Hepatitis C virus: epidemiology and immunology
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Chapter 5

Immunology in chronic HCV
Chapter 5.1

Skewing of hepatitis C virus (HCV)-specific CD4+ T cells to Core protein is associated with the presence of HCV RNA

Charlotte van den Berg, Nening Nanlohy, Thijs van de Laar, Maria Prins, Debbie van Baarle

Submitted.
Abstract

Individuals with a HCV-specific T-cell response mainly directed against Core protein seem more likely to develop chronic HCV infection. We hypothesized that persistent exposure to HCV by continuing risk behaviour leads to higher exposure to Core protein and may skew the HCV-specific T-cell response towards Core. To test this, we studied HCV-specific T-cell responses in HCV-seropositive drug users (DU) including HCV-RNA+ (n=10) and HCV-RNA- (n=8) injecting DU with a high frequency of injection drug use and sharing of needles and HCV-RNA+ DU that denied ever-injecting (n=9). HCV-specific CD4⁺ T-cell responses were measured using a sensitive assay based on expansion of specific memory T cells by HCV recombinant proteins and interleukin-2 (IL-2) and measuring interferon (IFN)-γ production after restimulation. The total HCV-specific CD4⁺ T-cell response was lower in never-injecting DU compared to both RNA+ and RNA- injecting DU (p=0.034). In addition, the response against nonstructural (NS) proteins was higher in injecting DU compared to never-injecting DU (p=0.045). Interestingly, the percentage of individuals with a CD4⁺ response directed against Core was lower in individuals without detectable HCV RNA (0%) than in those with HCV RNA (40-44%) (p=0.049). In conclusion, ongoing injecting risk behaviour is associated with higher overall CD4⁺ HCV-specific T-cell responses, but detectable HCV RNA is associated with a higher T-cell response directed against Core protein.
Introduction

Hepatitis C virus (HCV) is mainly transmitted via infected blood. After acute infection approximately 60-80% of individuals develop persistent viremia, which can lead to liver-related morbidity and mortality after years of chronic infection. Recently, we and others have described that HCV-specific T-cells directed against nonstructural proteins of HCV are associated with a favourable outcome of acute HCV in both injecting drug users (IDU) and men having sex with men (MSM). Individuals with a response mainly directed against Core protein are more likely to develop chronic HCV infection, and also in the chronic phase of infection a higher fraction of HCV-specific T cells are directed towards Core. Next to its role in formation of the HCV virion, Core has been shown to have immunomodulatory functions, thereby possibly supporting viral persistence. Thus Core is important in immunomodulation and it seems to induce T-cell responses which are not effective in clearance of the virus. Although we observe a higher proportion of the HCV-specific T-cell response to be focussed on Core protein in the majority of chronically HCV-infected patients, we do not observe this in all patients. We hypothesized that persistent exposure to HCV –by continuous risk behaviour, like injecting drug use or repeated sexual exposure– leads to enhanced exposure to mainly structural proteins and therefore leads to skewing of the HCV-specific T-cell response towards Core protein.

Therefore, we here studied the association between the frequency of recurrent exposure to HCV (measured as frequency of injecting drug use and number of shared needles in the previous 6 months) and the focus of the HCV-specific T-cell response as measured after 12 day expansion.

Materials and Methods

Study population

To evaluate the association between the HCV Core-specific CD4+ T-cell response and continuous exposure to HCV, we included HCV-infected injecting drug users (DU) from the Amsterdam Cohort Studies (ACS) among DU with the highest frequency of recent injecting drug use (i.e., in the past 6 months) and the highest number of shared needles in the past 6 months (n=18). Next to these, we included recently identified HCV-infected self-reported never-injecting DU (n=9).

Virological assays

All ACS participants with at least two visits between 1985 and November 2005 were tested for presence of HCV antibodies using a third generation commercial microparticle EIA system test (AxSym HCV version 3.0; Abbott, Wiesbaden, Germany). After HCV antibody screening, HCV-seropositive samples were additionally tested for the presence of HCV RNA. RNA isolation was performed on 100 μl of serum using the TriPure method (Roche Diagnostics, Almere, the Netherlands). Each RNA isolate was used as input for two nested multiplex RT-PCRs. The first PCR, which targets the conserved HCV core region, was devised as a genotyping system to differentiate subtypes 1a, 1b, 2a, 2b, 3a, 4, 5a and 6a. The second RT-PCR, which targets the NS5B region, was
used for genotype confirmation. Conditions and primers for both PCR have been described elsewhere. All ACS participants since 1985 (n=1640) were tested for HIV antibodies by enzyme linked immunosorbent assays (ELISA) at each study visit. Results were confirmed by Western blot (since 1986, by HIV Blot version 2.2, Genelab diagnostics, Singapore).

Immunological assays

Phenotyping of T cell subsets

Surface staining was performed to evaluate various T cell subsets present. To examine the presence of cytotoxic granules in CD8\(^+\) T cells, cryopreserved peripheral blood mononuclear cells (PBMC) were stained with a combination of monoclonal antibodies against CD3, CD4, CD8, perforin and granzyme B. The amount of immune activation was determined by staining PBMC with a combination of monoclonal antibodies against CD3, CD4, CD8, HLA-DR, CD38, PD-1 and CD57. Furthermore, we examined the proportion of naïve, memory and effector T cells in the PBMC pool, by staining with monoclonal antibodies against CD27, CD127, CCR7 and CD45RO. In short, PBMC were thawed, washed and stained with the different sets of fluorescently labelled monoclonal antibodies. In these analyses, PBMC from 10 healthy controls (unpaid blood bank donors) were included for comparison.

HCV-specific CD4\(^+\) T cell expansion

HCV-specific CD4\(^+\) T-cell responses were measured using a sensitive assay based on short-term culture of peripheral blood mononuclear cells (PBMC) previously described for detection of HCV, but also other antigen-specific T-cell responses and has been shown to be more sensitive than assays with a shorter stimulation period, like Elispot or CFSE. In short, PBMC were thawed and cultured for 12 days in the presence of recombinant HCV proteins (Core (C22-3), NS3 (C33c), NS4 (C100-3) and NS5A/B (NS5)) (kindly provided by Chiron/Novartis) and recombinant interleukin-2 (IL-2) (Chiron, Uxbridge, UK). After 12 days, cells were washed, pooled and rested overnight. On day 13, cells were restimulated using overlapping peptide pools (18-mers, 11 overlapping amino acids, Mimotopes, Australia) and the HCV-specific response was quantified by analysis of IFN-\(\gamma\) production using intracellular cytokine staining.

