Research Article

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RIPK1 protects hepatocytes from Kupffer cells-mediated TNF-induced apoptosis in mouse models of PAMP-induced hepatitis

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Background & Aims: The severity of liver diseases is exacerbated by the death of hepatocytes, which can be induced by the sensing of pathogen associated molecular patterns (PAMPs) derived from the gut microbiota. The molecular mechanisms regulating these cell death pathways are poorly documented. In this study, we investigated the role of the receptor interacting protein kinase 1 (RIPK1), a protein known to regulate cell fate decisions, in the death of hepatocytes using two *in vivo* models of PAMP-induced hepatitis.

Methods: Hepatitis was induced in mice by independent injections of two different bacterial PAMPs: lipopolysaccharide (LPS) and unmethylated CpG oligodeoxynucleotide (CpG-DNA) motifs. The role of RIPK1 was evaluated by using mice specifically lacking RIPK1 in liver parenchymal cells (*Ripk1*$^{LPC\text{-}KO}$). Administration of liposome-encapsulated clodronate served to investigate the role of Kupffer cells in the establishment of the disease. Etanercept, a tumor necrosis factor (TNF)-decoy receptor, was used to study the contribution of TNF-α during LPS-mediated liver injury.

Results: Whereas RIPK1 deficiency in liver parenchymal cells did not trigger basal hepatolysis, it greatly sensitized hepatocytes to apoptosis and liver damage following a single injection of LPS or CpG-DNA. Importantly, hepatocyte death was prevented by previous macrophage depletion or by TNF inhibition.

Conclusions: Our data highlight the pivotal function of RIPK1 in maintaining liver homeostasis in conditions of macrophage-induced TNF burst in response to PAMPs sensing.

Lay summary: Excessive death of hepatocytes is a characteristic of liver injury. A new programmed cell death pathway has been described involving upstream death ligands such as TNF and downstream kinases such as RIPK1. Here, we show that in the presence of LPS liver induced hepatic injury was due to secretion of TNF by liver macrophages, and that RIPK1 acts as a powerful protector of hepatocyte death. This newly identified pathway in the liver may be helpful in the management of patients to predict their risk of developing acute liver failure.

Keywords: Cytokine; Acute hepatitis; RIP kinase; RIPK1; TNF-α; LPS; CpG-DNA; PAMPs; DAMPs; Liver; Inflammation; Cell death.

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Introduction

The liver is constantly exposed to bacterial pathogen associated molecular patterns (PAMPs) such as unmethylated CpG-DNA motifs or lipopolysaccharides (LPS) originating from the microbiota of the gastrointestinal tract. In physiological conditions, the intestinal barrier prevents the translocation of large amounts of bacterial by-products to the liver [1]. The low quantities of bacterial debris reaching the liver are efficiently cleared by phagocytic hepatic cells, avoiding induction of inflammation and harmful response [2]. In chronic liver diseases of steatosis or alcoholic origin, this intestine barrier function is damaged [3,4], resulting in an abnormal elevation of PAMPs in the liver and impairing liver homeostasis [5]. An increase in PAMPs in the liver is one of the most common factors responsible for the outbreak of acute hepatitis on chronic liver failure background (ACLF for acute on chronic liver failure) [6]. According to the European Association for the Study of Liver Disease (EASL) and the Asian Pacific Association for the Study of the Liver (APASL), ACLF is an acute deterioration of a pre-existing chronic liver disease, and as a consequence, can provoke high short-term mortality. Thus today, ACLF is one of the most challenging fields in hepatology [7]. However, the molecular mechanisms responsible for the liver failure are not completely understood.

