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T-cell responses at baseline and during therapy with peginterferon-α and ribavirin are not associated with outcome in chronic hepatitis C infected patients☆

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ABSTRACT

Background: Since the association between hepatitis C virus (HCV)-specific T-cell responses both pre-treatment and during interferon-α based therapy and viral clearance is unresolved, a combined analysis of distinctive T-cell characteristics (proliferation and interferon-γ production) is important to clarify this issue.
Methods: Peripheral blood mononuclear cells (PBMC) collected in 22 chronic HCV infected patients at pre-treatment and at week 4 during pegIFN-α/ribavirin therapy, were stimulated with overlapping peptide pools in a [^{3}H]-thymidine assay, an interferon-γ-ELISA, and a sensitive 12-day T-cell expansion assay.
Results: Compared to the [^{3}H]-thymidine proliferation and interferon-γ secretion assays, the 12-day T-cell expansion assay was more sensitive in detecting T-cell responses. No significant association was demonstrated between pre-treatment HCV-specific CD4+ or CD8+ T-cell responses and either a sustained virological response (SVR) or a rapid virological response (RVR). However, a skewing of individual responses towards the non-structural antigens was observed. During pegIFN-α/ribavirin therapy, HCV-specific CD4+ and CD8+ T-cells declined similarly in both SVR/RVR and non-SVR/non-RVR patients.
Conclusion: No correlation was found between the magnitude of pre-treatment HCV-specific T-cell responses and the outcome of pegIFN-α/ribavirin therapy in terms of SVR and RVR. Moreover, the magnitude of HCV-specific T-cell responses declined in all patients early during treatment.

1. Introduction

Pegylated interferon α-2a or -2b (pegIFN-α) in combination with weight based ribavirin is currently the standard treatment for chronic hepatitis C virus (HCV) infected patients (Ghany et al., 2009). A sustained virological response (SVR), defined as an undetectable plasma HCV-RNA 24 weeks after cessation of therapy, is achieved in around 50% of patients infected with genotypes 1 and 4 (Hadziyannis et al., 2004). Pre-treatment characteristics like baseline viral load (Manns et al., 2001), liver cirrhosis (Aghemo et al., 2009), coinfection with the human immunodeficiency virus (HIV) (Torriani et al., 2004) and recently the interleukin-28B cc-genotype (Ge et al., 2009) are associated with outcome of therapy. Furthermore, achievement of a rapid virological response (RVR, i.e. plasma HCV-RNA with undetectable, i.e. <50 IU/ml at week 4 of therapy) is regarded as a strong on-treatment predictor for SVR (Ferenci, 2004; Zeuzem et al., 2006).

Abbreviations: aa, amino acid; CD, code of differentiation; DMSO, dimethylsulphoxide; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence activated cell sorting; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IFN-γ, interferon-γ; IL-2, interleukin-2; IL28B, interleukin-28B; IQR, interquartile range; IU/ml, international units/milliliter; kDa, kilodalton; ml, milliliter; ng/ml, nanogram/milliliter; ns, non-structural; PBMC, peripheral blood mononuclear cell; pegIFN-α, pegylated interferon α-2a or -2b; pg/ml, picogram/milliliter; PMA, phorbol myristate acetate; RNA, ribonucleic acid; RVR, rapid virological response; SD, standard deviation; SI, stimulation index; SVR, sustained virological response; U/ml, units/milliliter; μg/ml, microgram/milliliter; μl, microliter.

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It has been suggested that cellular immune responses, modulated by pegIFN-α and ribavirin, play a role in forced viral eradication, based on the immunological properties attributed to these anti-viral compounds (Brinkmann et al., 1993; Cramp et al., 2000; Luft et al., 1998; Tough et al., 1999). However, the role of HCV-specific T-cells before and during pegIFN-α/ribavirin therapy is still controversial (Ishii and Koziel, 2008; Thimme et al., 2008). Some studies have shown that achievement of a SVR is associated with high baseline CD4+ and/or CD8+ specific T-cell responses (Caetano et al., 2008; Hoffmann et al., 1995; Pilli et al., 2007; Rosen et al., 2007), while others have seen no such relationship (Barnes et al., 2009; Capa et al., 2007; Kamal et al., 2002; Kaplan et al., 2005; Tang et al., 2005). Similarly, contradictory results have been reported on the role of HCV-specific T-cells during pegIFN-α and ribavirin therapy showing either augmentation (Barnes et al., 2002; Cramp et al., 2000; Kamal et al., 2002) or decline (Barnes et al., 2009; Caetano et al., 2008; Pilli et al., 2007) of HCV-specific T-cells in relation to SVR. In patients achieving a RVR, higher percentages of baseline interferon-γ (IFN-γ) producing CD8+ T-cells have been demonstrated compared to non-RVR patients (Pilli et al., 2007).

There is no conclusive evidence whether HCV-specific immunity contributes to therapy-induced viral clearance for several reasons. Good animal models are unavailable (Boonstra et al., 2009), frequencies of circulating HCV-specific T-cells are very low (Gruner et al., 2000) and therefore hard to detect, and a consensus on the optimal ex vivo experimental cell culture protocols is lacking (Rehermann and Naoumov, 2007). A robust and sensitive assay able to detect low frequencies of HCV-specific T-cell responses is needed to resolve the above mentioned controversies.

