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Tumor Necrosis Factor-α: Life and Death of Hepatocytes During Liver Ischemia/Reperfusion Injury

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ABSTRACT

Background: Tumor necrosis factor-α (TNF-α) is a potent proinflammatory cytokine involved in a variety of disease pathologies, including ischemia/reperfusion (I/R) injuries in transplantation. The interaction of TNF-α with its cognate receptor TNF receptor I (TNFRI) results in the activation of signal transduction pathways that regulate either cell survival or cell death. Hepatocytes express TNFRI and respond to TNF-α released by resident Kupffer cells as well as leukocytes that migrate to the liver during I/R injury. Upon binding TNF-α, the hepatocyte proliferates or undergoes apoptosis or necroptosis. The decision by the cell to commit to one path or the other is not understood. The damaged tissue exhibits cell death and hemorrhaging from the influx of immune mediators. TNF-α inhibitors ameliorate the injury in animal models, suggesting that lowering (but not eliminating) TNF-α levels shifts the balance of TNF-α toward its beneficial functions.

Methods: We review TNF-α signal transduction pathways and the role of TNF-α in liver I/R injury.

Conclusions: Because TNF-α plays an important role in hepatocyte proliferation, complete inhibition of TNF-α is not desirable in treating liver I/R injury. The strategy for developing pharmacological therapies may be the identification of specific intermediates in the TNF-α/TNFR1 signal transduction pathway and directed targeting of proapoptotic and pronecroptotic events.

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INTRODUCTION

Orthotopic liver transplantation is the only treatment option for end-stage liver disease. National 1-year and 5-year patient survival rates have risen to 89% and 80%, respectively.[1] While treatment plans often focus on the recipient, 2 donor events directly affect the recipient's outcome. First, the condition of the donor liver has an impact on the success of the transplant. As of October 2012, 16,832 recipients awaited livers, but only 3,917 deceased donor livers were available and transplanted.[2] Thus, with recipients outnumbering donors 4 to 1, surgeons are increasingly turning toward the use of live donors, split livers, and marginal livers. Marginal livers include livers from older donors, donors with significant fatty livers, and donors with anticipated long cold ischemia times. Second, the degree of I/R injury caused by cold preservation of the excised liver and warm reperfusion upon implantation has an equal and profound effect on the outcome. While the donor liver condition and I/R injury involve multiple physiological events, the inflammatory response is a key mediator in both liver damage and liver regeneration. This review examines the mechanism of action by the cytokine TNF-α in regulating both hepatocyte death and survival pathways. Pharmacological interventions to inhibit TNF-α in vivo, therefore, must take into account the beneficial functions of TNF-α.

The table lists the molecules discussed and assists the reader with the numerous abbreviations used in this review.

TNF-α: STRUCTURE AND FUNCTION

The immune system responds to liver injury and/or stress by activating resident Kupffer cells and recruiting an influx of leukocytes to release proinflammatory cytokines, chemokines, and other factors.

Table. Definitions of Abbreviations Used in the Text

Abbreviation	Term	Alternative Name(s)/Terms(s)
ADAM17	a disintegrin and metalloprotease domain 17	
ALT	alanine aminotransferase	
APAF-1	apoptotic protease activating factor-1	
ATP	adenosine triphosphate	
BAK	Bcl-2 homologous killer protein	
BAX	Bcl-2-associated X protein	
BID	BH3 interacting death domain	
c-FLIP	cellular FLICE-like inhibitory protein (FLICE is a FADD-like IL-1β-converting enzyme)	Caspase-8 and FADD-like apoptosis regulator (CFLAR), caspase homolog (CASH), caspase-8–related protein (Casper), caspase-like apoptosis regulatory protein (CLARP), FADD-like antiapoptotic molecule (FLAME), inhibitor of FLICE (I-FLICE), Mach-related inducer of toxicity (MRIT), or Usurpin
$c\text{-}FLIP_L$	full-length or long form of c-FLIP	
$c\text{-}FLIP_R$	Raji form of c-FLIP	
$c\text{-}FLIP_S$	short form of c-FLIP	
cIAP1/2	cellular inhibitor of apoptosis-1 and -2	
cyto c	cytochrome c	
DAMP/PAMP	damage-associated and pathogen-associated molecular pattern molecules	
DC	dendritic cell	
DD	death domain	
DR	death receptor	
ECM	extracellular matrix	
ERK1/2	extracellular signal-related kinase-1 and -2	p44/42; MAPK
FADD	Fas-associated death domain	
IFNγ	interferon-γ	
IκB	inhibitor of κB	
IKK	IκB kinase	Subunits: IKKα, IKKβ, IKKγ
IL-1β	interleukin-1 beta	
IL-2	interleukin-2	
IL-6	interleukin-6	
IL-13	interleukin-13	
I/R	ischemia/reperfusion	
ITCH	itchy homolog	
JNK	c-Jun N-terminal kinase	MAPK
kDa	kiloDalton	
Lck	lymphocyte-specific protein tyrosine kinase	
MAPK	mitogen-activated protein kinase	
MAPKK	mitogen-activated protein kinase kinase	
MAPKKK	mitogen-activated protein kinase kinase kinase	
MIP-2	mouse macrophage inflammatory protein-2	CXCL2
MMP	matrix metalloproteinase	
mRNA	messenger RNA	
NEMO	NF-κB essential modulator	IKK-γ
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells	Family members: RelA, RelB, c-Rel, p50 (NF-κB1), p52 (NF-κB2)
NK	natural killer cells	
NKT	natural killer T cells	
PI	phosphoinositide	
RANTES	regulated upon activation, normal T cell expressed and presumably secreted	CCL5

