Hepatitis C virus (HCV) is the major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Antiviral therapy based on pegylated IFN (Peg-IFN) combined with ribavirin is successful in approximately 50% of patients and the response rate increases to over 70% when triple-combination therapy including inhibitors of the non-structural protein 3 (NS3) viral protease is used (Ghany et al., 2011). The clearance of HCV RNA from serum is usually accompanied by normalization of liver biochemical tests and improvement of liver histology. Generally accepted criteria for a sustained virological response (SVR) require the patient to remain negative for HCV RNA in serum for 6 months after termination of treatment when tested with an assay whose sensitivity is at least 100 viral copies ml⁻¹ (Saleh et al., 1994).

A late relapse to HCV infection in responders is rare and, according to published studies, ranges from 0 to 4% (Pearlman & Traub, 2011). However, significantly higher incidence rates were also reported, particularly when older treatment protocols using IFN monotherapy were employed (Saleh et al., 1994). While clear-cut viral relapses after SVR may be rare, several studies reported on the persistence of low-level virus in PBMCs and in the liver, and occasionally even in serum in a significant proportion of SVR patients (Pham et al., 2004; Radkowski et al., 2005). Nevertheless, a recent study questioned the presence of protracted HCV persistence among SVR patients and even of active HCV replication in PBMCs, suggesting that the virus is only passively adsorbed (Fujiwara et al., 2013). The latter is a well-recognized phenomenon that could contribute to differences between circulating and PBMC-derived viral variants (Laskus et al., 2000).

However, despite well-documented viral persistence in at least some SVR patients, it is unclear whether such low-level infection, which is often confined to the lymphoid system, is in any way consequential.

In the current study, we showed that, in patients responding to Peg-IFN and ribavirin therapy, long-term (>52 months) persistence of HCV RNA was associated with immune-cell activation, as determined by quantitative analysis of key cytokine and chemokine transcripts.

In total, 140 patients who completed antiviral therapy ≥50 months earlier and achieved a SVR were invited to participate in the study, and 49 (25 females and 24 males) were eventually enrolled. Forty-five patients were infected with HCV genotype 1b, three with genotype 1a and one patient had a mixed 4c/4d infection. The demographic, clinical and virological data of the patients in this study are presented in Table 1. Patients with human immunodeficiency virus (HIV) co-infection, autoimmune conditions and those receiving immunosuppressive drugs were excluded from the study.

Patients received treatment for 48 weeks with either 180 μg IFN-α-2a per week (Pegasys; Hoffmann-LaRoche) (n=23) or IFN-α-2b (PEG-Intron; Schering-Plough) (n=26) combined with ribavirin (Rebetol; Schering-Plough) at 1000 mg day⁻¹ if their body mass was <75 kg or 1200 mg day⁻¹ if their body mass was ≥75 kg. SVR was defined as undetectable HCV RNA in serum determined by a qualitative reverse
transcription-PCR (RT-PCR) assay (COBAS AMPLICOR HCV Monitor Test; Roche Diagnostics) (limit of detection 50 U ml\(^{-1}\)) at week 72.

The study was approved by the Internal Review Board (ref. no. KB-O/23/09), and each patient provided written consent. Serum and PBMC samples were tested for the presence of HCV RNA by an in-house RT-PCR assay following exactly a procedure described previously; this assay had a sensitivity of approximately 10 genomic equivalents (Radkowski et al., 2005). To maximize sensitivity, RNA extracted from 10^5 cells or 1 ml serum was tested in each reaction. Patients who were found to test positive for HCV in serum, PBMCs or both were retested using a new sample aliquot, and both results had to be concordant to define the sample as being positive. All HCV RNA-positive sera were retested with a commercial quantitative test (COBAS AmpliPrep/COBAS TaqMan HCV Test; Roche), which has a sensitivity limit of 15 IU ml\(^{-1}\). The assay was done according to manufacturer’s instructions. PBMC samples were also tested for the presence of the HCV RNA negative strand as described previously (Radkowski et al., 2005).

The HCV NS3 protein was detected in PBMCs using anti-NS3 mAb (Novocastra) as described elsewhere (Kisiel et al., 2014). In brief, acetone/chloroform-fixed slides were incubated for 60 min at room temperature with anti-NS3 (Novocastra) mAb diluted 1:25, washed twice in PBS for 5 min and incubated with Alexa Fluor 488-conjugated goat anti-mouse antibody (Life Technologies) diluted 1:1000 and with Hoechst reagent (Molecular Probes) diluted 1:10,000 for another 90 min. The results of staining were visualized using a Nikon Eclipse 80i microscope and a 450–480 nm UV-2A filter. Positive controls consisted of liver-tissue biopsy samples collected from HCV-infected patients, and negative controls consisted of PBMCs and liver samples from uninfected subjects. The specificity of staining was ascertained each time by a parallel staining procedure in which the primary antibody was omitted. The staining was considered positive when repeated in an independent experiment and when all negative controls tested in parallel were negative.

