Towards optimal treatment for chronic hepatitis C infection
Gelderblom, H.C.
Early and sustained HCV virological responses to therapy despite suppression of HCV specific T cells

Eleanor Barnes1*, Huub C Gelderblom1,2*, Isla Humphreys4, Nasser Semmo4, Henk W Reesink1, Marcel GHM Beld2, René AW van Lier3, Paul Klenerman4

*These authors contributed equally to the work.

1AMC Liver Center, Department of Gastroenterology and Hepatology, 2Section of Clinical Virology, Department of Medical Microbiology, and 3Department of Experimental Immunology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands 4Peter Medawar Building for Pathogen Research, Nuffield Department of Medicine, University of Oxford, Oxford, UK
Abstract

**Background/Aims:** The role of the adaptive immune response in sustaining hepatitis C viral control during therapy with interferon-α and ribavirin is unclear. We examined the effect of high dose interferon-α induction therapy on T-cell responses and addressed the hypothesis that a sustained virological response (SVR) after treatment is associated with a restoration of IL-2 and IFN-γ secreting HCV specific T-cells.

**Methods:** 31 treatment-naïve chronic HCV patients were treated with amantadine and ribavirin, combined with 6 weeks of high dose IFN-alpha2b induction therapy followed by weekly PEG-IFN-alpha-2b, for 24 or 48 weeks. Using IFN-γ and IL-2 ELISpots, we analysed the pattern of cytokine secretion by structural and non-structural HCV, CMV and influenza specific T-cells before, during and following therapy. CD4+ and CD8+ T-cell subsets were distinguished using CFSE proliferation assays. Viral kinetics were assessed using bDNA, PCR and TMA assays.

**Results:** 15/31 patients achieved SVR. HCV specific T-cell responses that secreted predominantly IFN-γ and correlated with ALT ($r^2=0.45$, $p=0.001$) were found in 10/15 SVR and 11/16 non-SVR patients before treatment. There was a striking loss of IFN-γ and IL-2 HCV specific T-cells during therapy seen predominantly in the SVR group, which recovered following cessation of therapy. Suppression of CMV and influenza T-cell responses in addition to total lymphocyte counts were also observed.

**Conclusions:** High dose IFN-α induction therapy leads to a profound decline in IL-2 and IFN-γ secreting HCV specific T-cells. This data indicates that restoration of T-cell responses is not causally linked to early or SVR to therapy.
Introduction

HCV is currently a worldwide epidemic. The development of antiviral therapies against HCV and understanding the mechanism of action of currently available successful therapies will be crucial in controlling this epidemic.

Combination therapy with PEG-α–IFN and ribavirin, currently the best available treatment, leads to long term resolution of infection in 50-80% of individuals depending on viral genotype [1]. The mechanism of action is not completely understood, but both drugs have wide ranging effects on the immune system [2, 3]. During acute HCV infection, robust T-cellular immune responses can be detected in the majority of individuals [4, 5]. These are maintained in those that spontaneously resolve infection [6]. However, once persistent HCV is established HCV specific cellular immune responses are generally weak and narrowly focused [7]. It is plausible then that combination therapy leads to long-term viral control through enhancement of HCV specific T-cell responses.

In support of this theory a number of studies have shown some enhancement of HCV specific T-cells during therapy. However, it remains controversial whether this is associated with an SVR to therapy [8-11]. Additionally, whether the re-emergence of HCV specific T-cell responses during therapy is simply a consequence rather than a cause of a falling viral load is currently unclear. Furthermore, studies of T-cell responses during successful treatment of acute HCV showed either a decline or no enhancement in T-cell responses despite the very high efficacy of therapy at this stage of infection. In one patient in this study subsequent treatment with OKT3 antibody led to the abolishment of detectable responses against HCV without viral recrudescence, suggesting that T-cell immunosurveillance may not be required to establish a SVR [12].

The quality as well as the magnitude of the HCV specific T-cell response both in the context of antiviral therapy and in the spontaneous resolution of HCV infection may also be critically important in determining a successful outcome. We have previously shown that persistent HCV viremia is associated with a significant loss of IL-2 secreting HCV specific T-cells compared to IFN-γ secreting T-cells, whilst in spontaneously resolved HCV infection IL-2 secretion is preserved [13]. Similarly, CD4 + T-cells in human immunodeficiency viremic patients that fail to respond to therapy have been shown to produce exclusively IFN-γ, whilst in aviremic patients the production of both IL-2 and IFN-γ is preserved [14]. Others have suggested the emergence of Th1 type responses during combination therapy are related to a beneficial outcome [8].

