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ORIGINAL RESEARCH—CLINICAL

Cirrhosis-Based Acute-on-Chronic Liver Failure Is Marked by Inflammation and Impaired Liver Regeneration Despite Stat3 Activation

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BACKGROUND AND AIMS: Acute-on-chronic liver failure (ACLF) is associated with excessive systemic inflammation, cell death, and organ failures. Yet, little is known about the hepatic histopathology of ACLF. Here, we assessed the histopathology and regenerative capacity of the liver in ACLF with or without cirrhosis. **METHODS:** Liver specimens of patients with compensated cirrhosis (N = 37), acute decompensation (N = 40), and ACLF with (N = 18) or without (N = 10) cirrhosis were assessed for morphological features and the pro-regenerative Stat3 pathway. **RESULTS:** ACLF was associated with high levels of lobular inflammation, tissue necrosis, and apoptosis. In patients with cirrhosis, the percentage of pStat3-positive hepatocytes was increasing with disease severity (3.5%/10.4%/21% for compensated cirrhosis/acute decompensation/cirrhosis-ACLF; $P < .001$), but lower in non-cirrhotic ACLF vs cirrhosis-ACLF (21% vs 13%; $P = .02$). A distinct pattern of the expression of the proliferation marker Ki-67, a downstream effector marker of pStat3, was observed. Ki-67–positive hepatocytes were more frequent in patients with cirrhosis-ACLF compared to compensated cirrhosis or acute decompensation (4.9% vs 1.3% vs 1.8%; $P < .05$), but much lower in cirrhosis-ACLF vs noncirrhotic ACLF (4.9% vs 13.5%; $P = .01$). The ratio of Ki-67–positive to pStat3-positive hepatocytes was lowest in cirrhosis-ACLF and predicted 3-month transplant-free survival accurately (area under the curve = 0.95, $P < .00001$). **CONCLUSION:** Our study identifies hepatic inflammation and Stat3 activation as hallmarks of ACLF. In cirrhosis-ACLF, Stat3 activation does not appear to translate in effective liver regeneration, which is distinct from non-cirrhotic ACLF.

Keywords: Liver Cirrhosis; Cell Death; Systemic Inflammation; Acute Decompensation

Acute-on-chronic liver failure (ACLF) is characterized by acute decompensation (AD) of cirrhosis in combination with distinct hepatic and extrahepatic organ failures.[1] ACLF is triggered by precipitating events such as infections or excessive alcohol consumption and is associated with high short-term mortality.[1] Decompensated cirrhosis and ACLF are associated with moderate and profound inflammation and activation of the immune system, respectively, which are evidenced, for example, by increased to excessive levels of danger-associated molecular patterns and pathogen-associated molecular patterns and proinflammatory and anti-inflammatory cytokines.[2] Of note, systemic inflammation in patients with decompensated cirrhosis and ACLF is accompanied by exhausted innate and adaptive cellular immune responses, which may explain the detrimental impact of infections on risk and course of ACLF.[3] It is believed that inflammation and immune dysfunction are important determinants of the pathogenesis of ACLF.

Cytokines which are strongly induced during the development of ACLF include—among others—interleukin (IL)-6, IL-22, or IL-1α/IL-1β.[2,4] While the inflammasome-driving cytokines IL-1α/IL-1β signal predominantly *via* the MYD88 pathway, IL-6 and IL-22 receptor signaling is mainly mediated through the Janus kinases (JAK)–signal transducer and activator of transcription (STAT) signaling pathway.[5,6] Both IL-6 and IL-22 have proinflammatory functions (which are strong in the case of IL-6) that are predominantly driven by Stat1 phosphorylation and which may be detrimental as drivers of ACLF-defining organ failures.[5,6] Yet, both cytokines also exert Stat3-mediated hepatoprotective effects by promoting liver regeneration and suppressing apoptosis.[7,8] Although the role of liver regeneration in the pathogenesis of ACLF is not clear, IL-22 might

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Abbreviations used in this paper: **ACLF, acute-on-chronic liver failure; AD, acute decompensation; AUC, area under the curve; CI, confidence interval; CLIF-OF, chronic liver failure–organ failure; HPF, high-power fields; MELD, model of end-stage liver disease; OR, odds ratio.**

Most current article

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be a promising therapeutic agent in advanced liver diseases, and intravenous IL-22 is under clinical evaluation in patients with alcoholic hepatitis.[9] Yet, it is unclear whether the assumed beneficial effects of IL-22 will translate into improved clinical outcomes, as high serum levels of IL-22 have been associated with adverse outcomes in patients with alcoholic and nonalcoholic liver disease and ACLF.[10,11] Importantly, IL-22 binding to its transmembrane receptor can be prevented by the IL-22 binding protein (IL-22BP), which is secreted excessively in patients with decompensated cirrhosis and ACLF.[12–16]

Despite these data, the role of Stat3-driven liver regeneration in the pathogenesis and outcome of ACLF is not clear. In the present study, we therefore assessed morphological features of liver specimens and the pro-regenerative Stat3 pathway in patients with the entire spectrum of advanced liver disease from compensated cirrhosis to ACLF.

Patients and Methods

Study Population

The present retrospective study included all consecutive adult patients with suspected liver cirrhosis in whom mini-laparoscopic liver biopsy was performed at the University Hospital Essen, Germany, from January 2010 to October 2018. Reasons for liver biopsies varied, that is, differentiation from drug-induced liver injury vs autoimmune hepatitis, confirmation of alcoholic hepatitis, and discrimination of acute liver failure from ACLF. Patients were excluded if no sufficient liver specimens were available (N = 2). Additional exclusion criteria were age below 18 years, presence of hepatocellular carcinoma or other active malignancies, infection with human immunodeficiency virus, or pregnancy.

The cohort was then categorized into 4 groups based on their clinical and histopathological features, namely (a) compensated cirrhosis, (b) AD of cirrhosis, (c) ACLF with cirrhosis according to the criteria of the European Foundation for the Study of Chronic Liver Failure consortium,[1] and (d) ACLF in patients with fibrosis grade 2–3 without cirrhosis. The presence of large ascites, hepatic encephalopathy defined by West-Haven criteria,[17] gastrointestinal hemorrhage, or bacterial infection (each) marked AD of cirrhosis. Liver-associated death or liver transplantation within 3 months after liver biopsy was defined as the primary endpoint.

The study was approved by the local ethic committee of the University Duisburg-Essen, Germany (reference number 18-8499-BO).

