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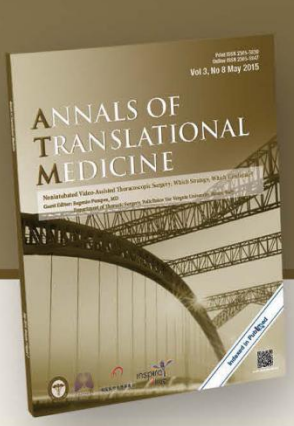

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Peer Review File

Article information: http://dx.doi.org/10.21037/atm-20-5162

Reviewer Comments

The manuscript ATM-20-5162-MS focuses on a very pertinent, actual and controversial topic, which fully justifies the possibility of its publication.

However, the way it was constructed and written, reveals a biased view, which is not well supported by the most current literature. In fact, the citation and discussion of several key manuscripts in the field is missing.

Some concerns:
The authors claim that necroptosis could occur when caspases are inhibited. But is this true, how the authors explain that necroptosis is activated in disease (as they state)? One conceivable explanation is that, under pathological conditions, augmented levels of RIPK3 or MLKL switch apoptosis to necroptosis in cells with low expression of these key necroptotic proteins. Particularly, RIPk3 is weakly expressed in adult human and murine tissues such as adult liver, brain, pancreas, heart and kidney under physiological conditions (Belizario et al., 2015; He et al., 2009), but its expression is increased after injury, correlating with human and experimental data supporting pathological activation of necroptosis. Indeed, several papers showed RIPk3 is weakly expressed in hepatic cells of healthy controls but is increased in livers from patients with chronic liver diseases, including NAFLD, alcoholic steatohepatitis, chronic viral hepatitis and PBC. Likewise, RIPK3 has been found induced in the liver of animal models of liver injury (Gautheron et al., 2014; Afonso et al 2015; Afonso et al 2016; Afonso et al 2018; Roychowdhury et al., 2013, Roychowdhury et al., 2016). Supporting the concept that an increase in RIPK3 levels causes a disturbance in cell homeostasis favoring necroptosis, the expression of RIP3 in different cell lines correlates with their responsiveness to induction of necrosis (He et al., 2009), while in vitro functional studies have shown that increased RIPK3 (Moujalled et al., 2013; Zhang et al., 2009a) or overexpression of a RIPK3 phospo-mimetic mutant (McQuade et al., 2013) can trigger necroptosis and bypass the requirement for RIPK1. Conversely, RIPK3 expression is often epigenetically silenced in cancer cells causing resistance to chemotherapy-induced necroptotic cell death, whereas treatment of these cells with hypomethylating agents restores RIPK3 expression and sensitivity to chemotherapeutics (Koo et al., 2015). Therefore, it is likely signals trigger up-regulation of RIPK3 during hepatic injury, promoting enhanced susceptibility to necroptosis.

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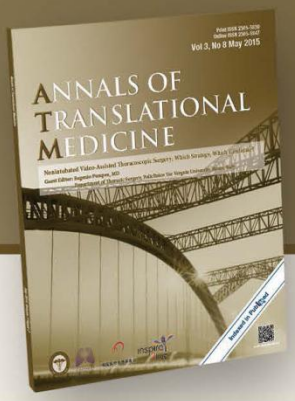

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So all the rhetoric that says that necroptosis is improbable in the liver because the expression of mediators is low does not take this into account.
Reply 1: Thank you for your helpful comments and we completely agreed with you. The revised content is as follows: We conclude that in order for primary hepatocytes to execute necroptosis, an additional sensitization step is required. It is possible that under pathological conditions, augmented levels of RIPK3 or MLKL switch apoptosis to necroptosis in cells with low expression of these key necroptotic proteins.
Changes in the test: page 9, line 2-5.

The authors should explain better the pathways that lead to necroptotic cell death, including RIPK1-independent necroptosis.
Reply 2: Thank you for your valuable suggestions. We have added the RIPK1-independent necroptosis signaling pathway as advised. The revised content is as follows: Despite the critical role of RIP1 in necroptosis, and researches suggest that there are RIP1-independent necroptosis pathways. It has been shown that virus-induced necroptosis depends on the DNA-dependent activator interferon-regulatory factor (DAI) and RIP3, independent of RIP1 function (26). Furthermore, interferons (IFNs) can also trigger necroptosis independent of RIP1 via the interferon-induced protein kinase PKR (27). In addition, RIP1 kinase does not contribute to TRIF-dependent necroptosis in some kind of cell types apart from macrophages (26).
Changes in the test: page 5, line 10-17.