The gut has a strong anatomical link with the liver, which plays a key role in the bacterial clearance, and explains why ~80% of macrophages are present in the liver [5,8]. These liver

resident macrophages, named Kupffer cells (KC) efficiently phagocytose bacteria and eliminate endotoxins in order to avoid systemic bacterial infection [5]. In addition to phagocytosis, KC are among the first cells to initiate the inflammatory response through the sensing of microbial products by the Toll-like receptors (TLRs) expressed at their surface or within their endosomes [8]. For example, CpG-DNA and LPS respectively activate TLR9 and TLR4. Activation of TLRs contributes to ACLF by triggering an inflammatory response. Thus, LPS has been shown to contribute to cirrhosis, autoimmune hepatitis, primary biliary cirrhosis, alcoholic and fatty liver diseases both in humans and mouse models [9–11,5]. Among pro-inflammatory cytokines released by LPS activated KC, TNF-α has emerged as a key factor in the inflammatory process, as levels of soluble TNF receptors correlate with endotoxin influx and liver injury [12,13]. In mice, LPS intraperitoneal administration induces a systemic inflammatory response that can lead to death by septic shock, without inducing liver injury [14]. However, administration of LPS in a dietary non-alcoholic steatohepatits (NASH) model or in combination with the hepatotoxic D-Galactosamine (D-GalN) in mice induces hepatocyte apoptosis in a TNF-α-dependent manner [15,16]. D-GalN is metabolized into an active metabolite exclusively in hepatocytes and inhibits transcription by hepatic uridine nucleotide depletion [17]. Thus, it has been widely described that in the liver, NF-κB or transcription inhibition sensitizes hepatocytes to TNF-α-mediated apoptosis [18,16,19]. However, because of the pleiotropic role of TNF-α, anti-TNF-α therapeutic strategies in patients with alcoholic hepatitis are associated with an increased risk of infection and mortality [20,21]. The receptor interacting protein kinase 1 (RIPK1) play key roles in the signaling of several death receptors (DR), such as TNFR1, by deciding the cell fate between survival and death [22]. It has been recently reported that RIPK1 is implicated in epithelial cell death in the intestine in a TNF-α and bacterial dependent manner [23]. As its role in the liver is poorly understood, here by taking advantage of a mouse line with RIPK1 expression knocked out in liver parenchymal cells (*Ripk1*$^{LPC-KO}$), we address the potential role of RIPK1 in hepatocyte death induced by PAMPs and relayed by TNF-α.

Materials and methods

Animals and treatment protocols

Ripk1^{LPC-KO} mouse model has been previously developed [24]. LPS (Sigma-Aldrich, #L6761) diluted at 2 μg/ml in PBS was administrated by intraperitoneal (i.p.) route (20 μg/kg body weight and 200 μl/20 g). Unmethylated CpG oligodeoxynucleotide (ODN) (InvivoGen, ODN 2395) diluted at 3.5 mg/ml in PBS was administrated by i.p. route (2 mg/kg body weight and 200 μl/20 g). Etanercept (Pfizer) was administrated by i.p. route 1 h before LPS injection (10 mg/kg body weight and 200 μl/20 g). Two injections of liposomes-encapsulated dichloromethylene bisphosphonate (Cl_2MBP) (see below for the preparation) were administrated by i.p. route in mice. The first injection was given with 200 μl/20 g body weight, and the second, 24 h after the first, with 100 μl/20 g body weight. Mice were administrated with LPS or control (PBS) 48 h after first injection of liposomes-encapsulated Cl_2MBP and sacrificed 8.5 h after LPS injection. In all experiments, genetically modified mice were systematically compared to their littermates. Mature mice at 10–12 weeks of age were used for each experiments. Animals were housed in individually ventilated cages at the VIB Inflammation Research Center (Ghent, Belgium) in conventional animal facilities. All experiments on mice were conducted according to institutional, national and European animal regulations. *In vivo* protocols were approved by the ethics committee of Ghent University.

Genotyping

Genotyping was routinely performed using DNA extracted from tails with nucleospin tissue kit (Macherey Nagel, #740952), and PCR with couple of primers for Alfp-Cre gene. Forward and reverse primers sequences were 5′-GCC TGC ATT ACC GGT CGA TGC AAC GA-3′ and 5′-GTG GCA GAT GGC GCG GCA ACA CCA TT-3′, respectively.

