Hepatitis C virus: epidemiology and immunology

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Chapter 4

Immunology in acute HCV
Chapter 4.1

HCV-specific T-cell responses in injecting drug users: evidence for previous exposure to HCV and a role for CD4\(^+\) T cells focussing on nonstructural proteins in viral clearance

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Abstract

To understand parameters associated with resolved Hepatitis C Virus (HCV)-infection, we analyzed HCV-specific T-cell responses longitudinally in 13 injecting drug users (IDU) with a prospectively identified acute HCV infection. Seven IDU cleared HCV and six IDU remained chronically infected. T-cell responses were followed in the period needed to resolve and a comparable time span in chronic carriers. Ex-vivo T-cell responses were measured using IFN-γ Elispot assays after stimulation with overlapping peptide pools spanning the complete HCV genome. CD4+ memory T-cell responses were determined after 12-day stimulation with HCV proteins. The maximum response was compared between individuals. The T-cell responses measured directly ex vivo were weak but significantly higher in resolvers compared to chronic carriers, whereas the CD4+ memory T-cell response was not different between resolvers and chronic carriers. However, HCV Core protein was targeted more often in chronic carriers compared to individuals resolving HCV infection. CD4+ T-cell responses predominantly targeting non-structural proteins were associated with resolved HCV-infection. Interestingly, the observation of memory T-cell responses present before documented HCV seroconversion suggest that reinfection in IDU occurs often, while the presence of these responses were not predictive for the outcome of infection. However, a transition of the HCV-specific CD4+ memory T-cell response from targeting Core to targeting non-structural proteins during onset of infection was associated with a favourable outcome. Therefore, the specificity of the CD4+ memory T-cell responses measured after 12-day expansion seems most predictive of resolved infection.
Introduction

Hepatitis C virus (HCV) is a positive-stranded RNA virus which is mainly transmitted through blood-contact.\(^1\) HCV infection persists in the majority of individuals resulting in liver fibrosis, cirrhosis and/or hepatocellular carcinoma.\(^2\) Acute HCV infection, however, is difficult to identify because it is usually asymptomatic and most infections occur among injection drug users (IDU).

Studies in humans have shown that spontaneous clearance of acute HCV infection is associated with strong and sustained CD4\(^+\) and CD8\(^+\) T-cell responses against several HCV-derived antigens.\(^3-10\) The mechanism by which the immune response fails to control the virus remains, however, unclear. Because identification of acute HCV infection is rare, only in a limited number of persons a longitudinal follow up of HCV-specific T-cell responses from the early phase of HCV infection has been reported.\(^6,9,11-13\) Most of these responses have been studied using a limited number of epitopes, mostly restricted by the HLA A2 haplotype.\(^7,9,14,15\) It is likely that this approach underestimates the complete T-cell immune response.\(^16\)

To study why some HCV-infected IDU fail to clear the virus, we analysed the HCV-specific immune response between IDU with a prospectively identified acute HCV infection who eventually resolved HCV or remained chronic. We followed ex vivo T-cell responses using HCV overlapping peptide pools covering the complete HCV genome. In addition, we measured memory CD4\(^+\) T-cell responses after stimulation with HCV proteins, using a sensitive and reproducible expansion assay\(^17\), in the period needed to resolve and a comparable time span in eventual chronic carriers.

Methods

Study subjects

Study subjects were recruited from the Amsterdam Cohort Studies (ACS) among drug users, an open, ongoing cohort study that started in December 1985 to study the epidemiology of HIV/AIDS and other blood borne or sexually transmitted diseases\(^18\), which was carried out in accordance with the Helsinki declaration and approved by the institutional Review Board. Participants visit the Amsterdam Health Service every 4-6 months to fill in a detailed questionnaire on injecting drug use and other risk behaviour. In addition, blood is drawn for prospective HIV-testing and storage of PBMC. By screening for HCV antibodies in stored serum, Beld et al. retrospectively established the HCV status of 358 drug users included between December 1985 and March 1996 and identified 19 HCV seroconverters among those at risk. Four were HIV positive before HCV seroconversion and two experienced an acute HIV infection at the time of HCV infection. Seven of 19 (39%) HCV seroconverters resolved HCV-infection.\(^19\) All HCV seroconverters were studied longitudinally for the presence of HCV RNA.\(^20\) Conversion from a negative to positive HCV-RNA test could be documented in 18 of them making it possible to estimate the date of infection (EDI). Thirteen HIV-negative acute HCV infected out of 18 HCV-RNA converters were selected for our study (Table 4.1.1).
Table 4.1.1  Clinical and laboratory characteristics.

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<tr>
<th>Subject (pubID)</th>
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<th>Age at EDI</th>
<th>Sex</th>
<th>Total follow up</th>
<th>Follow up since EDI</th>
<th>Interval of HCV-RNA conversion</th>
<th>HCV genotype</th>
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Seven of these 13 subjects resolved acute HCV infection and six remained chronically infected. During follow up, two of seven resolvers became re-infected with HCV and HIV-1, after which they remained chronically HCV-HIV co-infected. As all 13 individuals with an acute HCV infection were identified retrospectively from the ACS, designed for prospective follow up of HIV-1 infection, no active examination of the occurrence of an acute hepatitis was done. Examination of the medical files did not show any indication of symptomatic acute HCV infection.

Definitions

*HCV infection* was defined as the conversion from a negative to a positive HCV-RNA test (bDNA HCV 3.0 Bayer, lower limit of detection 615 IU/ml or HCV-RNA assay by transcription-mediated amplification (TMA), Versant HCV-RNA Qualitative assay, Bayer, lower limit of detection 5 IU/ml), documented over two consecutive visits in combination with HCV seroconversion (presence of antibodies to HCV by 3rd-generation Enzyme Immunoassay, EIA 3.0 Abbot Laboratories).

*Estimated date of infection* (EDI) was defined as the midpoint between the last negative and first positive HCV-RNA test date. EDI was determined with a variation of ±2 months in 11 of 13 subjects. In two subjects, EDI had a variation of ±19 months and ±12 months respectively.

*Date of HIV seroconversion* was defined as the midpoint between the last negative and first positive HIV antibody test (commercial EIA, Abbot) and confirmed by Western blot (Diagnostic Biotechnology, Belgium). HIV-RNA plasma concentration was determined by NASBA technology (lower quantification limit of 10^3 HIV-RNA copies/ml).

*Resolved infection* was defined as two consecutive visits with negative qualitative HCV-RNA assays after onset of HCV-infection.

The *resolving phase* was defined as the phase after EDI in which spontaneous clearance was possible and calculated as the time elapsed between EDI and the midpoint between the last positive and first negative HCV-RNA time point.

**HCV genotyping**

RNA was isolated using the TriPure method (Roche Diagnostics, Almere, the Netherlands) and subsequently amplified and genotyped using a nested RT-PCR based on the conserved Core region of the HCV genome as described by Ohno et al. Genotypes were confirmed by sequencing part of the NS5B region of the HCV genome.

**Peptides, peptide pools and proteins**

As peptide pools for stimulation of both CD4+ and CD8+ T-cell responses, panels of overlapping peptides (provided by NIH Aids Research Reagent Program) spanning the complete HCV genome corresponding to the HCV 1a genotype (H77 sequence, Genbank access AF009606) with a length of 18 amino acids (overlapping adjacent peptides by 11 aa) derived from the following HCV proteins were used: Core/envelope polyprotein (Core, E1, E2, p7 protein, aa 1-805, consisting of 116 peptides), NS2 protein (aa 806-1022, consisting of 31 peptides), NS3 protease/helicase (aa 1023-1645, consisting of 50 peptides), NS4 protein (aa 1646-1967, consisting of 49 peptides), NS5A protein (aa 1968-2415, consisting of 67 peptides) and NS5B protein (aa 2416-3011, consisting of 87 peptides). Peptides were dissolved in DMSO and 1 µg of total peptide pool mix (each peptide present in a representative amount, i.e. in a concentration of
1 µg divided by the number of peptides in the pool) was used in stimulations. The DMSO concentration never exceeded 1% in the final stimulation. Expansion of CD4+ T cells was performed using the HCV proteins Core, NS3, NS4 and NS5 (provided by Chiron).

**PBMC separation and storage**

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by density gradient centrifugation on Ficoll-Hypaque and cryopreserved using a computerized freezing system in liquid nitrogen within 24 hours of collection.

**Elispot assay for single cell IFN-γ-release**

IFN-γ producing antigen-specific T cells were enumerated using overnight IFN-γ specific Elispot assays as previously described using the anti-IFN-γ antibodies from Mabtech (Stockholm) and streptavidin poly-HRP from Sanquin (Amsterdam).24 PBMC were stimulated directly *ex vivo* in triplicate wells at 1 x 10^5 cells/well in the absence or presence of 1 μg/ml peptide pools. To provide us with the highest sensitivity, we have optimized our assay in such a way that the ratio background to specific response level is optimal at 100.000 cells input. Individual cytokine-producing cells were detected as dark purple spots after a reaction with TMB substrate (Sanquin, Amsterdam) and counted using the A.EL.VIS automated spot analyzer. The number of specific T cell responders per 10^6 PBMC was calculated after subtracting two times negative control values, which leads to the highest specificity as validated in our lab. A response of 50 spots/10^6 PBMC was regarded as positive (after subtraction of negative control values), based on values in healthy blood bank donors.

**Expansion of HCV-specific CD4+ T cells**

As direct responses towards HCV proteins did not result in detectable responses, we used an expansion assay prior to measurement of effector function. To expand HCV-specific CD4+ T cells, 3x10^6 PBMC were cultured for 12 days as previously described17,25 in the presence of the HCV proteins Core, NS3, NS4 and NS5. Culture medium consisted of RPMI 1640 (Gibco Life technologies, Breda, The Netherlands) supplemented with penicillin/ streptomycin and 10% human pool serum. Cells were cultured at 2x10^5 PBMC/well in 100 µl medium in 96-well round bottom plates, at 37°C and 5% CO₂. Protein (2 μg/ml) was added on days 0 and 6. IL-2 was added at 10 U/ml on days 3, 6, and 9. On day 12, cells were pooled, washed, counted and rested overnight in complete medium. On day 13 cells were restimulated for six hours with overlapping peptide pools, corresponding to the HCV proteins used to expand T cells, to assess effector function (IFN-γ production).

**Detection of IFN-γ-producing HCV-specific T cells after re-stimulation**

IFN-γ producing cells after restimulation with overlapping peptide pools were enumerated by intracellular cytokine staining (ICCS).26,27 As higher numbers of IFN-γ producing cells are expected after expansion than directly *ex vivo*, ICCS was chosen for better visualization. Briefly, 10^6 PBMC were stimulated for six hours with HCV Core/envelope (also including E1,E2 and p7 peptides), NS3, NS4 and NS5 peptide pools (at 2 μg/ml of each peptide) and both αCD28 (2 μg/ml) and αCD49d (1 μg/ml) as
co stimuli, in the presence of 1:1000 Brefeldin A (Golgiplug, BD Biosciences (BD), San José, California, USA). 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 and 2 μg/ml ionomycin. After stimulation, cells were washed, permeabilized (FACS Permeabilizing Solution, BD), washed again and stained with antibodies specific for CD3, CD4, CD8 and IFN-γ (BD). After fixation (Cellfix, BD) 200,000 events were acquired on a FACSCalibur flow cytometer (BD). Lymphocytes were gated by forward and sideward scatter and data analyzed using the software program CELL Quest (BD). Reponses were scored as positive when twice above the medium control value and expressed as the percentage of IFN-γ producing CD4+ T cells.

Calculation of the number of IFN-γ producing CD4+ T cells/10^6 PBMC input

To enable comparison of donors and patients with different CD4+ T cell numbers, a more absolute number of HCV-specific CD4+ T cells was determined by calculating the number of HCV protein-specific IFN-γ-producing CD4+ T cells recovered out of 10^6 PBMC put into culture on day 0. This is the combination of the initial number of specific cells present and their ability to survive, proliferate and differentiate in vitro. To this end we counted the number of cells after culture (the ones that survived and proliferated) and calculated the number of IFN-γ producing T cells grown out as a function of the input (initial frequency of specific cells) using the following equation: (number of cells grown out/number of input cells x % IFN-γ) x 10,000 = number of cells per 10^6 input PBMC. As this calculation takes into account all variables potentially influencing the end result, it results in a more reliable number than just the percentage of responding cells after 12 days (as previously shown in ref 17). Addition of no stimulus or control proteins (Chiron) during the 12-day culture did not lead to recovery of specific T cells after 12 days. In addition, stimulation with a mismatch antigen (e.g., HIV peptides), did not lead to detectable HCV-specific T-cell responses (either CD4 or CD8) after re-stimulation with HCV peptides.17

Statistical analysis

Data are presented as medians with minimum and maximum values. Comparisons between the peak T-cell responses of resolvers and chronic HCV infected patients were done by Mann-Whitney test using Statistical Product and Service Solutions (SPSS) for Windows, version 9.0 (SPSS Inc., Chicago, IL, USA).

