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Maintenance of Th1 HCV-specific responses in individuals with acute HCV who achieve sustained virological clearance after treatment

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None

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Abstract

Background and aim—T cell responses against hepatitis C are believed to be critical in achieving both natural and treatment induced clearance. However, rapid clearance of antigen with early treatment of primary infection may result in reduced or poorly sustained cellular immunity. This study longitudinally examined Th1 and Th2 HCV-specific cytokine production and T cell effector function from subjects enrolled in the Australian Trial in Acute Hepatitis C (ATAHC) comparing three groups: treatment-induced clearance (sustained virological response [SVR]), treatment non-response, and untreated spontaneous clearance.

Methods—HCV-specific T cell responses were characterised by HCV peptide ELISpot, *in-vitro* cytokine production and T cell flow cytometry assays.

Results—Treated subjects with a SVR displayed a better maintenance of HCV-specific Th1 responses compared to treatment non-responders (higher interferon (IFN)- and interleukin (IL)-2 magnitude week 24, broader IFN- responses week 24 and 48, $p < 0.05$), and significantly increased IFN- responses between screening and week 48 (magnitude $p = 0.026$, breadth $p = 0.009$). Treatment-induced viral clearance was also associated with a trend towards decreased IL-10 responses (screening to week 48 $p = 0.070$), higher expression of CD45RO ($p = 0.042$) and CD38 ($p = 0.088$) on CD4⁺ T cells and higher IFN- R expression (CD56⁺IFN- R⁺ $p = 0.033$) compared to treatment non-responders. Untreated subjects with viral clearance also displayed high magnitude and broad HCV-specific IFN- and IL-2 responses early in infection, however IFN- responses were not as well maintained compared to treated subjects with a SVR (week 48 magnitude, breadth $p = 0.064$).

Conclusion—Treatment-induced viral clearance of recent HCV infection is associated with maintenance of HCV-specific Th1 responses.

Keywords

Acute hepatitis C; injecting drug users; immune response; PEG-IFN- ; T cells

Introduction

Therapeutic intervention in acute HCV infection has been associated with improved treatment outcomes and less complex regimens than in chronic HCV, with 24-weeks of pegylated interferon- (PEG-IFN) monotherapy (1–4) or PEG-IFN combined with ribavirin (5–7) being the standard of care regardless of genotype.

In the absence of therapy, the likelihood of clearance from acute HCV infection has been shown to be influenced by the presence of functional CD4⁺ helper and CD8⁺ T cell responses (8, 9). Whether early treatment alters these HCV-specific T cell responses in acute infection is unclear. Some studies of acute HCV treatment-induced clearance have demonstrated sustained HCV-specific T cell responses (6, 10), detection of HCV-specific memory T cells for 1.5 years (11) and a sustained CD8⁺ T cell responses but not CD4⁺ T cell responses (12), whereas others have not (13–15). To add to the complexity, both Kamal et al (2004) and Rahman et al (2004) suggested a reduction in HCV RNA via HCV treatment was associated with increased HCV-specific T cell responses, albeit transiently.

A more recent study in acute HCV by Schulze zur Wiesch et al (2012) demonstrated broad HCV-specific CD4⁺ T cell responses early in infection, regardless of clinical outcome (16). In this study, persistent viremia was associated with a loss of HCV-specific responses, whereas restoration of CD4⁺ T cell responses was only possible through early treatment, which enhanced viral clearance (16). These data suggest initiation of treatment during acute HCV infection could play a role in the restoration and maintenance of HCV-specific CD4⁺ T cell responses important for viral clearance. Similarly, Badr et al (2008) found early therapeutic intervention could rescue polyfunctional T cell responses (11) and a later study from the same laboratory demonstrated the presence of multifunctional CD4⁺ and CD8⁺ memory T cells in spontaneous clearers and in those who cleared HCV through early treatment, whereas limited responses were detected in those who received therapy during chronic HCV infection (17). These studies suggest a narrow window may exist whereby early initiation of therapy may preserve HCV-specific T cell responses preventing T cell exhaustion, demonstrated in chronic HCV infection (18–20).

The aim of this study was to examine HCV-specific cytokine production and effector function of T cells from subjects with acute HCV with samples collected longitudinally (pre-, during and post-treatment) comparing three groups: treatment-induced clearance, treatment non-response, and untreated spontaneous clearance.

