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Soluble CD137 is a novel serum marker of liver cirrhosis in patients with hepatitis C and alcohol-associated disease etiology

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Defective T-cell functions play a role in the persistence of HCV infection. Activated T cells express CD137, which costimulates antivirus T-cell responses, and this activity is antagonized by soluble CD137 (sCD137). Here, we show that in sera of 81 patients with chronic HCV, sCD137 levels did not correlate with measures of viral infection, and did not decline after virus eradication using direct-acting antivirals. Thus, serum sCD137 was similar in patients infected with HCV and in uninfected controls. Of note, in HCV patients with liver cirrhosis and patients with mostly alcohol-associated liver cirrhosis, sCD137 was increased. A negative association of sCD137 and albumin existed in both cohorts. sCD137 concentrations were similar in hepatic and portal vein blood excluding the liver as the origin of higher levels. Recombinant sCD137 reduced Th1 and Th2 but not Th17 cell polarization in vitro, and accordingly lowered IFN-γ, TNF, and IL-13 in cell media. Serum sCD137 is associated with inflammatory states, and positively correlated with serum TNF in cirrhotic HCV patients following virus eradication. Our study argues against a role of sCD137 in HCV infection and suggests a function of sCD137 in liver cirrhosis, which yet has to be defined.

Keywords: Direct-acting antivirals · Foxp3 · liver cirrhosis · MELD score · TNF

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Hepatitis C virus (HCV) infection is a major cause for chronic liver diseases. Patients infected with HCV mostly progress to chronic disease, and less than 30% are able to clear the virus [1,2]. CD4 and CD8 T cells exert a central role in the elimination of the virus, and an impaired T-cell response is a major cause of chronic HCV infections. T-cell exhaustion develops upon persistent stimulation with an antigen, and significantly contributes to the chronicity of HCV infections [2].

The TNF receptor family member CD137 (TNFRSF9, 4-1BB) is an important costimulatory molecule, and is expressed by activated T cells [3,4]. This receptor was also found on NK cells, B cells, and monocytes/macrophages [5–7]. Activation of monocyte

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expressed CD137 by agonistic anti-CD137 antibodies induces an M1-like phenotype, which is characterized by increased phagocytic activity and production of inflammatory cytokines like TNF [7].

CD137 ligand (CD137L) is a transmembrane protein expressed by APCs. Interaction of CD137 and CD137L provides signals to the APC and the T cell, a process called bidirectional signaling [5]. Activation of CD137 signaling stimulates T-cell proliferation and antiviral T-cell responses. Agonistic CD137 antibodies enhanced the CD4 and CD8 cell immune response during HCV vaccination in an experimental study [3,8].

Only little is known about CD137 signaling pathways in the liver diseases. Agonistic CD137 antibodies induced hepatic myeloid cell IL-27 production and subsequent immigration of CD8 T cells into the liver. T-cell mediated tissue damage was blocked in C-C chemokine receptor type 2 null mice illustrating that recruitment of immune cells to the liver has a central role in CD137-induced hepatitis [9]. A recent study identified CD137 agonists with low or no hepatotoxicity [10], suggesting that the CD137–CD137L interaction does not induce liver injury.

In chronic HCV, T cells are functionally defective and do not appropriately respond to antigen stimulation [11,12]. CD137 is highly expressed on activated T cells and has a key role in the survival of these cells [3,13]. Yet, an attempt to improve the liver T-cell function of HCV patients by blockage of programmed death -1 and CD137 stimulation via CD137L failed [14]. Peripheral blood T cells showed a good response to this intervention [14]. Notably, there was an about twofold higher expression of CD137 on hepatic compared to peripheral HCV-specific CD8 T cells indicating that CD137 signaling in the liver cells is defective [14]. Another study described reduced CD137 expression of PBMCs in chronic HCV infection, which recovered within 24 weeks after efficient elimination of the virus [15]. Of clinical relevance, normalization of CD137 was not observed in patients, who did not respond to IFN/ribavirin treatment [15].

The almost complete eradication of HCV by direct-acting antivirals (DAAs) offers the unique opportunity to study recovery of T-cell function after antigen removal. CD8 T cells are a heterogeneous population and consist of memory-like and terminally exhausted cell subsets in chronic HCV. Whereas this latter population was lost after DAA therapy, the memory-like T cells persisted. These cells have acquired a molecular signature of exhaustion during long-term chronic HCV infection and prevent immune restoration [11,12].

In liver cirrhosis, abnormal immune cell function, systemic inflammation, and an impaired immune response are referred to as “cirrhosis-associated immune dysfunction” [16]. In chronic HCV patients with liver cirrhosis, immune dysfunction persists after efficient elimination of the virus [17]. CD8 T-cell activity was still dysfunctional at 24 weeks post-treatment and these cells displayed a high rate of cell proliferation and cytolytic activity [17]. Efficient elimination of HCV is associated with lower systemic inflammation, and levels of various cytokines decreased in serum [18,19]. Such a decline of inflammatory cytokines did not occur in patients with advanced liver fibrosis [17].

Soluble CD137 (sCD137) is generated by differential splicing and lacks the transmembrane domain [20,21]. A very recent study showed that sCD137 is also produced upon shedding by A Disintegrin and Metalloproteinases (ADAMs) 10 and 17 [22]. In-vitro data suggest that sCD137 inhibits T-cell costimulation through CD137 [4]. Delayed production of sCD137 relative to CD137 is in line with an immune-suppressive role of sCD137 [4,23]. Moreover, a direct immunoregulatory function of sCD137 on CD4 T cells has been shown [24]. sCD137 impairs the binding of T-cell-expressed CD137 to APC-expressed CD137L and thereby blocks bidirectional signaling [4]. Moreover, sCD137 suppressed CD4 T-cell activation in the absence of CD137L-expressing cells, and future research has to clarify the pathways involved herein [24].

Tregs are immunosuppressive and high production of sCD137 has a function herein [25]. Of note, Treg produced sCD137 downregulated the costimulatory CD137L on APCs [4].

sCD137 levels are relatively low in the serum of healthy donors and are strongly induced in serum of patients with autoimmune diseases or chronic lymphocytic leukemia [4,23,26,27]. We hypothesized that sCD137 may have a role in the immune suppression of chronic HCV. We, therefore, measured sCD137 in serum of patients with chronic HCV and noninfected controls. Analysis of sCD137 before therapy, at 4 and 12 weeks after start of DAA therapy and 12 weeks post-treatment complemented this investigation.

Results

Serum sCD137 levels in patients with HCV

Serum sCD137 levels were measured in 81 chronic HCV patients. Serum sCD137 levels were similar in female and male patients (Figure 1A). Serum sCD137 was essentially the same in normal weight, overweight, and obese patients (Figure 1B), and did not correlate with the body mass index (Table 1). Associations with patients´ age could not be detected (Table 1). The 18 patients with diabetes had serum sCD137 levels comparable to those of nondiabetic patients (Figure 1C).

