

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

T. A. Ruys¹, N. M. Nanlohy², C. H. S. B. van den Berg^{2,3}, E. Hassink¹, M. Beld⁴, T. van de Laar³, S. Bruisten³, F. Wit¹, A. Krol³, M. Prins³, J. Lange¹ and D. van Baarle² ¹Department of Internal Medicine, Division of Infectious Diseases, Tropical Medicine and AIDS, International Antiviral Therapy and Evaluation Center (IATEC) and Academic Medical Center, Amsterdam; ²Department of Immunology, University Medical Center Utrecht, Utrecht; ³Cluster of Infectious diseases, Department of HIV and STI research, Municipal Health Service, Amsterdam; and ⁴Department of Microbiology, Division of Virology, Academic Medical Center, Amsterdam, The Netherlands

Received August 2007; accepted for publication November 2007

SUMMARY. In order to understand the parameters associated with resolved hepatitis C virus (HCV)-infection, we analysed the HCV-specific T-cell responses longitudinally in 13 injecting drug-users (IDUs) with a prospectively identified acute HCV infection. Seven IDUs cleared HCV and six IDUs 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 interferon- 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 nonstructural proteins were associated with resolved HCV infection. Interestingly, observation of *memory*-T-cell responses present before the documented HCV-seroconversion suggests that reinfections in IDUs occur often. The presence of these responses however, 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 nonstructural 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.

Keywords: hepatitis C virus, human, injecting drug-user, T cells.

INTRODUCTION

Hepatitis C virus (HCV) is a positive-stranded RNA virus, which is mainly transmitted through blood-contact [1]. HCV infection persists in a 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 (IDUs).

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 on account of which the immune response fails to control the virus remains, however, unclear. Because, the 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 to the HLA A2 haplotype [7,9,14,15]. It is likely that this approach underestimates the complete T-cell immune response [16].

Abbreviations: ACS, Amsterdam Cohort Studies; EDI, estimate the date of infection; HCV, hepatitis C virus; ICCS, intracellular cytokine staining; IDU, injection drug-users; IFN, interferon; PBMC, peripheral blood mononuclear cell.

Correspondence: Dr D van Baarle, Department of Immunology, UMCU, Lundlaan 6, 3584 EA Utrecht, The Netherlands. E-mail: d.vanbaarle@umcutrecht.nl

To study why some HCV-infected IDUs fail to clear the virus, we analysed the HCV-specific immune response between IDUs with a prospectively identified acute HCV infection, who either eventually resolved the HCV infection 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], within the period needed either to resolve or 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 accor-

dance 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 IDU and other risky behaviours. In addition, blood is drawn for prospective HIV-testing and storage of peripheral blood mononuclear cells (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 1). Seven of these 13 subjects resolved acute HCV infection and six remained chronically infected. During follow-up, two of

Table 1 Clinical and laboratory characteristics

Subject (pubID)	Subject study ID	Age at EDI (years)	SEX	Total Follow-up (months)	Follow-up since EDI (months)	Interval of HCV-RNA conversion (months)	HCV genotype	Resolving phase (months)	HLA-class I A/B alleles
19854	R1	38	M	171	146	3.5	3a	14.6	A3,25 B 27, 51
18915	R2	37	F	110	15	4	1a	4.2	A2 B 44, 62
16991	R3	26	F	137	41	4	3a	4.0	A2,3 B7,60
16994	R4	29	F	56	46	4	1a	6.0	A24,34 B49,53
18885	R5	24	M	182	171	4	1a	4.8	A26,66 B27,41
18787	R6	26	M	119	108	5	3a	24.2	A30,68 B18,62
12905	R7	28	M	183	142	3.5	1a	3.6	A3,29 B 44,55
18898	C1	34	F	177	154	3	1a	Chronic	A1,28 B7,27
18886	C2	20	F	146	139	5	1a	Chronic	A2,32 B14,44
18877	C3	34	M	165	142	3.5	3a	Chronic	A2,28 B7,8
16941	C4	25	F	137	99	3.5	3a	Chronic	A24 B7,60
12970	C5	28	F	153	134	39	3a	Chronic	A2,23 B49,53
19927	C6	27	F	143	100	23	1a	Chronic	A2,3 B7

EDI, estimated date of infection; R, resolver; C, chronic.

seven resolvers became reinfected with HCV and HIV-1, after which they remained chronically HCV-HIV coinfectd. 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 performed. Examination of the medical files did not show any indication of symptomatic acute HCV-infection.

Definitions

Hepatitis C virus 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, 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 third-generation Enzyme Immunoassay; EIA 3.0 Abbot Laboratories, Abbott Park, IL, USA).

Estimated date of infection 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 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 [21] and calculated as the time elapsed between EDI and the midpoint between the last positive and first negative HCV-RNA time point.

HCV genotyping

The 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.* [22]. Genotypes were confirmed by sequencing part of the NS5B region of the HCV genome [23].

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 (aa) (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 μ g of total peptide pool mix (each peptide present in a representative amount, i.e. in a concentration of 1 μ g 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, Emeryville, CA, USA).

