Cellular immune responses to HCV core increase and HCV RNA levels decrease during successful antiretroviral therapy

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Abstract

BACKGROUND: Hepatitis C virus (HCV) infection is a major cause of morbidity in HIV infected individuals. Coinfection with HIV is associated with diminished HCV-specific immune responses and higher HCV RNA levels.

AIMS: To investigate whether long-term combination antiretroviral therapy (cART) restores HCV-specific T cell responses and improves the control of HCV replication.

METHODS: T cell responses were evaluated longitudinally in 80 HIV/HCV coinfected individuals by ex vivo interferon-gamma-ELISpot responses to HCV core peptides, that predominantly stimulate CD4(+) T cells. HCV RNA levels were assessed by real-time PCR in 114 individuals.

RESULTS: The proportion of individuals with detectable T cell responses to HCV core peptides was 19% before starting cART, 24% in the first year on cART and increased significantly to 45% and 49% after 33 and 70 months on cART (p=0.001). HCV-specific immune responses increased in individuals with chronic (+31%) and spontaneously cleared HCV infection (+30%). Median HCV RNA levels before starting cART were 6.5 log(10) IU/ml. During long-term cART, median HCV-RNA levels slightly decreased compared to pre-cART levels (-0.3 log10 IU/ml, p=0.02).

CONCLUSIONS: Successful cART is associated with increasing cellular immune responses to HCV core peptides and with a slight long-term decrease in HCV RNA levels. These findings are in line with the favourable clinical effects of cART on the natural history of hepatitis C and with the current recommendation to start cART earlier in HCV/HIV coinfected individuals.
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Cellular immune responses to HCV core increase and HCV RNA levels decrease during successful antiretroviral therapy

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the Swiss HIV Cohort Study

ABSTRACT

Background Hepatitis C virus (HCV) infection is a major cause of morbidity in HIV infected individuals. Coinfection with HIV is associated with diminished HCV-specific immune responses and higher HCV RNA levels.

Aims To investigate whether long-term combination antiretroviral therapy (cART) restores HCV-specific T cell responses and improves the control of HCV replication.

Methods T cell responses were evaluated longitudinally in 80 HIV/HCV coinfected individuals by ex vivo interferon-γ-ELISPOT responses to HCV core peptides, that predominantly stimulate CD4⁺ T cells. HCV RNA levels were assessed by real-time PCR in 114 individuals.

Results The proportion of individuals with detectable T cell responses to HCV core peptides was 19% before starting cART, 24% in the first year on cART and increased significantly to 45% and 49% after 33 and 70 months on cART (p=0.001). HCV-specific immune responses increased in individuals with chronic (+31%) and spontaneously cleared HCV infection (+30%). Median HCV RNA levels before starting cART were 6.5 log₁₀ IU/ml. During long-term cART, median HCV-RNA levels slightly decreased compared to pre-cART levels (−0.3 log₁₀ IU/ml, p=0.02).

Conclusions Successful cART is associated with increasing cellular immune responses to HCV core peptides and with a slight long-term decrease in HCV RNA levels. These findings are in line with the favourable clinical effects of cART on the natural history of hepatitis C and with the current recommendation to start cART earlier in HIV/HCV coinfected individuals.

INTRODUCTION

Hepatitis C is a major cause of morbidity and death in HIV infected individuals.¹ In the Swiss HIV Cohort Study (SHCS), 53% of HIV infected individuals are coinfected with the hepatitis C virus (HCV).² Coinfection with HIV accelerates the progression to liver cirrhosis³ and is associated with higher HCV RNA levels,⁴ ⁵ particularly in individuals with low CD4⁺ T cell counts.⁶ Cellular immune responses, crucial for the control of HCV infection,⁷ are severely diminished in HIV infected individuals; HCV-specific CD8⁺ and CD4⁺ T cell responses are weak in chronic hepatitis C and are further impaired in HIV coinfected individuals.⁸ ⁹ ¹⁰ The loss of cellular immune responses to HCV is particularly evident in individuals with low CD4⁺ T cell counts.¹¹ ¹²

Combination antiretroviral therapy (cART) reduces liver-related mortality in HIV/HCV coinfected individuals.¹³ ¹⁴ Potential favourable effects of cART on the course of hepatitis C include a reduction in immune activation and an increase in cellular immune responses. It is unclear to what extent a successful cART restores HCV-specific immune responses. Previous studies suggested that HCV RNA levels increase in the first 5–6 months...
after the start of cART. However, it is unclear whether immune restoration through cART improves the control of HCV replication long term. In this study, we longitudinally investigated the impact of HIV and of a successful cART on HCV-specific T cell responses and on HCV RNA levels.