The rate of viral decline may also impact on the emergence of T-cell responses during therapy. During the first few weeks of combination therapy two phases of viral decline occur. Mathematical modelling has proposed that the first more rapid decline in viral load is due to direct antiviral effects of therapy, whilst the second phase is due to immune pressure [15]. There is some experimental data to show that a rapid fall in viral load during therapy is more likely to be associated with restoration of T-cell responses than a more gentle decline [16]. In these studies causality is not established.
In this study we explore these issues using high dose IFN-α induction therapy. High dose IFN-α induction therapy has been shown to increase the likelihood of an early virological response [17, 18]. However data regarding the SVR rate is conflicting [17-20]. We focus on HCV specific CD4+ T-cell responses detectable with IFN-γ and IL-2 ELISpot as we find reproducible responses using this approach [9, 13]. We address two specific hypotheses. Firstly, that a rapid fall in viral load is causally linked with restoration of HCV-specific responses and a SVR to therapy. Secondly, that a SVR to treatment is associated with the restoration of IL-2 secreting HCV-specific T-cell responses as has been shown in the spontaneous resolution of HCV infection [13] and in the treatment of HIV infection [14].

Methods

Patients
The patients in this study were part of a larger clinical study of 100 HCV patients (previous non-responders to conventional interferon and ribavirin, and treatment naïve patients), designed to assess the efficacy of the treatment regime described in detail below (currently in submission).

Of these, 31 treatment naïve patients (21 males, 10 females) with persistent HCV genotype-1 infection were included in this immunological study (Table 1). In all patients, HCV RNA was detectable by RT-PCR in the serum on at least two consecutive occasions 6 months apart (Roche v2.0 Amplicor assay, Roche Diagnostics Ltd, Branchburg, NJ, USA) before treatment. All patients were HCV genotype-1 (TruGene® HCV genotyping assay, Bayer Diagnostics, Berkeley CA, USA) and negative for HIV and HBV antibodies. 5 patients were cirrhotic (4 biopsy proven and one on clinical grounds).

All patients were treated with triple therapy consisting of: amantadine hydrochloride 200 mg/d (Symmetrel®; Novartis, Basel, Switzerland) and ribavirin (Rebetol®; Schering-Plough, Kenilworth, NJ, USA) 1000 or 1200 mg/d (based on body weight) for a total of 24 or 48 weeks, combined with interferon alfa-2b induction (IntronA®; Schering-Plough) during the first 6 weeks, and thereafter combined with weekly pegylated interferon alfa-2b (Pegintron®; Schering-Plough), 1.5 μg/kg for a total of 24 or 48 weeks. The interferon induction scheme during the first 6 weeks was as follows: week 1 and 2: 18 MU/d in 3 divided doses; week 3 and 4: 9 MU/d in 3 divided doses; week 5 and 6: 6 MU/d in 2 divided doses.

Patients with a decrease of HCV RNA < 3 log at week 4 were treated for 48 weeks. Patients with a decrease of HCV RNA ≥ 3 log at week 4 were randomized at week 24 to stop treatment at 24 weeks or continue to 48 weeks. Treatment was stopped in all patients with HCV RNA > 615 IU/ml at week 24. All patients were followed for 24 weeks after completion of therapy. The study was approved by the institutional review board.
Samples
Peripheral blood mononuclear cells (PBMC) were isolated from blood by density gradient centrifugation using Lymphoprep (Nycomed, Oslo, Norway) and frozen immediately before treatment (week 0), 12 and 24 and 48 weeks after the start of treatment, and 24 weeks after completion of treatment (follow up-FU). Analysis of viral load, alanine transaminase (ALT) and full blood counts were performed at week 0, 1, 2, 3, 4, 6, 8, 12, 16, 20, 24, 48 and then weeks 12 and 24 after treatment.

Viral kinetics
Viral kinetics were assessed using the following three assays; quantitative bDNA (VERSANT® HCV 3.0 assay; Bayer Diagnostics; linear dynamic range 6.15 x 10^2-7.7 x 10^6 IU/ml), qualitative PCR (COBAS® Amplicor HCV Test v2.0; Roche diagnostics; Lower limit of detection (LLD) 50 IU/ml, and qualitative TMA (Transcription-Mediated Amplification; VERSANT® HCV qualitative assay; Bayer Diagnostics; LLD 5 IU/ml)

Clinical definitions
Clinical definitions are based on the highly sensitive TMA assay. SVR was defined as loss of HCV RNA from the serum that was maintained for 24 weeks after therapy. Non-responders had detectable HCV RNA levels in serum throughout treatment. Breakthrough was defined as initial loss of HCV RNA from the serum followed by recurrence of HCV RNA whilst on therapy. Relapse was defined as absence of viremia during therapy followed by detectable viremia within 24 weeks of completion of therapy. For the purpose of analyses non-SVR is defined as non-response, breakthrough or relapse.