Table. Continued.

Abbreviation	Term	Alternative Name(s)/Terms(s)
RIP1	receptor-interacting protein-1	
RIP3	receptor-interacting protein-3	
ROS	reactive oxygen species	
shRNA	small hairpin RNA	
SMAC	second mitochondrial-derived activator of caspases	direct IAP binding protein with low pI (DIABLO)
SODD	silencer of death domain	
sTNF-α	soluble TNF-α	
TACE	TNF-α-converting enzyme	a disintegrin and metalloprotease domain 17 (ADAM17)
TAK1	transforming growth factor-β–activated kinase-1	
tBID	truncated BID	
TCR	T cell receptor	
TIMP-3	tissue inhibitor of metalloproteinases-3	
TLR	toll-like receptor	
TNF-α	tumor necrosis factor-α	TNF-A, TNF superfamily member 1A (TNFSF1A), necrosin, macrophage cytotoxic factor (MCF), differentiation inducing factor (DIF), and cachectin
TNFR1	TNF-α receptor 1	CD120a; p55/p60
TNFR2	TNF-α receptor 2	CD120b; p75/p80
TRADD	TNFR1-associated death domain protein	
TRAF2/5	TNF receptor-associated factor-2 and -5	
XIAP	X-linked inhibitor of apoptosis protein	inhibitor of apoptosis protein-3 (IAP3)

Numerous immune proteins are involved; however, TNF-α is implicated as the primary mediator of inflammation during I/R injury in several tissues, including the lung, heart, liver, eye, kidney, and brain. TNF-α is a potent proinflammatory cytokine that targets various cell types through receptor-mediated signal transduction pathways. TNF-α is encoded by a 1,686-ribonucleotide mRNA that is translated into a 26 kDa, nonglycosylated, membrane-bound, precursor protein, mTNF-α.[3-6] The mTNF-α monomer is assembled at the cell surface as a homotrimer known as proTNF-α (Figure 1). Active, soluble TNF-α is generated by the enzyme activity of TACE/ADAM17 between Ala-76 and Val-77 of mTNF-α.[7,8] TACE/ADAM17 cleavage of mTNF-α releases the 51 kDa trimeric TNF-α (or sTNF-α), which contains three 17 kDa monomers. TACE/ADAM17 is a member of the zinc-dependent MMP family that degrades ECM.[9-11] MMPs have been implicated in the pathology of several diseases, including arthritis and cancer. Approximately 26 MMPs have been identified to date.[12]

Many activated immune cells express TNF-α, including neutrophils, B lymphocytes, CD4+ T lymphocytes, NK cells, NKT cells, and cells of the monocyte lineage. Resident macrophages—astroglia, microglia, Langerhans cells, Kupffer cells, and alveo-

