

Akt and mTOR mediate programmed necrosis in neurons

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Necroptosis is a newly described form of regulated necrosis that contributes to neuronal death in experimental models of stroke and brain trauma. Although much work has been done elucidating initiating mechanisms, signaling events governing necroptosis remain largely unexplored. Akt is known to inhibit apoptotic neuronal cell death. Mechanistic target of rapamycin (mTOR) is a downstream effector of Akt that controls protein synthesis. We previously reported that dual inhibition of Akt and mTOR reduced acute cell death and improved long term cognitive deficits after controlled-cortical impact in mice. These findings raised the possibility that Akt/mTOR might regulate necroptosis. To test this hypothesis, we induced necroptosis in the hippocampal neuronal cell line HT22 using concomitant treatment with tumor necrosis factor α (TNF α) and the pan-caspase inhibitor N-benzoyloxycarbonyl-Val-Ala-Asp-fluoromethylketone. TNF α /zVAD treatment induced cell death within 4 h. Cell death was preceded by RIPK1–RIPK3–pAkt assembly, and phosphorylation of Thr-308 and Thr473 of AKT and its direct substrate glycogen synthase kinase-3 β , as well as mTOR and its direct substrate S6 ribosomal protein (S6), suggesting activation of Akt/mTOR pathways. Pretreatment with Akt inhibitor viii and rapamycin inhibited Akt and S6 phosphorylation events, mitochondrial reactive oxygen species production, and necroptosis by over 50% without affecting RIPK1–RIPK3 complex assembly. These data were confirmed using small inhibitory ribonucleic acid-mediated knockdown of AKT1/2 and mTOR. All of the aforementioned biochemical events were inhibited by necrostatin-1, including Akt and mTOR phosphorylation, generation of oxidative stress, and RIPK1–RIPK3–pAkt complex assembly. The data suggest a novel, heretofore unexpected role for Akt and mTOR downstream of RIPK1 activation in neuronal cell death.

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Necroptosis is a form of programmed necrosis regulated by the kinase activity of receptor-interacting protein kinase 1 (RIP1; also known as RIPK1) and RIP3 (also known as RIPK3). Necroptotic stimuli induce TNF receptor 1 complex assembly involving TNFR-associated death domain protein, Fas-associated death domain protein, caspase-8, RIPK1, RIPK3, and various regulator molecules such as FADD-like interleukin-1 beta converting enzyme (FLICE)-like inhibitory protein and cylindromatosis protein, among others. The complex formed by RIPK1–RIPK3 defines the necrosome, which is required for initiation of necroptotic cell death.¹ Necroptosis contributes to neuronal death in experimental models of stroke and traumatic brain injury.^{2,3} Recently, intensive research effort has been directed towards elucidating mechanisms that contribute to necroptosis execution, revealing critical roles for the kinase activity of RIP1 and RIP3,

as well as the presence of the mixed lineage kinase domain-like protein,^{4,5} the mitochondrial fission protein regulator dynamin-related protein-1⁶ and reactive oxygen species (ROS).² RIPK1 activity and necrosome complex assembly is regulated by ubiquitylation and deubiquitylation reactions mediated by cylindromatosis protein⁷ and others. Although a number of reports have elucidated mechanisms of necroptosis initiation, little is known about the signaling and execution mechanisms downstream of the necrosome, and how necroptosis is governed in neuronal cells remains unexplored.^{8–10}

We recently reported that, surprisingly, combination-pharmacological inhibition of Akt and mechanistic target of rapamycin (mTOR) reduced necrotic cell death in CA3 and CA1 regions of hippocampus and improved functional outcome after controlled-cortical impact (CCI) in mice.¹¹

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Abbreviations: mTOR, mechanistic target of rapamycin; TNF, tumor necrosis factor; GSK, glycogen synthase kinase; S6, S6 ribosomal protein; ROS, reactive oxygen species; FLIP, FADD-like interleukin-1-beta converting enzyme (FLICE)-like inhibitory protein; CCI, controlled cortical impact; BHA, butylated hydroxyanisole; Nec-1, 7-Cl-O-methyl-necrostatin-1; LOX-1, LOXBlock-1; Bai, baicalin; HMGB1, high mobility box group-1 protein; CHX, cyclohexamide; PI, propidium iodide; siRNA, small inhibitory ribonucleic acid; EDTA, ethylenediaminetetraacetic acid; IP, immunoprecipitation; JNK, Jun-N-kinase; FOXO, Forkhead box class O; MAPK, mitogen-activated protein kinase

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These observations, along with our previous data suggesting the contribution of RIP1 kinase-dependent necroptosis to CCI,³ suggested a possible role for Akt/mTOR signaling in programmed necrosis in neuronal cells. These *in vivo* studies in the mouse CCI model prompted us to examine directly whether Akt and mTOR mediate programmed necrosis in neuronal cells. To this end, we used hippocampal HT22 cells to test the hypothesis that RIPK1–RIPK3 mediated necroptosis is regulated downstream of necrosome assembly by Akt and mTOR. Herein, we report activation of Akt/mTOR signaling pathways and neuronal cell death that are inhibited by pharmacologic or genetic inhibition of Akt and mTOR together. Inhibition of Akt/mTOR did not affect necrosome complex assembly but inhibited oxidative stress and cell death. The data suggest an unexpected role for Akt/mTOR in the regulation of neuronal necrosis. Given a large number of Akt and mTOR inhibitors currently under development, this

mechanism of acute neuronal cell death could be highly amenable for therapeutic intervention.

Results

TNF α /zVAD induces necrosis in HT22 cells. To assess signaling pathways that might mediate necroptosis downstream of RIPK1, we began by establishing conditions that induce necroptosis in HT22 cells using tumor necrosis factor- α (TNF α) and zVAD administration. Dose–response studies of TNF α and zVAD separately determined optimal concentrations of each reagent that together promote necroptosis (Figure 1). We found that 1 ng/ml TNF α and 50 μ M zVAD efficiently induced cell death and, therefore, these conditions were used subsequently in all experiments (Figures 1a–c). TNF α /zVAD treatment induced membrane permeability to propidium iodide (PI) (Figure 1c) as well as