Clinical Data Collection

Clinical data and laboratory values were obtained from medical records at the time of liver biopsy (baseline). Demographic and clinical characteristics included gender, age, body mass index, underlying cause of liver disease, presence of portal vein thrombosis or diabetes, active alcohol consumption, presence of ascites, presence and grading of hepatic encephalopathy, gastrointestinal hemorrhage, infections, use of kidney replacement therapy or vasopressors (including terlipressin), PaO2/FiO2, systolic, diastolic, and mean blood pressure, admission to an intensive care unit or intermediate care unit, and ACLF grade. Recorded laboratory parameters included red and white blood cell count, platelet count, hemoglobin, C-reactive protein, serum sodium, serum creatinine, total bilirubin, serum alanine aminotransferase, serum aspartate aminotransferase, serum gamma-glutamyltransferase, alkaline phosphatase, international normalized ratio, and serum albumin. Chronic liver failure–organ failure (CLIF-OF) scores and model of end-stage liver disease (MELD) scores were calculated. The occurrence of liver-associated death or liver transplantation within 3 months after biopsy was recorded.

Histopathological Examination of Liver Samples

Liver fibrosis and cirrhosis were diagnosed according to the grading system by Desmet et al.[18] Histopathological parameters were retrospectively assessed on hematoxylin and eosin– and Masson-Goldner-Elastica–stained liver tissues in a blinded fashion by 2 observers (J.R. and K.A.-J.). The following histopathological features were assessed: grade of fibrosis, percentage of macrovesicular steatosis, lobular inflammation as foci of at least 3 inflammatory cells per 10 high-power fields (HPF, 40× magnification), granulocytic or lymphocytic composition of lobular inflammation, presence of portal/septal inflammation and its composition (granulocytic vs lymphocytic), presence of interface hepatitis, number of apoptosis per 10 HPF (40×), percentage of necrosis assessing the whole tissue slide, hepatocyte ballooning (grade 0–2), Mallory-Denk bodies per 10 HPF (40×), nuclear inclusions (type 1 vacuoles as described by Schwertheim et al[19]) per 10 HPF (40×), and presence of ductal and ductular proliferation. More in detail, apoptotic cells were defined as eosinophilic condensated cells with a karyorrhectic or pycnotic nucleus or loss of an identifiable nucleus.

Ballooning of hepatocytes was graded as 0 = no ballooning, 1, or 2. Category 1 was defined as at least small clusters of enlarged foamy degenerated pale hepatocytes. Category 2 additionally included the enlargement of hepatocytes to twice the size of normal hepatocytes.

Immunohistochemistry

Paraffin sections were used for immunochemistry staining. Antibodies were tested in paraffin-embedded human hepatoma cell lines (HepG2 cells) stimulated with IL22 (100 ng/mL), interfern (50 ng/mL), or vehicle. The following antibodies were used (details in Table A1): anti-phospho-STAT3 (Tyr705, #CS9145), anti-STAT3 (#CS9139), anti-IL10 receptorβ (R&D #AF874), and anti-IL22 receptor α1 (#sc-134366). Cell proliferation was assessed using an antibody against Ki-67 (Roche Ventana 5278384001).

Nuclear staining (pSTAT3, Ki-67) was quantified by Leica image analysis software (Aperio ImageScope) after digitalizing the stained slides. The percentage of positive hepatocyte nuclei in relation to all hepatocyte nuclei was calculated.

For all other antibodies, only liver parenchyma (hepatocytes) without fibrotic areas was analyzed. The immunoreactivity of IL22Rα1 was determined as weak or strong. For IL10Rβ, the immunoreactivity of the cell membrane was analyzed by the following categories: 0%, <10%, and >10%. For STAT3, immunoreactivity of the cytoplasm was analyzed.

Table 1. Baseline Characteristics and Laboratory Results of Included Patients

Variable	Compensated cirrhosis (N = 37)	Acute decompensation (N = 40)	ACLF with cirrhosis (N = 18)	ACLF without cirrhosis (N = 10)[c]	*P*-value (all groups[a])	*P*-value (Decom. vs ACLF cirrhosis/ACLF with vs without cirrhosis[b])
Age (y), mean (SD)	54 (15)	53 (13)	54 (9)	40 (18)	.2	.8/**.09**
Male gender, N (%)	22 (59.5)	15 (37.5)	6 (33.3)	2 (20)	.06	.8/.5
BMI (kg/m^2), mean (SD)	27.3 (5.4) [N = 36]	25.2 (4.8) [N = 39]	26 (4.3)	27.3 (6.2) [N = 9]	.4	.5/.7
Diabetes, N (%)	12 (32.4)	2 (5)	1 (5.6)	0 (0)	.001	.2/.4
Underlying liver disease						
Alcohol, N (%)	8 (21.6)	8 (20)	9 (50)	1 (10)	.05	**.02/.03**
NASH, N (%)	6 (16.2)	2 (5)	0 (0)	0 (0)	.09	.3/1.0
HCV/HBV, N (%)	10 (27)	3 (7.5)	1 (5.6)	0 (0)	.02	.8/.4
Cholestatic, N (%)	1 (2.7)	1 (2.5)	0 (0)	0 (0)	.9	.5/1.0
AIH, N (%)	3 (8.1)	10 (25)	2 (11.1)	4 (40)	.05	.2/**.07**
Cryptogenic, N (%)	9 (24.3)	16 (40)	6 (33.3)	5 (50)	.4	.6/.4
Active alcohol consumption, N (%)	5 (13.5)	12 (30)	10 (55.6)	3 (30)	.01	.06/.2
Complications of liver cirrhosis, organ failures						
Portal vein thrombosis, N (%)	1 (2.7)	2 (5)	0 (0)	0 (0)	.7	.3/1.0
Gastrointestinal bleeding, N (%)	0 (0)	4 (10)	3 (16.7)	0 (0)	.07	.5/.2
Infection, N (%)	0 (0)	14 (35)	6 (33.3)	3 (30)	**.001**	.9/.9
Presence of ascites, N (%)	0 (0)	30 (75)	12 (66.7)	2 (20)	**<.000001**	.5/**.02**
Hepatic encephalopathy (HE)						
Grade 0, N (%)	37 (100)	39 (97.5)	6 (33.3)	0 (0)	**<.000001**	**<.000001/.04**
Grade I/II, N (%)	0 (0)	1 (2.5)	11 (61.1)	8 (80)	**<.000001**/.3	
Grade III/IV, N (%)	0 (0)	0 (0)	1 (5.6)	2 (20)	**.004**	.1/.2
Kidney and other organ failures						
Creatinine <2 mg/dL, N (%)	37 (100)	38 (95)	12 (66.7)	9 (90)	**.002**	**.004**/.2
Creatinine 2–3.4 mg/dL, N (%)	0 (0)	1 (2.5)	2 (11.1)	0 (0)	.1	.2/.3
Creatinine ≥3.5 mg/dL or RRT, N (%)	0 (0)	0 (0)	4 (22.2)	1 (10)	**.0009**	**.001**/.3
Circulatory failure, N (%)	0 (0)	0 (0) [N = 39]	5 (27.8) [N = 17]	2 (20)	**.0001**	**.0004**/.6
Respiratory failure, N (%)	0 (0)	0 (0)	1 (5.6)	1 (10)	.1	.1/.6
Admission to the ICU, N (%)	2 (5.4)	7 (17.5)	11 (61.1)	5 (50)	**.00001**	**.0009**/.6
Laboratory data						
Leucocytes (/nL), mean (SD)	5.48 (2)	7.08 (2.81)	10.8 (5.78)	13.74 (11.96)	**.0001**	**.02**/.7
Hemoglobin (g/dL), mean (SD)	12.4 (3.46)	11.28 (2.64)	11.46 (1.89)	12.12 (1.8)	.05	.9/.4
Platelets (/nL), mean (SD)	118.08 (54.07)	150.98 (83.27)	175.56 (92.31)	265.2 (138.72)	**.002**	.2/.2
CRP (mg/dL), mean (SD)	0.66 [N = 35]	4.04 (12.72)	3.28 (2.2)	2.5 (2.8)	**<.000001**	**.009**/.2
Sodium (mmol/L), mean (SD)	140.81 (3.05)	137.75 (4.27)	135.78 (4.97)	136.4 (4.22)	**.000006**	**.04**/.49
Bilirubin (mg/dL), mean (SD)	1.36 (0.77)	5.21 (4.91)	14.52 (9.55)	19.79 (5.72)	**.000000**	**.0001**/.1
AST (U/L), mean (SD)	105 (98) [N = 29]	129 (130)	970 (1647)	880 (925)	**.0006**	.07/.3
INR, mean (SD)	1.16 (0.13) [N = 36]	1.31 (0.28)	2.14 (1.06)	1.53 (0.48)	**.000002**	**.002**/.1
Albumin (g/dL), mean (SD)	3.97 (0.45) [N = 31]	3.29 (0.6) [N = 33]	3.73 (1.37) [N = 17]	2.97 (0.84) [N = 9]	**.00004**	.5/.4