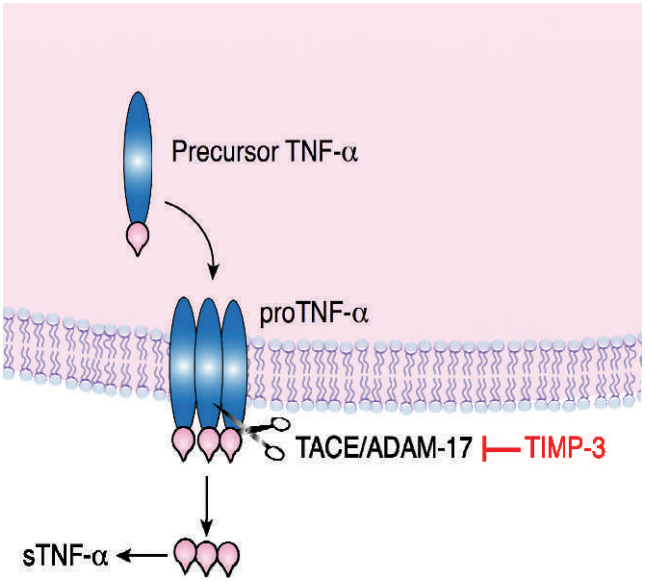

Figure 1. TNF-α is modified posttranslationally. TNF-α is expressed as a monomeric precursor protein of 233 amino acids, 26 kDa. The membrane-bound form (proTNF-α; mTNF-α) is a trimer. Cleavage between Ala-76 and Val-77 of proTNF-α by TACE/ADAM17 releases the active, soluble 51 kDa form that contains three 17 kDa monomers. TIMP-3 inhibits TACE/ADAM17, preventing the release of sTNF-α.

lar macrophages—are primary producers of TNF-α.[13] Binding TNF-α to one of its receptors, either TNFR1 or TNFR2, activates signal transduction pathways.[14-18] TNFR1 is expressed constitutively on the surface of all cell types as a trimer of 55 kDa subunits, and TNFR2 is expressed in activated immune cells as a trimer of 75 kDa subunits. Although TNF-α binds either receptor, TNF-α mediates its effects primarily through its interactions with TNFR1.[19]

TNF-α ACTIVATES PROGRAMMED DEATH PATHWAYS

TNFR1 is a membrane-bound protein that contains a DD in its cytoplasmic tail that is associated with the 60 kDa SODD protein. Soluble TNF-α binds TNFR1, resulting in the trimerization of TNFR1 and the release of SODD (Figure 2).[20,21] TRADD binds the trimeric DD of TNFR1, which recruits RIP1, TRAF2/5, and cIAP1/2 to form Complex 1.[22] Endocytosis of Complex 1 leads to the degradation of cIAP1/2 and the formation of proapoptotic Complex 2a or the dissociation of Complex 1 and the formation of pronecroptotic Complex 2b. Thus, Complex 2a leads to apoptosis (programmed cell death), and Complex 2b results in necroptosis (programmed necrosis) of the hepatocyte. The transition between Complex 1 and Complex 2a/2b has yet to be elucidated. Complex 2a consists of TRADD, RIP1, TRAF2/5, FADD, and procaspase-8 and -10. As zymogens, procaspases are inactive forms of the caspases (cysteine-aspartic proteases or cysteine-dependent aspartate-directed proteases) that consist of 2 groups: upstream initiator (apical) caspases and downstream effector (executioner) caspases.[23] Initiator caspase-2/8/9/10 cleaves and activates the effector caspase-3/6/7. From Complex 2, procaspase-8 and -10 are converted to caspase-8 and -10, which initiate apoptosis through caspase-3, -6, and -7 and the mitochondria death pathway. Caspase-8 and -10 cleave BID into the 15 kDa tBID.[24-26] tBID activates BAX and BAK to reassemble into heterodimeric pore units in the mitochondrial membrane, resulting in the release of cytochrome c and SMAC.[26-29] Cytochrome c activates caspase-9 either directly or through APAF-1 in an ATP-dependent manner, resulting in the binding of APAF-1 to caspase-9, a complex known as the apoptosome.[30-33] Whether APAF-1 is required for cytochrome c–dependent activation of caspase-9 is unclear. SMAC binds and blocks XIAP from binding to caspases.[34,35] Caspase-9 activates executioner caspase-3 and -7, leading to apoptosis.[36,37]

