atmosphere. All the experiments were performed on logarithmically growing cells.

Growth inhibition assay

The growth inhibitory effect of TNFα on L929 cells was measured by crystal violet staining. The cells were dispensed in 96-well plates with 5 × 104 cells/ml. After 48 h incubation, they were treated with or without Nec-1, 3MA, zVAD, SB 203580, PD 98059, SP 600125, or PDTC at given concentrations 1 h prior to the administration of TNFα for 24 h. The cells were then washed twice with phosphate-buffered saline (PBS) and stained with 0.5% crystal violet solution containing 20% ethanol at room temperature for 30 min. After washing three times with water, the retained dye was dissolved in 120 μl methanol for each well and the absorbance was measured at 620 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Tecan Spectra, Wetzlar, Germany). The percentage of cell growth inhibition was calculated as follows: Cell viability (%) = 100 − (A620, control − A620, experiment) / (A620, control − A620, blank) × 100.

Observation of morphological changes

L929 cells were seeded into 6-well culture plates and incubated with TNFα. The cellular morphology was observed by phase contrast microscopy (Leica, Nussloch, Germany), or staining the cells with the fluorescent DNA-binding dye acridine orange / ethidium bromide (AO/EB). After treatment with or without TNFα, the cells were washed with PBS three times and subsequently stained with 20 μg/ml AO/EB for 15 min. Finally, the nuclear morphology was observed under fluorescence microscope (Olympus, Tokyo).

Measurement of PI positive ratio (cell death ratio)

The L929 cells were treated with TNFα for the indicated time periods or co-incubated with the given inhibitors for 24 h. The collected cells were washed twice with PBS, after washing the cells were stained for DNA content with PI for 10 min. PI can be inserted into double-stranded DNA, but cannot be penetrated into integrated cell membrane. So it can mark dying cell but not living and apoptotic cell without fixation with 70% ethanol at 4°C overnight. The percentage of PI-positive ratio was
measured by FACScan flow cytometry.

**Transmission electron microscopy**

The L929 cells were treated with TNFα for the indicated time periods. The collected cells were fixed with PBS containing 3% glutaraldehyde and then postfixed with PBS containing 1% OsO4. The samples were dehydrated in graded alcohol, embedded in Durcupan resin, and sectioned. Ultrathin sections were stained with uranyl acetate and lead citrate and examined by a JEM-1230 transmission electron microscope (JEOL, Tokyo).

**Measurement of autophagy**

After incubation with TNFα for the indicated time periods, the cells were cultured with 0.05 mM monodansylcadaverine (MDC) at 37°C for 1 h. The cellular morphologic changes were observed under a fluorescence microscope. After transfection with green fluorescent protein–labeled LC3 (GFP-LC3) using Lipofectamine 2000 for 24 h, the cells were treated with TNFα for another 24 h. Then, the fluorescence of GFP-LC3 was observed under a fluorescence microscope.

**siRNA transfection**

The cells were transfected with siRNAs using Lipofectamine 2000 according to the manufacturer’s instructions. The transfected cells were used for subsequent experiments 24 h later.

**Western blot analysis**

The cells were treated with TNFα for 0, 6, 12, 24, and 36 h or co-incubated with the given inhibitors for 24 h. The cells were lysed in lysis buffer [20 mM Tris-HCl, pH 7.4, 1% Triton X-100, 10% glycerol, 150 mM NaCl, 1 mM phenylmenthanesulfonyl fluoride (PMSF), and 1% Triton X-100, 1% glycerol, 150 mM NaCl, 1 mM phenylmenthanesulfonyl fluoride (PMSF), and 1 μg/ml each leupeptin, antipain, chymostatin, and pepstatin A] on ice for 1 h and centrifuged (15 min, 9,500 × g). Equivalent amounts of total proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk powder in 0.1% Tween 20 in Tris-buffered saline (TBS) for 2 h and incubated with the primary antibodies at 4°C overnight. Membranes were washed three times for 10 min with 0.1% Tween 20 in TBS and incubated with the respective peroxidase-conjugated secondary antibodies for 2 h. After three times washing for 10 min, the proteins were visualized by enhanced chemiluminescent ECL reagents (Thermo Scientific, Rockford, IL, USA). If required, membranes were stripped in stripping buffer [100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl (pH 6.8)] at 55°C for 7 – 10 min for probing with different antibodies.