Results

Clinical and laboratory findings

We studied 13 HIV-negative IDU with documented onset of HCV infection (Table 4.1.1). Seven of these resolved HCV infection after a median of 4.8 months (the resolving phase of HCV-infection, interquartile range 3.6-24.2 months) (Table 4.1.1). Six of 13 subjects remained chronically infected as evidenced by the presence of high levels of HCV-RNA plasma concentrations beyond two years (Figure 4.1.1B). After the estimated date of infection (EDI), resolvers had lower HCV-RNA concentrations (median 3.53 log10 IU/ml, range 2.78-6.9) than chronic carriers (median 5.57 log10 IU/ml, range 4.34-6.12) (p=0.051, Figure 4.1.1). Onset of HCV infection was always associated with
self-reported injecting drug use (Figure 4.1.1A,B). In most cases, injecting drug use continued during follow up, which may lead to reexposure or reinfection with HCV.

T-cell analyses

T-cell responses were analysed in the time span needed to clear the acute infection, which we refer to as the resolving phase of HCV infection. To make the two groups, resolvers and chronic carriers, comparable, we have measured T-cell responses in chronic carriers within a comparable time span (within the first two years after EDI). The first time point analysed during follow up had a median of 3.5 months after EDI for resolvers (interquartile range 1-10 months) as well as chronic carriers (interquartile range 1-16 months). Comparison between groups was performed using the highest observed T-cell response. The median time to development of the highest observed T-cell response was 11 months (range 4-23) in resolvers and 19 months (range 15-24) in chronic carriers (Figure 4.1.1).

Resolvers have higher ex vivo HCV-specific T-cell responses than chronic carriers

Using an IFN-γ-Elispot assay after stimulation with pools of overlapping peptides, the total HCV-specific T-cell response directly ex vivo was estimated by the sum of the separate responses towards the different HCV-peptide pools (Figure 4.1.1A,B). At the time point at which the highest T-cell response was observed, resolvers had significantly higher HCV-specific T-cell responses (median 640 spots/million PBMC, range 83-5445) than chronic carriers (median 74 spots/million PBMC, range 0-230) (p=0.035) (Figure 4.1.2A). At that time point, a corresponding HCV-RNA measurement could be performed in 6 of 7 resolvers and all of them had plasma concentrations below 615 IU/ml (Figure 4.1.1A).

Next, we identified which antigens/peptide pools were targeted. Resolvers targeted a median of 5 antigens, (range 3-6) while chronic carriers targeted a median of two antigens, (range 0-5), suggesting that a larger breadth of the T-cell response was associated with resolved infection (Figure 4.1.2A). No differences for the specificity of the HCV-specific T-cell response to a particular peptide pool (cluster) was observed between resolvers and chronic carriers directly ex vivo.

We were able to analyse long-term HCV-specific T-cell responses in three resolvers and five chronic carriers, some at multiple time points. (see Figure 4.1.1) These long-term HCV-specific T-cell responses (median follow up 124 months, range 59-135) were repeatedly low in both groups (0 spots and median of 9 spots/million PBMC (range 0-47), respectively) (Figure 4.1.2B).

Total CD4⁺ memory T-cell responses are not significantly different between resolvers and chronic carriers

HCV-specific CD4⁺ T cells were quantified using a 12-day in vitro expansion assay, which was shown to give a proper reflection of a memory CD4⁺ T-cell response. Representative FACS plots of HCV-specific CD4⁺ T cells in one chronic carrier (Figure 4.1.3A) and one resolver (Figure 4.1.3B) after 12-day expansion with HCV proteins and re-stimulation with peptide pools show the percentage of CD3⁺CD4⁺ T cells producing IFN-γ (total CD4⁺ T-cell response 16.23% and 25.17% respectively). Subsequently the number of IFN-γ producing CD4⁺ T cells that grew out after 12 days was calculated in relation to the number of cells put into culture at day 0.
Follow up of injecting drug use and HCV-specific T-cell responses in 13 IDU.

Follow up (in months), relative to estimated date of HCV infection at t=0 months (vertical dashed line) is shown for seven resolvers (R1-R7, A) and six chronic carriers (C1-C6, B). Resolvers R6 and R7 became chronic HCV carriers after re-infection in combination with HIV-1. Viral RNA plasma concentrations are shown on a logarithmic scale on the y-axis (A, B upper panels) with lower limits of quantification of 615 IU/ml for HCV (•) and 1000 cp/ml for HIV-1 (○). A negative HCV RNA that was positive or negative in a qualitative assay is indicated with + or - respectively. Self reported injecting drug use is indicated by □ on the x-axis (A, B upper panels). The sum of HCV-specific T-cell responses as measured by IFN-γ Elispot assay against different HCV-peptide pools are shown as a solid vertical bar. For resolver R3, the time point with the highest observed HCV-specific T-cell response is indicated with *. HCV-specific T-cell responses that were measured but found to be less than 50 spots/10⁶ PBMC or undetectable are indicated with # on the x-axis (A, B lower panels).
A IFN-\(\gamma\) producing of HCV-specific T cells directly \textit{ex vivo}

\[ \text{resolvers} \quad \text{P}=0.035 \quad \text{carriers} \]

Figure 4.1.2. HCV-specific T-cell responses. The number of IFN-\(\gamma\) producing T cells, as measured by IFN-\(\gamma\) Elispot assay after stimulation with overlapping HCV peptide pools, are shown for 7 resolvers (2A, left panel) and 6 chronic carriers (2A, right panel) during acute HCV-infection. The T-cell response for resolver R3 is shown at a different y-axis (indicated on the right and encircled by a dashed line) (left panel) to be able to show the protein specificities of all resolvers clearly. HCV-specific T-cell responses \(<50\) spots/10\(^6\) PBMC are indicated with * (right panel). The kinetics of HCV-specific T-cell responses during long-term follow up (months) is shown for resolvers (2B, left panel) and chronic carriers (2B, right panel). The vertical dashed line at t=0 indicates estimated date of HCV infection.

This number of IFN-\(\gamma\) producing CD4\(^+\) T cells/million PBMC is a composite of the frequency of HCV-specific T cells within the total T cell pool and the ability of the cells to survive and proliferate \textit{in vitro}.

Although higher HCV-specific CD4\(^+\) T-cell responses were observed in resolvers (median 10663 IFN-\(\gamma\) producing CD4\(^+\) T cells/million PBMC, range 93-74197) than in chronic carriers (median 2604 IFN-\(\gamma\) producing CD4\(^+\) T cells/million PBMC, range 1278-15730), the differences were not significant (p=0.534) (Figure 4.1.4A). After a median follow up of 114 months (range 59-154 months), an HCV-specific CD4\(^+\) T-cell response could be assessed in one resolver (160 IFN-\(\gamma\) producing cells/million PBMC) and in five chronic carriers (median 3844 IFN-\(\gamma\) producing cells/million PBMC, range 484-25652) (data not shown).
Role of HCV-specific CD4+ T cells in viral clearance

Figure 4.1.3 HCV-specific CD4+ T-cell responses after a 12-day expansion period.
Representative FACS plots of the percentage of HCV-specific IFN-γ producing CD4+ T cells after 12-day expansion with NS3, NS4, NS5 or Core in a representative chronic carrier (C12, panel A) and resolver (R5, panel B). The percentage of CD3+CD4+ T cells producing IFN-γ (y-axis) upon 6-hour restimulation with NS3, NS4, NS5 and Core/envelope peptide pools is shown after 12-day expansion using proteins. In the upper right quadrants of each FACS plot the percentage of IFN-γ-producing CD4+ T cells is shown.
Chapter 4.1

Immunology in acute HCV

Resolvers have low CD4\(^+\) Core responses and high CD4\(^+\) nonstructural protein responses

We analyzed CD4\(^+\) T-cell responses that were directed against Core, NS3, NS4 and NS5 separately. The breadth of the CD4\(^+\) T-cell response against the separate proteins was the same in resolvers (median 3 proteins, range 1-4) and chronic carriers (median 3 proteins, range 2-4) (Table 4.1.2). However, the strength of the responses against the separate proteins (Core versus NS proteins) was different between resolvers and chronic carriers (Figure 4.1.4B). Resolvers had a significantly lower percentage of CD4\(^+\) T cells directed against Core (median 1%, range 0-14%) compared to chronic carriers (median 47%, range 2.3-68; \(p=0.008\)) (Figure 4.1.4C).

In resolvers the highest CD4\(^+\) T-cell response measurable was targeted against a cluster of 2 or 3 nonstructural proteins and in one (R7) against a single nonstructural protein (NS3) (Table 4.1.2). In contrast, Core protein was the most or second most dominantly targeted protein in four of six chronic carriers (Table 4.1.2). Noteworthy, a chronic carrier (C8) who generated a relative strong CD4\(^+\) T-cell response (8285 IFN-\(\gamma\) producing cells/million PBMC) against nonstructural proteins (98% of total response) (Table 4.1.2) during the early phase of chronic infection (Figure 4.1.1B), was capable of suppressing HCV-RNA plasma concentrations (<615 IU/ml). However, HCV-RNA levels became detectable again, despite a sustained magnitude of the total CD4\(^+\) T-cell response (7101 IFN-\(\gamma\) producing cells/million PBMC). However, the quality of the CD4\(^+\) T-cell response had changed by increasing the Core protein response from 2% to 22% (Figure 4.1.4B, right panel), with a concomitant decrease against the nonstructural proteins (data not shown).

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<td>C13</td>
<td>54</td>
<td>8514</td>
<td>28</td>
<td>4368</td>
</tr>
</tbody>
</table>

Table 4.1.2 CD4\(^+\)T-cell response against separate proteins as a percentage of total CD4\(^+\)T-cell peak response or as an absolute response (IFN-\(\gamma\) producing cells/million PBMC).
Figure 4.1.4  HCV-specific CD4\(^+\) T cell response.

A. The number of IFN-\(\gamma\) producing CD4\(^+\) T cells after 12 days of expansion calculated from the input PBMC (IFN-\(\gamma\) producing CD4\(^+\) T cells/10\(^6\) PBMC) as measured by ICCS after re-stimulation, is shown for 7 resolvers (left panel) and six chronic carriers (right panel) before and after acute HCV infection. The CD4\(^+\) T-cell response is shown for resolver R3 at the moment of the highest T-cell response. The low CD4\(^+\) T-cell peak response in resolver R7 (93 IFN-\(\gamma\) producing cells/10\(^6\) PBMC) is indicated with # (A, left panel). Separate protein-specific responses are shown by gray tones in the bars.

B. The kinetics of HCV-specific CD4\(^+\) T-cell responses directed against Core protein (as percentage of IFN-\(\gamma\) producing CD4\(^+\) T cells/10\(^6\) PBMC) is shown for resolvers (left panel) and chronic carriers (right panel) over onset of HCV infection and long term follow up. Estimated date of HCV infection is indicated by a vertical dashed line at month=0.

C. HCV-specific CD4\(^+\) T-cell responses against Core and NS-proteins, presented as a percentage of the total HCV-specific CD4\(^+\) T cell response (y-axis), are compared between resolvers and chronic carriers (x-axis) at the moment of the highest T-cell response.
HCV-specific T-cell responses before and after EDI

In four individuals (R2, 3, 7, C11) we were able to assess HCV-specific CD4$^+$ memory T cells and ex vivo T-cell responses before onset of infection at month -27, -52, -5, -14, respectively. A relative long interval before EDI was chosen in order to prevent analyses of HCV-specific T-cell responses during the window phase of an acute HCV infection. Surprisingly, ex vivo HCV-specific T-cell responses were easily detectable before EDI (in resolvers median 167 spots/million PBMC, range 55 to 255 and in the chronic carrier 200 spots/million PBMC). After onset of infection, HCV-specific T-cell responses increased in resolvers (varying from 2- to 20-fold) but decreased to undetectable levels in the chronic carrier during the same time span (Figure 4.1.1). These easily detectable T-cell responses before EDI coincided with self-reported injecting drug use in most IDU, but HCV RNA remained undetectable (Figure 4.1.1A,B). Noteworthy, in resolver R3 the T-cell response became undetectable when injecting drug use had stopped during the period before EDI. Also HCV-specific CD4$^+$ T-cell memory responses after 12-day expansion were easily detected well before EDI (median 2570, range 1025-17249 IFN-γ producing cells/million PBMC), while no CD4$^+$ T-cell responses were detectable after stimulating four healthy individuals who served as a control group (data not shown). Interestingly, Core protein-specific CD4$^+$ T-cell responses, which dominated before EDI, decreased over onset of infection to very low levels in all resolvers (Figure 4.1.4B, left panel) with a concomitant increase in responses against nonstructural proteins (data not shown). In contrast, after onset of infection the CD4$^+$ T-cell response to Core protein increased in the chronic carrier (Figure 4.1.4B, right panel).