ELISpot assays
Thawed PBMC were tested by IFN-γ (MABTECH, Stockholm, Sweden) and IL-2 (BD Biosciences, Oxford, UK) ELISpot assays as previously described [9]. PBMC from the same patient derived at multiple time-points were assessed concurrently. Briefly, PBMCs (0.2 million/well following exclusion of dead cells with tryphan blue) were plated in duplicate in anti–IFN-γ or anti-IL-2 precoated 96-well ELISpot plates. Antigens used included; HCV core-derived peptides spanning amino acids (AA) 1-191 in a single pool, NS5a derived peptides AA 2201-2410 in a second pool (each peptide 20AA in length overlapping by 10 AA, 10μg/ml final concentration, corresponded to HCV isolate H77 genotype 1a), recombinant proteins NS3, NS4, and NS5 (Chiron, Emeryville, CA, USA) (final concentration of 1 μg/ml) in a single pool as previously described [9, 13], CMV lysate (final concentration 0.05μg/ml; Virusys, North Berwick, ME, USA), medium alone as negative control, phytohemagglutinin (PHA; 4μg/ml) as a positive control and influenza vaccine (enzira split virion inactivated, Chiron). Plates were developed after 18-20 hours incubation and analyzed for spot forming units (SFU) using an ELISpot plate reader (AID Reader System, Strassberg, Germany, ELISpot 3.1 SR program).
A positive response was recorded where the probability of a spot appearing in the stimulated well was significantly different ($p < 0.05$) from the probability of a spot appearing in the negative control well (Excel BINOMDIST) [9, 13]. The frequency of T-cells was calculated by subtracting the average SFC in negative control duplicate wells from the average SFC in stimulated duplicate wells and expressed per 10^6 PBMC.

**CFSE based proliferation assays.**

PBMC ($1 \times 10^7$/ml) in PBS were incubated at 37°C for 7 min with 0.5μM CFSE (Molecular Probes, Eugene, Oregon, USA). PBMC were washed with PBS containing 10% pooled human serum and then PBS only. Cells were resuspended at $2 \times 10^6$/ml in RPMI containing 10% human serum. Stained cells ($1 \times 10^6$/well, 1ml) were cultured in 48-well plates with either medium alone, PHA, core peptide pools 1-4 (10μg/ml final concentration), CMV lysate (optimized final concentration 0.05μg/ml; Virusys) or influenza vaccine (enzira split virion inactivated). After six days cells were washed and stained at 4°C with: anti-human CD4-APC, CD8-PE and Viaprobe (7-AAD) (BD Pharmingen, Oxford, UK). Flow cytometric analysis was performed on a FACS Calibur and analysed using FlowJo (Treestar, Ashland, Oregon, USA). T-cell proliferation was determined by gating on the lineage-positive CFSE^{low} and CFSE^{high} subset. A stimulation index (SI) was derived by dividing the CD4+ proliferative frequency (%) in presence of antigen by the CD4+ proliferative frequency without antigen using the following formula: CD4+ proliferative frequency(%)=Number of CD4+ CFSE^{low} cells/(Number of CD4+ CFSE^{low} cells + number of CD4+ CFSE^{high} cells) x 100. A SI > 2 was considered positive. The CD4+ proliferative frequency (%) for a positive response was achieved by subtracting the proliferative frequency with antigen from the proliferative frequency without antigen.

**T-cell FACS analysis**

100,000 viable PBMC (following staining with tryphan blue) were incubated for 20’ at 4°C with CD3-PE, CD4-APC and CD8-FITC and acquired/analysed as above.

**Statistical analysis**

Significance of the proportion of viraemic patients was determined using the Fishers’ exact test. Comparisons between SVR and non-SVR patient cohorts pre-treatment were made using the non-parametric Mann-Whitney U test. Correlations were determined using the Pearson correlation coefficient. T-cell responses at each time point were compared with baseline (pre-treatment) values using the paired non-parametric Wilcoxon ranked sign test. P values <0.05 are considered significant.
SVR despite HCV specific T-cell decline

Results

Clinical outcome
SVR was observed in 15/31 (48%) patients. Sixteen patients failed to make a SVR. Of these 8/16 patients were non-responders, 2/16 had breakthrough viremia and 6/16 patients relapsed after therapy. There was no significant difference in the age or gender in SVR compared to non-SVR patients. (table 1)

Of the 15 patients who had a SVR to therapy 7/15 had undetectable viraemia by TMA at week 4, 13/15 at week 12 and 15/15 by week 24. This was in contrast to the non-SVR patients where only 1/16 had undetectable viraemia at week 4 (p=0.05), 6/16 at week 12 (p=0.009) and 10/16 at week 24 (p=0.02).

Figure 1a. HCV core specific T-cell responses, ALT and viral load before therapy in relation to outcome.

