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Acute and Chronic Immune Biomarker Changes During Interferon/Ribavirin Treatment in HIV-HCV Co-infected Patients

Mamta K. Jain¹, Beverley Adams-Huet², Darya Terekhova¹, Lauren E. Kushner⁴, Roger Bedimo^{1,3}, Xilong Li², and Mark Holodniy^{4,5}

¹Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX

²Department of Clinical Sciences, University of Texas Southwestern Medical Center, Dallas, TX

³Department of Internal Medicine, North Texas VA Medical Center, Dallas, TX

⁴AIDS Research Center, Veterans Affairs Palo Alto Health Care System, Palo Alto, CA

⁵Department of Medicine, Stanford University School of Medicine, Stanford, CA, United States

Abstract

Chronic viral infections lead to persistent immune activation, which is alleviated by eradicating or suppressing the infection. To understand the effects of interferon treatment on immune system activation by chronic infections, we evaluated kinetic patterns of a broad spectrum of serum biomarkers during HCV treatment in HIV/HCV co-infected patients. HCV viral load and 50 biomarkers were analyzed at baseline and 27 time points during pegylated interferon and ribavirin (IFN/RBV) treatment of 12 HIV/HCV co-infected patients. We evaluated biomarker changes from baseline for each time point, and biomarker correlations with clinical parameters, treatment response and liver histopathology. IL-1 α , IL-12p40, IL-1RA, IP-10, MIG, MIP-1 α /1 β , HGF, sCD40L, TRAIL and leptin increased in the first day. IL-12p70, IL-17A, IL-10, GRO α , IL-8, MCP-3, IL-4, and M-CSF peaked later during week 1. IL-1 α , HGF, IP-10, MIP-1 α , TRAIL, sCD40L, IL-10, IL-12p70, MCP-3, FGFb, ENA-78, TGF β , IL-2, IFN γ , IL-6, IL-15, IL-7, and PDGF-BB decreased below baseline over the course of treatment. Higher BMI, baseline HCV viral load and leptin levels were associated with lack of sustained virological response. ENA-78 was associated with sustained viral response. Positive correlations were found between liver inflammation and baseline CD4 count, sVCAM, and HGF; fibrosis stage and HGF; liver steatosis, BMI and leptin. Our findings suggest IFN/RBV treatment initially increases levels of several biomarkers, but eventually leads to a decline in many immune markers. These findings shed light on the relationship between IFN treatment and immune activation by chronic viral infections, such as HCV.

Corresponding author: Mamta K. Jain, MD UT Southwestern Medical Center 5323 Harry Hines Blvd. Dallas, TX 75390-9113
mamta.jain@utsouthwestern.edu Phone: 214-648-6703 Fax: 214-648-2741.

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Keywords

HIV; HCV; immune response; antiviral treatment; interferon

Hepatitis C virus (HCV) infection, like other chronic viral infections, can lead to chronic immune activation (1-3). Inflammation, which is part of chronic immune activation during HCV infection, has been suggested to contribute to progression of liver disease either through fibrosis activation or through direct cytotoxic effects of inflammatory mediators (4-6). Liver disease resulting from HCV infection is a leading cause of morbidity and mortality in human immunodeficiency virus (HIV)-infected patients (7). Until recently, the standard of care for HCV treatment in both HCV mono-infected and HIV/HCV co-infected patients was pegylated interferon and ribavirin (IFN/RBV). It has been shown that increased CD38 expression, as a marker of immune activation, is greater among HIV/HCV co-infected compared to HCV and HIV mono-infected patients, and decreases with IFN/RBV therapy (8). However, little is known about the mechanism of IFN/RBV treatment and its effect on chronic viral infection-induced immune activation.

Most studies on the role of cytokines and chemokines in HCV infection have been performed in HCV mono-infected patients and focused on comparing cytokine and chemokine profiles between HCV patients and uninfected controls, identifying markers that are predictive of response to IFN/RBV therapy, and exploring the role of cytokines in liver fibrosis to identify markers predictive of fibrosis progression (9-11). Substantially fewer studies of cytokine and chemokine patterns in HIV/HCV co-infected patients have been performed, and these studies have focused on the role of HIV suppression by antiretroviral therapy (ART) in hepatitis C progression and on determining the link between chronic inflammation and the tendency for HIV/HCV co-infected patients to have accelerated disease progression and an increased risk of comorbidities (12-14). Suppression of HIV viral load among HIV/HCV co-infected patients can decrease inflammation and immune activation and slow the progression of liver disease to a rate seen in patients with HCV alone (14). However, very little is known about the impact of IFN/RBV treatment on immune activation in HIV/HCV co-infected patients.

Recent developments in direct acting agents against HCV raise the possibility of IFN-free treatment for chronic HCV infection. However, studying the effects of IFN administration on the immune system is still relevant because it can provide insights into the resolution of immune system activation after chronic infection elimination or suppression. Additionally, IFN treatment has been reported to control HIV replication and decrease HIV integration, suggesting that it may be used in HIV suppression and/or eradication (15, 16). There have also been reports that IFN treatment may help increase CD4 cell counts, delay disease progression and prolong survival of asymptomatic HIV-infected individuals (17). In vivo studies suggested that IFN alpha enhance antiviral immune response rather than having a direct antiviral activity (18). To understand the mechanisms by which IFN alpha immunomodulation helps control or eradicate viral infections, immune system response to IFN alpha treatment needs to be studied.

