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ANTIGEN-SPECIFIC T LYMPHOCYTE PROLIFERATION DECREASES OVER TIME IN ADVANCED CHRONIC HEPATITIS C

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Abstract

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To evaluate T cell immunity in advanced liver disease, antigen-specific lymphoproliferative responses were prospectively studied in the context of the Hepatitis C Antiviral Long-term Treatment against Cirrhosis (HALT-C) trial. Peripheral blood responses to HCV, tetanus and *Candida* protein antigens were measured at baseline, Month 12 (M12), M24, M36 and M48 in 186 patients randomized to either low-dose PEG-IFN only or observation. Liver histology was evaluated at baseline, M24 and M48. Patients with cirrhosis (Ishak 5–6) were less likely to have positive lymphoproliferative responses to HCV at baseline than patients with fibrosis (15% vs 29%, $p=0.03$) and had lower levels of HCV c100 responses at baseline, M24 and M48 ($p=0.11$, $p=0.05$, $p=0.02$, respectively). For 97 patients with complete longitudinal data, the frequency of positive lymphoproliferative responses to HCV, tetanus and *Candida* antigens declined over time ($p<0.003$) and the slope of this decline was greater in the PEG-IFN treatment group than the observation group ($p < 0.02$). Lower levels of tetanus lymphoproliferative responses were associated with fibrosis progression and clinical outcomes ($p=0.009$). Poorer CD4+ T cell proliferative function is associated with more advanced liver disease in chronic hepatitis C, and may be further affected by long-term PEG-IFN treatment.

Keywords

Hepatitis C Virus; T cell; Cirrhosis; Fibrosis; Interferon-alpha; Lymphoproliferation

Hepatocyte infection by hepatitis C virus (HCV) *in vivo* is thought to be noncytopathic. As a consequence, the immune response to viral infection is believed to play a key role in the liver damage and ensuing fibrosis that occurs in chronic infection. However, specific information regarding immune function during advanced liver disease has been limited, and longitudinal studies have been rare, in part due to the protracted natural history of chronic hepatitis C. To evaluate the relationship between the immune response and liver disease progression, peripheral blood antigen-specific CD4+ lymphoproliferative (LP) responses were analyzed longitudinally over 48 months in a cohort of patients enrolled in the Hepatitis C Antiviral Long-term Treatment against Cirrhosis (HALT-C) trial.

The HALT-C trial was a randomized, controlled clinical trial of patients with chronic hepatitis C and advanced liver fibrosis (Ishak fibrosis score ≥ 3) who had not responded to prior interferon therapy (1). The main finding of the HALT-C trial was that long term low-dose peginterferon-alfa-2a (PEG-IFN) therapy had no effect in preventing cirrhosis and its complications in these patients compared to an untreated control group. During the course of the trial, approximately one-third of both treated and untreated patients developed either increasing hepatic fibrosis on a serial biopsy or a predefined clinical disease outcome (2). The HALT-C trial therefore represented an opportunity to prospectively investigate defects in cellular immune responses in patients with chronic liver disease, and their possible relationships with liver disease stage and IFN treatment.

MATERIALS AND METHODS

Patients and Study Design

The design and results of the HALT-C trial have been described (1, 2). Briefly, criteria for enrollment were chronic HCV infection with detectable HCV RNA, lack of virological response to previous interferon- α therapy (with or without ribavirin), and presence of bridging fibrosis or cirrhosis on liver biopsy within 12 months of enrollment. Blood samples for this ancillary study were prospectively collected as described (3); study protocols were approved by the IRBs of all participating institutions, and written informed consents were obtained from all study subjects.

All subjects in this study were treated for 20 weeks with peginterferon-alfa-2a (PEG-IFN) 180 µg SQ weekly and ribavirin 1000–1200 mg p.o. daily (depending on body weight) in 2 divided doses. At the Week 20 (W20) study visit, serum HCV RNA was tested in duplicate with the qualitative Roche COBAS Amplicor v. 2.0 test (Roche Molecular Systems). Patients with detectable serum HCV RNA at W20 were randomized at W24 to either low dose PEG-IFN (90 µg weekly) or observation for an additional 3.5 years (through month 48 [M48]). Patients with undetectable serum HCV RNA at W20 were excluded from this analysis.