PBMC separation and storage

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

Elispot assay for single cell IFN- γ release

Interferon- 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, Sweden) and streptavidin poly-HRP from Sanquin (Amsterdam, The Netherlands) [24]. PBMC were stimulated directly *ex vivo* in triplicate wells at 1×10^5 cells/well in the absence or presence of 1 μ g/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, The Netherlands) and counted using the A.EL.VIS automated spot analyser (Sanquin Reagents, Amsterdam, the Netherlands). 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 the measurement of effector function. To expand HCV-specific CD4⁺ T cells, 3×10^6 PBMC were cultured for 12 days as previously described [17,25] 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 2×10^5 PBMC/well in 100 μ l medium in 96-well round bottom plates, at 37 °C and 5% CO₂. Protein (2 μ g/mL) was added on days 0 and 6. Interleukin-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, the cells were restimulated for 6 h 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 restimulation

Interferon- γ -producing cells after restimulation with overlapping peptide pools were enumerated by intracellular cytokine staining (ICCS) [26,27]. As higher number of IFN- γ -producing cells are to be expected after expansion than directly *ex vivo*, ICCS was chosen for better visualization. Briefly, 10^6 PBMCs were stimulated for 6 h with HCV Core / envelope (also including E1,E2 and p7 peptides), NS3, NS4 and NS5 peptide pools (at 2 μ g/mL) and both α CD28 (2 μ g/mL) and α CD49d (1 μ g/mL) as costimuli, in the presence of 1:1000 Brefeldin A (Golgiplug, BD Biosciences, San José, CA, USA). As a negative control, PBMCs were stimulated with medium and co stimulation alone. As a positive control PBMCs were stimulated with 10 ng/mL phorbol myristate acetate and 2 μ g/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 analysed using the software program CELL QUEST (BD). Responses were scored as positive when 2 \times above the medium control value and expressed as the % 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:
$$\left(\frac{\text{Number of cells grown out}}{\text{Number of input cells}} \right)$$

$\times \% \text{IFN-}\gamma \times 10.000 = \text{number of cells per } 10^6 \text{ input PBMC}$. As this calculation takes into account all variables potentially influencing the end result, it results in a more reliable number than just the % of responding cells after 12 days as previously shown [17].

Nonaddition of either 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 restimulation 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 performed 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 IDUs with documented onset of HCV-infection (Table 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 1) Six of 13 subjects remained chronically infected as evidenced by the presence of high levels of HCV-RNA plasma concentrations >2 years (Fig. 1b). After the EDI, resolvers had lower HCV-RNA concentrations (median 3.53 log₁₀, range 2.78–6.9) than chronic carriers (median 5.57 log₁₀, range 4.34–6.12) ($P = 0.051$, Fig. 1). Onset of HCV infection was always associated with self-reported injecting drugs-use (Figs 1a,b). In most cases IDU continued during follow-up, which might be leading to re-exposure 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, the resolvers and the chronic carriers, comparable, we have measured T-cell responses in chronic carriers within a comparable time span (within the first 2 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 (Fig. 1).

