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High precision-cut liver slice model to study cell-autonomous antiviral defense of hepatocytes within their microenvironment

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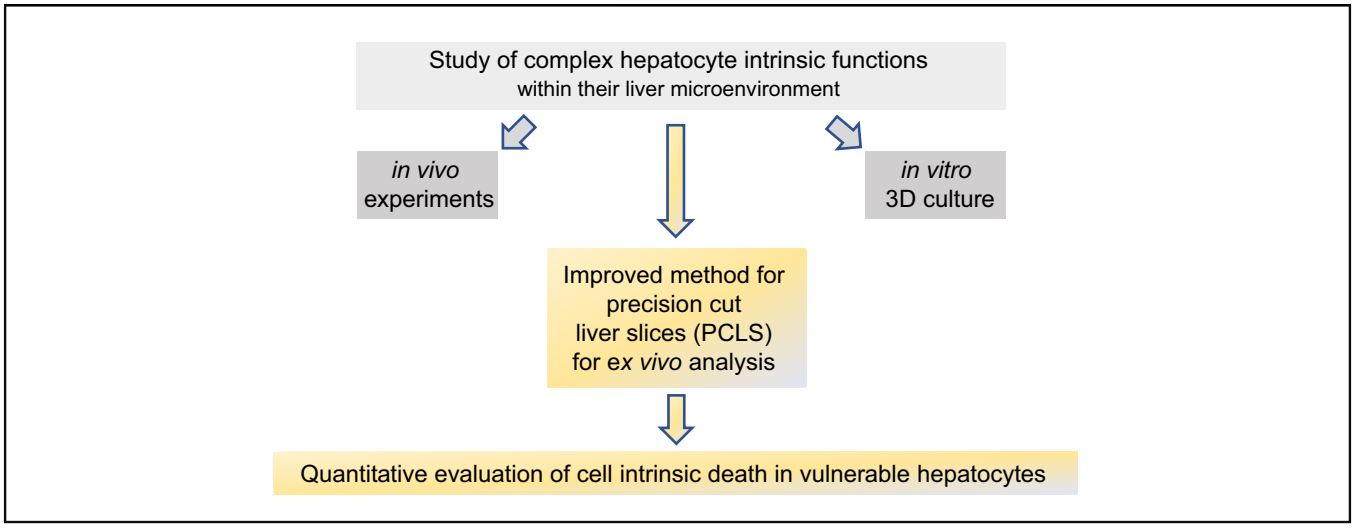
Graphical abstract



Highlights

- Precision-cut liver slices (PCLS) can be used to study hepatocyte-intrinsic regulation of cell death *ex vivo*.
- Increased induction of apoptosis is detected in PCLS from adenovirus- and LCMV-infected livers after TNF challenge *ex vivo*.
- Optimized protocol may enable the characterization of human hepatocyte sensitivity to apoptosis in PCLS from patients with liver diseases.

Lay summary

Virus-infected hepatocytes *in vivo* show an increased sensitivity towards induction of cell death signaling through the TNF receptor. Studying this hepatocyte-intrinsic antiviral immune surveillance mechanism has been hampered by the absence of model systems that reciprocate the *in vivo* finding of increased apoptosis of virus-infected hepatocytes challenged with TNF. Herein, we report that an optimized protocol for generation of precision-cut liver slices can be used to study this hepatocyte-intrinsic surveillance mechanism *ex vivo*.

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Short communication

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High precision-cut liver slice model to study cell-autonomous antiviral defense of hepatocytes within their microenvironment

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Background & Aims: Increased sensitivity towards tumor necrosis factor (TNF)-induced cell death in virus-infected hepatocytes has revealed a so far unrecognized hepatocyte-intrinsic antiviral immune surveillance mechanism, for which no *in vitro* or *ex vivo* model is available. We aimed to establish precision-cut liver slices (PCLS) as a model system to study hepatocyte-intrinsic regulation of apoptosis.

Methods: Preparation of PCLS from mouse and human liver tissue was optimized for minimal procedure-associated apoptosis. Functionality of liver cells in PCLS was characterized using extracellular flux analysis to determine mitochondrial respiration, and viral infection with recombinant adenovirus and lymphocytic choriomeningitis virus (LCMV) was used to probe for hepatocyte-intrinsic sensitivity towards apoptosis in PCLS. Apoptosis was detected by immunohistochemical staining for cleaved-caspase 3 and quantified by detection of effector caspase activity in PCLS.