Tregs express high levels of sCD137, and their cell number positively correlated with the viral load [25,28]. Serum sCD137 was, however, not associated with the viral titer (Figure 1D). T-cell responses to HCV are moreover genotype-specific [29]. The HCV genotypes 1a, 1b, and 3a were present in 24, 37, and 14 patients, respectively. The six patients with rare genotypes were integrated into a separate group. Serum levels of sCD137 did not vary among viral genotypes (Figure 1E).

Serum sCD137 in relation to hepatic steatosis and noninvasive liver fibrosis scores

Thirty-seven of the HCV patients had a diagnosis of liver steatosis. Serum sCD137 was comparable in HCV patients with and

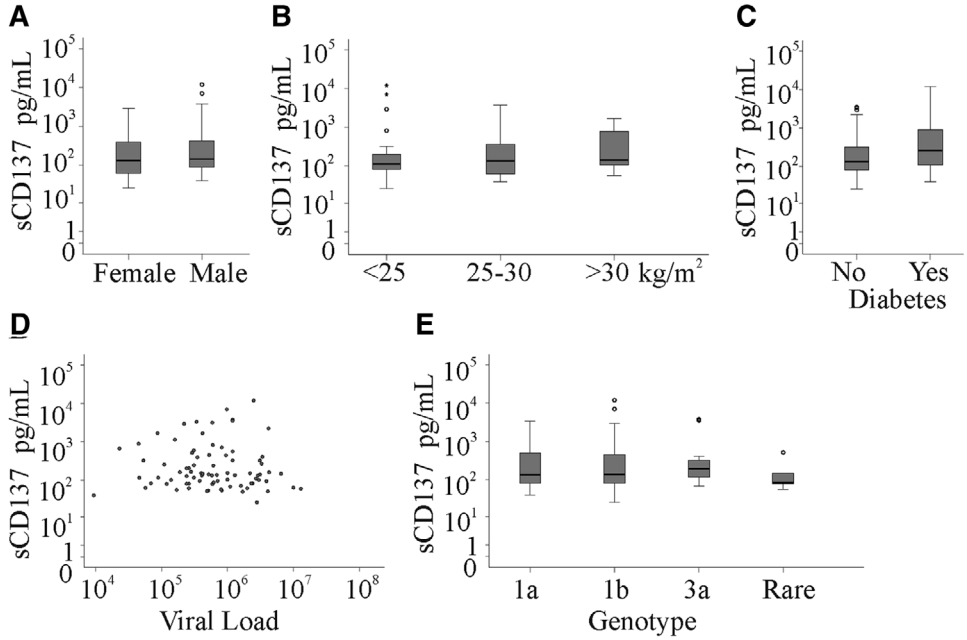

Figure 1. Serum sCD137 levels in 81 patients with chronic HCV. (A) Serum sCD137 in 33 female and 48 male HCV patients (Mann–Whitney U-test). (B) Serum sCD137 levels in patients stratified for body mass index (Kruskal–Wallis test). (C) Serum sCD137 levels in 18 patients with and 63 patients without diabetes (Mann–Whitney U-test). (D) Correlation analysis of serum sCD137 levels with viral load (Spearman correlation). (E) Serum sCD137 levels in patients stratified for HCV genotype (rare group signifies six patients with genotypes other than 1a, 1b, and 3a) (Kruskal–Wallis test). Outliers = values between 1.5 and 3 times the interquartile range are shown as circles and values more than 3 times the interquartile range are shown as stars.

without liver steatosis (Figure 2A). Serum sCD137 was not different between the groups when patients were stratified for fibrosis scores, which were evaluated by acoustic radiation force impulse, the fibrosis 4 (FIB-4) score [30], the Non-alcoholic fatty liver disease (NAFLD) score [31], and the aspartate aminotransferase/platelet ratio index (APRI) [30] (Figure 2B–E). Positive correlations of sCD137 existed with the FIB-4 scores ($r = 0.256$, $p = 0.021$) and the NAFLD scores ($r = 0.305$, $p = 0.009$). Serum

Table 1. Spearman correlation coefficients and *p*-values of the correlations of age, BMI, and laboratory parameters and sCD137 in the whole cohort, in HCV patients without, and HCV patients with liver cirrhosis before direct-acting antiviral (DAA) therapy and of patients with liver cirrhosis at SVR12

Parameters	Whole cohort (81 patients)	Noncirrhotic (44 patients)	Cirrhotic (37 patients)	Cirrhotic (30 patients at SVR12)
Age years	0.018 (0.871)	−0.230 (0.133)	0.152 (0.369)	0.204 (0.280)
BMI kg/m^2	0.198 (0.093)	0.203 (0.229)	0.329 (0.050)	0.280 (0.143)
ALT U/L	−0.168 (0.134)	0.112 (0.468)	**−0.347 (0.035)**	− 0.306 (0.100)
AST U/L	−0.041 (0.720)	−0.009 (0.952)	−0.091 (0.591)	0.025 (0.894)
MELD Score	**0.431 (<0.001)**	0.254 (0.096)	**0.405 (0.013)**	0.347 (0.060)
Bilirubin mg/dL	**0.366 (0.001)**	0.104 (0.502)	**0.395 (0.015)**	0.337 (0.074)[29]
Albumin g/L	**−0.261 (0.019)**	−0.010 (0.948)	−0.238 (0.156)	**−0.566 (0.001)**
INR	**−0.384 (<0.001)**	−0.225 (0.142)	0.277 (0.097)	0.236 (0.210)
Creatinine mg/dL	0.058 (0.608)	−0.024 (0.879)	0.133 (0.432)	0.057 (0.766)
Leukocytes n/L	−0.175 (0.118)	0.159 (0.302)	**−0.332 (0.045)**	**−0.392 (0.035)**
Platelets n/nL	**−0.321 (0.003)**	0.000 (0.999)	**−0.337 (0.041)**	**−0.437 (0.018)**[29]
CRP mg/L	0.138 (0.219)	0.079 (0.489)	0.203 (0.489)	0.170 (0.368)
HDL mg/dL	−0.003 (0.979)[77]	0.247 (0.125)	−0. 219 (0.198)[36]	−0.176 (0.369)[28]
LDL mg/dL	**−0.333 (0.003)**[77]	0.020 (0.902)	**−0.491 (0.002)**[36]	−0.367 (0.055)[28]

ALT, Alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; HDL, high-density lipoprotein; INR, international normalized ratio; LDL, low-density lipoprotein; MELD, model of end-stage liver disease.
Superscript numbers indicate the number of patients in case the laboratory values were not documented for the whole study group.
Significant correlations are in bold.