For this purpose we examined the magnitude and breadth of HCV-specific T-cell responses at baseline and during therapy in patients with a HCV mono-infection using a sensitive 12-day expansion assay as has been previously reported by our group (Piriou et al., 2005; Ruys et al., 2008; van den Berg et al., 2009). This assay measures both the IFN-γ production and the proliferative capacity of HCV-specific CD4+ and CD8+ T-cells simultaneously, allowing a more comprehensive analysis of the HCV-specific T-cell response. Using this assay, we found no relation between the magnitude of pre-treatment HCV-specific CD4+ or CD8+ T-cell responses and achievement of either a SVR or a RVR, albeit that skewing of these responses was demonstrated against HCV non-structural antigens. In addition, irrespective of treatment outcome, HCV-specific T-cell responses declined in all patients early during treatment.

2. Methods

2.1. Patients

In this multi-centre cohort study, 22 patients diagnosed with chronic HCV mono-infection were consecutively enrolled (Arends et al., 2009), and prospectively sampled during standard treatment with either peginterferon α-2a (40 kDa) (Pegasys® 180 μg/week; Roche, Basel, Switzerland) or peginterferon α-2b (12 kDa) (PegIntron® 1.5 μg/kg/week; Schering-Plough, Kenilworth, USA) in combination with weight based ribavirin (Copegus® or Rebetol®). Only patients with HCV genotypes 1 or 4 were included while patients with a coinfection with either hepatitis B or HIV were excluded. During treatment plasma HCV-RNA was measured using the qualitative Roche Amplicor® polymerase chain reaction (PCR) assay with a lower limit of detection of 50 IU/ml. A rapid viral response (RVR) is defined as a qualitative undetectable HCV-RNA at week 4 of treatment (<50 IU/ml) whereas an early virological response (EVR) is defined as achieving either a $\geq 2 \log_{10}$ viral load decrease from baseline or a qualitative undetectable (<50 IU/ml) HCV-RNA at week 12 after initiation of treatment. A sustained virological response (SVR) was defined as a negative HCV viral load (<50 IU/ml) 24 weeks after discontinuation of therapy. All patients provided written informed consent and institutional ethical review boards at participating centres approved the protocol.

2.2. PBMC processing

Peripheral blood (approximately 25 ml) was collected before and at week 4 during therapy. Within 24 h, peripheral blood mononuclear cells (PBMC) were isolated using a Ficoll–Hypaque density gradient centrifugation. Cells were re-suspended in RPMI 1640 (Gibco Life Technologies, Breda, the Netherlands) to which 20% fetal calf serum and 1% penicillin and streptomycin were added (hereafter called medium). After adding 10% DMSO, PBMC were frozen to −80 °C and stored thereafter at −180 °C until further use. Except for the proliferation assay, all experiments were performed using frozen PBMC.

2.3. T-cell assays

2.3.1. In vitro quantification of proliferation and cytokine production

Freshly isolated PBMC at a final concentration of 1×10^6 cells/ml were cultured in quadruplets in 96-well round bottom plates in 200 μl culture medium in the presence of overlapping peptide pools (spanning the core, NS3, NS4 and NS5a and NS5b HCV genome; clone J4 genotype 1b; BEI Resources, Manassas, USA), anti-CD3 antibody (1 μg/ml; OKT-3; orthoclone), cytomegalovirus lysate or no stimulus. All cultures were performed in the presence of anti-CD28 (1 μg/ml; CD28.2; eBioscience) and anti-CD49d antibody (1 μg/ml; 9F10; eBioscience). After culturing for 3 days, 100 μl supernatant was harvested and replaced by 100 μl fresh culture medium. IFN-γ production was determined by a commercially available ELISA kit (eBioscience, San Diego, USA). After stimulation for 5 days, the cells were pulsed with 0.5 μCi/well of [^{3}H]-thymidine (Radiochemical Centre, Amersham, Little Chalfont, UK), and harvested 16 h later. Proliferation was determined as counts per minute (cpm) via liquid scintillation counting of the harvested cells. The cut-off value for a positive response was set as the median plus 2× the standard deviation (SD) of the medium.

2.3.2. HCV-specific CD4+ and CD8+ T-cell expansion assay

To stimulate both CD4+ and CD8+ T-cells, overlapping peptide pools (http://www.mimotopes.com) with a length of 18 amino acids (aa) and an overlap of 11 aa, spanning the whole HCV genome, were used. The peptides were pooled as follows: core (core/E1/E2/p7, aa 1–805 with a total of 116 peptides), non-structural protein 2 (NS2) (aa 806–1022, with a total of 31 peptides), NS3 (aa 1023–1645, with a total of 90 peptides), NS4 (aa 1646–1967, with a total of 49 peptides), NS5A (aa 1968–2415, with a total of 67 peptides) and NS5B (aa 2416–3011, with a total of 87 peptides). Peptide pools were dissolved in DMSO.