Results: We established an optimized protocol for preparation of PCLS from human and mouse models using agarose-embedding of liver tissue to improve precision cutting and using organ-protective buffer solutions to minimize procedure-associated cell death. PCLS prepared from virus-infected livers showed preserved functional metabolic properties. Importantly, in PCLS from adenovirus- and LCMV-infected livers we detected increased induction of apoptosis after TNF challenge *ex vivo*.

Conclusion: We conclude that PCLS can be used as model system to *ex vivo* characterize hepatocyte-intrinsic sensitivity to cell death. This may also enable researchers to characterize human hepatocyte sensitivity to apoptosis in PCLS prepared from patients with acute or chronic liver diseases.

Lay summary: Virus-infected hepatocytes *in vivo* show an increased sensitivity towards induction of cell death signaling through the TNF receptor. Studying this hepatocyte-intrinsic antiviral immune surveillance mechanism has been hampered by the absence of model systems that reciprocate the *in vivo* finding of increased apoptosis of virus-infected hepatocytes challenged with TNF. Herein, we report that an optimized protocol for generation of precision-cut liver slices can be used to study this hepatocyte-intrinsic surveillance mechanism *ex vivo*.

Introduction

Immune effector cell populations like virus-specific T cells and natural killer cells, that recognize and kill virus-infected hepatocytes, contribute to successful immune surveillance in the liver.[1] The selective elimination of virus-infected hepatocytes was considered to rely entirely on the ability of virus-specific effector cell populations to execute their killing activity. Consequently, research aiming to understand successful or failing immune surveillance against viral infection in the liver has mainly focused on studying the numbers and the breadth of effector function of virus-specific T cells.[2,3] Recently it has become evident, however, that hepatocytes themselves contribute to immune surveillance by developing a unique state of responsiveness towards death signals.[4] Virus-infected but not healthy hepatocytes selectively respond to tumor necrosis factor (TNF) receptor signaling with induction of caspase-induced cell death, *i.e.*, apoptosis. This reveals the existence of hepatocyte-intrinsic antiviral surveillance that is independent of cytolytic T cell effector function and that leads to death of hepatocytes.[4,5]

Currently, there are no *in vitro* or *ex vivo* models in place to study the contribution and mechanistic aspects of this increased sensitivity to apoptosis in hepatocytes in liver diseases.

Keywords: TNF-induced apoptosis; precision-cut liver slices; anti-viral immunity.
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Hepatocytes function within their microenvironment, which may be important to sustain their metabolic and immune functions and may explain why certain functions observed *in vivo* are not observed *in vitro* in isolated primary hepatocytes.[6,7] The necessity to study organ context-dependent functions of cells has been recognized as a general challenge, and has led to the development of experimental model systems in many organ-specific research fields that allow for contextual analysis of cell functions in tissues.[8] For the liver, different 3D cell culture models have been developed, which simulate the hepatic microanatomy like liver scaffolds and facilitate the growth of different liver cell populations in defined 3D-structures.[9] Stem cell-derived liver cells were also used to construct human liver tissue.[10] Yet, all these approaches require sophisticated technologies. A more direct approach to generate 3D tissue models, which reflect the complexity of tissue organization, for the study of tissue context-dependent cell functions is the generation of precision-cut tissue slices.[11,12] Precision-cut liver slice (PCLS) models were used in the past to explore pathophysiological mechanisms underlying chronic liver damage,[13–15] and mechanisms underlying hepatic steatosis, steatohepatitis or drug metabolism.[16–21] Herein, we report on an improved PCLS model that enables the *ex vivo* analysis of hepatocyte-intrinsic mechanisms, which determine sensitivity of virus-infected hepatocytes towards death signaling.

Materials and methods

Mouse and human liver

C57BL/6J mice were purchased from Charles River (Germany). All mice used in the experiments were maintained under specific pathogen-free conditions according to the guidelines of the Federation of Laboratory Animal Science Association. Animal experiments were approved by local authorities (ROB-55.2.2532.Vet_02-18-16). Experiments with human liver samples were approved by and performed in accordance with the regulations of the Ethics committee at the Technical University of Munich (86/17S) and the ethical guidelines of the World Medical Association Declaration of Helsinki. Informed written consent was obtained from each patient.