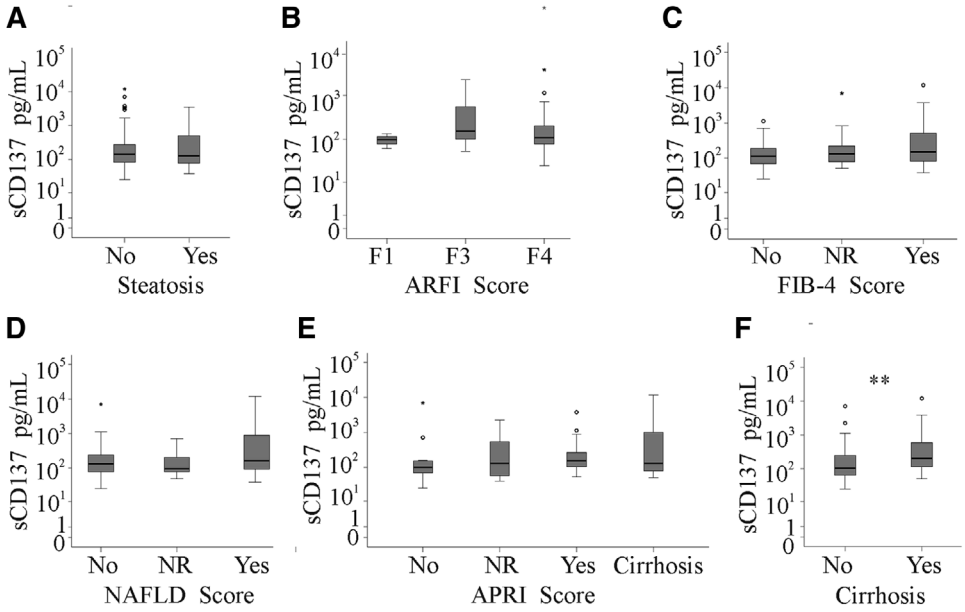

Figure 2. Serum sCD137 in relation to liver steatosis and fibrosis. (A) Serum sCD137 in 37 patients with and 44 patients without liver steatosis (Mann–Whitney U-test). Serum sCD137 levels in patients stratified for the fibrosis scores. (B) Acoustic radiation force impulse (ARFI) score (Kruskal–Wallis test). (C) Fibrosis-4 (FIB-4) score (Kruskal–Wallis test). (D) Nonalcoholic fatty liver disease (NAFLD) score (Kruskal–Wallis test); and (E) Aminotransferase/platelet (AST/PLT) ratio index (APRI) score (Kruskal–Wallis test). (F) Serum sCD137 in 37 patients with liver cirrhosis and 44 noncirrhotic patients diagnosed by ultrasound (Mann–Whitney U-test). (No fibrosis = No; not reliable values = NR, fibrosis = Yes) Outliers = values between 1.5 and 3 times the interquartile range are shown as circles and values more than 3 times the interquartile range are shown as stars. $^{**}p < 0.01$.

sCD137 levels were enhanced in the 37 patients with liver cirrhosis as diagnosed by ultrasound examination [32] (Figure 2F).

Association of serum sCD137 levels with clinical laboratory markers of inflammation and liver disease

Consistent with higher levels of sCD137 in liver cirrhosis (Figure 2F), a positive correlation with the model for end-stage liver disease (MELD) score existed (Table 1). Calculation of the MELD score uses bilirubin, international normalized ratio (INR), and creatinine [33]. sCD137 negatively correlated with INR and positively with bilirubin (Table 1). There were no associations with creatinine or aminotransferase levels (Table 1). sCD137 negatively correlated with albumin (Table 1).

Platelets are low in liver cirrhosis [34] (Supporting information Table S1), and were negatively correlated with sCD137 levels (Table 1). This may also apply to low-density lipoprotein (LDL), which is reduced in liver cirrhosis (Supporting information Table S1), and was negatively associated with serum sCD137 (Table 1). C-reactive protein or leukocyte counts were not related to sCD137 serum levels (Table 1).

Association of serum sCD137 levels with laboratory markers and spleen size in cirrhosis

Because sCD137 levels were increased in liver cirrhosis, associations with laboratory values were separately analyzed in cirrhosis and in noncirrhotic HCV patients. Of note, none of the associations identified in the whole cohort existed in noncirrhotic HCV. In the patients with liver cirrhosis, positive correlations with the MELD score and bilirubin were identified (Table 1). sCD137 negatively correlated with ALT, LDL, and platelet and leukocyte counts (Table 1).

Enlargement of the spleen was mainly observed in HCV and NAFLD-associated liver cirrhosis and may develop secondary to portal hypertension [35], a major risk factor for decompensation [33]. Spleen length was significantly increased in liver cirrhosis patients compared to HCV patients without cirrhosis (Supporting information Figure S1A). sCD137 positively correlated with spleen length in the whole cohort ($r = 0.366$, $p = 0.001$) and in liver cirrhosis patients ($r = 0.472$, $p = 0.004$; Supporting information Figure S1B) but not in patients without cirrhosis ($r = 0.016$, $p = 0.919$).

sCD137 during and after treatment with direct-acting antivirals (DAAs) and levels in uninfected controls

DAA therapy efficiently eliminated HCV in our cohort within 12 weeks, with an HCV virus load below the limit of detection after 4 weeks of therapy in all patients [36]. Serum sCD137 was determined at 4 weeks (79 patients) and 12 weeks (81 patients) after the start of DAA therapy and at 3 months post-treatment (69 patients). Serum sCD137 was not changed at any of these time points (Supporting information Figure S1C).

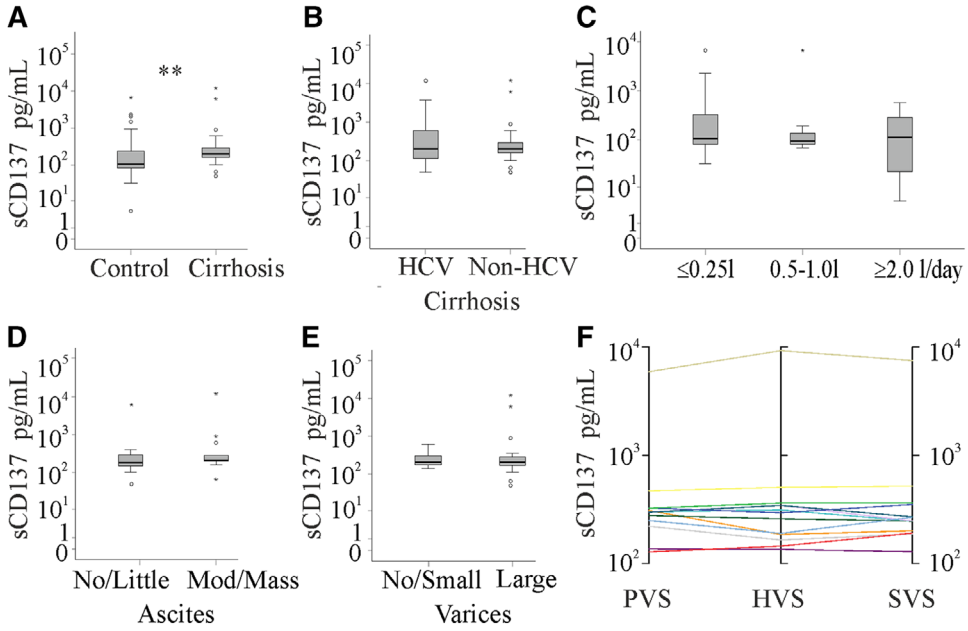

Figure 3. sCD137 levels in patients with non-HCV liver cirrhosis. (A) Serum sCD137 in the 41 noninfected controls with normal liver function and the 30 non-HCV-infected cirrhosis patients (Mann–Whitney U-test). (B) sCD137 in the 37 HCV-cirrhosis patients and the 30 non-HCV cirrhosis patients (Mann–Whitney U-test). (C) Serum sCD137 in the noninfected controls with normal liver function stratified for daily alcohol intake (Kruskal–Wallis test). (D) sCD137 in the 16 patients with no/little ascites and the 14 patients with modest/massive ascites (Mann–Whitney U-test). (E) sCD137 in the 11 patients with no/small varices and the 19 patients with large varices (Mann–Whitney U-test). (F) sCD137 in portal venous (PVS), hepatic venous (HVS), and systemic venous blood (SVS) of 11 non-HCV cirrhosis patients (paired Students´ t-test). Outliers = values between 1.5 and 3 times the interquartile range are shown as circles and values more than 3 times the interquartile range are shown as stars.**$p < 0.01$.