**Statistical analysis**

All the presented data and results were confirmed in at least three independent experiments. The data are expressed as the mean ± S.D. The data were analyzed by one-way ANOVA using Statistics Package for Social Science (SPSS) software (version 13.0; SPSS, Chicago, IL, USA), and the statistical comparisons were made by the least significant difference (LSD) post-hoc test. *P* < 0.05 was considered statistically significant.

**Results**

**TNFα induced necroptosis and autophagy in L929 cells**

Firstly, the ultrastructure of TNFα-treated L929 cells were observed by transmission electron microscopy. Compared with the control group, TNFα-treated L929 cells showed typical necrotic features, including a loss of plasma membrane integrity, obvious vacuolation, organelle swelling, and massive mitochondrial damage (Fig. 1A: a, b). AO is a vital dye that permeates all cells and makes the nuclei appear green, whereas EB is only taken up by the cells that have lost their membrane integrity and stains the nucleus red (18). The morphological changes were further confirmed by AO/EB staining of cell nuclei (Fig. 1B). These results suggested that TNFα induced L929 cell necrosis, but not apoptosis (Supplementary Fig. 1). Moreover, the necroptosis inhibitor Nec-1 completely blocked TNFα-induced cell growth inhibition (Fig. 1E) and cell death (Fig. 1F), indicating that TNFα-induced cell death was associated with necroptosis. It was reported that when necroptosis occurred, autophagy always accompanied it (19). As shown in Fig. 1A, c and d, the cells exhibiting necrotic characteristics also showed extensive cytoplasm vacuolization and some autophagic vacuoles that contained degraded organelles, suggesting that TNFα also induced L929 cell autophagy. These were also demonstrated by MDC staining (Fig. 1C), a specific fluorescent dye of autophagic vacuoles (11); GFP-LC3 localization (Fig. 1D) and Beclin 1 expression; and the conversion of LC3 I to LC3 II (Fig. 4C), commonly used markers for autophagy (20). Microtubule-associated protein LC3 is associated with the autophagosome membranes after processing, and the accumulation of LC3 II is correlated with the extent of autophagosome formation (21). It remains a controversy that autophagy is a downstream consequence (22) or a contributor factor of necroptosis (23). To answer this, MDC fluorescent intensity was detected after pretreated with Nec-1. As shown in Fig. 1G, pretreatment with Nec-1 completely inhibited autophagy. However, inhibition of autophagy with 3MA (Supplementary Fig. 2: available in the online version only) and Beclin 1 siRNA promoted TNFα-induced necroptosis in L929
Inhibition of p38 promoted TNFα-induced necroptosis

To examine the contribution of MAPK family kinases p38, JNK, and ERK to TNFα-induced necroptosis, L929 cells were pretreated with the p38 inhibitor SB 203580, JNK inhibitor SP 600125, and ERK inhibitor PD 98059. The results indicated that JNK and ERK were not involved in TNFα-induced cell death (Fig. 2A) (Supplementary Fig. 3: available in the online version only).

cells (Fig. 1: H and I), indicating that autophagy was a downstream consequence of necroptosis and had a negative-feedback function.

Inhibition of p38 promoted TNFα-induced necroptosis

To examine the contribution of MAPK family kinases
However, p38 played a protective role in TNFα-induced necroptosis (Fig. 2B). These results were further verified by using p38 siRNA (Fig. 2: C and D). Moreover, western blot analysis showed that the level of p-p38 was markedly decreased after TNFα administration (Fig. 2E), and Nec-1 significantly reversed the decrease (Fig. 2F). These results indicated that TNFα induced cell necroptosis by inhibiting p38-mediated survival signaling.