The 12-day expansion assay measured proliferative capacity and IFN-γ production as previously published (Ruys et al., 2008; van den Berg et al., 2009). Both pre-treatment and week 4 time points were assessed concurrently. PBMC were stimulated at 1×10^6/ml with 2 μg/ml peptide pool (the DMSO concentration was never more than 1% in the final stimulation). Thereafter, cells were incubated in 96-well round bottom plates at 2×10^5 cells/well at 37 °C and 5% CO_2 and interleukin-2 (IL-2) (20 U/ml) was added at days 3, 6 and 9. HCV peptides at a concentration of 2 μg/ml were added again on day 6. Cells were pooled, washed and counted on day 12, rested overnight in medium after which re-stimulation with HCV

Table 1
Characteristics of patients grouped by clinical outcome.

Feature	SVR ($n = 11$)	Non-SVR ($n = 11$)	p-Value
General characteristics			
Male/female	10/1	9/2	ns
Age (years)	48 (40–52)	47 (46–55)	ns
Weight (kg)	78 (63–86)	78 (62–95)	ns
Caucasian ethnicity	11 (100)	11 (100)	ns
HCV/liver related characteristics			
Genotype			ns
1	8 (73)	9 (82)	
4	3 (27)	2 (18)	
HCV-RNA (Log 10)	5.82 (5.55–6.57)	6.62 (5.50–6.68)	ns
ALT (IU/ml)	86 (30–135)	94 (48–120)	ns
Liver biopsy			
≤F2	6 (55)	3 (27)	ns
F3–F4	1 (9)	3 (27)	
NP	4 (36)	5 (45)	
Immunological characteristics			
CD38+/HLA-DR+			
CD4	0.30 (0.20–0.50)	0.60 (0.20–0.80)	ns
CD8	0.40 (0.30–0.70)	0.50 (0.20–1.30)	ns
CD8 subsets			
Naive (CD27+CD45RO−)	39.40 (22.7–57.7)	33.1 (16.2–42.4)	ns
Effector (CD27−CD45RO+)	37.6 (23.4–58.3)	19.9 (15.4–44.0)	ns
Memory (CD27+CD45RO+)	16.5 (11.8–34.4)	37.4 (27.8–44.8)	ns
Treatment outcome characteristics			
RVR	7 (64)	2 (18)	0.08
EVR	11 (100)	6 (55)	0.04

Continuous variables are shown as median values (interquartile range) while categorical variables are given as numbers (percentages). ns = not significant; NP = not performed.

peptide pools for 6 h in the presence of anti-CD28 (1 μg/ml) and anti-CD49d (1 μg/ml) as co-stimuli was performed. Concurrently, medium alone and PMA (10 ng/ml) with ionomycin (50 ng/ml) were used a negative and positive control, respectively. After 1 h, Brefeldin A (Golgiplug, BD Biosciences, San José, CA, USA) was added, followed by a further incubation period of 5 h. Thereafter, cells were washed, permeabilized (FACS Permeabilizing Solution, BD) and stained with specific antibodies against CD3 (Pacific Blue, eBioscience), CD4 (PE Cy7, eBioscience), CD8 (APC AlexaFluor750, eBioscience) and IFN-γ (FITC, BD) for 20 min at 4 °C. After washing, cells were fixed with cellfix (BD) and at least 100 000 cells were acquired by flow cytometry (FACS, LRSII, BD). Using the forward and sideward scatter, the lymphocyte population was gated and analyzed by FACSDiva software (BD). The HCV-specific T-cell response (i.e. IFN-γ production) was calculated by subtracting the IFN-γ production in the unstimulated control and subsequently multiplied with the proliferation ratio (fold increase) of the expansion assay, resulting in a number of HCV-specific T-cells per 10^6 PBMC, which is a combination of the (memory) T-cells initially present that survived, proliferated and differentiated into effector T-cell phenotype in each individual. A positive HCV-specific T-cell response was defined as a response of more than 200 out of 1 million PBMC in since T-cell responses in healthy controls after culture with HCV peptide pools never exceeded 200 per million PBMC. Therefore, this was taken as a 'cut-off' for a positive HCV-response.

All HCV-specific T-cell responses per patient (core, NS2, NS3, NS4 and NS5A/B) were summed and displayed as the total HCV-specific T-cell response. For the analysis, patients were divided into SVR/non-SVR or RVR/non-RVR.

2.4. Surface marker staining

Direct ex vivo surface marker expression for T-cell subsets (CD27/CD45RO) and T-cell activation (CD38/HLA-DR) were analyzed pre-treatment and at week 4. After thawing and washing, cells were incubated for 20 min at 4 °C with a combination of antibodies against CD3 (PerCP, BD), CD4 (PE Cy7, eBioscience), CD8 (APC Alexa Fluor 750, eBioscience), CD38 (PE Cy7, eBioscience), HLA-DR (FITC, eBioscience), CD27 (FITC, BD) and CD45RO (APC, BD). After washing, cells were fixed with cellfix and at least 200 000 cells were acquired by FACS (LRSII, BD). Using the forward and sideward scatter the lymphocyte population was gated and analyzed by FACSDiva software (BD).

2.5. Statistical analysis

Continuous data are presented as median values (with interquartile range (IQR)) analyzed using a Mann–Whitney *U*-test whereas categorical variables are given as number of cases (percentage) analyzed using a Wilcoxon signed-rank test. Comparison of categorical variables was done using a Fisher's exact test. Spearmann's Rank correlation and linear regression analysis were performed to examine the relationship between continuous variables and immunological parameters. A p-value ≤ 0.05 was considered as statistically significant and all tests used were two-sided. All data were analyzed using GraphPad Prism (version 5.0 for Windows, GraphPad Software, San Diego, USA).