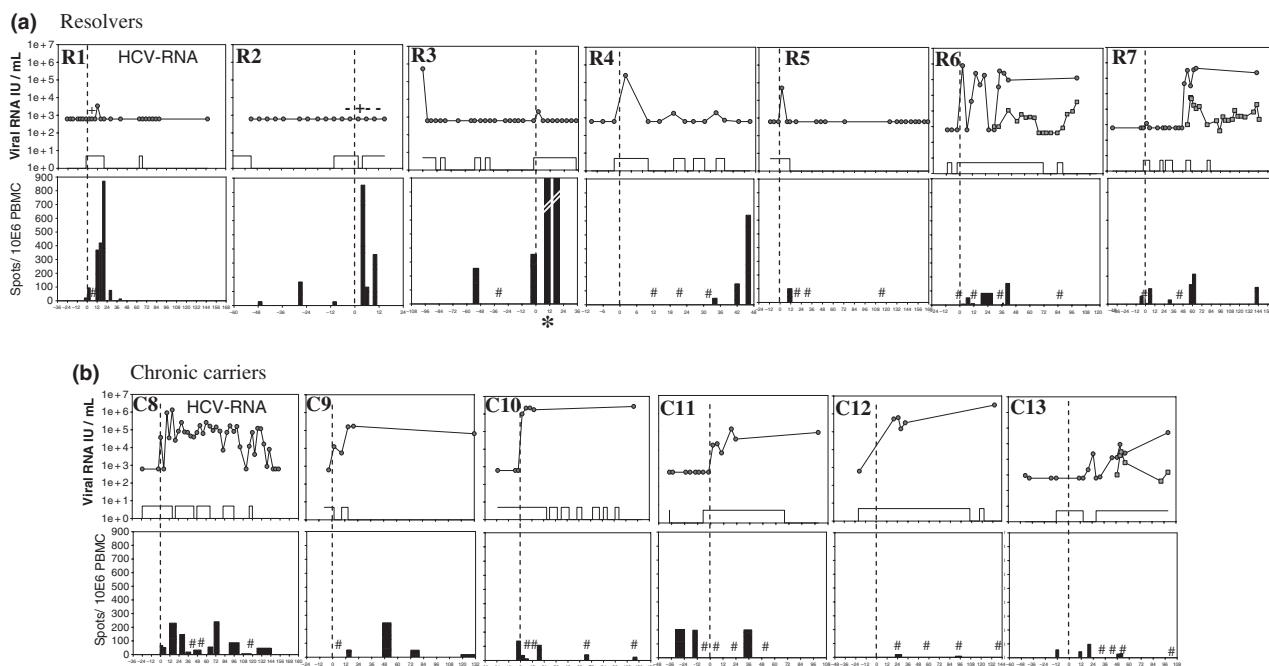


Fig. 1 Follow-up of injecting drug-use (IDU) and HCV-specific T-cell responses in 13 IDUs. Follow-up (months), relative to estimated date of HCV infection at $t = 0$ months (vertical dashed line) is shown for seven resolvers (R1–R7, a) and 6 chronic carriers (C1–C6, b). Resolvers R6 and R7 became chronic HCV-carriers after reinfection 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 IDUs 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 <50 spots/10⁶ PBMC or undetectable are indicated with # on the x -axis (A,B lower panels).

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 (Figs 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) ($P = 0.035$) (Fig. 2a). At that time point, a corresponding HCV-RNA measurement could be performed in six of seven resolvers and all of them had plasma concentrations below 615 IU/mL (Fig. 1a).

Next, we identified the specific antigens/peptide pools that were targeted. Resolvers targeted a median of five antigens, (range 3–6) while chronic carriers targeted a median of 2 antigens, (range 0–5), suggesting that a larger breadth of the T-cell response was associated with resolved infection (Fig. 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 Fig. 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) (Fig. 2b).

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

Hepatitis C virus-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 [17,28]. Representative FACS plots of HCV-specific CD4⁺ T-cells in one chronic carrier (Fig. 3a) and one resolver (Fig. 3b) after 12-day expansion with HCV proteins and restimulation 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

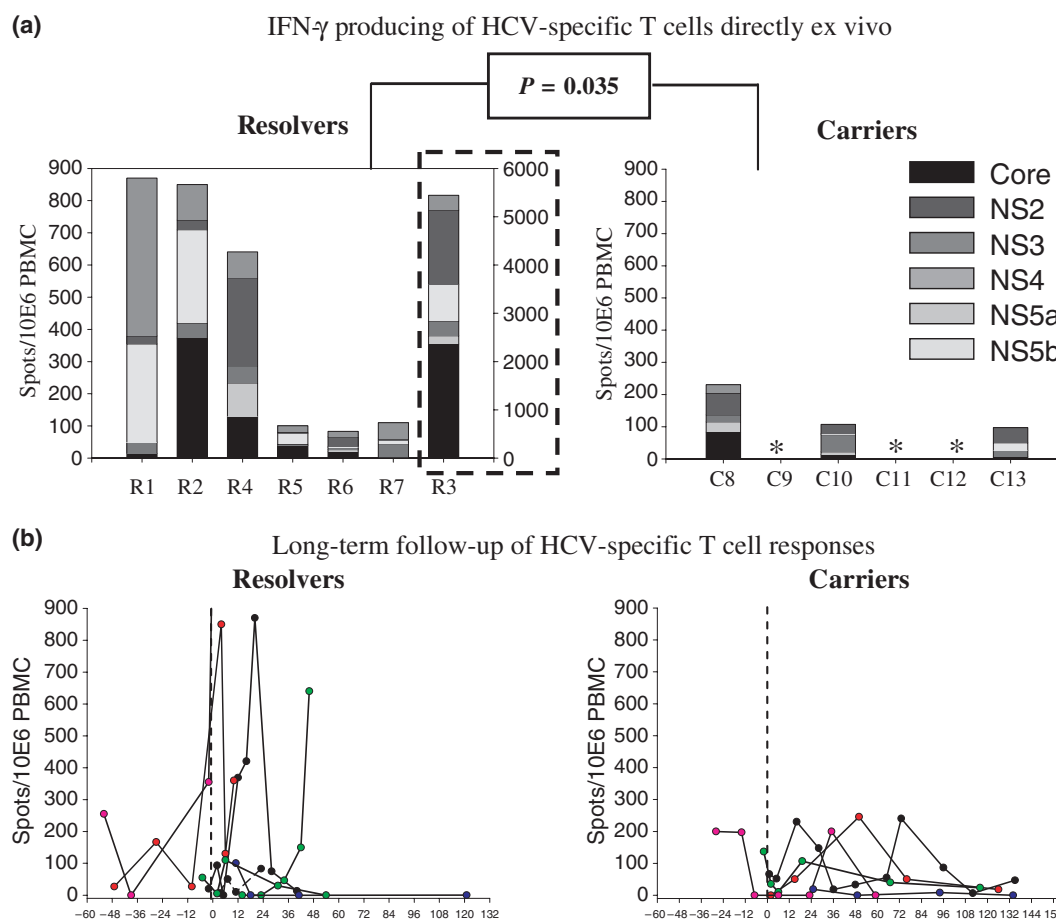


Fig. 2 HCV-specific T-cell responses. The number of IFN- γ -producing T cells, as measured by IFN- γ Elispot assay after stimulation with overlapping HCV peptide pools, are shown for 7 resolvers (a, left panel) and 6 chronic carriers (a, right panel) during acute HCV-infection. The T-cell response for 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/10E6 PBMC are indicated with * (right panel). The kinetics of HCV-specific T-cell responses during long-term follow up (months) is shown for resolvers (b, left panel) and chronic carriers (b, right panel). The vertical dashed line at $t = 0$ indicates estimated date of HCV infection.