Associations of sCD137 with the MELD score in patients with liver cirrhosis were no longer significant at Sustained virological response (SVR)12 (Table 1) most likely since fewer patients were included in this cohort. Of note, a negative correlation with albumin, which did not exist in patients with liver cirrhosis before DAA therapy, was identified (Table 1).

A recent study showed that patients with the development of hepatocellular carcinoma (HCC) after DAA therapy have lower sCD137 compared to patients who do not develop HCC [37]. During the follow-up of 3 months, six patients developed HCC in our cohort. Serum sCD137 before therapy start was 152.9 (25.2–11920.3) pg/mL in those patients without tumors and 335.2 (70.1–3522.7) pg/mL in HCC, and was similar in both groups ($p = 0.313$). Current preliminary observations suggest that serum sCD137 levels are not increased in patients with HCV.

To determine whether sCD137 levels differ between HCV-infected and -uninfected patients, sCD137 was measured in serum of 41 non-HCV-infected patients not suffering from severe liver diseases. Serum sCD137 was comparable in HCV-infected patients and uninfected controls (Supporting information Figure S2A). There was no difference in sCD137 when uninfected controls and HCV patients not suffering from liver cirrhosis were compared ($p = 0.545$, data not shown). In the uninfected controls, sCD137 was not correlated with age or BMI, was similar in both genders, and did not vary in patients with liver steatosis or type 2 diabetes (Supporting information Figure S2B and C and data not shown).

sCD137 levels in HCV and non-HCV cirrhosis

To find out whether increased sCD137 is a characteristic of HCV cirrhosis, sCD137 was also determined in serum of patients with mostly alcoholic liver cirrhosis (Supporting information Table S2). These patients had lower levels of ALT, AST, and higher C-reactive protein (CRP) levels. The MELD score of the non-HCV cirrhosis patients was lower than that of HCV-cirrhosis patients (Supporting information Table S2).

Serum sCD137 levels were again higher in the cirrhosis patients in comparison to the noncirrhotic controls not infected with HCV (Figure 3A). Patients with alcoholic and HCV-related liver cirrhosis had comparable sCD137 serum levels (Figure 3B), and this suggests that alcohol may not affect serum sCD137. Accordingly, sCD137 was comparable in noncirrhosis patients with a daily alcohol consumption of up to 0.25 l (40 patients), 0.5–1.0 l (9 patients), and those drinking 2 l and more (4 patients) (Figure 3C).

In the non-HCV cirrhosis patients, sCD137 did not correlate with the MELD score ($r = 0.169$, $p = 0.373$), bilirubin ($r = 0.066$, $p = 0.730$), or LDL ($r = -0.273$, $p = 0.258$). Notably, a negative association with albumin ($r = -0.500$, $p = 0.005$) existed. Platelet and leukocyte numbers, which correlated with sCD137 in HCV-cirrhosis (Table 1), were not documented for most of these patients. Ten of the 30 patients had diabetes, but sCD137 was similar in diabetic and nondiabetic patients ($p = 0.746$).