3. Results

Of the 22 chronic HCV genotype 1 and 4 infected patients treated with pegIFN-α/ribavirin, 11 patients reached a SVR (50%). Patient characteristics, shown in Table 1, are similar when grouped into SVR and non-SVR. Factors possibly influencing the HCV-specific T-cell response like age, gender and extent of liver injury were not of significant influence on the outcome of treatment. In patients reaching a SVR, 64% also achieved a RVR and all reached an EVR. In contrast, in the non-SVR group only 18% achieved a RVR ($p = 0.08$) and 55% reached an EVR ($p = 0.04$). Furthermore, the baseline values of activated CD4+ and CD8+ T-cells (CD38/HLA-DR double positive) as well as the percentages of CD8+ T-cell subsets (defined by CD27 and CD45RO) were not different between SVR patients and non-SVR patients (Table 1).

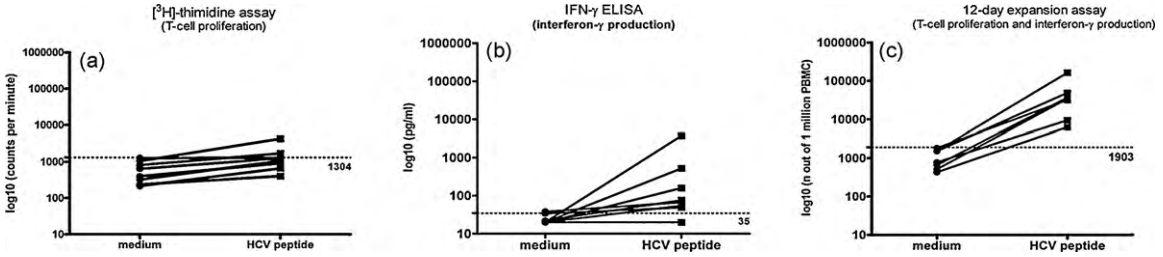

Fig. 1. Higher HCV-specific T-cell responses were detectable using the T-cell expansion assay compared to proliferation and cytokine assay. To measure HCV-specific T-cell responses, three different T-cell assays were compared: [^{3}H]-thymidine assay (panel a), IFN-γ-ELISA (panel b) and the 12-day T-cell expansion assay (panel c). PBMC were stimulated using the different protocols (see Section 2) without peptides (medium) (●) or with HCV peptides (■). All HCV peptide responses were total responses directed against the total pool of HCV peptides (i.e. core, NS2, NS3, NS4 and NS5A/B). On the *y*-axis a log transformation is performed to account for the different scales of each assay. The dotted line represents the threshold of each assay calculated as two times the standard deviation above the median of medium values. One patient's PBMC failed to grow in the expansion assay (no positive control) resulting in seven evaluable patients for this assay.

3.1. Higher HCV-specific T-cell responses were detectable using the T-cell expansion assay compared to proliferation and cytokine assay

Since direct ex vivo analyses have shown that very low numbers of HCV-specific T-cells are present (Gruner et al., 2000), stimulation assays have been used to circumvent this problem (Barnes et al., 2009; Chang et al., 2001; Pilli et al., 2007). In this study we compared the 12-day expansion assay with the [^{3}H]-thymidine proliferation assay and the IFN-γ-ELISA as a more sensitive way to measure HCV-specific T-cells. In a randomly selected subgroup of patients the majority (6 out of 8 patients) failed to show HCV-specific T-cell proliferation in the [^{3}H]-thymidine assay, although one of the remaining two patients displayed a relatively strong response (4304 cpm; Fig. 1a). Likewise, the IFN-γ-ELISA showed low IFN-γ levels in supernatant after HCV peptide stimulation around the cut-off value (35 pg/ml) in 5 out of 8 patients (Fig. 1b) with a stimulation index (SI) of ≤3. In the remaining three patients moderate to good IFN-γ production was detectable with values ranging between 159 and 3773 pg/ml (SI between 8 and 188). Finally, the 12-day expansion assay showed low background values and T-cell responses were detectable in all evaluable patients well above the cut-off value (SI ≥ 12; Fig. 1c). The 12-day expansion assay therefore allows for a sensitive detection of both HCV-specific CD4+ and CD8+ T-cell responses since it combines both production of IFN-γ and proliferation of (memory) T-cells into becoming effector T-cells.

3.2. Pre-treatment HCV-specific T-cell responses are not associated with outcome of therapy

To address the question whether pre-treatment HCV-specific T-cell responses were associated with therapeutic outcome, we measured HCV-specific CD4+ and CD8+ T-cells before start of therapy using the 12-day expansion assay in patients achieving a SVR (n = 11) and those without a SVR (n = 11). The pre-treatment HCV-specific CD4+ T-cell response was equally strong in patients reaching a SVR (9564 with IQR 6281–13 335) compared to those without a SVR (6870 with IQR 2699–14 001; p = 0.53; Fig. 2a). Simi-