The transcription factor NF-κB, a downstream factor of the TNF signaling pathway, mainly mediates cell survival signaling. In this study, we found that inhibition of NF-κB activation by using the NF-κB inhibitor PDTC or transfecting with p65 siRNA significantly increased the TNFα-induced cytocidal activity (Fig. 3A) and cell death ratio (Fig. 3: B and C). Further study showed that the expression of NF-κB was down-regulated, but there

p38–NF-κB pathway was involved in TNFα-induced L929 cell death
Inhibition of p38–NF-κB–induced L929 cells. Compared to the TNF-α was regulated by the p38–NF-κB (Fig. 3F), implicating that TNF-α-induced down-regulation of NF-κB expression and NF-κB pathways to augment TNFα-induced necroptosis and autophagy. To further investigate the relationship between p38 and NF-κB, western blot analysis was carried out. The results confirmed that SB 203580 further intensified TNFα-induced down-regulation of NF-κB expression (Fig. 3F), implicating that TNFα-induced necroptosis was regulated by the p38–NF-κB signaling pathway.

**Inhibition of p38–NF-κB promoted TNFα-induced L929 cell autophagy**

Next we examined the involvement of the autophagy-associated p38–NF-κB signaling pathway in TNFα-treated L929 cells. Compared to the TNFα-alone treatment group, interruption of p38 or NF-κB caused a significant increase in MDC positive ratio (Fig. 4: A and B) and Beclin 1 expression and the conversion of LC3 I to LC3 II (Fig. 4C). It is apparent that p38–NF-κB inhibited TNFα-induced autophagy.

**zVAD further inhibited p38–NF-κB pathways to augment TNFα-induced necroptosis and autophagy**

It is well known that in the presence of the pan-caspase inhibitor zVAD, TNFα induced L929 cell necroptosis (24, 25). To further confirm role of p38–NF-κB in necroptosis and autophagy, we co-administered TNFα and zVAD. Compared with TNFα-alone treatment, the cell viability was significantly decreased by pretreatment with 2.5 μM zVAD (Fig. 5A), zVAD also increased TNFα-induced cell death, as examined by cell death ratio after PI staining (Fig. 5B), indicating that zVAD exacerbated TNFα-induced necroptosis. Further study showed that zVAD augmented TNFα-induced autophagy, as assessed by increasing MDC positive ratio (Fig. 5C) and Beclin 1 expression and the conversion of LC3 I to LC3 II (Fig. 5D). It is well known that in the presence of the pan-caspase inhibitor zVAD, TNFα induced L929 cell necroptosis (24, 25). To further confirm role of p38–NF-κB in necroptosis and autophagy, we co-administered TNFα and zVAD. Compared with TNFα-alone treatment, the cell viability was significantly decreased by pretreatment with 2.5 μM zVAD (Fig. 5A), zVAD also increased TNFα-induced cell death, as examined by cell death ratio after PI staining (Fig. 5B), indicating that zVAD exacerbated TNFα-induced necroptosis. Further study showed that zVAD augmented TNFα-induced autophagy, as assessed by increasing MDC positive ratio (Fig. 5C) and Beclin 1 expression and the conversion of LC3 I to LC3 II (Fig. 5D).
To verify whether zVAD augmented TNFα-induced cell death was associated with p38 and NF-κB, p38, p-p38, and NF-κB levels were examined by western blotting (Fig. 5E). zVAD could further augment the decrease in p-p38 and NF-κB levels, whereas these decreases were markedly inhibited by Nec-1 (Fig. 5E). Moreover, p38 and p65 siRNA increased TNFα/zVAD-induced cell death (Fig. 5F). Taking the above results together, these findings suggested that the p38–NF-κB survival pathway played an important role in necroptosis and autophagy.

Discussion

Necroptosis is a basic cell death pathway recently defined by Degterev et al. (22). It was reported that even in the absence of caspase activation, TNFα could induce necroptosis (26). RIP1 kinase, which is inhibited by necrostatins, is a key upstream kinase involved in the activation of necroptosis (3). When necroptosis occurred, autophagy commonly accompanied it (19). The result of this study showed that TNFα-induced L929 cell autophagy accompanied by necroptosis, and autophagy, like necroptosis, could be completely blocked by the RIP1 inhibitor Nec-1. This idea was also supported by the work of Yu et al., which indicated that the activation of RIP induced autophagy when caspase-8 was inhibited (27). Additionally, inhibition of autophagy promoted TNFα-induced necroptosis. Taken together, these results implied that autophagy was a downstream consequence of necroptosis, yet being a negative feedback to necroptosis in L929 cells.