Each data-point represents a single patient. Bars = mean. (A) HCV core specific T-cell responses (pooled peptides spanning the entire core region) were tested pre-treatment by IFN-ELISpot in SVR (●) and non-SVR (▲) patients. (B) Viral load pre-treatment in patients with a subsequent SVR and non-SVR to treatment. (C) Correlation between viral load and HCV core specific T-cell responses pre-treatment by IFN-ELISpot. (D) Correlation between ALT and HCV core specific T-cell responses pre-treatment by IFN-ELISpot.
HCV specific T-cell responses, ALT and viral load before therapy in relation to outcome

Before therapy HCV core specific T-cell responses could be detected in 21/31 patients; 10/15 patients with a subsequent SVR and 11/16 patients with a non-SVR to therapy. The mean viral load, ALT and HCV core specific responses as detected by IFN-γ ELISpot were lower in patients who achieved a SVR (viral load IU/ml: 5.791 ± 0.1723 vs 5.990 ± 0.1181, ALT IU/L 118.4

<table>
<thead>
<tr>
<th>ID</th>
<th>Age</th>
<th>Sex</th>
<th>ALT pre-Rx (U/l)</th>
<th>Cirrhosis</th>
<th>Viral load (IU/ml) Pre-Rx</th>
<th>TMA at week 4</th>
<th>TMA at week 12</th>
<th>&gt; 3 log decrease in viral load at week 4</th>
<th>Treatment duration (weeks)</th>
<th>Response to treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>321</td>
<td>19</td>
<td>F</td>
<td>50</td>
<td>no</td>
<td>178000</td>
<td>+</td>
<td>–</td>
<td>yes</td>
<td>32</td>
<td>Breakthrough</td>
</tr>
<tr>
<td>354</td>
<td>42</td>
<td>M</td>
<td>208</td>
<td>yes</td>
<td>1380000</td>
<td>–</td>
<td>+</td>
<td>yes</td>
<td>24</td>
<td>Breakthrough</td>
</tr>
<tr>
<td>311</td>
<td>49</td>
<td>M</td>
<td>494</td>
<td>no</td>
<td>381000</td>
<td>+</td>
<td>+</td>
<td>no</td>
<td>48</td>
<td>Non-responder</td>
</tr>
<tr>
<td>322</td>
<td>42</td>
<td>F</td>
<td>55</td>
<td>no</td>
<td>2550000</td>
<td>+</td>
<td>+</td>
<td>no</td>
<td>24</td>
<td>Non-responder</td>
</tr>
<tr>
<td>324</td>
<td>67</td>
<td>F</td>
<td>96</td>
<td>yes</td>
<td>1290000</td>
<td>+</td>
<td>+</td>
<td>no</td>
<td>24</td>
<td>Non-responder</td>
</tr>
<tr>
<td>325</td>
<td>51</td>
<td>M</td>
<td>217</td>
<td>yes</td>
<td>5850000</td>
<td>+</td>
<td>+</td>
<td>no</td>
<td>48</td>
<td>Non-responder</td>
</tr>
<tr>
<td>330</td>
<td>47</td>
<td>F</td>
<td>34</td>
<td>no</td>
<td>1760000</td>
<td>+</td>
<td>+</td>
<td>no</td>
<td>24</td>
<td>Non-responder</td>
</tr>
<tr>
<td>336</td>
<td>47</td>
<td>M</td>
<td>72</td>
<td>no</td>
<td>313000</td>
<td>+</td>
<td>+</td>
<td>no</td>
<td>24</td>
<td>Non-responder</td>
</tr>
<tr>
<td>341</td>
<td>46</td>
<td>M</td>
<td>81</td>
<td>nd</td>
<td>5400000</td>
<td>+</td>
<td>+</td>
<td>no</td>
<td>24</td>
<td>Non-responder</td>
</tr>
<tr>
<td>346</td>
<td>46</td>
<td>M</td>
<td>43</td>
<td>nd</td>
<td>1060000</td>
<td>+</td>
<td>+</td>
<td>no</td>
<td>48</td>
<td>Non-responder</td>
</tr>
<tr>
<td>314</td>
<td>49</td>
<td>F</td>
<td>194</td>
<td>no</td>
<td>1350000</td>
<td>+</td>
<td>–</td>
<td>yes</td>
<td>24</td>
<td>Relapse</td>
</tr>