Discussion

Based on studies of acute hepatitis C in humans, clearance of hepatitis C infection is thought to be associated with the ability to mount strong T-cell responses. Our study shows that, although T-cell responses in general were low, resolvers had higher ex vivo HCV-specific T-cell responses during the resolving phase of HCV-infection compared to chronic carriers in a comparable time frame. In contrast, we show that the magnitude of the total HCV-specific memory CD4$^+$ T-cell response after expansion did not differ significantly between resolvers and chronic carriers. However, HCV-specific CD4$^+$ NS protein responses were significantly higher in resolvers compared to chronic carriers.

Using stimulation with overlapping peptide pools spanning the entire HCV genome, our study among IDU with a prospectively sampled HCV-infection confirms previous studies (which were often cross-sectional and in health care workers) that direct ex vivo T-cell responses are weak. But resolved HCV infection is associated with relatively stronger HCV-specific T-cell responses. Long-term HCV-specific T-cell responses observed directly ex vivo were weaker than reported by others. Obviously, our group of individuals may have been different in many ways compared to other groups. For one the frequency of (injecting) drug use, which by itself may have a negative influence on the cellular immune responses. In those in whom we assessed HCV-specific T-cell responses from before onset of infection, we observed that an increase of these responses over EDI was associated with a favourable outcome of infection.
As CD4+ T-cell responses in our hands were undetectable directly ex vivo using protein stimulation, we studied the role of CD4+ T-cell responses in HCV infection using a recently developed 12-day expansion assay. This assay was previously shown to detect antigen-experienced T cells that are able to proliferate and exert their function by cytokine production upon re-encounter with the antigen. It was shown in both HCV-infected and Plasmodium falciparum-infected individuals that protection against infection and/or clearance of the pathogen was associated with IFN-γ producing CD4+ T cells measured after ex vivo expansion.

We found that most resolvers had higher HCV-specific CD4+ T-cell responses and most chronic carriers had lower CD4+ T-cell responses, confirming the general picture of robust CD4+ T-cell responses in self-limiting HCV-infections. However, the difference in magnitude of the CD4+ T-cell response between resolvers and chronic carriers was not significant. This suggests that the outcome of HCV infection may not be determined by the magnitude of IFN-γ producing CD4+ T cells, as previously suggested in a study in chimpanzees. Other qualitative aspects of the HCV-specific CD4+ T-cell response could play a role in the outcome of HCV-infection. Interestingly, we found that HCV-specific CD4+ T-cell responses against nonstructural proteins were significantly higher in resolvers compared to chronic carriers. In addition, over onset of infection, the CD4+ T-cell responses against separate proteins showed an increasing CD4+ T-cell response against nonstructural proteins in resolvers. Moreover, recurrence of HCV-RNA after temporary control of HCV infection during the early phase of infection, was also associated with a shift towards a more dominant CD4+ Core protein response, suggesting that a chronic carrier state is associated with the appearance of a relative strong CD4+ Core protein response.

In an earlier study, a trend for CD4+ T-cell responses to Core protein was found to be more common in individuals who evolved to chronic hepatitis and in a more recent cross-sectional study it was found that T-cell responses in resolvers were more commonly targeting nonstructural proteins. However, other studies using similar techniques failed to support this finding and showed that CD4+ T-cell responses were broad, but not specifically targeting only NS proteins. In that light the Core protein-responses detected prior to seroconversion in subsequently resolving individuals is of interest as Core protein-responses are more abundant before onset of infection and shift to dominant NS-responses over seroconversion.

We assume that differences in reactivity of CD4+ T cells against Core during HCV infection is caused by differences in Core-antigen presentation to CD4+ T cells. We did observe lower HCV-RNA plasma concentrations in resolvers compared to chronic carriers, but it remains speculative whether higher HCV-RNA concentrations lead to more circulating Core protein. It has been suggested that serum of HCV-infected individuals contains virus particles with HCV Core epitopes exposed on their surfaces and that serum may contain free circulating Core proteins. Higher HCV replication could lead to higher quantities of Core antigen in plasma and this may negatively influence the development or maintenance of an effective T-cell response. This may further facilitate HCV replication, potentially pushing the balance between clearance and persistence into the direction of a chronic HCV infection.

Traditionally, acute hepatitis C is considered to run a course of approximately six months, which is based on the observation that most patients who spontaneously clear HCV, do so within the first three to four months of infection. However, most
observations were done in symptomatic patients who were subsequently referred to medical centres. Consequently, most analyses of a successful immune response were done in symptomatic HCV-infected patients, whom we assume were not repeatedly exposed to HCV, once they received medical care during the first six months of infection. In contrast, we assume that some of our asymptomatic injecting drug users were repeatedly re-infected with HCV during the acute phase of infection. As a consequence of this repeated and prolonged HCV exposure, some IDU probably needed more time to resolve HCV infection (Figure 4.1.1). On the other hand, we may have overestimated the time to viral clearance due to some wide HCV-RNA testing intervals. Late viral clearance (>24 months) has been reported before.

A possible caveat in our study might be the use of HCV peptide pools based on the genotype 1a consensus sequence, as 6 out of 13 patients were infected with genotype 3a strains, which may result in underestimation of T-cell responses in these patients. On the other hand genotype 3a was equally divided over the two groups studied. However, three of the chronic carriers who carried genotype 3a strains lacked responses against NS5 (Table 4.1.3). Whether this is due to their genotype difference or due to escape mutations has to be elucidated. The fact that the resolvers infected with genotype 3a did show NS5 responses, suggests that the three genotype 3a chronic carriers may have obtained additional mutations making them unresponsive to NS5 stimulation.

In addition, we were not able to investigate the T-cell response at the same time point in each individual. Although most individuals were studied within three months after EDI, three of the resolvers and two of the chronic carriers were studied after more than ten months. To adjust for this potential bias we compared the highest T-cell response instead of the earliest time point measured. However, these different approaches led to similar results. (This thesis, chapter 4.3) Furthermore, one chronic carrier had a long interval between last negative and first positive RNA samples and thus the EDI has a larger improbability. We cannot exclude that this has interfered with the results.

An unexpected finding was the detection of HCV-specific memory T-cell responses in resolvers as well as a chronic carrier before EDI, despite undetectable HCV RNA and HCV antibodies. These responses suggest that exposure to HCV occurs much more often than previously thought and can lead to induction of a cellular immune response without consistently detectable viremia or seroconversion and may influence subsequent outcome of infection. Indeed, in one of our subjects it was previously shown that HCV seroconversion after a “first” HCV infection was followed by loss of detectable antibodies after which re-seroconversion occurred after a “second” HCV infection. Clearance of the “second” HCV infection in R3 was associated with very strong T-cell response, suggestive of protective immunity and strikingly paralleling periods of intermittent injecting drug use. Alternatively, the dominant Core responses before acute HCV infection may be the result of exposure to Core particles instead of infectious virus. Conversely, we cannot assume that individuals in whom we could not demonstrate potential previous infection have truly not been in contact with HCV before follow up.

Intriguingly, the detectable T-cell responses before EDI in chronic carrier C11 were apparently not protective, suggesting that a CD4+ T-cell responses before EDI does not predict an effective immune response after re-exposure to high levels of HCV RNA. It has been reported that IDU who were previously infected were less likely to develop persistent HCV viremia than individuals infected for the first time, indeed suggesting that protective immunity may be acquired. However, a more recent study did not confirm
this and calculated the re-infection rate to be 41/100 PY. Therefore, the observed T-cell responses before onset of infection may be merely a reflection of exposure to the virus, as has been reported in homosexual men who seroconverted for HIV despite detectable HIV-specific cytotoxic T-lymphocyte (CTL) responses well before HIV seroconversion. Most likely T-cell responses decrease after clearance and can only be detected after recent (re)-infection. Interestingly, in resolver R3 the T-cell response became undetectable when injecting drug use had stopped during the period before EDI.

Surprisingly, in the chronic carrier and the resolvers, the memory responses measured before onset of infection were in part directed against Core protein. One would expect the memory response in the individuals whom resolve after (re)-infection, to resemble a protective response, which would be directed predominantly against nonstructural proteins. In resolvers the response became mainly NS-focused only after (re)-infection, suggesting that a rapid transition to a predominantly NS-response would provide the ability to gain a protective response.

In conclusion, during the resolving phase of HCV infection higher ex vivo HCV-specific T-cell responses and memory HCV-specific CD4+ T-cell responses targeting mainly nonstructural proteins are observed in resolvers compared to in chronic carriers. Memory T-cell responses present before documented HCV-seroconversion suggest that re-infection in IDU occurs often, while the presence of these responses were not predictive for the outcome of infection. Persistent HCV viremia was associated with increasing HCV-specific CD4+ T-cell responsiveness against Core protein, implicating a role for Core protein in negative modulation of the CD4+ T-cell response, which may have implications for the design of HCV vaccines.
References

Role of HCV-specific CD4+ T cells in viral clearance


23. van AL, Prins M. Infection with concurrent multiple hepatitis C virus genotypes is associated with faster HIV disease progression. AIDS 2004;18:2319-2324.


40. Yao ZQ, Eisen-Vandervelde A, Waggoner SN, Cale EM, Hahn YS. Direct binding of hepatitis C virus core to gC1qR on CD4+ and CD8+ T cells leads to impaired activation of Lck and Akt. J Virol 2004;78:6409-6419.


Chapter 4.2

Comprehensive longitudinal analysis of hepatitis C virus (HCV)-specific T-cell responses during acute HCV infection in the presence of existing HIV-1 infection

Charlotte van den Berg, Thomas Ruys, Nening Nanlohy, Suzanne Geerlings, Jan van der Meer, Jan-Willem Mulder, Joep Lange, Debbie van Baarle

Abstract

Objective
To study the development of HCV-specific T cell immunity during acute HCV infection in the presence of an existing HIV-1 infection in four HIV-1 infected men having sex with men (MSM).

Methods
A comprehensive analysis of HCV-specific T-cell responses was performed at two time points during acute HCV infection using a T-cell expansion assay with overlapping peptide pools spanning the entire HCV genome.

Results
Three patients with (near) normal CD4⁺ T cell counts (range 400-970x10⁶/l) either resolved (n=1) or temporary suppressed HCV RNA. In contrast, one patient with low CD4⁺ T cell counts (330x10⁶/l), had sustained high HCV RNA levels. All four patients had low HCV-specific CD8⁺ T-cell responses, and similar magnitudes of CD4⁺ T-cell responses. Interestingly, individuals with resolved infection or temporary suppression of HCV-RNA had HCV-specific CD4⁺ T-cell responses predominantly against nonstructural (NS) proteins. While the individual with high HCV RNA plasma concentrations had CD4⁺ T-cell responses predominantly directed against Core.

Conclusions
Our data show that an acute HCV infection in an HIV-1 infected person can be suppressed in the presence of HCV-specific CD4⁺ T-cell response targeting nonstructural proteins. However further research is needed in a larger group of patients to evaluate the role of HIV-1 on HCV-specific T-cell responses in relation to outcome of acute HCV infection.
Hepatitis C virus (HCV) leads to a chronic infection in the majority of patients. HCV-specific CD8+ T cells play a central role in the control of hepatitis C. A multi-specific CTL response during the first six months of infection is associated with resolved infection1,4 and the presence of strong polyclonal CTL responses is associated with lower HCV RNA plasma concentrations.5-7 Several studies have shown a close correlation between polyclonal, multi-specific proliferative CD4+ T-cell response and clearance of acute HCV infection.8-11 Furthermore, HCV-specific memory CD8+ and CD4+ T-cell responses were shown to be important for the control of re-infection with HCV in a serially infected chimpanzee.12,13 It has been suggested that such a protective HCV-specific memory response may exist in injecting drug users and that an effective HCV-specific immune response may be lost after co-infection with HIV-1.14 There is a general believe that HIV-1 infected patients have a lower clearance rate of acute HCV infection compared to persons with an acute HCV mono-infection.15 Therefore, we hypothesized that HIV-1 may influence HCV infection by impairing HCV-specific T-cell responses.

As a read-out of functionality of T cells several parameters can be analyzed. The ability of HCV-specific T cells to produce effector cytokines (e.g., IFN-γ and IL-2) after direct stimulation was shown to be low.16 Therefore we used a recently described sensitive and reproducible assay combining specific in vitro expansion and effector T cell analysis to determine the number of HCV-specific memory T cells.16,17 This assay is not just a measure of proliferation, but detects a combination of the survival of specific T cells, proliferative capacity of these cells and ability to differentiate into effector T cells and produce IFN-γ. Numbers of antigen specific T cells, capable of proliferation and subsequent IFN-γ production in response to re-exposure to antigen were shown to correlate with protection against HCV and malaria.18,19

To our knowledge, there are no longitudinal data available at this moment on the effect of already existing chronic HIV-infection on HCV-specific immunity after acute HCV infection. We had the unique opportunity to identify four men having sex with men (MSM) with an acute HCV infection in presence of an already existing HIV-1 infection and performed a comprehensive analysis of HCV-specific CD4+ and CD8+ T cells after stimulation with HCV overlapping peptide pools.