Quantification of IFN-\(\gamma\) production by HCV-specific T cells

As a read-out of effector function, IFN-\(\gamma\)-producing CD4\(^+\) T cells were enumerated using intracellular cytokine staining (ICCS) after HCV-specific T cell expansion. Briefly, PBMC were stimulated for six hours with HCV Core/E1/E2/p7, NS2, NS3, NS4 and NS5 peptide pools (2 \(\mu\)g/ml) and both \(\alpha\)CD28 (2 \(\mu\)g/ml) and \(\alpha\)CD49d (2 \(\mu\)g/ml) as co-stimuli, after 1h 1:1,000 Brefeldin A (Golgiplug, BD Biosciences (BD), San José, California, USA) was added. As a negative control, PBMC were stimulated with medium and co-stimulation alone. As a positive control PBMC were stimulated with 10 ng/ml PMA, 2 \(\mu\)g/ml ionomycin and co-stimulation. After stimulation, cells were washed, permeabilized (FACS Permeabilizing Solution, BD), washed again and stained with antibodies specific for CD3, CD4, CD8 and IFN-\(\gamma\) (BD). After fixation (Cellfix, BD) min. 50,000 events were acquired on LSRII flow cytometer (BD). Lymphocytes were gated by forward and sideward scatter and data analyzed using FACSDiva software (BD).
Results were expressed as the percentage of IFN-γ producing CD4+ T cells, which was calculated by subtracting the percentage of IFN-γ producing T cells in unstimulated conditions from the percentage of IFN-γ producing T cells in peptide pool restimulated conditions.

To enable comparison of DU with different CD4+ T cell counts, we did not only compare the percentages of IFN-γ-producing CD4+ T cells, but we also determined the proliferation rate, which we defined as the total number of cells recovered after six or twelve days of expansion divided by the total number of cells put in to culture at day 0.17

The number of HCV-specific effector T cells was calculated as the product of the percentage of IFN-γ producing T cells and the proliferation rate.

Statistical analyses

Non-parametric tests (Kruskal-Wallis) were used to compare medians between groups, if there was a significant difference Dunns’ post-test was used to compare medians between groups. Pearson χ² was used to compare proportions between groups. All performed statistical tests were two-sided. A p-value ≤0.05 was considered to be statistically significant. All statistical analyses were performed using Graphpad and SPSS (Graphpad version 5.0, SPSS version 15.0.1, SPSS Inc.).

Table 5.1.1 Demographic characteristics of drug users (never-injecting and injecting).

<table>
<thead>
<tr>
<th></th>
<th>Injecting DU</th>
<th>Injecting DU</th>
<th>Never-injecting DU</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HCV RNA-</td>
<td>HCV RNA⁺</td>
<td>HCV RNA⁺</td>
</tr>
<tr>
<td>n=8</td>
<td>n=10</td>
<td>n=9</td>
<td></td>
</tr>
<tr>
<td>Median age (range)</td>
<td>29 (21-39)</td>
<td>28 (24-38)</td>
<td>30 (24-38)</td>
</tr>
<tr>
<td>Male sex (%)</td>
<td>6 (75)</td>
<td>5 (50)</td>
<td>5 (55.6)</td>
</tr>
<tr>
<td>HIV prevalence (%)</td>
<td>0 (0)</td>
<td>2 (20)</td>
<td>2 (22.2)</td>
</tr>
<tr>
<td>HCV genotype distribution</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1a</td>
<td></td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>1b</td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>3a</td>
<td></td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>4d</td>
<td></td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>At least daily injection drug use in the past six months (%)</td>
<td>7 (88)</td>
<td>6 (60)</td>
<td>-</td>
</tr>
<tr>
<td>Frequency sharing needles in the six months preceding entry ACS (range)</td>
<td>9 (6-700)</td>
<td>25 (5-180)</td>
<td>-</td>
</tr>
</tbody>
</table>

Results

Study population and HCV RNA presence and genotype

In total, 22 HCV-antibody positive never-injecting DU were identified.13 Of these 22, 15 (68.2%) were HCV-RNA⁺ and 9/15 had PBMC available for T cell analyses. Furthermore, we included 18 injecting DU that reported recent injecting drug use (i.e., in the past 6 months). Most injecting DU reported at least daily injecting drug use (72.2%) and the median number of shared needles was 10 (5-700) in the six months preceding ACS entry. For those reporting injecting drug use at entry, the median time since first
injection was 7.3 years (IQR 3.5-9.1 years). Surprisingly, from these injecting DU only 55.6% (10/18) was HCV-RNA positive. In DU with detectable HCV RNA, genotype 3a was most common (7/9 never-injecting DU and 4/10 injecting DU). In the HCV-RNA negative injecting DU, no individuals were HIV positive, while in the two groups where HCV RNA could be detected, the HIV prevalence was 20-22% (ns). Other general characteristics were similar in the three groups (Table 5.1.1). Immune activation, as measured by HLA-DR and CD38 expression on CD4+ and CD8+ T cells, did not differ significantly between the three groups of DU. Furthermore, the distribution of CD4+ and CD8+ T cell subsets (defined by CD45RO and CD27 expression) was not different between individuals with HCV RNA and those without detectable HCV RNA or between the three groups (Table 5.1.2).

Table 5.1.2 Immune activation and subset differentiation of CD4+ and CD8+ T cells in HCV-RNA positive never-injecting DU, HCV-RNA positive IDU, and HCV-RNA negative IDU from the Amsterdam Cohort Studies.