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. This number of IFN- γ -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 *in vitro*.

Although higher HCV-specific CD4 $^{+}$ T-cell responses were observed in resolvers (median 10663 IFN- γ producing CD4 $^{+}$ T-cells/million PBMC, range 93–74 197) than in chronic carriers (median 2604 IFN- γ producing CD4 $^{+}$ T-cells/million PBMC, range 1278–15 730), the differences were not significant ($P = 0.534$) (Fig. 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- γ producing cells/million PBMC) and in five chronic carriers (median 3844 IFN- γ producing cells/million PBMCs, range 484–25652) (data not shown).

Resolvers have low CD4-Core responses and high CD4-NS protein responses

We analysed 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 2). However, the strength of the responses against the separate proteins (Core vs NS proteins) was different between resolvers and chronic carriers (Fig. 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). ($P = 0.008$) (Fig. 4c).

In resolvers, the highest CD4 $^{+}$ T-cell response measurable was targeted against a cluster of two or three nonstructural proteins and in one (R7) against a single nonstructural

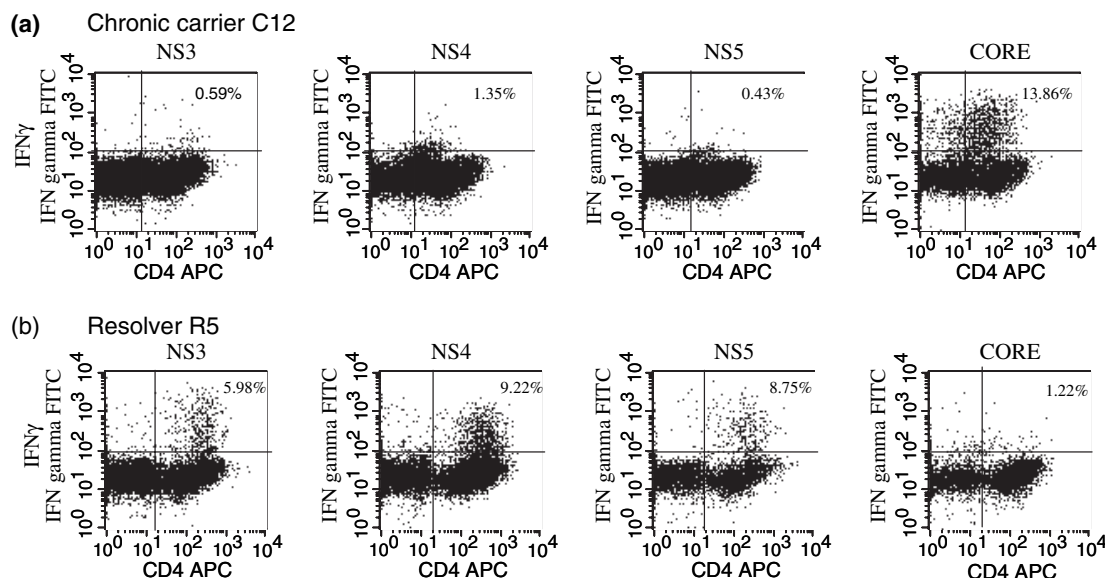


Fig. 3 HCV-specific CD4⁺ T-cell responses after a 12-day expansion period. Representative FACS plots of the percentage of HCV-specific IFN- γ -producing CD4⁺ T cells after 12-day expansion with NS3, NS4, NS5 or Core in a representative chronic carrier (C12, panel a) and resolver (R5, panel b). The percentage of CD3⁺ CD4⁺ T-cells-producing IFN- γ (y-axis) upon 6-h stimulation 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 % of IFN- γ -producing CD4⁺ T cells is shown.

protein (NS3) (Table 2). In contrast, Core protein was the most or second most dominantly targeted protein in four of six chronic carriers (Table 2). Noteworthy, a chronic carrier (C8) who generated a relative strong CD4⁺ T cell response (8285 IFN- γ producing cells/million PBMCs) against non-structural proteins (98% of total response) (Table 2) during the early phase of chronic infection (Fig. 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- γ producing cells/million PBMCs). However, the quality of the CD4⁺ T-cell response had changed by increasing the Core protein response from 2% to 22% (Fig. 4b, right panel), with a concomitant decrease against the nonstructural proteins (data not shown).

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 months -27, -52, -5, -14 respectively. A relative long interval before EDI was chosen 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 55–255 and in the chronic carrier 200 spots/million PBMC). After onset of infection, HCV-specific T-cell response increased in resolvers (varying from two to 20-fold) but decreased to undetectable levels in the chronic carrier during the same time

span (Fig. 1). These easily detectable T-cell responses before EDI coincided with self-reported IDU in most IDUs, but HCV-RNA remained undetectable (Figs 1a,b). Noteworthy, in resolver R3 the T-cell response became undetectable when IDU had stopped during the period before EDI (Fig. 1a).