Table 1. Continued

Variable	Compensated cirrhosis (N = 37)	Acute decompensation (N = 40)	ACLF with cirrhosis (N = 18)	ACLF without cirrhosis (N = 10)[c]	*P*-value (all groups[a])	*P*-value (Decom. vs ACLF cirrhosis/ACLF with vs without cirrhosis[b])
Scores						
CLIF-OF score, mean (SD)	6 .09 (0.28) (N = 35)	6.58 (0.89)	10 (1.93) (N = 17)	10 (2.6)	**<.000001**	**<.000001**/.3
MELD score (SD)	10 (2)	15 (4)	26 (7)	24 (6)	**<.000001**	**<.000001**/.3

γGT, γ-glutamyl transferase; AIH, autoimmune hepatitis; ALT, alanine aminotransferase; AP, alkaline phosphatase; AST, aspartate aminotransferase; BMI, body mass index; CRP, C-reactive protein; HBV, hepatitis B virus; HCV, hepatitis C virus; ICU, intensive care unit; INR, international normalized ratio; NASH, non-alcoholic fatty liver disease; RRT, renal replacement therapy; SD, standard deviation.
[a]Kruskal-Wallis test or chi^2 test, as appropriate.
[b]Wilcoxon-Mann-Whitney-U test or chi^2 test, as appropriate.
[c]4 and 6 patients had F2 and F3 fibrosis, respectively.

Granular positive signals in the cytoplasm were quantified as % of hepatocytes.

Statistical Analyses

Statistical analyses were performed using BiAS, Version 11.06, and Graphpad PRISM5. Group differences were assessed by means of χ^2 contingency tables, Wilcoxon-Mann-Whitney-U tests, or Kruskal-Wallis test, as appropriate. For statistical significance, a threshold of $P < .05$ was chosen. Moreover, univariate and multivariate regression models were assessed using a P value $> .10$ for removal from the model, and corresponding odds ratios (ORs) as well as 95% confidence intervals (CIs) were determined. In addition, the discrimination ability of CLIF-OF score, Meld score, and Ki-67/pSTAT3 ratio to predict 3-month survival and transplant-free survival was evaluated by calculating the area under the curve (AUC).

Results

Patient Characteristics

Overall, 105 patients who fulfilled the described selection criteria were included in our study. At the time of the liver biopsy, 37 (35.2%) patients had compensated cirrhosis, 40 (38%) patients had AD of cirrhosis, 18 (17%) patients had ACLF with cirrhosis, and 10 (10%) patients had ACLF without cirrhosis (but advanced fibrosis). Among patients with cirrhosis-ACLF, 61.1%, 16.7%, and 22.2% had ACLF grade 1, 2, and 3, respectively, whereas 80%, 0%, and 20% of patients with noncirrhotic ACLF had ACLF grade 1, 2, and 3, respectively. Infections were frequent in patients with AD, cirrhosis-ACLF, and noncirrhotic ACLF (35%, 33.3%, and 30%). During 3 months of follow-up, 0 patients (0%), 3 patients (7.5%), 3 patients (16.7%), and 1 patient (10%) with compensated cirrhosis, AD, cirrhosis-ACLF, and noncirrhotic ACLF died. In addition, 2 (5%) patients with AD and 2 patients with cirrhosis-ACLF underwent liver transplantation during 3 months of follow-up. Detailed baseline characteristics are shown in Table 1.

Histopathological Features of Cirrhosis-ACLF and Noncirrhotic ACLF

First, a detailed analysis of histopathological features of all stages of liver cirrhosis as well as of noncirrhotic ACLF was performed (Figure 1). A different magnitude and pattern of hepatic inflammation was observed between patient groups. Lobular inflammation foci were most frequently observed in patients with cirrhosis ACLF (19.94/10 HPF), compared to 6.08/HPF, 7.59/HPF, and 7.87/HPF in patients with compensated cirrhosis, AD, and noncirrhotic ACLF ($P = .00004$). Of note, lobular inflammation was frequently granulocytic in ACLF (88.9% in cirrhosis-ACLF and 70% in noncirrhotic ACLF), but less frequent in compensated cirrhosis (43.2%) or decompensated cirrhosis (40%); $P = .002$. In contrast,

