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

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Revised 31 March 2010
Accepted 13 April 2010
Published Online First
26 July 2010

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 $\log_{10}$ 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.

Significance of this study

What is already known about this subject?

- Coinfection with HIV accelerates the progression of liver fibrosis in individuals with chronic hepatitis C.
- Coinfection with HIV is associated with diminished hepatitis C specific T cell responses and with higher hepatitis C virus (HCV) RNA levels.

What are the new findings?

- Cellular immune responses to HCV core peptides increase significantly during successful combination antiretroviral therapy (cART).
- Immune responses to HCV core peptides during long term cART are comparable to those in HCV mono-infected individuals.
- HCV RNA levels slightly decrease long term in individuals with immune recovery through a successful cART.

How might it impact on clinical practice in the foreseeable future?

- cART can improve the immunological and viral control of HCV infection.
- This finding is in line with the current recommendation to start cART earlier in HIV/HCV coinfected individuals to reduce liver-related morbidity in HIV/HCV coinfected individuals.
- An earlier start of cART might also improve the response to HCV therapy, as lower HCV RNA levels are an important predictor of a sustained virological response.

INTRODUCTION

Hepatitis C is a major cause of morbidity and death in HIV infected individuals.[1] In the Swiss HIV Cohort Study (SHCS), 33% of HIV infected individuals are coinfected with the hepatitis C virus (HCV).[2] Coinfection with HIV accelerates the progression to liver cirrhosis[3] 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.[8–10] 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 3–6 months

after the start of cART.[15 16] However, it is unclear whether immune restoration through cART improves the control of HCV replication long term.[17]

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.[18 19] 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.

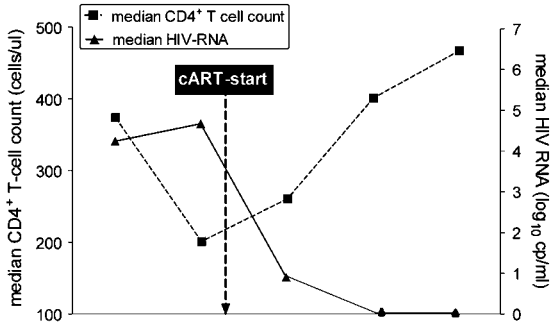
HCV-specific T cell responses and HCV RNA levels were assessed longitudinally during untreated HIV infection and during successful cART. Successful cART was defined as HIV-RNA levels below 400 copies/ml after 6 months from cART start and thereafter. Follow-up was censored at the first virological failure (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=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.

Table 1 Demographic characteristics

Characteristics	N (%)
Total*	141
Chronic HCV infection	119 (84)
Cleared HCV infection	22 (16)
Sex	
Male	94 (67)
Female	47 (33)
Ethnicity	
Caucasian	134 (95)
Non-Caucasian	7 (5)
Transmission mode	
Intravenous drugs	103 (73)
Heterosexual	30 (21)
MSM	5 (4)
Haemophilia	3 (2)
Age, median (IQR)	38 (34–42)

*Number of individuals with ≥1 viable peripheral blood mononuclear cells and/or plasma sample.
HCV, hepatitis C virus; MSM, men who have sex with men.



Median time from cART-start (months)	-35	-0.8	6	33	70
Number of individuals with assessable* ELISpot assays	N=16	N=64	N=50	N=64	N=37
- Chronic Hepatitis C	N=13	N=43	N=35	N=46	N=29
- Resolved Hepatitis C	N=3	N=21	N=15	N=18	N=8
Number of individuals with Hepatitis C RNA	N=30	N=90	N=98	N=114	N=61

*viable cells, experiment successful

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.

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.[9] 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.[9 20–22] Core peptides are therefore ideally suited to assess the influence of cART on HCV-specific T cell responses. The HCV antigens were 18 HCV core genotype 1 specific sequence peptides (20mers overlapping by 10) that cover amino acids 1–191 and were pooled to a final concentration of 10 μg/ml of each peptide. To further investigate whether the change in immune responses to cART was only restricted to core peptides, we included the recombinant HCV non-structural (NS) proteins NS3, NS4 and NS5. These proteins have been shown to induce immune responses especially during acute HCV infection or in individuals with spontaneous HCV clearance.[23 24] The recombinant HCV genotype 1 proteins NS3, NS4 and NS5 were pooled to a final concentration of 1 μg/ml for each antigen in the ELISpot assay. We used pooled recombinant NS proteins instead of overlapping peptides, as these predominantly stimulate CD4$^+$ T cell responses and to maximise the detection rate from fairly limited cell numbers. Recombinant HIV-p24 antigen, EBV and CMV control peptides were used at the same concentration of 1 μg/ml.

