Research Article

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Lipocalin 2 drives neutrophilic inflammation in alcoholic liver disease

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Background & Aims: Alcoholic steatohepatitis (ASH) is characterised by neutrophil infiltration that contributes to hepatic injury and disease. Lipocalin-2 (LCN2) was originally identified as siderophore binding peptide in neutrophils, which exerted tissue protective effects in several disease models. Here we investigate the role of LCN2 in the pathogenesis of alcohol-induced liver injury.

Methods: We compared hepatic LCN2 expression in ASH patients, alcoholic cirrhosis patients without evidence of ASH and patients with non-alcoholic fatty liver disease (NAFLD; i.e. simple steatosis). To mechanistically dissect LCN2 function in alcohol-induced liver injury, we subjected wild-type (WT) and *Lcn2*-deficient ($Lcn2^{-/-}$) mice to the Lieber-DeCarli diet containing 5% ethanol (EtOH) or isocaloric maltose. Adoptive transfer experiments were performed to track neutrophil migration. Furthermore, we tested the effect of antibody-mediated LCN2 neutralisation in an acute model of ethanol-induced hepatic injury.

Results: Patients with ASH exhibited increased hepatic LCN2 immunoreactivity compared to patients with alcoholic cirrhosis or simple steatosis, which mainly localised to neutrophils. Similarly, ethanol-fed mice exhibited increased LCN2 expression that mainly localised to leukocytes and especially neutrophils. $Lcn2^{-/-}$ mice were protected from alcoholic liver disease (ALD) as demonstrated by reduced neutrophil infiltration, liver injury and hepatic steatosis compared to WT controls. Adoptive transfers revealed that neutrophil-derived LCN2 critically determines hepatic neutrophil immigration and persistence during chronic alcohol exposure. Antibody-mediated neutralisation of LCN2 protected from hepatic injury and neutrophilic infiltration after acute alcohol challenge.

Conclusions: LCN2 drives ethanol-induced neutrophilic inflammation and propagates the development of ALD. Despite a critical role for LCN2 in immunity and infection, pharmacological neutralisation of LCN2 might be of promise in ALD.

Keywords: Alcoholic liver disease; Hepatic inflammation; Neutrophils; Lipocalin 2; Alcoholic steatohepatitis (ASH).

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Abbreviations: ALD, alcoholic liver disease; ALT, alanine-aminotransferase; ASH, alcoholic steatohepatitis; AST, aspartat-aminotransferase; CFU, colony forming units; CRP, C-reactive protein; CXCL1/KC, chemokine (C-X-C motif) ligand 1/keratinocyte chemoattractant; CXCL2/MIP-2, chemokine (C-X-C motif) ligand 2/macrophage inflammatory protein 2; DAPI, 4′,6-diamidino-2-phenylindole; EtOH, Ethanol; FFPE, formalin-fixed paraffin-embedded; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GGT, gamma-glutamyl-transferase; H&E, hematoxylin and eosin; HPF, high power field; IL, Interleukin; INR, international normalized ratio; KC, Kupffer cell; LCN2/NGAL, Lipocalin 2/neutrophil gelatinase-associated lipocalin; LPS, lipopolysaccharide; MELD, model for end-stage liver disease; MPO, myeloperoxidase; NAFLD, non-alcoholic fatty liver disease; NGAL, neutrophil gelatinase-associated lipocalin; TLR, toll-like receptor; TNFα, tumour necrosis factor alpha; WT, wild-type.

Introduction

Alcohol consumption causes 3.8% of deaths worldwide and substantially contributes to a social and economic burden [1]. Alcoholic liver disease (ALD) displays a spectrum of disease phenotypes that ranges from hepatic steatosis to fibrosis and cirrhosis all of which may result in an acute hepatic inflammatory condition termed alcoholic steatohepatitis (ASH). A substantial proportion of ALD patients, especially heavy drinkers, develop ASH with a very poor prognosis [2]. Mechanistically, it has been suggested that alcohol-induced hepatocyte injury and Kupffer cell (KC) activation initiates a cytokine storm, which in turn propagates the recruitment of leukocytes, e.g. neutrophils that perpetuate hepatic inflammation [3–5]. Despite our growing biological understanding of ALD and the advent of biologicals in many

inflammatory diseases, the effective treatment of ASH remains challenging [6,7].