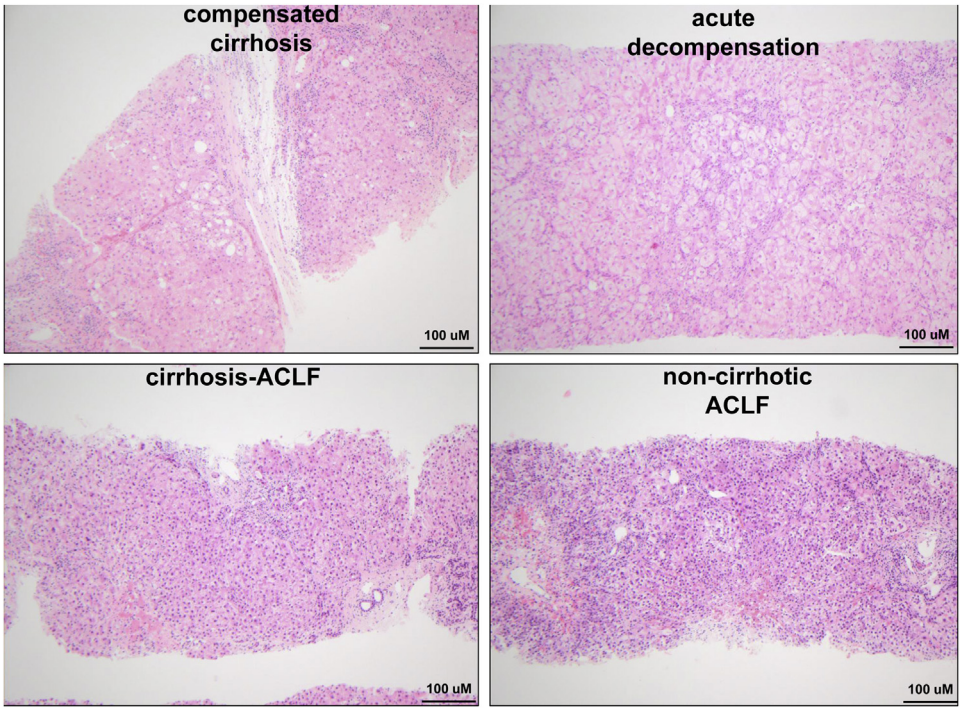

Figure 1. Histopathological features of cirrhosis-ACLF and noncirrhotic ACLF. Representative images of H&E-stained liver specimens of patients with compensated cirrhosis, acute decompensation, cirrhosis-ACLF, or noncirrhotic ACLF are shown. H&E, hematoxylin and eosin.

lymphocytic lobular inflammation was rarely observed in cirrhosis-ACLF (5.6%), compared to 91.9%, 77.5%, and 61.1% in patients with compensated cirrhosis, AD, and noncirrhotic ACLF ($P = .002$). In contrast to lobular inflammation, portal/septal inflammation was common in all patient groups. The extent of hepatocyte cell death was another distinctive feature of ACLF. Both apoptosis and necrosis were rare in compensated cirrhosis, more frequently observed in AD, and most frequent in patients with ACLF ($P = .00008$ for apoptosis and 0.002 for necrosis). Ductal and ductular proliferation was detected with increasing frequency from compensated cirrhosis to decompensated cirrhosis and cirrhosis-ACLF, but rarely in noncirrhotic ACLF. Mallory Denk bodies were most frequent in cirrhosis-ACLF and rare in noncirrhotic ACFL. No significant differences between patient groups were observed for macrovesicular steatosis, nuclear inclusions, and hepatocyte ballooning. Details of histopathological results are summarized in Table 2.

Dissociation of STAT3 Activation and Hepatocyte Proliferation (Ki-67) in Patients With Cirrhosis-ACLF and Noncirrhotic ACLF

Next, we quantified expression of Stat3 in hepatocytes, a key mediator of liver regeneration. Total Stat3 increased with severity of liver cirrhosis, with highest levels in patients with cirrhosis-ACLF, while total Stat3 levels were comparable in patients with cirrhosis-ACLF and noncirrhotic ACLF (Figure 2A). Since IL-22 is an important cytokine to mediate hepatic regeneration in liver diseases and a key inducer of pStat3—the active form of Stat3—we also quantified the components of the IL-22 receptor. Both the IL-22R1 and IL10-Rb chains were detected on the majority of hepatocytes, though no significant differences were observed between patient groups (Figure A1).

Compared to compensated liver cirrhosis (3.55% pos. hepatocytes), the frequency of hepatocytes with detectable pStat3 (ie, activated Stat3) was significantly higher in patients with AD (mean = 10.5%) and highest in patients with cirrhosis-ACLF (mean = 21%); $P < .00001$ (Figure 2B). Of note, pStat3 levels were significantly higher in cirrhosis-ACLF than those in noncirrhotic ACLF (21% vs 12.8%, $P = .02$) (Figure 2B). In contrast, a distinct pattern of the expression of Ki-67, a downstream effector marker of pStat3, was observed. While again a progressive increase of Ki-67–positive hepatocytes was observed in patients with compensated liver cirrhosis vs AD vs cirrhosis-ACLF (mean = 1.33% vs 1.9% vs 4.9%, $P = .03$ for AD vs cirrhosis-ACLF), patients with noncirrhotic ACLF had significantly higher frequencies of Ki-67–positive hepatocytes than patients with cirrhosis-ACLF (mean = 13.5% vs 4.9%, $P = .01$) (Figure 2C).

Due to the importance of infections and alcoholic hepatitis as precipitating events of ACLF, we assessed the influence of these scenarios on Stat3 and Ki-67 expression. Total Stat3, pStat3, and Ki-67 levels were similar in patients with or without active alcoholism (Figure A2A). In contrast, pStat3-positive hepatocytes were more frequently observed in patients with vs without infections, which, however, was not associated with a relevant increase of Ki-67 expression during infections (Figure A2B).

Collectively, these data suggest a dissociation of pStat3 and Ki-67 expression (ie, Stat3 activation not fully

Table 2. Histopathological Features of Patients With or Without ACLF

Variable	Compensated cirrhosis (N = 37)	Acute decompensation (N = 40)	ACLF with cirrhosis (N = 18)	ACLF without cirrhosis (N = 10)	*P*-value (all groups)	*P*-value (Decom. vs ACLF cirrhosis/ACLF with vs without cirrhosis)
Fibrosis grade 4, N (%)	37 (100)	40 (100)	18 (100)	0 (0)	**<.000001**	1.0/**<.000001**
Fibrosis grade 2–3, N (%)	0 (0)	0 (0)	0 (0)	10 (100)	**<.000001**	1.0/**<.000001**
Macrovesicular steatosis, mean % (SD)	7.7 (12.55)	9.74 (18.01) (N = 39)	9.56 (16.71)	5.1 (11.5)	.4	.9/.1
Mallory Denk bodies per 10 HPF (40×), mean (SD)	9.19 (7.22) (N = 36)	8.47 (7.37) (N = 38)	15.56 (13.71)	5.1 (3.57)	.07	.06/**.02**
Nuclear inclusions (type 1 vacuoles per 10 HPF), mean (SD)	5.58 (9.6) (N = 36)	4.21 (4.12) (N = 39)	2.83 (2.88)	1.9 (4.01)	.05	.2/.2
Presence of ductal and ductular proliferation, N (%)	16 (43.2)	24 (60)	13 (72.2)	2 (20)	**.03**	.5/.02
Presence and composition of lobular inflammation						
Lobular inflammation (number of foci per 10 HPF), mean (SD)	6.08 (4.5) (N = 36)	7.59 (6.19) (N = 39)	19.94 (17.98)	13.8 (7.87)	**.00004**	**.001**/.5
Granulocytic, N (%)	16 (43.2) (N = 34)	16 (40) (N = 39)	16 (88.9) (N = 17)	7 (70)	**.002**	**.0007**/.2
Lymphocytic, N (%)	18 (48.6) (N = 34)	23 (57.5) (N = 39)	1 (5.6) (N = 17)	3 (30)	**.002**	**.0002**/.2
Presence and composition of portal/septal inflammation						
Presence of portal/septal inflammation, N (%)	35 (94.6)	39 (97.5)	18 (100)	10 (100)	.6	.5/1.0
Granulocytic, N (%)	1 (2.7) (N = 35)	8 (20) (N = 39)	7 (38.9)	5 (50)	**.001**	.1/.6
Lymphocytic, N (%)	34 (91.9) (N = 35)	31 (77.5) (N = 39)	11 (61.1)	5 (50)	**.001**	.1/.6
Presence of interface hepatitis, N (%)	5 (13.5)	8 (20) (N = 39)	2 (11.1)	2 (20)	.8	.4/.5
Apoptosis/necrosis						
Apoptotic bodies per 10 HPF, mean (SD)	2.86 (2.09) (N = 36)	3.9 (2.56) (N = 39)	6 (2.87)	7.6 (4.97)	**.00008**	**.005**/.40
Percentage of necrosis in relation to liver tissue, mean (SD)	0.59 (1.09)	1.95 (3.59) (N = 39)	3.56 (4.82)	6 (6.02)	**.002**	.08/.3