Materials and Methods

Study design

The ATAHc study was a multicenter, prospective cohort study of the natural history and treatment of recent HCV infection (2). All study participants provided written informed consent. The study protocol was approved by St Vincent's Hospital, Sydney Human Research Ethics Committee and through local ethics committees. The study was registered with clinicaltrials.gov registry (NCT00192569) and conducted according to the Declaration of Helsinki and ICH/GCP guidelines.

The ATAHc study was a non-randomised evaluation of early HCV therapeutic intervention in HCV mono-infected and HIV/HCV co-infected subjects (2, 21, 22). Of 163 subjects enrolled, 111 initiated interferon-based therapy. Treated HCV mono-infected subjects received 24-weeks of PEG-IFN α 2a (180 micrograms weekly, wk0–24) and the SVRs were 55% and 72% by intention-to-treat and per-protocol analysis, respectively (2). Screening was a pre-treatment timepoint (~4–6 weeks pre-treatment), wk12 and wk24 denoted 12 and 24 weeks of treatment respectively, and wk48 was 24 weeks after treatment cessation. Treated subjects were designated as having a SVR if they were HCV RNA negative at wk48 and a non-response (NR) if they were HCV RNA positive from screening to wk48. Untreated subjects with spontaneous clearance (>2 consecutive negative qualitative HCV RNA assessments) were assessed at the same timepoints.

In this immunological sub-study subjects with the following criteria were included: HCV mono-infected, HCV RNA positive at screening, an estimated duration of HCV infection of <32 weeks at screening, and samples available (Table 1). Treated subjects were required to have received at least 80% of planned therapy and have a defined virological outcome (SVR or NR). Untreated spontaneous clearers were required to have detectable HCV RNA at screening, and two consecutive undetectable HCV RNA assessments during follow-up. Sub-study subjects were assessed for HCV-specific cytokine responses from screening to wk48.

Virological testing

HCV RNA assessment was performed with a qualitative HCV-RNA assay (TMA assay, Versant, Bayer, Australia, detection limit 9.6 IU/ml, (23)) and if detectable repeated on a

quantitative assay (Versant HCV RNA 3.0 Bayer, Australia, detection limit 615 IU/ml). HCV genotype (Versant LiPa2, Bayer) and HCV IL-28B genotyping (rs8099917, rs1297860 and rs12980275) was performed as previously described (24, 25).

HCV peptides

Immunological assays were performed using peptides (18aa, overlapping by 11aa) based on the HCV genotype 1a sequence (NIH AIDS Reference and Reagent Program, Division of AIDS, NIAID, NIH: HCV 1a H77 Peptides) covering the entire HCV coding region. Peptides were grouped into 10 pools for ELISpot assays (Core, E1, E2, p7, NS2, NS3, NS4a, NS 4b, NS5a, NS5b), and three pools for multiplex *in vitro* cytokine production assays (Core-p7, NS2-3, NS4-5).

IFN- γ and IL-2 ELISpot assays

ELISpot assays were performed following the manufacturer's protocol (Mabtech, Nacka, Sweden) with the exception of the coating antibody concentration (5 μ g/mL). PBMCs were added to triplicate wells at 1×10^5 cells/well with HCV peptide pools (1 μ g/mL) or controls (PHA 5 μ g/mL, CEF peptides 2 μ g/mL, anti-CD3 2 μ g/mL, negative control RPMI 10% FCS 0.8% DMSO), previously described (21, 25). Plates were incubated at 37°C, 5% CO₂ for 24h. Spot-forming cells (SFC) were evaluated using an automated ELISpot reader (AID version 3.2.3; Strasberg, Germany). Positive responses were at least twice background and the threshold, 50 SFC/ 10^6 PBMC, was previously determined using 15 seronegative blood donors (21).

IFN- γ and IL-10 ELISpot blocking and re-stimulation experiments

PBMC (1×10^5) were incubated with a HCV peptide pool (1 μ g/mL), or controls (PHA 5 μ g/mL, CEF 2 μ g/mL, RPMI 10% FCS 0.8% DMSO). Blocking antibodies (10 μ g/mL final, IFN- γ MAB285 or IL-10 MAB217 R&D systems, Minneapolis, MN) or recombinant proteins (10ng/mL final, IFN- γ 285-IF or IL-10 217-IL R&D systems) were added to IL-10 and IFN- γ ELISpot assays (respectively). Isotype controls (10 μ g/mL MAB002 or MAB004 R&D Systems) were used and ELISpot assays were performed (above methods) with known positive HCV peptides.