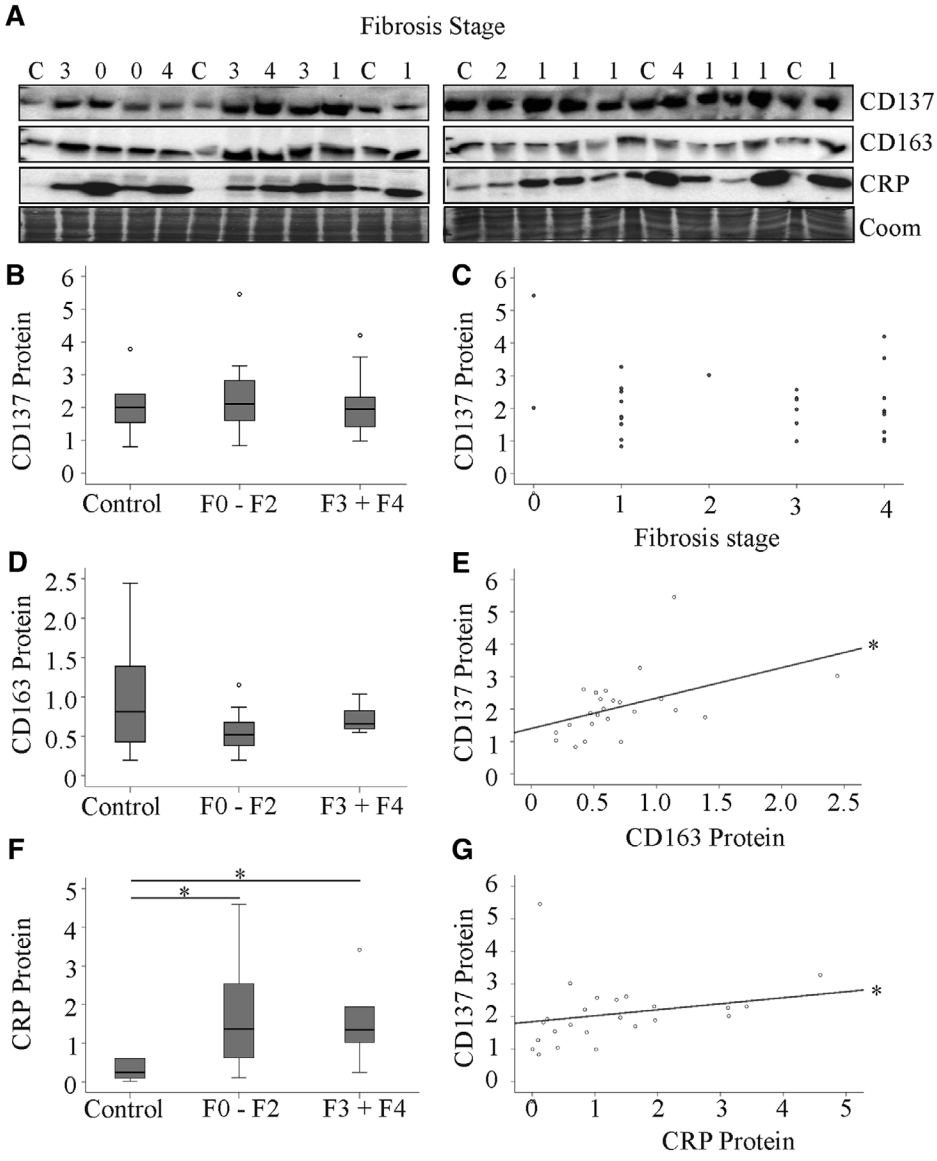

Figure 4. CD137 protein levels in the liver. (A) Immunoblot analysis of CD137, CD163, and CRP in the liver of patients with chronic liver diseases and the healthy liver of controls (C). The Coomassie-stained membrane was used as a loading control. The respective fibrosis stages are given as numbers above the immunoblots; (b) CD137 protein in the liver of six controls, 12 patients with fibrosis stage 0–2, and 16 patients with fibrosis stages 3 and 4 (Kruskal–Wallis test). (C) Correlation of CD137 with fibrosis stages in the 28 patients with chronic liver diseases (Spearman correlation). (D) CD163 protein in the liver of six controls, 12 patients with fibrosis stage 0–2, and six patients with fibrosis stages 3 and 4 (Kruskal–Wallis test). (E) Correlation of CD137 with CD163 protein in 18 patients with chronic liver diseases (Spearman correlation). (F) CRP protein in the liver of six controls, 12 patients with fibrosis stage 0–2, and six patients with fibrosis stages 3 and 4 (Kruskal–Wallis test). (G) Correlation of CD137 with CRP protein in 18 patients with chronic liver diseases (Spearman correlation); Outliers = values between 1.5 and 3 times the interquartile range are shown as circles.*$p < 0.05$.

Ascites and varices are complications of decompensated liver cirrhosis [33]. Serum sCD137 was similar in the 14 patients with modest/massive ascites in comparison to the 16 patients with no/little ascites (Figure 3D). Serum sCD137 was not changed in the 19 patients with large varices in relation to the 11 patients with no/small varices (Figure 3E). In line with these results, sCD137 did not correlate with the hepatic venous pressure gradient ($r = -0.218$, $p = 0.247$).

Higher serum sCD137 levels in liver cirrhosis suggest a hepatic origin. Increased hepatic production or impaired hepatic elimination of sCD137 may result in higher levels in the hepatic vein of patients with liver cirrhosis. Levels of sCD137 were, however, similar in hepatic vein, portal vein, and systemic vein blood of 11 patients with alcoholic liver cirrhosis (Figure 3F), and these preliminary data argue against the liver as the origin of increased serum sCD137 in these patients.

Association of hepatic CD137 with liver fibrosis, CD163, and CRP

sCD137 can be generated by shedding of CD137 [22] and ADAM17 was found induced in the fibrotic liver [38]. Increased levels of shed CD137 may decrease cellular levels. Therefore, the hepatic expression of CD137 in relation to fibrosis stages was analyzed. There was no difference in the hepatic CD137 protein levels between healthy and fibrotic liver. Accordingly, CD137 did not correlate with fibrosis stage in the patients with liver diseases (Figure 4A–C; original immunoblots with the molecular weight marker are shown in Supporting information Figure S3). Similarly, the macrophage-specific protein CD163 was not induced in the fibrotic liver (Figure 4D). Notably, there was a positive correlation of hepatic CD137 and CD163 protein levels (Figure 4E) and an association of CD137 with CRP

protein levels, which gradually increased with liver fibrosis stages (Figure 4F and G).

Association of sCD137 and proprotein convertase subtilisin/kexin type 9

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is well known for its role in LDL-receptor degradation [39]. The LDL-receptor binds to the TCR and regulates its recycling. PCSK9 lowers cholesterol uptake by T cells and TCR recycling and thereby suppresses CD8 T-cell function [40]. PCSK9 in serum has been determined in our HCV cohort [41].

Of note, serum sCD137 was negatively correlated with PCSK9 in the entire cohort ($r = -0.476$, $p < 0.001$) and in cirrhotic patients ($r = -0.405$, $p = 0.013$; Supporting information Figure S4A) but not in the noncirrhotic patients ($r = -0.282$, $p = 0.064$). PCSK9 correlated with the MELD score [41], and correlations of PCSK9 with sCD137 were insignificant when adjusted for the MELD score ($r = -0.122$, $p = 0.282$). Association of sCD137 and PCSK9 was, therefore, related to liver cirrhosis rather than reflecting an effect of PCSK9 on T-cell function. In non-HCV cirrhosis, a correlation between PCSK9 and the MELD score did not exist [42]. Accordingly, sCD137 did not correlate with PCSK9 in this cohort (Supporting information Figure S4B).

Soluble CD137 quenches immune activity by reducing cytokine secretion and limits helper T-cell polarization

To evaluate which effect sCD137 might have, the extracellular domain of CD137 as a recombinant protein, expressed in human embryonic kidney 293 cells, was employed. This sCD137 protein retains the ligand-binding capacity, lacks the cytoplasmic and the transmembrane domains, and thus, represents an ideal substitute for natural sCD137. Supplemented with a suboptimal dose of anti-CD3 agonistic antibody, healthy donors' PBMCs were cocultured with Daudi, a Burkitt lymphoma cell line that expresses high levels of CD137L, and that can costimulate CD137-expressing T cells. We hypothesized that if sCD137 competes with full-length CD137 on the surface of T cells for CD137L, the presence of sCD137 would dampen the T-cell activity. Indeed, sCD137 decreased the secretion of IFN-γ, TNF, and IL-13 (Figure 5A). However, the secretion of IL-17a and IL-10 remained unchanged (Figure 5A), indicating that sCD137 does not affect Th17 polarization or general immune suppression. TNF and IFN-γ were also significantly reduced when BSA was added instead of PBS to the control cells excluding unspecific effects of the added protein (Supporting information Figure S5). Notably, 1 and 5 μg/mL sCD137 had a similar repressive effect on IFN-γ, TNF, and IL-13 levels (Supporting information Figure S6) further arguing for a specific activity of the recombinant protein.

During priming, naïve T cells receive signals that not only activate but also polarize them. IFN-γ and TNF are indicative