Materials and Methods

Subjects

Between 2002 and 2004, four Caucasian men having sex with men (MSM I, II, III, IV) with known HIV-1 seropositivity varying from 1.5 to 10 years were identified with an acute hepatitis, as evidenced by markedly elevated liver enzymes, by primary HIV providers in Amsterdam, the Netherlands (Table 4.2.1). Three of four male subjects (I, III, IV) received highly active anti-retroviral treatment (HAART, range 15-118 weeks) when this acute hepatitis occurred. Because of the markedly elevated ALT levels and hence initial suspicion of HAART toxicity, HAART was stopped in 2 MSM (I, III) and HIV-RNA levels became detectable after 12 and 14 weeks. Because of continuing high ALT levels after cessation of HAART, suspicion on acute HCV arose and patients were tested for HCV antibodies and presence of HCV RNA. After confirmation of acute HCV
infection, earlier stored plasma samples were retrospectively tested for HCV antibodies and HCV RNA and HCV seroconversion was documented. The patients were followed regularly at the outpatient clinic. An alanine-aminotransferase (ALT) peak (median 1,056 IU/l, range 697-1,949) was observed a median of seven weeks (range 4-9 weeks) after the onset of HCV infection (Figure 4.2.1). All patients gave informed consent.

Definitions

Acute HCV infection was defined as detectable serum HCV RNA in the setting of documented seroconversion to HCV antibody positivity within the past six months. The onset of HCV infection was retrospectively determined and estimated to have occurred between the last HCV RNA negative and first positive serum sample, at the moment of sexual risk behaviour or the presence of a sexual transmitted disease. Resolved HCV infection was defined as undetectable HCV RNA (qualitative HCV RNA, TMA) at two consecutive visits, during a period of six months or more.

HCV-serology and RNA analyses

HCV antibody testing was performed with a third-generation enzyme immunoassay (Ortho Diagnostics, Rochester, NY). Serum HCV RNA was quantified by bDNA (Versant™ HCV 3.0, Bayer Diagnostics with a lower limit of detection of 615 IU/ml). Subsequently, HCV RNA was measured by means of a qualitative transcription mediated amplification (TMA) assay (Versant™ HCV RNA Qualitative assay, Bayer Diagnostics, with a lower limit of detection of 5 IU/ml), when HCV RNA was below the detection limit of the bDNA assay (615 IU/ml). For HCV genotyping RNA was isolated using the TriPure method (Roche Diagnostics, Almere, the Netherlands) and subsequently amplified using a nested RT-PCR based on the conserved Core region of the HCV genome as described by Ohno et al. Genotypes were confirmed by sequencing a part of the NS5B region of the HCV genome.

Assessment of HCV-specific T-cell responses

HCV-specific T-cell responses were assessed at two time points during the first 30 weeks after onset of acute HCV infection (first time point, range 6-14 weeks, second time point range 10-28 weeks). Panels of overlapping peptides spanning the complete HCV genotype 1a genome were used as stimulation (provided by NIH Aids Research Reagent Programme). Peptides had a length of 18 amino acids (overlapping adjacent peptides by 11 aa) derived from the following HCV proteins: Core polyprotein (Core, E1, E2, p7 protein, aa 1-805), NS2 protein (aa 806-1,022), NS3 protease/helicase (aa 1,023-1,645), NS4 protein (aa 1,646-1,967), NS5A protein (aa 1,968-2,415) and NS5B protein (aa 2,416-3,011). Peptides were pooled in a way that each single peptide in the pool was present at a concentration of 1 mg/ml.

ELIspot assay for single cell IFN-γ release

IFN-γ producing HCV-specific T cells were enumerated using IFN-γ specific ELIspot assays as previously described using the anti-IFN-γ antibodies from Mabtech (Stockholm) and streptavidin poly-HRP from Sanquin (Amsterdam). PBMC were stimulated in triplicate wells at 100,000 cells/well in the absence or presence of 2 μg/ml
Expansion of HCV-specific T cells

To expand HCV-specific T cells, PBMC were cultured for 12 days as previously described\textsuperscript{17} in the presence of overlapping peptide pools corresponding to the Core, NS2, NS3, NS4 and NS5 proteins. Peptide pools (2 μg/ml) were added on day 0 and 6. IL-2 was added at 360 IU/ml on days 3, 6, and 9. On day 12, cells were pooled, washed, counted and rested overnight in complete medium. On day 13 cells were restimulated to determine the number of IFN-γ-producing effector HCV-specific T cells.

Figure 4.2.1 Follow up of MSM during acute HCV infection. Kinetics of both HCV-RNA load (filled circles) and ALT-levels (open squares) are depicted in time (weeks) for four MSM (I-IV) who experienced acute HCV infection. Estimated date of acute HCV infection is depicted with the dotted vertical line. Periods on HAART (horizontal bars) or PEG-IFN/ribavirin therapy (vertical solid lines) are indicated in the figures.
### Table 4.2.1 General characteristics of the study population.

<table>
<thead>
<tr>
<th>MSM</th>
<th>Age</th>
<th>Time since HIV-1 infection</th>
<th>HAART*</th>
<th>Duration of HAART^</th>
<th>HIV-RNA</th>
<th>HCV genotype</th>
<th>CD4 count^</th>
<th>Nadir CD4 counts%</th>
<th>Outcome HCV-infection</th>
<th>HCV therapy</th>
<th>Outcome therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>37</td>
<td>3 years</td>
<td>yes</td>
<td>2 years</td>
<td>&lt;50 cp/ml</td>
<td>1</td>
<td>640</td>
<td>340</td>
<td>resolved</td>
<td>No</td>
<td>NA</td>
</tr>
<tr>
<td>II</td>
<td>37</td>
<td>1 year</td>
<td>no</td>
<td>-</td>
<td>&lt;50 cp/ml</td>
<td>4</td>
<td>970</td>
<td>550</td>
<td>chronic</td>
<td>No</td>
<td>NA</td>
</tr>
<tr>
<td>III</td>
<td>45</td>
<td>5.5 years</td>
<td>yes</td>
<td>5 years</td>
<td>&lt;50 cp/ml</td>
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<td>400</td>
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<td>Relapse</td>
<td></td>
</tr>
<tr>
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<td>&lt;50 cp/ml</td>
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<td>260</td>
<td>130</td>
<td>chronic +14 wks</td>
<td>Relapse</td>
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</tr>
</tbody>
</table>

^ Men who have sex with men (MSM), study subject; * age in years at onset acute HCV infection; ^ at onset acute HCV infection; ° CD4° T-cell numbers (x10^6/l), before onset of acute HCV infection; % nadir CD4° T-cell numbers (x10^6/l), during acute HCV infection; ~ First HIV-RNA during acute HCV.

### Table 4.2.2 Proliferation data CD4° T-cells after 12 day expansion.

<table>
<thead>
<tr>
<th>Patient</th>
<th>HCV antigen</th>
<th>Percentage IFN-γ^</th>
<th>Proliferation rate</th>
<th>Number of IFN-γ producing CD4° T cells/million PBMC input</th>
<th>% Core of total response</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSM I</td>
<td>CORE</td>
<td>0.23</td>
<td>0.089</td>
<td>204</td>
<td>5.5</td>
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<tr>
<td></td>
<td>NS2</td>
<td>0.10</td>
<td>0.050</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NS3</td>
<td>0.29</td>
<td>0.113</td>
<td>328</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NS4</td>
<td>0.85</td>
<td>0.079</td>
<td>668</td>
<td></td>
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<tr>
<td></td>
<td>NS5A+5B</td>
<td>2.20</td>
<td>0.112</td>
<td>2,473</td>
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<tr>
<td>TOTAL</td>
<td></td>
<td>3.67</td>
<td></td>
<td>3,723</td>
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<tr>
<td>MSM II</td>
<td>CORE</td>
<td>0.89</td>
<td>0.057</td>
<td>507</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>NS2</td>
<td>0.03</td>
<td>0.067</td>
<td>20</td>
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<tr>
<td></td>
<td>NS3</td>
<td>0.21</td>
<td>0.086</td>
<td>181</td>
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<tr>
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<td>0.070</td>
<td>182</td>
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<td>TOTAL</td>
<td></td>
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<td>0.303</td>
<td>1,607</td>
<td>56</td>
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<tr>
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<td>NS2</td>
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<td>0.082</td>
<td>91</td>
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<tr>
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<td>NS3</td>
<td>0.76</td>
<td>0.059</td>
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<td>NS4</td>
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<td>682</td>
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<td>NS5A+5B</td>
<td>0.04</td>
<td>0.086</td>
<td>34</td>
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<tr>
<td>TOTAL</td>
<td></td>
<td>2.11</td>
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<td>2,866</td>
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<tr>
<td>MSM IV</td>
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<td>0.102</td>
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<td>85</td>
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<tr>
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<td>0.075</td>
<td>30</td>
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<tr>
<td></td>
<td>NS3</td>
<td>0.04</td>
<td>0.066</td>
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<td></td>
<td>NS4</td>
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<td>0.049</td>
<td>178</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NS5A+5B</td>
<td>0</td>
<td>0.081</td>
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<td>TOTAL</td>
<td></td>
<td>1.73</td>
<td></td>
<td>1,553</td>
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</table>

^ Percentage IFN-γ^: percentage of IFN-γ producing cells after 12 day expansion and restimulation with peptide pools minus percentage of IFN-γ producing cells restimulated with medium; proliferation rate: number of cells recovered after 12 days of culture/number of cells put in to culture at day 0; number of # IFN producing CD4° T cells/million PBMC input: the product of the percentage of IFN-γ-producing T cells and the proliferation rate (x10.000 to express this number as a number per million PBMC input).
Detection of IFN-γ producing HCV-specific T cells after re-stimulation

IFN-γ producing cells after re-stimulation with overlapping peptide pools were enumerated by intracellular cytokine staining (ICCS). Briefly, 10^6 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 one 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 and 2 μg/ml ionomycin. After stimulation, cells were washed, permeabilized (FACS Permeabilizing Solution, BD), washed again and stained with antibodies specific for CD3, CD4, CD8 and IFN-γ (BD). After fixation (Cellfix, BD) min. 20,000 events were acquired on a FACS Calibur or LSRII flow cytometer (BD). Lymphocytes were gated by forward and sideward scatter and data analysed using the software program CELLQuest (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 restimulated conditions (Table 4.2.2). To determine the number of HCV-specific T cells per individual, enabling comparison of individuals with different T cell numbers, we calculated the number of HCV-protein-specific IFN-γ-producing T cells recovered out of 10^6 PBMC put into culture on day 0.17 To this end we determined the proliferation rate, i.e., 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 (Table 4.2.2). We called this rate a proliferation rate, since it is a measure of proliferation of HCV-specific T cells. However, another important determinant of this proliferation rate is the death rate of non-HCV-specific T cells, as can be seen in Table 4.2.2: the number of cells declines on average a factor 10 after 12 days of culture.

The number of HCV-specific T cells is the product of the percentage of IFN-γ-producing T cells and the proliferation rate. This number of HCV-specific effector T cells shown in Table 4.2.2, is therefore a combination of (memory) cells initially present in the PBMC pool and the ability of these cells to survive, proliferate and differentiate to (IFN-γ producing) effector T cells during the 12-day culture period. Thereby this assay is not restricted to measuring only one quality of (memory) T cells, but it is a combination of factors contributing to its effector function. It has been shown that Epstein-Barr virus (EBV)-specific cells measured after 12 days expansion were inversely correlated with antigen burden in case of EBV-infection.17

Results

Acute HCV infection and HIV-related determinants

Four MSM with acute HCV in the presence of HIV-1 infection were included, of whom three were infected with genotype 1. HIV-1 viral load was undetectable in all four MSM (Table 4.2.1). After onset of HCV infection in these four MSM, three different patterns in the course of HCV RNA levels were identified. First, resolved HCV infection in one MSM (I), as evidenced by undetectable HCV-RNA plasma concentrations (TMA) from week 14 onwards (Figure 4.2.1). Approximately half a year later MSM I became re-infected.
with HCV genotype 4, in the presence of high HIV-1 RNA levels and remained chronically HCV-HIV co-infected thereafter. A second pattern was characterized by transiently suppressed HCV RNA levels (<615 IU/ml, MSM II and III) during a period varying between four and ten weeks. The third pattern (MSM IV) was characterized by sustained high levels of HCV RNA since onset of infection (Figure 4.2.1). The number of CD4⁺ T cells prior to onset of HCV infection and during acute HCV infection (nadir) were highest in MSM who could resolve (I) or temporarily suppress HCV RNA (II, III) and lowest in MSM IV, in whom we observed sustained high HCV RNA levels (Table 4.2.1, Figure 4.2.1).