The authors state “RIPK3 staining of human liver cells leads to morphologically discrepant images, which raises substantial doubts about the specificity of the antibody for immunohistochemical analysis. This partly explains the many controversies regarding necroptosis in human liver diseases”. On what data do the authors strive to say this? IHC of p-MLKL in human tissue was already performed (Afonso et al 2016; Wang et al., 2014). Further there is other strategies to detect necroptosis in the liver that were not mentioned including necrosome assembly and RIP3-MLKL interactions through co-immunoprecipitation experiments (Cho et al., 2009; Koo et al., 2015) or microscopically using the DuoLink® proximity ligation assay (Roychowdhury et al., 2013). The aggregation status of RIP3 and MLKL can also be monitored by evaluating their retention in insoluble/soluble protein fractions (Ofengeim et al., 2015, Afonso et al 2015, Afonso et al 2016) or by co-staining of thioflavin T, a β-amyloid fibril marker, with RIP3 puncta (Li et al., 2012).
Reply 3: Thank you for your helpful comments. And we have deleted the corresponding statement and added necroptosis detection strategies and corresponding citation as advised to make it more precise.

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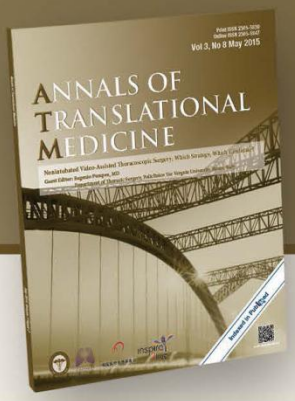

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The revised content is as follows: Nevertheless, there are other possible methods to detect necroptosis. For instance, necrosome assembly and RIP3-MLKL have been shown to interact through co-immunoprecipitation experiments or microscopically using the DuoLink® proximity ligation assay (37-39). The aggregation status of RIP3 and MLKL can also be monitored by evaluating their retention in insoluble/soluble protein fractions, or by co-staining of thioflavin T, a β-amyloid fibril marker, with RIP3 puncta (40-42).
Changes in the test: page 7, line 2-8.

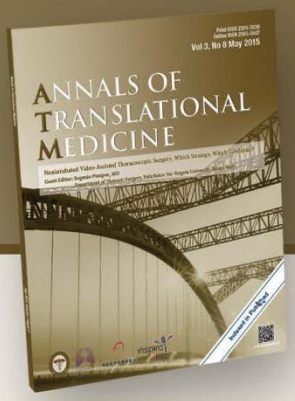
The abbreviation ACHBLF (pag 8) appeared first than the full name (pag 11).
Reply 4: We apologize for the mistake and we have added the full name in pag 8 and abbreviated in pag 11.
Changes in the test: page 8, line 12. Page 12, line 18.

Pag 9. The citation 52 was not indicated to describe the activation of necroptosis after common bile duct ligation. It should be Afonso et al 2016, Afonso et al 2018, Cubero et al 2018. And then several issues should be discussed regarding the differential pathophysiological role of necroptosis and apoptosis for cholestasis and fibrosis.
Reply 5: Thanks for your valuable suggestions. We are sorry for this mistake and we have exchanged the citation as advised.

41. Afonso MB, Rodrigues PM, Simao AL, Ofengeim D, Carvalho T, Amaral JD, et al. Activation of necroptosis in human and experimental cholestasis. Cell Death Dis. 2016;7(9):e2390.
62. Afonso MB, Rodrigues PM, Simao AL, Gaspar MM, Carvalho T, Borralho P, et al. miRNA-21 ablation protects against liver injury and necroptosis in cholestasis. Cell Death Differ. 2018;25(5):857-72.
63. Cubero FJ, Peng J, Liao L, Su H, Zhao G, Zoubek ME, et al. Inactivation of caspase 8 in liver parenchymal cells confers protection against murine obstructive cholestasis. J Hepatol. 2018;69(6):1326-34.
Changes in the test: page 10, line 11.

Pag 9. Is the reference 55 suitable to sustain the following sentence: “Recent years, several studies have shown that RIPK3-dependent necroptosis is an important pathway that regulates the fibrosis progression”?

Reply 6: Thanks for your valuable suggestions. We have added more compelling citation to support this argument as follows.



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12. Schwabe RF, Luedde T. Apoptosis and necroptosis in the liver: a matter of life and death. Nat Rev Gastroenterol Hepatol. 2018;15(12):738-52.
70. Xiao M, Chen W, Wang C, Wu Y, Zhu S, Zeng C, et al. Senescence and cell death in chronic liver injury: roles and mechanisms underlying hepatocarcinogenesis. Oncotarget. 2018;9(9):8772-84.
71. Gautheron J, Vucur M, Reisinger F, Cardenas DV, Roderburg C, Koppe C, et al. A positive feedback loop between RIP3 and JNK controls non-alcoholic steatohepatitis. EMBO Mol Med. 2014;6(8):1062-74.
72. Zhang Z, Xie G, Liang L, Liu H, Pan J, Cheng H, et al. RIPK3-Mediated Necroptosis and Neutrophil Infiltration Are Associated with Poor Prognosis in Patients with Alcoholic Cirrhosis. J Immunol Res. 2018;2018:1509851.
73. Jia Y, Wang F, Guo Q, Li M, Wang L, Zhang Z, et al. Curcumol induces RIPK1/RIPK3 complex-dependent necroptosis via JNK1/2-ROS signaling in hepatic stellate cells. Redox Biol. 2018;19:375-87.
Changes in the test: page 12, line 9.

