The Journal of Infectious Diseases

MAJOR ARTICLE

Direct-Acting Antiviral–Induced Hepatitis C Virus Clearance Does Not Completely Restore the Altered Cytokine and Chemokine Milieu in Patients With Chronic Hepatitis C

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Background. Persistent infection with hepatitis C virus (HCV) causes profound alterations of the cytokine and chemokine milieu in peripheral blood. However, it is unknown to what extend these alterations affect the progression of liver disease and whether HCV clearance normalizes soluble inflammatory mediators.

Methods. We performed multianalyte profiling of 50 plasma proteins in 28 patients with persistent HCV infection and advanced stages of liver fibrosis or cirrhosis and 20 controls with fatty liver disease. The patients were treated for 24 weeks with sofosbuvir and ribavirin and underwent sampling longitudinally. Ten patients experienced viral relapse after treatment cessation.

Results. The cytokine and chemokine expression pattern was markedly altered in patients with chronic HCV infection as compared to healthy controls and patients with nonalcoholic steatohepatitis. Distinct soluble factors were associated with the level of fibrosis/cirrhosis, viral replication, or treatment outcome. The baseline expression level of 10 cytokines distinguished patients with a sustained viral response from those who experienced viral relapse. While the majority of upregulated analytes declined during and after successful therapy, HCV clearance did not lead to a restoration of parameters that were suppressed.

Conclusions. Chronic HCV infection appears to disrupt the milieu of soluble inflammatory mediators even after viral clearance. Thus, HCV cure does not lead to complete immunological restitution.

Keywords. HCV; chronic hepatitis C; DAA; direct-acting antiviral; cytokines; chemokines; soluble immune mediators; cirrhosis.

Viral infections are controlled by a tightly regulated immune network. In addition to cellular components of the immune system, various soluble mediators are essential for eliminating the virus. Whether any of these soluble immune mediators (SIMs) can serve as a biomarker for predicting disease severity or response to antiviral treatment is so far unclear. It is well established that chronic viral infections cause major changes in the inflammatory cytokine and chemokine milieu [1]. Still, limited data are available about the extent to which imprints in the immune network are reversible once chronic infections are cleared. We address these questions in an analysis of patients who were persistently infected with hepatitis C virus (HCV) and who received novel interferon-free therapy with direct-acting antivirals (DAAs).

HCV is a human pathogenic virus, and viral replication takes place mainly in hepatocytes [2]. In the majority of cases, HCV causes a persistent infection. It is estimated that 64–102 million people are infected with HCV worldwide [3]. Chronic hepatitis C can lead to liver fibrosis, cirrhosis, and hepatic decompensation, as well as hepatocellular carcinoma [2]. Persistent HCV infection has been associated with suppression and exhaustion of HCV-specific immune responses [4]. Moreover, HCV infection leads to induction of excessive interferon-stimulated gene expression [5] and alterations in the overall systemic inflammatory milieu [6, 7], which subsequently have been linked to alterations in cytomegalovirus-specific and Epstein-Barr virus–specific T-cell responses, as well as natural killer–cell phenotype and function [8–10].

During the last few years, several new DAAs that interfere with viral replication or assembly were approved for the treatment of chronic hepatitis C [11–13]. These new treatment options are very potent, leading to cure rates of 90%–100% for most HCV genotypes [12]. The novel therapies enable for the first time direct investigations of the effects of cure from a persistent viral infection on the human immune system. Of note, the novel DAAs do

Received 14 June 2016; accepted 21 September 2016; published online 28 September 2016.
Presented in part: European Association for the Study of the Liver, Barcelona, Spain, April 2016.

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The Journal of Infectious Diseases® 2016;214:1965–74

DOI: 10.1093/infdis/jiw457

not exert direct immune-modulatory activity, in contrast to the previous treatment, which was based on administration of type I interferons, mainly pegylated interferon alfa-2 [14–16].

Until now, only a limited number of studies have been performed to investigate the inflammatory cytokine and chemokine milieu in acute [6] and chronic [17–19] HCV infections. So far, a single study investigated inflammatory mediators during DAA treatment of chronic hepatitis C, but only 4 parameters were investigated [18]. It therefore remains unclear whether potential alterations in the systemic inflammatory cytokine and chemokine milieu in patients with chronic hepatitis C are restored upon clearance of infection.

The aim of this study was to investigate how the inflammatory milieu, including cytokines, chemokines, growth factors, and adhesion molecules, is altered in chronic hepatitis C and whether these changes are associated with stage of liver disease. Moreover, we aimed to analyze whether distinct SIMs differ between patients who achieve a sustained viral response (SVR) and patients who experience viral relapse after treatment cessation. Finally, we aimed to study whether clearance of the infection by DAA treatment would restore the immunological imprints established upon chronic HCV infection.

MATERIAL AND METHODS

Patient Material

In this study, 53 subjects were evaluated, including 5 healthy controls, 20 patients with nonalcoholic steatohepatitis (NASH), and 28 patients persistently infected with HCV. Patients with chronic hepatitis C were monitored before, during, and after DAA treatment in the outpatient clinic of the Department of Gastroenterology, Hepatology, and Endocrinology at Hannover Medical School (Hannover, Germany). Patients with chronic hepatitis C were treated for 24 weeks with sofosbuvir (400 mg 4 times daily) and ribavirin (RBV; weight-based starting dose of 800–1200 mg daily). Peripheral blood samples were collected at baseline (before the start of therapy); week 4, week 12, and week 24 (end of treatment) during therapy; and 12 weeks after treatment cessation. Eighteen patients achieved a SVR, whereas 10 patients experienced viral relapse. All patients except 2 were negative for antibodies to human immunodeficiency virus (HIV), and none were coinfected with hepatitis B virus. HIV-positive patients were receiving antiretroviral therapy and had controlled HIV infection, with undetectable HIV RNA loads. Blood plasma was collected from ethylenediaminetetraacetic acid–treated peripheral blood samples and stored at −80°C for later analysis.

Patient characteristics are presented in Table 1. For all patients with chronic hepatitis C, clinical data on liver inflammation, liver fibrosis, and cirrhosis were collected from routine clinical diagnostic assays, as previously described in detail [20]. The diagnosis of NASH was based on liver histologic findings in all but 1 patient, who had evidence of steatosis and elevated liver enzyme levels in the absence of any other cause of liver disease. Patients gave informed written consent for the study of immunological parameters. The protocols for sample collection and investigations were reviewed and approved by the local ethics committee of Hannover Medical School (study number 2148-2014). Of the 28 patients with chronic hepatitis C, baseline samples were available for all 18 SVR patients but only for 8 (out of 10) patients that experienced a viral relapse.