<table>
<thead>
<tr>
<th>CD4+ T cells</th>
<th>Never IDU, RNA-</th>
<th>IDU, RNA+</th>
<th>IDU, RNA-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune activation</td>
<td>HLA-DR+/CD38+</td>
<td>0.7 (0.49-2.19)</td>
<td>2.3 (0.98-10.1)</td>
</tr>
<tr>
<td>Phenotyping</td>
<td>Memory (CD27+CD45RO+)</td>
<td>10.9 (5.66-17.5)</td>
<td>15.7 (9.56-24.4)</td>
</tr>
<tr>
<td></td>
<td>Naive (CD27+CD45RO+)</td>
<td>7.81 (6.84-35.8)</td>
<td>25.6 (17.7-34.7)</td>
</tr>
<tr>
<td></td>
<td>Effector (CD27+CD45RO+)</td>
<td>26.2 (7.11-54.3)</td>
<td>16.6 (8.16-27.9)</td>
</tr>
<tr>
<td></td>
<td>Memory effector (CD27+CD45RO+)</td>
<td>31.8 (26.9-69.3)</td>
<td>38.2 (16.4-48.9)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CD8+ T cells</th>
<th>Never IDU, RNA-</th>
<th>IDU, RNA+</th>
<th>IDU, RNA-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune activation</td>
<td>HLA-DR+/CD38+</td>
<td>1.17 (0.91-2.31)</td>
<td>1.39 (0.76-2.32)</td>
</tr>
<tr>
<td>Phenotyping</td>
<td>Memory (CD27+CD45RO+)</td>
<td>23.3 (11.1-26.0)</td>
<td>13.3 (11.8-17.2)</td>
</tr>
<tr>
<td></td>
<td>Naive (CD27+CD45RO+)</td>
<td>31.4 (19.9-41.6)</td>
<td>36.0 (24.3-48.0)</td>
</tr>
<tr>
<td></td>
<td>Effector (CD27+CD45RO+)</td>
<td>2.94 (1.83-6.51)</td>
<td>1.18 (0.61-3.87)</td>
</tr>
<tr>
<td></td>
<td>Memory effector (CD27+CD45RO+)</td>
<td>39.8 (31.0-64.7)</td>
<td>42.3 (32.1-53.5)</td>
</tr>
</tbody>
</table>

HCV Core-specific CD4+ T-cell response is associated with presence of HCV RNA

To study the association between ongoing risk behaviour and skewing of the T-cell response to Core, we measured HCV-specific CD4+ and CD8+ T-cell responses in DU with high-risk behaviour and never-injecting DU. An HCV-specific CD4+ T-cell response could be detected in 20/27 DU (using a cut-off value of 150 IFN-γ producing CD4+ T cells/million PBMC). Irrespective of HCV-RNA presence, injecting DU had higher total HCV-specific CD4+ T-cell responses (median 1372 and 1010 IFN-γ producing CD4+ T cells/million PBMC among HCV RNA+ and HCV RNA- injecting DU, respectively) compared to never-injecting DU (median 294 IFN-γ producing CD4+ T cells/million PBMC, p=0.012) (Figure 5.1.1a). Especially, the NS5-specific CD4+ T-cell response was higher in injecting DU compared to never-injecting DU (p=0.0006). An HCV-specific CD4+ T-cell response was present in 6/9 HCV RNA-positive never-injecting DU (66.7%),
in 9/10 (90%) HCV RNA-positive injecting DU, and in 5/8 HCV RNA-negative injecting DU (62.5%). The breadth of the HCV-specific CD4\(^+\) T-cell response was not different between groups.

Interestingly, none of the HCV RNA-negative injecting DU had detectable response against Core protein, compared to 4/10 HCV RNA-positive injecting DU and 4/9 HCV RNA-positive never-injecting DU. The median Core-specific T-cell response in those with a detectable response was 28.7% (range 11.9-71.2%) of the total HCV-specific response, and was not different in RNA-positive injecting DU compared to RNA-positive never-injecting DU (Figure 5.1.1B).

All 4 DU that were HIV co-infected had detectable HCV RNA. Only one of them had a low, but detectable, HCV-specific CD4\(^+\) T-cell response (targeting NS4, 237 HCV NS4-specific CD4\(^+\) T cells/million PBMC) (Figure 5.1.1A, HIV-infected DU indicated with asterisk). CD4 counts ranged from 210 to 900 CD4\(^+\) T cells/mm\(^3\).

Figure 5.1.1A  HCV-specific CD4\(^+\) T-cell response per drug user (* indicates HIV-infected DU).
Discussion

Here we studied the association between the frequency of recurrent exposure to HCV (measured as frequency of injecting drug use and number of shared needles in the previous six months) and the focus of the HCV-specific T-cell response as measured after 12 day expansion. We show that HCV-seropositive injecting DU with a high frequency of injection and sharing of needles have higher total HCV-specific CD4+ T-cell responses compared to HCV-infected never-injecting DU irrespective of the presence of HCV RNA. Furthermore, we show that the presence of HCV RNA is associated with a higher HCV Core-specific CD4+ T-cell response in both injecting and never-injecting DU compared to HCV-RNA negative injecting DU. Thus, continuous exposure does not influence the focus of the T-cell response, but rather the presence of HCV RNA affects the focus of the T-cell response to HCV.

Frequent injecting and sharing of needles in IDU can be seen as a proxy for frequent exposure to HCV. Although HCV re-infection may not necessarily have occurred after each shared needle, it is plausible to assume that the chance of exposure was very high, since HCV prevalence ranged between 63 and 93% in Amsterdam in the 20 years since the start of the ACS.20 This continuous exposure is likely to have boosted the immune system, which has led to higher HCV-specific T-cell responses in injecting DU. Although the frequency of injecting drug use and the number of shared needles was very high in the selected DU, 44.4% of them were HCV-RNA negative at the first ACS visit, suggesting they were able to control HCV. However, longitudinal HCV-RNA testing is necessary to conclude they had spontaneously cleared HCV. Furthermore, it is possible that this high HCV-RNA negative proportion is due to survival bias, since these DU had a long history of injection drug use. And although, 44.4% is a high proportion of spontaneous clearance, it is still within the range of spontaneous clearance we found in HCV seroconverters in the ACS (clearance rate 33.0%, Pearson χ², p=0.35).21 Larger
numbers are needed to evaluate whether continued exposure is associated with viral clearance.

The HCV Core protein is synthesized as a polypeptide of ~22 kDa and is one of the structural proteins of HCV. It functions as the nucleocapsid of the HCV virion. The amino acid sequence of Core is highly conserved among different HCV genotypes. It has been shown that nonenveloped HCV nucleocapsids occur naturally in the serum of HCV-infected individuals and that Core protein is abundantly present in serum of HCV-infected patients (and chimpanzees). The amount of Core protein present in serum is correlated with the height of HCV viral load. Therefore, targeting of Core protein by the adaptive immune system might be associated with unfavourable outcome of infection, since HCV-RNA and HCV Core protein are higher in individuals with persistent infection compared to those with a self-limiting course of infection. Indeed, we show here that HCV-specific T cells targeting Core protein were only present in individuals with detectable HCV RNA.