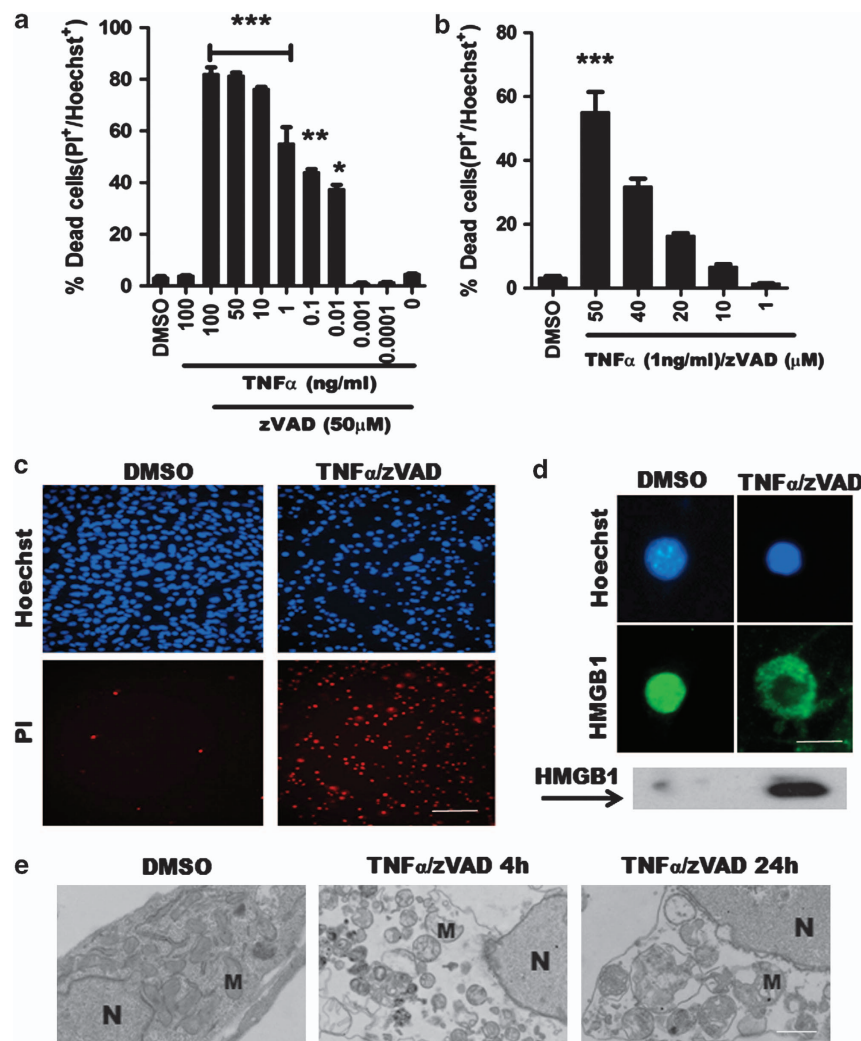


Figure 1 TNF α /zVAD induces necrosis in HT22 cells. (a, b) TNF α and zVAD dose–response curves. Cell death was assessed by propidium iodide (PI) and Hoechst staining. (c) Representative images of HT22 cells treated with DMSO or TNF α (1 ng/ml)/zVAD (50 μ M) for 4 h. (d) HMGB1 translocation was detected in cells (blue: Hoechst dye; green: HMGB1 antibody), and in culture media by western blot. (e) Transmission electron microscopy of HT22 cells treated with TNF α /zVAD for 4 or 24 h shows necrotic morphology including swollen mitochondria, cytoplasmic clearing, membrane damage, and chromatinolysis. M, mitochondrion; N, nucleus. (All data are presented as mean \pm S.E.M. from 3–5 independent experiments. * P < 0.05; ** P < 0.01; *** P < 0.001 versus DMSO group.) Scale bars: c, 100 μ m; d, 10 μ m; e, 1 μ m

early translocation of high mobility box group-1 protein (HMGB1) from nucleus to cytosol (Figure 1d), suggesting necrosis. Electron microscopy confirmed ultrastructural features of necrosis including swollen mitochondria, cytoplasmic clearing, membrane damage, and characteristic nuclear changes (Figure 1e). Thus, HT22 cells die by necrosis in response to TNF α /zVAD.

TNF α /zVAD-induced cell death in HT22 cells is necroptosis. To determine whether TNF α induces necroptosis, for example, RIPK1/RIPK3-dependent programmed necrosis, HT22 cells were treated with TNF α /zVAD in the presence or absence of the potent and specific RIPK1 inhibitor 7-Cl-O-necrostatin-1 (referred to as Nec-1 hereafter), an optimized analog of necrostatin-1 that showed no off-target inhibitory activity in a screen of over 400 human kinases.^{12–14} Nec-1 dose dependently inhibited cell death in response to TNF α /zVAD (Figures 2a and b). To further

validate the role of Nec-1, we used an *N*-methyl inactive analog of 7-Cl-O-necrostatin-1 that lacks inhibitory activity¹² and showed that cell death was no longer inhibited, strongly suggesting that HT22 cell death was necroptosis. To further pursue necroptosis as the specific mode of death we employed genetic knockdown of RIPK3 using specific small inhibitory ribonucleic acid (siRNA). Figure 2 shows robust reduction of RIPK3 protein after 72h treatment with RIPK3-specific siRNA and potent inhibition of TNF α /zVAD-induced cell death, strongly suggesting involvement of both RIPK1 and RIPK3 together in HT22 necrosis (Figures 2c and d). Necroptosis in non-neuronal cells requires formation of a RIPK1–RIPK3 necrosome complex that is dependent on the kinase activity of RIPK1 and RIPK3.^{15–17} To assess for necrosome assembly, we performed immunoprecipitation (IP) experiments that confirmed interaction between RIPK1–RIPK3. RIPK1/RIPK3 complex formation was inhibited by Nec-1, as previously reported in non-neuronal cell lines.¹⁵