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 PBMCs), 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 (Fig. 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 (Fig. 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

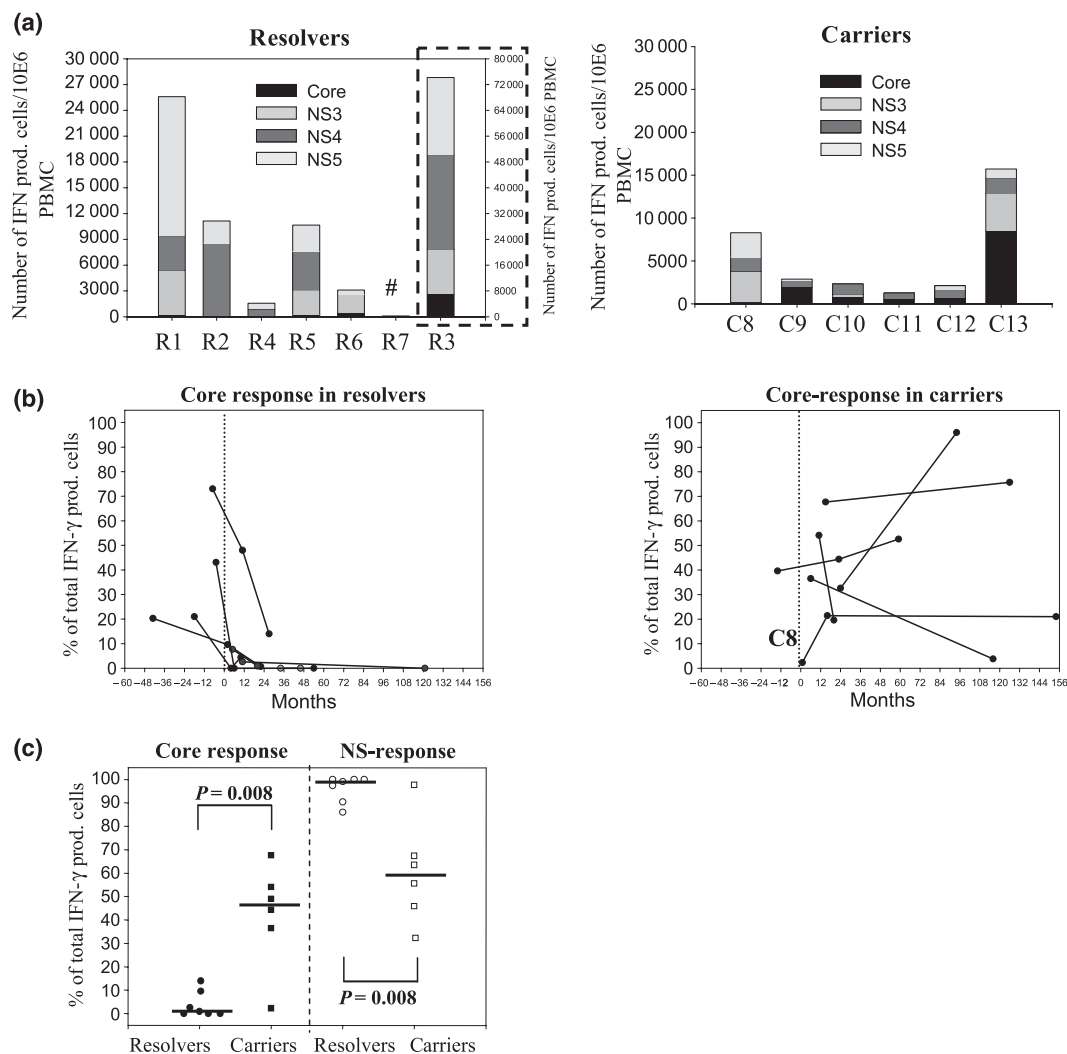


Fig. 4 HCV-specific CD4+ T-cell response. (a) The number of IFN- γ -producing CD4+ T cells after 12-days of expansion calculated from the input PBMCs (IFN- γ -producing CD4+ T cells/10E6 PBMC) as measured by ICCS after restimulation, are shown for 7 resolvers (left panel) and 6 chronic carriers (right panel) before and after acute HCV-infection. The CD4+ T-cell response is shown for resolver R3 at the right y-axis (encircled a dashed line) (left panel), as this individual has a much higher T-cell response. The low CD4+ T-cell peak response in resolver R7 (93 IFN-producing cells/10E6 PBMC) is indicated with # (a, left panel). Separate protein-specific responses are shown by grey tones in the bars. (b) The kinetics of HCV-specific CD4+ T-cell responses directed against Core protein (as % of IFN- γ -producing CD4+ T cells/10E6 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.