Presence of HCV RNA in serum implies active viral replication in the liver, which means that there is a continuous antigen production. When more Core antigen is produced, this will probably results in skewing of the T-cell response. Since HCV is translated as a polypeptide, it is unlikely that more Core protein than NS proteins is produced. However, since it has been shown that nonenveloped HCV nucleocapsids can be present in serum, T cells might be more exposed to Core than to NS proteins. Since all individuals in this study with supposed continuous exposure to HCV did all show a high CD4+ T-cell-response targeting HCV nonstructural proteins and did not show a significant response targeting Core protein, future vaccine development should focus preferably on nonstructural proteins.
References

Exposure to HCV and Core-specific T-cell responses


Chapter 5.2

Stronger decline in HCV-specific T-cell response in genotype 3 compared to genotype 1 HCV/HIV co-infected patients during treatment with pegylated interferon and ribavirin suggests no role for HCV-specific T cells in forced viral clearance

Charlotte van den Berg, Joop Arends, Nening Nanlohy, Thomas Ruys, Joep Lange, Andy Hoepelman, Debbie van Baarle

Submitted.
Abstract

It has been suggested that HCV clearance during treatment with pegylated interferon (PEG-IFN) and ribavirin (RBV) is dependend on killing of HCV-infected hepatocytes by HCV-specific T cells. In this study we analyzed the role of HCV-specific CD4+ and CD8+ T cells in viral clearance during therapy in HIV co-infected patients, in whom lower rates of sustained viral responses have been reported, with different HCV genotypes. HCV-specific T-cell responses were measured in the first 12 weeks of treatment using a sensitive assay for detection of antigen-specific memory T cells using overlapping peptide pools corresponding to the infecting genotype of the patient. In total, 28 HCV/HIV co-infected patients were included, of whom 18 were infected with HCV genotype 1 and 10 were infected with HCV genotype 3. During the first 12 weeks of HCV treatment, we observed a decline in both magnitude and breadth of HCV-specific CD4+ and CD8+ T-cell responses. Even more, genotype 3 infected patients tended to have a stronger decline in HCV-specific T-cell responses than genotype 1 infected patients, which paralleled the decline in viral load. This indicates that even in individuals infected with a genotype associated with a good response to therapy the immune response is not enhanced. In addition, a higher percentage of perforin-expressing CD8+ T cells at baseline in genotype 3 patients was observed, suggesting there is a difference in general host immune effector function between genotype 1 and 3 patients. Although the number of patients in our study is small and does not allow for definite conclusions, augmentation of HCV-specific T cells does not appear to play a major role in forced viral clearance.
Introduction

Spontaneous viral clearance of hepatitis C virus (HCV) occurs in 15-40% of individuals. Treatment of chronic HCV infection with pegylated interferon (PEG-IFN) and ribavirin (RBV) leads to sustained viral response (SVR, defined as undetectable HCV-RNA load 6 months after cessation of therapy) in 50-90% of patients. The most important baseline predictor for SVR known so far is HCV genotype; both HCV mono-infected and HCV/HIV co-infected individuals with genotype 2 and 3 respond better to therapy than those with genotype 1 and 4. The underlying mechanism is not yet understood. Decline of HCV-RNA levels during combination therapy is biphasic and mathematical modeling of HCV viral kinetics during therapy has suggested that the first slope of HCV-RNA decline is most likely determined by the half life of free virus (i.e., blocking of new virus production), while the second slope is determined by the half life of infected cells (i.e., killing of infected hepatocytes by cytotoxic T lymphocytes (CTL)). Individuals with an impaired immune system, like those co-infected with HIV, have higher rates of treatment failure suggesting that the host immune system plays an important role in treatment-induced viral clearance. HCV-specific CTL can recognize infected hepatocytes and have different mechanisms to kill them. Next to Fas-mediated apoptosis, CTL can induce apoptosis using cytotoxic granules containing perforin and granzymes. In HCV mono-infected patients it has been shown that higher proliferative capacity of HCV-specific CD8+ T cells before therapy and higher pretreatment IFN-γ production by CD4+ T cells were associated with SVR, which may reflect better capacity to kill infected target cells during forced clearance of HCV. Approximately 30% of hepatitis C virus-infected patients are HIV co-infected due to shared routes of transmission. HCV/HIV co-infection is associated with lower rates of SVR after treatment of HCV with PEG-IFN and RBV. Furthermore, HCV/HIV co-infection is associated with faster progression to liver-related morbidity and mortality. Interferons play an important role in the human antiviral response. In acute HCV it has been shown that a strong HCV-specific T-cell response (predominantly IFN-γ production) targeting multiple HCV proteins is associated with spontaneous viral clearance. We aimed to investigate whether HCV-specific T-cell responses play a role in 'forced' viral clearance during treatment, and whether these responses are inferior in HCV/HIV co-infected individuals which may explain the lower response rates. Since there is a large disparity in efficacy of PEG-IFN and RBV treatment between HCV genotypes, we studied individuals infected with HCV genotypes that respond good (i.e., HCV genotype 3) and genotypes that are less responsive to HCV treatment (i.e., HCV genotype 1) to examine whether these individuals display different T-cell responses.

Materials and Methods

Patients and HCV treatment

To analyze the dynamics of HCV-specific T-cell responses in HCV/HIV co-infected individuals, 28 HIV-infected patients with chronic HCV genotype 1 or 3 co-infection were studied prospectively. Of those, 18 patients were included in a randomized, open label, pilot study and were treated for chronic HCV infection during HIV co-infection with combination therapy consisting of PEG-IFN and RBV. 10 patients were treated with...
PEG-IFN-α2b (Pegintron, Schering-Plough) 1.5μg/kg/wk (standard-arm) and 10 patients were treated with induction PEG-IFN-α2b (regimen: 3.0 μg/kg/wk during the first four weeks, 2.0 μg/kg/wk during the next four weeks and 1.5μg/kg/wk during the remaining 40 weeks) plus oral RBV at a daily dose of 1000 mg when bodyweight was less than 75 kg or 1200 mg when it was equal or more than 75 kg. The remaining 8 patients were included from another study (Privicop) that was designed for immunological and viral studies during HCV treatment in which patients were treated with standard doses PEG-IFN-α2a (180 μgr/wk, Pegasys, Roche) and weight-based RBV. Institutional review boards at participating centers approved the protocol. All patients provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines.