METHODS

Participants and study design

Study participants were included from the Swiss HIV Cohort Study (SHCS), a prospective multicentre study carried out at seven major Swiss hospitals and their local affiliated centres. Written informed consent, including for genetic testing, was mandatory for inclusion, and the study was approved by all local ethical committees. Demographic and clinical characteristics were extracted from clinical databases.

HCV-specific T cell responses and HCV RNA levels were assessed longitudinally during untreated HIV infection and during successful cART. Successful cART was defined as HIV-RNA levels below 400 copies/ml after 6 months from cART start and thereafter. Follow-up was censored at the first virological failure (HIV-RNA above 1000 cp/ml). Low level viral replication (HIV-RNA 400–1000 cp/ml) post-cART was present in 2% of measurements. In these instances, follow-up was not censored as these events were not considered immunologically relevant.

SHCS participants fulfilling the following criteria were included: (i) anti-HCV seropositivity (using ELISA and confirmed by immunoblot or recombinant immunoblot assay (RIBA)) and detectable HCV RNA, assessed by quantitative or qualitative assays; and (ii) availability of frozen peripheral blood mononuclear cells (PBMCs) and/or plasma samples before commencement of cART (median 0.8 (IQR 4 to 0.2) months pre-cART) and after more than 2 years of successful cART (median 33 (IQR 30 to 36) months on cART). In some individuals, analyses were performed additionally at enrolment, during the first year and after more than 4 years on successful cART. We restricted our analyses to individuals infected with HCV-genotype 1 (n=22) of unknown HCV genotype (n=73) or 3 (n=46). For cellular analyses we also included individuals with spontaneous HCV clearance (n=22) of unknown HCV genotype (genotyping was not possible because clearance had already occurred when entering the SHCS). Spontaneous HCV clearance was defined as HCV seropositivity and undetectable HCV-RNA. All analyses were before HCV therapy. Table 1 shows the characteristics of the study participants. Figure 1 shows the number of individuals with available viable cells and/or plasma samples at each time-point, and the median CD4+ T cell counts and HIV-RNA levels at these time-points.

Laboratory methods

Cellular assays

Peptides and proteins

We used the same experimental approach as in previous studies to investigate the influence of cART on HCV-specific T cell immunity. We have shown in several studies, that responses to the core peptides are reproducibly detectable in a reasonable fraction of patients, that these are dominated by CD4+ T cell responses, and that they are rarely associated with virus escape.

Figure 1 Study population, CD4+ T cell counts and HIV-RNA levels. Median CD4+ T cell counts and HIV-RNA levels at the different study time-points are shown. The table below the graph indicates the number of individuals with available viable peripheral blood mononuclear cells for cellular assays, and with plasma samples for the measurement of hepatitis C virus (HCV) RNA levels. cART, combination antiretroviral therapy.

<table>
<thead>
<tr>
<th>Time (months)</th>
<th>N=10</th>
<th>N=12</th>
<th>N=22</th>
<th>N=30</th>
<th>N=44</th>
<th>N=50</th>
<th>N=66</th>
<th>N=77</th>
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</thead>
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<td>Median HIV-RNA (log10 cp/ml)</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Number of individuals with detectable core peptides</td>
<td>10</td>
<td>12</td>
<td>22</td>
<td>30</td>
<td>44</td>
<td>50</td>
<td>66</td>
<td>77</td>
</tr>
<tr>
<td>Number of individuals with undetectable core peptides</td>
<td>10</td>
<td>12</td>
<td>22</td>
<td>30</td>
<td>44</td>
<td>50</td>
<td>66</td>
<td>77</td>
</tr>
</tbody>
</table>