Mitogen-activated protein kinase pathways, including ERK1/2, JNK, and p38, are involved in various biological responses such as differentiation, proliferation, and cell death (28). The functional roles of these kinases are often controversially discussed. In this study, we found that ERK and JNK were not involved in TNFα-induced cell death, supported by the fact that ERK and JNK inhibitor did not affect TNFα-induced cell growth inhibition, and the levels of ERK, JNK, and their phosphorylated forms were not changed. However, inhibition of p38 activity by a p38-specific inhibitor or siRNA increased TNFα-induced necroptosis and autophagy. We also demonstrated that the pan-caspase inhibitor zVAD further decreased the expression of p-p38 in TNFα-treated L929 cells. These results suggested that p38 played a vital role in TNFα or TNFα/zVAD-induced necroptosis and autophagy. It was reported that zVAD-induced autophagic cell death and necroptosis required ERK and JNK activation in L929 cells (29, 30). TNFα/zVAD-induced RIP1-mediated necroptosis was associated with the inhibition of adenine nucleotide translocase dependent ADP/ATP exchange, but not in TNFα or
zVAD-alone treatment (31). Whether p38 was involved in zVAD-induced necroptosis and autophagy of L929 cells remained to be elucidated. Our findings, taken together with these results, supported the idea that necroptosis and autophagy were induced in different conditions, for instance, treatment with TNFα, zVAD, or TNFα/
zVAD. Even if the induction mechanisms were different, all of these had been clarified to be associated with RIP. Therefore, we could compare the difference among the three models to find out the common and crosstalk molecular mechanisms between necroptosis and autophagy.

Nuclear transcription factor NF-κB plays an important role in inflammatory and immune responses, as well as cell proliferation and survival (32, 33). Typically, in most unstimulated cells, NF-κB is sequestered in the cytoplasm by binding to the inhibitor of NF-κB (I-κB). In response to a variety of stimuli, degradation of I-κB proteins via phosphorylation of I-κB on two N-terminal residues leads to the release of NF-κB (34), and subsequently, activation of NF-κB increases expression of anti-apoptotic genes such as Bcl-2 to inhibit the apoptotic pathway (35). In this study, we showed that inhibition of NF-κB with PDTC or siRNA increased TNFα-induced cell growth inhibition and cell death ratio; and NF-κB level was decreased in a time-dependent manner after TNFα administration in L929 cells, which was remarkably blunted by Nec-1, suggesting that NF-κB had a protective role in TNFα-induced necroptosis. This was also supported by the work of Wu et al. (30), which indicated that NF-κB pathway had a protective function during zVAD-induced necroptosis. However, the expression level of I-κB was not changed, and the cytosolic Bax (pro-apoptotic protein) did not translocate to mitochondria and the levels of Bcl-2 (anti-apoptotic protein) in the mitochondria were also not changed (Supplementary Fig. 4: available in the online version only), suggesting that the release of NF-κB might not be controlled by the phosphorylated I-κB in our models. Saha et al. reported that p38 regulated the transcriptional activity of NF-κB via direct acetylation of p65 in human astrocytes (36). In this study, inhibition of p38 further decreased the level of NF-κB, showing that p38-mediated the activation of NF-κB. NF-κB activation might be partially through acetylation of p65 by p38. The activation of NF-κB has been reported to mediate the repression of autophagy, which is a cell death mechanism in TNFα-treated Ewing sarcoma cells (37). In this study, we demonstrated that inhibition of NF-κB increased TNFα-induced autophagy. Likewise, zVAD further decreased the level of NF-κB induced by TNFα. As pointed out above, NF-κB played an important role in necroptosis and autophagy.

In summary, our results established that TNFα induced autophagy negative feedback to necroptosis. Moreover, the p38–NF-κB survival signaling pathway is a negative regulator of necroptosis and autophagy, which can be reduced by TNFα and TNFα/zVAD. These findings provide new evidence for the further understanding of more detailed molecular mechanisms between necroptosis and autophagy.

References