Table 1. Baseline Characteristics of Healthy Individuals, Patients With Nonalcoholic Steatohepatitis (NASH), and Patients With Chronic Hepatitis C

Characteristic	Healthy Group (n = 5)	NASH Group (n = 20)	Chronic Hepatitis C Group (n = 28)
HCV RNA load, IU/mL	. . .	. . .	1 624 000 (1600–7 600 000)
Gender			
Male	4	12	15
Female	1	8	13
Age, y	38.8 (27–58)	48.5 (23–72)	56.8 (41–72)
Outcome			
SVR	. . .	. . .	18
Relapse	. . .	. . .	10
Liver stiffness, kPa[a]	. . .	8.0 (3.6–23.5)	22.4 (5.8–48)
ALT level, U/L	. . .	101.6 (29–242)	106.3 (31–415)
HCV genotype			
1	. . .	. . .	12
2	. . .	. . .	1
3	. . .	. . .	13
4	. . .	. . .	2

Abbreviations: ALT, alanine aminotransferase; HCV, hepatitis C virus; SVR, sustained virologic response.

[a] By Fibroscan.

Cytokine and Chemokine Measurements

We performed multianalyte profiling of 50 cytokines, chemokines, adhesion molecules, and growth factors in the blood plasma of all samples, using the LUMINEX-based multiplex bead technology (BioPlex Pro Human Cytokine Panel; Bio-Rad, Hercules, CA). The assay was conducted by following the manufacturer's recommendations [8] and according to optimized protocols used in various previous studies [8, 21–23]. Of note, all samples were analyzed in 1 run. The beads were acquired on the LUMINEX instrument, using BioPlex Manager 6.0 software.

Statistical Analyses

Data were analyzed using GraphPad Prism v6.0b (Graph Pad Software, La Jolla, CA). All data were evaluated for their statistical distribution by using the Kolmogorov-Smirnov test or the D'Agostino-Pearson test. In general, quantitative comparisons were performed using the parametric Student *t* test (for

normally distributed values) and the nonparametric Wilcoxon test or Mann–Whitney test (for values that did not show a normal distribution). The statistical test used for each analysis is mentioned in the respective figure legend. Principal component analysis (PCA) was performed using Qlucore Omics Explorer v3.2 (Qlucore, Lund, Sweden). Repetitive *t* testing was used in PCA to compare groups of patients to each other. For analysis, values were set to *P* values of .05 and a *Q* value of < 0.2. Correction for multiple *t* testing was performed using the false-discovery rate (FDR) approach, with a desired FDR (*Q*) of 10%.

RESULTS

Altered Inflammatory Cytokine and Chemokine Milieu in Patients With Chronic Hepatitis C

The expression pattern of all SIMs analyzed in plasma specimens differed strongly between patients with chronic hepatitis C, patients with NASH, and healthy individuals (Figure 1*A*). The degree of biochemical disease activity was similar between patients with NASH and patients with chronic hepatitis C, with a median alanine aminotransferase (ALT) value of 100 U/L, but patients with chronic hepatitis C had higher liver stiffness values than patients with NASH (Table 1). In

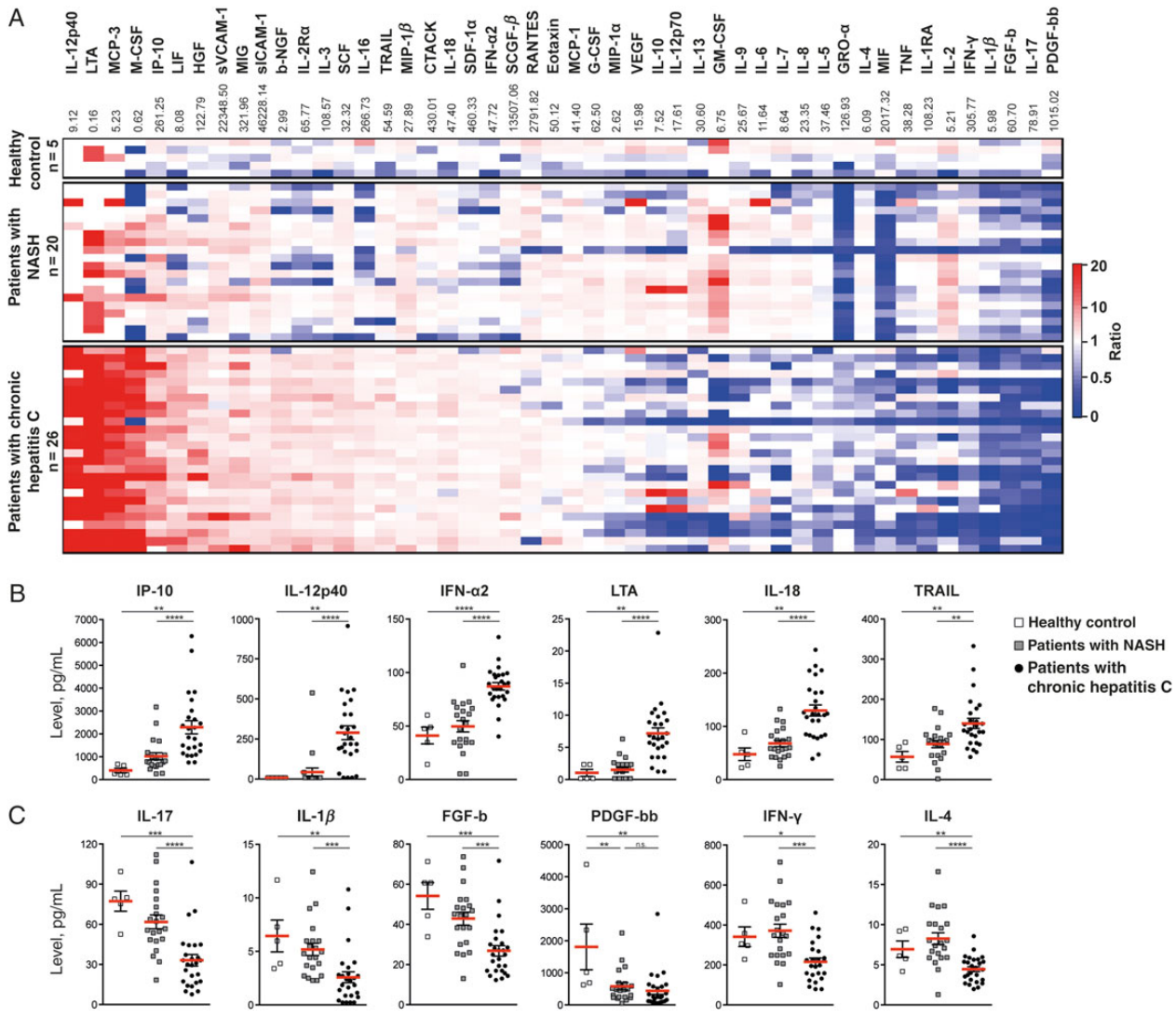

Figure 1. Chronic hepatitis C yields a cytokine and chemokine milieu that is altered as compared to that in healthy individuals and patients with nonalcoholic steatohepatitis (NASH). *A*, Heat map showing the expression pattern of 48 cytokines and chemokines normalized to the median value of all healthy individuals. Data are presented for 5 healthy controls, 20 patients with NASH, and 26 patients with chronic hepatitis C. Cytokines and chemokines are ordered on the basis of their expression level in patients with chronic hepatitis C. Values shown above heatmap are median values of all healthy controls. Interleukin 1α (IL-1α) and IL-15 were excluded, as the median value of healthy individuals for these cytokines was <0.1. *B*, Expression level of several cytokines and chemokines that were significantly upregulated during chronic hepatitis C. *C*, Expression level of all 6 cytokines and growth factors that were significantly reduced in patients with chronic hepatitis C as compared to healthy individuals. Horizontal bars represent means and standard errors of the mean. **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001, by multiple *t* testing with a false-discovery rate (*Q*) of 10%. Abbreviations: G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; TNF, tumor necrosis factor.