ELISPOT assay for interferon γ secretion

ELISPOT assays were performed as previously described. PBMCs (100 000 per well) were used and plates were read with an AID plate reader. Each sample was tested in duplicate against HCV antigens, HIV-p24, Epstein–Barr virus (EBV) and cytomegalovirus (CMV) antigens. Phytohaemagglutinin (PHA) was used as a positive control.
a positive control. Samples without any detectable PHA response were excluded. A test was considered positive if the probability of a spot appearing in the test well was significantly different (p=0.05) from the probability of a spot appearing in the control well (background), assuming a binomial distribution for each test antigen (Excel BINOMDIST statistics program, Microsoft). The mean number of spot forming units (SFU) in control wells was subtracted from the mean SFU number in the test wells to give a final reading.

HCV RNA measurement

RNA extraction

For the preparation of RNA from plasma samples we used the EasyMag Magnetic extraction kit according to the manufacturer’s instructions (Biomerieux, Geneva, Switzerland). In brief, 200 µl of plasma was incubated with 2 ml lysis buffer containing guanidin thiocyanate and 2.5 µl carrier RNA (1 µg/µl) for 10 min at room temperature. After adding 550 µl NucliSens easyMAG magnetic silica and incubation for another 10 min at room temperature, several wash steps (using NucliSens easyMAG extraction buffer 1 and 2) were performed, and finally the purified RNA was eluted with 110 µl NucliSens extraction buffer 3. HCV RNA standards were obtained from AcroMetrix (OptiQuant HCV RNA quantification panel, AcroMetrix, Netherlands) and extracted according to the same procedure as described above, together with clinical samples.

Viral load assay

Based on the method previously published by Castelain et al., a real-time RT-PCR assay using TaqMan (fluorescence-based real-time PCR) and minor groove binding (MGB) probes was designed for quantitative determination of HCV RNA in clinical samples. The specific reverse transcription of HCV was performed in 20 µl reaction mixture containing 10.5 µl of eluted RNA and reverse transcription reagents including MultiScribe TM reverse transcriptase, dNTP (2.5 mM), MgCl₂ (2.5 mM), RNA and reverse transcription reagents including MultiScribe TM reverse transcriptase, dNTP (2.5 mM), MgCl₂ (2.5 mM), 10× buffer with RNasin (20 U/µl), and random hexamers (Applied Biosystems, Rotkreuz, Switzerland). The products were hybridised by incubating at 25°C for 10 min, incubated at 48°C for 30 min for reverse transcription, followed by heating to 95°C for 5 min to deactivate the reverse transcriptase. To increase specificity, a second round of reverse transcription at a higher temperature and subsequent cDNA amplification was performed using 96-well plates in duplicate reactions requiring 6 µl of cDNA template in 20 µl reactions containing rTH DNA polymerase (2.5 U/µl), AmpErase UNG, dATP, dCTP, dUTP and a 5× buffer containing a passive reference (6-carboxyxyrhomidine labelled with ROX) (EZ-RT PCR kit, Applied Biosystems, Rotkreuz, Switzerland) forward primer (500 nM) HCV-S1 (nucleotides: 127 to 145; 5'-TCCCGGAGACGCC-ATAGT-3'), reverse primer (1 µM) HCV-AS2 (nucleotides: 202 to 185; 5'-TCCAGAAGGGGCCCRTG-3') and 1 µM TaqMan minor groove binding (MGB) probe labelled with 6-carboxyfluorescein (nucleotides: 147 to 161; 5'-FAM-TCTGCCGAAACGGCTGT-MGB-3').

