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Intrahepatocytic necroptosis is dispensable for hepatocyte death in murine immune-mediated hepatitis

Letter to the Editor:

Hundreds of millions of people globally are affected by acute or chronic hepatitis, for which there are no effective treatments. Hepatocyte death is a key element in both initiation and progression of these liver diseases. Necroptosis emerged recently as one of the possible cell death programs involved in liver damage. This pathway is a regulated modality of necrosis that occurs in the presence of caspase inhibition. It involves RIP1/3 (receptor interacting protein) kinases and a final effector, the pseudokinase MLKL (mixed lineage kinase domain-like protein).[1] Many studies have investigated the role of these necroptosis-associated proteins in different liver injury models, sometimes with conflicting results.[2] Thus, the involvement of necroptosis in different liver diseases remains controversial and a hot topic in hepatology research.

In 2016, Günther *et al.* reported that necroptosis mediates hepatocyte death in a murine model of immune-mediated hepatitis.[3] Indeed, using mice with a total ablation of *Mlkl* ($Mlkl^{-/-}$ mice) challenged by Concanavalin A (ConA), a lectin triggering severe inflammation and T-cell mediated liver injury, they found that $Mlkl^{-/-}$ mice are completely protected from ConA-induced damage compare to wild-type mice.[4] The authors concluded that the pseudokinase MLKL is an essential mediator of hepatocellular necrosis in ConA-induced hepatitis in mice.

To deepen the understanding of the role of MLKL in ConA-induced hepatitis and particularly in the hepatocyte itself, we have generated a new mouse line with conditional ablation of *Mlkl* specifically in liver parenchymal cells ($Mlkl^{LPC\text{-}KO}$ mice). As expected, these $Mlkl^{LPC\text{-}KO}$ mice have reduced levels of MLKL in the whole liver and a total absence of MLKL in isolated hepatocytes, as shown by western blotting analyses (Fig. 1A). Importantly, intravenous treatment with ConA at a dose of 12 mg/kg for 6 h, 11 h and 16 h did not reveal any differences in levels of serum transaminases and degree of necrotic damage of the liver between $Mlkl^{LPC\text{-}KO}$ mice and $Mlkl^{fl/fl}$ littermate controls (Fig. 1B and 1C). These results show that $Mlkl^{LPC\text{-}KO}$ mice are not protected from ConA-induced hepatitis demonstrating that hepatocyte-intrinsic MLKL is dispensable for ConA-induced hepatolysis in contrast to what has been advanced by Günther *et al.*[3]

The contradictory results obtained with the $Mlkl^{-/-}$ and the $Mlkl^{LPC\text{-}KO}$ mice, explained by the use of a constitute knockout model on the one hand and specific knockout model on the other hand, suggest that necroptosis occurring in other cell population(s) is involved in hepatolysis in response to ConA injection, in which immunity is essential to hepatocyte damage.