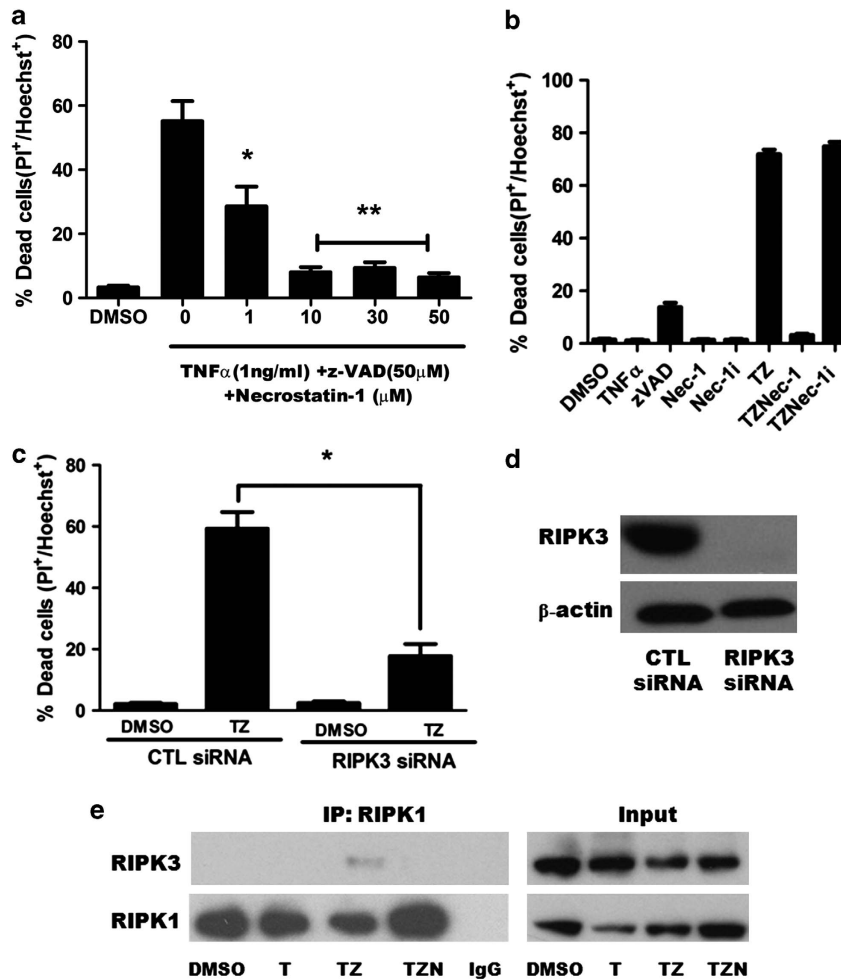


Figure 2 Cell death induced by TNF α /zVAD in HT22 cells is necroptosis. (a) Dose dependent inhibition of TNF α /zVAD-induced cell death by necrostatin-1. Data are mean \pm S.E.M. from three independent experiments. * P < 0.01; ** P < 0.001 versus TNF α (1 ng/ml)/zVAD (50 μ M) (TZ) alone. (b) The specific RIPK1 kinase inhibitor, 7-Cl-O-necrostatin-1 (Nec-1) but not inactive analog (7-Cl-O-Nec-1; Nec-1i) prevented cell death induced by TNF α and zVAD. p = ns TZ versus TZ Nec-1i. (c) Knockdown of RIPK3 by specific siRNA suppressed TNF α /zVAD-induced cell death. * P < 0.001 versus negative siRNA treated with TNF α /zVAD. (d) Western blot analysis of RIPK3 knockdown. (e) Representative immunoprecipitation experiment (n = 3) showing RIPK1–RIPK3 complex assembly in TNF α /zVAD-treated HT22 cells. Complex assembly was inhibited by Nec-1. T, TNF α (1 ng/ml); TZN, TNF α (1 ng/ml)/zVAD (50 μ M)/Nec-1 (30 μ M). Data are mean and S.E.M. of three independent experiments

Inhibition of RIPK1/RIPK3 interaction by Nec-1 was not explained by a reduction in protein level as shown by western blot of input sample homogenates (Figure 2e). From these experiments, we conclude that HT22 cell death in response to TNF α /zVAD is necroptosis, in this case dependent upon both RIPK1 and RIPK3, and that at least part of the protective effects of necrostatin-1 involves inhibition of RIPK1/RIPK3 complex assembly.

Necroptosis in HT22 cells is dependent on ROS. Necroptosis in some, but not all, cell lines has been shown to depend on generation of ROS.^{2,16,18} To determine whether generation of ROS has a role in TNF α /zVAD-induced necrosis in HT22 cells we used MitoSox red, which detects mitochondrial ROS as well as a number of other potential ROS species.¹⁹ MitoSox-positive cells were increased by TNF α /zVAD treatment and this increase was strongly inhibited by Nec-1, indicating that ROS are generated downstream of RIPK1 (Figures 3a and b). ROS generation was also inhibited by Akt viii/rapamycin, suggesting Akt and mTOR signaling is upstream of ROS production (Figures 3a and b). ROS generation (not shown) and HT22 cell death (Figure 3c) was dose dependently inhibited by the antioxidant betahydroxyanisole, and cell death was blocked by a number of other antioxidants including rotenone (a mitochondrial complex I inhibitor) and the lipoxygenase inhibitor baicalein (Bai; Figure 3c). Another lipoxygenase inhibitor

that does not have antioxidant activity itself but is known to reduce oxidative stress-related cell death in HT22 cells, LOXBlock-1 (LOX-1),^{20,21} also protected against TNF α /zVAD to the same extent as bai and rotenone. Thus, ROS generation is required for necroptosis in HT22 cells.

TNF α /zVAD activates Akt and mTOR signaling pathways in HT22 cells. Curiously, previous studies in cancer cells suggested that Akt activity may promote, rather than inhibit ROS-induced cell death due to transcriptional downregulation of antioxidant defenses. Our recent study suggested that in a specific case of mouse fibrosarcoma L929 cell line Akt and mTOR activation contributes to necroptosis.^{22–24} However, this was not observed in a number of other cell lines, where Akt was linked to TNF α synthesis but not cell death, suggesting that pronecrotic signaling may be limited to L929 cells. However, given our previous data regarding the roles of Akt and mTOR in CCI, we assessed for activation of Akt and mTOR pathways in TNF α /zVAD-treated HT22 cells at 2 h. We chose this early time point because it is before the onset of detectable cell death in our model system. In response to TNF α /zVAD, Akt was phosphorylated at the 473 and 308 sites along with glycogen synthase kinase (GSK)-3 β (Ser9), a direct substrate of Akt, and mTOR and its direct substrate S-6 (Figures 4a and c). In contrast to L929 cells induced to necroptosis by TNF α alone in which Akt phosphorylation was transient early on but sustained several