HCV-specific T-cell responses during acute HCV infection

HCV-specific T-cell responses were assessed after stimulation with HCV peptide pools using Elispot assay for IFN-γ production directly ex vivo at two different time points in MSM I, II and III. Overall, low to undetectable HCV-specific T-cell responses were observed at the earliest time point (range 6-11 weeks, range 0-307 spots/million PBMC, which equals 0-0.031%) (Figure 4.2.2A) and last assessed time point during acute infection (range 14-28 weeks, range 0-13 spots/PBMC, 0-0.0013%) (data not shown). Because of these low HCV-specific T-cell responses measured by Elispot-assay, we analyzed HCV-specific T cells after a 12 day expansion period using overlapping peptide pools spanning the entire HCV genome. Especially CD4⁺ T cells were shown to grow out during the 12-day stimulation period, which made analyses of CD8-responses more difficult.

Overall, HCV-specific CD4⁺ T-cell responses were stronger than CD8⁺ T-cell responses and ranged from 1.73 to 3.67% IFN-γ producing CD4⁺ T cells (Table 4.2.2). When taking the proliferation rate into account (Table 4.2.2), numbers ranged from 1,527-3,723 IFN-γ producing CD4⁺ T-cells/million PBMC at the first time point (Figure 4.2.2C, Table 4.2.2) and from 1,164-3,862 IFN-γ producing CD4⁺ T cells/10⁶ PBMC at the second time point during acute HCV infection (data not shown).

After restimulation the percentage of HCV-specific CD8⁺ T cells varied between 0 and 0.29% at the earliest assessed time point, and between 0 and 0.45% at the last assessed time point (data not shown). When incorporating the proliferation rate and thereby calculating the number of HCV-specific T cells (see section Methods), IFN-γ producing CD8⁺ T cells ranged from 0 and 318 at the earliest assessed time point (Figure 4.2.2B) and varied between 17 and 237 at the last assessed time point (data not shown).

Resolved HCV infection is associated with an HCV-specific CD4⁺ T-cell response against nonstructural proteins

By comparing CD4⁺ T-cell responses directed against HCV proteins separately at the earliest assessed time point, MSM I, who resolves HCV infection showed an HCV-specific CD4⁺ T-cell response almost completely targeted against nonstructural proteins (3,520 IFN-γ producing CD4⁺ T cells/10⁶ PBMC, 95% of total CD4⁺ T-cell response) (Figure 4.2.2C). MSM II and III, who could suppress HCV RNA temporarily during the early phase of acute HCV infection, showed more pronounced CD4⁺ T-cell responses directed against Core protein (507 IFN-γ producing CD4⁺ T cells/10⁶ PBMC, 33% of total CD4⁺ T-cell response and 1,607 IFN-γ producing CD4⁺ T cells/10⁶ PBMC, 56% of total CD4⁺ T-cell response, respectively). While in MSM IV, in whom elevated HCV RNA levels persisted, a CD4⁺ T-cell response that was predominantly targeted against Core-
HCV-specific T cells in HIV-infected MSM

protein (1,319 IFN-γ producing CD4⁺ T cells/10⁶ PBMC, 85% of total CD4-response) (Table 4.2.2, Figure 4.2.3). When analysing a later time point during acute HCV infection (range 14-28 weeks), CD4⁺ T-cell responses show a similar pattern as the earlier assessed time point with relative low CD4⁺ T-cell Core responses in MSM I with resolving HCV infection (13%) compared to higher CD4⁺ T-cell Core responses in chronic carriers MSM II (59%), MSM III (77%) and MSM IV (60%) (data not shown).

During follow up of MSM I infected with HCV genotype 1, HCV RNA levels remained undetectable with normalized ALT levels until week 41. Thereafter, HCV RNA became detectable again, however, from a different genotype (genotype 4). In the absence of anti-HIV therapy during this HCV re-infection, MSM I developed chronic infection as evidenced by persistent high levels of HCV RNA (Figure 4.2.3). In contrast to a predominantly nonstructural protein directed CD4⁺ T-cell response, as observed during the first acute infection, the development of chronic HCV infection was associated with a CD4⁺ T-cell response predominantly directed against Core protein (range 58-69% of total CD4⁺ T-cell response) (Figure 4.2.3).

A  

B  

C  

Figure 4.2.2  HCV-specific T cells during acute HCV infection.

HCV-specific T cells measured after stimulation with HCV overlapping peptide pools corresponding to the Core (black) and NS-protein regions (grey tones) are indicated in MSM I, II, III and IV. A) HCV-specific T cells (spots per 10⁶ PBMC) measured directly ex-vivo using IFN-γ Elispot assay B) HCV-specific CD8⁺ T cells and C) HCV-specific CD4⁺ T cells measured after 12 days expansion and expressed as number of IFN-γ producing T cells/10⁶ PBMC input.
T-cell responses during anti HCV-therapy

Because of persistently elevated HCV-RNA levels, MSM III and IV were treated for acute HCV infection with pegylated interferon-α (PEG-IFN) plus ribavirin (RBV). Treatment was not successful in either of them. In MSM IV we assessed HCV-specific T-cell responses during treatment. Throughout temporary suppression of HCV RNA a low CD4⁺ T-cell response (640 IFN-γ producing CD4⁺ T cells/10⁸ PBMC), only targeting nonstructural proteins (NS3 75%, NS4 25%) was observed (Figure 4.2.3). However,
HCV RNA relapsed during treatment and after re-appearance of HCV RNA the HCV-specific CD4+ T-cell response had shifted back towards Core (1,034 IFN-γ producing CD4+ T cells/10^6 PBMC, 47% of total CD4 response) (Figure 4.2.3). MSM III was treated for HCV after (immunological) follow up in this study.

Discussion

To our knowledge this is the first study in which HCV-specific T-cell responses were analyzed during acute HCV infection in the presence of an already existing HIV-1 infection. HIV-1 seropositivity itself did not seem to be a dominant factor for the outcome of acute HCV infection. However, continuous HIV-1 replication seemed associated with a negative outcome of acute HCV infection, which may suggest that uncontrolled HIV-1 infection may impair the development of an effective HCV-specific T-cell response. We found that the individuals with permanent or transient suppression of HCV RNA during acute HCV had higher CD4+ T cell numbers, which may suggest that low CD4+ T cell numbers impaired the development of an effective immune response. This finding seems in line with recent cross-sectional studies in which the magnitude and breadth of HCV-specific CD8+ T-cell response depended on CD4+ T cell count in HIV/HCV co-infected individuals.4,24,25 However, in our study only low ex vivo HCV-specific T-cell responses were observed. It has been shown that HCV-specific T cells during acute infection are impaired in IFN-γ production.4 Although it is possible that these cells make other cytokines, like IL-2, this is not likely in the light of a bulk of reports in both mice and human showing that mainly IL-2 production (and consequently proliferative capacity) is disturbed during chronic viral infections, and that IFN-γ responses are actually the last to wane in the face of chronic high antigen levels.26,27 As CD8+ memory T cells measured after expansion were also low, this suggests that either also proliferative capacity is impaired or that high frequencies of antigen specific CD8+ T cells may not be necessary in order to resolve acute HCV infection in HIV co-infected patients. Recent data on the occurrence of HCV-specific T-cell responses in HCV-HIV co-infected patients suggest however that HCV-specific immune responses occur in frequencies not different from HCV mono-infected patients.24,25,28 The assay we use is based on a combination of proliferative capacity and initial frequency of memory T cells and thereby may analyze a different function of T cells compared to other studies. We have chosen this assay as it has proven to be reproducible, specific and sensitive, being able to detect HCV-specific T-cell responses in individuals with low direct ex vivo responses. Furthermore, by taking into account a combination of functional properties instead of analysing only one function of memory T cells, like proliferation (e.g., CFSE dye dilution or ^3H uptake) or cytokine production (e.g., IFN-γ production), it provides better insight in the capacity of memory T-cell responses to control infection.

Using this assay we also studied HCV-specific T-cell responses before and after acute HCV mono-infection in injecting drug users. The height of CD4+ T-cell responses in the MSM in this study were comparable to that in mono-infected injecting drug users that develop chronic HCV infection. Similarly, the transition of the HCV-specific CD4+ memory T-cell response from targeting Core to targeting nonstructural proteins during onset of infection was associated with a favourable outcome in both IDU and MSM.16 However, we believe it is difficult to make a comparison between these two groups with acute HCV infection. The mode of transmission is very different, and possibly also the
size of the inoculum. While injecting drug users get frequently (re-)exposed to HCV parenterally during sharing of needles and/or syringes, the mode of transmission in MSM is still unclear. Acute HCV in MSM has been associated with co-infection with ulcerative sexually transmitted diseases (e.g., lymphogranuloma venereum and syphilis) and with rough sexual techniques, suggesting that HCV may be transmitted sexually. Furthermore, HCV infection in MSM seems to occur mainly within the HIV-positive population, which further complicates comparisons. In this setting, the dramatic drop of the number of CD4+ T cells, especially in the mucosa of the gut, has also been suggested to play a significant role in the susceptibility to HCV in HIV-1 infected MSM.

A maintained CD4+ T-cell response is a prerequisite for prolonged HCV RNA suppression and protection from secondary infection13, while loss of CD4+ T-cell reactivity in the acute phase of infection is accompanied with re-occurrence of HCV RNA29,30. We observed CD4+ T-cell responses of comparable magnitudes in these MSM. However, in individuals who resolved HCV infection or temporarily suppressed HCV RNA a HCV-specific CD4+ T-cell response mainly targeted nonstructural proteins, and the individuals developing chronic HCV infection or HCV RNA relapse a relative strong CD4+ T-cell response against Core polyprotein was seen, as we have previously also shown in Ruys et al. and as was also reported by others.16,31-34

Qualitative differences in reactivity of CD4+ T cells against Core during acute infection may be caused by higher levels of Core-antigen presentation to CD4+ T cells35, possibly because of higher quantities of Core-antigen in plasma.36 In addition, HCV Core is thought to play a role in modulating immune responses by affecting the function of virus-specific T cells.37 It has been reported that HCV-HIV co-infected patients have higher HCV-RNA plasma concentrations compared to HCV mono-infected patients, in theory leading to more Core-antigen presentation and potentially causing a stronger negative effect on the development of an effective T-cell response. An ineffective T-cell response may further facilitate HCV replication.38

Recent studies in HCV mono-infected39 and HCV/HIV co-infected patients40 suggest that early treatment of acute HCV is much more effective compared to treatment in a later and chronic phase. We observed disappointing results of early treatment of HCV infection in our two treated patients which is in line with another recent study in HCV/HIV co-infected patients.41 It has to be noted that in MSM IV HCV viral load in the first 10 weeks of infection was comparable to HCV viral load in MSM II and III. Therefore this individual might have been able to clear HCV spontaneously or temporarily suppress HCV RNA in the absence of treatment early after infection. In one of the treated individuals we were able to analyze T-cell responses during treatment. CD8+ T-cell responses became undetectable soon after the treatment was started in line with a recent study in which HCV-specific CD8+ T-cell responses also decreased with successful treatment and increased transiently with recrudescence of viremia in those who failed to achieve a sustained viral response.42

The small size and heterogeneity of these patients will not allow for firm conclusions, however, these longitudinal findings add to the limited data available on HCV-specific T-cell responses during acute HCV in HIV-1 co-infected persons and encourage the search to identify these patients to analyze larger groups. It is important to understand immunological factors associated with viral clearance, especially since HCV/HIV co-infection is associated with lower rates of HCV treatment success and with more complications during HCV treatment. Furthermore, the spread of HCV in HIV infected MSM is continuing, and is becoming a major public health problem.43,44

Our data may suggest that an acute HCV infection in an HIV infected person can be at least temporarily suppressed in the presence of HCV-specific CD4+ T-cell response.
targeting nonstructural proteins, despite low HCV-specific CD8⁺ T-cell responses. However, further research is needed in a larger group of patients to evaluate the role of HIV on HCV-specific T-cell responses in relation to the outcome of acute HCV infection.
References


HCV-specific T cells in HIV-infected MSM


Chapter 4.3

HCV-specific CD4\(^+\) T-cell responses in HIV and HCV seroconverters are influenced by the sequence of HIV and HCV infection and outcome of previous HCV infection

Charlotte van den Berg, Thomas Ruys, Nening Nanlohy, Thijs van de Laar, Marcel Beld, Maria Prins, Debbie van Baarle

Submitted.
Abstract

Objective
To elucidate the role of HIV infection on HCV-specific CD4\(^+\) T-cell responses in acute HCV infection.

Design
We analyzed HCV-specific CD4\(^+\) T cells in 14 injecting drug users (IDU) from the prospective Amsterdam Cohort Study among drug users shortly after HCV seroconversion in the presence or absence of acute HIV infection. In addition, we analyzed the influence of HIV infection on pre-existing HCV-specific CD4\(^+\) T-cell responses in already HCV-infected IDU who subsequently acquired HIV.