Preparation of PCLS

Murine livers were removed from anesthetized mice (2.5% isoflurane) and human liver tissue was obtained from patients undergoing liver resection for colorectal tumor metastasis. PCLS were generated as described here in short. After resection, liver tissue was immediately stored in the organ-preservation buffer solution *custodiol*® at 4°C (Köhler, Germany) and was immersed in 4% low-melting agarose. Agarose-stabilized liver tissue enabled the generation of PCLS using a vibratome Leica VT 1000S (Leica Biosystems, Germany). The freshly generated PCLS were maintained in Williams E Medium (PAN-Biotech, Germany), with gentle orbital shaking (80 times/min) at 37°C in a CO_2 incubator to ensure tissue oxygenation and consistent exposure to reagents during experiments (please visit our detailed protocol for generation of PCLS in the supplementary information).

Results

Improved preparation of mouse and human PCLS

In vivo the induction of apoptosis in hepatocytes infected with a recombinant adenovirus coding for the marker genes GFP and luciferase (Ad-CMV-GL, $5x10^8$ infectious units per mouse) occurred within 2 hours after TNF challenge (Fig. 1A,B). However, TNF-induced cell death after *in vitro* infection of hepatocytes with Ad-CMV-GL, required more than 5 hours, measured by loss of electrical impedance of the hepatocyte cell layer in a 96-well plate (Fig. 1C). Since it is not possible to isolate viable virus-infected hepatocytes for mechanistic *in vitro* studies of induction of cell death,[4,5] we set out to establish a PCLS model that could enable the *ex vivo* characterization of the unique responsiveness of virus-infected hepatocytes *in vivo* within the liver to die by apoptosis.

We improved the generation of PCLS by stabilizing mouse and human liver tissue in a low-melting agarose to allow for gentle precision cutting of liver tissue into slices and by reducing tissue damage during this procedure through use of an organ-protective buffer solution (see detailed protocol for preparation of PCLS). Histomorphological analysis demonstrated that neither generation of PCLS nor subsequent incubation at 37°C for a period of 2 hours led to detectable cell damage in mouse and human PCLS (Fig. 1D). Consequently, we did not detect a release of lactate dehydrogenase, a marker for cell death, from PCLS after cutting or after incubation at 37°C (Fig. 1E). To confirm the absence of apoptotic cell death in the improved PCLS, we quantified induction of apoptosis by measuring cleaved-caspase 3, which is the apoptosis inducing active form of the effector caspase 3. We found very few cleaved-caspase 3-positive cells by immunohistochemistry in murine and human PCLS (Fig. 1F,G) compared to a higher number of apoptotic cells in PCLS generated by the conventional method (Fig. S1). Numbers of cleaved-caspase 3-positive hepatocytes were slightly higher in human PCLS (0.54% ± 0.27) compared to murine PCLS (0.23% ± 0.5). Taken together, these results demonstrated that the improved method for generating PCLS yielded viable liver tissue without generation of procedure-associated apoptotic cells (Fig. 1).

PCLS as *ex vivo* model system to study hepatocyte sensitivity to apoptosis

Establishing PCLS that did not bear high numbers of apoptotic cells allowed us to investigate whether these PCLS could be employed to study *ex vivo* the apoptotic response of infected hepatocytes observed *in vivo*. We first evaluated whether hepatocytes infected *in vivo* were detected in PCLS *ex vivo*. To this end, we generated a recombinant adenovirus coding for the fluorescence reporter gene iRFP720 (Ad-CMV-GIRO) that enables the detection of *in vivo* fluorescence activity of Ad-CMV-GIRO-infected hepatocytes due to the high tissue-penetration of light with high wavelength (>700 nm) emitted from iRFP720. *In vivo* fluorescence imaging of Ad-CMV-GIRO-infected mice demonstrated successful adenoviral transduction of the liver (Fig. 2A). Importantly, iRFP720-fluorescence was also *ex vivo* detected in PCLS from livers of mice infected with Ad-CMV-GIRO (Fig. 2B,C). No fluorescence signal was detected from uninfected livers *in vivo* or from PCLS prepared from these livers *ex vivo* (Fig. 2A-C). Histomorphological analysis of PCLS from infected livers compared to non-infected livers by H&E staining or staining for apoptotic cells (detection of cleaved-caspase 3) did not reveal tissue damage after infection (Fig. 2D). Taken together, these results demonstrated that virus-infected cells were present in PCLS generated from Ad-CMV-GIRO-infected liver.