Multiplex in-vitro cytokine production

Multiplex *in-vitro* Th1 and Th2 cytokine assays (IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-13, IFN- γ , TNF- α , GM-CSF plus IFN- γ) were performed following the manufacturer's protocol (Bio-plex Human Cytokine Assay; Bio-Rad, CA, USA), previously described (21, 25). Briefly, PBMCs were cultured in 384-well microplates (7.5×10^5 /well), stimulated with HCV peptide pools (1 μ g/mL) or controls (PHA 5 μ g/mL, CEF peptides 2 μ g/mL, RPMI 10% FCS 0.8% DMSO). Cells were incubated at 37°C for 48h, before 50 μ L of supernatant was assayed in a bead-based enzyme-linked immunosorbent assay.

Expression of cellular markers

Cellular marker expression on CD4⁺ and CD8⁺ T cells was assessed with HCV Core-p7 (Core, E1, E2 and p7 peptides) peptide pool stimulated wk12 PBMC from SVR and NR subjects who had previously demonstrated a positive IFN- γ response to these peptides. One million PBMC were stimulated in 24 well plates for 24h prior to phenotyping with CD3 PerCP, CD4 APC and CD8 FITC antibodies with a PE antibody for CD45RA, CD45RO, CD62L, CD38, CD69, Ki-67 and IFN- γ and IL-10 receptors (BD Biosciences, NSW, Australia). IFN- γ R (CD119) and IL-10R (CDw210) expression was also examined on CD14⁺, CD19⁺ and CD56⁺ cells. Cells were assessed on a BD FACSCalibur (collecting

>100,000 events) and analyzed using BD FACSDiva software (version 4.1.2) and Gategistic (Inivai, Melbourne, Australia).

Statistical Analysis

Non-parametric analyses were performed using Mann-Whitney or Wilcoxon sign rank tests, and Fisher's exact test or chi-square test for categorical analyses, as appropriate. Correlations were performed with Spearman's statistic (Stata/IC 10.0 for Windows, TX, USA). A significance level of 0.05 was used for all analyses.

Results

Treated subjects

Clinical characteristics were similar between SVR (n=10) and NR subjects (n=4, $p>0.05$, Table 1), with the majority of subjects reporting injecting drug use (IDU, SVR 80%, NR 100%). All SVR subjects had undetectable HCV RNA by wk12 (mean VL screening 5.50 ± 1.07 log₁₀ HCV RNA UI/mL, wk12 0.00 ± 0.00 , $p<0.001$), with seven subjects (70%) having a rapid virological response (undetectable HCV RNA wk4). NR subjects remained HCV RNA positive throughout the study. Both groups significantly reduced ALT between screening and wk12 (mean SVR screen 242 ± 197 IU/mL wk12 57 ± 39 , $p=0.045$, NR screen 299 ± 123 IU/mL wk12 74 ± 34 , $p=0.021$).

Untreated subjects

In comparison to treated SVR subjects, untreated clearers (n=4) had a lower duration of infection (mean weeks clearers 16 ± 10 , SVR 22 ± 4), VL (median clearers 2.42 IQR 2.1–3.9 IU/mL, SVR 4.95 IQR 4.1–5.7) and ALT (median clearers 71 IQR 24–952, SVR 227 IQR 42–387 IU/mL) at screening ($p>0.05$, Table 1). Other clinical characteristics were similar between the cohorts and the majority of subjects reported IDU (clearers 75%, SVR 80%). Untreated clearers were HCV RNA negative by wk24, with three subjects clearing by wk4 (75%). In comparison, treated SVR subjects had undetectable HCV RNA by wk12.