<tr>
<td>316</td>
<td>35</td>
<td>M</td>
<td>145</td>
<td>nd</td>
<td>368000</td>
<td>+</td>
<td>–</td>
<td>yes</td>
<td>48</td>
<td>Relapse</td>
</tr>
<tr>
<td>319</td>
<td>49</td>
<td>M</td>
<td>243</td>
<td>no</td>
<td>1390000</td>
<td>+</td>
<td>+</td>
<td>yes</td>
<td>48</td>
<td>Relapse</td>
</tr>
<tr>
<td>326</td>
<td>48</td>
<td>M</td>
<td>94</td>
<td>yes</td>
<td>140000</td>
<td>–</td>
<td>+</td>
<td>yes</td>
<td>48</td>
<td>Relapse</td>
</tr>
<tr>
<td>327</td>
<td>50</td>
<td>M</td>
<td>67</td>
<td>no</td>
<td>854000</td>
<td>+</td>
<td>–</td>
<td>yes</td>
<td>24</td>
<td>Relapse</td>
</tr>
<tr>
<td>339</td>
<td>47</td>
<td>M</td>
<td>65</td>
<td>no</td>
<td>1420000</td>
<td>+</td>
<td>–</td>
<td>yes</td>
<td>48</td>
<td>Relapse</td>
</tr>
<tr>
<td>305</td>
<td>55</td>
<td>M</td>
<td>415</td>
<td>no</td>
<td>3270000</td>
<td>+</td>
<td>–</td>
<td>yes</td>
<td>48</td>
<td>SVR</td>
</tr>
<tr>
<td>313</td>
<td>41</td>
<td>M</td>
<td>62</td>
<td>no</td>
<td>3100000</td>
<td>+</td>
<td>–</td>
<td>yes</td>
<td>24</td>
<td>SVR</td>
</tr>
<tr>
<td>315</td>
<td>40</td>
<td>F</td>
<td>31</td>
<td>no</td>
<td>296000</td>
<td>+</td>
<td>–</td>
<td>no</td>
<td>48</td>
<td>SVR</td>
</tr>
<tr>
<td>317</td>
<td>35</td>
<td>M</td>
<td>170</td>
<td>no</td>
<td>2920000</td>
<td>–</td>
<td>–</td>
<td>yes</td>
<td>48</td>
<td>SVR</td>
</tr>
<tr>
<td>318</td>
<td>32</td>
<td>F</td>
<td>79</td>
<td>nd</td>
<td>132000</td>
<td>–</td>
<td>–</td>
<td>yes</td>
<td>24</td>
<td>SVR</td>
</tr>
<tr>
<td>320</td>
<td>50</td>
<td>M</td>
<td>205</td>
<td>no</td>
<td>1590000</td>
<td>–</td>
<td>–</td>
<td>yes</td>
<td>24</td>
<td>SVR</td>
</tr>
<tr>
<td>323</td>
<td>47</td>
<td>M</td>
<td>51</td>
<td>no</td>
<td>2910000</td>
<td>–</td>
<td>–</td>
<td>yes</td>
<td>24</td>
<td>SVR</td>
</tr>
<tr>
<td>329</td>
<td>40</td>
<td>F</td>
<td>37</td>
<td>no</td>
<td>2850000</td>
<td>+</td>
<td>+</td>
<td>no</td>
<td>48</td>
<td>SVR</td>
</tr>
<tr>
<td>333</td>
<td>32</td>
<td>F</td>
<td>82</td>
<td>no</td>
<td>196000</td>
<td>–</td>
<td>–</td>
<td>yes</td>
<td>24</td>
<td>SVR</td>
</tr>
<tr>
<td>334</td>
<td>55</td>
<td>F</td>
<td>49</td>
<td>no</td>
<td>161000</td>
<td>+</td>
<td>–</td>
<td>no</td>
<td>48</td>
<td>SVR</td>
</tr>
<tr>
<td>335</td>
<td>39</td>
<td>M</td>
<td>263</td>
<td>no</td>
<td>915000</td>
<td>+</td>
<td>–</td>
<td>yes</td>
<td>48</td>
<td>SVR</td>
</tr>
<tr>
<td>340</td>
<td>34</td>
<td>M</td>
<td>58</td>
<td>no</td>
<td>1880000</td>
<td>+</td>
<td>–</td>
<td>yes</td>
<td>24</td>
<td>SVR</td>
</tr>
<tr>
<td>343</td>
<td>49</td>
<td>M</td>
<td>77</td>
<td>yes</td>
<td>239000</td>
<td>+</td>
<td>+</td>
<td>no</td>
<td>48</td>
<td>SVR</td>
</tr>
<tr>
<td>347</td>
<td>48</td>
<td>M</td>
<td>121</td>
<td>nd</td>
<td>119000</td>
<td>–</td>
<td>–</td>
<td>yes</td>
<td>24</td>
<td>SVR</td>
</tr>
<tr>
<td>350</td>
<td>50</td>
<td>M</td>
<td>76</td>
<td>no</td>
<td>322000</td>
<td>–</td>
<td>–</td>
<td>yes</td>
<td>24</td>
<td>SVR</td>
</tr>
</tbody>
</table>
SVR despite HCV specific T-cell decline