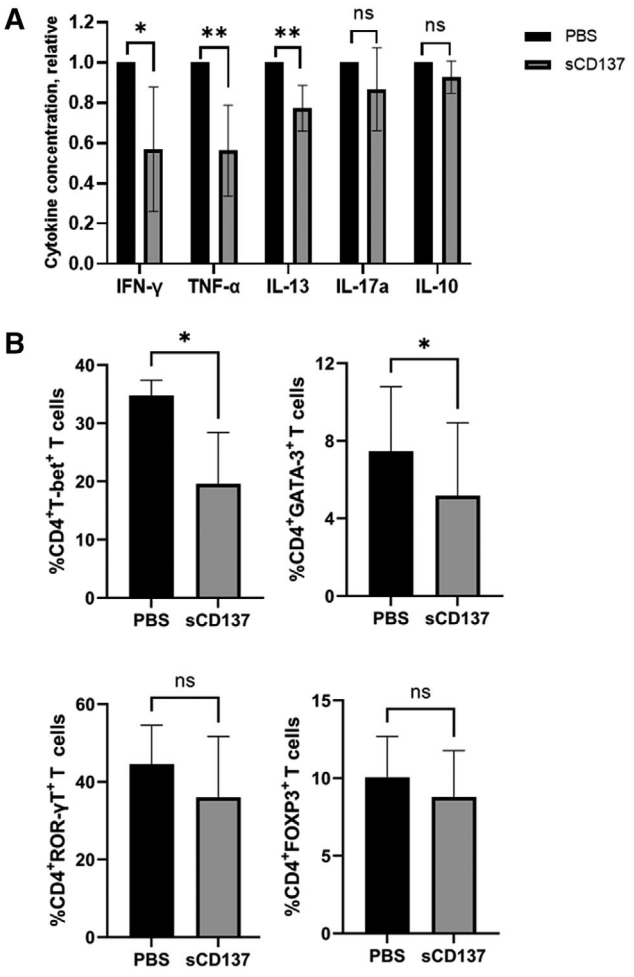

Figure 5. Recombinant sCD137 reduces immune activity and lowers cytokine levels in cell media and affects helper T-cell (Th) polarization. Daudi cells (4×10^4) were cocultured with 2×10^6 healthy donors' PBMCs (ratio of 1 : 5), supplemented with 0.1 ng/mL anti-CD3 antibody, and either 1 μg/mL recombinant human CD137 extracellular domain (sCD137) or PBS. After 3 days, cytokine concentrations in supernatants were determined by ELISA (A), and expression of transcription factors driving T-cell polarization was determined by flow cytometry (B). Cells were gated on live, single, $CD3^+$ cells. Depicted are means ± standard deviations of data from four independent donors (biological replicates). Two-tailed Student's unpaired (A) or paired (B) t-tests were used to calculate the statistical significance between PBS and sCD137 treatment; ns nonsignificant. $^*p < 0.05$, $^{**}p < 0.01$.

of a type 1 polarization, while IL-13 and IL-17 are the signature cytokines for a type 2 and a type 17 polarization, respectively [43]. Based on the observed changes in cytokine levels, we hypothesized that the presence of sCD137 would impair a Th1 and Th2 polarization, while not affecting the Th17 or Treg subpopulations. This was indeed confirmed by intracellular staining for transcription factors that are characteristic for the different subsets. The percentages of cells expressing T-box expressed in T-cells and GATA binding protein (GATA)-3, the master transcription factors for a Th1 and Th2 polarization, respectively, were decreased by sCD137 while the populations expressing retinoic acid receptor-related orphan nuclear receptor (ROR)-γt and

forkhead-box-protein (Foxp)3, the master transcription factors for a Th17 and Treg polarization [44], respectively, remained unchanged (Figure 5B).

Soluble CD137 correlates with TNF in patients with liver cirrhosis at SVR12

The in-vitro studies described above indicate a role for sCD137 in TNF levels. Serum sCD137 and serum TNF were not correlated in HCV patients before DAA therapy ($r = 0.145$, $p = 0.210$). It is well established that eradication of HCV causes a decline of circulating cytokines, and serum TNF was reduced from 11.4 (2.4–110.5) pg/mL to 10.5 (0–75.1) pg/mL after the virus elimination ($p = 0.022$) (Supporting information Figure S7A). Notably, sCD137 positively correlated with TNF in the whole cohort ($r = 0.248$, $p = 0.045$) at SVR12. This association existed in patients with liver cirrhosis ($r = 0.570$, $p = 0.001$) but not in the non-cirrhosis patients ($r = 0.041$, $p = 0.811$) at SVR12 (Supporting information Figure S7B and C). TNF did, however, not differ between noncirrhosis and cirrhosis patients before therapy started or at SVR12 (Supporting information Figure S7D and data not shown).

Discussion

Here, we have shown that sCD137 is increased in serum of patients with HCV and alcohol-associated liver cirrhosis. In HCV-cirrhosis, MELD score, FIB-4 score, NAFLD score, ultrasound findings, platelet count, and spleen size, as the essential clinical markers of advanced fibrosis and cirrhosis, showed a correlation with sCD137. In the smaller cohorts of HCV cirrhosis patients at SVR12 and the non-HCV cirrhosis patients, associations of sCD137 with the MELD score did not exist. Strong negative correlations with serum albumin were identified in both cohorts of patients with liver cirrhosis. Albumin was shown to improve immune cells function [45] and future research may identify a role of sCD137 herein.

CD137 agonists are promising drugs for the therapy of viral infections and cancers [3]. Activation of CD137 signaling and simultaneous inhibition of coinhibitory pathways involved in T-cell exhaustion were effective in experimental models of lymphocytic choriomeningitis and colon cancer [46,47]. However, an attempt to restore hepatic T-cell responses to HCV by blocking programmed death-1 and enhancing T-cell signaling by CD137L failed [14]. These findings indicate that CD137 is not pivotal for the elimination of HCV. We showed that sCD137 did not correlate with viral genotype or load in the HCV patients, and did not decline after elimination of HCV. Current observation is in accordance with a recent study also showing that sCD137 did not change after DAA treatment [37]. Also, sCD137 did not differ in serum of HCV-infected patients and uninfected controls. In summary, at present, there is no indication for a crucial function of CD137 signaling in HCV infection.

There is some evidence that DAA therapy partly restores the function of circulating T cells [11,12,17]. CD8 T-cell dysfunction did, however, not recover in patients with liver cirrhosis at 24 weeks post-treatment. The abnormal CD8 T-cell distribution increased with a higher grade of fibrosis and was not normalized after successful virus eradication [17]. This indicates that T-cell abnormalities are, at least in part, related to liver cirrhosis. Serum sCD137 was indeed induced in HCV patients with liver cirrhosis. Moreover, compared to liver-healthy controls, higher serum sCD137 was also detected in patients with mostly alcoholic liver cirrhosis.

Of note, sCD137 modestly correlated with measures of liver disease and the MELD score in the HCV patients with liver cirrhosis. Associations of serum sCD137 with the MELD score were, however, not observed in non-HCV cirrhosis. The lack of significance may be attributed to the smaller cohort size. Indeed, there was no positive correlation of the MELD score and sCD137 at SVR12, where few patients were included.

Actually, sCD137 was similar in serum of patients with HCV and alcoholic liver cirrhosis although the latter group had a lower MELD score. This could indicate that alcohol induces serum sCD137 independent of liver disease severity. Chronic alcohol consumption leads early to impaired Th1 and Th2 activity in experimental models [48,49]. The effect of alcohol intake on serum sCD137 was not studied in great detail so far. Preliminary data obtained here do not support an effect of alcohol intake on sCD137 levels.

The more likely explanation for the different findings in the two cohorts of patients with liver cirrhosis is that the relatively modest associations of sCD137 with markers of liver injury identified in HCV were not found in alcoholic cirrhosis because of the smaller cohort size. Notably, serum albumin was negatively correlated with sCD137 in HCV patients at SVR12 and in patients with mostly alcoholic liver cirrhosis. Such an association did not exist in cirrhosis patients infected with HCV. Albumin serum levels improve after viral eradication and were significantly increased at SVR12 in the HCV cohort analyzed herein [36]. There is evidence that albumin can improve immune cell function partly by reducing the bioavailability of immune-suppressive lipids such as prostaglandin E2 [45]. The strong negative correlation of sCD137 and albumin suggests a role of sCD137 herein which has to be analyzed in the future.