HCV-specific IFN- γ and IL-2 ELISpot responses

Treated subjects—Subjects with a SVR showed a trend for better maintenance of HCV-specific IFN- responses compared to treatment NR, with significantly higher IFN- responses at wk24 (magnitude $p=0.043$, breadth $p=0.048$) and wk48 (breadth $p=0.031$, Figure 1a–b). SVR subjects displayed a peak in IFN- responses at wk48 (24wks post-treatment and >36wks post-clearance, magnitude 421 ± 413 SFC, breadth 2 ± 1 pools), which was a significant increase in magnitude ($p=0.026$) and breadth ($p=0.009$) from screening (Figure 1a–b). In contrast, NR subjects displayed a peak in HCV-specific IFN- responses during treatment at wk12 (magnitude 180 ± 202 SFC, breadth 1 ± 1 pools) and a decrease at wk24 (below background, magnitude 20 ± 34 SFC, breadth 0 ± 0 pools). The specificity of the IFN- responses was largely directed towards E2 and NS5b.

HCV-specific IL-2 responses were of a lower magnitude and breadth than IFN- responses, with few SVR subjects and no NR subjects having positive HCV-specific IL-2 responses from screening to wk48 (Figure 1c–d). Despite the low magnitude, SVR subjects maintained HCV-specific IL-2 responses to wk48 (>50 SFC wk12–wk48), with significantly higher magnitude of IL-2 responses at wk24 compared to treatment NR ($p=0.043$). The specificity of IL-2 responses was largely directed towards E2, p7 and NS5b.

There were no significant differences in magnitude or breadth of HCV-specific IFN- and IL-2 responses between subjects infected with genotype 1 and those infected with non-

genotype 1 strains (IFN- γ magnitude $p=0.689$, breadth $p=1.000$, IL-2 magnitude $p=0.195$, breadth $p=0.456$).

Treated SVR subjects and untreated clearers—HCV-specific IFN- γ responses were of a high magnitude and breadth in both cohorts early in infection, particularly in untreated clearers (screen magnitude treated 111 ± 100 SFC untreated 412 ± 289 SFC $p=0.038$, breadth treated 1 ± 1 untreated 2 ± 3 $p=0.091$ Figure 2a–b). IFN- γ responses began to wane in untreated clearers after viral clearance, with a trend for better maintenance in treated SVR subjects at wk48 (magnitude treated 425 ± 410 SFC, untreated 90 ± 90 SFC $p=0.064$, breadth treated 3 ± 1 , untreated 1 ± 1 $p=0.064$). Treated SVR subjects also increased IFN- γ responses from screening to wk48 (SVR magnitude $p=0.026$, breadth $p=0.009$, Figure 2a–b).

There was a trend for HCV-specific IL-2 responses to be lower in magnitude and breadth in treated SVR subjects compared to untreated clearers (screening magnitude treated 33 ± 45 SFC untreated 130 ± 60 SFC $p=0.086$), significant at wk24 (magnitude treated 66 ± 74 SFC untreated 260 ± 270 SFC $p=0.050$, breadth treated 0 ± 0 untreated 2 ± 2 $p=0.007$, Figure 2c–d). HCV-specific IL-2 responses were maintained in both cohorts after viral clearance.

Representative examples of a subject from each cohort, demonstrating HCV-specific IFN- γ and IL-2 responses are shown in Figure 3a–f.

Blocking and re-stimulation experiments

HCV-specific IFN- γ production was significantly increased in treated SVR subjects (screening $p=0.043$, wk48 $p=0.043$) through the addition of IL-10 blocking antibody (Supplementary Figure 1a). Whilst, addition of recombinant IL-10 protein significantly decreased IFN- γ responses (screening $p=0.043$, wk48 $p=0.043$, Supplementary Figure 1b). Conversely, IFN- γ blocking antibody significantly increased HCV-specific IL-10 production (screening, wk48 $p=0.042$, Supplementary Figure 1c), while the recombinant IFN- γ protein significantly decreased IL-10 production (screening $p=0.043$, wk48 $p=0.042$, Supplementary Figure 1d). There were too few cells to analyze the NR cohort, however the same significant trends were seen in untreated subjects with viral clearance and persistence ($p<0.05$, data not shown) and in individual cell populations for IFN- γ ($CD4^+$ and $CD8^+$ T cells) and for IL-10 production ($CD4^+$ and $CD8^+$ T cells, $CD14^+$ cells, data not shown). The single cell analysis was also concurrent with our previously published findings of HCV-specific IFN- γ and IL-10 producing cells in acute HCV (ELISpot cell depletion studies and ICS (21)).