Viral assays

Serum HCV RNA was quantified by bDNA (Versant™ HCV 3.0, Bayer Diagnostics) during screening, at baseline and thereafter at week 2, 4, 8, 12, 24, 48 and 72 as long as HCV RNA remained detectable. Below the detection limit of the bDNA assay (615 IU/ml), HCV RNA was measured by means of a qualitative transcription mediated amplification (TMA) assay (lower limit of detection 10 IU/ml, Versant™ HCV RNA Qualitative assay, Bayer Diagnostics). In Privicop patients, samples below the detection limit were measured using a qualitative Roche Amplicor polymerase chain reaction (PCR) assay (lower limit of detection 50 IU/ml, Roche Molecular Systems, Pleasanton, California).

Response definitions

Rapid viral response (RVR) was defined as undetectable viral load at week four of treatment using a qualitative assay. Early viral response (EVR) was defined as undetectable viral load or at least 2 log HCV-RNA load decline at week 12 of treatment compared to baseline. Sustained viral response (SVR) was defined as undetectable HCV RNA at week 24 after stopping of treatment.

Flow cytometry and phenotyping of PBMC

Six color fluorescence analysis was performed. Cryopreserved peripheral blood mononuclear cells (PBMC) were thawed and up to 1 x 10⁶ cells were stained using different sets of monoclonal antibodies. At least 100,000 events were acquired using a LSRII flowcytometer (BD Biosciences). Lymphocytes were gated by forward and sideward scatter and data analyzed using the software program FACSDiva (BD Biosciences).

Degranulation activity and cytotoxic granules (containing perforin and granzymes) generally define CD8⁺ T cells with cytotoxic function. Perforin expression was determined by an intracellular staining. In short, cryopreserved PBMC were thawed, washed, permeabilized (FACS Permeabilizing Solution, BD), washed again and stained with antibodies specific for CD3, CD8 and perforin (eBioscience). After fixation (Cellfix, BD) min. 50,000 events were acquired on a LSRII flow cytometer (BD). Lymphocytes were gated by forward and sideward scatter and data analyzed using FACSDiva software (BD).
HCV-specific T cells during HCV treatment

HCV-specific T cells during HCV treatment

HCV peptide pools
For stimulation and expansion of HCV-specific T cells, pools of peptides were used. To study differences in T-cell responses between HCV genotype 1 and 3 infected individuals, pools of overlapping peptides spanning the complete HCV genotype 1a and 3a were used, respectively (kindly provided by www.beiresources.com). The pools consisted of 14- to 18-mers, overlapping by 11 amino acids. Peptides were pooled in six pools (corresponding to Core, NS2, NS3, NS4, NS5A and NS5B region of HCV) in such a way that each peptide was present in the final pool at a concentration of 1 mg/ml.

HCV-specific T-cell expansion
Since the frequency of HCV-specific T cells in HCV/HIV co-infected patients is usually very low, we used an assay that has proven to be very sensitive to show the presence of HCV- and other antigen-specific T cells. Furthermore, to study the difference in T-cell response between individuals infected with genotypes 1 and 3, we stimulated PBMC with overlapping peptide pools from the corresponding HCV genotype.

To expand HCV-specific memory T cells, cryopreserved PBMC were thawed and subsequently expanded during a period of 12 days using overlapping HCV peptide pools corresponding to Core, NS2, NS3, NS4, NS5A and NS5B region of the HCV genome (2 μg/ml) in the presence of recombinant interleukin-2 (rIL-2, 360 IU/ml). After 12 days, expanded cells were pooled, washed, counted and rested overnight. On day 13 cells were restimulated using peptide pools from the corresponding HCV genotype to assess effector function (i.e., IFN-γ production).

Detection of IFN-γ producing HCV-specific T cells after (re-)stimulation
As a read-out of effector function, IFN-γ producing T cells were enumerated using intracellular cytokine staining (ICCS). Briefly, PBMC were stimulated for six hours with HCV Core, NS2, NS3, NS4 and NS5 peptide pools (2 μg/ml) and both αCD28 (2 μg/ml) and αCD49d (2 μg/ml) as co-stimuli, after 1 hour 1:1,000 Brefeldin A (Golgiplug, BD Biosciences (BD), San José, California, USA) was added. As a negative control, PBMC were stimulated with medium and co-stimulation alone. As a positive control PBMC were stimulated with 10 ng/ml PMA, 2 μg/ml ionomycin and co-stimulation. After stimulation, cells were washed, permeabilized (FACS Permeabilizing Solution, BD), washed again and stained with antibodies specific for CD3, CD4, CD8 and IFN-γ (eBioscience). After fixation (Celfix, BD) min. 25,000 events were acquired on a LSRII flow cytometer (BD). Lymphocytes were gated by forward and sideward scatter and data analyzed using FACSDiva software (BD).

Results were expressed as the percentage of IFN-γ producing T cells, which was calculated by subtracting the percentage of IFN-γ producing T cells in medium from the percentage of IFN-γ producing T cells in peptide pool (re-)stimulated conditions. To enable comparison of patients with different baseline CD4+ T cell counts, we did not only compare the percentages of IFN-γ-producing T cells, but we also determined the proliferation rate, which we defined as the total number of cells recovered after 12 days of expansion divided by the total number of cells put in to culture at day 0. Subsequently we calculated the number of HCV-specific T cells as the product of the percentage of IFN-γ-producing T cells and the proliferation rate. For easier understanding of this number, we expressed the number of IFN-γ producing T cells per
million PBMC. This number of HCV-specific T cells is a combination of (memory) cells initially present and the ability of these cells to survive, proliferate and differentiate to (IFN-γ-producing) effector T cells during the 12-day culture period.9

Statistical analysis

All tests performed were 2-sided. A p-value ≤0.05 was considered to be statistically significant. Pearson χ² test was used for comparison of proportions. Non-parametric tests were used for comparison between groups (Kruskal-Wallis). Statistical analyses were performed using SPSS and STATA (v9.2, Statacorp, Collegestation, Texas, USA).

Results

Baseline characteristics of the patient population

In total, 28 HIV-infected patients were included. Of those, 18 were co-infected with HCV genotype 1 and 10 were co-infected with HCV genotype 3. The patients were mainly male, and mainly of Caucasian ethnicity. In each group, five patients were treated with induction treatment and the remaining patients were treated with standard dose regimens of PEG-IFN-α2a or PEG-IFN-α2b.