ELISpot assay for interferon γ secretion

ELISpot assays were performed as previously described.[9] 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. 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 ($p \leq 0.05$) from the probability of a spot appearing in the control well (background), assuming a binominal 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 (Biomérieux, Geneva, Switzerland). In brief 200 µl of plasma was incubated with 2 ml lysis buffer containing guanidinthiocyanate 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*,[25] 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), $MgCl_2$ (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, dGTP, dCTP, dUTP and a 5× buffer containing a passive reference (6-carboxyrhodamine labelled with ROX) (EZ-RT PCR kit, Applied Biosystems, Rotkreuz, Switzerland) forward primer (500 nM) HCV-S1 (nucleotides: 127 to 145; 5′-TCCCGGGAGAGCCATAGTG-3′), reverse primer (1 µM) HCV-AS2 (nucleotides: 202 to 185; 5′-TCCAAGAAAGGACCCRGT-3′) and 1 µM TaqMan minor groove binding (MGB) probe labelled with 6-carboxyfluorescein (nucleotides: 147 to 161; 5′-FAM-TCTGCGGAACCGGTG-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 $\log_{10}$ 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 $\log_{10}$ 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) $\log_{10}$ 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±0.3) and 5.1 (SD±0.2) $\log_{10}$ 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.[26] 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.[27]