Type 1 and 2 in-vitro cytokine production in treated subjects

SVR subjects displayed a trend towards higher HCV-specific Th1 cytokine production and better maintenance of the specificity of HCV-specific cytokines compared to NR, although this did not reach significance ($p>0.05$). SVR subjects displayed a trend of decreased IL-10 production screening to wk48 (median screen 351 pg/mL, wk48 206 pg/mL $p=0.070$). In contrast, NR subjects demonstrated maintenance of IL-10 production screening to wk48 (median screen 467 pg/mL, wk48 431 pg/mL Figure 4a). Representative examples of HCV-specific IL-10 production by SVR and NR subjects are shown in Figure 4b–c.

Cellular marker expression in treated subjects

Following HCV-stimulation, SVR subjects displayed a trend towards higher CD45RO and CD38 expression on $CD4^+$ T cells ($CD3^+CD4^+CD45RO^+$ cells $p=0.042$, $CD3^+CD4^+CD38^+$ cells $p=0.088$, Figure 5a–b) compared to NR subjects. CD69 and Ki-67 expression was low in both cohorts (range 0.1–1%, Figure 5b) compared to control donors (HCV RNA negative, $CD4^+$ T cells 1–4%, $CD8^+$ T cells 7–13%, data not shown).

IFN- γ expression was higher in SVR subjects, significantly on NK cells (CD56 $^{+}$ $p=0.033$, Figure 5c) compared to NR subjects. IFN- γ was expressed highest on CD8 $^{+}$ T cells and NK cells and IL-10R was expressed highest on CD8 $^{+}$ T cells and CD14 $^{+}$ cells in both cohorts (Figure 5c–d). This is concurrent with our previously published findings of HCV-specific IFN- γ and IL-10 producing cells in acute HCV (ELISpot cell depletion studies and ICS (21)).

Cellular marker expression on unstimulated cells from SVR and NR subjects showed similar trends (Supplementary Figure 2a–d). Representative FACS plots illustrating the detection of cellular markers are shown in Supplementary Figure 3.

Correlations with clinical parameters

Clinical characteristics were not correlated with magnitude or breadth of cytokine production at screening in SVR subjects, NR subjects or untreated clearers ($p>0.05$).

Discussion

Few studies have examined the influence of early therapeutic intervention on the development and maintenance of T cell responses in acute HCV infection. Our findings demonstrate treatment-induced clearance was associated with a better maintenance of HCV-specific Th1 responses, which may have implications for protection against HCV reinfection.

Subjects who achieved a SVR had a higher breadth, magnitude and maintenance of HCV-specific IL-2 and IFN- γ responses compared to treated subjects with NR, despite the latter having high magnitude IFN- γ responses early in infection. Untreated clearers also displayed high magnitude and broad IL-2 and IFN- γ responses, however IFN- γ responses waned post viral clearance suggesting better maintenance in treatment-induced clearance. Both IL-2 and IFN- γ have been demonstrated to be a key cytokines for clearance of acute and chronic HCV (19, 21, 26, 27). IL-2 potentially due to its role in the differentiation of CD8 $^{+}$ T cells into effector and long lived memory cells (28, 29) and IFN- γ for its role as an anti-viral cytokine which can inhibit viral replication (30). Several studies have shown IFN- γ producing CD8 $^{+}$ T cells to be associated with clearance of HCV (8, 31–33) and similar to our study some have reported these responses to drop after viral clearance in untreated subjects (8, 33). Thus, the T cell priming environment and maintenance of HCV-specific Th1 responses is likely to be important for acute HCV clearance.

The expression of IFN- γ was highest on CD8 $^{+}$ T cells and NK cells from SVR subjects, concordant with our previous findings of cells producing HCV-specific IFN- γ (21). NK cells are large producers of IFN- γ and can play a vital role in activation and immunoregulation of T cells, B cells and DC (30). Previous studies have shown an increase in NK activity (34) and expression of particular KIR receptors (KIR2DL3 (35)) to be associated with HCV clearance. Thus, increased IFN- γ producing cells and IFN- γ levels early in HCV infection may be linked to increased NK activity, contributing to early immune activation and potential clearance of HCV.