Table 2. Continued

Variable	Compensated cirrhosis (N = 37)	Acute decompensation (N = 40)	ACLF with cirrhosis (N = 18)	ACLF without cirrhosis (N = 10)	*P*-value (all groups)	*P*-value (Decom. vs ACLF cirrhosis/ACLF with vs without cirrhosis)
Hepatocyte ballooning (score 0–2)						
0, N (%)	1 (2.7)	5 (12.5) (N = 39)	2 (11.1)	2 (20)	.3	.9/.7
1, N (%)	29 (78.4)	25 (62.5) (N = 39)	10 (55.6)	7 (70)	.3	.5/.5
2, N (%)	7 (18.9)	9 (22.5) (N = 39)	6 (33.3)	1 (10)	.5	.4/.2

SD, standard deviation.
Significant values are indicated in bold.

translating into hepatocyte proliferation, as assessed by Ki-67 staining) in patients with cirrhosis-ACLF compared to noncirrhotic ACLF. For further illustration, ratios of Ki-67–positive to pStat3-positive hepatocytes were calculated. One can note lower ratios of Ki-67/pStat3 in patients with AD (mean = 0.36) and cirrhosis-ACLF (mean = 0.29) than in patients with compensated cirrhosis (mean = 1.7; $P <$ .001 each), while the Ki-67/pStat3 ratio was significantly higher in patients with noncirrhotic ACLF than that in patients with cirrhosis-ACLF (mean = 1.28 vs 0.29, $P = .003$) (Figure 2D).

Upregulated pSTAT3 and Inadequately Low Ki-67 Levels Are Associated With Adverse Outcomes

We next assessed the association between pStat3 and Ki-67 levels with 3-month survival. In the entire cohort, a significant inverse association with 3-month survival (Figure 3A) as well as transplant-free survival (Figure 3B) was observed, whereas Ki-67 expression was not associated with survival. Yet, in the subgroup of patients with ACLF, Ki-67 expression was significantly higher in survivors than that in patients who died or received a liver transplant within 3 months, whereas pStat3 expression was high in both survivors and nonsurvivors in the ACLF group (Figure 3). As a consequence, Ki-67/pStat3 ratios were significantly associated with survival (mean ratio = 0.97 vs 0.13 for survivors vs death, $P = .008$) and transplant-free survival (mean ratio = 1.0 vs 0.19 for survivors vs death or Tx, $P = .009$) in the entire cohort, as well as in the subgroup of ACLF patients (mean ratio = 0.74 vs 0.05 for survivors vs death, $P = .008$; and 0.78 vs 0.14 for survivors vs death or Tx, $P = .01$) (Figure 4). In view of the strength of this association, AUCs for 3-month transplant-free survival were calculated for Ki-67/pStat3 ratio, MELD scores, and CLIF-OF scores. As shown in Figure 4C, AUCs were comparable for these predictors in the entire cohort, while the Ki-67/pStat3 ratio outperformed both the CLIF-OF and MELD scores in the subgroup of patients with ACLF (AUC = 0.85, $P = .0006$ for CLIF-OF; AUC = 0.75, $P = .09$ for MELD; AUC = 0.95, $P <$ .00001 for pStat3/Ki-67 ratio; Figure 4D). Furthermore, moderate and strong correlations between the Ki67/pStat3 ratio and MELD scores/CLIF-OF scores and CLIF C ACLF scores, respectively, were observed (Table A3).

Finally, logistic regression analysis of 3-month transplant-free survival was performed. Univariate analysis revealed significant associations of presence of ACLF (OR = 4.14, 95% CI = 1.12–15.1, $P = .03$), MELD score (OR = 1.11, 95% CI = 1.04–1.20, $P = .02$), and Ki-67-to-pStat3 ratio (OR = 0.61, 95% CI = 0.41–0.89, $P = .01$) with the risk of death or liver transplantation within 3 months. Furthermore, the MELD score ($P = .02$) and Ki-67-to-pStat3 ratio ($P = .05$) remained as independent predictors of 3-month mortality or transplantation in multivariate analysis (Table A2).