Clinical and laboratory data were collected using standardized procedures. Serum HCV RNA levels and genotype were analyzed at a central laboratory as previously described (4). Liver histology was evaluated and graded by a central panel of pathologists using the Ishak scoring system at baseline and at M24 and M48. Predefined clinical outcomes for the HALT-C trial have been described (1).

Lymphoproliferation (LP) assays

Peripheral blood mononuclear cells (PBMC) were isolated and tested within 24 hours of blood draw, after overnight shipment of blood to the University of Washington. PBMC (10^5 per well) were tested in quadruplicate wells with superoxide dismutase (SOD)-recombinant HCV protein antigens (generously provided by Kevin Crawford and Michael Houghton, Chiron Corp.) and control antigens in a 6 day ^3H -thymidine incorporation assay as previously described (3). Antigens included yeast-derived SOD (negative control), SOD-c22 (HCV aa 2–120), SOD-c100 (HCV aa 1569–1931), SOD-NS5 (HCV aa 2054–2995), and E. coli-derived SOD-c33c (HCV 1192–1457). Antigens were aliquoted and kept frozen at -20°C or -80°C as recommended by the manufacturer until immediately prior to assay, undergoing a total of 2 freeze/thaw cycles. Responses to PHA (positive control, $1.6\mu\text{g ml}^{-1}$, Remel), *Candida albicans* ($20\mu\text{g ml}^{-1}$, Greer Laboratories, Lenoir, NC), and tetanus toxoid (12 Lf ml^{-1} , Wyeth-Ayerst Laboratories, Marietta, PA) were also tested. The stimulation index (S.I.) to individual HCV antigens was calculated as (mean cpm in presence of antigen) $\div$ (mean cpm in presence of appropriate control antigen). Assays were considered valid for analysis only if the S.I. value for PHA was ≥ 3 . LP assays were scored as positive if the S.I. value for any HCV antigen or for tetanus or *Candida* was ≥ 4 , as previously described (3).

Statistics

Standard statistical methods (chi-square test, Fisher Exact test, t-test, and Wilcoxon rank sum test) were performed with SAS® (Statistical Analysis Software, Cary, NC) version 9.1. Generalized linear mixed models (SAS proc Mixed or proc GLIMMIX) were used to analyze the changes over time in the $\log_{10}$ S.I. or the percentage of subjects who had positive LP results. All error bars shown in the figures are 95% confidence intervals for the means from these models; their width depends on the number of observations for each group at each time point and does not reflect outliers within a particular group. To evaluate the association of clinical and histological outcomes with $\log_{10}$ S.I. we included a time varying covariate with two categories: whether or not the patient met the definition of an outcome at or before the date of the visit at which the LP response was measured. All analyses were two-tailed with $\alpha = 0.05$.

RESULTS

The data in Table 1 illustrate the baseline clinical, demographic, and laboratory parameters of the 186 HCV-infected subjects included in this analysis. Proliferative responses to *Candida albicans*, tetanus toxoid and HCV protein antigens were prospectively measured as described in Materials and Methods. Over all visits, positive LP responses were found most

commonly to c100 (HCV NS4) > c22 (HCV core) > NS5 or c33c (HCV NS3) HCV antigens; this hierarchy of responsiveness to specific HCV antigens did not vary over time (data not shown). Among all patients tested at baseline, 44/186 (24%) patients had LP responses to any HCV antigen, 150/186 (81%) had LP responses to tetanus, and 162/186 (87%) had LP responses to *Candida*.

The percentage of positive LP responses declined over time during the HALT-C trial

The percentage of subjects with positive LP responses to HCV antigens declined significantly over time according to study visit ($p < 0.0001$, Figure 1), as did responses to the recall antigens, *Candida* and tetanus ($p < 0.0001$). The *Candida* protein antigen was obtained periodically from the manufacturer on an as needed basis (range 3–10 months per lot, median every 5 months). The HCV antigens and tetanus toxoid were obtained at the beginning of the study and used for the duration of follow-up. Patients were enrolled into the HALT-C trial over a period of 2.5 years and tests were performed over a period of 7 years. To determine the impact of antigen storage on our results, we investigated whether the frequency of positive LP responses to tetanus and *Candida* antigens varied during the ~2.5 year window for each study visit. No significant changes in the percentage of LP-positive responses over the 2.5 years were found for either tetanus or *Candida* antigens (data not shown). Time-dependent changes in the LP results were found when the data were analyzed over serial visits or according to the year in which the data were generated; however, the decline over serial visits remained significant after adjusting for the year in which the assay was performed ($p = 0.009$ for both tetanus and *Candida*). The same statistical analysis could not be performed for HCV antigen responses because of the substantially lower frequency of positive responses.