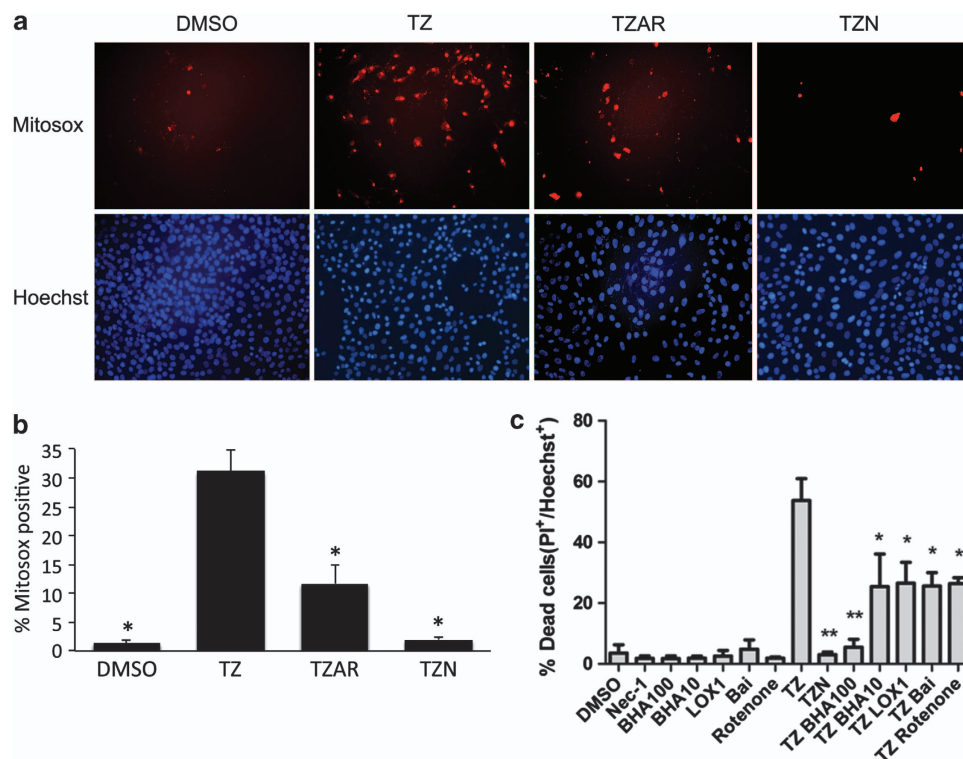


Figure 3 Cell death induced by TNF α /zVAD is associated with oxidative stress. (a) Representative $\times 200$ photomicrographs showing MitoSox Red and Hoechst fluorescence in HT22 cells 4 h after treatment with dimethylsulfoxide vehicle (DMSO), TNF α /zVAD (TZ), or TNF α /zVAD treatment in the presence or absence of Akt/mTOR inhibitors (TZAR, TZ + Akt inhibitor VIII (10 μ M) + rapamycin (100 nM)) or necrostatin-1 (30 μ M; TZN). (b) MitoSox-positive cells were quantitated by fluorescence microscopy. ANOVA $P < 0.0001$. * $P < 0.05$ versus TZ for all groups. Data are from four independent experiments. (c) Protective effects of betahydroxyanisole (BHA), LOXBlock-1 (LOX-1) inhibitor, baicalein, and rotenone on TNF α /zVAD-induced cell death. Data are from three independent experiments. * $P < 0.05$; ** $P < 0.01$ versus TNF α /zVAD (TZ) group. TZN, TNF α /zVAD/Nec-1 (30 μ M)

hours later,²³ Akt and mTOR phosphorylation in HT22 cells was detectable as early as 30 min after addition of TNF α /zVAD and was sustained throughout the entire experimental period (Figures 4a and c and data not shown). Phosphorylation of Akt and mTOR and their related substrates was not induced by TNF α or zVAD alone but required specific necroptotic signaling by TNF α /zVAD together (Figures 4b and d). Necrostatin-1 pretreatment inhibited the increase in Akt-308 and -473, as well as that of mTOR and Akt and downstream mTOR substrates, confirming that phosphorylation and activation of Akt/mTOR in response to TNF α /zVAD is RIPK1 dependent and represent sequelae of necroptosis signaling downstream from RIPK1 (Figures 5b and c). Notably, treatment of HT22 cells with TNF/cyclohexamide (CHX) induced widespread death by 18 h but did not induce Akt phosphorylation at 2 h (Figure 4e) or 18 h (data not shown), suggesting that Akt activation in HT22 cells treated with TNF/zVAD is specific for necroptosis.

To test functionality of Akt and mTOR activation, we used two different approaches. First, we treated HT22 cells with Akt inhibitor viii, rapamycin, or both and assessed cell death by PI staining and pathway inhibition by western blot. Administration of Akt and mTOR inhibitors together reduced HT22 cell death to a greater extent than Akt or mTOR inhibitors alone,

suggesting that both pathways participate in necroptosis signaling (Figure 5a). Cell death was also reduced by treatment with Akt inhibitor viii and torin II, a more specific inhibitor of mTOR than rapamycin (data not shown). Western blot and densitometric analyses confirmed inhibition of phosphorylation of Akt and mTOR and their substrates by Akt inhibitor viii and rapamycin, respectively (Figures 5b and c).

In a second approach to mitigate the potential problem of off-target effects that could confound interpretation of pharmacological experiments, we used specific siRNA to knockdown Akt 1 and 2 (Akt 3 was not detected in HT22 cells; Figure 6a) and mTOR (Figures 6b and c), and assessed the effect of genetic inhibition of each in TNF α /zVAD-induced cell death. In each case robust knock down of protein was obtained, and HT22 cells administered siRNA against Akt1/2 and mTOR together had reduction in TNF α /zVAD-induced death similar in magnitude to treatment with chemical Akt and mTOR inhibitors (Figure 6d). Thus, chemical and genetic approaches each suggested a role for Akt and mTOR activation in programmed necrosis of HT22 cells.

Akt/mTOR inhibition does not affect RIPK1–RIPK3 complex assembly in TNF α /zVAD-treated HT22 cells. Previous studies have demonstrated an essential role for