Viral infection leads to rapid expression of viral genes that might cause changes in cellular metabolism.[22] We therefore investigated whether metabolic mitochondrial activity was

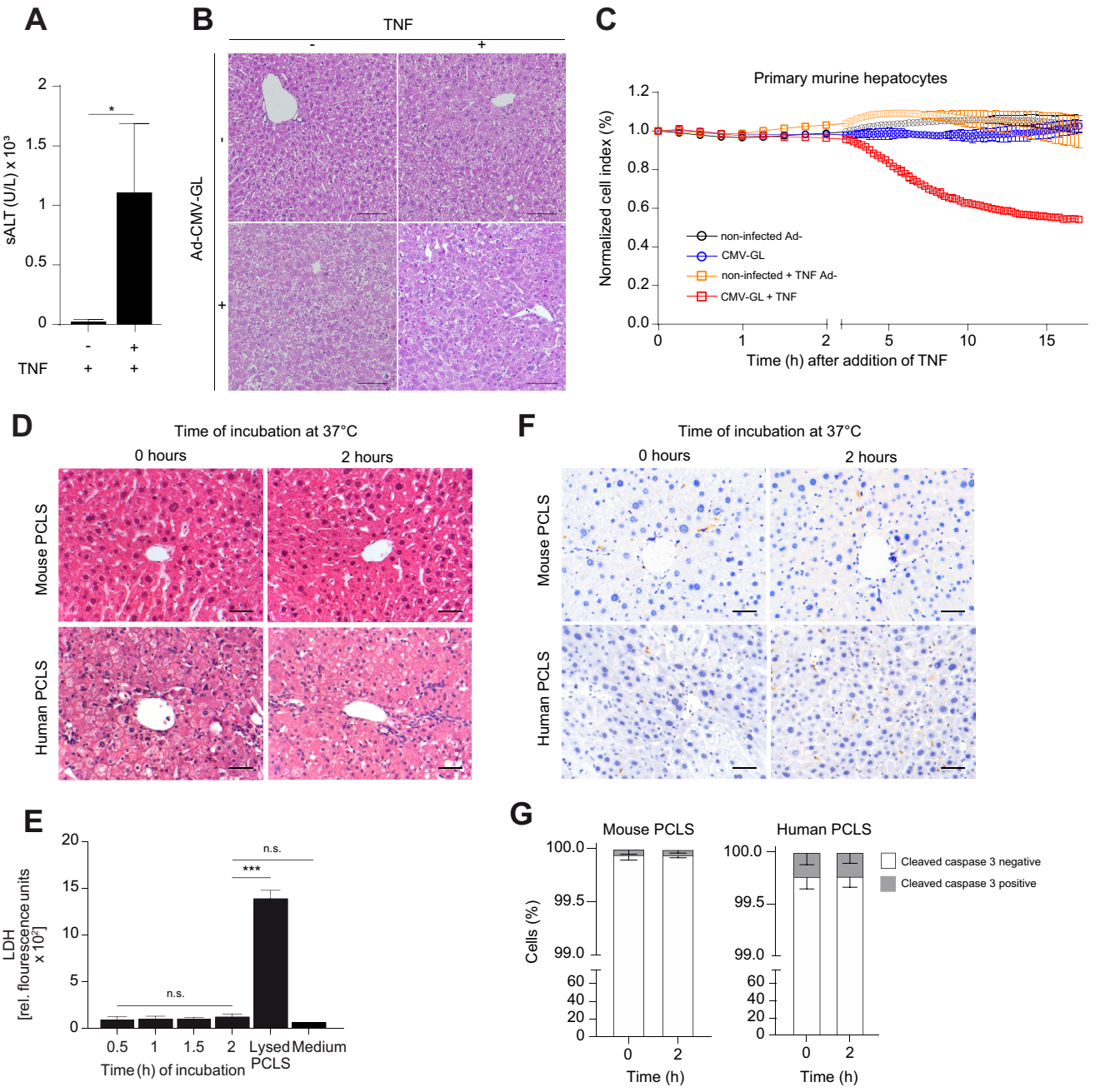

Fig. 1. Absence of cell death in murine and human PCLS. (A) Serum ALT levels in mice after injection of TNF (400 ng/mouse) at d2 after infection with Ad-CMV-GL ($5x10^8$ infectious units/mouse). (B) H&E staining of liver sections at 4 h after TNF-injection in Ad-CMV-GL-infected mice, scale bar: 100 μm. (C) Primary mouse hepatocytes were grown to confluence in 2D-culture and were infected with Ad-CMV-GL before challenge with TNF 2 days later; time kinetics of hepatocyte death after TNF challenge was determined by measuring change in electrical impedance of healthy, healthy/TNF challenged, Ad-CMV-GL-infected and Ad-CMV-GL-infected/TNF challenged hepatocytes. (D) H&E staining of murine and human PCLS directly after preparation and after 2 h of incubation at 37°C, scale bar 100 μm. (E) Time kinetics of LDH release from murine PCLS after incubation at 37°C; lysed PCLS as positive control and culture medium alone as negative control. (F) Immunohistochemistry of PCLS for detection of cleaved-caspase 3 to identify apoptotic cells directly after preparation and after 2 hours of incubation at 37°C. (G) Quantification of cleaved-caspase 3-positive cells in human and murine PCLS (≥3,150 hepatocytes analyzed for each parameter) from (F). (A-G) Representative data from at least 3 separate experiments are shown as mean ± SEM. Statistical significance was calculated using unpaired *t* test, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$. LDH, lactate dehydrogenase; PCLS, precision-cut liver slices; sALT, serum alanine aminotransferase; TNF, tumor necrosis factor.

different in PCLS prepared from Ad-CMV-GIRO-infected livers compared to healthy livers. There was no difference in mitochondrial respiration activity measured by extracellular flux analysis of PCLS prepared from Ad-CMV-GIRO-infected compared to healthy livers (Fig. 2E, Fig. S2). In the absence of a change in mitochondrial respiration after Ad-CMV-GIRO infection, we next characterized the response of Ad-CMV-GIRO-infected cells to TNF challenge. Strikingly, in PCLS prepared