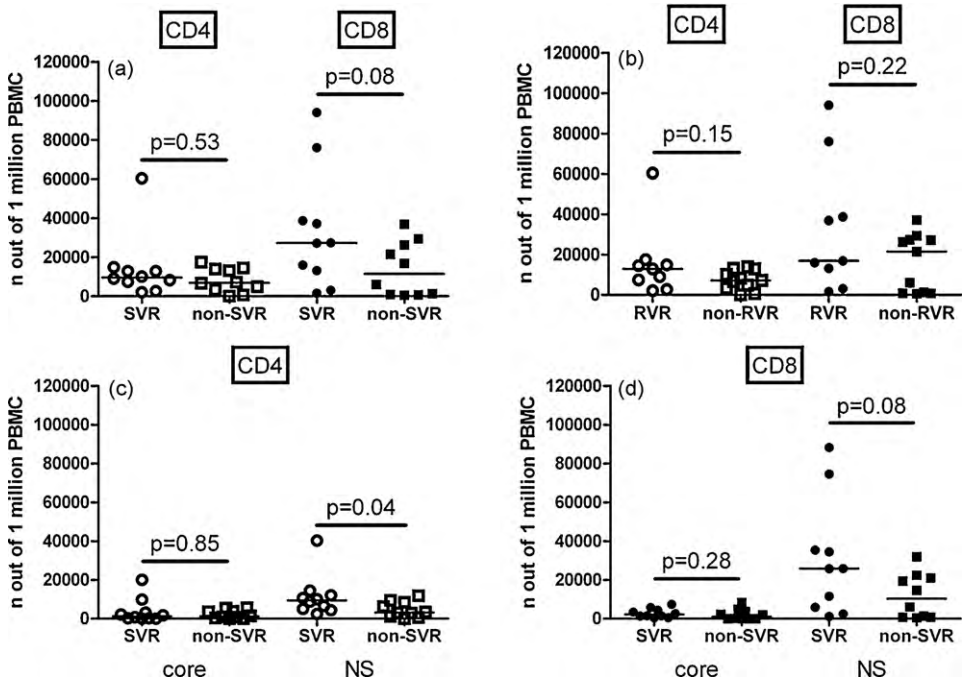

Fig. 2. Pre-treatment total and individual HCV-specific T-cell responses in relation to outcome of therapy; Total pre-treatment HCV-specific CD4+ (○/□) and CD8+ (●/■) T-cell responses (sum of core, NS2, NS3, NS4 and NS5A/B), measured with the 12-day T-cell expansion assay, are shown in relation to achieving a SVR (panel a) or a RVR (panel b) with pegIFN-α/ribavirin therapy. Responses against individual HCV antigens (core or non-structural (ns, sum of NS2, NS3, NS4 and NS5A/B)) are shown for CD4+ (panel c) and CD8+ T-cells (panel d). On the *y*-axis the number of IFN-γ producing T-cells per million PBMC is shown. Of one patient in the SVR group too few pre-treatment PBMC were available for analysis while of one patient in the non-SVR group the PBMC failed to grow in the expansion assay (no positive control).