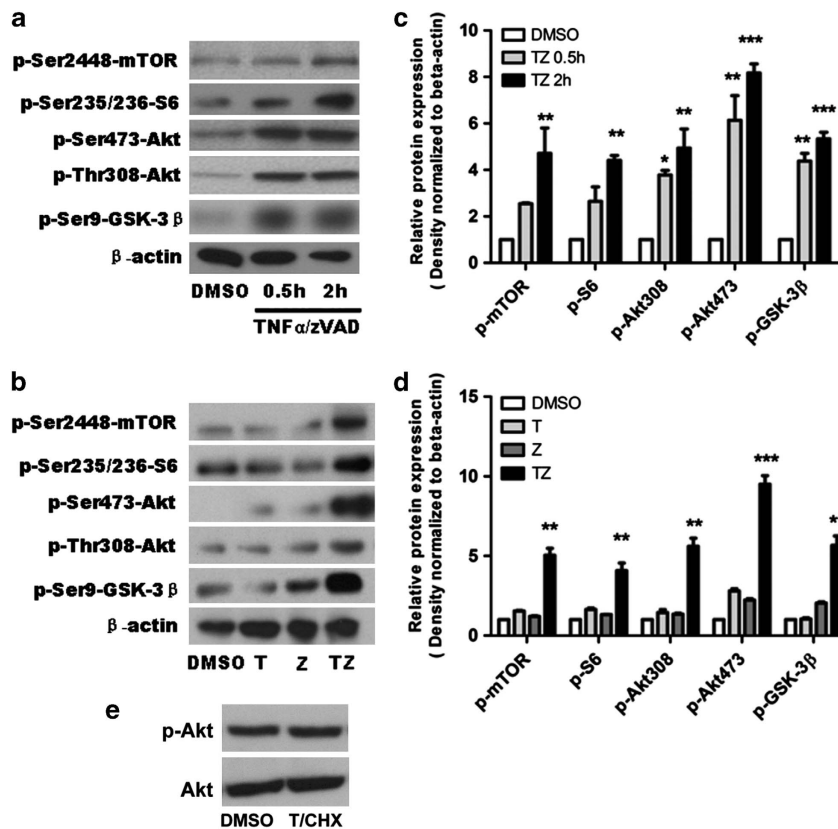


Figure 4 Activation of Akt/mTOR after TNF α /zVAD treatment. Representative immunoblots (a, b) and densitometry results (c, d) of Akt/mTOR activation in HT22 cells after TNF α /zVAD or vehicle dimethylsulfoxide treatment. (a) Time course of phosphorylation events downstream of Akt/mTOR ($n=3$ independent experiments). (c) Densitometry data from a. (b) Synergistic effect of TNF α and zVAD is required to activate Akt/mTOR signaling in HT22 cells. (d) Densitometry data from b. (e) Representative western blot analysis of Akt phosphorylation 2 h after treatment with TNF/cyclohexamide to induce apoptotic cell death. No change in pAkt-473 or total Akt was observed using apoptotic stimuli. * $P<0.05$; ** $P<0.01$; *** $P<0.001$ versus DMSO group. Data are from $n=3$ independent experiments. Abbreviations: phospho-Ser-473-Akt (p-Akt-473), phospho-Ser308-Akt (p-Akt-308), phospho-Ser9-GSK-3 β (p-GSK-3 β), phospho-Ser2448-mTOR (p-mTOR) and phospho-Ser235/236 S6 (p-S6)

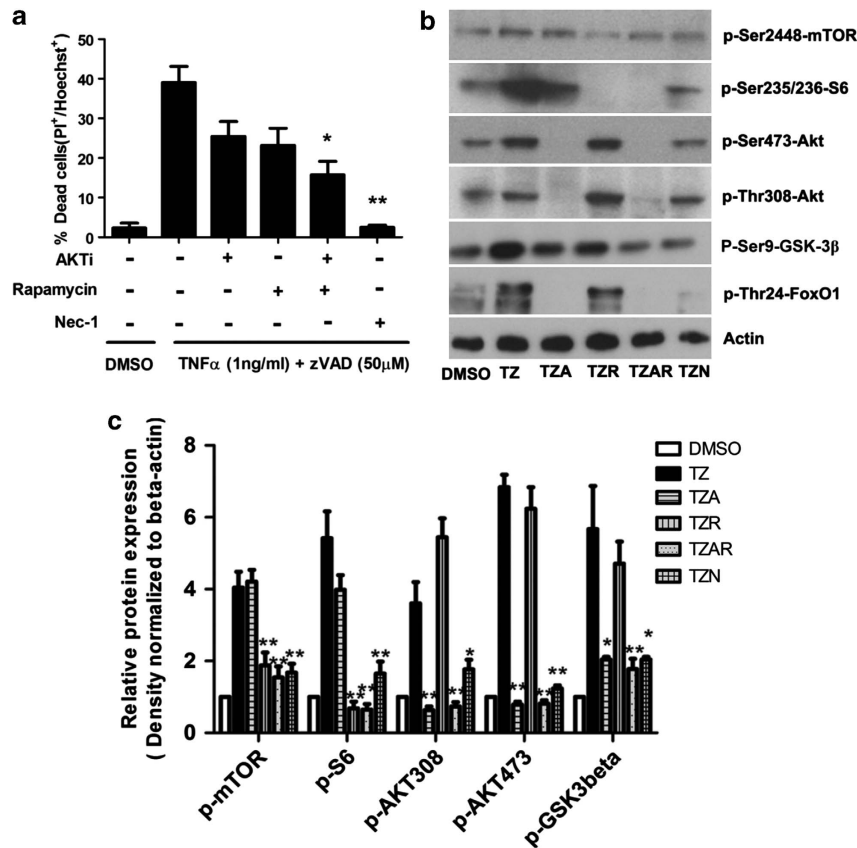


Figure 5 Dual inhibition of Akt and mTOR inhibits necroptosis induced by TNF α /zVAD. (a) Akt and mTOR inhibitors reduced cell death induced by TNF α /zVAD as detected by propidium iodide (PI) and Hoechst staining. (b, c) Representative immunoblots and densitometry for phosphorylated Akt, GSK-3 β , FoxO1, mTOR, and S6 in HT22 cells after TNF α /zVAD in the presence or absence of Akt inhibitor VIII (10 μ M) and rapamycin (100 nM) (data are three independent experiments, * P < 0.01; ** P < 0.001 compared with TZ group). Abbreviations: TNF α (1 ng/ml)/zVAD (50 μ M)/Akt inhibitor VIII (10 μ M) (TZA), TNF α (1 ng/ml)/zVAD (50 μ M)/rapamycin (100 nM) (TZR), TNF α (1 ng/ml)/zVAD (50 μ M)/Akt inhibitor VIII (10 μ M)/ rapamycin (100 nM) (TZAR)

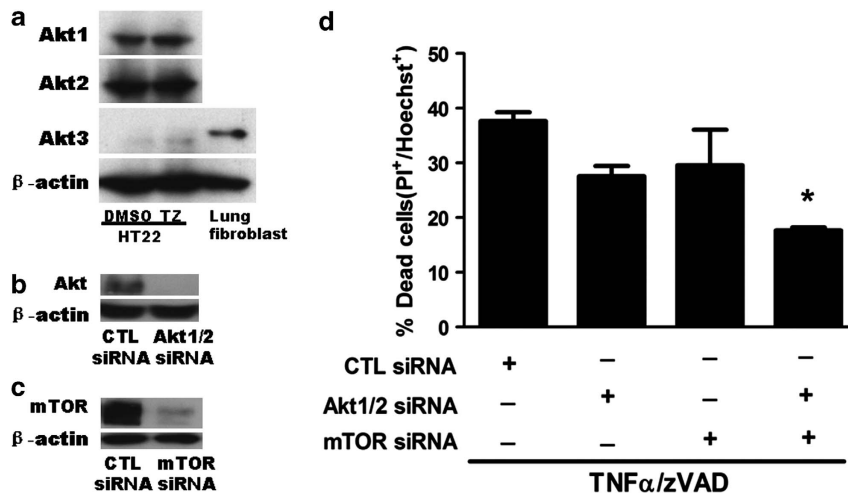


Figure 6 Knockdown of Akt and mTOR inhibits necroptosis induced by TNF α /zVAD. (a) HT22 cells expressed Akt1 and Akt2 but not Akt3 determined by immunoblot. Mouse lung fibroblasts were used as Akt3 positive control. (b, c) siRNA-mediated knockdown of mTOR, Akt1, and Akt2 in HT22 cells was confirmed by immunoblot using reagents identifying total Akt and mTOR. (d) Cell death was determined in control cells and in cells with knockdown of Akt1/2, mTOR, or triple knockdown of mTOR and Akt1/Akt2 together. Data are representative of three independent experiments, * P < 0.05 versus control (CTL) siRNA

kinase activity of RIPK1/RIPK3 in assembly of the necrosome complex.^{4,15–17} To determine whether Akt/mTOR activation might also have a role in RIPK1/RIPK3 complex

assembly, HT22 cells were treated with TNF α /zVAD in the presence or absence of Akt inhibitor viii and rapamycin, and RIPK1–RIPK3 interaction was assessed by IP. Akt/mTOR