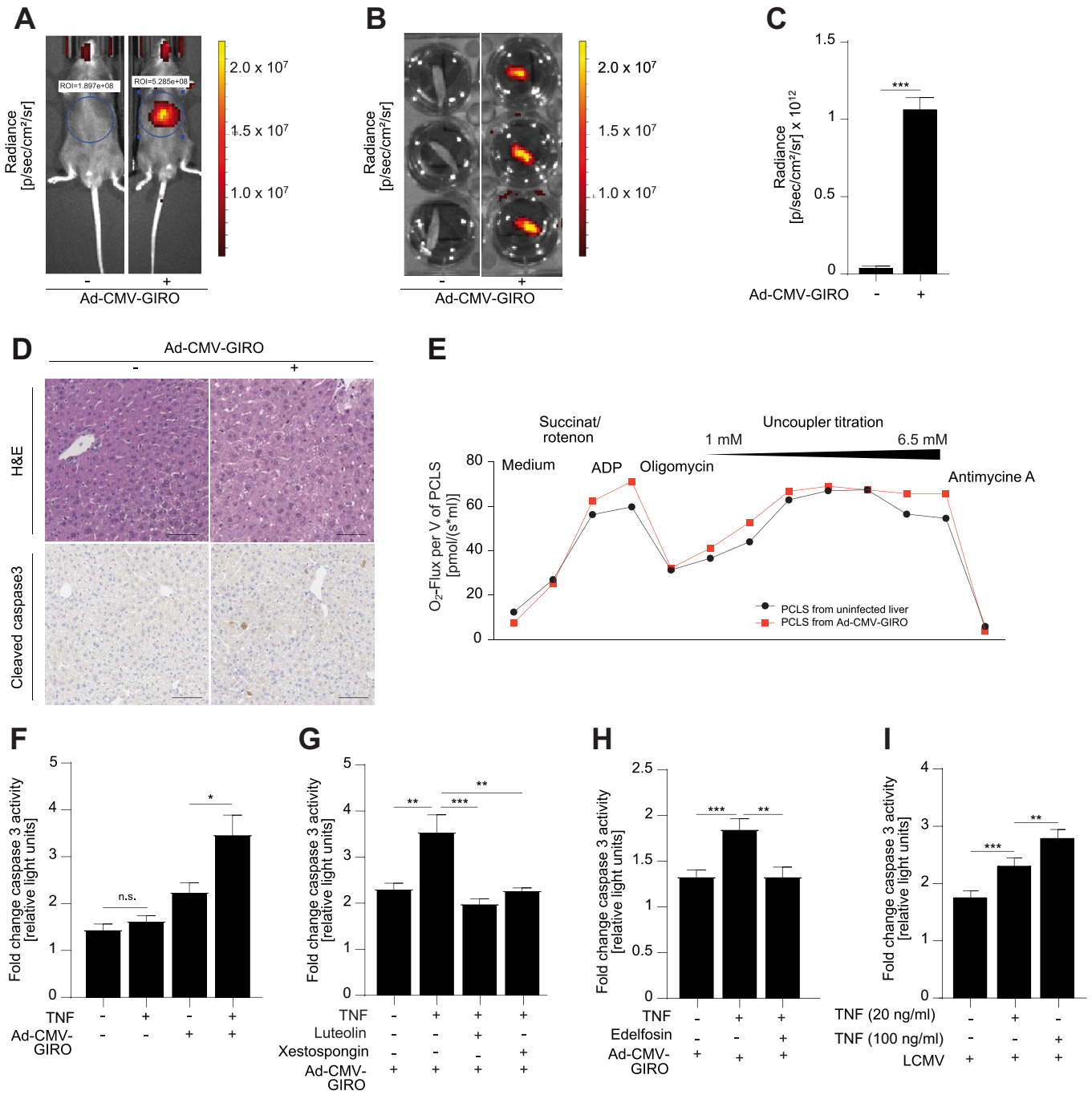

Fig. 2. TNF mediates cell death in PCLS from virus-infected liver. (A) *In vivo* fluorescence imaging day 2 post infection (Ad-CMV-GIRO, $5x10^8$ infectious units/mouse). (B) Fluorescence images of PCLS prepared from murine liver at day 2 post infection with Ad-CMV-GIRO ($5x10^8$ infectious units/mouse). (C) Quantification of fluorescence intensity (radiance) from PCLS (B). (D) H&E staining and immunohistochemistry for cleaved-caspase 3 in PCLS prepared from Ad-CMV-GIRO-infected livers or healthy livers. (E) Mitochondrial stress test of PCLS prepared from Ad-CMV-GIRO-infected livers ($5x10^8$ infectious units/mouse) or healthy livers. (F) Quantification of caspase 3 activity by luminescence detection assay in PCLS prepared from Ad-CMV-GIRO-infected liver ($5x10^8$ infectious units/mouse) at 2 h after *ex vivo* TNF challenge (20 ng/ml). (G, H) Quantification of caspase 3-activity in PCLS prepared from Ad-CMV-GIRO infected liver ($5x10^8$ infectious units/mouse) after incubation with pharmacological inhibitors of ROS (luteolin), IP_3 receptor signaling (xestospongin), PLCg-signaling (edelfosin) and *ex vivo* TNF challenge (20 ng/ml). (I) Quantification of caspase 3-activity in PCLS prepared from LCMV strain WE-infected livers at 2 h after *ex vivo* TNF challenge (20 ng/ml and 100 ng/ml). (A-I) Representative data from at least 3 separate experiments are shown as mean ± SEM. Statistical significance was calculated using unpaired *t* test, $^*p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$. IP_3, inositol-3-phosphate; LCMV, lymphocytic choriomeningitis virus; PCLS, precision-cut liver slices; PLCg, phospholipase C gamma; ROS, reactive oxygen species; TNF, tumor necrosis factor.