The median log baseline HCV viral load was 6.23 IU/ml (interquartile range (IQR) 5.58-6.41 IU/ml), and was somewhat lower in HCV genotype 3 infected individuals compared to HCV genotype 1 infected individuals (median (IQR) 5.78 (5.39-6.23) and 6.34 (5.97-6.48), respectively, p=0.069). For 13/28 patients, liver biopsies were performed prior to baseline. Of these patients, 6 (46.2%) showed significant fibrosis. ALT levels were lower in the group of patients that were treated with PEG-IFN-α2a, median 55 IU/ml than in the groups treated with PEG-IFN-α2b median 101 IU/ml and 115 IU/ml for standard and induction arm, respectively (p=0.048).

Of the 28 HIV-infected patients, 19 were treated with highly active antiretroviral therapy (HAART). The median CD4 count for all included patients was 430 cells/ml (IQR 280-740 cells/ml), and was somewhat higher in patients not receiving HAART (median 477 cells/ml, IQR 390-655 cells/ml) compared to those on HAART (372 cells/ml, IQR 270-740 cells/ml) (p=0.44, data not shown).

Outcome of therapy

Of 28 included patients, 22 (78.6%) achieved EVR and of those, 11 (50%) achieved SVR. As expected, patients infected with HCV genotype 3 were more likely to achieve EVR (10/10 genotype 3 infected patients vs. 12/18 genotype 1 infected patients, p=0.039) and SVR (6/10 genotype 3 infected patients vs. 5/18 genotype 1 infected patients, p=0.090) compared with individuals infected with HCV genotype 1 (Table 5.2.1).

The median decline of log10 viral load in the first four weeks of treatment was somewhat lower in genotype 1 infected patients (2.11 (IQR 1.66-3.56)) than in genotype 3 infected patients (3.10 (IQR 2.15-4.39), p=0.17), in the first 12 weeks of treatment this was 3.62 (IQR 2.13-4.46) and 4.50 (3.49-4.89), respectively (p=0.15).
Table 5.2.1 Baseline characteristics of included patients.

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<th>HCV genotype 1 n=18</th>
<th>HCV genotype 3 n=10</th>
<th>P value*</th>
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<td>Median age (IQR)</td>
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<td>EVR (%)</td>
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<td>SVR (%)</td>
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<td>Median baseline HCV viral load (IQR)</td>
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<td>5.78 (5.39-6.23)</td>
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<td>Undetectable HIV viral load (n)</td>
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* Pearson χ² for proportions, Kruskall-Wallis for continuous variables.

Baseline HCV-specific T-cell responses

Since in HCV mono-infected individuals it has previously been shown that both baseline proliferative capacity of HCV-specific CTL and IFN-γ production of CD4⁺ T cells was associated with SVR, we analyzed HCV-specific T-cell responses using a previously described sensitive assay that measures a combination of these properties. The experiments could be carried out in 21 of 28 (75%) patients that had sufficient PBMC available. HCV-specific CD4⁺ T-cell responses could be detected in 13/21 (61.9%) and HCV-specific CD8⁺ T-cell responses in 14/20 (70.0%) individuals. 11/20 (55.0%) individuals had both HCV-specific CD4⁺ and CD8⁺ T-cell responses. 7/13 (53.8%) HCV genotype 1 infected individuals had both HCV-specific CD4⁺ and CD8⁺ T-cell responses, compared to 4/7 (57.1%) HCV genotype 3 infected individuals (p=0.89, χ²). The median height of the total HCV-specific CD4⁺ T-cell response was not different between treatment groups (median (IQR) 685 (0-3544), 1006 (0-4175), and 468 (144-1164) IFN-γ producing CD4⁺ T cells/million PBMC for standard PEG-IFN-α-2a, standard PEG-IFN-α-2b and induction PEG-IFN-α-2b, respectively (Kruskall Wallis, p=0.98)) (Figure 5.2.1A). The median height of the total HCV-specific CD8⁺ T-cell response was also similar for patients in the different treatment groups (median (IQR) 1339 (677-18416), 412 (8-1361), and 1242 (180-2823) IFN-γ producing CD8⁺ T cells/million PBMC for standard PEG-IFN-α-2a, standard PEG-IFN-α-2b and induction PEG-IFN-α-2b, respectively (Kruskall Wallis, p=0.32)) (Figure 5.2.1A). Although ALT levels were lower in the group treated with standard PEG-IFN-α-2a than the other groups, baseline HCV-specific T-cell responses were comparable, therefore we subsequently analyzed the groups together. Baseline HCV viral load did not differ...
significantly between groups, however HCV viral load tended to be lower in genotype 3 infected patients (p=0.069).

In the analyzed patients, only three individuals did not achieve an EVR. In patients that did achieve an EVR the observed median CD4⁺ T-cell responses (686 IFN-γ producing CD4⁺ T cells/million PBMC (IQR 45-2482)) were similar to those who did not (144 IFN-γ producing CD4⁺ T cells/million PBMC (0-10371), p=0.87) (Figure 5.2.1B). The HCV-specific CD8⁺ T-cell response was not significantly different between those who achieved EVR (median 884 IFN-γ producing CD8⁺ T cells/million PBMC (IQR 116-3613) and those who did not achieve EVR (1912 IFN-γ producing CD8⁺ T cells/million PBMC (IQR 0-1958), p=0.91) (Figure 5.2.1B).

The median height of the total HCV-specific CD4⁺ T-cell response was 685 IFN-γ producing CD4⁺ T cells/million PBMC (IQR 45-3528). This was not significantly different between HCV genotype 1 and HCV genotype 3 infected patients (345 IFN-γ producing CD4⁺ T cells/million PBMC (IQR 0-3114) and 1006 IFN-γ producing CD4⁺ T cells/million PBMC (IQR 57-4175), respectively, p=0.39) (Figure 5.2.1C). The median HCV-specific CD8⁺ T-cell response for all included patients was 1100 IFN-γ producing CD8⁺ T cells/million PBMC (IQR 116-3613). This did not significantly differ between genotype 1 infected patients and genotype 3 infected patients, but tended to be lower in genotype 3 infected individuals: median 1912 (IQR 429-12477) and 658 (IQR 54-1242) IFN-γ producing CD8⁺ T cells/million PBMC, respectively, p=0.10) (Figure 5.2.1C).