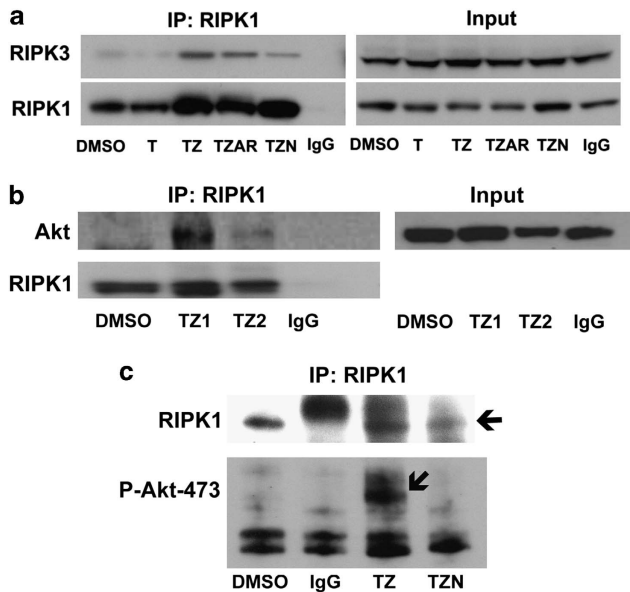


Figure 7 RIPK1–RIPK3 complex assembly occurs upstream of Akt/mTOR activation and reactive oxygen species (ROS) generation after TNF α /zVAD treatment. Interaction between RIPK1–RIPK3 (a) and RIPK1–Akt (b) was detected by immunoprecipitation (IP) and western blot analysis. (c) RIPK1–pAkt-473 interaction (arrow) was also detected at 2 h after TNF/ZVAD (TZ) treatment. Detection of p-Akt-473 was abolished by pretreatment with necrostatin-1 (TZN, 30 μ M). Control experiments showed the presence of RIPK1 in immunoprecipitants from all of the groups except those immunoprecipitated with irrelevant IgG. DMSO, dimethylsulfoxide control. Data are representative of three independent experiments

inhibition did not affect RIPK1–RIPK3 interaction (Figure 7a), suggesting that Akt/mTOR activation occurs downstream of RIPK1–RIPK3 complex assembly and prior to necroptosis steps that produce oxidative stress. These data also raised the possibility of direct interaction of Akt with the necrosome and regulation of its phosphorylation and activation by RIPK1. Indeed, total Akt as well as phospho–Akt–473 was detected in RIPK1 immunoprecipitants in TNF/ZVAD-stimulated HT22 cells, and phospho–Akt–473 interaction with RIPK1 was undetectable with pretreatment of cells with necrostatin-1 (Figures 7b and c). Inhibition of pAkt–RIPK1 interaction by Nec-1 was not due to lack of RIPK1 protein as confirmed by IP (Figure 7c). These data suggest that Akt complexes with RIPK1 and RIPK3, and that its activity during necroptosis may be regulated by necrosome activity.

Discussion

Elucidating mechanisms of neuronal necroptosis is an important area of investigation considering the growing number of reports suggesting its involvement in acute central nervous system injury, including ischemia/reperfusion, trauma, and intracerebral hemorrhage.^{2,3,25,26} Despite a significant research effort to characterize the molecular machinery of necroptosis, little is known regarding the components of necroptosis signaling downstream of necrosome assembly, and almost nothing is known about the regulation of necroptosis in neuronal cells.^{8–10} Necroptosis in HT22 cells exhibited all of the cellular and biochemical

features of necrosis reported in non-neuronal cells including assembly of a RIPK1–RIPK3 necrosome complex, generation of ROS, sensitivity to Jun-N-kinase (JNK) and ROS inhibitors,^{2,23,27,28} loss of mitochondrial membrane potential and adenosine triphosphate depletion, and characteristic ultrastructural features.^{2,15–17} Protection by the specific RIPK1 inhibitor 7-Cl-O-necrostatin-1 and by RIPK3 knock-down confirmed necroptotic cell death in this model system.

We previously reported that pharmacological inhibition of Akt and mTOR reduced early cell death in CA1 and CA3 after CCI in mice, suggesting a novel role for Akt and mTOR in the pathogenesis of traumatic brain cell death.¹¹ These observations prompted us to examine the role of Akt and mTOR in necroptosis signaling in neuronal cells. We found that administration of TNF α /zVAD (but not TNF α or zVAD alone) induced rapid and sustained phosphorylation of Akt on Thr-308 and Ser-473 and mTOR, as well as phosphorylation of direct substrates of Akt (GSK-3 β , forkhead box class O (FOXO)-1) and mTOR (S6 ribosomal protein (S6)). All of these phosphorylation events were inhibited by necrostatin-1, a potent and highly specific RIPK1 inhibitor.¹² IP experiments showed that TNF/ZVAD induced RIPK1–Akt and RIPK1–pAkt-473 interaction. Necrostatin-1 treatment rendered RIPK1–pAkt-473 interaction undetectable, consistent with inhibition of phosphorylation of Akt by the necrosome. Alternatively, necrostatin-1 may prevent binding of Akt to RIPK1. To our knowledge, the data are the first to show a direct interaction of Akt with RIPK1, and provide a molecular link between necrosome assembly and Akt activation during neuronal necroptosis.

Akt/mTOR inhibition did not affect RIPK1–RIPK3 interaction but inhibited ROS production and cell death. Importantly, genetic inhibition of Akt and mTOR together reduced cell death by a magnitude similar to that of pharmacological inhibitors, confirming a specific role for Akt and mTOR. Taken together, the data indicate that Akt/mTOR mediate necroptosis signaling downstream of the necrosome assembly and before the production of ROS and onset of plasmalemma damage. The observation that concomitant inhibition of Akt and mTOR together was required for maximal protection is reminiscent of our findings in the CCI model, and suggests divergent roles for Akt and mTOR in necroptotic signaling in neuronal cells. In contrast, Akt activation was not involved in apoptosis of HT22 cells treated with TNF and CHX (Figure 4e).