SVR subjects also showed a trend for decreasing HCV-specific IL-10 production, whilst treated NR subjects showed a trend for maintenance of IL-10 responses. Kaplan et al (2008) demonstrated a correlation of HCV-specific IL-10 responses with elevated liver enzymes, increased viremia and suppressed HCV-specific CD4 $^{+}$ T-cell proliferation in acute HCV infection, where IL-10 responses persisted in chronic HCV infection (36). Similarly in untreated subjects, we have previously demonstrated high IL-10 to be associated with viral persistence (21). In the current study we demonstrated a detrimental effect of IL-10 upon the

production of HCV-specific IFN- responses (similar to Kaplan et al (36)). IL-10 is an immunoregulatory cytokine, where excess IL-10 has the ability to suppress Th1 cytokine production and proliferation by CD4⁺ and CD8⁺ T cells (37, 38). Thus suggesting IL-10 may have a role in viral persistence, with a low level required for immune regulation, but higher levels detrimental for viral clearance (21, 39–41).

This study showed a trend for increased expression of CD45RO and CD38 on T cells from SVR subjects compared to NR. Previous studies in chronic HCV have demonstrated a lower expression of activation markers (CD38, HLA-DR, CD69) and CD127 and an increased expression of inhibitory receptors (2B4, CD160, KLRG1, PD-1) on T cells (compared to acute infection), which have been associated with T cell exhaustion (20, 42–46). Although a more comprehensive analysis is required, our preliminary results may suggest a trend towards the development of a more chronic HCV effector phenotype, also described as the 'stunned' phenotype (18). This may stem from a poor priming environment (decreased Th1 cytokines, lack of CD4 help, increased IL-10), which could decrease the effector function of T cells and their ability to become activated.

It is also important to note some potential limitations of this study. Firstly, genotype 1a peptides were used as the majority of participants were genotype 1a. Despite cross-reactive responses being detected, the recent availability of peptide sets from other genotypes will allow more precise definition of cytokine production and cross genotype responses in future studies. Secondly, phenotype analysis was performed following HCV peptide stimulation. Despite similar trends present before and after peptide stimulation, we cannot dismiss that this technique may cause an amplification of a cell phenotype not directly representative of the cells in-vivo. Future studies using HLA tetramers would be a beneficial addition to study the phenotype of T cells. Thirdly, we cannot discount the maintenance of HCV-specific T cell responses seen in SVR after viral clearance are not attributed to by reappearance of HCV RNA (detectable by PCR) as recently illustrated by Veerapu et al (2011) (47).

This study suggests that the early therapeutic intervention in acute HCV can positively assist the generation and maintenance of HCV-specific Th1 responses, and has provided evidence for the importance of maintaining polyfunctional HCV-specific Th1 T cells for treatment-induced clearance of HCV. Spontaneous clearers of HCV also displayed a high magnitude of Th1 responses early in acute infection, however the IFN- response waned after viral clearance. A better understanding of the mechanisms behind clearance of acute HCV, and the effect of therapy on the cellular immune response early in HCV, is important to guide current therapeutics in acute HCV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

aa	amino acids
ALT	alanine aminotransferase
ATAHC	Australian Trial in Acute Hepatitis C
GM-CSF	granulocyte monocyte colony stimulating factor
h	hours
HCV	Hepatitis C virus
IDU	injection drug users
IFN-	Interferon-gamma
IL-	Interleukin-
NR	non-responder
PBMC	peripheral blood mononuclear cell
PEG-IFN	pegylated-interferon
SFC	spot forming cells
SVR	sustained virological response
TNF-	tumor necrosis factor-alpha
VL	viral load
wk	week

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Figure 1. Longitudinal analysis of the magnitude and breadth of HCV-specific IFN- and IL-2 cytokine responses in SVR (n=10) and NR (n=4) subjects from screening to week 48