Studies in the past 3 years have shown that necrosis can occur in a genetically encoded, regulated manner similar to apoptosis, known as necroptosis or programmed necrosis.[38] Unlike apoptosis, necroptosis does not require caspases but, rather, the kinases RIP1 and RIP3. The formation of Complex 2b, or the necrosome, during TNF-α signaling has only recently been described.[39] RIP1 is found in Complexes 2a and 2b, and an antiapoptotic domain within RIP1 may control whether RIP1 participates in apoptosis or necroptosis.[40] The association of RIP3 with RIP1 leads to phosphorylation of both kinases; however, the identity of the activating kinases is not known. He et al[39] suggest that RIP3 undergoes autophosphorylation, while Cho et al[41] suggest that an as-yet-to-be-determined kinase phosphorylates RIP3. It is not clear which kinase phosphorylates RIP1. Necroptosis has been identified as a mechanism of cell death in renal, cardiac, and retinal I/R injuries.[42-45] We have shown that rat livers undergoing I/R injury are characterized by massive necrosis that may be caused by TNF-α–mediated necroptosis.[46-48] Characterization of the processes involved to activate necroptosis during liver I/R injury has not yet been undertaken.

TNF-α ACTIVATES CELL SURVIVAL AND PROLIFERATION

Engagement of sTNF-α with TNFR1 activates cell survival and proliferation pathways if Complex 1 is retained on the cell membrane. Complex 1 leads to either of 2 signal transduction pathways: the canonical (classical) NF-κB pathway or the MAPK pathway (Figure 3). The polyubiquitination of RIP1 and TRAF2/5 by cIAP1/2 results in the recruitment of NEMO to the complex.[49-52] NEMO is associated with TAK1, a member of the MAPKKK family. TAK1 activates IKK, which phosphorylates IκB.[53] IκB becomes polyubiquitinated, releasing NF-κB, and IκB is targeted to the proteasome for degradation. NF-κB translocates to the nucleus and activates transcription of genes that regulate cell survival and proliferation. The canonical NF-κB pathway has been well studied, and Hayden and Ghosh[54] have provided a recent overview of the progress made in NF-κB research.

Alternatively, Complex 1 leads to the activation of the MAPK signal transduction pathway through TRADD and TRAF2/5. TRAF2/5 oligomerizes, resulting in the binding of TAK1 (a MAPKKK) to TRAF2/5.[55,56] Activated MAPKKK follows the classical MAPK phosphorylation cascade by activating a MAPKK that, in turn, phosphorylates the 3 terminal MAPKs: p38 MAPK, JNK, and ERK1/2. Phosphorylated MAPKs translocate into the nucleus to activate transcription factors. The MAPK pathway has been the focus of intense efforts in designing and applying pharmacological inhibitors in vivo and in vitro with some inhibitors advancing to clinical trials for a variety of pathologies, including inflammatory diseases and