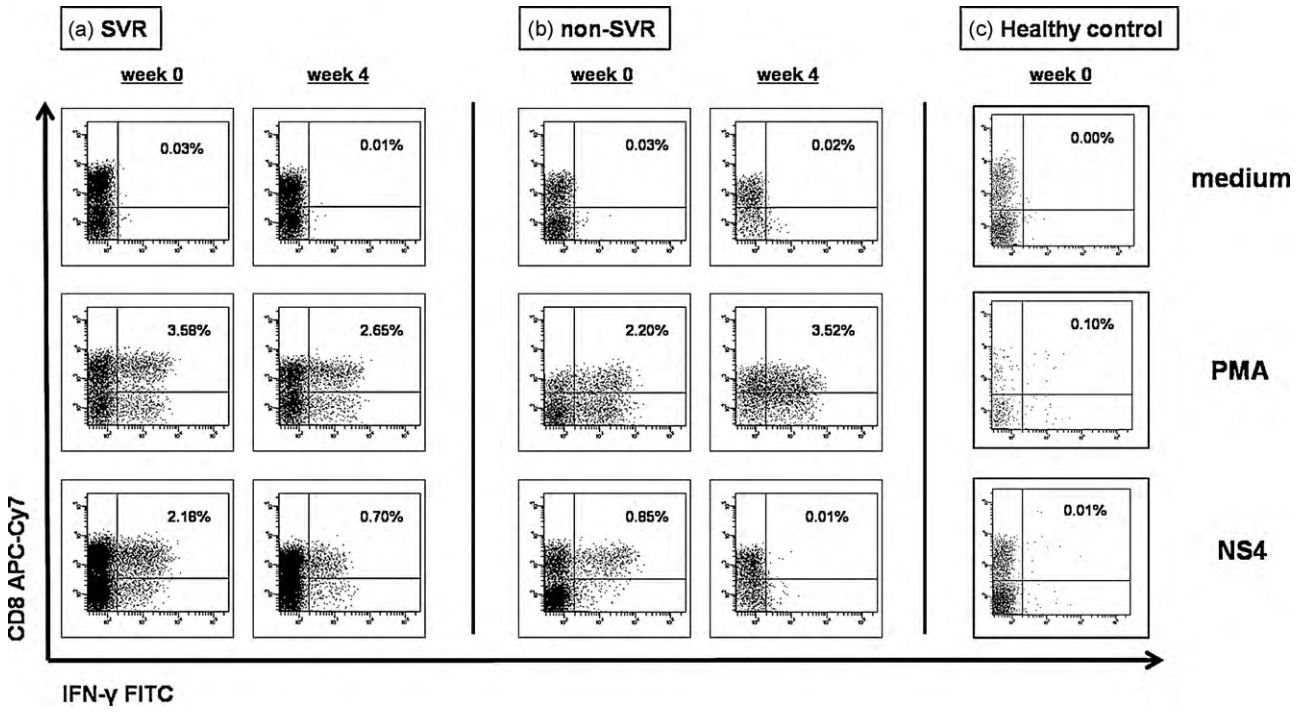

Fig. 3. The effect of pegIFN-α/ribavirin therapy on the percentage of IFN-γ producing HCV-specific CD4+ and CD8+ T-cell responses against HCV-NS4; flow cytometry dot plot showing a longitudinal analysis of the percentage of IFN-γ producing HCV-specific CD8+ T-cell responses against HCV-NS4 pre-treatment and at week 4 in a representative patient achieving a SVR (panel a), patient without a SVR (panel b) and a healthy control (panel c). On the *y*-axis, CD8 APC-Cy7 and on the *x*-axis IFN-γ FITC is shown. The upper row displays the negative control (medium), the middle row is the positive control (PMA) and the bottom row shows the HCV-NS4 peptide.

larly, no difference in HCV-specific CD8+ T-cell responses was noted between SVR and non-SVR patients (27 237 (IQR 10 643–48 021) and 11 491 (IQR 829–26 992); $p = 0.08$). Likewise, when patients were grouped according to achievement of a RVR or non-RVR, HCV-specific T-cell responses between the groups were not significantly different (Fig. 2b). Irrespective of treatment outcome, HCV-specific CD8+ T-cells responded better to stimulation with HCV antigens than HCV-specific CD4+ T-cells.

Subsequently, we assessed the focus of the T-cell response directed against individual HCV antigens in relation to the outcome of therapy. In patients achieving a SVR, preferential targeting of the non-structural HCV peptides by CD4+ T-cells was demonstrated ($p = 0.04$) when compared to those patients not achieving a SVR (Fig. 2c). NS4 and NS5 being most significant for HCV-specific CD4+ T-cells ($p = 0.009$ and $p = 0.05$, respectively).

Several studies have analyzed the interdependence of CD4+ and CD8+ T-cell responses in the same individual either via depletion studies (Chang et al., 2001; Gruner et al., 2000) or direct ex vivo quantification (Capa et al., 2007) showing a correlation between both responses. In agreement with these findings, we also found a positive correlation between individual HCV-specific CD4+ and CD8+ T-cell responses ($r = 0.47$ with $p = 0.04$; Spearman Rank correlation; data not shown). There was no correlation between the HCV-specific CD4+ or CD8+ T-cell responses on the one hand and other baseline characteristics like ALAT and plasma HCV-RNA on the other hand (data not shown).

3.3. HCV-specific T-cell responses decline during pegIFN-α/ribavirin treatment regardless of outcome of therapy

Next, we investigated the role of HCV-specific CD4+ and CD8+ T-cells in eradication of HCV during pegIFN-α/ribavirin therapy by quantifying the number of HCV-specific T-cells at week 4 of therapy. This time point is used to define RVR which is an important

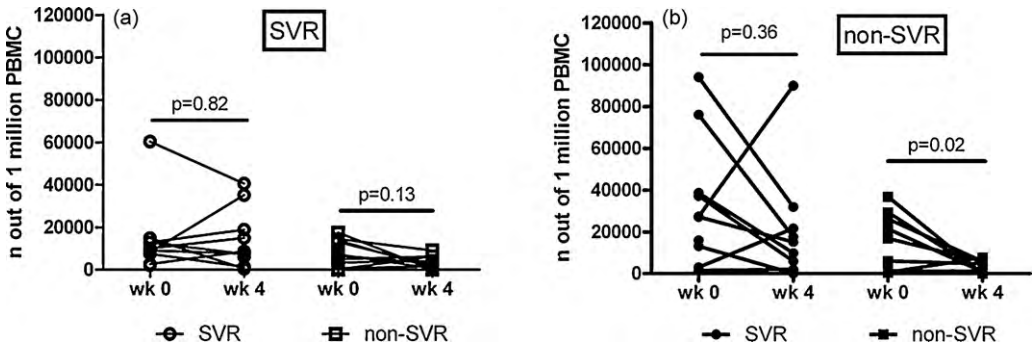

Fig. 4. HCV-specific T-cell responses decline during treatment regardless of achieving a SVR; longitudinal total HCV-specific T-cell responses, measured with the 12-day T-cell expansion assay, are displayed pre-treatment and at week 4 of pegIFN-α/ribavirin therapy for CD4+ (panel a) and CD8+ (panel b) T-cells. The *y*-axis shows the number of IFN-γ producing T-cells per million PBMC. On the *x*-axis the different time point are shown (pre-treatment and week 4) while patients are grouped into SVR (○/●) or non-SVR (□/■). Removing the SVR patient with the high HCV-specific CD8+ T-cell response at week 4 of therapy from the analysis resulted in a trend towards a significant decrease in T-cell responses at this time point ($p = 0.08$). Of two patients (one SVR and one non-SVR) the PBMC failed to grow in the expansion assay (no positive control).

virological parameter determining the chances of SVR (Ferenci, 2004; Zeuzem et al., 2006). Consequently, at this time point patients with detectable and those without detectable HCV-RNA were present allowing us to investigate the relationship between antigen presence and HCV-specific T-cell responses.