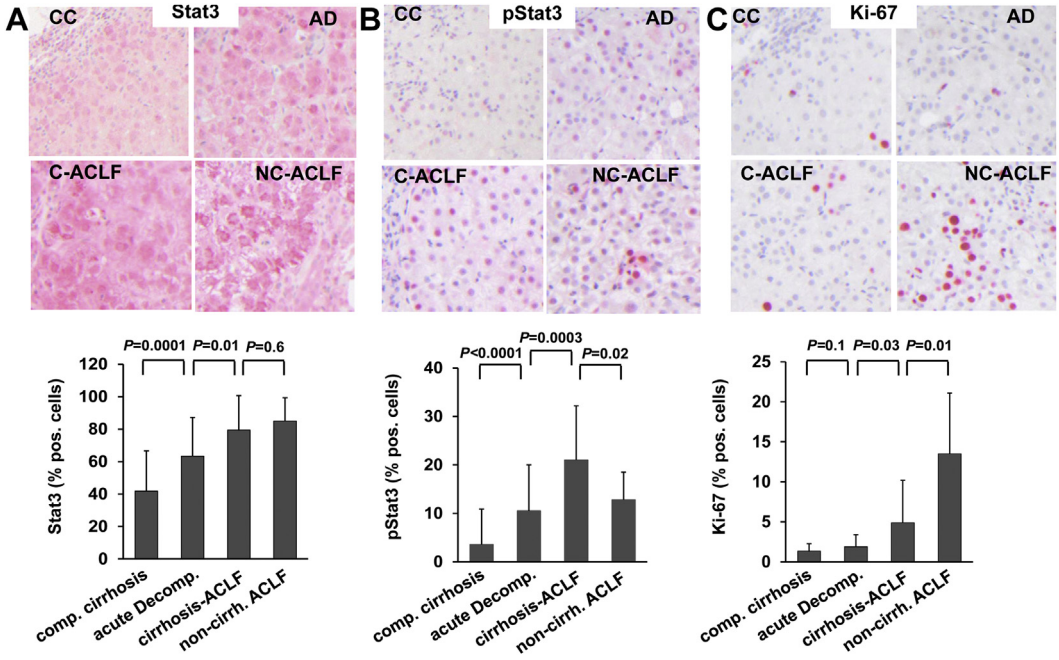

Figure 2. Dissociation of Stat3 activation and liver proliferation in cirrhosis-ACLF vs noncirrhotic ACLF. Representative images of immunhistochemical staining of liver specimens (upper panel) and mean values of positively stained hepatocytes for total Stat3 (A), pStat3 (B), and Ki-67 (C) (lower panel) of patients with compensated cirrhosis, acute decompensation, cirrhosis-ACLF, or noncirrhotic ACLF. The ratio of Ki-67–positive to pStat3-positive hepatocytes is shown in (D). C-ACLF, cirrhosis-ACLF; CC, compensated cirrhosis; NC-ACLF, noncirrhotic ACLF.

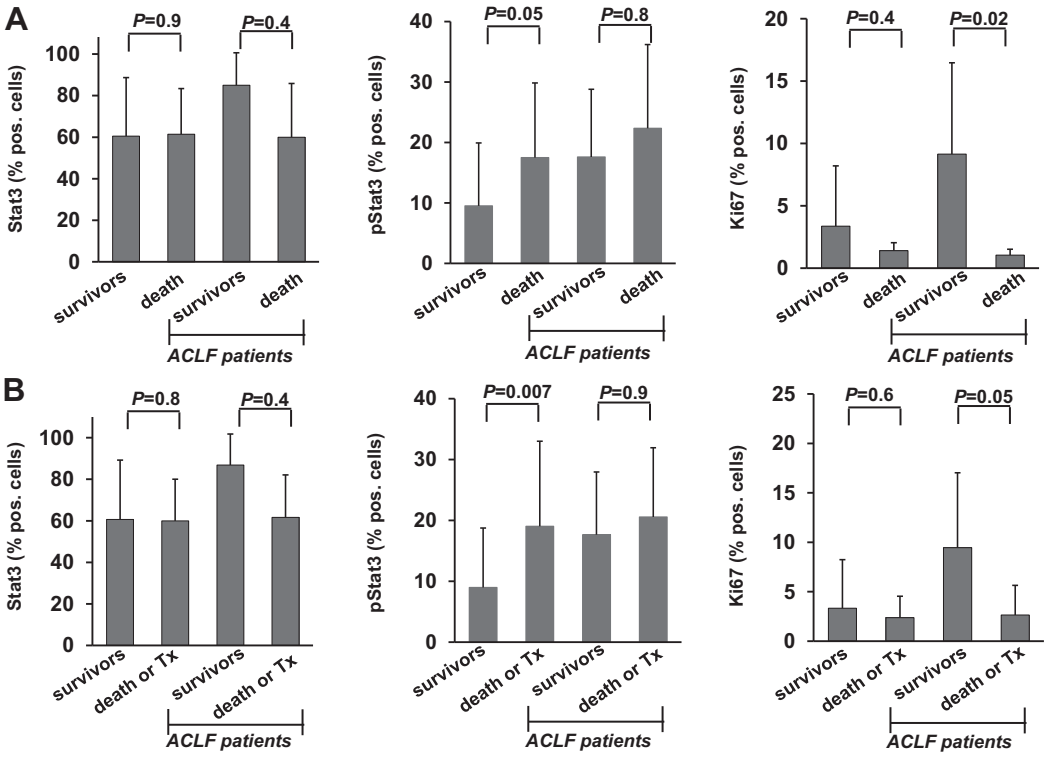

Figure 3. Stat3 activation and low Ki-67 staining are associated with mortality. Mean values of positively stained hepatocytes for total Stat3, pStat3, and Ki-67 are shown according to 3-month survival (A), as well as for 3-month transplant-free survival (B). Data are shown for the entire cohort (left bars of the graphs), as well as for the subgroup of patients with ACLF only (right bars of the graphs). *P* values were calculated with the Wilcoxon-Mann-Whitney-U test. Tx, transplantation.