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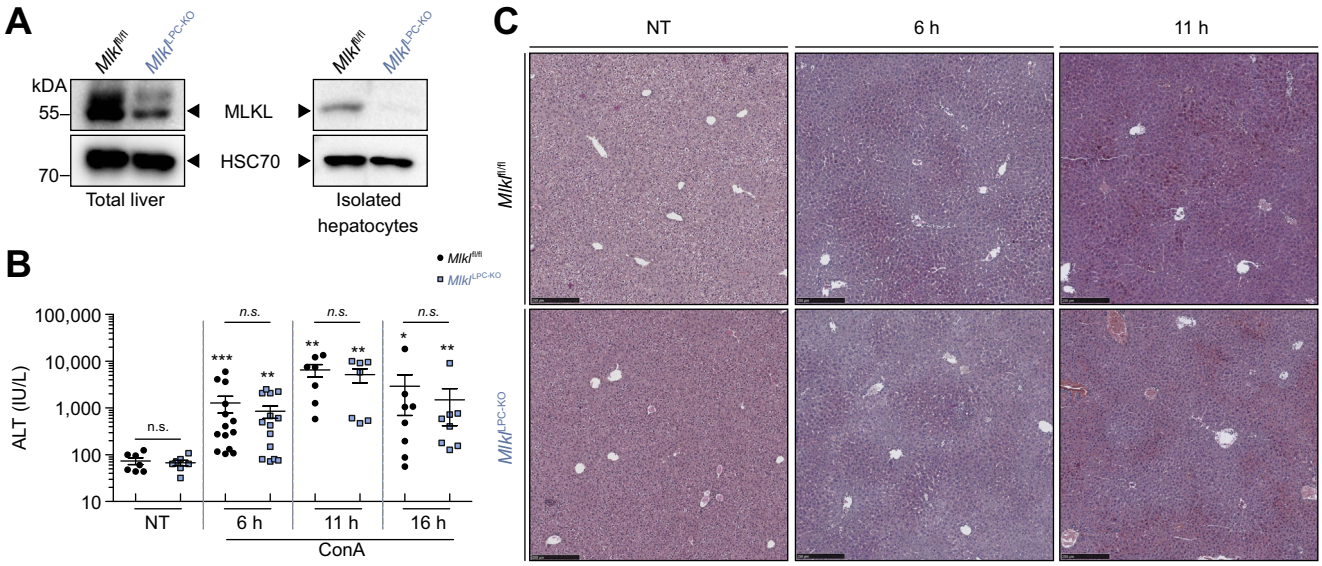

Fig.1. *Mlkl*$^{LPC-KO}$ mice are not protected from ConA-induced hepatitis. Mice with conditional ablation of *Mlkl* in liver parenchymal cells (*Mlkl*$^{LPC-KO}$ mice) were generated by crossing *Mlkl*-floxed mice with *Alfp*-cre transgenic mice. (A) MLKL depletion in parenchymal liver cells is efficient. Western blot analyses were performed on whole liver or isolated hepatocyte protein extracts using anti-Mlkl antibody and anti-Hsc70 antibody as loading control. (B and C) Lack of MLKL in parenchymal cells does not confer protection against ConA-induced hepatitis. *Mlkl*$^{LPC-KO}$ mice and their littermate controls (*Mlkl*$^{fl/fl}$) were injected intravenously with vehicle (PBS, 1 mM $CaCl_2$, 0.5 mM $MnCl_2$) (NT: non-treated; 7 mice per genotype) or with 12 mg/kg of ConA and euthanized at 6 h, 11 h or 16 h post-injection (14, 7 or 8 mice per genotype, respectively). (B) Levels of serum alanine aminotransferase. Each dot and square represent an individual; error bars are expressed as mean ± SEM; each time point of ConA-treatment was compared to the NT related mouse strain and comparisons were made between the 2 mouse strains at each time point. Mean differences between experimental groups were assessed using the non-parametric Mann–Whitney *U* test. Significance is shown as follows: $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001; ns: non-significant. (C) Representative pictures of liver tissue sections analyzed by hematoxylin and eosin staining. Black bars represent 250 μm. ConA, Concanavalin A.

Günther *et al.* excluded the involvement of immune cells by performing bone marrow graft experiments.[3] However, further investigations studying the effect of ConA in mice lacking MLKL in other intrahepatic cell populations are needed to better understand the liver damage process. Liver sinusoidal endothelial cells are potentially interesting candidates to investigate, since necroptosis could be induced in endothelial cells.[5]

Moreover, our new data show that other types of cell death than necroptosis can lead to hepatocyte death during ConA-induced hepatitis. Like Günther *et al.*, we did not observe apoptotic cells following ConA injection either in *Mlkl*$^{fl/fl}$ or in *Mlkl*$^{LPC-KO}$ mice (data not shown), in contrast to what we already observed in immune-mediated hepatitis in *Ripk1*$^{LPC-KO}$ mice.[6,7] Other cell death pathways must now be considered. Luan *et al.*, in 2018, suggested that pyroptosis could be one of the cell death programs involved in ConA-induced hepatitis.[8] We think that we have to reconsider the research on hepatocyte death, taking into account the fact that different pathways could act in synergy and compensate each other as addressed in the new concept of PAN-optosis.[9,10]