both cohorts of patients with liver disease (ie, those with chronic hepatitis C and those with NASH), a specific and unique profile of inflammatory mediators was observed, with a distinct pattern of proteins upregulated or downregulated in comparison to healthy controls. In total, the expression level of 25 analytes was significantly altered in patients with chronic hepatitis C as compared to healthy individuals (Figure 2), with expression of 17 SIMs increased in the chronic hepatitis C group. The expression level of the chemokine IP-10 (CXCL10) and the cytokines interleukin 12p40 (IL-12p40), interferon α2 (IFN-α2), LTA, interleukin 18, and TRAIL were also upregulated in comparison to levels in patients with NASH (Figure 1*B*). Interestingly, only 6 of 8 SIMs with reduced levels were significantly lower in patients with chronic hepatitis C after performance of additional individual *t* testing, including the 4 cytokines interleukin 17 (IL-17), interleukin 1β, IFN-γ, and interleukin 4 (IL-4), as well as the 2 growth factors FGF-basic and PDGF-bb. Of note, only the expression level of PDGF-bb was decreased in patients with NASH to the same extent as in patients with chronic hepatitis C, while reductions in IL-17, interleukin 1β, IFN-γ, IL-4 and FGF-b expression were largely specific for patients with chronic hepatitis C (Figure 1*C*). Thus, patients persistently infected with HCV had a disrupted milieu of cytokines and chemokines as compared to healthy individuals and patients with NASH.

P < .0001	*P* < .001	*P* < .01	*P* < .05	Not Significant
IFN-α2	SCF	M-CSF	IL-7	GRO-α
MCP-3	IL-17	PDGF-bb	IFN-γ	IL-1α
β-NGF	FGF-b	sICAM-1	HGF	IL-1RA
CTACK	LIF	IL-18	MIF	IL-2
	SDF-1α	LTA		IL-5
	IL-3	IL-2Ra		IL-6
		IL-4		IL-8
		IL-1β		IL-9
		IP-10		IL-10
		IL-12p40		IL-12p70
		TRAIL		IL-13
				IL-15
				IL-16
				Eotaxin
				G-CSF
				GM-CSF
				MCP-1
				MIG
				MIP-1α
				MIP-1β
				RANTES
				SCGF-β
				sVCAM-1
				TNF
				VEGF

Figure 2. Soluble immune mediators that are expressed differently between patients with chronic hepatitis C and healthy individuals. Levels in patients with chronic hepatitis C were measured at baseline. Comparisons were made using the Mann–Whitney test. Abbreviations: G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

Severity of Liver Damage Affects the Inflammatory Milieu in Patients With Chronic Hepatitis C

During chronic hepatitis C, liver fibrosis and cirrhosis may also influence levels of SIMs. Accordingly, we aimed to investigate whether the expression levels of SIMs correlated with liver stiffness values. All patients with chronic hepatitis C were grouped on the basis of the stage of liver damage into patients with fibrosis (stiffness level, <14.5 kPa), mild cirrhosis (14.5–25 kPa), and severe cirrhosis (>25 kPa). The expression levels of the soluble form of the adhesion molecules VCAM-1 (CD106; sVCAM-1) and ICAM-1 (CD54; sICAM-1) positively correlated with the Fibroscan values, whereas the expression of the growth factor PDGF-bb correlated negatively (Figure 3*A*). Expression levels of the 2 adhesion molecules sVCAM-1 and sICAM-1 increased gradually with the severity of cirrhosis (Figure 3*B*). In contrast to this, the expression of PDGF-bb, FGF-basic, IL-17, and IL-4 decreased in patients with chronic hepatitis C as compared to healthy individuals (Figure 1*C*) and showed a stepwise reduction correlating with the severity of liver stiffness (Figure 3*B*). Thus, of the 6 SIMs with reduced expression in patients with chronic hepatitis C as compared to healthy individuals, 4 were associated with the cirrhosis stage. Together, these findings reveal that not only HCV infection but also the stage of liver cirrhosis affects the expression of distinct analytes. Notably, only the expression of the adhesion molecules correlated positively with the severity of liver stiffness.

PCA Identifies Distinct Pretreatment Patterns of SIMs Distinguishing Patients With a SVR From Those With Viral Relapse

The patients were treated with sofosbuvir and RBV only in this study. Thus, we had the unique chance to study whether inflammatory mediators may be predictive of viral clearance with suboptimal interferon-free therapies. Patients with a SVR and those with viral relapse clearly differed from each other in the expression levels of SIMs before the start of DAA therapy (Figure 4*A*). A distinct clustering of both patient groups could be seen in the PCA plot, which represents the strength of difference based on all significantly different values ($P = .05$ and $Q < 0.2$). To identify on which SIMs the clustering was based, a heat map displaying all significantly different parameters was generated (Figure 4*B*). Expression of 12 parameters, comprising 10 cytokines, 1 chemokine (SDF-1α; CXCL12), and 1 growth factor (β-NGF), were significantly different between the patient groups, based on the PCA analysis (Figure 4*C*). Ten of those were confirmed by correction for multiple t tests (Figure 4*C*). The most contrasting cytokine in both analyses was IL-12p40 ($P = .0002$ and $Q = 0.0102$, by PCA; $P = .0002$, by multiple *t* testing).