The aim of this study was to examine a wide array of cytokines and chemokines during IFN/RBV treatment and elucidate the impact of this treatment on immune activation in HIV/HCV co-infected patients.

Material and Methods

Patient Population and Treatment

Sixty-two patients were recruited prospectively from the HIV and Liver Clinics at Parkland Health and Hospital Systems, a teaching hospital associated with the University of Texas Southwestern (UTSW) Medical Center, for a study of viral kinetics in patients with HCV mono-infection or HIV/HCV co-infection. Of these 62 patients, twelve consecutive HIV/HCV co-infected individuals with available serum samples were selected for the present study (Table 1). All patients signed an informed consent form before starting the study, and the Institutional Review Board at UTSW approved the study protocol and informed consent.

Patient selection criteria were previously described (19). The study included patients between 18 and 65 years of age who were anti-HCV antibody reactive, naïve to IFN/RBV treatment, and infected with HCV genotype 1 with serum HCV RNA >1,000 IU/mL. In addition, these patients were HIV-1 positive, had no evidence of AIDS-defining illness, and if on ART were on a stable ART regimen with CD4 T-cell counts >200 cells/mm³ for at least 12 weeks before study enrollment and initiation. Exclusion criteria included presence of other liver diseases or evidence of liver decompensation, uncontrolled diabetes mellitus or psychiatric illness, current use of >50 g of alcohol per day, and injection drug use anytime within the six months prior to enrollment. Race, ethnicity, and use of tobacco, alcohol, or intravenous drugs were self-reported.

All patients began a standard HCV treatment regimen that included weekly injections of pegylated-interferon-alpha-2a (180 µg) and twice daily administration of weight-based ribavirin (either 1,000 mg for body weight <75 kg or 1,200 mg for body weight >75 kg). Sustained virologic response (SVR) patients were those who received 48 weeks of treatment and had undetectable HCV viral load 24 weeks after treatment. The non-SVR group included non-responder patients that did not achieve undetectable HCV RNA levels during 24 weeks of treatment and discontinued treatment after 24 weeks, as well as patients who relapsed after completing 48 weeks of treatment.

Sample Collection and Laboratory Evaluation

Blood samples were collected from participants at baseline and hours 4, 8, 12, 18, 24, 28, 32, 48; days 3, 4, 7, 9, 11; and weeks 2, 3, 4, 6, 8, 12, 16, 20, 24, 30, 36, 42, 48, and 60 after the start of treatment. IL28B gene polymorphism was determined at baseline by sequence analysis of genomic DNA extracted from blood samples as previously described (20). HCV viral loads at each time point were measured by VERSANT® HCV RNA 3.0 assay with detection limit of 43 copies/ml (Siemens Medical Solutions Diagnostics, Tarrytown, NY). HIV viral loads were measured by COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test with detection limit of 48 RNA copies/ml (Roche Molecular Systems, Branchburg, NJ).

Fifty serum biomarkers were analyzed at each time point using the Luminex® 50-plex assay (Luminex, San Diego, CA) according to manufacturer's instructions (protocol found at: <http://iti.stanford.edu/research/himc-protocols-immunoassays.html>). The serum biomarkers assessed included interferons, pro-inflammatory and anti-inflammatory cytokines, chemokines, growth factors, endothelial markers, apoptosis-inducing factors, and factors involved in immune cell regulation and proliferation (Table 2).

Liver biopsy data were accessed from study participants' medical records. All subjects had a liver biopsy within 2 years of starting HCV treatment. Trained pathologists evaluated biopsies as part of standard of care at Parkland Health and Hospital Systems, Dallas, TX. The Batts and Ludwig grading scale (21) was used to assess inflammation and fibrosis in liver biopsies. Extent of steatosis was estimated as the percentage of the liver biopsy that consists of fat cells.

Statistical Analysis

Demographic and clinical characteristics and serum marker levels at baseline were compared between SVR and non-SVR patients using Mann-Whitney test or Fisher's exact test, as appropriate. Associations between serum biomarker levels and liver histopathology were examined using Spearman correlation for all 12 patient samples at baseline. Changes in serum biomarker levels from baseline were evaluated for each time point by mixed model repeated measures analysis of all available samples. Serum biomarker level differences between SVR and non-SVR were examined using both Mann-Whitney test and repeated measures analysis. Biomarker variables were log transformed prior to parametric analyses. A two-sided p-value <0.05 was considered statistically significant. All statistical tests were considered hypothesis generating; they were not adjusted for multiple testing due to small sample size and exploratory nature of the analyses. Statistical analysis and data visualization were performed using SAS version 9.2 (SAS Institute, Cary, NC), GraphPad Prism version 5.04, and STATA version 12.1 edX/Harvard/MIT edition.