Methods
HCV-specific CD4\(^+\) T-cell responses were analyzed after 12 days of expansion using recombinant HCV proteins. Interferon-\(\gamma\) was measured as read-out of effector function.

Results
CD4\(^+\) T-cell responses against nonstructural (NS) HCV proteins, especially T-cell responses against NS5, were higher among HCV mono-infected IDU who clear HCV infection (n=6) compared to IDU with persistent HCV viremia both in the presence (n=4) and absence of HIV co-infection (n=4). Interestingly, IDU with acute co-infection who were previously able to clear HCV mono-infection (n=2) had higher HCV-Core-specific CD4\(^+\) T-cell responses than those without evidence of previous exposure to HCV (n=2).

Conclusions
Both acute HCV/HIV co-infected IDU who were previously exposed to HCV and mono-infected IDU who developed chronic HCV infection tended to have higher responses to Core protein compared to IDU that clear HCV. Furthermore, the HCV-specific T-cell responses were also skewed to unfavourable Core in 3/5 IDU after HIV seroconversion. These results suggest that the effect of HIV infection on HCV-specific T-cell responses is not clear-cut, but is influenced by the sequence and outcome of previous HCV infection.
Introduction

HIV and hepatitis C virus (HCV) infection are both highly prevalent in injecting drug users (IDU) due to shared routes of transmission, and HIV/HCV co-infection is common. CD4⁺ T cells play an important role in controlling acute HCV infection in humans and have been shown to be crucial for HCV clearance in chimpanzees. High and broad HCV-specific CD4⁺ T-cell responses are associated with clearance of acute HCV mono-infection in humans. Cross-sectional studies have shown that HIV co-infection is associated with loss of control of HCV viremia, suggesting that HIV co-infection negatively influences HCV-specific T-cell responses. Indeed it has been shown that chronic HIV co-infection is associated with lower HCV-specific CD4⁺ T-cell responses in acute HCV. However, to our knowledge no studies have been done comparing HCV-specific CD4⁺ T-cell responses in IDU with acute HIV/HCV co-infection and in IDU with acute HCV mono-infection.

There are different assays one can use to measure antigen-specific T cells by fluorescence-activated cell sorting (FACS). One of the frequently used assays to measure effector function is intracellular cytokine staining (ICCS) directly ex vivo after short-term (6-hour) stimulation. Unfortunately, HCV-specific CD4⁺ T cell frequencies measured directly ex vivo are very low. Another frequently used assay is the carboxyl fluorescent succinimidyl ester (CFSE) dye dilution assay, with which proliferative capacity of antigen-specific T cells can be visualized by FACS analysis after 6-day stimulation with antigen. Furthermore, a previously described assay in which T cells were expanded for 12 days can be used to measure effector function after expansion. This assay has been shown to be a reproducible and sensitive assay for measuring Epstein-Barr virus-specific memory T-cell responses, and has also been used for detection of HCV-specific responses. In this paper we compared results from this T cell line-based assay with direct stimulation and expansion assays with a stimulation period comparable to CFSE dye dilution (i.e., six days).

To study factors associated with viral clearance and the influence of HIV co-infection, we examined HCV-protein-specific CD4⁺ T-cell responses using the T cell line based expansion assay shortly after acute HCV infection in the presence or absence of acute HIV infection in IDU with documented HCV seroconversion. To further explore the influence of HIV infection during chronic HCV infection, we also examined HCV-specific CD4⁺ T-cell responses longitudinally in HCV infected IDU who later acquire HIV infection.

Methods

Study population

From the prospective Amsterdam Cohort Studies among drug users (ACS), we have studied IDU with documented HCV infection and HCV seroconversion during follow up. HCV infection was estimated as the midpoint between a negative and a positive HCV-RNA test independent of the presence of HCV antibodies, documented over two consecutive visits (bDNA HCV 3.0 Bayer or HCV RNA assay by transcription-mediated amplification (TMA), Versant HCV RNA Qualitative assay, Bayer Diagnostics). Date of HIV or HCV seroconversion (i.e., appearance of antibodies to HIV, EIA; Abbot
Laboratories, confirmation by Western blot (Diagnostic Biotechnology, Herent, Belgium) or appearance of antibodies to HCV, EIA 3.0 Abbot Laboratories, respectively) was estimated as the midpoint between the last negative and first positive HIV or HCV antibody test.

We analyzed HCV-specific CD4+ T-cell responses in 14 IDU with acute HCV infection in the presence (n=4) or absence (n=10) of acute HIV infection. The IDU were divided into three groups: IDU who became infected with HIV and HCV at approximately the same time and who all developed chronic HCV viremia (group co-CH, n=4), HCV mono-infected IDU who cleared HCV (group mono-CL, n=6) and HCV mono-infected IDU who developed chronic infection (group mono-CH, n=4). HCV clearance was defined as two consecutive visits with negative qualitative HCV-RNA assays after the onset of HCV infection. In group co-CH, two IDU previously cleared HCV without HIV co-infection (CO-1 and CO-2) and two IDU had acute co-infection without evidence of an earlier HCV infection (CO-3 and CO-4). None of these IDU were treated with antiretroviral therapy or anti-HCV treatment during acute HCV infection.

To study the influence of HIV infection on pre-existing HCV-specific memory CD4+ T-cell responses, we studied five IDU who acquired HCV infection a considerable period before HIV seroconversion, and who had PBMC available before and after HIV seroconversion. Characteristics of the study population are shown in Tables 4.3.1A and 4.3.1B.

### Direct stimulation

To measure effector function directly ex vivo, cryopreserved PBMC were thawed and stimulated for six hours in the presence of overlapping HCV peptide pools, corresponding to the Core/E1/E2/p7, NS2, NS3, NS4, and the NS5AB regions of the HCV genome (peptides were 18-mer peptides, overlap 11 amino acids; kindly provided by NIH AIDS Research Reagent Program).

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of IDU</th>
<th>Median age (range)*</th>
<th>Median CD4 count (range)*</th>
<th>HCV genotype (%)#</th>
<th>Median log HCV viral load (copies/ml)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO</td>
<td>4</td>
<td>25 (27-32)</td>
<td>830 (810-980)</td>
<td>1a (75%), 3a (25%)</td>
<td>2.7 x 10^5</td>
</tr>
<tr>
<td>CL</td>
<td>6</td>
<td>31 (23-38)</td>
<td>1115 (600-1580)</td>
<td>1a (33%)</td>
<td>1.9 x 10^3</td>
</tr>
<tr>
<td>CH</td>
<td>4</td>
<td>26 (21-33)</td>
<td>750 (590-1550)</td>
<td>Undetectable/ND (67%)</td>
<td>3.8 x 10^5</td>
</tr>
</tbody>
</table>

*: at time of acute HCV infection, #: at time of T cell analysis, ND: not done.
Table 4.3.1B General characteristics of five chronically HCV-infected IDU with prospective HIV infection.

<table>
<thead>
<tr>
<th>IDU</th>
<th>Sex</th>
<th>Age (years) at first HIV positive visit</th>
<th>Outcome</th>
<th>Age at previous HCV infection</th>
<th>CD4 count at first HIV positive visit</th>
<th>HCV genotype at first HIV positive visit</th>
<th>Log HCV viral load at first HIV positive visit</th>
</tr>
</thead>
<tbody>
<tr>
<td>18787</td>
<td>Male</td>
<td>48</td>
<td>Clearance</td>
<td>800</td>
<td>1a</td>
<td>5.53</td>
<td></td>
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<tr>
<td>18783</td>
<td>Male</td>
<td>41</td>
<td>Chronic</td>
<td>1120</td>
<td>1a</td>
<td>6.69</td>
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</tr>
<tr>
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<td>700</td>
<td>1a</td>
<td>3.52</td>
<td></td>
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<tr>
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<td>3a</td>
<td>5.51</td>
<td></td>
</tr>
<tr>
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<td>31</td>
<td>Chronic</td>
<td>690</td>
<td>1a</td>
<td>4.95</td>
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</tr>
</tbody>
</table>

HCV-specific T-cell expansion

To expand HCV-specific memory T cells, cryopreserved PBMC were thawed and subsequently expanded during a period of six or twelve days using recombinant HCV proteins Core (C22-3), NS3 (C33c), NS4 (C100-3) and NS5A/B (NS5) (2 μg/ml; kindly provided by M. Houghton and K. Crawford, Chiron) in the presence of IL-2 (180 IU/ml). After expansion, cells were pooled, washed, counted and rested overnight. On day 7 or 13, cells were restimulated using peptide pools 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 CD4+ T cells were enumerated using intracellular cytokine staining. Briefly, PBMC were stimulated for six hours with HCV Core/E1/E2/p7, 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 one hour 1:1000 Brefeldin A (Golgiplug, BD Biosciences (BD), San José, California, USA) was added. As negative control, PBMC were stimulated with medium and co-stimulation alone. As 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 (BD). After fixation (Cellfix, BD) min. 20,000 events were acquired on a FACSCalibur or LSRII flow cytometer (BD). Lymphocytes were gated by forward and sideward scatter and data analyzed using CELLQuest software (BD).

Results were expressed as the percentage of IFN-γ producing CD3+CD4+ T cells, which was calculated by subtracting the percentage of IFN-γ producing T cells in medium from that in peptidepool restimulated conditions. To enable comparison of IDU 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 (Table 4.3.2). Subsequently we calculated the number of HCV-specific effector 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 (as shown in Table 4.3.2), is therefore 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. It has been shown that these cells were inversely correlated with antigen burden in case of EBV infection.
Chapter 4.3

Immunology in acute HCV

Table 4.3.2 Comparison of different assays for measuring HCV-specific CD4+ T cells in two IDU (CL2 and CH2) with different outcome of primary HCV infection, clearance vs. persistent viremia. After short term stimulation (i.e., six hours) or short term culture (i.e., six days) no HCV-specific T-cell responses can be detected. However, after 12-day expansion there is clear proliferation and effector function in response to HCV proteins.

<table>
<thead>
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<th>CH2</th>
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<td>IFN-γ producing CD4+ T cells*</td>
<td>Number</td>
<td></td>
<td></td>
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<tr>
<td>Core</td>
<td>0.00</td>
<td>0.00</td>
<td>0.03</td>
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<td>0</td>
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<tr>
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<td>0.00</td>
<td>0.08</td>
<td>0</td>
<td>428</td>
</tr>
<tr>
<td>NS5</td>
<td>0.00</td>
<td>0.00</td>
<td>0.03</td>
<td>0</td>
<td>96</td>
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<table>
<thead>
<tr>
<th>Antigen</th>
<th>CL2</th>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IFN-γ producing CD4+ T cells*</td>
<td>Number</td>
<td></td>
<td></td>
<td></td>
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</table>

*: Expressed as percentage IFN-γ producing CD3+CD4+ T cells of total number of PBMC. Data for six hour stimulation with NS2 peptide pool not shown, no recombinant protein available for NS2.

Statistical analysis

All statistical tests were two-sided. A p-value ≤0.05 test was considered to be statistically significant. Non-parametric tests (Mann-Whitney (two groups) or Kruskal-Wallis (>2 groups)) were used to test differences between groups. All statistical analysis were performed using SPSS software version 15.

Results

General characteristics

To study factors associated with viral clearance and the influence of HIV infection heron, we examined HCV-specific CD4+ T-cell responses in 14 IDU with acute HCV infection in the presence or absence of acute HIV infection. The median interval between first HCV-RNA positive visit and visit at which PBMC were analyzed was 4.3 months (range 0-32.3 months). Six IDU cleared HCV and eight stayed chronically HCV infected. The three groups were comparable in age, CD4 count and genotype at the estimated moment of HCV infection. However, HCV-RNA load in group mono-CL was lower than in the other two groups (p=0.011) (Table 4.3.1A).

Furthermore, we examined HCV-specific CD4+ T-cell responses in five HCV-infected IDU before and after HIV seroconversion to evaluate the effect of HIV infection on earlier established HCV-specific CD4+ T-cell responses. The median interval between HCV and HIV infection was 2.4 years, 3/5 IDU had chronic HCV infection and were infected with HIV thereafter, 2/5 IDU first cleared HCV and got re-infected with HIV/HCV at approximately the same time and subsequently developed chronic co-infection (Table 4.3.1B). At the time of HIV infection all had HCV genotype 1 infection, except IDU CO-2.
who was infected with HCV genotype 3a. CD4 counts were all ≥450/mm$^3$ at the moment of acute HCV infection.

Comparison of different assays to measure HCV-specific T cells

To compare sensitivity of several assays to measure virus-specific proliferative and effector responses, we performed 6-hour, 6-day and 12-day stimulation assays. Direct ex vivo (6-hour stimulation) or after expansion (6 or 12 days) cells were restimulated to assess effector function with IFN-$\gamma$ as read-out. Due to limited availability of PBMC, these experiments were performed in two IDU (CL-2 and CH-2).

