TNF-α-mediated cardiorenal injury after rhabdomyolysis in rats

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Homsi E, Andreazzi DD, Lopes de Faria JB, Janino P. TNF-α-mediated cardiorenal injury after rhabdomyolysis in rats. Am J Physiol Renal Physiol 308: F1259–F1267, 2015. First published November 12, 2014; doi:10.1152/ajprenal.00311.2014.—The TNF-α serum level increases after rhabdomyolysis and is involved in the subsequent cardiorenal injury. In the present study, we investigated the TNF-α-dependent cell signaling pathways implicated in cellular injury in these organs. Rhabdomyolysis was induced by intramuscular glycerol injection in rats. Renal function, cardiac and renal pathology, and activation of caspases were evaluated during the first 24 h after glycerol injection. TNF-α blockade with infliximab reduced tubular necrosis and cardiorenal apoptosis. Cellular Fas-associated protein with death domain-like IL-1β-converting enzyme inhibitory protein (cFLIP), an inhibitor of caspase-8, was overexpressed in the kidney but not in the heart. The inhibitory effect of cFLIP blunted caspase-8 activation in the kidney. In this condition, the cellular response to the TNF-α stimulus was driven to receptor-interacting protein-1 (RIP1)-mediated necroptosis. Treatment with RIP1 inhibitor (necrostatin-1) isolated or in combination with infliximab showed a similar reduction in tubular necrosis, underscoring the importance of TNF-α-mediated tubular necroptosis in this model. TNF-α played a positive regulatory role in the transcription of proapoptotic Bax and p53-upregulated modulator of apoptosis (PUMA) proteins. Infliximab treatment reduced caspase-9-mediated apoptosis in both organs. Treatment with a caspase-8 inhibitor showed that caspase-8 participated in the process of apoptosis only in the heart, upstream of caspase-9 activation. TNF-α-mediated necroptosis is the predominant form of tubular injury observed in the glycerol model. TNF-α upregulates Bax and PUMA proapoptotic proteins, resulting in activation of the intrinsic pathway of apoptosis in the kidney and heart. Acute kidney injury; apoptosis; necroptosis; cardiac injury; tumor necrosis factor-α; rhabdomyolysis

Rhabdomyolysis elicits a systemic inflammatory response that may contribute to distant organ injuries (3, 35, 42). Acute kidney injury (AKI) is the most well-known distant organ injury frequently observed after severe rhabdomyolysis (2, 19). A large amount of evidence supports the role of renal ischemia and myoglobinuria as the main mechanisms involved in the renal injury after rhabdomyolysis (9, 41). Apart from these mechanisms, Shulman et al. (34) showed many years ago that treatment with anti-TNF-α-neutralizing antibody conferred renal protection to rats subjected to rhabdomyolysis.

More recently, Kelly (12) showed an increased serum level and cardiac expression of TNF-α after renal ischemia-reperfusion injury (IRI) in rats. Cardiac function was depressed after renal IRI, and the author detected a significant increase in cardiomyocyte apoptosis 48 h after renal IRI. Treatment with anti-TNF-α polyclonal antibody simultaneously with renal IRI significantly attenuated cardiomyocyte apoptosis.

Renal tubular cells express cell surface “death receptors” of the TNF family, including TNF receptor type 1 (TNFR1), which is the main target of TNF-α (4). Upon interaction with its specific receptor, TNF-α forms signaling complex I, composed of TNFR1-associated death domain, TNFR-associated factor (TRAF)2 and TRAF5, receptor-interacting protein (RIP1), and cellular inhibitor of apoptosis (cIAP) proteins, which leads to NF-κB activation and the transcription of many inflammatory cytokines as well as several antiapoptotic proteins (20). Alternatively, after a process of deubiquitination of the protein RIP1, this complex can be internalized into the cell, where it incorporates Fas-associated protein with death domain-like IL-1β-converting enzyme inhibitory protein (cFLIP), a third pathway of TNF-α-mediated necroptosis. In this condition, the cellular response to the TNF-α stimulus was driven to receptor-interacting protein-1 (RIP1)-mediated necroptosis. Treatment with RIP1 inhibitor (necrostatin-1) isolated or in combination with infliximab showed a similar reduction in tubular necrosis, underscoring the importance of TNF-α-mediated tubular necroptosis in this model. TNF-α played a positive regulatory role in the transcription of proapoptotic Bax and p53-upregulated modulator of apoptosis (PUMA) proteins. Infliximab treatment reduced caspase-9-mediated apoptosis in both organs. Treatment with a caspase-8 inhibitor showed that caspase-8 participated in the process of apoptosis only in the heart, upstream of caspase-9 activation. TNF-α-mediated necroptosis is the predominant form of tubular injury observed in the glycerol model. TNF-α upregulates Bax and PUMA proapoptotic proteins, resulting in activation of the intrinsic pathway of apoptosis in the kidney and heart.