A) There was a trend for better maintenance of IFN- responses in SVR compared to NR subjects screening to wk48, with SVR subjects having significantly higher IFN- production at wk24 compared to NR ($p=0.043$, Mann-Whitney). Subjects with an SVR had a significant increase in IFN- responses at wk48, comparing pre- and post-treatment responses ($p=0.026$, Mann-Whitney). B) The breadth of IFN- responses showed a similar trend for a better maintenance of responses in subjects with a SVR. SVR subjects had significantly broader IFN- response at wk24 ($p=0.048$, Mann-Whitney) and wk48 compared to NR ($p=0.031$, Mann-Whitney). Subjects with an SVR also significantly increased the breadth of IFN-

responses at wk48, comparing pre- and post-treatment responses ($p=0.009$, Mann-Whitney). The magnitude C) and breadth D) of HCV-specific IL-2 responses were low in both cohorts with few SVR and no NR subjects having positive HCV-specific IL-2 responses. SVR subjects had a significantly higher magnitude of IL-2 responses at wk24 compared to NR ($p=0.043$, Mann-Whitney). Scatter plots represent each participant's HCV-specific response with the median denoted by a horizontal line. SVR subjects are represented by black circles and NR subjects by grey triangles. One asterisk represents $p<0.05$.

A)

B)

C)

D)

Figure 2. Longitudinal analysis of the magnitude and breadth of HCV-specific IFN- and IL-2 cytokine responses in subjects with a SVR (n=10) and untreated clearers (n=4) from screening to week 48

A) Early in infection untreated clearers had a higher magnitude of IFN- responses compared to treated subjects with an SVR (screening $p=0.038$, Mann-Whitney). There was a trend for better maintenance of IFN- responses in treated subjects with an SVR compared to untreated clearers (wk48 $p=0.064$, Mann-Whitney). Subjects with an SVR significantly increased their IFN- responses at wk48, comparing pre- and post-treatment responses ($p=0.026$, Mann-Whitney). B) There was a trend for broader IFN- responses at screening in untreated clearers compared to treated subjects with an SVR ($p=0.091$, Mann-Whitney).

This breadth was not maintained in untreated clearers with a trend for lower breadth at wk48 compared to treated subjects with an SVR ($p=0.064$, Mann-Whitney). Subjects with an SVR significantly increased the breadth of IFN- responses at wk48, comparing pre- and post-treatment responses ($p=0.009$, Mann-Whitney). C) HCV-specific IL-2 responses were lower in magnitude in treated subjects with an SVR compared to untreated clearers (screening $p=0.086$, wk24 $p=0.050$ Mann-Whitney). D) There was trend for the breadth of IL-2 responses to be higher in untreated clearers compared to treated subjects with an SVR, significant at wk24 ($p=0.007$, Mann-Whitney). Scatter plots represent each participant's HCV-specific response with the median denoted by a horizontal line. SVR subjects are represented by black circles and untreated clearers by clear. One asterisk represents $p<0.05$.

Figure 3. Representative example of IFN- and IL-2 HCV-specific in subjects with a treatment non-response (NR n=1), subjects with a treatment-induced sustained virological response (SVR n=1) and in a spontaneous clearer (untreated clearer n=1)

A) NR subjects show a trend of low level of IFN- production which decreased longitudinally screen to wk48. B) Subjects with a SVR demonstrate a higher magnitude of IFN- responses compared to NR and a trend for maintenance of IFN- production after treatment ceases and after viral clearance. C) Untreated clearers have an early high magnitude IFN- response, however this does not seem to be as well maintained as those with treatment-induced clearance. D) The IL-2 response in subjects with a treatment NR are very low to undetectable. E) Subjects with a treatment-induced SVR display a higher

magnitude IL-2 response, however it is still quite low. F) Untreated clearers demonstrate a higher magnitude IL-2 response compared to NR and SVR. This response is high during viral clearance and there is a trend for it to be maintained after viral clearance. Each plot shows the HCV RNA levels (left hand y axis) and HCV-specific cytokine responses (right hand y axis) from a single subject from each cohort. Each subject's HCV RNA depicted by the black line with adjoining points. The HCV-specific cytokine responses are shown in dark grey bars. Treatment is illustrated by a light grey bar and timepoints of PBMC collection are indicated by black arrows.