Figure 1b. T-cell proliferation

T-cell proliferation was assessed following 6 days of stimulation with medium alone, HCV core pooled antigens, CMV lysate and PHA following incubation with CFSE. FACS analysis of two representative patients (322 and 324) is shown following gating on the viaprobe negative T-cell subset. The % in the upper left quadrants = number of CD4+CFSElow/total CD4+ cells x100.

± 27.25 vs 134.9 ± 29.44, core responses SFU/10^6 PBMC 85.07 ± 31.58 vs 187.9 ± 94.09), although this did not reach statistical significance. There was no significant association between the magnitude of the core specific response and the viral loads before treatment. However, there was a significant correlation between the IFN-γ core specific HCV response and both ALT (Pearson correlation r²=0.45, p=0.001)(Figure 1a) and AST levels (Pearson correlation r²=0.42, p=0.001) before treatment.

Figure 2a: HCV core specific T-cell responses by IFN-ELISpot in all patients at each time point.

A single data-point at each time point represents total HCV core specific T-cell responses (using peptides spanning the entire core region) in a single patient assessed by IFN-ELISpot. Values at each time point are compared with baseline (Wilcoxon signed rank test). Bars = mean.
In 13 patients (5 SVR and 8 non-SVR) with HCV core specific T-cell responses that were detected by IFN-γ ELISpot, sufficient PBMC were available pre-treatment for further analysis using CFSE assays. Using this assay 9/13 patients showed CD4+ T-cell proliferation to HCV core antigens. In 2 of these patients proliferative responses were also observed for the CD8+ T-cell subset, consistent with previous studies [13] showing T-cell responses to core were...
largely CD4+. Generally proliferative responses to HCV core antigens were very weak (mean CD4+CFSE low/total CD4+ =1.15%) compared to proliferative responses that were observed using CMV (mean CD4+CFSE low/total CD4+ =42.96%) and influenza (mean CD4+CFSE low/total CD4+=30.58%) antigens consistent with a previous study [21]. Two representative patients are shown (Figure 1b). The magnitude of the HCV proliferative response pre-treatment did not differ between patients with a SVR and a non-SVR to treatment (data not shown).

**Effect of therapy on HCV core IFN-γ responses**

HCV core specific responses were assessed in all patients by IFN-γ ELISpot before treatment, weeks 12, 24 and 48 weeks during treatment, and 24 weeks after the completion of treatment. Overall there was a significant decline in IFN-γ T-cell responses at weeks 12 (week 0 vs week 12 p=0.007) and 24 (week 0 vs week 24 p=0.009) compared to pre-treatment, that recovered after therapy (Figure 2a). Further subgroup analysis of patients with a SVR and a non-SVR to therapy showed that the decline in T-cell responses was statistically significant only in patients with a SVR to therapy (p= 0.02 week 0 vs. 12, p= 0.019 week 0 vs. 24, Figures 2bA and 2bB). The viral kinetics during therapy for these subgroups are shown (Figures 2bC and 2bD). The viral kinetics and HCV specific T-cell responses are detailed in 4 representative patients; 311, 341, 335 and 333 (Figure 3).

**Figure 3:** Kinetics of HCV core specific T-cell responses and viral loads in individual patients.
Chapter 5

Effect of therapy on HCV core IL-2 responses

The effect of therapy on HCV core specific T-cell responses was assessed using IL-2 ELISpot. IL-2 secretion could be detected before therapy in 8/31 patients. The mean magnitude of IL-2 production by HCV core specific T-cells was significantly weaker than IFN-γ production (35.72±14.38 vs. 138.1±50.97 SFU/10⁶PBMC) (p=0.030) before treatment and remained lower at all time points during therapy compared to IFN-γ secretion. Similar to the decline in IFN-γ response, a decrease in IL-2 production was seen during therapy, which recovered after the cessation of therapy (Figure 4). This was true for both SVR and non-SVR groups (data not shown). The mean number of IL-2 secreting HCV core specific T-cells was higher at the follow up time point in both the SVR and the non-SVR group compared to pre-treatment. However, this did not reach statistical significance (data not shown).

Effect of therapy on HCV non-structural specific T-cell responses

IFN-γ and IL-2 production was then assessed by ELISpot following stimulation with HCV proteins NS3-5. Responses were detected in fewer patients (5/31; 3 patients with a SVR and 2 with a non-SVR) and were significantly weaker (IFN-γ mean 42.4±8.9 SFU/10⁶PBMC) pre-
SVR despite HCV specific T-cell decline
treatment than those observed using core peptides (IFN-\(\gamma\) mean 138.1±50.97 SFU/10\(^6\)PBMC, consistent with our previous studies [13]). Responses to NS3-5 did not increase during therapy, and were lower at all time points during therapy compared to pre-treatment, though this did not reach statistical significance. There was no significant increase in HCV NS3-5 IL-2 secretion during therapy. In a subset of 6 patients we assessed T-cell responses to a pool of overlapping peptides spanning NS5a before treatment and at week 12. No T-cell responses to NS5a were observed at either time point.

Effect of therapy on CMV and Influenza specific T-cell responses
To determine if the suppression of T-cell responses observed during therapy was specific to HCV we assessed the magnitude of CMV specific T-cell responses during therapy. CMV responses could be detected in 14/31 patients by IFN-\(\gamma\) ELISpot before treatment (mean magnitude 461.4±46.31 SFU/10\(^6\)PBMC) There was a marked decline in the magnitude of the CMV specific T-cell response at 12 weeks (mean 178.2±49.86 SFU/10\(^6\)PBMC p=0.003) and 24 weeks p=0.037 (mean 236.0±80.0 SFU/10\(^6\)PBMC) into therapy compared to baseline. These recovered again after the cessation of therapy (mean 307.5±60.02 SFU/10\(^6\)PBMC p=n/s) (Figure 5). As both CMV and HCV are persistent viral infections and as IFN-\(\alpha\) has broad anti-viral effects it was unclear if therapy was exerting suppressive effects directly on T-cells or indirectly through suppression of antigen. We therefore examined the effect of therapy on influenza specific T-cells, where there is no circulating antigen, in a subgroup of 6 patients (Figure 6). In these six patients a significant fall in the influenza specific T-cell response was observed between the pre-treatment and 12 week time point (p=0.03).