Results

Patient Characteristics and Response to Treatment

Twelve consecutive patients were selected for this analysis based on serum sample availability. Their demographic and clinical characteristics are listed in Table 1. Mean patient age was 44 years (33-57 range), 75% were male, 67% were white and 33% were black, and 17% were of Hispanic ethnicity (Table 1). IL28B genotype CC was found in 40% of study participants. History of alcohol abuse was reported in 9% of patients, while 27% had a history of drug abuse and 82% had a history of psychiatric illness (Table 1).

Of the twelve study participants, six achieved SVR, three were non-SVR (two treatment nonresponders and one relapser), and three dropped out of the study before SVR or non-SVR status could be ascertained (at day 7, week 4, and week 8 after starting treatment). HIV viral loads were undetectable in all patients prior to HCV treatment except for two patients. One patient was a long-term non-progressor, was not on ART, and had an HIV viral load of 53 copies/mL prior to HCV treatment; his HIV RNA became undetectable during treatment.

The second patient had an HIV viral load of 64 copies/mL prior to treatment which remained <200 copies/mL until week 36 when he had a HIV viral breakthrough to 838 copies/mL and then dropped to 447 copies/mL at week 48. Both of these patients achieved an SVR.

The demographic or clinical parameters that were significantly different between SVR and non-SVR at baseline were HCV viral load ($p=0.02$) and body mass index (BMI) ($p=0.02$) (Table 1).

Serum Biomarker Kinetics during Treatment

For this analysis, all 12 patients were included, although not all patients had samples available for every time point. Seven patients completed 48 weeks of treatment and 12 to 24 weeks of follow-up. Of these seven, five were SVR patients with samples available through week 60, another was an SVR patient with samples available through week 36, and the last was a non-SVR patient (relapser) with samples available through week 48. Of the remaining five patients, two were non-SVR patients who received only 24 weeks of treatment and had samples available through week 30 and 36. The last three patients completed 7 weeks, 4 weeks, and 9 days of treatment, respectively. Samples for drop-out patients were available up to the time of drop out. Figure 1 shows kinetics of selected serum biomarkers. Table 3 lists serum biomarkers whose levels were found to change significantly from baseline ($p<0.05$).

Three major patterns of serum biomarker changes were observed during treatment. First, one group of biomarkers increased significantly either during the first 24 hours of treatment (Figure 1A) or within the first 7-10 days (Figure 1B), but then returned to baseline later during treatment. The early peaks within 24 hours were observed for leptin, IL-12p40, IL-1RA, MCP-1, and MIG (Figure 1A, Table 3). Later peaks within the first week included IL-8, IL-17A, GRO- α , M-CSF, and IL-4 (Figure 1B, Table 3).

Next, a second group of biomarkers initially peaked but then continually decreased to levels below baseline values. This group included IL-1 α , IP-10, MIP-1 α , and MIP-1 β , sCD40 ligand, TRAIL, and HGF, all of which peaked within 24 hours (Figure 1C, Table 3). IL-12p70, IL-10, and MCP-3 peaked later by day 7, but also decreased below baseline during treatment (Figure 1D, Table 3). Interestingly, endothelial factor sVCAM-1 followed the pattern of IFN- α , the treatment drug (Figure 1E, Table 3).

The third group contained biomarkers that did not significantly change in the first week but then decreased below baseline over the course of the study. These included FGFb, IFN- γ , ENA-78, PDGF-BB, TGF- β , IL-2, IL-7, IL-15, and IL-6 (Figure 1F, Table 3).

The fourth group contained biomarkers that did not change significantly during treatment: eotaxin, sFas ligand, G-CSF, GM-CSF, sICAM-1, IFN- β , IL-1 β , IL-5, IL-13, IL-17F, LIF, NGF, RANTES, resistin, PAI-1, SCF, TGF- α , TNF- α , and VEGF (results not shown).

Serum Biomarker Levels Significantly Different between SVR and Non-SVR Patients

To identify potential markers of treatment outcome, serum marker levels of SVR and non-SVR patient groups were compared at baseline and selected time points during treatment. Leptin was the only marker in which median serum levels were significantly different between the SVR and non-SVR at several time points, including baseline (87 pg/mL vs. 872 pg/mL), 4 hours (87 pg/mL vs. 1364 pg/mL), 12 hours (135 pg/mL vs. 1803 pg/mL), 24 hours (147 pg/mL vs. 528 pg/mL), 32 hours (144 pg/mL vs. 1525 pg/mL) and 7 days (91 pg/mL vs. 633 pg/mL). Statistically significant differences in serum leptin levels between SVR and non-SVR patients were not detected at later time points; however, the qualitative differences persisted for the duration of the study (Figure 2A).

Median serum levels of ENA-78 differed between SVR and non-SVR patients throughout the study (Figure 2B), though a statistically significant difference was only observed at 4 hours ($p=0.02$, 42 pg/mL vs. 108 pg/mL). Statistical power is limited because of the small sample size.

Correlations between Serum Biomarkers and Liver Histopathology

Correlations between median baseline serum biomarker levels, demographic and clinical factors, and histopathology parameters (inflammation, fibrosis, and steatosis) were examined using Spearman correlation analysis. Table 4 shows statistically significant correlations between serum markers and histopathology.