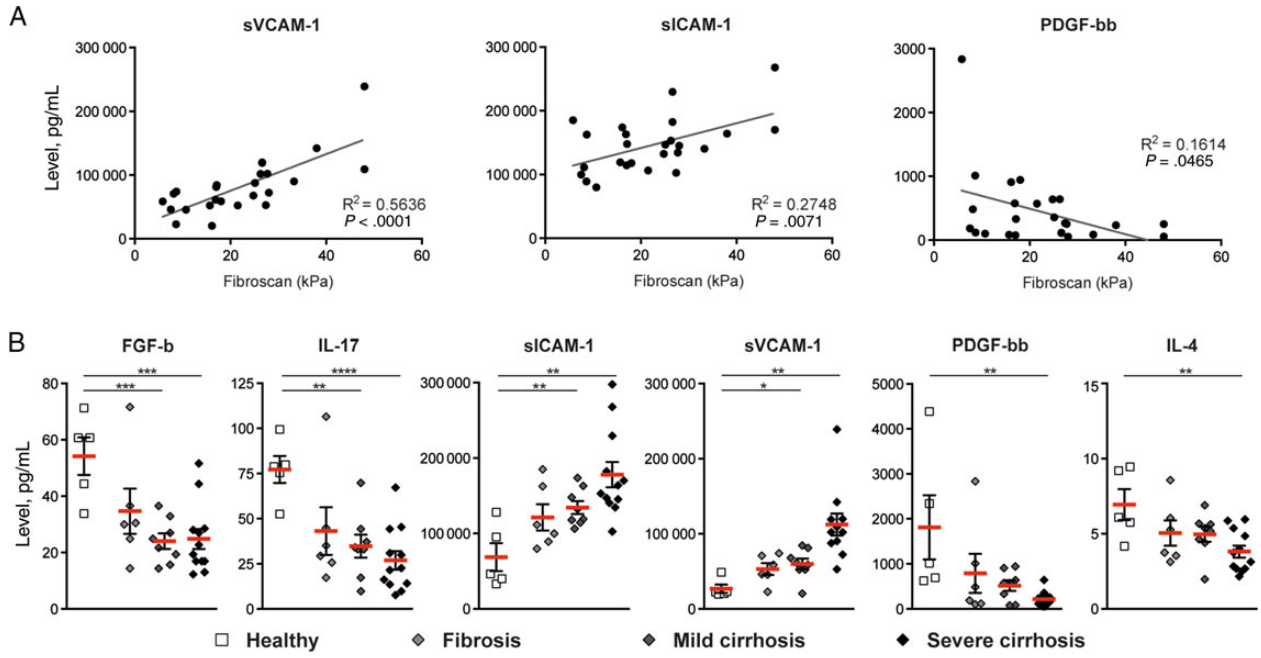

Figure 3. The expression level of several analytes is affected by progression of liver fibrosis and cirrhosis. *A*, Correlation analysis of sVCAM-1, sICAM-1, and PDGF-bb with Fibroscan values. *B*, Expression of adhesion molecules, cytokines, and growth factors with significantly different regulation in healthy (n = 5) as compared to fibrotic chronic hepatitis C patients (Fibroscan value, <14.5 kPa; n = 6) or cirrhotic chronic hepatitis C patients (Fibroscan value, 14.5–25 kPa [n = 8] or >25 [n = 12]). Horizontal bars represent means and standard errors of the mean. **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001, by multiple *t* testing with a false-discovery rate (*Q*) of 10%. Abbreviation: IL, interleukin.

Noticeably, the expression level of each of these analytes was higher in patients who had viral relapse as compared to those with a SVR (Figure 4*C*). IP-10 expression was not statistically different between these 2 groups. Thus, pretreatment identification of patients with chronic hepatitis C who may not achieve a SVR with suboptimal DAA treatment may be possible by studying expression levels of distinct inflammatory mediators.

DAA Therapy Restores Some but Not All Mediators in Patients With a SVR

As shown in Figure 5*A*, viral loads and ALT levels, a marker for liver inflammation, declined rapidly upon DAA treatment initiation. Moreover, liver stiffness values also improved upon HCV clearance. Because of these alterations, we investigated whether the imprints on the cytokine and chemokine milieu acquired during chronic HCV infection (baseline) disappear upon viral clearance.

Both groups differed not only at baseline (Figure 4) but also during and after therapy as patients with viral relapse showed higher expression levels of most SIMs increased during chronic hepatitis C (Figure 5*B*). The expression level of 22 analytes decreased significantly from baseline to 12 weeks after treatment cessation in patients with a SVR (Figure 6), including 17 SIMs whose expression was significantly higher in all patients with chronic hepatitis C as compared to healthy controls (Figure 2). This is shown in Figure 5*B* for the 6 analytes shown in Figure 1*B*. Despite the drastic decline in levels of expression in patients with a SVR, levels similar to those in healthy controls were not reached (Figure 5*C*). Expression of 4 chemokines (GRO-α [CXCL], interleukin 8 [CXCL8], MIP-1β [CCL4], and RANTES [CCL5]) and TNF decreased significantly during therapy (Figure 6), even though their expression was not enhanced in patients with chronic hepatitis C (Figure 1*A*). Notably, only the expression of HGF was lowered significantly in patients with a SVR and those with viral relapse at follow-up week 12. IP-10 was the only analyte associated with viral relapse, as the level of its expression decreased rapidly and increased again upon reappearance of the virus in patients with viral relapse (Figure 5*C*). In contrast, the expression levels of all significantly decreased SIMs in patients with chronic hepatitis C (Figure 1*C*) were not restored during DAA treatment (Figure 5*D*) in either patient group. These findings show that the majority of inflammatory mediators with increased expression in patients with chronic hepatitis C had significantly decreased expression during and after therapy, but levels were not comparable to those in healthy individuals. The imprint on the 6 suppressed SIMs was not altered 36 weeks after treatment initiation.

DISCUSSION

The introduction of IFN-free therapy of hepatitis C allows for the first time investigation the immunological consequences