CD137 and sCD137 levels are induced by proinflammatory cytokines [5,50]. A positive association of sCD137 and serum TNF was observed in HCV cirrhosis patients at 3 months post-treatment suggesting that inflammation may contribute to higher sCD137. Such an association did, however, neither exist in noncirrhosis HCV patients nor in patients before DAA therapy excluding a strong association between serum TNF and sCD137 levels.

TNF was found increased in serum of HCV-infected patients, and in this cohort, levels were similar in patients with mild and advanced liver fibrosis [51]. A separate study described that serum TNF was positively correlated with liver inflammation, and was higher in patients with alcohol- than HCV-related liver disease [52]. It was also reported that plasma TNF was

higher in HCV than alcohol-induced liver cirrhosis [53]. Altogether, these data show that the severity of liver disease and disease etiology impacted inflammatory cytokines such as TNF [52]. In our cohort, TNF declined after DAA treatment, but did not differ between noncirrhosis and cirrhosis patients before and after therapy. Besides the concentration, the cellular origin of TNF is important because TNF from different cells has different functions [54,55]. Thus, further analysis is needed to clarify the role of TNF for sCD137 levels in serum of patients with liver cirrhosis.

Since both, CD137 and CD137L are expressed as transmembrane proteins on the cell surface and signal bidirectionally, sCD137 is likely to affect the activity of CD137-expressing T cells and CD137L-expressing APCs. In this study, we unequivocally proofed the influence on T cells by measuring T-cell cytokines (IFN-γ and IL-13). TNF is also reduced by sCD137 but can be released by T cells as well as APCs. However, in this study we have not explored the effects of sCD137 on APCs in very detail. Indeed, here it was not studied whether the effect of sCD137 involves CD137L and/or CD137. Blockage of CD4 T-cell activation by sCD137 did not require CD137L [24] and experiments using inhibitory antibodies may clarify this issue.

Positive associations of TNF and sCD137 do not fit with the immunosuppressive role of sCD137. sCD137 antagonizes CD137 signaling and suppresses CD137-costimulated immune responses [4]. Current in-vitro analysis showed that sCD137 lowers IFN-γ, TNF, and IL-13 further providing evidence for an anti-inflammatory function of this soluble protein. Whether increased sCD137 has a role in systemic inflammation in patients with liver cirrhosis cannot be evaluated by a retrospective cohort study like ours. It is well known that liver cirrhosis is associated with inflammation and, at the same time, immunosuppressive pathways are also active [16] and cell-type specific analysis may be more successful in the evaluation of the pathophysiological role of sCD137 in cirrhosis.

For the in-vitro experiments, recombinant sCD137 at 1 μg/mL was used which is higher than sCD137 concentrations found in vivo (mean values of up to 1500 pg/mL have been reported in serum of healthy controls [56]). However, it is a well-known fact that concentrations of soluble mediators are profoundly higher locally at the site of inflammation than the systemic concentration in serum [57]. Also, in-vivo mediators are produced continuously, while in experimental systems they are generally added once.

One physiological source of systemic sCD137 are hepatic immune cells. CD137 protein was, however, similar in the healthy and the cirrhotic liver, and did not change with increasing fibrosis stages. This illustrates that at least hepatic CD137 protein levels are not associated with liver fibrosis. Delayed production of sCD137 relative to CD137 has been reported [4], and thus, normal CD137 protein in liver cirrhosis argues against the liver as the major origin of increased sCD137. In line with this, sCD137 levels were comparable between the portal and the hepatic vein. Enhanced hepatic production or impaired hepatic elimination is reflected by higher levels of these proteins in the hepatic vein [33], which was not observed for sCD137 levels. Nonhepatic cells, therefore, seem to be the origin of serum sCD137 in liver cirrhosis.

It is noteworthy that hepatic CD137 protein positively correlated with CD163 protein being exclusively expressed by macrophages [58]. Notably, stimulation of CD137 by agonistic anti-CD137 antibodies downregulated CD163 protein in these cells [7]. Moreover, CD137 protein in the liver was positively associated with hepatic CRP protein. CRP in serum was, however, not correlated with serum sCD137. These findings suggest a relation between CD137, sCD137, CD163, and CRP in patients with chronic liver disease, which warrants further investigations.

It has to be noted that sCD137 did not further increase in patients with alcoholic liver cirrhosis and secondary complications such as massive ascites or large varices. There was, however, a positive correlation of sCD137 and spleen size in HCV cirrhosis. Portal hypertension contributes to spleen enlargement, ascites, and varices [33,35]. Whether this preliminary observation suggests that sCD137 is associated with decompensation in only HCV cirrhosis needs further study.

Patients with liver cirrhosis have a high risk to develop severe infections [59]. CD137-deficient mice were resistant to Gram-negative bacteria but had reduced survival when infected with Gram-positive bacteria [60]. CD137 also contributes to fungal clearance [61] and high levels of sCD137 may be related to fungal infections. Early recognition of inappropriate immune responses is essential to prevent complications, slow disease progression, and reduce mortality. Serum sCD137 has the potential to become a predictive and prognostic biomarker of bacterial and fungal infections.

Liver cirrhosis is, moreover, a risk factor for HCC [62]. Agonistic anti-CD137 antibody therapy led to tumor regression in an orthotopic HCC model [63]. Activation of CD137 promotes tumoricidal effects in monocytes and T cells [7,64]. Unexpectedly, serum sCD137 levels were low in HCV-infected patients who developed HCC within 4 years after DAA therapy [37]. This difference in sCD137 levels was, however, no longer apparent at 3 months after viral eradication [37]. In the cohort studied herein, six patients developed HCC during the 3 months follow-up, and pretreatment sCD137 levels did not differ in this subgroup. Thus, further analysis is required to clarify the functional relationship between sCD137 and HCC.

The levels of sCD137 determined in the current study were 102 (5–6672) pg/mL in uninfected controls, 105 (25–7009) pg/mL in noncirrhotic HCV patients, 203 (50–11 920) pg/mL in HCV patients with liver cirrhosis, and 206 (50–12 053) pg/mL in patients with alcoholic cirrhosis. In a separate analysis, sCD137 was 200 (27–681) pg/mL in healthy controls and 957 pg/mL in patients with rheumatoid arthritis [65]. In serum, levels were 3 (1.3–5.3) pg/mL, 180 pg/mL, or had a mean value of 1500 pg/mL [23,56,66]. Altogether, sCD137 levels of controls were highly variable, and absolute levels determined in different studies are not comparable. Standardized tests for sCD137 measurement are, therefore, needed for clinical studies.

Levels of sCD137 were increased in subcutaneous adipose tissues of obese patients undergoing cholecystectomy [67]. This

suggests that serum sCD137 may be altered in the obese. In the HCV patients and the uninfected controls, sCD137 levels were not higher in the overweight/obese patients. Moreover, serum sCD137 was neither induced in patients with liver steatosis nor in patients with diabetes. Thus, sCD137 is not associated with the prevalence of fatty liver and diabetes in the noninfected patients and patients infected with HCV.