In the current study, antagonism of Akt and mTOR together was required to maximally inhibit necroptosis in HT22 cells. These findings are in agreement with our previous *in vivo* study in which Akt and mTOR inhibitors together were required to reduce necrotic cell death and improve postinjury cognitive function after cerebral contusion in mice.¹¹ Thus, regulation of necroptosis by Akt and mTOR together may be a unique property of neuronal cells, or may depend on the exact stimulus used to initiate necroptosis. Akt is activated and is essential for necroptosis in mouse L929 fibroblasts stimulated with TNF α or zVAD, but not for necroptosis of Fas-associated protein with death domain-deficient Jurkat T lymphocytes treated with TNF α .²³ In mouse lung fibroblasts and macrophages, Akt exhibited control over necroptosis-associated TNF α production but did not have a role in cell death.²³

Thus, Akt activation mediates necroptosis in some but not all non-neuronal cell types, and as such is not a uniform defining feature of necroptosis. This idea is supported by data showing partial or complete inhibition of cell death by various antioxidant agents and inhibitors of oxidative stress enzymes (Figure 3c). Akt is activated during necroptosis in Jurkat cells but ROS production does not occur, and Akt inhibitors block TNF production but not cell death in this line.^{2,23} Thus, no simple relationship exists between Akt/mTOR activation, ROS production and necroptosis in all cell types.

IP studies performed herein suggest that phosphorylation of Akt may be required for its incorporation into the necrosome complex, as treatment with necrostatin-1 abolished detection of phospho-Akt-473-RIPK1 interaction. These findings suggest that Akt phosphorylation might regulate necroptosis at the level of the necrosome. In the case of L929 cells, Akt Ser-473 was not increased or involved in cell death; however, plasmalemma localization and selective phosphorylation of Akt Thr-308 was required to link RIPK1 to downstream JNK signaling, autocrine TNF α production, and death.²³ Although the exact mechanism of Thr-308 phosphorylation remains unknown, inhibition of phosphatase 2A (a phosphatase that dephosphorylates Thr-308; 45 MnA) had no effect.²³ *In vitro*, recombinant RIPK1 can phosphorylate Akt on Thr146, 195/197, 435, and Ser381 but whether Akt is an endogenous substrate of RIPK1 is not clear.²³ Further studies are needed to better understand how Akt is regulated by phosphorylation during necroptosis.

Prevailing wisdom assigns a prosurvival role for Akt in CNS injury paradigms including stroke and traumatic brain injury.²⁹⁻⁴⁰ Antiapoptotic activity of Akt has been well documented, through a number of mechanisms including inhibition of caspase 9, Bad, and GSK-3 β , and induction of mitochondrial hexokinase and nuclear factor kappa B-dependent antiapoptotic gene expression.⁴¹⁻⁴³ Nonetheless, several recent reports illustrate an emerging role for Akt as a 'death kinase' and a key regulator of programmed necrosis, including neuronal cell types,⁴⁴ depending on injury context. Activation of phosphatidylinositol 3 kinase/Akt has been linked to cytotoxic cell death⁴⁵⁻⁴⁸ and nuclear translocation of Akt has been reported to sensitize cancer cells to death by chemotherapeutic agents.^{49,50} Akt inhibitors also protect cultured neurons against photodynamic induced necrosis.⁴⁴ Data from the current study raise the question of which Akt/mTOR substrates might be critical regulators of necroptosis in neuronal cells. Our data suggest a possible role for mTOR complex1-dependent regulation as previously reported by McNamara *et al.*,²³ as well as the Akt substrates FOXO1, GSK-3 β , and others. Although further studies are needed to determine the specific mediators downstream of Akt and mTOR, taken together the present data and our previous report¹¹ suggest that Akt may promote necrosis in neuronal cell types following acute CNS injury.

How might Akt promote necrotic death? Although limited activation of Akt is antiapoptotic, sustained Akt activity may promote cell death by a number of distinct mechanisms. Strong activation of Akt increases oxygen consumption by increasing glycolysis and oxidative phosphorylation, resulting in generation of ROS and oxidative stress.^{24,42} Sustained Akt activation also induces phosphorylation of forkhead box

subclass O (FOXO) transcription factors required for upregulation of antioxidant defenses such as manganese superoxide dismutase, catalase and sestrin-3.^{51,52} Phosphorylated FOXO transcription factors bind to 14-3-3 protein and are excluded from the nucleus, thus rendered transcriptionally inactive,⁵³ and sustained phosphorylation promotes their proteosomal degradation.^{54,55} Our finding that the inhibition of Akt/mTOR strongly inhibited phosphorylation of FOXO1, reduced generation of ROS and inhibited death in HT22 cells stimulated to necroptosis suggests that Akt/mTOR activation may increase lethal oxidative stress after acute injury to neuronal cells. Finally, sustained Akt activation can constitutively activate cyclin-dependent kinase 2 leading to its cytoplasmic sequestration, cell cycle arrest at G2-M, and induction of cell death.^{56,57} Future studies aimed at pinpointing the exact mechanisms of Akt/mTOR-induced necroptosis are warranted as Akt and mTOR may not be optimal therapeutic targets for neuroprotection because of their protean functions in cells.

Herein, we show that Akt and mTOR together account for ~50% of the cell death in the conditions used in necroptosis assays. The other mechanism(s) that account for the remainder of necroptotic cell death remain to be discovered. A JNK inhibitor reduced necroptosis in HT22 cells by ~50%; however, we did not observe an additive effect of Akt/mTOR/JNK inhibitors and no effect of extracellular-regulated kinase or P38 mitogen-activated protein kinase (MAPK) inhibition, consistent with a similar lack of a role for these MAPK in necroptosis of non-neuronal cell types.^{23,28} Interestingly, we previously reported that intracerebroventricular administration of necrostatin-1 reduced necrotic cell death in dentate gyrus and cortex after CCI in mice.³ However, these brain regions were not protected by Akt/mTOR inhibitors in our subsequent study,¹¹ consistent with the findings of the current study that necrostatin-1 is fully and Akt/mTOR inhibitors only partially protective against necrotic cell death in HT22 cells.

In conclusion, we demonstrate for the first time a role for Akt/mTOR signaling governing necroptosis in a neuronal cell line. The data are consistent with a need for dual inhibition of Akt and mTOR to reduce traumatic brain cell death *in vivo*.¹¹ Elucidation of additional necroptosis signaling mechanisms downstream of necrosome assembly will be important to fully understand how necroptosis is regulated in neuronal cells. Such information is likely to yield new therapeutic targets to reduce cell death and improve functional outcome after stroke, traumatic brain injury, and other forms of acute CNS injury.