C)

Sustained Virological Responder HCV-specific IL-10 Production

Figure 4. Longitudinal analysis of HCV-specific IL-10 production in SVR and NR subjects from screening to week 48 measured in the multiplex *in-vitro* cytokine array

A) Subjects with a SVR (n=10) displayed a trend for decreased IL-10 production from screening to wk48 (mean screen 664 $\pm$ 824 pg/mL, wk48 196 $\pm$ 111, p=0.070), whereas NR (n=4) displayed a trend for the maintenance of IL-10 production (mean screen 448 $\pm$ 236 pg/mL, wk48 431 $\pm$ 144 pg/ml). B) A representative example of IL-10 production in a NR (n=1). NR demonstrated a trend for maintenance of high IL-10 production screen to wk48. C) A representative example of IL-10 production in a SVR (n=1). Subjects with an SVR demonstrated a trend for a lower level of IL-10 production and a reduction in IL-10 production after viral clearance. In graph A scatter plots represent each participant's HCV-specific response with the median denoted by a horizontal line and SVR subjects are represented by black circles and NR subjects by grey triangles. In graphs B and C each plot shows the HCV RNA levels (left hand y axis) and HCV-specific IL-10 production (right hand y axis) from a single subject from each cohort. Each subject's HCV RNA depicted by the black line with adjoining points. The HCV-specific IL-10 production is shown in dark grey bars. Treatment is illustrated by a light grey bar and timepoints of PBMC collection are indicated by black arrows.

A) Expression of CD45RA, CD45RO and CD62L on HCV peptide stimulated CD4⁺ and CD8⁺ T cells

B) Expression of CD38, CD69 and Ki-67 on HCV peptide stimulated CD4⁺ and CD8⁺ T cells

Figure 5. Expression of cellular markers on Core-p7 HCV peptide stimulated CD4⁺ T cells and CD8⁺ T cells from treated subjects with a SVR (n=6) and NR (n=4)

A) There was significantly higher expression of CD45RO⁺ on CD4⁺T cells in SVR subjects compared to NR (p=0.042, Mann Whitney) B) There was a trend for the expression of CD38 to be higher on CD4⁺ T cells in subjects with a SVR (p=0.088, Mann-Whitney) compared to NR. C) IFN- receptor was expressed highest on CD8⁺ T cells and CD56⁺ cells and was significantly higher on CD56⁺ cells in subjects with a SVR compared to NR (p=0.033, Mann-Whitney). D) IL-10 receptor expression was highest on CD8⁺ T cells and CD14⁺ cells. Bar graphs represent the mean and standard deviation with SVR subjects denoted by white bars and NR by grey bars. One asterisk represents p<0.05.

Table 1

Demographic and clinical characteristics of acute treated subjects and untreated clearers

Clinical Characteristic	Treated SVR	%	Treated NR	%	Untreated Clearers	%
Total participants, (n)	10		4		4	
Sex						
Male	8	80	2	50	3	75
Female	1	10	2	50	1	25
Transgender	1	10	-	-	-	-
Mean age, years (SD)	34 (33)	-	26 (10)	-	25 (1)	-
Ethnicity						
Caucasian	8	80	3	75	4	100
Other	2	20	1	25	-	-
Mode of HCV acquisition						
Injecting drug use	8	80	4	100	3	75
Sexual	1	10	-	-	-	-
Other	1	10	-	-	1	25
Estimated duration of infection (weeks)						
Median (IQR)	24 (17–27)	-	16 (10–21)	-	12 (8–27)	-
Mean (SD)	22 (4)	-	16 (6)	-	16 (10)	-
Presentation of recent HCV						
Acute clinical (symptomatic)	6	60	3	75	1	25
Acute clinical (ALT >400 IU/mL)	1	10	-	-	1	25
Asymptomatic seroconversion	3	30	1	25	2	50
HCV RNA (IU/L)						
Log ₁₀ HCV RNA, Median (IQR)	4.95 (4.1–5.7)	-	4.02 (3.3–4.9)	-	2.42 (2.1–3.9)	-
<400,000 IU/mL	7	70	3	75	4	100
>400,000 IU/mL	3	30	1	25	-	-
ALT (IU/L)						
Median (IQR)	227 (42–387)	-	277 (198–421)	-	71 (24–952)	-
<100 IU/L	3	30	-	-	2	50
100 IU/L	7	70	4	100	2	50

Clinical Characteristic	Treated SVR	%	Treated NR	%	Untreated Clearers	%
HCV genotype						
Genotype 1	7	70	2	50	3	75
Genotype 3	3	30	2	50	1	25