Effect of therapy on global T-cell populations
As HCV, CMV and Influenza specific T-cell responses fell during therapy and recovered following therapy we assessed the impact of therapy on global T-cell populations at week 0 and week 12 by FACS analysis. We selected 6 patients where HCV specific T-cell responses were detectable at week 0 but undetectable at week 12 by IFN-\(\gamma\) ELISpot (Figure 7a). FACS analysis of PBMC showed a fall in the CD4+ and CD8+ T-cell subsets within the total PBMC population of approximately 50% in both T-cell subsets (Figure 7b and 7c, representative patient Figure 7d). Analysing the blood lymphocyte counts in all patients at week 0 and week 12 we observed the mean in the SVR group was 5.8 10\(^9\)/ml pre-treatment and this declined in 13/15 patients by week 12 to a mean count of 3.3 10\(^9\)/ml. Similarly in the non-SVR group the mean count before treatment was 6.4 10\(^9\)/ml declining in 14/15 patients (data not available in 1 patient at 12 week time point) to a mean count of 3.3 10\(^9\)/ml (Figure 7e). Thus although 200,000 cells were used in each ELISpot well, the T-cell fraction within each well would, at least in some cases, be approximately 50% less at week 12 compared to pre-treatment. However, this observation cannot explain the complete loss of HCV specific T-cell responsiveness observed in many patients during the first 12 weeks of therapy.
A number of studies have shown a successful outcome with combination therapy is associated with enhanced cellular immune responses [8, 10]. However, others have failed to demonstrate such a clear association [9, 11] and a causal relationship between enhanced T-cell responses and viral load decline has not been demonstrated.

In this study then we adopted a treatment regime that used high dose IFN-α in the early weeks of therapy, followed by conventional doses of PEG-IFN and ribavirin with the expectation that this may increase SVR rates, either through direct anti-viral effects or through enhancement of HCV-specific T-cell responses. It was therefore surprising that the main observation in this study was that there was a profound decline in HCV specific T-cell responses during the first 12 weeks of therapy, that was maintained at 24 weeks and that was more pronounced in those patients that had a subsequent SVR to therapy. Whilst a decrease in T-cell responses has been observed during resolving primary HCV infection in

**Discussion**

A number of studies have shown a successful outcome with combination therapy is associated with enhanced cellular immune responses [8, 10]. However, others have failed to demonstrate such a clear association [9, 11] and a causal relationship between enhanced T-cell responses and viral load decline has not been demonstrated.

In this study then we adopted a treatment regime that used high dose IFN-α in the early weeks of therapy, followed by conventional doses of PEG-IFN and ribavirin with the expectation that this may increase SVR rates, either through direct anti-viral effects or through enhancement of HCV-specific T-cell responses. It was therefore surprising that the main observation in this study was that there was a profound decline in HCV specific T-cell responses during the first 12 weeks of therapy, that was maintained at 24 weeks and that was more pronounced in those patients that had a subsequent SVR to therapy. Whilst a decrease in T-cell responses has been observed during resolving primary HCV infection in
parallel with a declining HCV viral load [12], this has not been previously described in the context of HCV antiviral therapy.

It is tempting to speculate that high dose IFN-α leads to a rapid decline in viral load and that as antigen is rapidly lost, antigen-dependent T-cell responses wane in the absence of ongoing antigenic stimulation. Mathematical modelling predicts that a very rapid loss of antigen below a critical threshold during therapy may lead to a failure of T-cell antigen recognition [22]. The finding that a decline in T-cell response at 12 weeks is most evident in those that achieve a SVR is significant. Of 15 patients who developed a SVR only 2/15 had detectable viraemia using the highly sensitive TMA assay at 12 weeks, and 0/15 patients at 24 weeks whereas viraemia was observed in 9/16 patients at 12 weeks and 10/16 at 24 weeks who failed to make a SVR, suggesting that indeed T-cell responses (whose decline is greatest in SVR patients at week 12 and 24) parallel a decline in HCV viral load.