The ABI 7900 HT real-time PCR detection system (Applied Biosystems) was used for analysis. Thermal cycling conditions were designed as follows: 50°C for 2 min, for contamination control with AmpErase UNG, followed by 60°C for 30 min for continued reverse transcription with rTH DNA polymerase, then 95°C for 15 sec and 60°C for 60 sec. Fluorescent measurements were recorded during each annealing step. At the end of each PCR run, data were automatically analysed by the system and amplification plots were generated. The HCV copy number was determined by reading from the standard curve (OptiQuant HCV RNA quantification panel, AcroMetrix, Netherlands, range 50 to 5 Mio IU/ml). Mean cycle threshold (CT) values for the main range of HCV RNA levels (5 to 7 log₁₀ IU/ml) were 28.5 (SD±0.5), 25.2 (SD±0.5) and 23.5 (SD±0.1). To avoid inter-assay variability, all samples from one patient were measured within the same assay. The standard transcripts, the controls and all study samples were run in duplicate. Replicate samples varying by more than 5% of their quantification cycle were repeated. To assess inter-assay variability, we included in each run plasma from one individual with a known HCV RNA level (5.0 log₁₀ IU/ml, assessed by the Roche Cobas assay). RNA levels from this individual measured by our in-house assay were comparable and showed low inter-assay variability over 15 runs (mean quantity 5.1 (SD±0.25) log₁₀ IU/ml). In addition, control plasma samples with a wide range of HCV RNA levels provided by Roche Diagnostics, Switzerland, were included in each run and also showed low inter-assay variability in both the low and high range (mean quantity 2.1 (SD±0.5) and 5.1 (SD±0.2) log₁₀ IU/ml, respectively). HCV-negative plasma sample obtained from AcroMetrix (OptiQuant HCV RNA quantification panel, AcroMetrix, Netherlands) and distilled water were included as non-template controls in every run. All negative controls were always amplified after CT values of 40.

Statistical analysis

Cross-sectional comparisons were performed using a robust variance estimation model for cluster-correlated data to consider data points representing repeated measurements. Longitudinal analyses were done by using the non-parametric paired Wilcoxon signed rank test. Statistical analyses were performed in STATA V.10.0 software and the figures were drawn using Graphpad Prism 5.01 software.

RESULTS

HCV-specific T cell responses to HCV core peptides

We quantified longitudinally HCV-specific T cell responses to HCV core peptides by ex vivo interferon γ (IFNγ) ELISpot assays in untreated and treated HIV infection. In cross-sectional analyses, the proportion of individuals with detectable HCV-specific immune responses to HCV core peptides was significantly higher during successful cART compared to untreated HIV infection (figure 2A). During untreated HIV infection there was no significant change in HCV-specific immune responses (13% (2/16) vs 19% (12/64), p=0.54). However, during successful cART, the proportion of individuals with detectable T cell responses increased significantly: 24% (12/50) during the first year, 45% (29/64) after more than 3 years and 49% (18/37) after more than 5 years of successful cART (p=0.001). Accordingly, the frequency of spot forming cells was significantly higher after initiation of HIV therapy (figure 2B). The increase in responses to HCV core peptides on cART was observed for chronic and spontaneously cleared HCV infection (figure 2D) and for both HCV genotypes (figure 2C). This is in line with the high sequence similarity of the HCV core region with 94% identity between genotypes 1 and 3.

Neither CD4⁺ T cell counts at the time of the experiments nor nadir CD4⁺ T cell counts correlated significantly with the presence or absence of HCV-specific T cell responses (p>0.1 for all comparisons). At baseline, the relative CD4⁺ T cell count was higher in individuals with detectable HCV-specific T cell responses to HCV core peptides, compared to those without detectable responses (22% vs 15%, p=0.05), while there was no...
A statistically significant difference with regard to absolute CD4⁺ T cell counts. There was no significant correlation of relative or absolute CD4⁺ T cell counts with HCV-specific T cell responses after starting cART.

In a subgroup of 54 individuals (supplementary table 1) with available viable PBMCs 0.8 months before starting HIV therapy and after a median of 33 months on successful cART, we assessed longitudinally the evolution of HCV-specific T cell responses. The proportion of individuals with detectable T cell responses and the frequency of IFN-γ producing T cells per million peripheral blood mononuclear cells recognizing HCV-core peptides significantly increased after initiation of a successful cART (B). The increase in responses to HCV core peptides on cART was observed for chronic (C) and spontaneously cleared HCV infection (D) and for both HCV genotypes (C). Only p-values <0.05 are shown. SFC, spot forming cells.