from virus-infected livers we detected higher caspase 3 activity than in PCLS from uninfected livers at 2 hours after *ex vivo* TNF challenge (Fig. 2F). Since induction of apoptosis through TNF receptor signaling in virus-infected hepatocytes *in vivo* requires NADPH-oxidase and generation of reactive oxygen species (ROS),[4] we addressed the question of whether blockade of ROS formation would rescue TNF-induced apoptosis in PCLS. Clearly, incubation of PCLS prepared from Ad-CMV-GIRO infected livers with the ROS-blocking agent luteolin prevented TNF-induced caspase activation (Fig. 2G). To further evaluate the involvement of signaling events downstream of ROS formation, we studied the relevance of phospholipase C gamma (PLCg) activation and inositol-3-phosphate (IP_3)-receptor signaling for induction of apoptosis in Ad-CMV-GIRO-infected hepatocytes. Incubation with the pharmacological inhibitor of IP_3-receptor signaling (xestospongin) prevented TNF-induced caspase 3 activation in PCLS prepared from Ad-CMV-GIRO-infected livers (Fig. 2G). Furthermore, pharmacological inhibition of PLCg-activation with edelfosin equally prevented caspase 3 activation in PCLS prepared from Ad-CMV-GIRO-infected livers at 2 hours after *ex vivo* TNF challenge (Fig. 2H). To demonstrate that increased sensitivity of hepatocytes to apoptosis in PCLS was not restricted to infection with recombinant adenoviruses, we used the lymphocytic choriomeningitis virus (LCMV) strain WE to infect the liver. PCLS prepared from LCMV-infected livers also showed caspase 3 activation at 2 hours after *ex vivo* TNF challenge and the extent of liver damage increased with higher TNF doses (Fig. 2I). Together, these results demonstrated that, in PCLS prepared from virus-infected livers *ex vivo*, a TNF challenge induced caspase 3 activation, which was prevented by inhibition of ROS formation and by interfering with PLCg-activation or IP_3-receptor signaling (Fig. 2).

Discussion

Studying the response of hepatocytes within their liver microenvironment to signaling processes requires either an *in vivo* disease model, complex 3D cultures of hepatocytes mimicking the complex liver microenvironment for *in vitro* studies, or viable liver slice cultures for *ex vivo* studies. Herein, we demonstrate an improved method for preparation of PCLS that yields liver tissue slices that could be used to characterize *ex vivo* the hepatocyte-intrinsic response to induction of apoptosis. Improved cutting of liver tissue through immobilization of liver tissue in agarose blocks together with the use of a tissue-preservation solution used in liver transplantation enabled the preparation of PCLS from mouse and human liver tissue that showed almost no procedure-associated cell death. However, PCLS prepared from surgically resected human liver tissue showed few apoptotic cells, which may be related to longer time spans of tissue hypoxia before the start of PCLS preparation. In accordance with this assumption, we did not find a further increase in numbers of apoptotic cells over time after incubation of human PCLS.

Next, we investigated whether PCLS can be used to study liver cell functions *ex vivo*. Fluorescence activity in hepatocytes infected *in vivo* with recombinant Ad-CMV-GIRO was preserved *ex vivo* in PCLS prepared from these livers, which pointed towards conservation of functions of virus-infected hepatocytes. Similarly, mitochondrial respiration was intact in PCLS as determined by extracellular flux analysis of single mouse PCLS, and showed similarity to mitochondrial respiration detected from hepatocytes analyzed *ex vivo*,[4] which further corroborates that liver cell functionality was maintained in PCLS. Most importantly, development of liver cell death was observed in PCLS prepared from virus-infected livers after TNF challenge *ex vivo*, which resembled the rapid response of virus-infected hepatocytes to TNF *in vivo*.[4] Such sensitivity to apoptosis of liver cells in PCLS was not restricted to infection with adenoviruses, a DNA virus, but was also observed after infection with an RNA virus (LCMV) suggesting that the unique sensitivity of hepatocytes to apoptosis after TNF challenge might also be present during infection with other viruses. The development of an improved method to prepare PCLS may therefore allow for the characterization of the underlying mechanism of hepatocyte-intrinsic regulation of apoptosis induction in molecular detail using inhibitors that would exert systemic toxicity *in vivo*. Furthermore, since we have also shown that human PCLS can be generated with this protocol, PCLS-based analysis of hepatocyte sensitivity to cell death may be extended to human diseases like acute or chronic viral hepatitis,[23,24,25] non-alcoholic steatohepatitis[26] or drug-induced liver injury,[27] where liver cell death is observed.

Abbreviations

IP_3, inositol-3-phosphate; LCMV, lymphocytic choriomeningitis virus; PCLS, precision-cut liver slices; PLCg, phospholipase C gamma; ROS, reactive oxygen species; TNF, tumor necrosis factor.

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Conflict of interest

The authors declare no conflict of interest.

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

Authors' contributions

MB, SL, AS, KM, ML performed experiments; NH and DH provided essential study material; MB, ML, KS, DW, PK analyzed the data; MB, SE, PK designed the study; MB, DW and PK wrote the manuscript, all authors approved the manuscript.

Data availability statement

The data generated and analyzed are available from the corresponding author, MB, upon reasonable request.

Acknowledgments

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2022.100463.

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Author names in bold designate shared co-first authorship.

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