There was a strong positive correlation between liver inflammation grade and fibrosis stage ($\rho=0.74$, $p=0.006$). Additionally, liver inflammation grade correlated positively with CD4 T cell counts ($\rho=0.83$, $p=0.006$), sVCAM-1, and HGF, and negatively with IL-1 β and LIF (Table 4) at baseline. Liver fibrosis stage also correlated positively with aspartate aminotransferase ($\rho=0.70$, $p=0.04$) and negatively with IFN α , IFN- γ and LIF (Table 4). The extent of liver steatosis correlated positively with BMI ($\rho=0.68$, $p=0.04$) and leptin.

Discussion

We observed several distinct patterns of change in serum cytokine and chemokine concentrations during IFN/RBV treatment in HIV/HCV co-infected patients. These changes can be divided into two functional categories: immune biomarkers whose concentrations peaked early in treatment, which may be a result of IFN- α induction (immediate immune activation by IFN- α), and immune biomarkers whose serum levels decreased later in treatment, which may be a result of decreased HCV viral loads and an immune system which has returned to a less active state.

Within hours after the start of treatment, we observed a peak in pro-inflammatory (IL-1 α , IL-12p40) and anti-inflammatory (IL-1RA) cytokines, chemokines associated with inflammation (IP-10, MCP-1, MIG, MIP-1 α , and MIP-1 β), as well as TRAIL, HGF, sCD40 ligand, and leptin. This was followed by a delayed peak in additional pro-inflammatory (IL-17A and IL-12p70) and anti-inflammatory (IL-10) cytokines, chemokines involved in inflammation (GRO- α , IL-8, and MCP-3), and immune cell proliferation/regulation (M-CSF) and regulatory factors (IL-4). Several of these biomarkers (IL-1RA, IP-10, and

TRAIL) have been previously shown to be directly induced by interferons and IFN- α specifically (22-24), suggesting that early biomarker changes may be a result of IFN- α activation through the JAK/STAT pathway. The immune markers induced by IFN- α may play a role in controlling HIV replication and integration and HIV reservoir. Further studies of interferon treatment in HIV-infected patients are needed to understand this mechanism.

The most striking example of IFN- α induction in our study was sVCAM-1, whose serum levels increased with increasing IFN- α levels and dropped after IFN- α treatment was stopped. Serum levels of vascular endothelial factors, sVCAM-1 and s-ICAM-1, have been shown to increase upon IFN α introduction and remain high until the end of IFN α treatment (25).

Many serum biomarkers decreased significantly below baseline over the course of the study. These included pro-inflammatory cytokines and chemokines (IL-1 α , ENA-78, IL-12p70, MIP-1 α , and MIP-1 β), suggesting a decrease in inflammatory activity. A major pro-inflammatory cytokine, TNF- α , also decreased over time, but this change was not statistically significant. Significant decreases in levels of IL-6, an important regulator of both pro- and anti-inflammatory activity, and IL-10, a major anti-inflammatory cytokine, suggest that not only inflammatory, but anti-inflammatory activity decreases as well. IL-6 has been shown to stimulate hepatocyte proliferation and liver regeneration in response to injury (26), so decreased levels of IL-6 may suggest a decreasing acute liver injury response. Serum levels of the antiviral IFN- γ , chemokines IP-10 and MCP-3, and immune cell proliferation and regulation factors sCD40 ligand, IL-2, IL-7, and IL-15 all decreased, perhaps reflecting a decrease in immune activity. Pro-fibrotic factor FGFb levels started decreasing from the beginning of treatment, suggesting that this may be a result of IFN- α introduction; indeed, IFN- α has been shown to inhibit FGFb expression (27). Decreasing FGFb levels can be an indication of decreasing fibrotic activity due to IFN- α treatment which has been reported previously (28-30). Although many serum biomarker levels decreased significantly as a result of IFN/RBV treatment, previous work by our group indicates that at the end of HCV treatment in those who achieve SVR still have elevated levels of many biomarkers, including inflammatory cytokines compared to those who were exposed to HCV and spontaneously cleared the infection without treatment (31).

A number of serum biomarkers that decreased below baseline levels by the end of our study have been previously shown to play a role in HCV pathogenesis and be predictive of IFN/RBV treatment outcome. IL-1 α is an important pro-inflammatory cytokine that is also known to be profibrogenic (32). ENA-78 was shown to contribute to inflammation and liver injury during chronic alcoholic liver disease, and higher levels of ENA-78 have been associated with worse prognosis (33). IFN- γ exacerbates liver damage by increasing inflammation and fibrogenesis (34). IP-10 was shown to correlate with hepatic inflammation and fibrosis and to be predictive of IFN/RBV treatment response both in HCV mono-infected in HCV/HIV co-infected patients (13, 35). HGF has been correlated with liver inflammation, fibrosis and cirrhosis in HCV infection (36). TGF- β has been shown to be induced by HCV in vivo and in vitro (37) and may play a role in liver fibrosis (38), so it is possible that decreased TGF- β levels observed at the end of this study is related to decreased HCV viral load as a result of treatment. Decreased serum levels of all of these markers –

IL-1 α , ENA-78, IFN- γ , IP-10, HGF and TGF- β - as a result of IFN/RBV treatment may contribute to a decrease or reversal in fibrosis.