For the analysis of cytokines transcripts, approximately 3 × 10^5–1 × 10^6 PBMCs were isolated from blood by centrifugation over density gradients, and RNA was extracted by means of a modified guanidinium thiocyanate/phenol/chloroform technique using a commercially available kit (TRIZOL LS; Gibco-BRL). Extracted RNA was incubated for 30 min at 37 °C in a 15 μl reaction mixture containing 25 pM random hexamers, 1 × PCR Buffer II (Perkin Elmer), 5 mM MgCl2, 5 mM DTT, 1 mM dNTPs and 10 U Moloney murine leukemia virus reverse transcriptase. The enzyme was deactivated by heating to 99 °C for 10 min. Real-time PCR was performed with LightCycler FastStart DNA Master SYBRGreen I (Roche Applied Sciences). Two microlitres of the above reverse-transcription product was diluted 1:5 in water and directly added to 18 μl real-time PCR mix containing 0.5 μM each primer pair, 1.25 mM MgCl2 and 2 μl Master SYBRGreen I. Amplification was run in a LightCycler (Roche Diagnostics) as described elsewhere (Wilkinson et al., 2010). Gene transcripts for the following cytokine/chemokines were quantified: IL-1α, IL-1β, IL-3, IL-4, IL-6, IL-8, IL-10, IL-12, IL-15, IL-16, IL-18, TNF-α, human leukocyte antigen DR1 (HLA-DRA), IFN-α, IFN-β, IFN-inducible protein 10 (IP-10/CXCL10), granulocyte–macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), monocyte chemotactic proteins 1 and 2 (MCP-1/CCL2 and MCP-2/CCL8), macrophage inflammatory protein 1x and 1β (MIP-1x/CCL3 and MIP-1β/CCL4), regulated on activation normal T-cell expressed and secreted (RANTES/CCL5), transforming growth factor β (TGF-β) and myxovirus resistance protein A (MxA). The primer sequences have been described previously (Wilkinson et al., 2010). Histone 3 was used as a housekeeping gene (forward primer, 5′-ACGACCTCGGTCTTCCAGAG-3′ and reverse primer, 5′-GTAATCACGCCCTCTCCG-3′) to normalize gene expression. Gene expression level was calculated with the 2^(-ΔΔCT) method (Livak & Schmittgen, 2001).

In our analysis, we included a wide range of both pro-inflammatory response mediators, such as IL-1, IL-6, IL-12, IL-15, IL-18, GM-CSF, M-CSF, MCP-1 and MCP-2, and

Table 1. Demographic, clinical and virological data of 49 patients with SVR >52 months after the end of treatment

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HCV RNA detectable in serum and/or PBMCs (n=23)</th>
<th>HCV RNA not detectable (n=26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (m/f; no. of patients)</td>
<td>12/11</td>
<td>12/14</td>
</tr>
<tr>
<td>Age (years; mean ± SD)</td>
<td>50.3 ± 10.4</td>
<td>52.4 ± 9.6</td>
</tr>
<tr>
<td>Viral genotype (no. of patients)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1b</td>
<td>21</td>
<td>24</td>
</tr>
<tr>
<td>1a</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Mixed 4c/4d</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Viral load at treatment initiation (IU ml(^{-1}); mean ± SD)</td>
<td>(9.4 ± 1.1) × 10^5</td>
<td>(1.1 ± 1.3) × 10^6</td>
</tr>
<tr>
<td>Current ALT level (normal ≤ 45 IU l(^{-1}); mean ± SD)</td>
<td>36.5 ± 13.2</td>
<td>32.0 ± 10.1</td>
</tr>
</tbody>
</table>
anti-inflammatory mediators, such as IL-4, IL-10 and TGF-β. Simultaneous activation of both pro- and anti-inflammatory arms of the immune system is not unexpected, and this process is tightly regulated at each stage of the immune response (Viganò et al., 2012). We also included MxA, which is an antiviral protein induced by type 1 IFNs (Haller et al., 1998); type 1 IFNs (IFN-α and IFN-β); and a number of chemokines, such as IL-8, lymphocyte chemoattractant factor (IL-16) and IP-10.

HCV RNA was detected in sera from 11 patients (22.4 %) and in PBMCs from 23 patients (46.9 %). All subjects who were HCV RNA positive in serum were also HCV RNA positive in PBMCs, but in 12 patients, viral sequences were confined to PBMCs. Two out of 11 positive sera samples were also positive when tested with a commercial quantitative test, although the viral load was low and close to the detection limit (15 IU ml⁻¹). PBMCs from all 49 patients were stained for the presence of HCV NS3 protein; however, only two patients (both were HCV RNA positive in PBMCs) were positive (Fig. 1). Both of these patients were found to be HCV RNA positive in serum by the in-house assay but negative by the commercial assay. Viral negative-strand RNA was not detected in any of the analysed samples, which is consistent with our earlier study showing that this replicative intermediate is rarely present in uncultured PBMCs from SVR patients (Radkowski et al., 2005). Thus, despite the presence of HCV RNA in PBMCs, direct evidence of viral replication was scant, which could be due to very low replication levels in SVR patients; alternatively, viral replication could be non-productive in this setting.