The pathogenesis of ASH appears to require multiple hits acquired during progression of disease. Alcohol-induced intestinal barrier dysfunction leads to systemic bacterial translocation (endotoxemia), which may present an early hit [8]. Bacterial products, such as lipopolysaccharide (LPS), activate hepatic resident macrophages, Kupffer cells (KCs), via Toll-like receptors (TLRs) [9]. Activated KCs produce high amounts of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNFα) and interleukin-1 beta (IL-1β) [10,11]. In response to TNFα and IL-1β, IL-8 and macrophage inflammatory protein 2 (MIP-2) guide neutrophil infiltration thereby promoting hepatic tissue inflammation [4]. In line with this, depletion of KCs protects against alcohol-induced liver injury [12], and antibody-mediated neutrophil depletion ameliorates alcoholic liver injury [13].

Lipocalin 2 (LCN2), also termed neutrophil gelatinase-associated lipocalin (NGAL), is an acute phase protein [14,15], highly expressed in myeloid and epithelial cells upon TLR or cytokine stimulation or ischemia/reperfusion injury [16–18]. Hepatocytes appear to express high levels of LCN2 upon stress [19,20], which may act as a danger signal to recruit immune cells such as neutrophils to the site of inflammation [21,22]. LCN2 protects against infections with certain Gram-negative bacteria by limiting siderophore-mediated bacterial iron acquisition [16,17]. In line with this, *Lcn2*$^{-/-}$ mice are more susceptible to sepsis [21], and LCN2 is critically involved in innate immune responses during infections [23]. In models of acute and chronic liver injury, LCN2 clearly exhibits protective functions [19,24], and *Lcn2*-deficient mice are more susceptible to non-alcoholic fatty liver disease (NAFLD) induced by a methionine-choline-deficient diet [25].

On the basis of a protective function of LCN2 in hepatic diseases, we hypothesized that *Lcn2*-deficiency renders mice susceptible to the development of alcohol-induced liver injury. Here, we demonstrate that LCN2^{+} neutrophils are increasingly found in human and mouse ALD. However, *Lcn2*-deficiency protects from ethanol-induced liver injury as LCN2 orchestrates hepatic infiltration of neutrophils. In line with this, pharmacological neutralisation of LCN2 blocks ethanol-induced hepatic infiltration of neutrophils and ameliorates liver injury.

Material and methods

Selection of patients

A total of eight randomly selected patients with histologically confirmed ASH included in the Infliximab study [26] were studied, and systemic and hepatic LCN2 expression was compared with nine NAFLD patients [27] and 10 patients with alcoholic cirrhosis without evidence of ASH (from now on referred to as cirrhosis). Histologically, all studied NAFLD patients exhibited simple steatosis without evidence of inflammation. Studies were performed in accordance with the Declaration of Helisinki and Austrian law and approved by the ethics committee of the Medical University Innsbruck.

Animal studies

C57BL/6 wild-type ('WT', CD45.1 or CD45.2) and *Lcn2*$^{-/-}$ mice [16], kindly provided by Dr. Shizu Akira, Osaka University, Japan, were bred in the specific pathogen free animal facility in Innsbruck and experiments were performed in compliance with the respective ethics approval (BMWF-66.011/0061-WF/II/3 b/2014). 6 to 8 week-old female WT and *Lcn2*$^{-/-}$ mice were subjected to the Lieber-DeCarli diet [28] (BioServ) with increasing ethanol concentrations up to 5% (vol/vol; 36% ethanol-derived calories; further termed "ethanol diet" or "EtOH-fed") or the control diet, which contained isocaloric amount of maltose (further termed "control diet" or "pair-fed"), both *ad libitum* for 2 weeks. Mice were weighed every other day and drinking amounts were monitored daily. In the acute alcohol model, mice received an intragastric dose of ethanol (31.5% (vol/vol); 5 g/kg body weight) using a 24-gauge stainless steel feeding tube as described previously [28]; control mice were administered an isocaloric amount of maltose dextrin (45% (wt/vol); 9 g/kg body weight) resolved in tap water. In LCN2 neutralisation experiments, WT mice were i.v. treated with the anti-LCN2-antibody MAB1857 (75 μg/mouse; R&D) or control antibody 30 min before gavage [29]. Mice were sacrificed four hours post-gavage. In all experiments mice were anaesthetized with xylazine (5 mg/kg) and ketamine (100 mg/kg) and blood and liver samples were collected. Plasma and liver samples were stored at −80 °C or in RNAlater (Qiagen) at −20 °C until further work-up or fixed in 10%-buffered formalin for histology.