Since in spontaneous viral clearance there is an association with breadth of the HCV-specific T-cell response, we evaluated how many HCV proteins were targeted per patient. At baseline of treatment a median of two proteins was targeted by HCV-specific CD4⁺ T-cell responses, while HCV-specific CD8⁺ T cells targeted a median of 1.5 proteins (data not shown). The median number of targeted proteins was not significantly different between individuals infected with genotype 1 compared with those infected with genotype 3. Only a minority of individuals had a detectable CD4⁺ or CD8⁺ HCV-specific T-cell response targeting Core protein (5/21 and 6/20, respectively) (Figure 5.2.2B and 5.2.2C).
Figure 5.2.1  
A) Baseline HCV-specific CD4+ and CD8+ T-cell response as measured after 12 day expansion in the three different study groups: standard arm: treated with PEG-IFN-α2b and RBV, induction arm: treated with PEG-IFN-α2b induction scheme and RBV, and Privicop: treated with PEG-IFN-α2b and RBV. 
B) Baseline HCV-specific CD4+ and CD8+ T-cell response as measured after 12 day expansion in patients achieving an early virological response (EVR) and those who do not. 
C) Baseline HCV-specific CD4+ and CD8+ T-cell response as measured after 12 day expansion in patients infected with HCV genotype 1 and 3.
Chapter 5.2
Immunology in chronic HCV

Fate of HCV-specific T-cell responses during treatment

It is likely that differences in augmentation of HCV-specific T-cell responses will be most obvious shortly after initiation of treatment when these responses are boosted by PEG-IFN and RBV treatment. Therefore, longitudinal HCV-specific T-cell responses were examined at baseline, week 4 and week 12 of treatment, corresponding to time points of RVR and EVR, respectively.

During HCV-treatment both HCV-specific CD4+ T cell and HCV-specific CD8+ T-cell responses declined. This decline was clear in both HCV genotype 1 infected and genotype 3 infected individuals (Figure 5.2.2A and 5.2.2B). In genotype 1 infected patients the HCV-specific CD4+ T-cell responses declined from 468 to 68 CD4+ T cells/million PBMC (IQR 0-912 CD4+ T cells/million PBMC) at week 4 and 900 CD4+ T cells/million PBMC (IQR 22-2794) at week 12. The HCV-specific CD8+ T cells declined from 1912 to 103 CD8+ T cells/million PBMC (IQR 0-1144 CD8+ T cells/million PBMC) at week 4 and 173 CD8+ T cells/million PBMC (IQR 0-1995 CD8+ T cells/million PBMC) at week 12 in genotype 1 infected patients (Figure 5.2.2A).

In genotype 3 infected patients, the decline in the first four weeks was more pronounced, the HCV-specific CD4+ T-cell responses declined from 922 to 214 CD4+ T cells/million PBMC (IQR 58-350 CD4+ T cells/million PBMC) at week 4 and to 50 CD4+ T cells/million PBMC (IQR 33-70) at week 12. HCV-specific CD8+ T cells in genotype 3 infected patients declined from 658 to 517 CD8+ T cells/million PBMC (IQR 307-747 CD8+ T cells/million PBMC) at week 4 and 243 CD8+ T cells/million PBMC (IQR 24-372 CD8+ T cells/million PBMC) at week 12 (Figure 5.2.2B).

Interestingly, in HCV genotype 1 infected individuals HCV-specific CD4+ and CD8+ T-cell responses (≥200 IFN-γ producing T cells/million PBMC) could still be detected after 12 weeks of treatment in five and three patients, respectively (Figure 5.2.2A). This in contrast to HCV genotype 3 infected patients, where in all individuals HCV-specific T cells had declined to undetectable levels (Pearson χ² for CD4+ p=0.037 and for CD8+ p=0.59, Figure 5.2.2B). The total HCV-specific CD4+ and CD8+ T-cell response at week 12 was lower for genotype 3 infected patients (50 (IQR 33-70) IFN-γ producing CD4+ T cells) compared to genotype 1 infected patients (900, IQR 22-2794 IFN-γ producing CD4+ T cells), although this not reach statistical significance (Figure 5.2.3). Although a CD8+ T-cell response above the detection limit was observed more often in individuals with genotype 3, no difference in median CD8+ T-cell responses was found between genotype 1 (173, IQR 0-1995 IFN-γ producing CD8+ cells/million PBMC) and genotype 3 infected patients (243, IQR 24-372 IFN-γ producing CD8+ T cells/million PBMC) (Figure 5.2.1C).

Not only the magnitude of the total HCV-specific T-cell response declined over time, but also the breadth of the response decreased in most individuals with a detectable HCV-specific CD4+ (n=13) or CD8+ (n=14) T-cell response at baseline. The breadth of the CD4+ T cell decreased in nine individuals and remained the same in four individuals, while the breadth of the CD8+ T-cell responses decreased in six, remained equal in four and increased in one patient. In patients who achieved an EVR and had a detectable baseline response, the number of proteins targeted by the CD4+ and CD8+ T-cell response decreased, in 8/9 and 6/10 patients, respectively. (Figure 5.2.2B and 5.2.2C)

Interestingly, patient C19 showed a decline in HCV-specific CD4+ T-cell responses at week 4, however the HCV-specific CD4+ T cell response rebounded to baseline level at week 12. This patient showed an initial viral response to treatment (with detectable HCV-RNA, but under the detection limit of the bDNA assay (615 IU/ml) at week 4) however HCV-RNA relapsed and was above the bDNA limit from week 8 onwards. In
the genotype 3 group, while all patients decreased considerably, patient C17 showed still detectable T-cell responses at week 4. Although the T-cell response declined afterwards to undetectable levels, this patient had a relapse after stop of therapy.

Figure 5.2.2A Fate of HCV-specific CD4⁺ T (upper panel) and CD8⁺ T (middle panel) cells in genotype 1 infected patients as measured after 12 day expansion at baseline, week 4 and week 12. In the lower panel log10 viral load is shown. ND=not done.
Figure 5.2.2B  Fate of HCV-specific CD4⁺ (upper panel) and CD8⁺ T (middle panel) cells in genotype 3 infected patients as measured after 12 day expansion at baseline, week 4 and week 12. In the lower panel log10 viral load is shown. ND=not done.
HCV-specific T cells during HCV treatment

Figure 5.2.3 Median height and interquartile range of HCV-specific CD4⁺ and CD8⁺ T-cell response at week 12 after start of treatment.