To conclude, our data demonstrate that intrahepatocytic MLKL and then necroptosis are dispensable for hepatocyte death in this model of immune-mediated hepatitis. Moreover, our results open new insights on the involvement of other intrahepatic cell populations in hepatocellular necrosis in this context. Thus, the design of therapeutic approaches integrating prevention of liver tissue cell death will have to consider the disparity of induced death types and their interconnections.

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Conflict of interest

The authors have no conflicts of interest to declare.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Conceptualization, A.H., C.P.P., M.T.D.B., M.S. and J.L.S.; Methodology, A.H., C.P.P., M.T.D.B., M.S. and J.L.S.; Validation, A.H., C.P.P., M.T.D.B., M.S. and J.L.S.; Formal Analysis, A.H.; Investigation, A.H., C.P.P. and J.L.S.; Data Curation, A.H.; Writing – Original Draft Preparation, A.H.; Writing – Review & Editing, M.S. and J.L.S.; Visualization, A.H.; Supervision, M.S. and J.L.S.; Project Administration, A.H., C.P.P., M.T.D.B., M.S. and J.L.S.; Funding Acquisition, M.T.D.B., M.S. and J.L.S.

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Supplementary data

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The risk-benefit assessment of liver biopsy in times of non-invasive screening for liver fibrosis

To the Editor:

During the past few decades medical research in almost every field has focused on developing diagnostic methods that are not only less invasive and time-consuming, but also more cost-effective, which has led to a potential oversight when it comes to the irreplaceable role that a liver biopsy as we know it today still has.

Therefore, with intense and consuming interest we read the meta-analysis by Serra-Burriel *et al.*[1] and found ourselves in full agreement on the idea of "preventive hepatology" by creating and implementing a unique diagnostic algorithm that would include a non-invasive assessment of patients at risk at the primary care level. Although it has many advantages, one should bear in mind the economic setting and healthcare structure in which the research was conducted. For example, healthcare systems in developing countries are facing numerous challenges, some of which are reflected in timely and widespread implementation of modern technology, such as transient elastography, mostly due to funding shortages. Consequently, under such circumstances, despite being invasive, "traditional" diagnostic tools should not be neglected easily, especially those we are familiar with.

Our preliminary, prospective, single-center study included a total of 123 consecutive patients, who underwent the ultrasonography-assisted percutaneous liver biopsy during the patients' hospital stay. Indications included grading of inflammation and staging of fibrosis, while in the majority of patients, biopsy was used to confirm a suspected diagnosis and exclude overlapping etiology. Each patient gave their written consent and was given a carefully constructed questionnaire to fill in, which encompassed demographic data, a visual analogue scale (VAS) of both anticipated and experienced pain, questions regarding post-procedural pain and its duration, presence of fear and its nature prior to the intervention itself. Patients were taught how to use the VAS scoring from 0 to 10, to grade the intensity of pain. On that scale, the left endpoint, 0, was defined as no pain, while the right endpoint, 10, was considered to be the greatest pain the patient could imagine. A complete blood count was completed several hours following the procedure, while control abdominal ultrasonography was performed the day after. For continuous variables, the mean and standard deviation or median and range were calculated depending on the normality of data distribution. Categorical data were presented as frequencies. Wilcoxon test was used to determine a statistical difference in anticipated and experienced pain. Our group of patients comprised 78 females (63.41%) and 45 males (36.59%), with a mean age of 51 (±14.88) and a mean body mass index of 25.92 (±4.93). Pathohistological findings included non-alcoholic steatohepatitis in 44.71% (n = 55), toxic lesion in 16.26% (n = 20), primary biliary cholangitis in

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