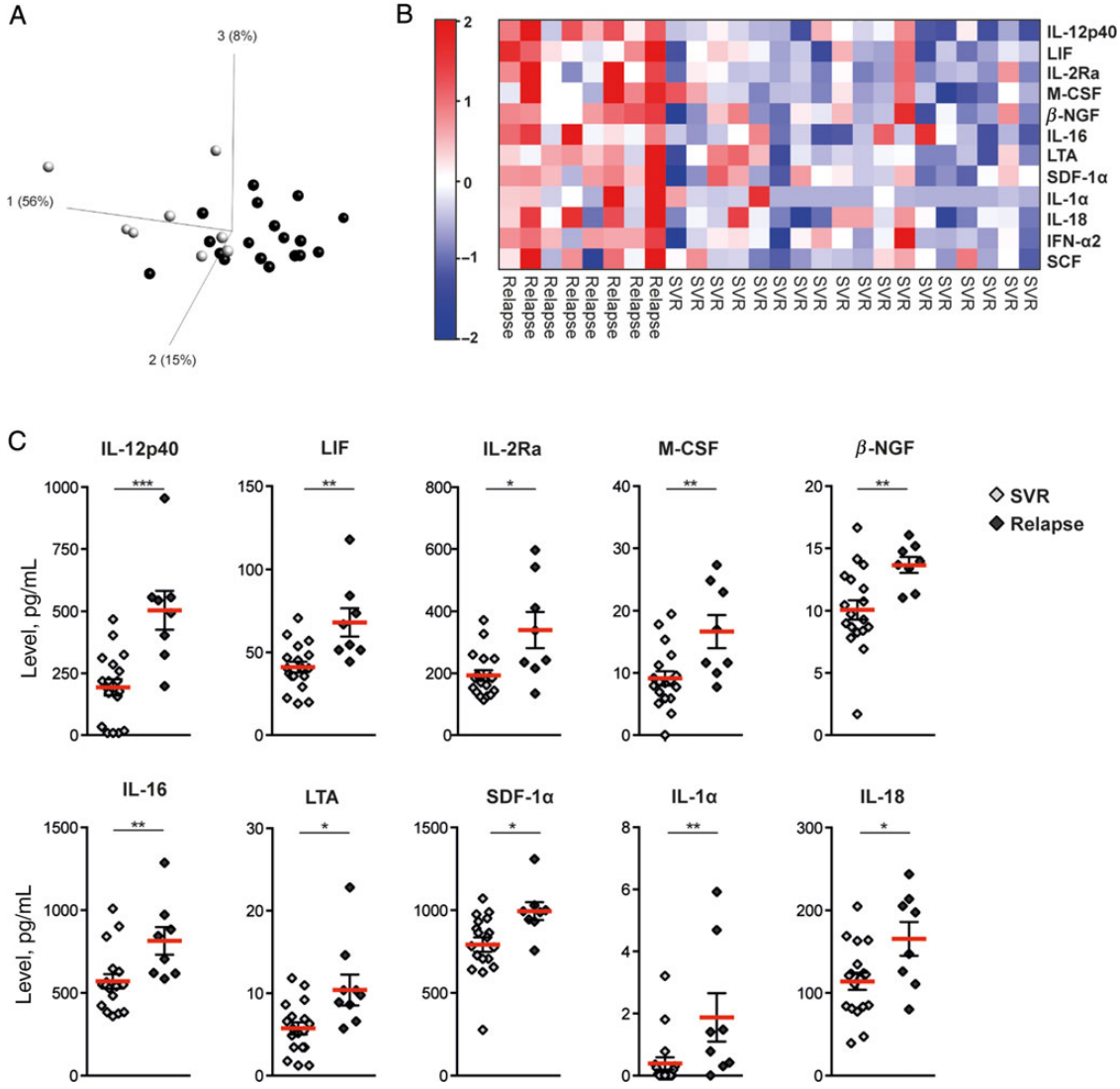

Figure 4. The cytokine and chemokine milieu differs before the start of direct-acting antiviral therapy (DAA) therapy between patients with a sustained viral response (SVR) and those with viral relapse. *A*, Principal component analysis (PCA) showed a distinct clustering of patients with chronic hepatitis C with a SVR (n = 18) upon DAA treatment and patients with viral relapse (n = 8). The PCA plot was calculated using baseline values of all measured soluble immune mediators and shows how the patients cluster together on the basis of their treatment outcome (SVR and relapse). *B*, Heat map summarizing the cytokines and chemokines that differ significantly in their expression levels upon PCA between the SVR and relapse groups. *C*, Levels of cytokines and chemokines that were significantly different expressed upon PCA were analyzed by multiple *t* testing with a false-discovery rate (*Q*) of 10%. Patients with chronic hepatitis C who experienced a viral relapse after treatment cessation show higher expression levels of these analytes. Horizontal bars represent means and standard errors of the mean. **P* < .05, ***P* < .01, and ****P* < .001, by multiple *t* testing. Abbreviation: IL, interleukin.

of clearance of a chronic viral infection in humans that, in most individuals with HCV infection, has been persistent for decades. We here show in a large, multianalyte profiling study of 50 SIMs that (1) the expression pattern of SIMs differed strikingly between patients with chronic hepatitis C, healthy individuals, and patients with a nonviral inflammatory liver disease; (2) distinct analytes, in particular those that were downregulated in HCV infection, correlated with the severity of liver fibrosis and cirrhosis; (3) patients who cleared HCV infection with otherwise suboptimal treatment consisting of sofosbuvir plus RBV could be distinguished from patients with viral relapse after therapy, based on their pretreatment cytokine and chemokine profile; and maybe most importantly, (4) the altered inflammatory milieu did not normalize upon viral clearance, even though levels of most proinflammatory parameters with increased expression partially declined while factors found to be suppressed in patients with chronic hepatitis C remained at low levels for up to 8 months after viral clearance.

It is well established that HCV infection is associated with a profound activation of the interferon system by induction of interferon-stimulated gene expression [5]. In our study, we also found upregulation of 22 SIMs in patient with chronic hepatitis C as compared to healthy controls. Levels of most of those parameters were also higher in patients with chronic hepatitis C