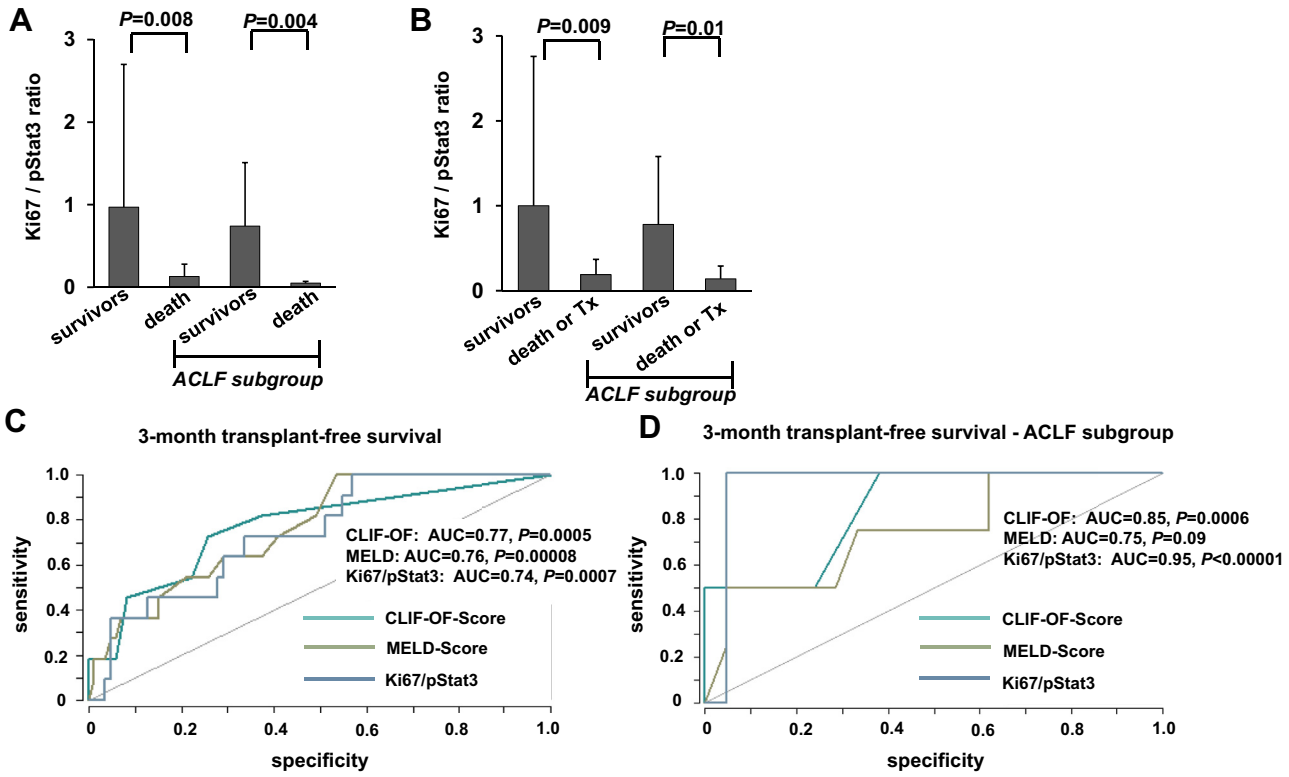

Figure 4. The Ki-67-to-Stat3 ratio is predictive for survival. The ratio of Ki-67–positive to pStat3-positive hepatocytes is shown according to 3-month survival (A), as well as for 3-month transplant-free survival (B). Data are shown for the entire cohort (left bars of the graphs), as well as for the subgroup of patients with ACLF only (right bars of the graphs). *P* values were calculated with the Wilcoxon-Mann-Whitney-U test. AUCs for 3-month transplant-free survival were calculated for the entire cohort (C) and for the subgroup of patients with ACLF (D) for the CLIF-OF score, the MELD score, and the Ki-67/pStat3 ratio. Tx, transplantation.

Discussion

In the present study, we provide a detailed analysis of histomorphological features as well as of components of the pro-regenerative Stat3 pathway throughout the entire spectrum of liver cirrhosis. The main results of our study are that (i) on a morphological basis, ACLF is associated with increased lobular inflammation and cell death compared to compensated liver cirrhosis and AD, (ii) the frequency of pStat3-positive hepatocytes is strongly increasing in patients with cirrhosis from compensated cirrhosis to AD and finally ACLF—which is paralleled by a less pronounced increase of cell proliferation, (iii) as a consequence, a low ratio of Ki-67/pStat3 is strongly predictive for death or need for liver transplantation, and (iv) ACLF in patients with advanced fibrosis appears to be distinct from ACLF on the basis of complete cirrhosis by having a higher regenerative capacity, as assessed by the Ki-67/pStat3 ratio.

Systemic inflammation and immune paralysis as well as impaired liver regeneration are considered as important components of the pathogenesis of ACLF.[20] Yet, histopathological data of livers of patients with ACLF are sparse. An important study published in 2009 identified histological features including bilirubinostasis, cholangiolitis, the presence of Mallory bodies, and hepatocyte ballooning associated with ACLF in patients with alcoholic cirrhosis.[21] In contrast, lobular and portal inflammation as well as the number of proliferating hepatocytes was not associated with ACLF compared to chronic hepatic decompensation. However, in this study, the more recently established EASL CLIF definition of ACLF has not been applied. This may explain differences to our study, where in particular, lobular inflammation, apoptosis, and necrosis were characteristic of ACLF according to the EASL CLIF definition. The finding that local hepatic inflammation (and not only well-described systemic inflammatory responses) is a hallmark of ACLF adds an important piece to the understanding of the pathogenesis of ACLF, and future studies should aim at a detailed understanding of the cellular composition and action of inflammatory infiltrates in ACLF. In this regard, promising examples were the previous identification of an accumulation of immunosuppressive MERTK+ monocytes in ACLF, as well as of a specific Kupffer cell subset in the distinct entity of acute liver failure.[22,23]

There is also a crosslink between inflammation and impaired liver regeneration, which was shown, for example, in a recent elegant study in infection-triggered murine ACLF, where the IL-22/IL-6 Stat3 pathway was shifted toward adverse Stat1 activation upon bacterial infection.[24] This

phenomenon could be rescued in animals by administration of recombinant IL-22Fc.[24] In our study, infections were associated with increased Stat3-phoshorylation, but not with increased cell proliferation, as determined by Ki-67 staining. In this regard, our observation of massive activation of Stat3 in cirrhosis-ACLF, which is not accompanied by an appropriate increase of Ki-67–positive hepatocytes as a measure of liver regeneration, adds a hint for possible future therapeutic strategies to enhance liver regeneration in cirrhosis-ACLF. Such strategies may need to overcome yet to be specified inhibitory (likely inflammatory) regulators of Stat3 signaling and liver regeneration. Yet, it is important to acknowledge that the underlying mechanism of insufficient liver regeneration in cirrhosis-ACLF despite Stat3 activation remains to be elucidated. Stat3 can be engaged by pro-regenerative cytokines such as IL-22 or IL-6, but also by proinflammatory or anti-inflammatory cytokines like interfern-γ or IL-10. Stat3 activation is therefore possible in cirrhosis-ACLF rather integrated proinflammatory than pro-regenerative stimuli, although the expression of the IL-22 receptor was not impaired in these patients.

Interestingly, the presence of cirrhosis appears to be an important determinant of suboptimal liver regeneration, as patients with ACLF on the basis of advanced fibrosis (but in the absence of complete cirrhosis, here termed "non-cirrhotic ACLF") show a remarkably higher frequency of Ki-67–positive hepatocytes as well as a higher ratio of Ki-67- to pStat3-positive hepatocytes than patients with cirrhosis-ACLF. Beyond their potential pathophysiological and therapeutic implications, our findings may also provide an argument that the (recently debated) prerequisite of the presence of liver cirrhosis for the EASL CLIF definition of ACLF is pathophysiologically justified.[25] In this regard, it is worth mentioning that also in a study of patients with ACLF according to the APASL definition, low numbers of Ki-67–positive hepatocytes were associated with mortality, though in this study, no comparison between advanced fibrosis and cirrhosis has been provided.[26]

Our study has limitations. First of all, no stored serum samples are available for this cohort. Hence, we are not able to measure cytokines (namely IL-6 and IL-22) inducing Stat3 phosphorylation and cannot directly correlate Stat3 phosphorylation with serum concentrations of these cytokines. Yet, it is very well known that both IL-6 and IL-22 are moderately increased in patients with AD compared to compensated cirrhosis and strongly increased in patients with ACLF.[2,16] Another limitation of our study is the relatively small number of patients with cirrhosis-ACLF and noncirrhotic ACLF, which, for example, does not allow a reliable subanalysis of different etiologies of ACLF. However, there is no general indication for liver biopsy in these liver diseases, making it difficult to establish a larger tissue bank even in liver centers. Finally, it is important to note that not all patients with AD or ACLF have received a liver biopsy in our center. Hence, the described retrospective cohort represents a selected cohort of patients in whom liver biopsy was performed due to various reasons, representing a possible source of selection bias.