As shown in Table 4.3.2 and in line with previous experiments, after 6-hour stimulation or 6-day expansion and subsequent restimulation no HCV-specific response could be detected in these IDU as the percentage of IFN-$\gamma$-producing CD4$^+$ T cells in the peptide stimulated conditions did not exceed the percentage of IFN-$\gamma$ producing CD4$^+$ T cells in unstimulated conditions. However, after 12 days we could clearly distinguish an IFN-$\gamma$-producing CD4$^+$ T cell population (range 0.03-0.14% of total PBMC) which is HCV-specific, since there was no IFN-$\gamma$ production after restimulation with medium and co-stimulation alone (Table 4.3.2). Additional control experiments (including culture and restimulation of cord blood and PBMC from healthy blood-bank donors with HCV proteins, and culture with HCV-NS3 protein and restimulation with HCV-NS4 peptide pool) showed that the observed IFN-$\gamma$ production after 12 days in HCV-infected individuals indeed was HCV specific (data not shown). Because we show here that this assay is capable of detecting HCV-specific CD4$^+$ T cells in individuals that do not show HCV-specific T-cell responses after shorter stimulation and/or culture, we have used this assay to examine the effect of HIV co-infection on the quality and quantity of HCV-specific CD4$^+$ T-cell responses in IDU.

Next to IFN-$\gamma$ production after 12 days, we calculated the proliferation rate (see methods section). This estimate not only depends on the true proliferation of cells, but also on death of cells. At day 0, the proliferation rate is per definition one as the number of cells put into culture at that moment is the same as the number of cells recovered. After six days the median proliferation rate decreased to 0.26 and 0.22 for IDU CL-2 and IDU CH-2, respectively (Figure 4.3.1). After 12 days the proliferation rate had increased again. Interestingly, the proliferation rate in IDU CL-2 --able to spontaneously clear HCV-- increased to 0.74 in contrast to IDU CH-2 --not able suppress HCV-- in which the proliferation rate increased to 0.52 (Figure 4.3.1). These data indicate that most proliferation took place after the sixth day of culture (Figure 4.3.1).

Comparison of HCV-specific CD4$^+$ T-cell responses in acute HCV infection in the presence or absence of acute HIV infection

Using the assay based on 12-day expansion and subsequent restimulation, we measured HCV-specific T-cell responses in three groups of IDU. The total HCV-specific T-cell response was higher in IDU that cleared HCV infection (mono-CL, median 9,474 IFN-$\gamma$ producing CD4$^+$ T cells/million PBMC input) when compared to IDU with chronic HCV infection (mono-CH, median 2,223 IFN-$\gamma$ producing CD4$^+$ T cells/million PBMC input) or group IDU with HIV and HCV infection at approximately the same time (co-CH, median 2,749 IFN-$\gamma$ producing CD4$^+$ T cells/million PBMC input), although this did not reach statistical significance (Figure 4.3.2A). Interestingly, only 1/4 co-infected and none of the chronically infected IDU had responses against more than three proteins, in contrast to IDU who cleared HCV, of whom 4/6 had responses against more than three
proteins (Figure 4.3.2B). Furthermore, responses against Core protein tended to be lower in mono-CL than in mono-CH (p=0.087). All IDU in group mono-CL had higher T-cell responses against nonstructural proteins than against Core protein in the same IDU (Figure 4.3.1A). In contrast, in group mono-CH the responses against nonstructural proteins were lower than responses against Core protein in the same IDU (Figure 4.3.1A). Responses against NS3 protein tended to be higher in co-CH than in mono-CH (p=0.076). Responses against NS3 protein tended to be higher in mono-CL than in mono-CH (p=0.062), but not higher than in co-CH. Median responses against NS4 protein were higher in mono-CL then in the other groups, although this was not statistically significant. Responses against NS5 protein were significantly lower in co-infected IDU and in mono-CH than in mono-infected IDU who eventually clear HCV infection (mono-CL) (p=0.010 and p=0.019, respectively) (Figure 4.3.1B). Thus, HCV-specific CD4+ T-cell responses against nonstructural proteins tended to be higher in mono-CL when compared to mono-CH and co-CH. And of note, IDU with acute HIV/HCV co-infection showed no to very limited HCV-specific responses to NS5.

In Figure 4.3.3a, the individuals who contract HIV and HCV at approximately the same time (CO-1, CO-2, CO-3 and CO-4) are shown in more detail including their injection drug use. It can be seen that injection drug use and HIV and HCV seroconversion coincide. In the second panel, log10 HCV and HIV viral load are shown, HCV load is always higher than HIV load. In the third panel, the decline of the CD4 count is shown in relation to the moment of HIV seroconversion. The CD4 count drops to $\leq 350$ cells/mm$^3$ in varying time periods (range 0.62-10.4 years). In the lowest panel HCV-specific CD4+ T-cell responses are shown.

![Figure 4.3.1](image-url)  
Figure 4.3.1  
Proliferation rate as measured directly ex vivo and after six or twelve days culture in the presence of HCV peptidepools (ex vivo) or proteins (for 6-day or 12-day culture) in patients CL2, who recovers spontaneously from HCV infection and CH2, who develops persistent HCV viremia. The proliferation rate was calculated as the number of cells recovered out of culture on day 6 or 12 divided by the number of cells put into culture on day 0. Therefore the proliferation rate for direct stimulation is per definition 1.
Figure 4.3.2A Total number of HCV-protein-specific IFN-γ producing CD4⁺ T cells/million PBMC input as measured by intracellular cytokine staining after 12 days culture with IL-2 and HCV recombinant proteins in injecting drug users (IDU) with acute HCV infection in the presence or absence of acute HIV infection. Abbreviations: CO=IDU with HIV and HCV infection at approximately at the same time; CL=IDU with acute HCV mono-infection who clear HCV infection; CH=IDU with acute HCV mono-infection who develop chronic HCV infection.

Figure 4.3.2B Number of HCV-protein specific IFN-γ producing CD4⁺ T cells/million PBMC input as measured by intracellular cytokine staining after 12 days culture with IL-2 and HCV proteins in injecting drug users (IDU) with acute HCV infection in the presence or absence of acute HIV infection. Abbreviations: CO=IDU with HIV and HCV infection at approximately at the same time; CL=IDU with acute HCV mono-infection who clear HCV infection; CH=IDU with acute HCV mono-infection who develop chronic HCV infection.
Interestingly, CO-1 and CO-2 were previously able to clear HCV infection in the absence of HIV infection (clearance indicated with *). However, they were not able to clear HCV infection again in the presence of HIV infection. After HIV infection, we observed higher Core responses (2,629 and 934 IFN-γ producing CD4+ T cells/million PBMC input) compared to CO-3 and CO-4 (0 and 245 IFN-γ producing CD4+ T cells/million PBMC input; Figure 4.3.2B). In contrast, despite the high responses directed against NS3 observed in CO-3 and CO-4, these IDU were not able to control HCV viremia (Figure 4.3.2B and 4.3.3). These data suggest that the influence of HIV co-infection on the HCV-specific T-cell response depends on the order and outcome of precedent HCV infection(s).

HCV-specific CD4+ T-cell responses before and after HIV infection

To study the direct influence of acute HIV co-infection on HCV-specific CD4+ T-cell responses, we studied T-cell responses longitudinally in five IDU. Two IDU from group co-CH were included, CO-1 and CO-2, and three additional IDU, CO-5, CO-6 and CO-7 (Figure 4.3.3A and 4.3.3B). The latter three IDU became HIV infected after having contracted earlier chronic HCV infection. PBMC ranging from 1.9 to 7.6 months before and 1.9 to 13.3 months after HIV seroconversion were analyzed. In IDU with a prior chronic HCV infection, we observed lower HCV-specific CD4+ T-cell responses (537, 623 and 1174, respectively) compared to IDU who were able to clear an earlier HCV infection 3114 and 3745 CD4+ T cells, respectively (Figure 4.3.1C). Interestingly, in IDU previously able to clear HCV infection, we observed higher responses against Core protein after HIV infection, suggesting that HIV infection negatively influences HCV-specific CD4+ T-cell responses by skewing the response away from nonstructural proteins. However, in acute co-infection without evidence of an earlier HCV infection, we do not observe this skewing of the HCV-specific T-cell response towards Core protein, suggesting that the effect of HIV is not straightforward, but is influenced by the host's history of previous HCV infection. After HIV seroconversion we observed a decline of responses against nonstructural proteins in 4/5 IDU and a decline in overall height of HCV-specific CD4+ T-cell responses in 3/5 IDU (Figure 4.3.3).
Figure 4.3.3  IDU with documented HIV and HCV seroconversion in whom HCV-specific CD4⁺ T-cell responses were studied longitudinally. Grey vertical line represents estimated HCV infection, grey dotted vertical line represents estimated HIV seroconversion. In the upper panel injection drug use is represented by gray bars. In the second panel HIV viral load (in copies/ml, indicated by dotted line and grey dots) and HCV viral load (in IU/ml, indicated black line and white dots) are shown on a log scale. In the third panel CD4 count is shown (10⁸ cells/mm³) over calendar time. In the last row CD4⁺ T-cell responses as measured by 12-day culture and subsequent restimulation are shown.
Figure 4.3.3 Part 2.
Figure 4.3.3 Part 3.
Discussion

High and broad HCV-specific CD4^+ T-cell responses during acute HCV infection have been associated with viral clearance.6,8,21,22 We also observed that mono-infected IDU who cleared HCV infection tended to have higher CD4^+ T-cell responses and responses against a larger number of proteins than IDU that develop chronic HCV viremia in the presence or absence of HIV infection. Interestingly, most IDU in this study that cleared HCV infection had NS3-specific memory CD4^+ T-cell responses, while only one IDU from the mono-CH group had a NS3-specific memory CD4^+ T-cell response. NS3 has been described as highly conserved and immunodominant and responses against NS3 are associated with viral clearance;23-25 however two IDU that were simultaneously infected with HCV and HIV were not able to clear HCV despite high NS3 responses. In addition, we observed a striking absence of T-cell responses against NS5 protein --specifically targeted in IDU that cleared infection-- in IDU with acute HIV/HCV co-infection that develop chronic HCV infection and also in mono-infected IDU that develop chronic HCV viremia. NS5 is a variable region of HCV, and development of chronic infection might be due to the inability to raise T-cell responses against NS5. A possible explanation for this may be that the primary responses against NS5 cannot be maintained and chronic HCV infection develops because of escape mutations away from the original NS5 sequence.26

Higher responses against Core protein were observed in acute phase of infection in IDU that were not able to spontaneously control HCV replication. This suggests that responses against Core are either associated with a less favourable outcome of infection or with exposure to higher levels of Core. HIV co-infection is associated with higher HCV viral load and HCV viral load is associated with the amount of Core protein in serum.15,24,27-29 Also in IDU previously able to clear HCV, we observe an increase of Core response and a decrease of responses against nonstructural proteins after HIV seroconversion indicating that HIV infection influences HCV-specific T-cell responses in an unprofitable way. In IDU that were chronically HCV infected before HIV seroconversion, we did not observe similar kinetics. This could be due to repeated exposure to HCV at the moment of infection with HIV, evoking HCV-specific memory CD4^+ T-cell responses next to development of new strain-specific T-cell responses. Alternatively, virus characteristics might explain these findings.