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

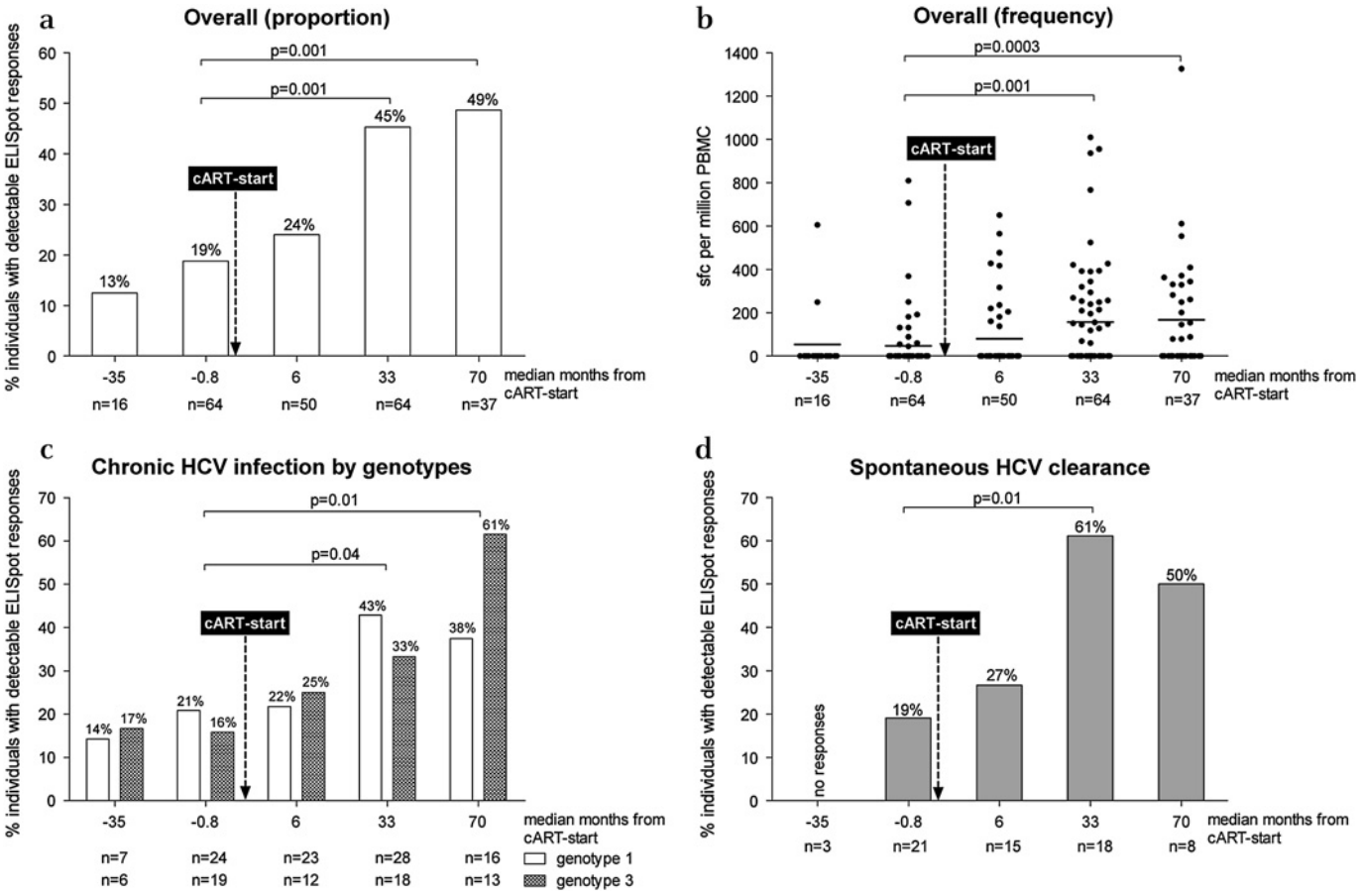


Figure 2 T cell ELISpot responses to hepatitis C virus (HCV) core peptides before and on successful combination antiretroviral therapy (cART). The proportion of individuals with detectable ELISpot responses to HCV core peptides increased significantly during successful cART (A). Peak frequency of interferon-γ 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.

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 IFNγ producing T cells increased significantly during cART (22% vs 44%, p=0.02) (supplementary figure 1). Emergence of detectable T cell responses was much more frequent than loss of responses during cART (31% vs 9%). Individuals with increases in immune responses had lower $CD4^+$ T cell counts at baseline compared to individuals without detectable T cell responses to HCV core peptides pre- and on-cART (150 vs 225 $CD4^+$ T cells/μl; p=0.03). However, this difference was driven by two individuals with increases in T cell responses who started cART with $CD4^+$ T cell counts below 5 cells/μl, and the relevance of this difference remains uncertain. 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

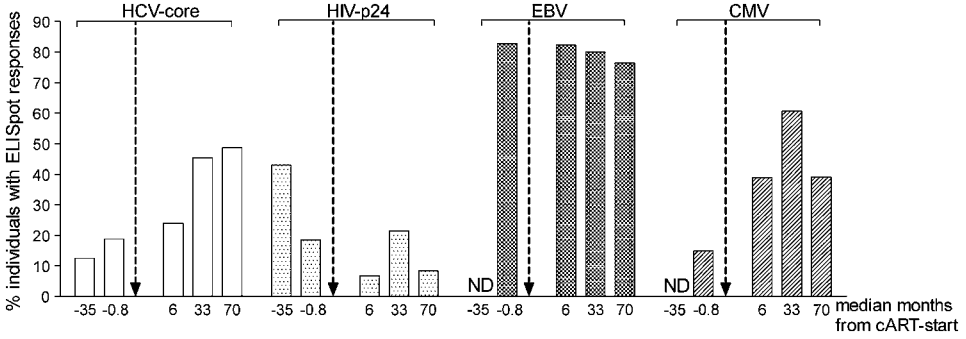


Figure 3 Proportion of individuals with detectable ELISpot responses to hepatitis C virus (HCV) core peptides and to different control peptides. Arrows denote the time-point of start of combination antiretroviral therapy (cART). ND, no data.

NS3-5 peptides. Conversely, 11 of 12 (92%) individuals with responses to NS3-5 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 23] Although some NS3-5 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) (figure 3). 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).

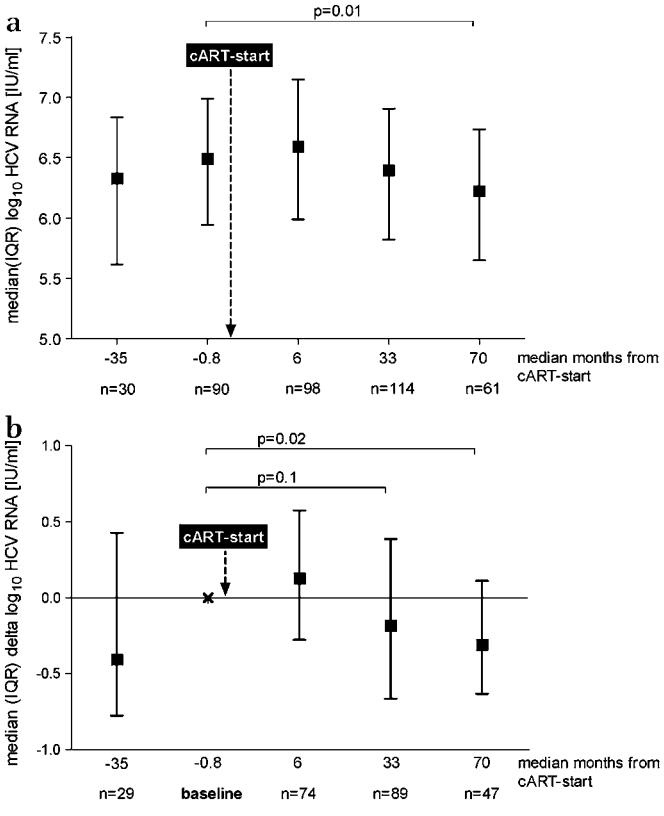


Figure 4 Impact of combination antiretroviral therapy (cART) on hepatitis C virus (HCV) RNA levels. Median HCV RNA levels at the different study time-points are shown in (A) in cross-sectional analysis. The change in HCV RNA levels within individuals in untreated and treated HIV infection is outlined in (B). Only p-values ≤0.1 are shown.