Acute kidney injury; apoptosis; necroptosis; cardiac injury; tumor necrosis factor-α; rhabdomyolysis

MATERIALS AND METHODS

Animal protocols. Experiments were performed in accordance with the guidelines established by the Brazilian College for Animal Experimentation. Rhabdomyolysis was induced in male Wistar rats (200–250 g) after an overnight fast by a single intramuscular injection of 50% glycerol (7 ml/kg) divided into both lower hindlimbs. Every time rhabdomyolysis was induced, a similar number of sham (glycerol)-injected rats that had been subjected to the same procedures before and after injection was used to serve as control groups. After glycerol injection, rats were immediately treated with saline (sham treatment) or with a single dose of infliximab (5 mg/kg ip, Centocor BV, Leiden, Holland) (40). In a separate series of experiments, using the same model, rats were treated with a single intraperitoneal dose of the RIP1 inhibitor necrostatin-1 (1.65 mg/kg, Sigma-Aldrich, St. Louis, MO) (16) or infliximab (5 mg/kg, Centocor BV), or their combination at the same doses used alone. Finally, other rats were treated with a single intraperitoneal dose of the caspase-8 inhibitor Ac-AEVD-CHO (4 mg/kg, Enzo Life Sciences, Farmingdale, NY) or vehicle (1% DMSO) immediately after glycerol injection.

Rats were euthanized 6 or 24 h after glycerol injection. At death, the heart and kidneys were harvested, a small sample of tissue was
separated for protein extraction and subsequent Western blot analysis, and the rest of the tissue was fixed and embedded in paraffin for histological and immunohistochemical experiments. Serum levels of creatine phosphokinase 6 h after glycerol injection and serum levels of creatinine 24 h after glycerol injection were used to evaluate the intensity of rhabdomyolysis and renal function (automatic analyzer Cobas-Mira, Roche, Basel, Switzerland, respectively).

**Histological analysis.** Paraffin-embedded slides from kidneys harvested 24 h after glycerol injection were stained with hematoxylin and eosin. Numbers of totally necrotic and desquamated tubules (absence of nuclei) were quantified in 15 random cortical high-power fields (HPFs; ×400) for each rat.

Thus detection of apoptotic cells was performed using a TUNEL assay. Briefly, longitudinal sections of embedded tissues were de-waxed, rehydrated, and had endogenous peroxidase blocked in 3% H2O2. Tissue was incubated with proteinase K (20 mg/ml) for 15 min at room temperature and then incubated with a solution containing the enzyme terminal deoxynucleotidyl transferase (1:50) and biotinylated dUTP (1:50) in terminal deoxynucleotidyl transferase buffer for 60 min at room temperature (GenScript, Piscataway, NJ). Apoptotic nuclei that had incorporated biotinylated dUTP were visualized using the enhanced chemiluminescence detection kit (Amersham, Arlington Heights, IL). Immunoreactive bands were visualized using the enhanced chemiluminescence detection kit (Amersham, Arlington Heights, IL).