Our results further suggest that leptin and ENA-78 may be predictive of SVR. Both biomarkers had lower serum levels in SVR patients compared to non-SVR patients at baseline and throughout the study. Leptin correlates positively with BMI and liver steatosis, an observation made in this and previous studies (39). We demonstrated, like others, that lower leptin levels and lower BMI correlate with SVR (40). Because BMI and leptin are confounded, we suggest considering leptin as a surrogate biomarker of BMI rather than directly involved in an interferon-induced HCV eradication mechanism. To date, only two reports have evaluated the role of ENA-78 in patients with HCV, and ENA-78 was not found to be predictive of SVR or to correlate with fibrosis in either of them (41). However, in patients with other inflammatory liver diseases, such as alcoholic liver disease, higher levels of ENA-78 were associated with increased inflammation and worse prognosis of liver disease (33). In addition, ENA-78 serum levels were reported to decrease during IFN treatment, consistent with our findings (42). Thus, ENA-78 appears to be involved in liver inflammation rather than fibrosis. Higher ENA-78 levels observed in non-SVR patients in our study may be associated with higher liver inflammation, while decreasing levels of ENA-78 may be associated with decreasing liver inflammation as a result of HCV treatment (43, 44). We propose ENA-78 as a potential novel predictive marker for the extent of liver inflammation and for outcomes of chronic HCV treatment; further studies are needed to prove this hypothesis.

Clinical parameters and serum biomarkers that we found to correlate with different aspects of liver pathology (inflammation, fibrosis, and steatosis) were generally consistent with the corresponding mechanisms of pathology and previously published findings. Inflammation grade correlated positively with CD4 cell count, which confirms previous associations found between higher CD4 count and increased inflammation: immune reconstitution due to ART may result in rapidly increased liver inflammation in patients with hepatitis B virus or HCV co-infection (45). Our finding of a positive correlation of liver inflammation with serum sVCAM-1 and HGF levels is consistent with pathological mechanisms: sVCAM-1 is an endothelial factor that increases blood vessel permeability during inflammation, and HGF is induced by inflammation (46). Liver fibrosis stage correlated positively with inflammation grade and negatively with IFN- α and IFN- γ levels, consistent with previous findings in HIV/HCV co-infected patients (47-50).

There are some important limitations of this study, which include small sample size, limited follow-up samples after week 24 in some patients, exploratory analyses, and multiple comparisons, which could produce spurious results. The results may not be directly translatable to HCV mono-infected patients, as the HIV/HCV patients are likely to have a greater degree of cytokine dysregulation than those with HCV alone. In fact, we have previously demonstrated that there are significant differences in baseline biomarker levels between HCV mono-infected and HIV/HCV co-infected groups (31). However, most of the patients in our study had undetectable HIV viral loads while receiving ART and during HCV treatment, thus lowering the potential for HIV replication as being contributory to ongoing inflammation. The strengths of the current study include longitudinal sampling,

multiple early sampling time points, and the breadth of serum markers examined. Although interpretation of the large number of biomarker examined and their interactions may be complex, our study provides novel observations on biomarker kinetics during IFN/RBV treatment in HIV/HCV co-infected patients.

In conclusion, this is the first study to examine changes in a wide range of serum cytokines and chemokines at multiple early time points during IFN/RBV treatment of HIV/HCV patients. Measurements of these biomarkers provide insight into how interferon- α may activate early immune responses and suggest that the gradual decrease in cytokine and chemokine serum levels during treatment reflects the impact of HCV viral clearance on the immune system. Studies examining direct-acting antiviral agent regimens (both with and without interferon) may also shed light on how the changes in the cytokine milieu may be related to interferon treatment and/or clearance of HCV.

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Figure 1. Serum biomarker kinetics during IFN/RBV treatment of HIV/HCV co-infected patients

A – Markers whose levels peaked within 24 hours from beginning treatment and returned to baseline; B – Markers whose levels peaked within 7-10 days from beginning treatment and returned to baseline; C - Markers whose levels peaked within 24 hours from beginning treatment and then decreased below baseline; D - Markers whose levels peaked within 7-10 days from beginning treatment and then decreased below baseline; E - sVCAM-1 follows IFN- α kinetics; F - Serum markers that started decreasing after 1 week of treatment.

Figure 2. Leptin and ENA-78 kinetics during IFN/RBV treatment of HIV/HCV co-infected patients

A - Leptin serum levels differ between SVR and non-SVR patients throughout the course of treatment; B – ENA-78 levels differ between SVR and non-SVR patients throughout the course of treatment.

Table 1

Demographic and Clinical Profile of Study Participants.