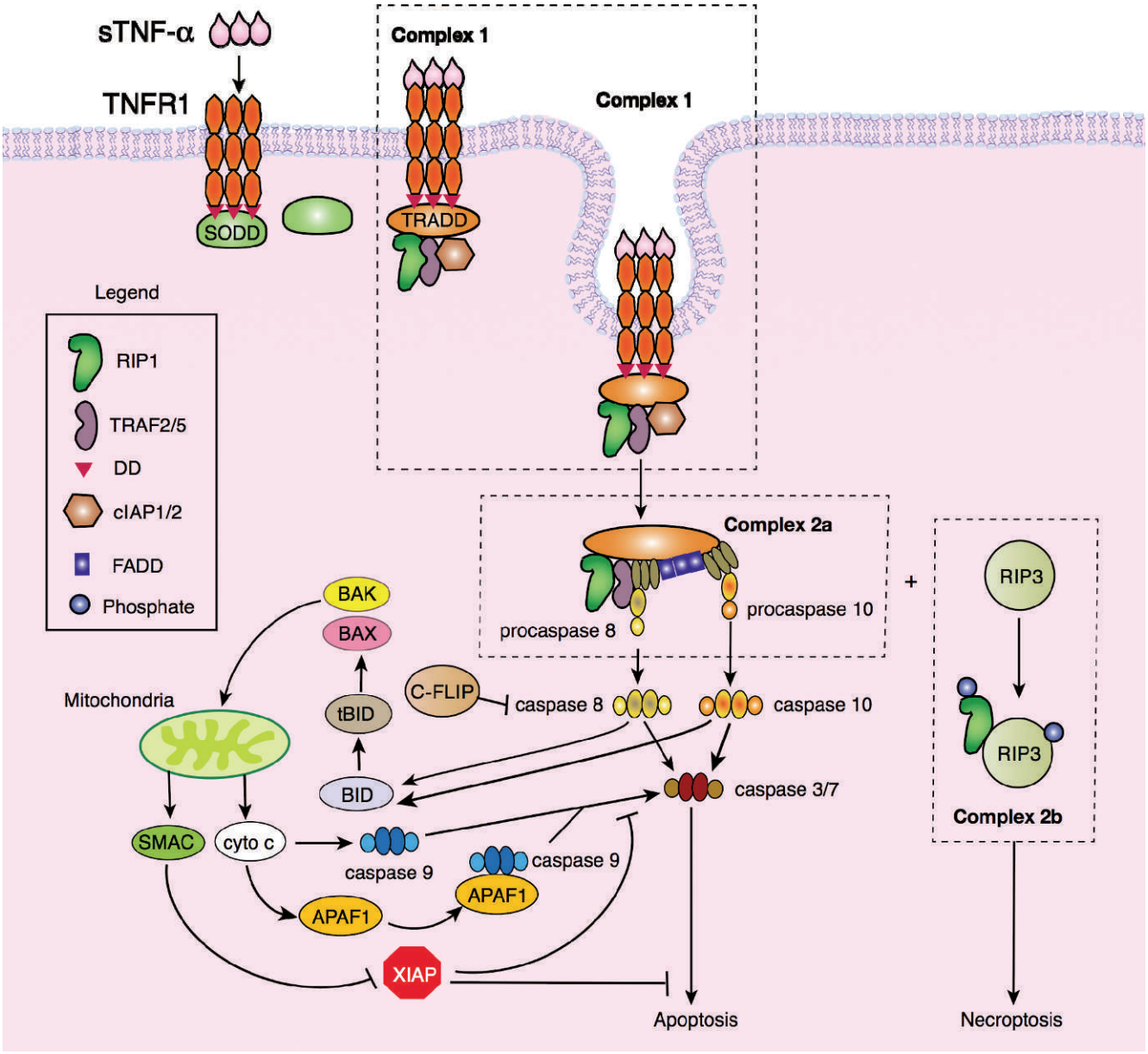

Figure 2. Signal transduction by TNF-α leads to apoptosis or necroptosis. Invagination of Complex 1 results in the formation of Complex 2a (apoptosome) or 2b (necroptosome). Soluble TNF-α binds to its cognate receptor, TNFR1, which is bound to SODD via the TNFR1 DD. Binding of TNF-α to TNFR1 releases SODD, enabling the binding of TRADD, followed by the assembly of RIP1, TRAF2/5, and cIAP1/2. Endocytosis of Complex 1 leads to the degradation of cIAP1/2 and the formation of Complex 2a, which consists of TRADD, TRAF2/5, RIP1, FADD, and the zymogens procaspase-8 and -10. Procaspase-8 and -10 are cleaved, and caspase-8 and -10 cleave BID into tBID, activating the mitochondrial death pathway. Caspase-8 and -10 also activate caspase-3, -6, and -7, leading to apoptosis.

cancer.[57] As with NF-κB, MAPK signaling pathways are an active area of research, and excellent reviews have been published that provide the recent progress in MAPK studies.[58]

Although genes that upregulate cell division are transcribed through NF-κB and MAPK signal transduction, antiapoptotic genes are also expressed. NF-κB induces the expression of c-FLIP.[59] Three isoforms of FLIP have been identified: c-FLIP$_L$, c-FLIP$_S$, and c-FLIP$_R$. All three regulate caspase-8 activation and DR-induced apoptosis. However, c-FLIP consists of FLIP$_S$ and FLIP$_L$ in the literature.[60-64] Recent data indicate that c-FLIP has pro- and antiapoptotic functions and is regulated by its intracellular stoichiometry.[65] Low, moderate, or no levels of c-FLIP mediate apoptosis, while high levels of c-FLIP may stimulate prolifera-