**Immunohistochemistry:** 8-hydroxydeoxyguanosine, cleaved caspase-3, and macrophages (ED-1). Tissues were fixed in 10% formalin [except for 8-hydroxydeoxyguanosine (8-OHdG), which was fixed in methacarn] and embedded in paraffin. Slides were incubated overnight at 4°C with the following primary antibodies: anti-8-OHdG (1:100, Japan Institute for the Control of Aging, Osaka, Japan), anti-cleaved caspase-3 (1:100, Cell Signaling Technology, Danvers, MA), and anti-ED-1 (1:200, AbD Serotec, Oxfordshire, UK). The next morning, tissue was incubated with specific biotinylated secondary antibodies (1:200), the ABC detection kit (Dako), and diaminobenzidine tetrahydrochloride color developer (Dako). Macrophages (ED-1 positive) and cleaved caspase-3-positive cells were scored in 10 random outer medullary HPFs (×400). 8-OHdG-positive tubular cell nuclei were scored in 10 cortical HPFs (×400).

**Western immunoblot analysis.** Renal fragments used to extract proteins were collected from the outer medulla 6 h after glycerol injection except for TNF-α (24 h after glycerol). Fifty micrograms of the homogenate protein was loaded in the SDS-polyacrylamide gel and electrophoresed in Laemmli solution. Proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The following primary antibodies were incubated overnight at 4°C: goat anti-rat TNF-α (1:500), mouse anti-rat Bcl-2 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit anti-rat phosphorylated (p)-IκBα (Ser15, 1:1,000), rabbit anti-rat Bax (1:500), rabbit anti-rat p53-upregulated modulator of apoptosis (PUMA; 1:1,000), rabbit anti-rat cleaved caspase-8, rabbit anti-rat cleaved caspase-9, rabbit anti-rat cleaved caspase-3, mouse anti-rat p-p65 NF-κB (1:1,000), rabbit anti-rat p-JNK (1:1,000), rabbit anti-rat p-p38 MAPK (1:1,000), and rabbit anti-rat FLIP (1:1,000, Cell Signaling Technology). After samples were washed with buffer, horseradish peroxidase-conjugated secondary antibodies were incubated for 1 h at room temperature. Immunoreactive bands were visualized using the enhanced chemilu-

### Table 1. Effect of treatments on the serum level of creatine phosphokinase

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>Glycerol-Induced Rhabdomyolysis Group</th>
<th>Glycerol-Induced Rhabdomyolysis + Infliximab Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatine phosphokinase, IU</td>
<td>699 ± 167</td>
<td>4,865 ± 736*</td>
<td>5,593 ± 153*</td>
</tr>
<tr>
<td>Control</td>
<td>Glycerol-Induced Rhabdomyolysis Group</td>
<td>Glycerol-Induced Rhabdomyolysis + Necrostatin-1 Group</td>
<td></td>
</tr>
<tr>
<td>Creatine phosphokinase, IU</td>
<td>465 ± 47</td>
<td>3,222 ± 409*</td>
<td>2,453 ± 390*</td>
</tr>
<tr>
<td>Control</td>
<td>Glycerol-Induced Rhabdomyolysis Group</td>
<td>Glycerol-Induced Rhabdomyolysis + ac-AEVD-CHO Group</td>
<td></td>
</tr>
<tr>
<td>Creatine phosphokinase, IU</td>
<td>129 ± 79</td>
<td>5,043 ± 760*</td>
<td>6,255 ± 619*</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE; n = 5 rats/group. *P < 0.01 vs. the respective control group.

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minesine method (Super Signal CL–HRP Substrate System, Pierce, Rockford, IL). β-Actin (Santa Cruz Biotechnology) was used as the load control for immunoblot analysis. Quantification of the bands was done by optical densitometry using ImageJ software (version 1.37, National Institutes of Health, Bethesda, MD).

**Enzyme immunoassay for rat TNF-α.** Plasma samples from control and rats subjected to rhabdomyolysis rats 6 and 24 h after glycerol injection were immediately assayed using a sandwich ELISA kit (Abcam, Cambridge, MA).

**Statistical analysis.** Data are expressed as means ± SE. ANOVA and Student-Newman-Keuls tests were used to test parametric values, and Kruskal-Wallis-Dunn tests were used to test nonparametric values. *P* values of <0.05 were considered significant.

**RESULTS**

**TNF-α expression after rhabdomyolysis.** The TNF-α serum level peaked 6 h after glycerol injection and returned to normal after 24 h (Fig. 1A). Renal expression of TNF-α protein was increased 24 h after glycerol injection. This increase was attenuated by infliximab treatment (Fig. 1B).