**Figure 2** T cell ELISpot responses to hepatitis C virus (HCV) core peptides before and on successful combination antiretroviral therapy (cART). The proportion of individuals with detectable ELISpot responses to HCV core peptides increased significantly during successful cART (A). Peak frequency of interferon-γ producing T cells per million peripheral blood mononuclear cells recognising HCV-core peptides significantly increased after initiation of a successful cART (B). The increase in responses to HCV core peptides on cART was observed for chronic (C) and spontaneously cleared HCV infection (D) and for both HCV genotypes (C). Only p-values <0.05 are shown. SFC, spot forming cells.

For the remaining characteristics, there were no significant differences among the four groups.

**T cell responses to further peptides**

In contrast to the responses to HCV core peptides, there was no significant change in the response to recombinant HCV NS3-5 proteins. From the 48 individuals with at least one response to HCV core peptides, 11 (23%) also responded to recombinant HCV NS3-5 proteins.

**Figure 3** Proportion of individuals with detectable ELISpot responses to hepatitis C virus (HCV) core peptides and to different control peptides. Arrows denote the time-point of start of combination antiretroviral therapy (cART). ND, no data.
NSS-5 peptides. Conversely, 11 of 12 (92%) individuals with responses to NSS-5 proteins also responded to core peptides. This is in line with previous findings that responses to core proteins are detected more frequently than responses to NS proteins.9 25 Although some NSS-5 epitopes are shared between genotype 1 and 3 sequences,28 30 other epitopes are clearly genotype-specific.29 31 Therefore, it is likely that some genotype 3 specific responses to the NS proteins were missed. Responses to HIV-p24 decreased, while responses to CMV peptides increased significantly during cART (p=0.001). Twenty-eight of the 48 (58%) individuals with responses to HCV core peptides also responded to CMV peptides.

HCV RNA levels

HCV-RNA levels were evaluated in 119 HIV/HCV coinfected individuals. In cross-sectional analysis, median HCV RNA levels during untreated HIV infection were 6.3 (IQR 5.6–6.8) log$_{10}$ IU/ml at enrolment and 6.5 (IQR 5.9–7.0) log$_{10}$ IU/ml just before starting cART. During the first year on successful cART, median HCV-RNA levels slightly increased to 6.6 (IQR 6.0–7.2) log$_{10}$ IU/ml. During long-term cART, median HCV-RNA levels decreased to 6.4 (IQR 5.9–6.9) and 6.2 (IQR 5.6–6.7) log$_{10}$ IU/ml after 3 and 5 years, respectively (figure 4A). HCV clearance was not observed despite long-term cART.

In individuals with longitudinal samples during untreated HIV infection, the median change in HCV-RNA levels was +0.4 log$_{10}$ IU/ml. In the first year on successful cART, there was a slight increase of HCV-RNA levels (+0.1 log$_{10}$ IU/ml). During long-term cART, median HCV-RNA levels slightly decreased compared to pre-cART levels (−0.3 log$_{10}$ IU/ml, p=0.02; figure 4B).

**DISCUSSION**

Coinfection with HIV substantially diminishes the immunological control of HCV. Previous studies have shown that HCV-specific immune responses are reduced in HIV infected individuals.9 10 Furthermore, the high HCV RNA levels observed in HIV infected individuals indicate a loss of control of HCV replication.9 Here, we show that successful cART partially restores T cell responses to HCV-core peptides. The proportion of individuals with detectable immune responses on long-term cART (49%) was very similar to that observed by Harcourt et al in HCV monoinfected individuals (52%) using the same experimental approach.9 A successful cART also increased cellular immune response to HCV core peptides in individuals that spontaneously cleared HCV infection. This finding is in line with the observation that cellular immunity is maintained long term in individuals who spontaneously clear infection.32

We have shown previously using CD8$^+$ T cell depleted PBMCs,20 in addition to previous experiments using flow cytometry,22 33 that the majority of responses to the HCV antigens used here were from CD4$^+$ T cells. Therefore, the measured increase in T cell responses largely represents CD4$^+$ T cell reactivity. High sensitivity techniques to isolate T cell subsets would have required larger cell numbers and ideally fresh samples, that were not available due to the long-term follow-up in this study.