Figure 5.2.4 A) Representative dotplot of perforin staining in CD3⁺ T cells as measured by intracellular cytokine staining in four patients. B) Perforin expression at HCV therapy baseline as measured by intracellular cytokine staining. Shown are median and range.
Perforin content in CTL

Perforin is an important molecule for CTL to mediate killing of infected cells and is upregulated after activation in effector T cells. Since PEG-IFN and RBV treatment is believed to enhance immune responses, we also analyzed perforin expression of T cells directly ex vivo. In Figure 5.2.4A, representative dotplots of perforin staining in two patients with HCV genotype 1 infection and two patients with HCV genotype 3 infection are shown. Median perforin expression in 10 HCV-uninfected healthy donors was 0.97% (IQR 0.42-4.0%) (data not shown). Interestingly, the median percentage of perforin+ CD8+ T cells was higher in both genotype 1 (3.10%, IQR 1.50-11.3%) and genotype 3 (22.3%, IQR 8.20-31.6%) infected patients. Surprisingly, the perforin content in CD8+ T cells in genotype 3 infected patients was higher compared to patients infected with genotype 1 (p=0.019, Figure 5.2.4B). No decline in perforin expression was observed during treatment (data not shown).

Discussion

Based on mathematical modeling of HCV viral kinetics during treatment with PEG-IFN and RBV, it was proposed that the second phase of the decline of HCV is caused by (HCV-specific) CTL. However, so far only one study was able to show an augmentation of HCV-specific T-cell responses during treatment with PEG-IFN and ribavirin, the difference between this study by Kamal et al. and other studies is that it was performed on fresh PBMC. Other studies have shown associations between SVR and either proliferative capacity of HCV-specific CD8+ T cells at baseline, or pretreatment IFN- secretion by HCV-specific CD4+ T cells. However, no augmentation of HCV-specific T-cell responses was observed. Since HIV/HCV co-infection is associated with lower treatment success rates, we conducted a thorough analysis of the HCV-specific T-cell responses early during treatment to investigate whether immunological predictors for EVR, which is associated with achieving SVR could be identified. Furthermore, since HCV genotype 3 is more responsive to treatment with PEG-IFN and RBV, we analyzed HCV-specific T-cell responses using overlapping peptide pools corresponding to the infecting genotype of the patient.

Although the used assay has proven to be very sensitive for the detection of low frequencies of antigen-specific T cells, we did not observe an increase in HCV-specific T-cell responses during treatment. On the contrary, we observed a decrease of HCV-specific T cells during treatment in nearly all included patients. This suggests that the immunomodulatory properties of PEG-IFN and RBV on HCV-specific T cells might actually be limited in forced viral clearance, at least in HIV/HCV co-infected patients. Even more, we observed undetectable HCV-specific T-cell responses more often in HIV/HCV genotype 3 compared to HIV/HCV genotype 1 infected patients at week 12 after start of treatment. This implies that the immune response declines in parallel with the viral load, and that there is no boosting of the immune response in these patients. This is not completely unexpected, since HIV-specific T-cell responses have also been shown to decrease after initiation of HAART.
Interestingly, we observed a higher percentage of CD8$^+$ T cells expressing perforin in HCV genotype 3 than in HCV genotype 1 HIV co-infected patients. This cytotoxic quality of CTL was measured in the total CD8$^+$ T cell population, and not in HCV-specific T cells, therefore the observed difference might not be HCV specific. The fact that we do not observe a concurrent decline of perforin with HCV viral load, also suggests that it is a reflection of the general immune activation state of the patients. Interestingly, HCV genotype 3 infection has been associated with higher prevalence of steatosis of the liver, and perhaps the high perforin expression in T cells is associated with this development of steatosis, but larger longitudinal studies are needed to explore this association. Furthermore, in our study we observed higher ALT levels in genotype 3 infected patients, however, this seemed to be caused by lower ALT levels in patients treated with standard PEG-IFN-α2-a, who were all infected with HCV genotype 1. Possibly this disparity is related to a different distribution of fibrosis and/or inflammation in both treatment studies. Since we have only liver biopsies in 14 out of 28 patients, we cannot exclude this. Furthermore, different HAART regimens in both treatment studies could have led to the observed difference in ALT levels.

Although chronic HCV infection has been associated with HCV-specific T-cell responses targeting Core protein in acute and chronic HCV infection, this was not observed in our cohort of hospital-recruited patients. Perhaps skewing of HCV-specific T cells towards Core protein is caused by higher exposure to the virus due to continuing risk behaviour (e.g., injecting drug use or sexual risk behaviour) in combination with high HCV-RNA load (i.e., antigenic pressure). These hospital-recruited patients may only have high antigenic pressure. This should be a focus of further study.

The experiments described in this study were performed on peripheral T cells, it would be interesting to study the dynamics of intrahepatic T cells, especially since Neumann-Haefelin et al. have shown an enrichment of HCV-specific T cells in the liver compared to the peripheral compartment. Although liver biopsies are performed in the Netherlands according to AASLD and national guidelines, a biopsy is not mandatory before start of treatment. Future studies might consider fine needle aspiration biopsies, which are much less invasive for the patient.

In conclusion, we did not observe an enhancement of HCV-specific T-cell responses in this cohort of HIV/HCV genotype 1 and 3 co-infected patients. We did observe different kinetics of the HCV-specific T-cell responses in genotype 1 and 3 infected individuals, and these responses paralleled the decline in HCV viral load. Furthermore, we observed a higher perforin content of CD8$^+$ T cells at baseline in genotype 3 patients, possibly indicating that there is a difference in general host immune activation between HCV genotype 1 and 3 infected patients, for which the cause is unknown, but which may be associated to the degree of liver inflammation. Although the number of patients in our study is small and does not allow us to draw firm conclusions, augmentation HCV-specific T cells does not appear to play a major role in forced viral clearance.
References


13. van den Berg CHSB, Ruys TA, Nanlohy NM, van der Laar TJW, Beld MGHM, Prins M, van Baarle D. HCV-specific CD4+ T-cell responses in HIV-1 and HCV seroconverters are influenced by the sequence of HIV-1 and HCV infection and the outcome of previous HCV infection. 2008 submitted.