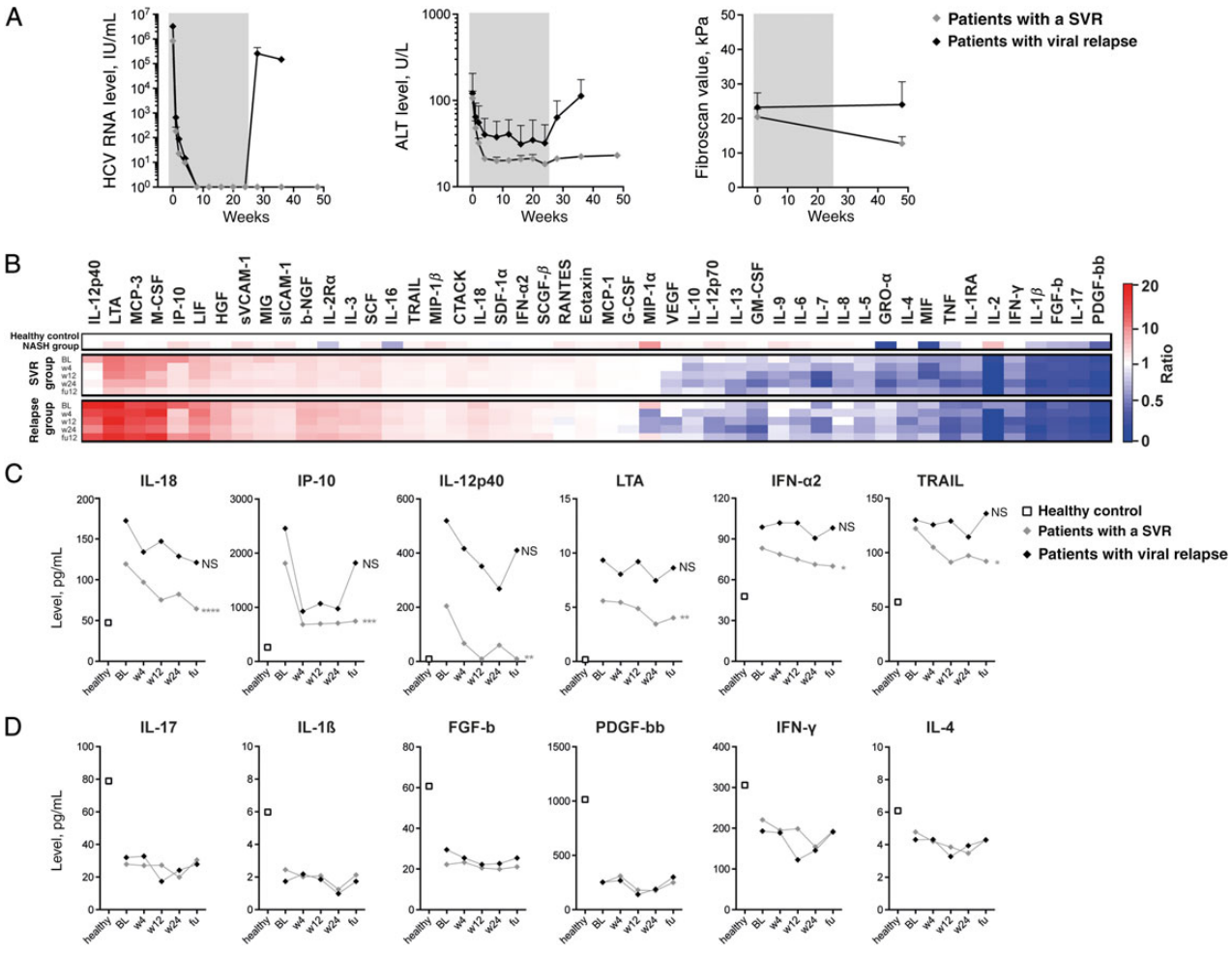

Figure 5. The cytokine and chemokine milieu improves but does not normalize upon successful direct-acting antiviral (DAA) treatment. *A*, Hepatitis C virus (HCV) RNA levels and alanine transferase (ALT) levels are shown for all 28 patients with chronic hepatitis C before, during, and after treatment with DAAs. *B*, Heat map showing the expression of all soluble immune mediators during DAA treatment of patients with chronic hepatitis C, normalized to the median value of all healthy individuals. The heat map summarizes data for 5 healthy individuals, 20 patients with nonalcoholic steatohepatitis (NASH), and 28 patients with chronic hepatitis C, of whom 18 cleared the infection upon therapy (the sustained viral response [SVR] group) and 10 experienced viral relapse. The expression of interleukin 12p40 (IL-12p40), MCP-3, M-CSF, and LTA was extrapolated as the expression level was >15-fold greater in patients with chronic hepatitis C. IL-1α and IL-15 were excluded as the median value for healthy individuals was <0.1. *C* and *D*, Patients who clear the infection are shown as gray diamonds (SVR group; n = 18), and patients who experienced viral relapse are shown as black diamonds (n = 10). *C*, Mean expression levels of SIMs with significantly elevated levels in patients with chronic hepatitis C as compared to healthy controls and significantly decreased levels upon successful DAA treatment (from baseline to follow-up week 12). Means and standard errors of the mean are presented. **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001, by the Wilcoxon test *D*, Mean expression levels of analytes that are significantly reduced in patients with chronic hepatitis C. Abbreviations: G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; NS, not significant; TNF, tumor necrosis factor.

than in patients with NASH. No study has yet performed a broad profiling analysis of cytokines, chemokines, and growth and adhesion factors in patients with chronic hepatitis C and individuals with fatty liver disease. We identified distinct differences between patients with chronic hepatitis C and patients with NASH for various other parameters, including granulocyte-macrophage colony-stimulating factor, interleukin 2, interleukin 16, and β-NGF. IL-12p40, the cytokine with the most significantly increased expression in patients with chronic hepatitis C as compared to healthy controls, was not increased in patients with NASH. Notably, IL-12p40 needs to form a heterodimer with interleukin 12p35 to be as functionally active as interleukin 12p70. However, interleukin 12p70 expression was not increased in patients with chronic hepatitis C, suggesting that the proinflammatory cytokine interleukin 23, the heterodimer between IL-12p40 and interleukin p90, may be responsible for this effect. In this context it is important to note that the degree of liver inflammation was similar between the NASH group and the chronic hepatitis C group, with median ALT values of approximately 100 U/L in both groups; however, Fibroscan values were higher in patients with chronic hepatitis C as compared to patients with NASH. Thus, both the stage of liver disease and HCV infection may have contributed to the differences in cytokine and chemokine patterns.

$P < .0001$	$P < .001$	$P < .01$	$P < .05$	Not Significant
IL-18	IP-10	M-CSF	IL-8	IL-1β
	MIP-1β	LIF	RANTES	IL-1RA
	GRO-α	MIG	IFN-α2	IL-2
	SCGF-β	sICAM-1	TNF	IL-3
	HGF	SCF	TRAIL	IL-4
	MCP-3	sVCAM-1		IL-5
		IL-12p40		IL-6
		IL-2Ra		IL-7
		LTA		IL-9
		IL-1α		IL-10
				IL-12p70
				IL-13
				IL-15
				IL-16
				IL-17
				β-NGF
				CTACK
				FGF-b
				G-CSF
				GM-CSF
				IFN-γ
				MIF
				MCP-1
				MIP-1α
				PDGF-bb
				SDF-1α
				VEGF

Figure 6. Analytes with levels that decreased from baseline to follow-up in patients with a sustained viral response. Comparisons were made using the Wilcoxon test. Abbreviations: G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

While the majority of analytes studied here were increased in patients with chronic hepatitis C as compared to healthy controls, 6 SIMs were downregulated. Levels of these parameters were also lower in HCV-infected individuals than in patients with NASH, except PDGF-bb. Of note, 4 of these analytes with decreased expression were inversely associated with the severity of liver cirrhosis (IL-4, IL-17, FGF-basic, and PDGF-bb). The role of some of these factors, such as PDGF or FGF, in liver fibrogenesis is well established, even though altered plasma levels may frequently be a consequence rather than the cause of a pathological condition. For example, intrahepatic expression of IL-17 promotes liver fibrogenesis by activation of stellate cells [24]. On first view, our finding of reduced circulating plasma levels of IL-17, in particular in patients with advanced cirrhosis, is therefore surprising. However, downregulation of IL-17 production may be the result of a regulatory loop to prevent further fibrosis progression. On the other hand, the strong correlation between distinct adhesion molecules (sVCAM-1 and sICAM-1) and the stage of liver fibrosis could indicate a direct pathophysiological link as higher expression of these markers could recruit proinflammatory immune cells to the liver, which can promote fibrogenesis [25]. Importantly, associations between histological disease activity and serum levels of sICAM-1, sVCAM-1, and IL-4 have already been described previously in patients with hepatitis C [26].