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 IDUs with a prospectively sampled HCV-infection confirms previous studies (which were often cross-sectional and in healthcare workers) that direct *ex vivo* T-cell responses are weak [29]. 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 [30]. In those in whom we assessed HCV-specific T-cell responses from before onset of infection, we observed that an increase of these responses

Table 2 CD4+ T-cell response against separate proteins as a percentage of total CD4+ T-cell peak response or as an absolute response (IFN- γ producing cells/million PBMC)

Subject	Cor0065% absolute		NS3% absolute		NS4% absolute		NS5% absolute	
R1	1	221	20	5212	16	3999	63	16158
R2	0	0	0	0	77	8537	23	2595
R3	0	0	0	0	57	892	43	682
R4	9	7113	19	13903	40	29287	32	23894
R5	3	272	27	2867	42	4498	28	3026
R6	14	436	70	2186	0	0	16	492
R7	0	0	100	93	0	0	0	0
C8	2	187	44	3612	19	1604	35	2882
C9	68	1949	0	0	27	791	5	2877
C10	37	851	12	296	51	1183	0	0
C11	44	567	0	0	56	711	0	0
C12	33	689	0	0	46	972	21	454
C13	54	8514	28	4368	12	1820	6	1028

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 [17]. 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 [28] and *Plasmodium falciparum*-infected individuals [31] 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 [32]. 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 [33]. 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 [5] and in a more recent cross-sectional study it was found that T-cell responses in resolvers were more commonly targeting nonstructural proteins [34]. However, other studies using similar techniques failed to support this finding and showed that CD4+ T-cells responses were broad, but not specifically targeting only NS proteins [35]. 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 [36]. 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 [37] and that serum may contain free circulating Core proteins [38]. In addition, HCV Core is thought to play a role in modulating immune responses by affecting the function of virus-specific T cells [36,39,40]. 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 [41], 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 6 months, which is based on the

observation that most patients who spontaneously clear HCV, do so within the first 3–4 months of infection [42]. However, most observations were performed in symptomatic patients who were subsequently referred to medical centres. Consequently, most analyses of a successful immune response were performed in symptomatic HCV-infected patients [7], who we assume were not repeatedly exposed to HCV, once they received medical care during the first 6 months of infection. In contrast, we assume that some of our asymptomatic IDUs were repeatedly reinfected with HCV during the acute phase of infection [43,44]. As a consequence of this repeated and prolonged HCV-exposure, some IDUs probably needed more time to resolve HCV infection (Fig. 1). On the other hand, we may have overestimated the time required for viral clearance on account of some wide HCV-RNA testing intervals. Late viral clearance (>24 months) has been reported before [45].

A possible caveat in our study might be the use of HCV-peptide pools based on the genotype 1a consensus sequence, as six 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 2). Whether this is on account of their genotype difference or because of 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 3 months after EDI, three of the resolvers and two of the chronic carriers were studied after more than 10 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. (Van den Berg, unpublished data) 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 not interfered with the results.

An unexpected finding was the detection of HCV-specific *memory*-T-cell responses in the 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, leads to induction of a cellular immune response without consistently detectable viraemia or seroconversion [9,15,46], and may influence subsequent outcome of infection [47]. Indeed, in one of our subjects it was previously shown [20] that HCV-seroconversion after a 'first' HCV-infection was followed by loss of detectable antibodies after which reseroconversion occurred after a 'second' HCV-infection. Clearance of the 'second' HCV-infection in R3 was associated with very

strong T-cell responses, suggestive of protective immunity [47] and strikingly paralleling periods of intermittent IDU. 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 probable previous infection have not been truly 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 response before EDI does not predict an effective immune response after re-exposure to high levels of HCV-RNA.

It has been reported that IDUs who were previously infected were less likely to develop persistent HCV-viraemia than individuals infected for the first time, indeed suggesting that protective immunity may be acquired [47]. However, a more recent study did not confirm this and calculated the reinfection rate to be 41/100 PY [45]. 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-cell responses well before HIV-seroconversion [48]. 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 IDU had stopped during the period before EDI.

Surprisingly, in the chronic carriers 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 who resolve after (re)infection, to resemble a protective response, which would be directed predominantly against nonstructural proteins. In resolvers, the response became mainly NS-focussed only after reinfection, 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 non-structural proteins are observed in resolvers compared to in chronic carriers. *Memory*-T-cell responses that are present before documented HCV-seroconversion suggest that reinfection in IDUs occurs often, while the presence of these responses was not predictive for the outcome of infection. Persistent HCV-viraemia 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.

ACKNOWLEDGEMENTS

These studies were performed as part of the Amsterdam Cohort Studies on AIDS and HIV-1 infection and financially

supported by NWO, VWS and AIDSfonds (4141). We are indebted to Sjoerd Rebers of the Dept. of Microbiology, Division of Virology for HCV-RNA data. We thank Prof. Dr. F. Miedema, Prof. Dr. B. Berkhout and Drs Arends for critically reading the manuscript. There were no conflicts of interest for any of the authors.