To illustrate the HCV-specific T-cell responses over time, flow cytometry dot plots of IFN-γ producing CD8+ T-cells against NS4 are shown (Fig. 3) for both a SVR and a non-SVR patient. The percentage of IFN-γ producing HCV-specific CD8+ T-cells declined during therapy in both the SVR patient (from 2.18% at baseline to 0.70% at week 4) and the non-SVR patient (from 0.85% at baseline to 0.01% at week 4). To account for the difference in T-cell proliferation, total HCV-specific T-cell responses were calculated by combining these T-cell proliferation ratios with the IFN-γ production. Regardless of the outcome of therapy, a decline in HCV-specific CD4+ and CD8+ T-cell responses was observed from baseline to week 4 (Fig. 4a and b). This decrease was more prominent in the CD8+ T-cell responses in non-SVR patients (median decline from 11 491 (IQR 829–26 992) to 1011 (IQR 351–5904); $p = 0.02$). The patient who showed a marked increase in HCV-specific CD8+ T-cell responses from baseline (27 345) to week 4 (89 956 per million PBMC) had no distinctive clinical, virological or immunological abnormalities that could explain this sharp increase (Fig. 4b). Similarly, when patients were grouped according to achievement of a RVR, a decline in HCV-specific CD4+ and CD8+ T-cell responses was observed as seen in SVR/non-SVR patients (data not shown).

Since at baseline skewing towards the NS peptides was associated with SVR, we evaluated the breadth of the response by the number of HCV NS-antigens targeted per patient at baseline and at week 4 of therapy. We observed that for both the CD4+ and CD8+ T-cell response the median number of targeted NS-antigens remained relatively conserved from baseline to week 4 in both SVR patients (3–4 and 4–3, respectively) and non-SVR patients (3–2 and 3–2, respectively).

4. Discussion

This study evaluated the role of HCV-specific T-cell responses in PBMC of chronic HCV genotype 1 and 4 patients before and during pegIFN-α/ribavirin therapy using a sensitive 12-day expansion assay. We are able to demonstrate that irrespective of clinical outcome, pre-treatment HCV-specific CD4+ and CD8+ T-cell responses exist and that they markedly decline during pegIFN-α/ribavirin therapy in both SVR and non-SVR patients. Moreover, a similar pattern of HCV-specific T-cell responses was observed in patients achieving a RVR when compared to those without a RVR. Finally, skewing of the T-cell response against the NS-antigens was found to be associated with reaching a SVR.

T-cell proliferation and IFN-γ production are important parameters of an effective anti-viral response (Neumann-Haefelin et al., 2005) which was generally analyzed using separate assays (Hoffmann et al., 1995; Kaplan et al., 2005; Lasarte et al., 1998; Lauer et al., 2005; Rosen et al., 2007). In recent years several groups have used T-cell expansion in different assays trying to combine both features (Barnes et al., 2009; Chang et al., 2001; Pilli et al., 2007). The 12-day T-cell expansion used in our study also combines both features of proliferation and IFN-γ production into one assay allowing simultaneous detection of CD4+ and CD8+ T-cell responses. The assay was first described by Reece et al. (2004) as an ex vivo expansion assay in which IFN-γ secretion was assessed by ELISPOT. Our group has previously modified this 12-day expansion assay to allow the IFN-γ secretion to be measured by flow cytometry validating this method for Epstein–Barr virus infection (Piriou et al., 2005; Scherrenburg et al., 2008), HIV (Jansen et al., 2006a) and HCV infections (Ruys et al., 2008; van den Berg et al., 2009). It combines the proliferation (and/or survival) of precursor (memory) HCV-specific T-cells after HCV antigen stimulation and IFN-γ secretion upon specific re-stimulation for both CD4+ and CD8+ T-cell responses simultaneously. Compared to proliferation and IFN-γ production assays, the 12-day expansion assay showed an improved detection of HCV-specific T-cell responses. Although, this assay provides an estimate of the total HCV-specific T-cell response in a given patient, irrespective of HLA-type, it does not allow to dissect the specific epitopes which are recognized and results cannot be confirmed using tetramer-staining.

Compared to previous studies showing either no or low pre-treatment HCV-specific T-cell responses (Aberle et al., 2007; Barnes et al., 2002; Cramp et al., 2000; Hoffmann et al., 1995; Kamal et al., 2002; Kaplan et al., 2005) studies demonstrating strong and multi-specific T-cell responses to HCV at baseline associated with or without achievement of SVR (Barnes et al., 2009; Burton et al., 2008; Caetano et al., 2008; Capa et al., 2007; Marinho et al., 2004; Nelson et al., 1998; Pilli et al., 2007; Rosen et al., 2007; Tang et al., 2005), our study was distinctive. First, in this prospective study the use of a sensitive expansion assay greatly improved the detection of HCV-specific T-cell responses. Second, no association was found between either pre-treatment or on-treatment HCV-specific CD4+ and CD8+ T-cell responses and the outcome of pegIFN-α/ribavirin therapy in terms of achieving a RVR, although the number of included patients was small. Furthermore, T-cell responses decline during anti-HCV treatment in all patients irrespective of clinical outcome.