Immune responses can contribute to the control of HCV replication in patients receiving interferon-free therapy with novel DAAs [27]. The role of innate and adaptive immunity likely increases in importance as the effectiveness of the antiviral drug regimen decreases. We here had the unique opportunity to study potential immune correlates of viral response in patients with chronic hepatitis C who received only sofosbuvir and RBV without combination with other DAAs. These patients were treated during the first half of 2014, when only sofosbuvir was approved in Europe, although other potent DAAs became available later that year. As a consequence, 10 patients relapsed after completing 24 weeks of treatment with sofosbuvir plus RBV, while 18 patients achieved a SVR. Interestingly, 10 SIMs were differently expressed before therapy in the 2 groups of patients, with all parameters being higher in individuals who did not clear HCV infection. These markers included various cytokines, such as IL-12p40, Il-2Ra (sCD25), and IFN-α2. Thus, a more activated immune system was predictive of treatment failure. Overall, these findings are in line with the concept of an overexhausted interferon system in chronic hepatitis C failing to control HCV, which has been also associated with a lower response to the previous pegylated interferon alfa–based therapies [5]. In contrast to findings during pegylated interferon alfa-2 therapy, there was no difference in the pretreatment IP-10 expression level between the SVR and relapse groups during interferon-free therapy. This observation is also in line with a previous study investigating the role of IP-10 in patients infected with HCV genotype 2 or 3 who were treated with sofosbuvir und RBV [18]. In interferon-containing therapies, the relative increase in IP-10 levels during therapy was indicative of responsiveness to pegylated interferon alfa-2, which was mainly observed in patients with low IP-10 levels before therapy. In contrast, the interferon system, including IP-10, is not boosted during IFN-free therapy for hepatitis C. The intrahepatic and peripheral interferon-stimulated gene expression declines with HCV eradication [28]. Thus, the lack of association between pretreatment IP-10 levels and treatment outcome in sofosbuvir plus RBV therapy may not be surprising.

Successful treatment of hepatitis C is associated with complete virological cure and rapidly declining liver inflammation during therapy. It was previously shown that levels of cytokines and chemokines decline during interferon-free treatment of hepatitis C, including blood IP-10, MCP-1, MIP-1β, and interleukin 18 levels [18] and intrahepatic interferon-stimulated genes [28]. Indeed, we here could confirm the decline of the same plasma SIMs in patients with a SVR, except for the

chemokine MCP-1. However and importantly, most of the parameters that were elevated before treatment did not normalize during further follow-up. HCV RNA becomes undetectable within 2–8 weeks of sofosbuvir therapy, and liver enzyme levels also normalize rapidly. Thus, viral replication was absent for 6–8 months in most patients in this study. Continuous inflammation despite viral suppression is a major topic of debate in HIV infection [29]. Longer follow-up is required to answer the important question of whether the inflammatory parameters will further decline over time in successfully treated patients with HCV infection and whether long-term restoration of the interferon system is achieved after HCV clearance.

Intriguingly, all 6 cytokines and growth factors with significantly decreased levels in patients with chronic hepatitis C as compared to healthy controls were similarly affected in patients with a SVR and those with viral relapse and did not normalize upon DAA treatment. This phenomenon was also observed for one immune cell subset in the peripheral blood, mucosal-associated invariant T (MAIT) cells [30, 31]. It has been shown that MAIT cells have a decreased frequency and an impaired functional capacity in patients with chronic hepatitis C as compared to healthy individuals. This suggests that not only SIMs but also immune cell subsets in the peripheral blood that are downregulated during chronic hepatitis C are not able to recover during viral clearance, but the exact mechanism for this remains unclear. The degree of liver cirrhosis decreases upon successful DAA treatment [32], but the decreased MAIT cell frequency does not recover up to 1 year after treatment cessation [30], indicating that the improvement of liver stiffness does not seem to affect this immune cell subset.

A strength of our study is the longitudinal sampling before, during, and after DAA treatment of patients with chronic hepatitis C. Moreover, we had the unique possibility to investigate how patients with a SVR and those with viral relapse differ with regard to their cytokine and chemokine profile, as patients were treated with sofosbuvir and RBV only, a treatment regimen that is meanwhile considered as being suboptimal for most HCV genotypes. It has to be considered that most HCV-infected patients already had advanced-stage liver disease and that the mean age of the HCV-infected patients was higher than that of the 5 healthy donors. A larger control cohort would be preferable. Moreover, all patients were treated with ribavirin and we cannot exclude distinct effects of RBV on SIMs.

In conclusion, persistent HCV infection is associated with profound alterations in levels of soluble inflammatory mediators, which are associated with liver disease progression, treatment outcome, and viral presence. Importantly, these changes were not fully reversible upon clearance of viral infection.

Notes

Acknowledgments. We thank Kerstin Daemen and Jana Keil for excellent technical assistance.

J. H. was responsible for the study concept and design, acquisition of data, analysis and interpretation of data, statistical analysis, and drafting and final approval of the manuscript. C. S. F. was responsible for the acquisition of data and final approval and revision of the manuscript. V. S. was responsible for the study concept and design, interpretation of data, statistical analysis, and drafting and final approval of the manuscript. K. D. was responsible for the acquisition of data and final approval of the manuscript. M. P. M. was responsible for the drafting and final approval of the manuscript. M. C. was responsible for the interpretation of data and drafting and final approval of the manuscript. H. W. was responsible for the study concept and design, interpretation of data, and drafting and final approval of the manuscript.

Financial support. This work was supported by the International Research Training Group 1273 supported by the German Research Foundation (DFG), the Center Research Grants 738 (projects B2 and B3) and 900 (project A5) supported by the DFG, and the IFB-Tx (BMBF 01EO1302) and the German Center for Infectious Diseases TTU Hepatitis and TTU-IICH.

