Liver Cell Adaptation: Death versus Survival

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Cellular Adaptation

• Hepatocytes in their microenvironment are in a steady state or “homeostasis”
• Changes in homeostasis due to chemicals, toxins, injury, signals, can cause irreversible damage leading to cell death
• The elimination of damaged or infected cells is critical to the normal development and homeostasis of the organ and the multicellular organism
• The liver can regenerate and replace damaged hepatocytes thereby restoring homeostasis
Cellular Adaptation

Irreversible Damage
Death Signal

Failure to respond (surpass a threshold) to stressor/cytokine or toxin/hazard

Cell Death
Failure to Adapt

Reversible Injury
Death Signal

Modulating cell function, gene expression, stress responses

Adaptation and Survival
The liver microenvironment has evolved a very particular innate immune system, which is characterized by tolerance toward a vast array of self and non-self antigens. The liver enjoys a state of privilege and participates in the induction of peripheral immune-tolerance. If immune tolerance is compromised, hepatocytes are targeted by immune cells.
Death Receptors

• Death receptors are ubiquitously expressed in the liver
  – Fas (also known as CD95)
  – TNF-α receptor 1 (TNF-R1)
  – Death receptor 4 and 5 (DR4 and DR5, also known as TRAIL-R1 and TRAIL-R2, respectively)
• Their ligands (FasL/CD95L, TNF-α, and TRAIL) are mainly expressed by cells of the immune system
• Apoptosis in the liver is the predominant model of cell death and executed by death receptors (this is highly context specific)
• Since IDILI is adaptive immune mediated the resultant hepatotoxicity likely involves death receptors and the mode of cell death is apoptosis

TNF signaling survival vs death

Complex IIa
- TRADD
- FADD
- Casp8
- FlipL

Complex IIb
- RIPK1
- RIPK3
- FADD
- Casp8
- FlipL

Activation of NFkB Cellular Survival

- Multifaceted regulator of cell survival and function (transcription factor)
- When released from IkB to nucleus and binds to IkB elements turning on genes that control inflammation, proliferation and survival (dampening of pro-apoptotic signals)
- P65/RelA -/- mice and IKKβ -deficient mice die at e15 (hepatocyte apoptosis)
- Anti apoptotic and pro-survival effectors:
  - cIAP1, cIAP2, XIAP, cFLIP, Bcl-XL, A1/Bfl-1

Downstream signaling
Survival/Apoptosis/Necroptosis and Inflammation

Pasparkis, Nature. 2015 Jan 15;517(7534):311-20
Necroptosis

- “Programmed Cell Death” (-optosis), morphologically similar to necrosis
- Evolved as an alternative death pathway when apoptosis was inhibited (in the presence of caspase inhibition)
- Loss of caspase 8 (or FADD) is embryonic lethal due to unleashing of necroptosis (endothelial cell death of yolk sac vasculature)
- Occurs in certain cell types: L929 mouse fibroblasts, MEF, Jurkat T cells, macrophages
- In some cell lines in addition to a caspase inhibitor (e.g., ZVAD-fmk), IAP antagonists are needed to induce necroptosis
- All cells that undergo necroptosis express RIPK3 and MLKL

Caspase 8 inhibition is necessary for necroptosis

Necroptosis is a form of programmed cell death carried out by receptor interacting protein kinase RIPK1, RIPK3, and the pseudokinase mixed lineage kinases domain-like (MLKL)

Newton, Annu. Rev. Biochem. 2016. 85:4.1–4.21
TNF driven necroptosis

- The ubiquitination status of RIPK1 influences cell survival
- Necrostatins block the kinase activity of RIPK1 (required for necroptosis) in vivo models
- RIPK1-/- is embryonic lethal
- RIPK3-/- and MLKL-/- are viable without any phenotype
- RIPK3 inhibitors do not block death, rather switch mode of death to apoptosis

Does Necroptosis Occur in DILI?

• APAP DILI is a form of necrotic liver cell death
• It is regulated, involves signaling (ASK1, MLK3, JNK, Sab, MPT)
• Necrostatin (RIPK1 kinase inhibitor) has been shown to protect against APAP by multiple labs
• RIPK3 activation and MLKL membrane translocation define necroptosis and RIPK3-/- and MLKL-/- mice do NOT undergo necroptosis
• So we decided to test APAP toxicity in RIPK1 knockdown and RIPK3-/- and MLKL-/- mice

RIPK1 knockdown protects against APAP

RIPK3-/- and MLKL-/- mice are NOT protected from APAP

![Graph showing serum ALT levels for WT, RIPK3-/-, and MLKL-/- mice at 6hs and 24hrs.](image)

RIPK1 KD protects RIPK3-/- mice

Is RIPK3 expressed in the liver?
Non specific antisera
RIPK3 in APAP Liver

RIPK3 KO APAP ABGENT AB
WT APAP ABGENT AB

RIPK3 KO APAP PROSCI AB
WT APAP PROSCI AB

RIPK3 KO APAP RABBIT IGG
WT APAP RABBIT IGG

RIPK3 Ab Prosci Whole liver

RIPK3 Ab Abgent Whole liver

RIPK3 Ab Abgent Whole liver

RIPK3 Ab Abgent Whole liver

RIPK3 Ab Abgent Whole liver

55 KD

Non specific antisera
RIPK3 in APAP Liver

RIPK3 KO APAP ABGENT AB
WT APAP ABGENT AB

RIPK3 KO APAP PROSCI AB
WT APAP PROSCI AB

RIPK3 KO APAP RABBIT IGG
WT APAP RABBIT IGG

RIPK3 Ab Prosci Whole liver

RIPK3 Ab Abgent Whole liver

RIPK3 Ab Abgent Whole liver

RIPK3 Ab Abgent Whole liver
We tested 9 commercially available antisera

<table>
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<tr>
<th>Antibody</th>
<th>Origin</th>
<th>Reacts</th>
<th>Application</th>
<th>Target</th>
<th>Recognizes +</th>
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<td>1. Abcam # AB6233</td>
<td>Rabbit anti-M</td>
<td>M,R</td>
<td>WB, IF, IHC</td>
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<td>2. Abgent #AP7819b</td>
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<td>3. Abgent #AP7184b</td>
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<td>5. Prosci #2283</td>
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</table>
RIPK3 protein is present in total liver lysate but NOT increased with APAP using monoclonal Ab

No RIPK3 protein in PMH even after APAP

Abbreviations:
MEF: Mouse embryonic fibroblasts
PMH: Primary mouse hepatocytes
KC: Kupffer cells
LSEC: Liver sinusoidal endothelial cells
WBC: White blood cells
WT: Wild type

PMH express MLKL

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Conclusions

• Non-parenchymal cells maintain a tolerogenic microenvironment in the liver
• Hepatocytes triggered by death receptors can either die of apoptosis or adapt and survive (NFkB)
• Acute DILI from APAP is a form of regulated necrosis involving RIPK1 (and JNK-data not shown) leading to MPT, but not necroptosis since MLKL-/- and RIPK3-/- are not protected
• Mouse hepatocytes do not express RIPK3 and do not undergo necroptosis during acute APAP DILI
Unresolved issues

- Is RIPK3 induced in hepatocytes in IDILI or other liver diseases?
- Are there conditions in which intrinsic stress activates RIPK3 and/or MLKL?
- In the absence of a caspase inhibitor does necroptosis occur in vivo?
- Since NPCs express RIPK3 and MLKL is necroptosis important in certain situations (LSEC death, IDILI, NPC death)?
- What is the function of hepatocyte MLKL and can it be activated without RIPK3?
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