**Treatment effects on muscle injury.** The creatine phosphokinase serum level was significantly increased 6 h after glycerol injection compared with controls, and its level was unaffected by treatments, as shown in Table 1.

**Effect of infliximab treatment on renal injury after rhabdomyolysis.** Renal function was evaluated by the level of serum creatinine, which was measured 24 h after glycerol injection. Rats treated with infliximab showed a smaller increase in serum creatinine, demonstrating that TNF-α blockade can attenuate the renal dysfunction in this model (Fig. 2A).

The main renal injury observed after rhabdomyolysis is cortical acute tubular necrosis. As shown in Fig. 2B, infliximab treatment reduced the severity of cortical tubular necrosis 24 h after glycerol injection. Objective quantification of the number of necrotic tubules per HPF (×400) showed a significant reduction (48%) in the number of necrotic tubules in treated rats (Fig. 2C). This significant reduction in tubular necrosis suggested that TNF-α-regulated necrosis (necroptosis) plays a fundamental role in the glycerol model. In cellular experiments, TNF-α-mediated cellular death switches from apoptosis to necrosis when a pancaspase inhibitor is added to the system (43). We observed a similar condition in vivo: the expression of cleaved caspase-8 was severely downregulated in the kidney after rhabdomyolysis (Fig. 2D). The NF-κB transcript cFLIP is a natural antagonist of caspase-8 (13, 14, 29), and expression of p-p65 (NF-κB active form) and cFLIP were increased in the kidney 6 h after glycerol injection (Fig. 2E). To highlight the importance of necroptosis in the glycerol model as well as the role of TNF-α in this process, a separate series of experiments was conducted (Fig. 3, A and B), in which rats were allocated to treatment with the RIP1 inhibitor necrostatin-1, infliximab, and a combination of both. The RIP1 inhibitor and infliximab significantly reduced the magnitude of tubular necrosis to approximately one-half of that observed in untreated rats, confirming the importance of necroptosis. The combined...
therapy was not more efficient than the isolated treatments. Necrostatin-1 treatment did not affect the activation of caspase-3 in the kidney (Fig. 3C).

We observed a significant increase in the number of TUNEL-positive cells in the outer medulla (Fig. 4, A and B) and cleaved caspase-3-positive tubular cells in the corticomedullary junction (Fig. 4, A and C), respectively, 6 and 24 h after glycerol injection, and infliximab treatment significantly reduced the numbers of these positive cells. As previously reported, TUNEL-positive cells are very rare in the renal cortex in the glycerol model (7). The same pattern of cleaved caspase-3 expression in renal tissue was observed using Western blot assays 6 h after glycerol injection (Fig. 4D). Renal expression of cleaved caspase-9, the initiator of the intrinsic pathway of apoptosis, was increased 6 h after glycerol injection, and this increase was significantly attenuated by infliximab treatment (Fig. 4D). The intrinsic (or mitochondrial) pathway is regulated by the transcription factor p53 (26). An upstream activator of p53 is the TNF-α-activated protein kinase JNK (31). In fact, renal p-JNK and p-p53 were found to be overexpressed after rhabdomyolysis, but neither p-JNK nor p-p53 expression were affected by infliximab treatment (Fig. 4E). The transcripts encoded by p53, such as Bax and PUMA, act in the mitochondrial membrane, increasing the permeability of the outer membrane to intermembrane space proteins such as cytochrome c and second mitochondria-derived activator of caspases/direct IAP-binding protein with low pl (Smac-DIABLO), which are released into the cytosol, where they activate caspase-9 (21, 25). We evaluated the renal expression of the proapoptotic transcripts that are positively regulated by p53 (Bax and PUMA) and the antiapoptotic component of the family that is repressed by p53 (Bcl-2). Bax and PUMA expressions were significantly increased after rhabdomyolysis, and infliximab therapy effectively reduced their expressions. Bcl-2 expression did not change after rhabdomyolysis (Fig. 4F).

The glycerol model is characterized by increased renal oxidative stress (22, 33). In the present study, we used an immunochemistry assay to detect 8-OHdG, a marker of nucleic acid oxidative stress, and noticed a significant reduction in oxidative stress in treated rats (Fig. 5, A and B). Bax and PUMA can induce the production and release of mitochondrial ROS (18, 28), and the downregulation of these proteins could be the reason for the reduction in renal oxidative stress in treated rats.