REFERENCES

- Lauer GM, Walker BD. Hepatitis C virus infection. *N Engl J Med* 2001; 345(1): 41–52.
- Hoofnagle JH. Course and outcome of hepatitis C. *Hepatology* 2002; 36(5 Suppl. 1): S21–S29.
- Chang KM, Thimme R, Melpolder JJ *et al.* Differential CD4(+) and CD8(+) T-cell responsiveness in hepatitis C virus infection. *Hepatology* 2001; 33(1): 267–276.
- Cooper S, Erickson AL, Adams EJ *et al.* Analysis of a successful immune response against hepatitis C virus. *Immunity* 1999; 10(4): 439–449.
- Diepolder HM, Zachoval R, Hoffmann RM *et al.* Possible mechanism involving T-lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis C virus infection. *Lancet* 1995; 346(8981): 1006–1007.
- Gerlach JT, Diepolder HM, Jung MC *et al.* Recurrence of hepatitis C virus after loss of virus-specific CD4(+) T-cell response in acute hepatitis C. *Gastroenterology* 1999; 117(4): 933–941.
- Lechner F, Wong DK, Dunbar PR *et al.* Analysis of successful immune responses in persons infected with hepatitis C virus. *J Exp Med* 2000; 191(9): 1499–1512.
- Shoukry NH, Grakoui A, Houghton M *et al.* Memory CD8⁺ T cells are required for protection from persistent hepatitis C virus infection. *J Exp Med* 2003; 197(12): 1645–1655.
- Thimme R, Oldach D, Chang KM, Steiger C, Ray SC, Chisari FV. Determinants of viral clearance and persistence during acute hepatitis C virus infection. *J Exp Med* 2001; 194(10): 1395–1406.
- Day CL, Lauer GM, Robbins GK *et al.* Broad specificity of virus-specific CD4⁺ T-helper-cell responses in resolved hepatitis C virus infection. *J Virol* 2002; 76(24): 12584–12595.
- Cox AL, Mosbruger T, Lauer GM, Pardoll D, Thomas DL, Ray SC. Comprehensive analyses of CD8⁺ T cell responses during longitudinal study of acute human hepatitis C. *Hepatology* 2005; 42(1): 104–112.
- Kamal SM, Rasenack JW, Bianchi L *et al.* Acute hepatitis C without and with schistosomiasis: correlation with hepatitis C-specific CD4(+) T-cell and cytokine response. *Gastroenterology* 2001; 121(3): 646–656.
- Thimme R, Bukh J, Spangenberg HC *et al.* Viral and immunological determinants of hepatitis C virus clearance, persistence, and disease. *Proc Natl Acad Sci USA* 2002; 99(24): 15661–15668.
- Gruener NH, Lechner F, Jung MC *et al.* Sustained dysfunction of antiviral CD8⁺ T lymphocytes after infection with hepatitis C virus. *J Virol* 2001; 75(12): 5550–5558.
- Takaki A, Wiese M, Maertens G *et al.* Cellular immune responses persist and humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C. *Nat Med* 2000; 6(5): 578–582.
- Wong DK, Dudley DD, Afdhal NH *et al.* Liver-derived CTL in hepatitis C virus infection: breadth and specificity of responses in a cohort of persons with chronic infection. *J Immunol* 1998; 160(3): 1479–1488.
- Piriou ER, van DK, Nanlohy NM, van Oers MH, Miedema F, van BD. Novel method for detection of virus-specific CD4(+) T cells indicates a decreased EBV-specific CD4(+) T cell response in untreated HIV-infected subjects. *Eur J Immunol* 2005; 35(3): 796–805.
- van den Hoek JA, Coutinho RA, van Haastrecht HJ, van Zadelhoff AW, Goudsmit J. Prevalence and risk factors of HIV infections among drug-users and drug-using prostitutes in Amsterdam. *AIDS* 1988; 2(1): 55–60.
- Beld M, Penning M, McMorris M, Gorgels J, van den HA, Goudsmit J. Different hepatitis C virus (HCV) RNA load profiles following seroconversion among injecting drug users without correlation with HCV genotype and serum alanine aminotransferase levels. *J Clin Microbiol* 1998; 36(4): 872–877.
- Beld M, Penning M, van PM *et al.* Low levels of hepatitis C virus RNA in serum, plasma, and peripheral blood mononuclear cells of injecting drug users during long antibody-undetectable periods before seroconversion. *Blood* 1999; 94(4): 1183–1191.
- Heller T, Rehermann B. Acute hepatitis C: a multifaceted disease. *Semin Liver Dis* 2005; 25(1): 7–17.
- Ohno O, Mizokami M, Wu RR *et al.* New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a. *J Clin Microbiol* 1997; 35(1): 201–207.
- van AL, Prins M. Infection with concurrent multiple hepatitis C virus genotypes is associated with faster HIV disease progression. *AIDS* 2004; 18(17): 2319–2324.
- Piriou E, van Dort K, Nanlohy NM, Miedema F, Van Oers MHJ, van Baarle D. Altered EBV viral load setpoint after HIV seroconversion is in accordance with lack of predictive value of EBV load for the occurrence of AIDS-related non-Hodgkin lymphoma. *J Immunol* 2004; 172: 6931–6937.
- Piriou E, van Dort K, Nanlohy NM, Van Oers MHJ, Miedema F, van Baarle D. Loss of EBNA1-specific memory CD4⁺ and CD8⁺ T cells in HIV-infected patients progressing to AIDS-related non-Hodgkin lymphoma. *Blood* 2005; 106(9): 3166–3174.
- Bitmansour AD, Douek DC, Maino VC, Picker LJ. Direct ex vivo analysis of human CD4⁺ memory T cell activation requirements at the single clonotype level. *J Immunol* 2002; 169: 1207–1218.
- Kostense S, Ogg GS, Manting EH *et al.* High viral burden in the presence of major HIV-specific CD8⁺ T cell expansions: evidence for impaired T cell effector function. *Eur J Immunol* 2001; 31(3): 677–686.
- Godkin AJ, Thomas HC, Openshaw PJ. Evolution of epitope-specific memory CD4(+) T cells after clearance of hepatitis C virus. *J Immunol* 2002; 169(4): 2210–2214.
- Lauer GM, Barnes E, Lucas M *et al.* High resolution analysis of cellular immune responses in resolved and persistent hepatitis C virus infection. *Gastroenterology* 2004; 127(3): 924–936.
- Rios-Olivares E, Vila LM, Reyes JC *et al.* Impaired cytokine production and suppressed lymphocyte proliferation activity