In summary, the current study describes increased serum sCD137 in HCV patients and alcoholics with liver cirrhosis. There is no evidence that sCD137 levels are associated with HCV infection, viral load, or viral genotype. sCD137 is an anti-inflammatory molecule, and whether it has a role in "cirrhosis-associated immune dysfunction" has to be evaluated in the future.

Materials and methods

Study cohort

The HCV-infected study cohort was used before for analysis of chemerin and PCSK9 [36,41]. This cohort initially included 82 patients [36,41] and serum of 81 patients was available for this study. The study was performed at the Department of Internal Medicine I at the University Hospital of Regensburg from October 2014 to September 2019.

Fasting serum was collected in the morning and aliquots were stored at −80°C. Serum sCD137 was measured by ELISA in 81 patients with chronic HCV infection. Cirrhosis diagnosis by ultrasound was made when the liver appeared comparatively small, had a nodular surface, and coarse parenchyma [32]. Details of HCV patients without and with liver cirrhosis are given in Supporting information Table S1.

Patients were treated with DAAs with one of the following regimens: sofosbuvir/daclatasvir, sofosbuvir/ledipasvir, sofosbuvir/velpatasvir, glecaprevir/pibrentasvir, or elbasvir/grazoprevir. Treatment was performed according to international guidelines [68].

The noninfected controls included patients referred to ultrasound imaging. In this control group, patients with cancers, severe liver diseases, or liver cirrhosis were excluded. The age of these 41 controls was 64 (25–80) years, BMI was 26 (18–43) kg/m^2 and 20 of the 41 patients were males. Age, BMI, and gender distribution were comparable between HCV patients and noninfected controls (data not shown). Data shown in Figure 3B are from these 41 controls and 12 additional noninfected controls with normal liver function. These latter patients had higher daily alcohol intake. Thirty patients with clinically diagnosed liver cirrhosis were also included in the study. Etiology of liver disease was alcoholic in 25, hepatitis C infection in 3, and of other reasons in 2 patients. Ascites volume was defined as: little (ascites only detectable by ultrasound), massive (extensive and bulging ascites), and modest (in between little and massive). Small varices disappeared in endoscopy during air insufflation and large varices did not. Parts of these sera were analyzed in previous studies [33].

Paratumorous tissues of HCC patients infected with HBV (9 patients) or HCV (10 patients), from patients with nonviral disease etiology (9 patients) and 6 controls without any severe liver diseases were obtained from resections. Experimental procedures were according to the guidelines of the charitable state controlled foundation Human Tissue and Cell Research.

All patients gave informed consent prior to inclusion in the studies. The studies were approved by the local ethical committee of the University Hospital of Regensburg (14-101-0049; 15-101-0052; 4/99) and were performed according to the updated guidelines of good clinical practice and updated Declaration of Helsinki.

ELISA

DuoSet ELISA to measure human sCD137 was from R&D Systems (Wiesbaden, Nordenstadt, Germany) and was performed as recommended by the distributor. Serum was diluted 1:2-fold for analysis. The concentration of IFN-γ in the cell culture supernatants was determined with the Human IFN-γ DuoSet ELISA kit (R&D Systems), according to the manufacturer's instructions.

The concentrations of TNF, IL-10, IL-13, and IL-17a in the cell culture supernatants were determined with human ELISA kits (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions.

Immunoblot experiments

Immunoblots were performed as already described with the modification that 40 μg protein for each lane was used [42]. The CD137 and CRP antibodies were from R&D Systems. CD163 antibody was from Cell Signalling (Frankfurt am Main, Germany). Coomassie-stained membrane was used as the loading control. Quantification was done with ImageJ [69].

Cell culture and preparation

Daudi cells (ATCC, Wesel, Germany) were cultured in RPMI 1640 medium, supplemented with 10% FBS (Gibco, Thermo Fisher Scientific), at 37°C and 5% CO_2. Anti-CD3 agonistic antibody was from Biolegend (clone OKT3, Ultra-LEAF; San Diego, CA) and added to the culture to achieve a final concentration of 0.1 ng/mL.

Healthy human donors' PBMCs were isolated by density gradient centrifugation using Ficoll–Paque PLUS (GE Healthcare, Chicago, IL). The protocol was approved by the National University of Singapore (NUS) IRB number B15–320E.

Where indicated, 4×10^4 Daudi cells were cultured with 2×10^6 PBMCs, supplemented with 0.1 ng/mL anti-CD3 antibody and either PBS or 1 μg/mL recombinant human CD137 extracellular domain (R&D Systems), for a duration of 3 days. This recombinant protein consists of the extracellular domain of

CD137 coupled to a His-tag (catalogue number 9220–4B), which is very similar to sCD137 released from T cells that exists as a trimer and higher order multimers [70].

Flow cytometry

To determine the expression of cell surface markers, cells were blocked with human Fc receptor blocking reagent (Miltenyi Biotec) and then stained with fluorochrome-conjugated antibodies diluted in flow cytometry staining buffer (PBS containing 2% FBS). To stain for intracellular transcriptional factors, Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific) was used. The following fluorochrome-conjugated antibodies were applied: anti-CD3-eFlour450 (clone UCHT1, Thermo Fisher Scientific), CD4-PE (clone RPA-T4, Biolegend), CD4-FITC (clone RPA-T4, Thermo Fisher Scientific), T-bet-FITC (clone ebio4B10, Thermo Fisher Scientific), GATA-3-AF488 (clone TWAJ, Thermo Fisher Scientific), ROR-γT-PE (clone AFKJS-9, Thermo Fisher Scientific), Foxp3-FITC (clone 150D/E4, Thermo Fisher Scientific). For flow cytometry, we have adhered to the guidelines for the use of flow cytometry and cell sorting in immunological studies [71]. The gating strategy is shown in Supporting information Figure S8.

Statistical analysis

Unless not indicated, otherwise data are given as a median value, and the minimum and maximum values in brackets, and are presented as boxplots. Small circles or asterisks above or below the boxes mark outliers. Statistical differences were analyzed by Chi-squared test, Kruskal–Wallis test, Mann-Whitney U-test, Spearman correlation, or two-tailed partial correlation (SPSS Statistics 25.0 program, IBM, Leibniz-Rechenzentrum, München, Germany). Paired samples were analyzed by Student´s *t*-test (MS Excel). A value of $p < 0.05$ was regarded as significant.

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Abbreviations: **ADAMs**: A disintegrin and metalloproteinases · **APRI**: aspartate aminotransferase/platelet ratio index · **CD137L**: CD137 ligand · **DAA**: direct-acting antivirals · **FIB-4**: fibrosis 4 · **Foxp**: forkhead-box-protein · **HCC**: hepatocellular carcinoma · **INR**: international normalized ratio · **LDL**: low-density lipoprotein · **MELD**: Model of end-stage liver disease · **PCSK9**: proprotein convertase subtilisin/kexin type 9 · **sCD137**: soluble CD137 · **SVR**: sustained virological response

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