In conclusion, our study reveals hepatic lobular inflammation as well as hepatocellular Stat3 activation as hallmarks of ACLF. Yet, in cirrhosis-ACLF, pStat3 activation is accompanied by inadequately low liver regeneration, which is distinct to noncirrhotic ACLF.

Supplementary Materials

Material associated with this article can be found in the online version at https://doi.org/10.1016/j.gastha.2022.03.005.

References

1. Moreau R, Jalan R, Gines P, et al. Acute-on-chronic liver failure is a distinct syndrome that develops in patients with acute decompensation of cirrhosis. Gastroenterology 2013;144:1426–1437, 1437.e1-9.
2. Claria J, Stauber RE, Coenraad MJ, et al. Systemic inflammation in decompensated cirrhosis: characterization and role in acute-on-chronic liver failure. Hepatology 2016;64:1249–1264.
3. Lange CM, Moreau R. Immunodysfunction in acute-on-chronic liver failure. Visc Med 2018;34:276–282.
4. Monteiro S, Grandt J, Uschner FE, et al. Differential inflammasome activation predisposes to acute-on-chronic liver failure in human and experimental cirrhosis with and without previous decompensation. Gut 2021;70:379–387.
5. Bode JG, Albrecht U, Haussinger D, et al. Hepatic acute phase proteins–regulation by IL-6- and IL-1-type cytokines involving STAT3 and its crosstalk with NF-kappaB-dependent signaling. Eur J Cell Biol 2012;91:496–505.
6. Dudakov JA, Hanash AM, van den Brink MR. Interleukin-22: immunobiology and pathology. Annu Rev Immunol 2015;33:747–785.
7. Wolk K, Witte E, Witte K, et al. Biology of interleukin-22. Semin Immunopathol 2010;32:17–31.
8. Feng D, Kong X, Weng H, et al. Interleukin-22 promotes proliferation of liver stem/progenitor cells in mice and patients with chronic hepatitis B virus infection. Gastroenterology 2012;143:188–198.e7.
9. Arab JP, Sehrawat TS, Simonetto DA, et al. An open-label, dose-escalation study to assess the safety and efficacy of IL-22 agonist F-652 in patients with alcohol-associated hepatitis. Hepatology 2020;72:441–453.
10. Liu Y, Verma VK, Malhi H, et al. Lipopolysaccharide downregulates macrophage-derived IL-22 to modulate alcohol-induced hepatocyte cell death. Am J Physiol Cell Physiol 2017;313:C305–C313.
11. Kronenberger B, Rudloff I, Bachmann M, et al. Interleukin-22 predicts severity and death in advanced liver cirrhosis: a prospective cohort study. BMC Med 2012;10:102.
12. Huber S, Gagliani N, Zenewicz LA, et al. IL-22BP is regulated by the inflammasome and modulates tumorigenesis in the intestine. Nature 2012;491:259–263.

13. Kotenko SV, Izotova LS, Mirochnitchenko OV, et al. Identification, cloning, and characterization of a novel soluble receptor that binds IL-22 and neutralizes its activity. J Immunol 2001;166:7096–7103.
14. Pelczar P, Witkowski M, Perez LG, et al. A pathogenic role for T cell-derived IL-22BP in inflammatory bowel disease. Science 2016;354:358–362.
15. Xu W, Presnell SR, Parrish-Novak J, et al. A soluble class II cytokine receptor, IL-22RA2, is a naturally occurring IL-22 antagonist. Proc Natl Acad Sci U S A 2001; 98:9511–9516.
16. Schwarzkopf K, Ruschenbaum S, Barat S, et al. IL-22 and IL-22-binding protein are associated with development of and mortality from acute-on-chronic liver failure. Hepatol Commun 2019;3:392–405.
17. Blei AT, Cordoba J. Practice parameters Committee of the American College of G. Hepatic Encephalopathy. Am J Gastroenterol 2001;96:1968–1976.
18. Desmet VJ, Gerber M, Hoofnagle JH, et al. Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology 1994;19:1513–1520.
19. Schwertheim S, Kalsch J, Jastrow H, et al. Characterization of two types of intranuclear hepatocellular inclusions in NAFLD. Sci Rep 2020;10:16533.
20. Engelmann C, Mehta G, Tacke F. Regeneration in acute-on-chronic liver failure - the phantom lost its camouflage. J Hepatol 2020;72:610–612.
21. Katoonizadeh A, Laleman W, Verslype C, et al. Early features of acute-on-chronic alcoholic liver failure: a prospective cohort study. Gut 2010;59:1561–1569.
22. Bernsmeier C, Pop OT, Singanayagam A, et al. Patients with acute-on-chronic liver failure have increased numbers of regulatory immune cells expressing the receptor tyrosine kinase MERTK. Gastroenterology 2015; 148:603–615.e14.
23. Kolodziejczyk AA, Federici S, Zmora N, et al. Acute liver failure is regulated by MYC- and microbiome-dependent programs. Nat Med 2020;26:1899–1911.
24. Xiang X, Feng D, Hwang S, et al. Interleukin-22 ameliorates acute-on-chronic liver failure by reprogramming impaired regeneration pathways in mice. J Hepatol 2020; 72:736–745.
25. Gottlieb A, Kottmann M, Manka P, et al. How to define acute liver failure patients with pre-existing liver disease without signs of cirrhosis. Dig Dis 2019; 37:147–154.
26. Shubham S, Kumar D, Rooge S, et al. Cellular and functional loss of liver endothelial cells correlates with poor hepatocyte regeneration in acute-on-chronic liver failure. Hepatol Int 2019;13:777–787.

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Authors' Contributions:
The authors have contributed to the manuscript by planning the study (Christian M. Lange and Hideo A. Baba), collecting the data (Christian M. Lange, Kawther Al-Juboori, Martin Schlattjan, JS; Dorothe Moellmann, Sabrina Guckenbiehl, Katharina Willuweit, Ali Canbay, and Hideo A. Baba), analysis and interpretation of data (Christian M. Lange, Kawther Al-Juboori, Martin Schlattjan, Josephine Rawitzer, Ali Canbay, and Hideo A. Baba), and preparation and revision of the manuscript (all authors).

Conflicts of Interest:
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Ethical Statement:
The corresponding author, on behalf of all authors, jointly and severally, certifies that their institution has approved the protocol for any investigation involving humans or animals and that all experimentation was conducted in conformity with ethical and humane principles of research.

Data Transparency Statement:
Data, analytic methods, and study material will be made available to other researchers upon request to the corresponding author.