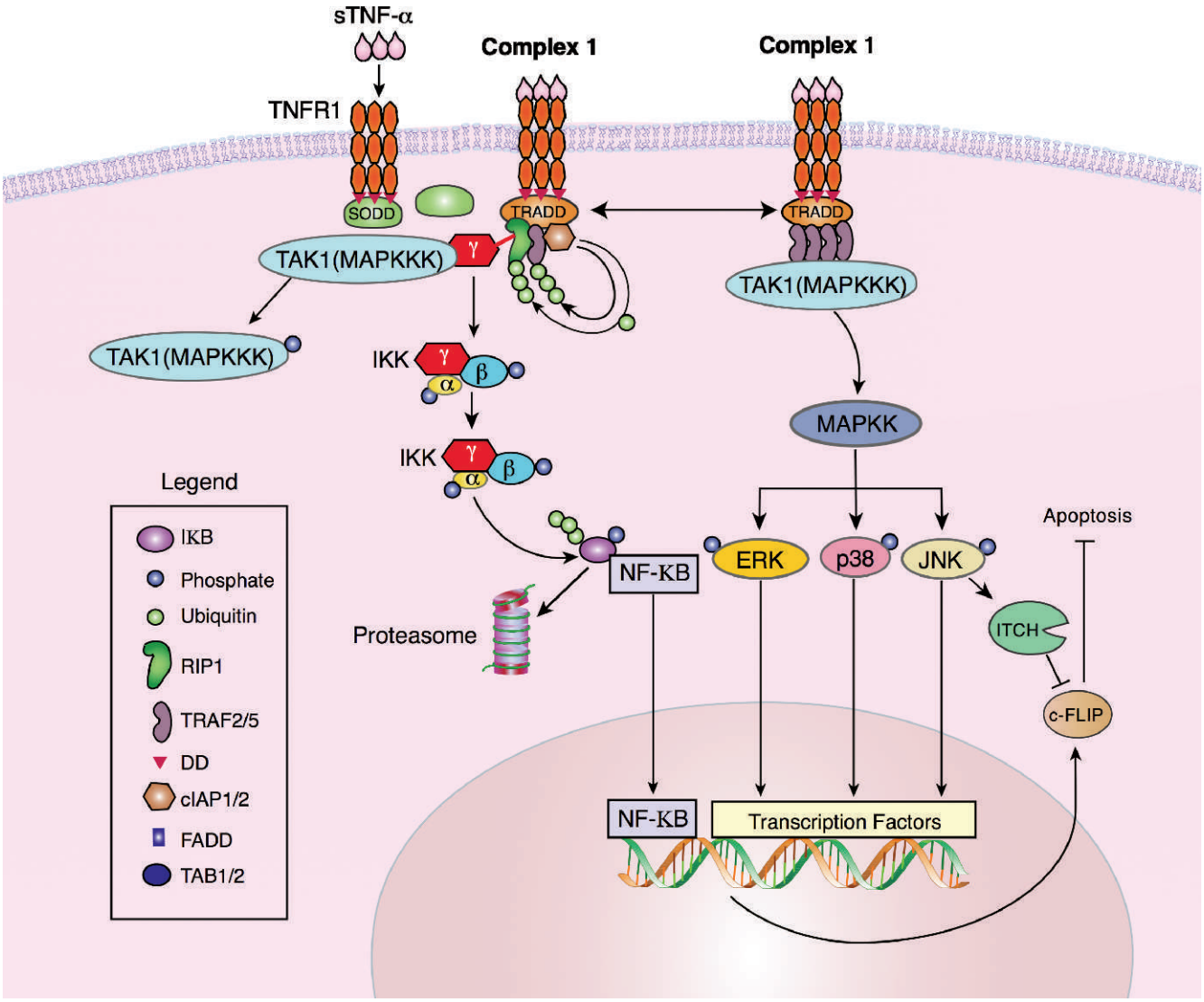

Figure 3. Signal transduction by TNF-α leads to cell survival and proliferation. Retention of Complex 1 on the cell surface commits the cell to proliferate via 2 signal transduction pathways: NF-κB or MAPK. Polyubiquitination of RIP1 releases NEMO or IKK and recruits both TAK1 (MAPKKK) and IKK. Active TAK1 phosphorylates the IKK complex (IKK-α, -β, and -γ), which phosphorylates IκB. Phospho-IκB is ubiquitinated, released from the transcription factor NF-κB, and targeted for degradation by the proteasome. NF-κB translocates to the nucleus to activate transcription. Binding of TNF-α to TNFR1 may also form another version of Complex 1 that consists of oligomers of TRAF2/5 bound to TNFR1. TRAF2/5 recruits TAK1 to the complex. TAK1, a MAPKKK, phosphorylates MAPKK, which in turn phosphorylates the terminal MAPKs: p38 MAPK, JNK, and ERK1/2. Phospho-JNK and phospho-ERK1/2 translocate to the nucleus to activate transcription.

tion.[62,66,67] Interestingly, JNK phosphorylates the E3 ubiquitin ligase ITCH, which ubiquitinates c-FLIP to induce c-FLIP degradation, leading to apoptosis.[68] Thus, JNK antagonizes NF-κB during TNF-α–mediated Complex 1 signal transduction.