Characteristic		All (n=12) ^a	SVR (n=6) ^a	Non-SVR (n=3) ^a	p value
Age, years (mean ± SD)		44 ± 8	42 ± 7	48 ± 9	0.55 ^b
Race	White (%)	8 (67%)	5 (83%)	2 (67%)	1.00 ^c
	Black (%)	4 (33%)	1 (17%)	1 (33%)	1.00 ^c
Ethnicity, Hispanic (%)		2 (17%)	2 (33%)	0 (0%)	0.50 ^c
Gender, Male (%)		9 (75%)	6 (100%)	1 (33%)	0.08 ^c
IL28B genotype, CC (%)		4 (40%) ^d	3 (60%) ^e	0 (0%)	0.20 ^c
BMI (mean ± SD)		26 ± 5	24 ± 2	34 ± 5	0.02 ^b
HCV viral load Log10 IU/mL at baseline (mean ± SD)		5.8 ± 0.8	5.2 ± 0.7	6.4 ± 0.3	0.02 ^b
Alanine aminotransferase (ALT) IU/L at baseline (mean ± SD)		67 ± 25	73 ± 26	64 ± 27	0.55 ^b
Aspartate aminotransferase (AST) IU/L at baseline (mean ± SD)		54 ± 17	52 ± 19	67 ± 15	0.26 ^b
CD4 count/mm ³ at baseline (mean ± SD)		649 ± 263	639 ± 226	694 ± 298	1.00 ^b
Liver inflammation grade: Batts and Ludvig (mean ± SD)		1.9 ± 0.9	1.9 ± 0.9	1.7 ± 1.2	0.68 ^b
Liver fibrosis stage: Batts and Ludvig (mean ± SD)		1.8 ± 1.2	1.5 ± 1.4	2.3 ± 1.3	0.42 ^b
Liver steatosis, % (mean ± SD)		10 ± 19 ^f	4 ± 6	30 ± 30	0.36 ^b
Smoking (%)		8 (73%) ^f	4 (67%)	1 (33%)	0.52 ^c
History of alcohol abuse (%)		1 (9%) ^f	0 (0%)	0 (0%)	1.00 ^c
History of drug abuse (%)		3 (27%) ^f	0 (0%)	2 (67%)	0.08 ^c
History of psychiatric disorders (%)		9 (82%) ^f	4 (67%)	3 (100%)	0.50 ^c

^a of the 12 patients in the study, 6 achieved SVR, 3 were non-SVR and 3 stopped treatment too early for the outcome to be determined^b based on Mann-Whitney test.^c based on Fisher's exact test.^d n=10.^e n=5.

$f_{n=11}$.

Table 2

Serum biomarkers assessed in the study by their functional category.

Function	Serum Biomarkers
Antiviral factors	interferon alpha (IFN- α), interferon beta (IFN- β), interferon gamma (IFN- γ)
Pro-inflammatory markers	interleukin-1 alpha (IL-1 α), interleukin-1 beta (IL-1 β), interleukin-12p40 (IL-12p40), interleukin-12p70 (IL-12p70), interleukin-17A (IL-17A), interleukin-17F (IL-17F), tumor necrosis factor alpha (TNF- α), tumor necrosis factor beta (TNF- β)
Anti-inflammatory markers	interleukin-1 receptor antagonist (IL-1RA), interleukin-10 (IL-10), interleukin-13 (IL-13)
Both pro-inflammatory and anti-inflammatory cytokine	interleukin-6 (IL-6)
Chemokines	epithelial-derived neutrophil-activating peptide 78/CXCL5 (ENA-78), eotaxin/CCL11, growth related oncogene alpha/CXCL1 (GRO- α), interleukin-8/CXCL8 (IL-8), interferon gamma-induced protein 10/CXCL10 (IP-10), monocyte chemoattractant protein 1/CCL2 (MCP-1), monocyte chemoattractant protein 3/CCL7 (MCP-3), monokine induced by gamma interferon/CXCL9 (MIG), macrophage inflammatory protein 1 alpha/CCL3 (MIP-1 α), macrophage inflammatory protein 1 beta/CCL4 (MIP-1 β), regulated upon activation normal T cell expressed and secreted/CCL5 (RANTES)
Endothelial factors	soluble intercellular adhesion molecule 1 (sICAM-1), platelet-derived growth factor-BB (PDGF-BB), soluble vascular cell adhesion molecule 1 (sVCAM-1), vascular endothelial growth factor (VEGF)
Hypercoagulation	factors fibroblast growth factor basic (FGFb), plasminogen activator inhibitor 1 (PAI-1)
Apoptosis inducers	soluble Fas ligand (sFasL), TNF-related apoptosis-inducing ligand (TRAIL)
Growth factors	hepatocyte growth factor (HGF), nerve growth factor (NGF), tumor growth factor alpha (TGF- α), tumor growth factor beta (TGF- β), stem cell factor (SCF)
Immune cell proliferation and regulation factors	soluble CD40 ligand (sCD40L), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 7 (IL-7), interleukin 15 (IL-15), leukocyte migration inhibition factor (LIF), macrophage colony-stimulating factor (M-CSF)
Fat metabolism markers	leptin, resistin

Table 3

Median (25th, 75th percentile) serum biomarker levels at selected time points, pg/ml.