The HCV-specific T-cell response in chronic HCV patients is highly regulated. It has become clear that multi-factorial mechanisms negatively regulate the HCV-specific T-cell response via mechanisms involving immunosuppressive cytokines and regulators such as PD-1 (Urbani et al., 2008; Walker et al., 2010). The enhanced sensitivity of the 12-day T-cell expansion assay could rely on lifting the regulation during the culture period, possibly as a result of the addition or production of growth factors, such as IL-2. Furthermore, when analyzing the different HCV peptides in relation to SVR, a skewing of reactivity towards the non-structural HCV peptides is observed. This is in agreement with previous studies (Rosen et al., 2002; Vertuani et al., 2002) showing an association between a vigorous T-cell response against NS3 and successful outcome of therapy. Furthermore, albeit not performed in our study, some studies have shown via tetramer analysis that specific epitopes in core and NS3 correlate with SVR (Barnes et al., 2002; Caetano et al., 2008). Therefore, rather than looking at the total HCV-specific response, future studies, possibly using tetramers, should focus more on the detailed poly-functionality and quality as well as regulation of HCV-specific T-cell responses to identify pre-treatment factors responsible for successful therapy.

By evaluating the role of HCV-specific T-cells during pegIFN-α/ribavirin therapy in both SVR and non-SVR patients a similar decline in HCV-specific T-cell responses from baseline to week 4 was demonstrated in both patient groups. Immunological parameters like T-cell activation or a shift in the numbers of T-cell subsets could not explain the therapy-induced decline of the HCV-specific T-cell response. This is in accordance with some other studies showing a decline in HCV-specific T-cell responses using IFN-γ production as direct effector function of T-cell activity (Barnes et al., 2009; Caetano et al., 2008; Pilli et al., 2007). In contrast, others have suggested augmentation of T-cells during therapy using T-cell proliferation assays with fresh PBMC and [^{3}H]-thymidine incorporation, thereby mainly describing T-cell memory function (Barnes et al., 2002; Cramp et al., 2000; Kamal et al., 2002). A possible explanation is that due to the decrease in viral load and thereby loss of antigen-triggering of HCV-specific T-cells, these T-cell responses decline. This would suggest that, similar to other infections like HIV (Benito et al., 2003; Jansen et al., 2006b), loss of antigen leads to a reduction of virus-specific T-cells and demonstrates no role

for HCV-specific T-cell responses in forced viral clearance during pegIFN-α/ribavirin therapy. This argument is supported by the observation that T-cell responses recover after treatment discontinuation in patients experiencing a relapse of HCV viremia (Barnes et al., 2002, 2009; Burton et al., 2008). One of our patients also experienced a relapse at the end of therapy with an increase in HCV-RNA levels subsequently followed by an increase in HCV-specific T-cell responses (data not shown).

Homing of HCV-specific T-cells to the liver during treatment is mentioned as an explanation for the low or absent T-cell responses measured in blood (Kaplan et al., 2005; Penna et al., 2002). This hypothesis is supported by the observation that in cross-sectional studies higher percentages of HCV-specific T-cells are found in the liver than in peripheral blood (Grabowska et al., 2001; Wong et al., 1998). However, peripheral T-cell responses are easily measurable in patients spontaneously clearing acute HCV (Day et al., 2002; Gruner et al., 2000; Missale et al., 1996). Moreover, a recent study in chronic HCV patients demonstrated that several of the measured intra-hepatic epitope-specific T-cell responses were also found in PBMC only after ex vivo expansion of these T-cells (Neumann-Haefelin et al., 2008; Penna et al., 2002). This indicates that these responses present in the liver can also be measured in peripheral blood albeit at low frequencies. Therefore, the use of a sensitive expansion assay, as used in our study, enabled us to detect these small frequencies of peripheral HCV-specific T-cells. However, extrapolation of findings obtained using peripheral blood to the functionality of intra-hepatic T-cells should be made with caution, since the microenvironment of the liver may modulate the phenotype and activity of these cells (Claassen et al., 2010).

In conclusion, using a sensitive expansion assay, we found no correlation between pre-treatment HCV-specific T-cell responses and outcome (SVR or RVR) of pegIFN-α/ribavirin therapy by assessing the total response to peptides spanning the entire HCV genome. However, skewing of the individual T-cell responses towards the NS-antigens was observed. Furthermore, irrespective of treatment outcome, HCV-specific T-cell responses declined during treatment in all patient groups. Although this suggests that total HCV-specific T-cell responses do not play a role in forced viral eradication, it is possible that HCV-specific T-cells at baseline initially maintain (or even increase) their effector function in the first weeks of therapy after which a decline sets in. Further studies to assess HCV-specific T-cell poly-functionality, kinetics and regulation in detail are needed.

Conflict of interest

A.I.M. Hoepelman (advisory board of Tibotec, Pfizer, BMS and Gilead and received grants from Roche, Gilead, Pfizer and Bayer), J.E. Arends (advisory board of Merck) and L.C. Baak (speakers' bureau, Roche). The other authors have no conflict of interest to report.

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