Over time, LP responses decreased in patients randomized to low dose PEG-IFN treatment compared to observation

To investigate the impact of long-term PEG-IFN therapy on antigen-specific LP responses, we analyzed data from 97 patients (44 control and 53 treatment) with available LP data at all of the following study visits: baseline, M12, M24, M36 and M48. The frequency of positive LP responses declined over time in both treated and untreated patients ($p < 0.003$); however, fewer PEG-IFN-treated patients were found to have positive LP responses to HCV, tetanus toxoid, and *Candida albicans* protein antigens over all study visits except baseline (Figure 2A, $p = 0.002$; B, $p = 0.0002$; and C, $p = 0.02$). Note that patients who had a positive LP result to any of 4 HCV antigens tested were considered among those with positive HCV responses (Figure 2A). Statistically significant differences between treated and untreated groups were also obtained when LP responses to HCV c100 (only), *Candida* and tetanus antigens were analyzed as the mean of the $\log_{10}$ Stimulation Index (S.I.) of all samples at each study visit (including those with $S.I. < 4.0$) (Figure 2D, $p = 0.009$; E, $p = 0.0008$; and F, $p = 0.09$).

Clinical associations with LP assays at baseline and during the randomized phase of the HALT-C trial

At baseline, a smaller percentage of patients with cirrhosis had positive HCV-specific responses (15%) compared to those with fibrosis (29%, $p = 0.03$). At M24 and M48, the other visits at which patients underwent liver biopsies, the frequency of HCV LP-positive responses was not statistically significantly different between patients with fibrosis and cirrhosis as measured at those study visits (Figure 3A). However, an analysis of the mean $\log_{10}$ S.I. to HCV c100 antigen demonstrated statistically significant differences between the fibrosis and cirrhosis groups at M24 ($p = 0.0497$) and M48 ($p = 0.02$), respectively (Figure 3B). A similar analysis of the mean $\log_{10}$ S.I. to HCV c22 (core), the next most frequently detected HCV-specific response, did not reveal any relationship with cirrhosis (data not

shown). No differences in the proportion of fibrotic and cirrhotic patients with LP-positive responses to either tetanus or *Candida* were found at any study visit.

To further evaluate an association between lack of lymphoproliferation and advanced liver disease, we investigated the relationship between LP responses and the occurrence of previously defined clinical outcomes (including hepatic decompensation) or a 2 point increase of the Ishak fibrosis score in serial biopsies. No statistically significant associations were found in these analyses using positivity thresholds for the LP data (S.I. 4, data not shown). We also analyzed the data using the mean $\log_{10}$ S.I. at each study visit and a time-varying covariate according to whether patients experienced the combined disease outcomes or not (Figure 4). These results indicate that patients who experienced fibrosis progression or other disease outcomes had lower mean LP responses to tetanus over time ($p=0.009$) compared to those without outcomes (Figure 4B and E). No statistically significant differences in HCV or *Candida* LP responses were detected (Figures 4A, C, D and F).

We then evaluated the dataset for relationships between circulating platelet counts and LP results, because a decreased platelet count is a commonly observed manifestation of severe liver disease/portal hypertension, and has been used in clinical models to predict the likelihood of cirrhosis (5, 6). Only patients who had positive LP responses at baseline were included in this analysis. We then compared platelet values at M12, M24, M36, and M48 according to LP status (positive or negative) at those study visits (Supplemental Table 1). Mean platelet counts were consistently lower in patients who lost LP responses to *Candida* and tetanus antigens compared to those who did not, although the results were only statistically significant at M36 for *Candida* and M24 for tetanus in the untreated control group. Similar results were found for the treated groups who lost or maintained LP responses, but this could have been influenced by the thrombocytopenic effects of PEG-IFN. HCV-specific responses were not analyzed because of the much smaller sample size of those with positive responses.