	baseline (n=12)	4 h (n=12)	12 h (n=12)	1 d (n=12)	7 d (n=12)	4 w (n=11)	12 w (n=9)	48 w (n=6)
Antiviral factors								
IFN-α	0.6 (0.29, 5.33)	0.6 (0.3, 13.4)	6.9 (0.5, 27.9) **	11.6 (0.3, 24) *	12.5 (1.2, 35.8) **	21.9 (18.2, 37.9) **	30.1 (27, 43.5) **	24.7 (15, 39.6) *
IFN-γ	9.9 (7.5, 23)	9.9 (8.3, 22.6)	9.5 (7.4, 20.4)	9.1 (6.9, 21.9)	11.8 (7.8, 26.9)	10.1 (7.2, 24.1)	8.8 (6.2, 22.7) *	9.1 (5.4, 21.9) *
Pro-inflammatory markers								
IL-1α	29.8 (24.4, 47)	40.1 (24.7, 50.8) *	44.2 (31.2, 61.3) ***	34 (30.2, 55.2) *	31.7 (24.6, 58)	22.4 (19.6, 45.1)	22.6 (18.3, 41.9) *	18.6 (14.2, 41.3) *
IL-12p40	12.6 (8.3, 19.1)	13.7 (8.5, 16.5)	16.4 (10.2, 23.8) **	16.4 (13.9, 18.3) **	14.1 (7.4, 20.3)	9.8 (5.9, 12.1)	10.2 (6.3, 14.7)	10 (6.2, 27.3)
IL-12p70	12.1 (7.8, 18.7)	10.9 (9.1, 20.3)	12.5 (9.1, 19.6)	13.7 (9.4, 17.5)	15.6 (10, 22.2) *	9 (7.2, 11.7)	7.4 (6.4, 12.1) *	7.1 (5.9, 16.5)
IL-17A	1.09 (0.58, 1.7)	1.21 (0.63, 2.23)	1.49 (0.66, 2.13)	1.15 (0.64, 1.97)	1.69 (0.91, 3.18) *	0.86 (0.64, 3.1)	0.7 (0.58, 1.26)	0.84 (0.53, 2.13)
Anti-inflammatory markers								
IL-1RA	72 (48, 106)	113 (72, 240)	842 (328, 2206) ***	249 (168, 475) ***	7 (52, 108)	87 (51, 96)	70 (55, 83)	59 (50, 102)
IL-10	3.39 (1.75, 8.06)	2.67 (2.18, 7.67)	3.58 (2.19, 8.27)	3.47 (2, 6.38)	3.28 (2.28, 9.82) *	3.26 (2.16, 8.45)	2.38 (1.59, 6.45) *	1.72 (1.51, 7.38)
Both pro- and anti-inflammatory cytokine								
IL-6	6.5 (3.6, 10.7)	7.1 (3.9, 9.5)	5.8 (4.3, 12.1)	7.1 (2.9, 10.8)	5.6 (4.7, 16.1)	5 (2.4, 8.1) *	3.3 (1.8, 5.9) *	1.5 (0.3, 7.4) *
Chemokines								
ENA-78	68 (36, 114)	78 (33, 105)	72 (32, 108)	56 (32, 120)	51 (35, 79)	31 (18, 41)	27 (16, 38) ***	28 (19, 36) **
GRO-α	26.4 (10.7, 31.8)	24.6 (12.5, 33.2)	25.4 (17.8, 30.3)	25.4 (20.2, 33.5)	34.5 (19.9, 42.9) **	28.9 (17.3, 37.3)	22.5 (20.6, 26.1)	21.1 (12.3, 37.7)
IL-8	0.85 (0.5, 1.75)	0.8 (0.35, 1.61)	1 (0.48, 2.56)	1.02 (0.61, 1.81)	1.95 (0.85, 3.34) *	1.05 (0.49, 1.75)	0.86 (0.61, 1.08)	0.77 (0.49, 1.68)
IP-10	44.8 (28, 63.5)	69.1 (46.2, 140.6) ***	146.5 (84.2, 233.2) ***	100.6 (69.1, 134.8) ***	45.7 (37.5, 66.1)	24.6 (18.8, 43.4) **	28 (25.1, 38.7) *	31.8 (21.3, 43.8)
MCP-1	12.9 (8.9, 21.6)	15.2 (9.5, 24.5)	17.6 (11.2, 31.1) **	13.1 (10.7, 20.5)	13.7 (10.6, 27.9)	11.6 (9.5, 22.1)	11.5 (9.6, 16.3)	10.5 (8.2, 27.9)