TNF-α is a potent chemoattractant to leukocytes and promotes the expression of adhesion molecules on endothelial cells (11). As expected, treated animals showed a reduction in macrophage infiltration (Fig. 5, A and C). As leukocytes are the primary source of TNF-α synthesis, the reduction in macrophage infiltration in the kidney could be responsible for the reduction in renal expression of TNF-α in treated rats (Fig. 1B).

Cardiac apoptosis after rhabdomyolysis. Cardiac tissue examined 6 h after glycerol injection showed impressive cardiomyocyte apoptosis, as evaluated by either TUNEL assay or cleaved caspase-3 immunostaining. This cardiac injury was efficiently prevented by infliximab treatment (Fig. 6, A–C). Different from renal tissue, both cleaved caspase-8 and caspase-9 were overexpressed in the rat heart after rhabdomyolysis. However, only cleaved caspase-9 expression was reduced by infliximab treatment. In the heart, we found that cFLIP expression was not increased after rhabdomyolysis; thus, caspase-8 cleavage and activation were not impaired by the action of this antagonist (Fig. 6D).

Role of the extrinsic apoptosis pathway in different organs. Because we observed caspase-8 activation in the heart but not in the kidney, we performed a separate series of experiments to confirm the reliability of this finding. Rats were
treated with Ac-AEVD-CHO (caspase-8 inhibitor) simultaneously with glycerol injection. In consonance with the observed pattern of cleaved caspase-8 expression in these organs, the caspase-8 blockade significantly reduced caspase-3 activation in the heart but failed to do the same in the kidney (Fig. 7, A and B).

DISCUSSION

The pharmacological blockade of TNF-α reduced renal tubular and cardiac injury after rhabdomyolysis. The renal function was partly preserved in treated rats, and both tubular necrosis and apoptosis were significantly reduced by infliximab treatment. In renal tissue, but not in the heart, we observed overexpression of cFLIP, an antiapoptotic protein transcriptionally regulated by various stimuli, some of them activated in our model, such as NF-κB and p53 tumor suppressor protein (13, 29). cFLIP can disrupt interactions among caspase-8, FADD, and other components of the death-inducing signaling complex (complex II), thus blocking the activation of caspase-8 (13, 14, 29). Active caspase-8 can cleave RIP1, an adaptor protein that is recruited after TNF-α receptor 1 (TNFR1) activation, and interact with FADD, leading to the formation of the death-inducing signaling complex (complex I) (23). Downregulation of cleaved caspase-8, as ob-
served in the kidney after rhabdomyolysis, frees RIP1/RIP3 from inhibition and initiates the process of necroptosis (Fig. 8) (6). The relevance of necroptosis in our model was confirmed in rats treated with the inhibitor of RIP1 (necrostatin-1). Necrostatin-1, infliximab, or combined therapy produced a similar reduction (50%) in tubular necrosis, suggesting that TNF-α engagement to the death receptor is the main event that triggers necroptosis in the glycerol model and that TNF-α is not involved in other mechanisms that may contribute to tubular necrosis. Recent data indicate that necroptosis may also be important in other models of AKI. Grenz et al. (5) showed that infliximab-treated or TNF-α-deficient mice had reduced tubular necrosis and preserved renal function after IRI. Linkermann et al. (16, 17) treated mice with necrostatin-1 and showed an efficient preservation of renal function as well as reduced tubular necrosis in two models of experimental AKI: IRI and cisplatin nephrotoxicity. Different cytokines, endotoxins, and damage-associate molecular patterns that interact with other receptors (e.g., Toll-like receptors) can also trigger the necroptotic pathway (6) and may be important in other AKI models.

Renal vasoconstriction is an important determinant of the glomerular filtration rate reduction after rhabdomyolysis (39). Several mechanisms may contribute to this hemodynamic response, such as tubuloglomerular feedback, renal oxidative stress, and the release of diverse vasoactive inflammatory mediators and cytokines in renal tissue, including TNF-α (30). TNF-α blockade efficiently reduced the tubular injury; thus, these complex mechanisms involved in renal vasoconstriction were affected, making it impossible to ascertain the contribution of TNF-α alone in this process. The similar pattern of renal protection observed in animals treated with necrostatin, infliximab, or both argued against a distinguished hemodynamic protective effect in rats treated with infliximab.