In contrast to the findings by Lauer et al., who describe the absence of proliferative CD4^+ T-cell responses against HIV and HCV in chronically co-infected individuals,30 we observed low --but measurable-- T-cell responses in acutely co-infected patients who later develop chronic infection. This may be explained by the fact that in contrast to Lauer et al. we analyzed PBMC shortly after HCV infection and not in the later course of chronic infection, when it has been shown that both proliferative responses and the capacity to secrete IFN-γ are reduced.30 Even more, ongoing antigenic exposure has been shown to diminish antigen-specific T-cell responses and for example during HAART the HIV-specific proliferative responses were shown to increase again.31,32 An alternative explanation may be a difference in sensitivity of the assay used. We show that the used assay can pick up HCV-specific responses in individuals that do not show IFN-γ production after direct ex vivo stimulation or after restimulation following six days culture. The difference in sensitivity of the assays can be explained by the assumption that cells that are not HCV-specific will die in the first days of culture, while HCV-specific cells will survive and start to proliferate. After addition of extra antigen on day 6 in the
presence of IL-2 HCV-specific cells will expand further and differentiate to an effector phenotype capable of IFN-γ production upon re-encounter with antigen. Although our study was performed in only a limited number of patients, they present an exceptional chance to study the role of HCV-specific CD4+ T cells in acute HCV infection, because of the prospective sampling which was independent of clinical symptoms. This is very different from patients sampled after presentation at a clinic weeks to months after the start of clinical symptoms, which could introduce a delay in sampling and selection bias, since clinical symptoms have been associated with a more vigorous immune response. Indeed, Thimme et al. described five health care workers that were sampled prospectively from the moment of exposure and found that the symptomatic infected patient who cleared HCV had vigorous T-cell responses. In conclusion, in this small group of IDU we show that HCV mono-infected IDU who clear HCV infection have higher memory CD4+ T-cell responses against NS5 protein compared to IDU that develop chronic HCV infection in the presence or absence of HIV during acute infection. Furthermore, we show that the effect of HIV infection on HCV-specific CD4+ T cells in IDU is dependent on the history and outcome of previous HCV infection. To enable a clearer understanding of the complex interaction between HIV and HCV and their corresponding immune responses, future studies should address this issue with a focus on prospectively identified infected individuals --preferably with information on previous exposure to HCV and/or HCV infection-- to minimize selection bias towards those with clinical symptoms.
References


Chapter 4.4

Detectable HCV-specific T cells in men having sex with men years before manifest HCV infection

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

Manuscript in preparation.
Introduction

Since 2000 there have been reports on outbreaks of acute hepatitis C virus (HCV) in HIV-infected men having sex with men (MSM).\textsuperscript{1,4} Phylogenetic analyses indicate that there has been introduction of multiple HCV strains, and that the introductions have occurred between 1995 and 2000.\textsuperscript{1,2} These ongoing epidemics are becoming a major public health problem.\textsuperscript{5} Adaptive immunity plays a major role in spontaneous viral clearance of HCV, however the exact correlates of protection are still unknown.\textsuperscript{6} HIV coinfection and probably the weakening of the immune system, is associated with loss of control of viral load and with lower HCV-treatment success rates.\textsuperscript{7,8} Here, we examined HCV-specific CD4\textsuperscript{+} T-cell responses in three HIV-infected MSM before HCV-antibody seroconversion and subsequent chronic HCV infection took place. Unexpectedly, HCV-specific T-cell responses were already present years before HCV seroconversion in all three MSM, indicating that these individuals had been exposed to HCV, but did not get (chronically) HCV infected until years later. These findings have implications for our understanding of correlates of protection and for future vaccine development.

In risk groups with parenteral exposure risk to HIV and HCV (like drug users (DU)), HCV usually precedes HIV infection. In the recently described HCV outbreaks among MSM mainly HIV-infected individuals are affected. HCV-specific T-cell responses seem to develop differently in the context of HIV co-infection, and it has been suggested that the development of the T-cell response is hampered by HIV co-infection.\textsuperscript{6} Previously, we and others have reported on HCV-specific T-cell responses in HIV-uninfected DU before actual HCV infection occurred, suggesting previous exposure to HCV.\textsuperscript{10-13} We were interested whether such exposure without apparent infection also occurs in MSM before they acquire HCV infection through sexual transmission. This would be unexpected, since the HCV prevalence and incidence in injecting DU populations is very high, while --until recently-- in MSM prevalence and incidence remained low.\textsuperscript{14} Therefore, the probability of exposure to HCV per ‘risky’ behaviour is much higher in DU than in MSM (e.g., sharing needles or syringes vs. unprotected high-risk sexual behaviour) and therefore also the probability of identifying multiple exposed uninfected individuals. Furthermore, most individuals were already HIV-infected at time of HCV seroconversion and it has been shown that HIV co-infection negatively influences HCV-specific T-cell responses.\textsuperscript{9,15,16}

Materials and Methods

Study population

In the ACS among MSM, eight HCV seroconverters in HIV-infected MSM were identified before 2003.\textsuperscript{1,17} From these men, three had cryopreserved peripheral blood mononuclear cells (PBMC) available before HCV seroconversion (MSM 1, 2, and 3).

HCV antibody, RNA and genotyping

HCV seroconversion was defined as occurrence of HCV antibodies in a previously negative individual. HCV antibodies were detected using a third generation EIA system (AxSym HCV version 3.0; Abbott, Wiesbaden, Germany). RNA detection and genotyping based on phylogenetic analysis was performed as described in Van de Laar et al.\textsuperscript{1}
HCV peptide pools

Synthetic peptides (14-18-mers, overlapping by 11 amino acids; Mimotopes, Australia) were dissolved in DMSO and then pooled. The peptides were divided in six pools, corresponding to core, NS2, NS3, NS4, NS5A and NS5B regions of HCV. The peptides were dissolved in such a way that the final concentration of each individual peptide was 1 mg/ml in the pool.

HCV-specific T cell expansion

Since the frequency of HCV-specific T cells direct ex vivo is very low, we use an assay for detection of HCV-specific T-cell responses based on expansion of these cells for 12 days in the presence of recombinant interleukin-2 (IL-2) and peptide pools. In short, cryopreserved PBMC were thawed, washed and counted. After that cells (2*10^6 cells/ml) were put into culture in a 96-wells plate for 12 days in the presence of overlapping HCV peptide pools and IL-2 (360 IU/ml). On day six extra peptide pool was added. On day two, six and eight extra IL-2 was added.

Detection of IFN-γ producing HCV-specific T cells after (re-)stimulation

IFN-γ-producing CD4^+ T cells were enumerated using intracellular cytokine staining (ICCS). Briefly, after 12-day expansion cells were pooled, washed and counted. PBMC were stimulated for six hours with HCV Core, NS2, NS3, NS4, NS5A and NS5B peptide pools (2 μg/ml) and αCD28 (2 μg/ml) and αCD49d (2 μg/ml) as co-stimuli. After one hour 1:1,000 Brefeldin A (Golgiplug, BD Biosciences (BD), San José, California, USA) was added. As negative control, PBMC were stimulated with medium and co-stimulation alone. As 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 fluorescently labelled monoclonal antibodies specific for CD3-pacific blue, CD4-PE-Cy7, CD8-APC-Cy7 and IFN-γ-FITC (CD3, CD4 and CD8 monoclonal antibodies from eBioscience, IFN-γ-FITC from BD). After fixation (Cellfix, BD) at least 25,000 events were acquired on an 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^+ or CD8^+ T cells, which was calculated by subtracting the percentage of IFN-γ producing T cells in medium from that in peptide pool restimulated conditions. Next to the percentage of IFN-γ-producing T cells, 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 effector 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 input. This number is a combination of (memory) cells initially present in the PBMC pool and the ability of these cells to survive, proliferate and differentiate to (IFN-γ-producing) effector T cells during the 12-day culture period. The observed response after expansion and restimulation is HCV-specific since we do not observe IFN-γ production after restimulation with medium. Furthermore, when expanding PBMC from a healthy (HCV-negative) blood bank donor or cord blood, expansion is less and we do not observe IFN-γ production. Also when expanding PBMC from HCV-infected individuals using peptide pool corresponding to the NS3 region of the
HCV genome and restimulating with NS4 peptide pool no IFN-γ production is observed (data not shown).

Results

In the Amsterdam Cohort Study among MSM, eight individuals with HCV seroconversion during follow up were identified by Van de Laar et al.\textsuperscript{1} Of these eight individuals, three had PBMC available before HCV seroconversion. To see whether HCV-specific T-cell responses were present in these men, we measured memory function of HCV-specific T cells approximately one year before HCV seroconversion. General characteristics of the MSM are shown in Table 4.4.1. The median age was 33 years, and the median duration of HIV infection at the time of HCV infection 5.3 years. Although the men were HIV-infected for some time, CD4\textsuperscript{+} T cell numbers were relatively high at time of HCV seroconversion (median 580 cells/ml). MSM 1 and 2 were on different regimens of HAART and HIV-1 viral load was undetectable in MSM 2 (Table 4.4.1). The presence of HCV-specific CD4\textsuperscript{+} and CD8\textsuperscript{+} T-cell responses was studied using a sensitive assay for detection of antigen-specific responses with a low frequency ex vivo. This assay is based on in vitro expansion of antigen-specific T cells in the presence of overlapping peptide pools and IL-2 and subsequent restimulation with peptide pools (see methods section).

To our surprise, we observed both CD4\textsuperscript{+} and CD8\textsuperscript{+} HCV-specific T-cell responses in all three MSM. In Figure 4.4.1, a representative dotplot of HCV-specific IFN-γ production after 12 days expansion is shown.

![Figure 4.4.1](image-url) Representative dot plot of IFN-γ production after restimulation with Core peptide pool. HCV-specific T cells were first expanded for 12 days in the presence of overlapping HCV peptide pools and interleukin-2. After that cells were pooled, washed, counted and rested overnight. On the next day cells were restimulated with co-stimulation and a. medium, b. PMA/IONOMycin or c. corresponding peptide pool.
Table 4.4.1 Univariate associations between general characteristics, drug use characteristics, sexual risk behaviour characteristics, and HIV and HCV seroconversion among DU in the ACS.

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<th>Age at first positive sample (years)</th>
<th>Duration HIV infection (years)</th>
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Restimulation with Core peptide pool or PMA/ionomycin and co-stimulation (α-CD28 and α-CD49d) reveals clear IFN-γ production by both CD4⁺ and CD8⁺ T cells after 12 days expansion, whereas there is no IFN-γ produced after restimulation with medium and co-stimulation. Although there is a large variation in the magnitude of both CD4⁺ (MSM 1: 1,046; MSM 2: 8,088; and MSM 3: 1,243 IFN-γ producing CD4⁺ T cells/million PBMC) and CD8⁺ T-cell responses (MSM 1: 1,319; MSM 2: 14,133; and MSM 3: 643 IFN-γ producing CD8⁺ T cells/million PBMC) (Figure 4.4.2a and 4.4.2b), the CD4⁺ T-cell response in MSM 1 and 3 is comparable to the response after seroconversion in 4 HIV-infected MSM with acute HCV co-infection we described earlier (range 1527-3723, Figure 4.4.3).\(^1\)

In contrast, in MSM 2 we observed a much higher response that is comparable to HCV-specific T-cell response in HIV-uninfected individuals with acute HCV (range 93-74197 IFN-γ producing CD4⁺ T cells/million PBMC).\(^1\)

Interestingly, we observed high Core-specific CD4⁺ T-cell responses in MSM 1 and MSM 2 (37.2% and 32.9% of the total HCV-specific CD4⁺ T-cell response, respectively), although these responses have been associated with viral persistence after HCV seroconversion. Possibly this Core-specific response is triggered by repeated exposure to HCV. Unfortunately we do not have additional information on sexual risk behaviour (as a proxy for continuing exposure to HCV) in these MSM.

Figure 4.4.2  A: HCV-specific CD4⁺ T-cell responses expressed as the number of IFN-γ producing CD4⁺ T cells/million PBMC input. B: HCV-specific CD8⁺ T-cell responses expressed as the number of IFN-γ producing CD8⁺ T cells/million PBMC input.
HCV-specific T cells in HIV-infected MSM

Discussion

HCV-specific T-cell responses have been reported previously in persistently antibody and RNA negative individuals that were exposed to HCV sexually. Also HIV-specific T-cell responses before infection have been described in MSM and Kenyan female sex workers with high-risk sexual behaviour who appeared resistant to HIV infection.\textsuperscript{20-22} We can only speculate on the reason why the enormous increase in HCV prevalence in HIV-infected MSM occurred now (recently an HCV prevalence of 18% was found in HIV-infected MSM in a cross-sectional study from the Amsterdam outpatient clinic for sexually transmitted infections)\textsuperscript{5}, while HCV has always circulated at a low level in the MSM population. There are different potential explanations for the recent outbreaks of HCV, and most likely it has been a combination of the following options. Biological explanations include the simultaneous increase in prevalence of ulcerative sexually transmitted infections (e.g., lymphogranuloma venereum (LGV), syphilis) which might facilitate HCV transmission by breaking down the mucosal barrier of the gut. It has also been described that shortly after HIV infection, CD4\textsuperscript+ T cells are depleted especially in the gut. Furthermore, introduction of more virulent HCV strains or HCV strains that are more easily transmitted sexually might contribute to the rapid spread of HCV, although this seems less likely since there have been introductions of multiple strains at...
approximately the same point in time.\textsuperscript{4} In addition, a change in risk behaviour might contribute to the increased incidence of HCV. Interestingly, some HIV-infected individuals have a very slow HCV antibody seroconversion, and HCV-specific T cells might be triggered long before HCV antibody seroconversion.\textsuperscript{23} Although most individuals described by Thomson \textit{et al.} had detectable levels of HCV viremia, there was an association between height of viremia and time to seroconversion with individuals with lower viral load taking longer to seroconvert. So it is possible that some individuals in our study had very low level HCV viremia --as has been described for HIV in high-risk seronegative MSM-- inducing an antigen-specific T-cell response but not (yet) an antibody response.\textsuperscript{23,24} Larger cohorts of HIV-infected individuals at risk for HCV infection are needed to further disentangle factors associated with protection from HCV and the role of HCV-specific T-cell responses before HCV-antibody seroconversion. Furthermore, identification of additional patients like those described here may enhance our understanding on why the outbreak of sexually transmitted HCV occurred since 2000.
References


15. van den Berg CHSB, Ruys TA, Nanlohy NM, van de 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.