Quantitative expression of cytokine and chemokine transcription levels in PBMCs was analysed in patients who harboured viral sequences (23 patients) and in those in whom viral RNA was undetectable (26 patients). Transcript levels of the analysed cytokine/chemokine genes were normalized against expression of the histone 3 gene and were found to be higher in the former group than in the latter, and the differences reached statistical significance (P ≤ 0.05) in a Mann–Whitney U test for IL-1α, IL-6, IL-8, IL-12, IL-18, TNF-α, M-CSF and MIP-1β (Fig. 2). However, since multiple independent tests were run simultaneously, we used the Benjamini–Hochberg step-up procedure (Benjamini & Hochberg, 1995) to control for the false discovery rate (at level 0.05). Using this post-hoc procedure for 25 simultaneous tests, only differences in expression for IL-6, IL-8, IL-12, TNF-α and MIP-1β transcripts remained statistically significant.

Among the patients analysed, the presence of HCV RNA was not accompanied by abnormal liver function tests, and the viral loads were very low. Nevertheless, this residual low-level viral persistence seemed to be sufficient to stimulate the immune system as evidenced by the elevation of transcripts for some crucial immune factors. We found statistically significant elevation of IL-6, IL-8, IL-12, TNF-α and MIP-1β transcripts in PBMCs, and all of these cytokines were found previously to be elevated in HCV-infected patients. For example, IL-6 was reported to be stimulated by HCV antigens and is commonly elevated in sera of patients with chronic hepatitis C (Machida et al., 2006). Similarly, IL-12 is a pro-inflammatory cytokine.

Fig. 1. Immunostaining of PBMCs for the presence of HCV NS3 in HCV RNA-positive SVR patients (Pt 13 and Pt 18). Neg., negative controls (PBMCs from HCV-negative subjects).
Fig. 2. Comparison of expression levels of cytokine/chemokine genes normalized to expression of histone 3 mRNA between HCV RNA-negative patients (−, filled circles) and HCV RNA-positive patients (+, open circles). Asterisks indicate statistically significant differences in gene expression between HCV RNA-infected patients and uninfected individuals: *$P < 0.05$ determined by Mann–Whitney U test only; **$P < 0.05$, using the Benjamini–Hochberg procedure to correct for multiple testing (Benjamini & Hochberg, 1995).
facilitating the cytotoxic activity of T-cells and NK cells (Akdis et al., 2011). IL-12 levels are elevated in patients with chronic hepatitis C, and these elevated levels positively correlate with necro-inflammatory processes in the liver (Quiroga et al., 1998).

Strong induction of IL-8 and TNF-α is a common phenomenon in viral infections, including HCV infection. The IL-8 chemokine is an important mediator of the innate immune response, whereas TNF-α stimulates acute-phase responses, induces apoptotic cell death and inhibits viral replication (Akdis et al., 2011). MIP-1β is another member of the chemokine family influencing migration of leukocytes; in particular, it recruits T-cells and monocytes, increasing their concentration around the site of inflammation (Moser & Loetscher, 2001). In HCV-infected patients, MIP-1β serum levels were reported to be significantly elevated when compared with those in healthy controls (Jimenez-Sousa et al., 2010).

In a previous study, Pham et al. (2009) reported that the PBMC-derived gene transcription profiles in SVR patients were different from those in healthy individuals. While the study analysed only nine different cytokines, and SVR patients with residual HCV infection were not compared with those who cleared the virus, it pointed out for the first time that HCV replicating at low levels in immune cells continues to trigger responses for years after clinical clearance. In agreement with our findings, PBMCs from patients with SVR displayed greater mRNA levels for IL-12 and TNF-α. Interestingly, the same cytokines were also higher among SVR patients than among untreated patients with chronic hepatitis C (Pham et al., 2009).

Chronic infection with HCV is associated with multiple extrahepatic complications, such as B-cell lymphoma, cryoglobulinaemia and vasculitis, as well as with neurological abnormalities, such as peripheral neuropathy, fatigue, depression and cognitive disorders (Jacobson et al., 2010). The underlying mechanisms of these conditions are not entirely clear, but the role of the immune system seems pivotal, as immune complexes, unspecific immune stimulation and viral invasion of cells of the immune system have been implicated in their pathogenesis (Zignego et al., 2012).

Our findings of immune activation accompanying persistence of HCV RNA in PBMCs after ostensibly successful treatment of chronic HCV infection raises the possibility that some patients might be at increased risk of developing immune-mediated extrahepatic complications of HCV infection. However, currently no published follow-up studies are addressing this issue.

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**References**