TNF-α AND THE IMMUNE RESPONSE IN HEPATIC I/R INJURY

Hepatic I/R injury occurs in numerous clinical settings, including but not limited to liver hemorrhage and shock, surgical resection, and transplantation. Although the pathophysiology of I/R injury involves multiple pathways, inflammatory cells and soluble factors are key mediators. Two general immune mechanisms have been identified during liver transplantation. The lack of ATP production because of glycogen consumption and oxygen depletion triggers the surface expression of DAMP/PAMP during ischemia (Figure 4). Kupffer cells and DCs express TLRs that bind the endogenous DAMPs/PAMPs expressed by the ischemic cells in the liver.[69-72] The Kupffer cells and DCs become activated and respond with a

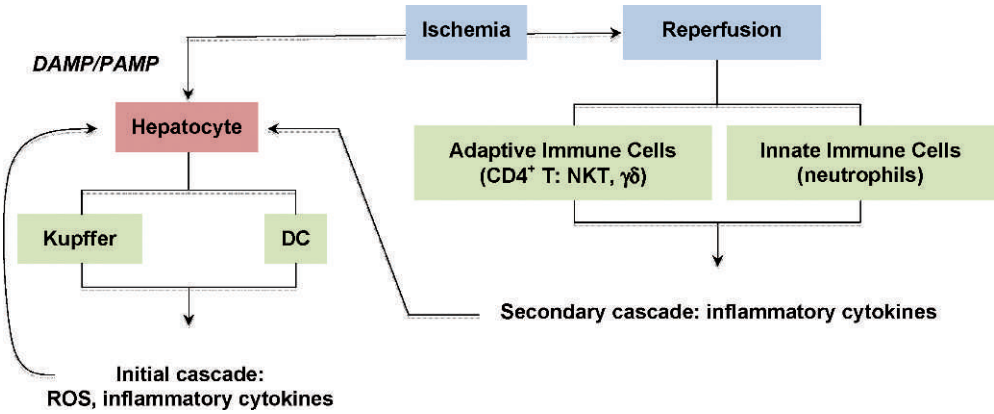

Figure 4. I/R injury to hepatocytes is mediated by immune cells. The initial immune cascade is caused by the expression of DAMP/PAMP on the surfaces of cells, including hepatocytes. The resident macrophages, Kupffer cells, and dendritic cells express TLRs that bind DAMPs/PAMPs and activate the immune cells, releasing ROS, cytokines, and chemokines. Reperfusion activates adaptive immune cells (CD4+ T cells, γδ T cells, and NKT cells) and innate immune cells (primarily neutrophils) that are recruited to the site of tissue injury. The cells release a second cascade of mediators. TNF-α is produced in both the initial and secondary cascades. Hepatocytes express TNFRI in response to the massive influx of TNF-α.

classic inflammatory reaction cascade, producing ROS and proinflammatory cytokines such as TNF-α.[73-77]

A second immune-mediated response occurs during the reperfusion phase. The initial inflammatory response during ischemia leads to the recruitment of leukocytes, particularly neutrophils and CD4+ T cells. These cells activate and secrete a secondary wave of cytokines and chemokines, amplifying the immune reaction at the site of I/R injury.[78-83] T cell–deficient mice have reduced I/R injury, and systemic treatment with immunosuppressive drugs attenuates I/R injury in various organs, suggesting that decreasing T cell function is beneficial to organ survival.[84-86] The activated T cells constitutively express their surface stimulatory molecules CD28 and CD154, which are recognized by B7 and CD40, respectively, on antigen-presenting cells during I/R injury.[80,87-90] The costimulation of the CD4+ T cell's CD28 and CD154 leads to the phosphorylation of CD28 by Lck and the activation of the PI signaling pathway. The phosphorylation events initiating from these kinases, as well as signal transduction events from the antigen-TCR complex and cytokine/cytokine receptor complexes, result in gene transcription, including TNF-α, which leads to additional T cell proliferation and cytokine and chemokine production, further damaging the tissue.