Potential conflicts of interest. K. D. has received lecture fees from Gilead, AbbVie, and Merck. M. P. M. has received grants and personal fees from AbbVie, Biotest, Boehringer Ingelheim, BristolMyers Squibb, Gilead, GlaxoSmithKline, Janssen, Merck (MSD), Novartis, and Roche and personal fees from Achillion. M. C. has received lecture fees from AbbVie, Bristol-Myers Squibb, Boehringer Ingelheim Pharma, Gilead, Janssen-Cilag, MSD Sharp & Dohme/Merck, Roche Diagnostic, Roche Pharma, and Siemens; advisory board fees from AbbVie, Bristol-Myers Squibb, Boehringer Ingelheim Pharma, Gilead, Roche Diagnostic, and Roche Pharma; and data safety board fees from Janssen-Cilag. H. W. has received grants from AbbVie, Gilead, Roche, Roche Diagnostics, Abbott, Myr, and Eiger and consulting fees or honoraria from AbbVie, Abbott, BMS, Boehringer Ingelheim, Eiger, Gilead, Janssen, Novartis, MSD/Merck, Roche, Roche Diagnostics, and Transgene for work under consideration for publication. Outside the submitted work, H. W. has received money for board memberships from AbbVie, Abbott, BMS, Boehringer, Eiger, Gilead, Myr, Novartis, and Roche; for consultancy from Eiger, Janssen, and Siemens; and for lectures, including service on speakers bureaus, from the Falk Foundation and OmnisMed. All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Beltra JC, Decaluwe H. Cytokines and persistent viral infections. Cytokine **2016**; 82:4–15.
2. Protzer U, Maini MK, Knolle PA. Living in the liver: hepatic infections. Nat Rev Immunol **2012**; 12:201–13.
3. Gower E, Estes C, Blach S, Razavi-Shearer K, Razavi H. Global epidemiology and genotype distribution of the hepatitis C virus infection. J Hepatol **2014**; 61: S45–57.
4. Klenerman P, Thimme R. T cell responses in hepatitis C: the good, the bad and the unconventional. Gut **2012**; 61:1226–34.
5. Heim MH, Thimme R. Innate and adaptive immune responses in HCV infections. J Hepatol **2014**; 61:S14–25.
6. Duffy D, Mamdouh R, Laird M, et al. The ABCs of viral hepatitis that define biomarker signatures of acute viral hepatitis. Hepatology **2014**; 59:1273–82.
7. Marra F, Tacke F. Roles for chemokines in liver disease. Gastroenterology **2014**; 147:577-94.e1.
8. Owusu Sekyere S, Suneetha PV, Hardtke S, et al. Type I interferon elevates co-regulatory receptor expression on CMV- and EBV-specific CD8T cells in chronic hepatitis C. Front Immunol **2015**; 6:270.
9. Golden-Mason L, Waasdorp Hurtado CE, Cheng L, Rosen HR. Hepatitis C viral infection is associated with activated cytolytic natural killer cells expressing high levels of T cell immunoglobulin- and mucin-domain-containing molecule-3. Clin Immunol **2015**; 158:114–25.
10. Rehermann B. Pathogenesis of chronic viral hepatitis: differential roles of T cells and NK cells. Nat Med **2013**; 19:859–68.
11. Gane EJ, Stedman CA, Hyland RH, et al. Nucleotide polymerase inhibitor sofosbuvir plus ribavirin for hepatitis C. N Engl J Med **2013**; 368:34–44.
12. Lawitz E, Mangia A, Wyles D, et al. Sofosbuvir for previously untreated chronic hepatitis C infection. N Engl J Med **2013**; 368:1878–87.

13. Foster GR, Irving WL, Cheung MC, et al. Impact of direct acting antiviral therapy in patients with chronic hepatitis C and decompensated cirrhosis. J Hepatol **2016**; 64:1224–31.
14. Wiegand J, Cornberg M, Aslan N, et al. Fate and function of hepatitis-C-virus-specific T-cells during peginterferon-alpha2b therapy for acute hepatitis C. Antivir Ther **2007**; 12:303–16.
15. Barnes E, Harcourt G, Brown D, et al. The dynamics of T-lymphocyte responses during combination therapy for chronic hepatitis C virus infection. Hepatology **2002**; 36:743–54.
16. Missale G, Pilli M, Zerbini A, et al. Lack of full CD8 functional restoration after antiviral treatment for acute and chronic hepatitis C virus infection. Gut **2012**; 61:1076–84.
17. Jablonska J, Pawlowski T, Laskus T, et al. The correlation between pretreatment cytokine expression patterns in peripheral blood mononuclear cells with chronic hepatitis C outcome. BMC Infect Dis **2015**; 15:556.
18. Carlin AF, Aristizabal P, Song Q, et al. Temporal dynamics of inflammatory cytokines/chemokines during sofosbuvir and ribavirin therapy for genotype 2 and 3 hepatitis C infection. Hepatology **2015**; 62:1047–58.
19. Wandrer F, Falk CS, John K, et al. Interferon-mediated cytokine induction determines sustained virus control in chronic hepatitis C virus Infection. J Infect Dis **2016**; 213:746–54.
20. Deterding K, Honer Zu Siederdissen C, Port K, et al. Improvement of liver function parameters in advanced HCV-associated liver cirrhosis by IFN-free antiviral therapies. Aliment Pharmacol Ther **2015**; 42:889–901.
21. Wranke A, Heidrich B, Ernst S, et al. Anti-HDV IgM as a marker of disease activity in hepatitis delta. PLoS One **2014**; 9:e101002.
22. Gisa A, Suneetha PV, Behrendt P, et al. Cross-genotype-specific T-cell responses in acute hepatitis E virus (HEV) infection. J Viral Hepat **2016**; 23:305–15.
23. Lemke A, Noriega M, Roske AM, et al. Rat renal transplant model for mixed acute humoral and cellular rejection: weak correlation of serum cytokines/chemokines with intragraft changes. Transpl Immunol **2015**; 33:95–102.
24. Hammerich L, Tacke F. Interleukins in chronic liver disease: lessons learned from experimental mouse models. Clin Exp Gastroenterol **2014**; 7:297–306.
25. Williams MJ, Clouston AD, Forbes SJ. Links between hepatic fibrosis, ductular reaction, and progenitor cell expansion. Gastroenterology **2014**; 146:349–56.
26. Patel K, Remlinger KS, Walker TG, et al. Multiplex protein analysis to determine fibrosis stage and progression in patients with chronic hepatitis C. Clin Gastroenterol Hepatol **2014**; 12:2113-20.e1-3.
27. Rehermann B, Bertoletti A. Immunological aspects of antiviral therapy of chronic hepatitis B virus and hepatitis C virus infections. Hepatology **2015**; 61:712–21.
28. Meissner EG, Wu D, Osinusi A, et al. Endogenous intrahepatic IFNs and association with IFN-free HCV treatment outcome. J Clin Invest **2014**; 124:3352–63.
29. Klatt NR, Chomont N, Douek DC, Deeks SG. Immune activation and HIV persistence: implications for curative approaches to HIV infection. Immunol Rev **2013**; 254:326–42.
30. Hengst J, Strunz B, Deterding K, et al. Nonreversible MAIT cell-dysfunction in chronic hepatitis C virus infection despite successful interferon-free therapy. Eur J Immunol **2016**; 46:2204–10.
31. Spaan M, Hullegie SJ, Beudeker BJ, et al. Frequencies of circulating MAIT cells are diminished in chronic HCV, HIV and HCV/HIV co-infection and do not recover during therapy. PLoS One **2016**; 11:e0159243.
32. Deterding K, Schlevogt B, Port K, Cornberg M, Wedemeyer H. Letter: can persisting liver stiffness indicate increased risk of hepatocellular cell carcinoma after successful anti-HCV therapy? - authors' reply. Aliment Pharmacol Ther **2016**; 43:546–7.