The absence of a significant increase in responses to HCV NSS-5 proteins in our HIV/HCV coinfected patients is in accordance with previous studies where these responses were not detectable in the majority of HCV monoinfected individuals.9 Non-structural HCV proteins are highly polymorphic and the consensus sequence that is used to generate the peptide libraries contains in many instances escaped variants that are poorly immunogenic.31 34 35 Viral adaptation to T cell pressure through mutational escape is more likely in polymorphic proteins, which might explain why T cell responses to NSS-5 peptides are more difficult to detect compared to responses to the conserved core peptides. Because we used different methods (core peptides versus non-structural proteins), the responses to core and NS are not directly comparable. However, a comparison of the relative importance of immune responses to different peptides and proteins was beyond the scope of this study. Instead, we used an optimised, reproducible and well studied approach to investigate the influence of cART on the evolution of T cell responses. Future studies are underway and will aim to assess the relationship between responses to structural and non-structural gene products in more detail.

Concurrently with the increase of cellular immune responses during cART, we observed a slight but significant decrease in HCV RNA levels. This effect was particularly evident in a small
subgroup of individuals with increasing HCV specific immune responses. The temporal association of increasing HCV specific T cell responses and decreasing HCV RNA levels might potentially indicate that immune reconstitution through successful cART improves the control of HCV replication, although it is difficult to show cause and effect in this case. A transient increase in HCV RNA levels during the first year of cART has been observed previously (reviewed in Cooper and Cameron17). Suggested mechanisms for such an increase include increased hepatocyte lysis through restored T cell immunity, and a decrease in interferon levels through treatment of HIV.15

Clearance of HCV infection during cART has been reported in only a few cases.25–41 We did not observe HCV clearance despite a substantial increase in T cell responses on cART. This suggests that in most cases, immunological HCV clearance cannot be achieved by restoring T cell responses through successful cART.

Our study has strengths and limitations. A main strength is the long-term follow-up and the longitudinal setting that largely avoids confounding through clinical and demographic characteristics. Additionally, we could assess cellular immune responses and HCV RNA levels in the same cohort and therefore simultaneously evaluate the impact of HIV and cART on T cell responses and viral loads. Through the restriction to successful and uninterrupted cART, we could avoid biases through treatment failures and interruptions. A limitation of the study is the restriction of cellular experiments through the use of stored PBMCs. Due to the limited number of frozen PBMCs, we could not assess CD8+ or regulatory T cell responses, or perform an in-depth analysis of HLA-restricted immune responses. In addition, viable cells and/or plasma were not available for all participants at all study time-points. We acknowledge that we might have missed many HCV specific T cell responses that could have been detected after in vivo culture, as shown recently.24 However, we explicitly wanted to assess the influence of an increased immune pressure in-vivo and therefore avoided additional in-vitro stimulation that can strongly influence T cell responses, as shown by Schnuriger et al.24 Additional culturing could have masked the main study question about the effects of cART on HCV-specific T cell reactivity. Ex-vivo ELISpot responses probably underestimate the total number of HCV-specific cells that can be generated after culturing. However, this does not alter the main conclusion that cART can increase HCV-specific T cell responses. As HCV specific T cell responses were not detectable in most individuals, we could not reliably correlate changes in the frequency of T cell responses with HCV RNA levels and establish possible cause and effect.

Our findings are in line with the beneficial clinical effects of cART on the natural course of hepatitis C in HIV infected individuals13 14 and with the current recommendation to start cART earlier in HCV/HIV coinfected individuals.42 43 However, it remains unclear to what extent the increases in cellular immune responses during cART result in beneficial effects on liver disease progression. Other favourable effects of cART on liver disease include a reduction in immune activation and in bacterial translocation. Although one study found that higher hepatitis C RNA levels were associated with increased mortality from end stage liver disease,44 most reports did not find a correlation between HCV RNA levels and liver disease.45 46 It is therefore unlikely that the modest effect of cART on HCV viraemia results in large clinical effects. However, as viral load is an important predictor of the response to HCV therapy,47 a reduction in HCV RNA levels through cART might result in better response rates to HCV therapy. Future studies should clarify whether starting cART early in the course of HIV infection would lower the risk of progression to end-stage liver disease and improve HCV treatment responses.

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