The controversy about the role of necroptosis in NASH deserves a much deeper explanation and discussion. For eg. Authors should discuss why RIP3 deficiency protects from liver disease in MCD-fed mice (Gautheron et al., 2014, Afonso eat al 2015), whereas deletion of Rip3 exacerbates liver damage and steatosis, and glucose intolerance induced by a high-fat diet (Roychowdhury et al., 2016).

Reply 7: Thanks for your valuable suggestions. We have added corresponding discuss in manuscripts as follows: RIP3 deficiency protects from liver disease in MCD-fed mice (40, 71), whereas deletion of RIP3 exacerbates liver damage and steatosis, and glucose intolerance induced by a high-fat diet (83). These conflicting results may be due to different modeling methods. Compared with liver injury induced by HFD, mice on MCD diet will show more severe lipidosis, fibrosis, and more active oxidative stress. At the same time, there are differences in modeling performance with different fat composition and proportions (85, 86). Furthermore, these data imply that RIP1 and RIP3 cannot be simply considered part of a single functional unit. Indeed, much of the literature in acute liver injury is hindered by conflicting reports between laboratories (58).
Changes in the test: page 15, line 7-15.

And what about the controversy regarding acetaminophen overdose? (Deutsch et al., 2015; Ramachandran et al., 2013). (Li et al., 2014). (Dara et al., 2015)…

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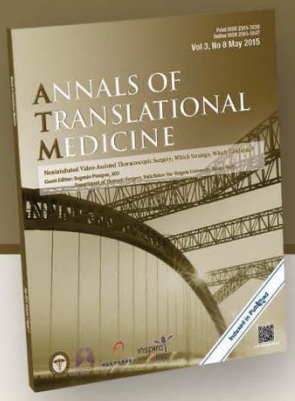

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Reply 8: Thank you for your helpful comments. We have discussed related questions as follows: In fact, there are many controversies arising from the use of acetaminophen (APAP)-overdosed mouse models. Generally, we believe that inhibition of RIP1 or RIP3 can alleviate APAP-induced acute liver injury, but the role of MLKL in APAP-induced acute liver injury is still uncertain (57-60).
Changes in the test: page 10, line 2-5.

And what about the discussion about the necroptosis -independent functions of RIPK3 and MLKL (Xiaoquin et al 2020)?

Reply 9: Thank you for your helpful comments. We have added related discussion as follows: It is reported that RIPK3 can promote NLRP3 inflammasome and IL-1β inflammatory responses independent of MLKL and necroptotic cell death (65). Furthermore, in addition to causing necroptosis by rupturing the membrane, MLKL controls the transport of endocytosed proteins, thereby enhancing the degradation of receptors and ligands. Thus, the extracellular release of phosphorylated MLKL within vesicles serves as a mechanism for self-restricting the necroptotic activity of this protein (66). Wong et al. showed that RIPK1 and RIPK3 can facilitate the production of multiple cytokines, independent of their role in necroptosis (67). They further found that the kinase activities of RIPK1 and RIPK3, in addition to their roles in regulating necroptosis, have a physiological role in the tumor microenvironment, in particular tumor cell extravasation and remodeling by altering the downstream signaling pathways of permeability factors (68).
Changes in the test: page 11, line 10-21.

In general, I think that to completely miss the expectations of the title “Double-edge Sword”, the potential deleterious effects of necroptosis activation in disease should be discussed, as well the possible secondary effects of necroptosis inhibition. The interplay between apoptosis and necroptosis in liver disease should be maintained but more linked with the possible pathophysiological role of each cell death pathway. For intance, the role of apoptosis and necroptosis in fibrosis should be paralled in the same section and not described in 2 different sections.

Reply 10: Thank you for your opinion and valuable suggestions. Generally, we believe that necroptosis plays different roles in different diseases or different stages of liver disease. We have discussed in the manuscripts that necroptosis may have deleterious effects in disease as show below:
5.1 Necroptosis promotes inflammation in acute liver disease (the deleterious effects of necroptosis activation in acute liver disease.)

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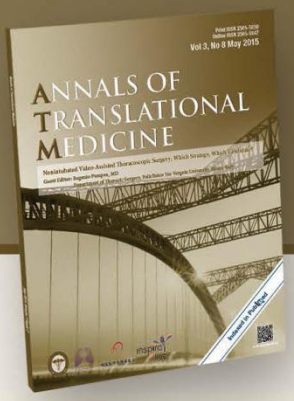

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5.2 Inhibiting necroptosis can alleviate chronic liver disease and cirrhosis (the deleterious effects of necroptosis activation in chronic liver disease and cirrhosis.)
5.4 Necroptosis exerts both pro- and anti-tumoral effects during liver cancer (the deleterious and protective effects of necroptosis activation in liver cancer.)