Platelets also express CD40 that binds to the T cell's CD154 receptor and mediates tissue damage following I/R injury.[91-93] The infiltration of NKT cells into renal and hepatic I/R injured tissue recruits neutrophils, and activated NKTs produce various cytokines, including IFN-γ, IL-2, IL-13, and TNF-α.[94-97] NKT cells are found in high quantities in the liver, and the production of TNF-α by NKT cells is yet another level of redundancy by the immune system in response to I/R injury. CD8+ T cells have been implicated in renal and intestinal I/R injury. Mice that underwent renal I/R exhibited increased IL-1β, IL-6, TNF-α, IFN-γ, MIP-2, and RANTES expression.[98,99] CD8-deficient mice showed lower cytokine expression levels, but kidney histology was unchanged after I/R induction, suggesting a chronic effect of CD8+ T cell infiltration.[99] Another type of inflammatory cells, mast cells, has not been shown to be involved in I/R injury.[100] Thus, multiple immune system events generate proinflammatory cytokines that initiate and amplify the responses that lead to tissue injury.

PHARMACOLOGICAL TARGETS OF TNF-α-MEDIATED SIGNAL TRANSDUCTION IN HEPATIC I/R INJURY

The dual roles of TNF-α present a conundrum when using inhibitors against TNF-α. Complete knockout of the TNF-α, TNFR1, or TIMP-3 gene in mice results in the inability of the liver to regenerate after tissue damage.[101-103] In the case of TIMP-3 knockout in mice, the deregulation of TACE/ADAM17 leads to sustained production of soluble TNF-α, which leads to increased inflammation and increased cell death.[102] Thus, TIMP-3 is critical to maintaining the homeostasis of the liver by regulating TNF-α release. Monoclonal antibodies (etanercept, infliximab, adalimumab, golimumab, and certolizumab pegol) against

TNF-α have been approved for inflammatory diseases, including rheumatoid arthritis, psoriatic arthritis, juvenile idiopathic arthritis, psoriasis, ankylosing spondylitis, and inflammatory bowel disease.[104,105] Applications of TNF-α monoclonal antibodies in liver I/R studies appear to attenuate tissue injury.[75,106,107] We used recombinant TIMP-3 in a rat I/R model and showed that liver damage is dramatically decreased in TIMP-3–treated animals prior to I/R induction as compared to untreated animals.[46-48] Hernandez et al[108] recently generated shRNAs to silence the TNF-α gene in a mouse liver I/R model. Although the data showed a correlation between decreased liver injury and shRNA pretreatment, the ALT levels in shRNA-treated mice were higher than in the control group, suggesting that tissue damage was still occurring. We suggest that this result may be caused by the lack of TNF-α for cell survival signal transduction that is required for liver regeneration.

Some researchers have targeted TNF-α's proliferative effects by using specific inhibitors of downstream signal transduction proteins. The rationale of these efforts is to diminish or inhibit immune cell proliferation. A review of the literature indicates that MAPK inhibitors, especially those targeting p38 MAPK, provide insights into the contrasting roles of MAPK in I/R injury. MAPK induces gene expression that leads to cell proliferation, and data in I/R injury studies indicate that upregulation of immune cell proliferation is a direct result. Studies assessing the effect of MAPK induction with small molecules in I/R injury are well documented in the retina, heart, kidney, lung, brain, and liver and show mixed results. In myocardial I/R injury, p38 MAPK aggravates lethal injury but can also protect the heart under certain circumstances, although this theory remains controversial.[109-117]

In liver I/R injury, p38 MAPK inhibitors appear to attenuate tissue damage in animals.[118-120] However, these studies have not determined the mechanism of action by the inhibitor, whether immune cells and/or hepatocytes are targeted. Nilotinib, a second-generation receptor tyrosine kinase inhibitor, protects against liver I/R injury in the mouse by reducing p38 MAPK in liver nonparenchymal cells and reducing JNK activation in hepatocytes.[121] Nilotinib did not inhibit p38 MAPK in bulk liver and may be selective for nonparenchymal cells that are involved in TLR signaling.[70] Interestingly, nilotinib did not inhibit its known receptor tyrosine kinases and may be exerting its effects through another pathway.

CONCLUSIONS

TNF-α's opposing functions—survival versus death—present a challenge in understanding the mechanism of I/R injury and designing treatments to prevent tissue damage, particularly during liver transplantation. The regenerative capability of the liver must be retained, and TNF-α plays an important role in hepatocyte proliferation. Thus, complete inhibition of TNF-α is not desirable in treating liver I/R injury. Identification of specific intermediates in the TNF-α/TNFR1 signal transduction pathway and directed targeting of proapoptotic and pronecroptotic events may be the strategy for developing pharmacological therapies in liver I/R injury.

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