However, this does not explain the observation that in many individuals T-cell responses recover once therapy has stopped whilst HCV viral loads remain undetectable (Figure 4 representative patients 335 and 333). In this study, there was a decline in blood lymphocyte counts between baseline and 12 weeks in almost all patients. Furthermore, we observed not only a decline in HCV specific T-cells but also in CMV and influenza specific T-cell responses during therapy. The decline in influenza T-cell responses is particularly informative as any effect on T-cell responses must be independent of viral load, since influenza virus does not persist. FACS analysis of PBMC at week 0 and 12 weeks into therapy confirmed that there was a decline in the CD4+ and CD8+ T-cell subsets. Taken together, this data suggests that the observed decline in T-cell responses is due, at least in part to a direct effect of therapy on T-cells through direct suppression of their generation (IFN-α is known to exert suppressive effects on bone marrow), increased apoptosis or more speculatively through T-cell redistribution away from the peripheral blood compartment.

We analysed HCV specific T-cell responses using antigen pools. The first of these, a core peptide pool generates a reproducible response in a number of previously studied cohorts [9, 13, 23]. This approach is analogous to the use of overlapping peptide pools for CMV pp65 or HIV gag in other antiviral studies. It is not a comprehensive analysis but provides a target population of T-cells to track. To add breadth we also used an NS3-5 whole antigen pool as has been used extensively elsewhere [9, 13]. Finally to provide data on peptide specific responses against a non-structural protein we also analysed an NS5a peptide pool. Importantly, no enhancement of these responses was observed during treatment.

Whilst we showed no association between the magnitude of the HCV specific T-cell responses before therapy and the subsequent response to therapy we did observe a highly significant correlation between liver inflammation as assessed by both ALT, AST and the HCV specific T-cell response. Whilst it is widely assumed that liver inflammation in HCV is due to an influx of HCV specific T-cells this is the first time that this association has been clearly made.

In defining a successful immune response simple quantification of cellular responses is likely to represent an over-simplistic approach. The phenotype and cytokine profile of viral specific T-cells clearly differs between different viral infections. Furthermore we have previously shown that the functional status of HCV specific T-cells is important in HCV control [13].
In individuals that resolve HCV, CD4+ HCV specific T-cells maintain IL-2 secretory capacity compared to IFN-γ secretion, whilst in persistent infection IL-2 secreting CD4+ T-cells are rarely observed. Such populations have a relatively low proliferative capacity as assessed using CFSE assays [24]. Furthermore in HIV infection it has been shown that in the presence of high viral loads HIV specific CD4+ T-cell populations do exist, but lack proliferative capacity and secrete IFN-γ but are unable to secrete IL-2 in response to antigenic stimulation. Once HIV antigen loads decline through drug therapy IL-2 secretion and proliferative capacity is restored [14]. In this study then we addressed the hypothesis that successful combination therapy is associated with the restoration of IL-2 secreting cellular immune responses. We observed that IL-2 secretion by HCV specific T-cells was significantly weaker than IFN-γ secretion before treatment and declined further during treatment in both the SVR and non-SVR groups. Following therapy IL-2 secretion was restored to pre-treatment levels in those with a SVR but was not enhanced above baseline.

Overall, in this study we demonstrate that rather than enhancing the T-cell response, as might have been predicted, high dose α-interferon induction therapy is associated with a profound loss of IL-2 and γ-IFN secreting HCV specific T-cells. This effect is probably mediated both through loss of antigenic stimulation of HCV specific T-cells and also through direct immunosuppressive effects of high dose IFN-α therapy. Although the magnitude of this effect is of most significance in the setting of high dose IFN-α, the significance of these observations is much more general. Firstly, the very rapid virological response seen can readily occur in the absence of immune restoration—indeed in the context of loss of HCV specific T-cells. This strongly implies that this phase of viral dynamics is independent of T-cell responses. Secondly, long term antiviral effects are also obtained whilst T-cell responses are suppressed. Again, this supports an argument that any change in T-cell responses seen during conventional doses of IFN-α therapy are not causally related to clinical outcome.

The challenge remains to develop new efficacious antiviral therapies for HCV. It is probable that HCV protease and polymerase inhibitors will be used in clinical practice in the next few years. These specific antiviral therapies will shed further light on the relationship between HCV viral loads and T-cell responses during therapy. Additionally, the fact that T-cell responses induced during current antiviral therapies appear not to influence virological outcomes does not mean that further direct manipulation of such responses would not confer some further benefit. In the meantime further evaluating the host pathways which contribute to successful outcomes for antiviral therapy remains an important objective.

References


Acknowledgements

E.B. is an M.R.C.(U.K.) clinician scientist, I.H. is funded by the M.R.C. (U.K.), P.K. is a Senior Wellcome clinician scientist. We would like to thank Ester Remmerswaal and Nelly Baylon for technical assistance. We would also like to thank the patients for their participation in this study.

Conflict of interest statement:

E.B., H.C.G., I.H., N.S., R.A.WVL., P.K: none to declare
H.W.R. is a consultant for Schering-Plough, he has received unrestricted grants from Hoffmann-La Roche, Schering-Plough and Vertex Pharmaceuticals. M.G.H.M.B. is a consultant for Bayer Diagnostics.

Grant support

This study was supported by the Medical Research Council (UK), Wellcome trust, Schering-Plough and Bayer Diagnostics