	baseline (n=12)	4 h (n=12)	12 h (n=12)	1 d (n=12)	7 d (n=12)	4 w (n=11)	12 w (n=9)	48 w (n=6)
MCP-3	28.3 (16.9, 33.3)	24.3 (17.4, 30.5)	25.1 (17.4, 37.2)	23.7 (20.2, 32.5)	27.2 (22.6, 41.8) *	21.9 (16.9, 32.9) *	24.8 (14.2, 28.5)	16.6 (12.5, 24.7) *
MIG	71 (31, 128)	58 (35, 132)	99 (60, 158) **	89 (59, 148) *	57 (36, 82)	33 (24, 39)	38 (24, 70)	57 (43, 74)
MIP-1α	8.8 (3.6, 14.3)	8.5 (3.6, 16.3)	11.9 (5.6, 20.8) **	8.1 (5.7, 14.1)	8.2 (4, 13.9)	5 (3.1, 11.3)	3.9 (2.7, 9.2) ***	4.6 (2.5, 12.1) *
MIP-1β	105 (64, 155)	100 (55, 142)	117 (85, 218) **	97 (68, 129)	78 (60, 157)	51 (44, 129) **	54 (38, 92) **	38 (26, 83) **
Endothelial factors								
PDGF-BB	369 (231, 568)	332 (245, 487)	340 (287, 449)	290 (216, 488)	336 (284, 503)	351 (156, 521) *	330 (137, 400) *	198 (90, 307) *
sVCAM-1	1284 (894, 2474)	1215 (912, 2654)	1423 (1077, 4484) **	1742 (944, 4544) ***	1784 (1399, 3904) **	2009 (1411, 4909) *	2063 (1586, 3766) **	2366 (1340, 3406) **
Hypercoagulation factors								
FGFb	369 (221, 548)	322 (202, 516)	345 (262, 404)	297 (207, 494)	360 (268, 453)	351 (173, 494)	351 (139, 373) *	191 (87, 312) **
Apoptosis inducers								
TRAIL	25.4 (15.9, 34.9)	30.3 (13.6, 36.1) *	34.5 (23.7, 43.2) **	26.9 (21, 37.6)	27.3 (16.5, 45.4)	19 (11.4, 24.9)	12.8 (11, 21.7) *	14.1 (10.2, 24.1) *
Growth factors								
HGF	19.1 (12.3, 27.2)	17.5 (13.7, 30.3)	30.7 (20.4, 40.9) **	29.5 (14.4, 36.3)	15.8 (13.7, 23.5)	16.3 (9.8, 22.3)	15.1 (11, 19.4) *	17.3 (11.7, 20.6)
TGF-β	11.8 (8.5, 21.6)	9.3 (8.4, 18.7)	9.4 (8.5, 20.5)	10.8 (7.7, 19.6)	10.9 (9.5, 29.8)	9.2 (8.3, 19)	8.8 (7.4, 12.7) *	8.5 (4.6, 27.5) *
Immune cell proliferation and regulation factors								
sCD40L	18.9 (12.8, 51.1)	23.7 (8.6, 61.5)	37.6 (19.2, 67.5) *	27.4 (20.1, 30.2)	19.1 (6.7, 40.7)	10.9 (8.1, 20.7) *	8.1 (1.2, 9.6) *	10.8 (8.5, 37.3)
IL-2	10.2 (7.9, 18.1)	9.6 (7.6, 18.2)	9.4 (7.3, 15.7)	8.9 (7.6, 16.3)	11.4 (8.1, 23.3)	8.9 (7.2, 20.2)	9.1 (6.4, 17.1) *	7.7 (4.5, 17.5) *
IL-4	5.12 (4, 9.96)	4.77 (3.95, 8.51)	5.06 (4.65, 9.13)	4.87 (4.57, 7.43)	5.52 (4.34, 12.45) *	4.69 (3.42, 10.39)	4.28 (3.65, 7.89)	4.37 (2.43, 10.51) *
IL-7	49.5 (41.8, 71.9)	40.6 (36.3, 61)	42.9 (37.5, 62.1)	43.5 (34.1, 61.6)	50.8 (44.1, 87.2)	38.9 (33.7, 79.2)	38.8 (34.9, 53.1) *	34.6 (22.3, 91.5) *
IL-15	19.9 (15.7, 38.8)	18.2 (16.2, 33.6)	19.3 (16.3, 35.7)	20.2 (18.3, 30.3)	19.9 (16.2, 45.5)	17 (15.2, 32.5)	17 (13.9, 24.9) *	13.6 (11.8, 39.8) *
M-CSF	2.2 (0.3, 15)	1.6 (0.2, 15.7)	3 (0.2, 20.2)	1.8 (0.1, 19.5)	8.5 (3, 22.3) *	3.5 (2.3, 9.1)	1.7 (0.3, 11.5)	1.3 (0.3, 8.2)

	baseline (n=12)	4 h (n=12)	12 h (n=12)	1 d (n=12)	7 d (n=12)	4 w (n=11)	12 w (n=9)	48 w (n=6)
Fat metabolism markers								
Leptin	172 (74, 583)	126 (73, 828)	217 (119, 1175) *	260 (102, 795) *	116 (73, 528)	87 (32, 183) **	143 (41, 182)	54 (37, 300)

* - p<0.05,
** - p<0.01,
*** - p<0.001.

Table 4

Correlations between liver histopathology parameters and serum biomarkers.

Serum marker ^a	Correlation coefficient ρ	p value
Liver inflammation grade (Batts and Ludvig) and:		
sVCAM	0.634	0.027
HGF	0.678	0.015
IL-1 β	-0.755	0.004
LIF	-0.600	0.039
Liver fibrosis stage (Batts and Ludvig) and:		
IFN- α	-0.721	0.008
IFN- γ	-0.636	0.026
LIF	-0.625	0.030
Liver steatosis (% of surface area) and:		
Leptin	0.811	0.002

^a – only markers with statistically significant correlations ($p < 0.05$) are shown