HCV RNA levels slightly decreased on successful cART in both HCV-genotype 1 (−0.2 $\log_{10}$ IU/ml) and HCV-genotype 3 infected individuals (−0.3 $\log_{10}$ IU/ml). The increase in absolute $CD4^+$ T cell counts on successful cART was not significantly associated with the change in HCV RNA levels (p=0.1).

In the subgroup of individuals with increasing HCV-specific immune responses a more marked decline of HCV-RNA levels on cART, we observed compared to individuals without any detectable ELISpot responses to HCV-core peptides (figure 5). In individuals with increasing HCV-specific T cell responses to HCV-core peptides, the median HCV RNA change from baseline was −0.5 and −0.8 $\log_{10}$ IU/ml. For individuals without T cell responses, the median change from baseline after 3 and 5 years of successful cART was −0.2 and −0.1 $\log_{10}$ IU/ml. However, these differences were not statistically significant.

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.[8 9 11] Furthermore, the high HCV RNA levels observed in HIV infected individuals indicate a loss of control of HCV replication.[1] 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 large 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 NS3-5 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 NS3-5 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

Gut 2010;**59**:1252–1258. doi:10.1136/gut.2009.205971

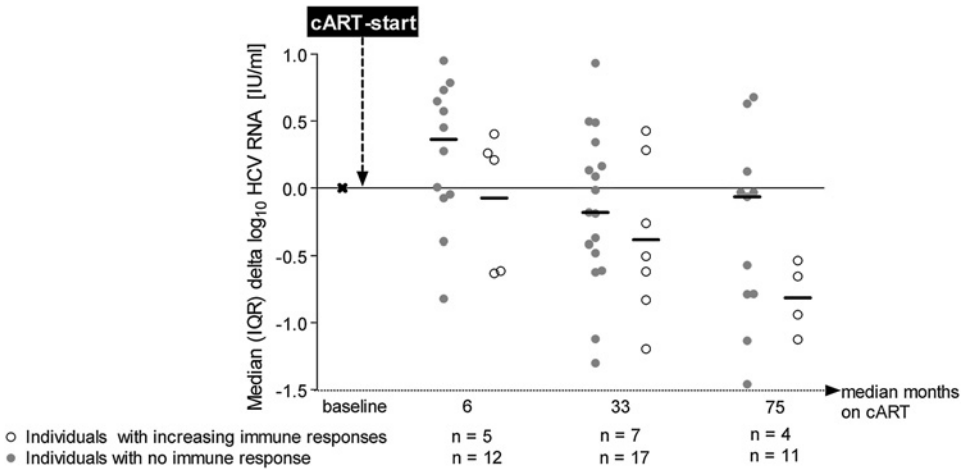


Figure 5 Change in hepatitis C virus (HCV) RNA levels by HCV-specific immune responses. The change in HCV RNA levels on combination antiretroviral therapy (cART) is shown for the subgroup of individuals with increases in HCV-specific cellular immune responses during cART and for those without immune responses pre- and on-cART.

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 Cameron[17]). 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.[36–41] 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 individuals [13 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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Acknowledgements We would like to thank all the participating patients, the physicians and the study nurses of all clinical centres for excellent patient care, and the SHCS core laboratories for their invaluable processing, storage and retrieval of the samples. We also thank the virology lab technicians from the Institute of Infectious Diseases, University of Berne.

Funding This study has been financed in the framework of the Swiss HIV Cohort Study, supported by the Swiss National Science Foundation (SNF grants #3345-062041 and #324730-116862), the Wellcome Trust and the James Martin School for 21st Century Oxford, the NIHR Biomedical Research Centre Program Oxford and the National Health and Medical Research Council Program, Western Australia.

The study sponsors had no role in the study design, data collection, analysis, interpretation of data and writing of this manuscript.

Competing interests None.

Ethics approval This study was performed in the framework of the Swiss HIV Cohort Study (www.shcs.ch). Written informed consent, including for genetic testing, was mandatory for inclusion, and the study was approved by all local ethical committees.

Provenance and peer review Not commissioned; externally peer reviewed.

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