Despite the lack of caspase-8 activation, TNF-α blockade was able to reduce renal tubular apoptosis in our model. Previous studies (27, 32) have already described some interventions in vitro where caspase-8-independent TNF-α-mediated apoptosis was observed. Renal expressions of Bax, PUMA, and cleaved caspase-9 were increased in rats with rhabdomyolysis and were all significantly reduced by infliximab. Previously, we observed a very similar pattern of apoptosis mitigation in this model when the transcriptional factor p53 was blocked using a specific p53 inhibitor (pifithrin) (8). However, in the present study, TNF-α blockade did not reduce p-p53 overexpression, and, therefore, TNF-α played an independent regulatory effect in the transcription of Bax and PUMA proteins. This finding suggests that in rhabdomyolysis-induced AKI, both TNF-α and p53 must act together to encode proapoptotic members of the Bcl-2 family and trigger the
intrinsic pathway of apoptosis (Fig. 8). A similar finding has already been described in vitro, where sarcoma cells were more susceptible to the apoptotic effect of TNF-α/H9251 in the presence of active p53 (24).

In the heart, both initiator caspases were activated after rhabdomyolysis. cFLIP expression was not increased, and, therefore, caspase-8 activation was not blunted. The dual pathway activation in the heart led to a higher level of caspase-3 activation and TUNEL-positive cells in the heart compared with the kidney. TNF-α/H9251 blockade did not reduce caspase-8 activation but efficiently reduced the expression of cleaved caspase-9 and apoptosis. To further explore the role of caspase-8 activation in the heart, we treated rats with a caspase-8 inhibitor and observed that this intervention efficiently reduced the expression of cleaved caspase-9 and apoptosis. To explore the role of caspase-8 activation, we used a caspase-8 inhibitor and observed that this intervention efficiently reduced the expression of cleaved caspase-9 and apoptosis.

In conclusion, TNF-α/H9251 plays an important role in cardiorenal injury after rhabdomyolysis. TNF-α-mediated necroptosis is the main component of the acute tubular injury in the glycerol model. Our findings suggest that overexpression of cFLIP blunts caspase-8 activation in the kidney, which tips the signaling pathway triggered by TNF-α toward RIP1-mediated necroptosis. TNF-α also exerts a positive regulatory effect in the transduction of proapoptotic Bcl-2 members, such as Bax and PUMA, which increase the mitochondrial permeability, oxidative stress, and caspase-9-mediated apoptosis.

**GRANTS**

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

REFERENCES


Fig. 7. Role of the extrinsic apoptosis pathway. A and B: quantification of cleaved caspase-3-positive cells in the heart (A) and kidney (B) 6 h after glycerol injection. n = 5 rats/group. *P < 0.05 vs. other groups; #P < 0.05 vs. the control group.


Fig. 8. Mechanisms involved in TNF-α-mediated renal tubular injury in the glycerol model. Systemic and renal increases in the level of TNF-α were observed after rhabdomyolysis. The engagement of TNF-α to TNF-α receptor (TNFR) type 1 (TNFR1) in the tubular cell forms the signaling complex I in the membrane, which is sequentially internalized and converted to a death-inducing complex (complex II). This passage through complex I may participate in the activation of the transcriptional factor p-65 NF-κB. Activated p-65 NF-κB and p53 encode the caspase-8 antagonist cFLIP. cFLIP overexpression inhibits the activation of caspase-8, leaving its target RIP1 unrestrained. The persistent kinase activity of RIP1 [complexed with RIP3 and mixed lineage kinase domain-like protein (MLKL)] triggers the necroptosis pathway of cell death. Additionally, in a TNFR1-independent manner, TNF-α and p53 encode proapoptotic Bcl-2 members (Bax and PUMA) that trigger the caspase-9-mediated mitochondrial pathway of apoptosis. TRAF, TNFα-associated factor; cIAP, cellular inhibitor of apoptosis; TRADD, TNFR1-associated death domain.