Materials and Methods

Reagents. The following materials were purchased from commercial companies: TNF α (PeroTech; Rocky Hill, NJ, USA); pan-caspase inhibitor z-VAD-fmk (Abcam, Cambridge, MA, USA). InSolution Akt Inhibitor viii isozyme-selective, Akti-1/2 and InSolution rapamycin were obtained from Calbiochem (San Diego, CA, USA). MitoSox Red was obtained from Invitrogen (Carlsbad, CA, USA). Hoechst 33258, butylated hydroxyanisole (BHA) and rotenone were obtained from Sigma (St. Louis, MO, USA). Nec-1 (5-(7-chloro-1H-indol-3-ylmethyl)-3-methylimidazolidine-2,4-dione), the inactive analog of necrostatin-1 analog (Nec-1i; 5-(7-chloro-1H-indol-3-ylmethyl)imidazolidine-2,4-dione),^{3,12,13} was a kind gift from Dr. Greg Cuny. LOX-1 was obtained from Chembridge (compound 5680672; San Diego, CA, USA), and Bai was from Cayman Chemicals (Ann Arbor, MI, USA).

Antibodies were obtained from commercial sources: anti-pAkt-473, anti-p-Akt-308, anti-p-GSK-3 β , anti-p-mTOR, anti-p-S6, anti-mTOR, anti-Akt1, anti-Akt2, anti-Akt3, and anti-Akt from Cell Signaling (Danvers, MA, USA); anti-RIPK1 from BD Transduction Laboratories (Lexington, KY, USA); anti-RIPK3 from ProSci Incorporated (San Diego, CA, USA); anti-HMGB1 and β -actin were obtained from Abcam.

Cell culture and treatment protocols. HT22 murine hippocampal neuronal cells were maintained in Dulbecco's modified essential medium supplemented with 10% (v/v) FBS and antibiotics (penicillin-streptomycin) and incubated at 37 °C under 5% CO₂. To test the effects of necrostatin-1 (30 μ M), Nec-1i (30 μ M), BHA (100 and 10 μ M), LOX-1 (5 μ M), Bai (10 μ M), Akt inhibitor VIII (10 μ M), and rapamycin (100 nM), cells were pretreated with each of the reagents at the mentioned concentrations for 15 min before the addition of TNF α (1 ng/ml) and zVAD (50 μ M). Control groups were treated with vehicle (DMSO) only. For experiments examining apoptosis, HT22 cells were treated for 2 or 18 h with TNF (10 ng/ml) and CHX (10 μ g/ml).

Detection of cell death using propidium iodide. Propidium iodide (1 μ g/ml; Sigma) and Hoechst 33258 (10 μ g/ml; Sigma) were added to media and incubated with HT22 cells for 5 min. For quantification of PI⁺ cells, photographs were randomly taken from three individual \times 200 fields per well. There were three wells per experimental condition. PI⁺ cells were expressed as a percentage of Hoechst⁺ cells.

Immunocytochemistry. For HMGB1 staining, HT22 cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and permeabilized with 0.1% Triton X-100 in PBS. After blocking with 1% bovine serum albumin, cells were incubated with primary antibody to HMGB1 (1:100; Cell Signaling) overnight at 4 °C, followed by incubation with a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:500) at room temperature for 1 h. Hoechst dye was used for nuclear staining.

Transmission electron microscopy. HT22 cells were fixed in PBS containing 2% paraformaldehyde/2% glutaraldehyde for 60 min. After washing in the same buffer, cells were gently scraped off with a rubber policeman, centrifuged, and postfixed with 1% OsO₄, 0.8% potassium ferricyanide, and 5 mM CaCl₂ in 0.1 M cacodylate buffer. Samples were washed twice in the same buffer, dehydrated in acetone and embedded in PolyBed 812. Ultrathin sections were stained with uranyl acetate and lead citrate and observed in a FEI Morgagni transmission electron microscope operating at 80 kV.

RNA interference. siRNA for mouse Akt1, Akt2, mTOR, and RIPK3 (ON-TARGETplus SMARTpool) was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Twenty nmol of siRNA with lipofectamine 2000 was transfected into HT22 cells. The same concentration of nontarget siRNA (from Thermo Fisher Scientific) was used as control. Cells were cultured for 3 days and targeted gene expression was examined by western blot.

Western blotting. Cells were lysed in radio-immunoprecipitation assay buffer (10 mM HEPES buffer, pH 7.6, 42 mM KCl, 5 mM MgCl₂, 1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA (ethylenediaminetetraacetic acid), 1 mM ethylene glycol tetraacetic acid, 1 mM dithiothreitol, 1.5 μ M pepstatin, 2 μ M leupeptin, and 0.7 μ M aprotinin). Protein concentration was determined using the DCTM protein assay (Bio-Rad, Hercules, CA, USA). Thirty to fifty micrograms of protein was applied for polyacrylamide gel electrophoresis. The primary antibodies against HMGB1 (1:500), RIPK3 (1:1000), Akt1 (1:500), Akt2 (1:500), Akt3 (1:500), Akt (1:500), mTOR (1:500), phospho (Ser-473)-Akt (1:500), phospho (Ser308)-Akt (1:500), phospho (Ser9)-GSK-3 β (1:1000), phospho (Ser235/236)-S6 (1:1000), and phospho (ser2448)-mTOR (1:1000) were obtained from Cell Signaling. Horseradish peroxidase conjugated secondary antibodies (1:10 000) were used for ECL-plus (GE Healthcare, Pittsburgh, PA, USA) detection. The results were normalized to β -actin (1:5000).

Immunoprecipitation. For co-immunoprecipitation experiments, a total of 1.8 mg of protein from HT22 cells was used. Cells were stimulated with TNF α (1 ng/ml) and zVAD (50 μ M) in the presence or absence of Necrostatin-1 (30 μ M), Akt Inhibitor VIII (10 μ M), or rapamycin (100 nM) for 2 h. Cells were collected, washed in PBS and lysed in a buffer containing 20 mM Tris-HCl (pH 8), 137 mM

NaCl, 10% glycerol, 1% Nonidet P-40 (NP-40), 2 mM EDTA, and protease inhibitor cocktail. Pre-cleaning was achieved with 50 μ l of protein A/G agarose beads (Santa Cruz, Dallas, TX, USA) for 1 h, and lysates were incubated overnight at 4 °C in presence of 50 μ l of protein A/G agarose or magnetic beads and 1–2 μ g mouse anti-RIP1 antibody (BD Transduction) before analysis by western blotting. The same amount of irrelevant mouse IgG (Santa Cruz) was used to control for nonspecific IgG binding to RIPK1.

Measurement of ROS. HT22 cells were incubated with 5 μ M MitoSOX Red for 10 min at 37 °C. Cells were washed in PBS and analyzed by fluorescence microscopy. Mitosox-positive cells were expressed as a percentage of total cells in 3–6 \times 200 fields/well, three wells per experiment. Experiments were repeated four times.

Statistical analysis. All values were expressed as mean \pm S.E.M. Data from more than two groups were assessed by one-way ANOVA, followed by Tukey's multiple comparison test using Graph Pad Prism V software (La Jolla, CA, USA). For all experiments, $P < 0.05$ was considered statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

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