Alanine aminotransferase (ALT) assay

Serum ALT was measured by a kinetic kit (BQ Kits). Briefly, plasma specimens were thawed on ice and 100 μl of ALT reagent was added to 10 μl of sample. Absorbance at 340 nm and 37 °C was measured five times at one minute intervals and mean absorbance change per minute was calculated (DA/min) to assess ALT activity.

RNA and protein expression analysis

RNA was purified after homogenization of tissue samples using TRIzol® reagent (Life Technologies). cDNA was transcribed with the Reverse Transcription System (Invitrogen). qPCR was performed using SybrGreen (Eurogentic) and the Mx3000 qPCR cycler (Agilent Technologies). PCR primer sequences are available upon request. Gene expression was normalised to *β-Actin* or *GAPDH* as indicated and means were visualised as hierarchically clustered heat maps (complete linkage algorithm, euclidean distance) using Genesis software [30].

For Western blot, hepatic protein was isolated using T-PER tissue protein extraction reagent supplemented with HALT proteinase inhibitor cocktail (Thermo Fisher Scientific Inc.). Protein concentrations were measured by BCA Protein Assay (Pierce), separated by SDS-PAGE (BioRad) and blotted onto Hybond-P PVDF membranes (GE Healthcare). The SNAP i.d. protein detection system (Millipore) was used for blocking, washing and antibody (anti-LCN2 goat polyclonal antibody, R&D) incubation. A chemiluminescent substrate visualised immunoreactivity on Amersham Hyperfilms (GE Healthcare). Band intensity was quantified with ImageJ and normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Serum (human study) and plasma (mouse study) samples were analysed for LCN2 by ELISA as recommended in the respective manual (mouse and human Lipocalin-2/NGAL DuoSet, R&D).

Histological analysis

Human and mouse liver sections were stained with hematoxylin and eosin (H&E) and analysed by an independent pathologist blinded to sample identity (M.D.); Oil Red O staining was performed on 5 μm frozen liver sections as specified in the respective manual (Amresco). Briefly, dried sections were fixed in 10% formalin and incubated in 100% Propylene Glycol for 10 min each. Slides were incubated in 60 °C pre-heated Oil Red solution (0.5%) for 10 min and then in 85% Propylene Glycol. Slides were counterstained in Mayer's Hematoxylin Solution (Sigma-Aldrich) for 30 s. Images were captured on a Zeiss AxioCam and analysed with ImageJ.

Immunohistochemistry and confocal immunofluorescence

FFPE sections were deparaffinised and rehydrated according to standard protocols. Sections were retrieved in Tris-EGTA-buffer (10 mM Tris, 0.5 mM EGTA, pH 9.0) for 30 min in a conventional steamer and peroxidase- (Dako) and serum-free protein blocked (Dako) each for 10 min at room temperature. Sections were then incubated with anti-LCN2 (polyclonal, R&D, Sigma-Aldrich), anti-Ly-6B.2 (clone 7/4, AbD Serotec), anti-MPO (clone MPO455-8E6, eBioscience) or anti-CD68 (clone PG-M1, Dako) for 1 h and incubated with secondary antibody (Vector laboratories, Dako). ImmPACT AMEC (Vector Laboratories) was used to visualise immunoreactivity. Sections were counterstained with Hematoxylin QS and images were captured with a Zeiss AxioCam and analysed with ImageJ. For confocal immunofluorescence, secondary antibodies coupled with Alexa Fluor 488 and 568 (Life Technologies) were used to visualise co-localisation on a Zeiss microscope and images were acquired with a Zeiss AxioCam.