Preparation of liposomes-encapsulated Cl_2MBP (Lip-Cl_2MBP)

Liposomes-encapsulated Cl_2MBP were prepared according to Rooijen *et al.* [25]. In brief, 86 mg of phosphatidylcholine (Sigma-Aldrich) and 9 mg of cholesterol (Sigma-Aldrich) were dissolved in chloroform in a round bottom flask. After 4 h on a gentle rotation on a rotary evaporator at 37 °C and under reduced pressure, the thin film formed was resuspended with 10 ml of PBS, and 1 g of Cl_2MBP (Sigma-Aldrich). After vigorous shaking for 1 min, liposomes in the saturated Cl_2-MBP solution were extruded through 1 μm diameter pores, followed by centrifugation at 10,000 g for 1 h. The Lip-Cl_2MBP were washed 3 times with PBS with centrifugations at 22,000 g during 30 min, and resuspended with 4 ml of PBS. The size measurement of Lip-Cl_2MBP was checked by Dynamic Light Scattering (DLS) technology (Zeta Sizer Nanoseries, Malvern) and as expected, 95% of the liposome had 1 μm diameter. The Lip-Cl_2MBP solution was diluted by half in PBS just before injection.

Histopathological and biochemical studies

Fragments of mouse livers were fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemistry (IHC) and hematoxylin and eosin (H&E). For histopathology, H&E staining of liver tissues was carried out to investigate liver injury. Serum alanine (ALT) and aspartate (AST) transaminases were measured according to the IFCC primary reference procedures using Olympus AU2700 Autoanalyser® (Olympus Optical, Tokyo, Japan).

Immunolocalization in liver tissues

For immunolocalization of cleaved caspase-3 (Cell Signaling) or myeloperoxidase (MPO) (Dako, #A0398) in liver tissues, paraffin-embedded mouse liver sections (5 μm) were dried 1 h at 58 °C, followed by antigen retrieval and incubated with primary antibody (Cell Signaling, 9661S) in a Ventana automated instrument (Ventana Medical Systems, USA). Revelation of primary antibody was carried out using horseradish peroxidase (HRP)-conjugated secondary antibody (Dako, USA) and DAB substrate kit (Ventana, #760-124). Slides were then counterstained with hematoxylin.

TUNEL analysis was performed on paraffin-embedded mouse liver sections (5 μm), incubated after antigen retrieval with a mix, composed of terminal transferase (Roche, #3333566011) and digoxigenin-11-UTP (Roche, #1558706) followed by HRP-anti-digoxigenin (Ventana, #760-4822). Revelation was done by Discovery Rhodamine kit (Ventana #760-233) according to the manufacturer guidelines, followed by nucleus labelling with DAPI.

All paraffin-embedded mouse liver sections were scanned with a digital slide scanner (Hamamatsu, Nanozoomer 2.0-RS) and files were analysed with the NDP viewer software.

TNF-α and F4/80 immunofluorescence staining were performed on cryosections of mouse liver tissues (7 μm). After fixation with 4% paraformaldehyde and NH_4Cl treatment, liver slides were blocked with 4% of BSA and then incubated with rabbit-anti-murine TNF-α (Abcam, #ab6671) and rat-anti-murine-F4/80 (eBioscience, #47-4801-80) at 4 °C overnight. Revelation of primary antibodies was carried out using DyLight 649-conjugated anti-Rabbit and DyLight 549 anti-rat IgG secondary antibodies (Jackson ImmunoResearch Laboratories). Nuclei were stained with Hoechst (Invitrogen, H3570). Slides were observed with a fluorescence microscope (Nikon's Eclipse Ni-E).

Isolation of liver F4/80 positive cells and RNA analysis

Liver from PBS or LPS treated mice was collected 1.5 h post-injection and crushed on a 70 μm filter. Liver immune cells were isolated after sedimentation and cell fractionation on a 35% Percoll layer and red blood cells were lysed with the ammonium-chloride-potassium (ACK) buffer. In order to isolate F4/80 positive cells, we used the MagniSort Mouse F4/80 positive selection kit (eBioScience, #8802-6863-74) and followed the manufacturer protocol. Quickly, liver immune cells were incubated with anti-F4/80 antibodies and biotin-magnetic beads. To