- in HCV-infected cocaine and heroin ("speedball") users. *Drug Alcohol Depend* 2006; 85(3): 236–243.
- 31 Reece WH, Pinder M, Gothard PK *et al.* A CD4(+) T-cell immune response to a conserved epitope in the circumsporozoite protein correlates with protection from natural *Plasmodium falciparum* infection and disease. *Nat Med* 2004; 10(4): 406–410.
 - 32 Grakoui A, Shoukry NH, Woollard DJ *et al.* HCV persistence and immune evasion in the absence of memory T cell help. *Science* 2003; 302(5645): 659–662.
 - 33 Thomson M, Nascimbeni M, Havert MB *et al.* The clearance of hepatitis C virus infection in chimpanzees may not necessarily correlate with the appearance of acquired immunity. *J Virol* 2003; 77(2): 862–870.
 - 34 Semmo N, Barnes E, Taylor C *et al.* T-cell responses and previous exposure to hepatitis C virus in indeterminate blood donors. *Lancet* 2005; 365(9456): 327–329.
 - 35 Schulze zur WJ, Lauer GM, Day CL *et al.* Broad repertoire of the CD4+ Th cell response in spontaneously controlled hepatitis C virus infection includes dominant and highly promiscuous epitopes. *J Immunol* 2005; 175(6): 3603–3613.
 - 36 Eisen-Vandervelde AL, Yao ZQ, Hahn YS. The molecular basis of HCV-mediated immune dysregulation. *Clin Immunol* 2004; 111(1): 16–21.
 - 37 Andre P, Perlemuter G, Budkowska A, Brechot C, Lotteau V. Hepatitis C virus particles and lipoprotein metabolism. *Semin Liver Dis* 2005; 25(1): 93–104.
 - 38 Okada K, Takishita Y, Shimomura H *et al.* Detection of hepatitis C virus core protein in the glomeruli of patients with membranous glomerulonephritis. *Clin Nephrol* 1996; 45(2): 71–76.
 - 39 Eisen-Vandervelde AL, Waggoner SN, Yao ZQ, Cale EM, Hahn CS, Hahn YS. Hepatitis C virus core selectively suppresses interleukin-12 synthesis in human macrophages by interfering with AP-1 activation. *J Biol Chem* 2004; 279(42): 43479–43486.
 - 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(12): 6409–6419.
 - 41 Reherrmann B, Chang KM, McHutchison JG, Kokka R, Houghton M, Chisari FV. Quantitative analysis of the peripheral blood cytotoxic T lymphocyte response in patients with chronic hepatitis C virus infection. *J Clin Invest* 1996; 98(6): 1432–1440.
 - 42 Gerlach JT, Diepolder HM, Zachoval R *et al.* Acute hepatitis C: high rate of both spontaneous and treatment-induced viral clearance. *Gastroenterology* 2003; 125(1): 80–88.
 - 43 Herring BL, Page-Shafer K, Tobler LH, Delwart EL. Frequent hepatitis C virus superinfection in injection drug users. *J Infect Dis* 2004; 190(8): 1396–1403.
 - 44 van de Laar TJW, Ruys TA, Prins M *et al.* Frequent HCV Reinfection and Superinfection among Active Injecting Drug Users in Amsterdam. Poster on 13th International meeting on hepatitis C virus and related viruses. Cairns, 2006.
 - 45 Micallef JM, Macdonald V, Jauncey M *et al.* High incidence of hepatitis C virus reinfection within a cohort of injecting drug users. *J Viral Hepat* 2007; 14(6): 413–418.
 - 46 Freeman AJ, Ffrench RA, Post JJ *et al.* Prevalence of production of virus-specific interferon-gamma among seronegative hepatitis C-resistant subjects reporting injection drug use. *J Infect Dis* 2004; 190(6): 1093–1097.
 - 47 Mehta SH, Cox A, Hoover DR *et al.* Protection against persistence of hepatitis C. *Lancet* 2002; 359(9316): 1478–1483.
 - 48 Koning FA, Jansen CA, Dekker J *et al.* Correlates of resistance to HIV-1 infection in homosexual men with high-risk sexual behaviour. *AIDS* 2004; 18(8): 1117–1126.