DISCUSSION

These studies demonstrate that HCV-specific, as well as recall antigen-specific LP responses declined over time in a large group of prospectively monitored patients with advanced chronic hepatitis C who were IFN-nonresponders, regardless of whether a positivity threshold was applied or whether all proliferative responses were considered using $\log_{10}$ -transformed S.I. values. Treatment with PEG-IFN appeared to substantially augment this anti-proliferative and potentially immunosuppressed state. Importantly, we describe for the first time a relationship between measures of advanced liver disease and LP responses. Lack of and lower levels of HCV-specific LP responses were associated with the presence of cirrhosis, and lower levels of lymphoproliferation stimulated by tetanus toxoid were associated with fibrosis progression and clinical outcomes. Loss of tetanus and *Candida* LP responses over multiple years of follow-up (as indicators of a general decline in CD4+ T cell memory responses) was also associated with lower platelet counts, a surrogate measure of advanced liver disease.

Despite the sizable cohort enrolled in this study ($n=186$ at baseline), many of the analyses were hindered by insufficient sample size. Specifically, the analyses were limited by the number of patients with positive LP responses to various antigens at each study visit, the number of patients who developed clinical or fibrosis outcomes and had serial LP results available, and the need to analyze treated and untreated patients separately to account for confounding effects of PEG-IFN. For example, serial HCV c100-specific responses could be analyzed over time (Figures 3B and 4) but trends in LP responses to the other HCV antigens could not be analyzed because of the low frequency of positive responses. Moreover,

because this study was not specifically designed to address the loss of LP responses to tetanus and *Candida* antigens over time, we did not perform longitudinal LP assays on a comparison group of HCV-uninfected subjects. Inclusion criteria for the HALT-C trial, such as prior non-response to IFN treatment, could also limit the generalizability of the findings. Despite these limitations, we report novel observations regarding serial measurements of a critical function of CD4+ T cells in a sizable population of HCV-infected patients with advanced hepatic fibrosis prospectively followed for 48 months.

Clinical observations and studies of *ex vivo* T cell function have indicated that patients with chronic hepatitis C are not globally immunosuppressed (7, 8). However, attenuation of HCV-specific immune responses has clearly been demonstrated (9, 10). Past studies of antigen-specific lymphoproliferation utilized peripheral blood samples from treatment-naïve chronic hepatitis C patients, of whom only a small minority had advanced hepatic fibrosis (11–13). By contrast, the patients in this study were a group of IFN-experienced patients who had previously demonstrated a lack of virologic response to treatment, with an interval of 0.8 to 12 years (median 1.8 years) between last IFN and baseline testing. These patients were also required to have a minimum of bridging fibrosis (Ishak fibrosis score ≥ 3) to enter the study. Despite this past history of treatment and virologic nonresponse, ~25% of patients exhibited an HCV-specific LP response at baseline and 94% of patients had a recall memory response to either tetanus or *Candida* and 74% to both. By these measures, the frequency of LP responsiveness at baseline was generally consistent with past reports indicating a diminished HCV-specific response but otherwise intact immunity. At this baseline study visit, when our sample size was largest, a lack of HCV-specific LP responses was significantly associated with the presence of cirrhosis on liver biopsy ($p=0.03$, Figure 3). Although more patients with cirrhosis also lacked HCV-specific LP responses at later study visits, these differences did not reach statistical significance. This may have been related to the low number of positive HCV-specific LP responses at these later study visits (4/49 vs 15/81 at M24 and 1/52 vs 4/58 at M48 in cirrhotic vs fibrotic patients respectively). However, an analysis of HCV c100-specific lymphoproliferation using S.I. values rather than positivity (Figure 3B), further reinforced the association between lack of proliferation and cirrhosis.

During the 42-month follow-up period, approximately one-third of the 186 patients evaluated developed liver fibrosis progression or clinical outcomes/hepatic decompensation (combined disease outcomes). No association was found between LP results and combined disease outcomes using a defined threshold for positive LP results. However, an association was identified between lower tetanus S.I. (a measure of memory T cell responses) and combined disease outcomes (Figure 4). Since greater power is usually obtained with continuous rather than categorical variables, the absence of an association with dichotomous (positive/negative) LP results may have been related to sample size limitations. Other indirect measures of liver disease progression such as low platelet counts were also found to be associated with loss of lymphoproliferation to recall antigens. Together, these results suggest that lack or loss over time of lymphoproliferative function may be associated with the presence of cirrhosis.

Our data also provide a functional correlate for a recent report describing the association of abnormally low CD4+ T cell counts (but *not* CD4+ T cell percentages) with splenomegaly or thrombocytopenia among a heterogeneous group of cirrhotic patients (all HIV-seronegative) (14). CD4+ T cell frequencies were not measured in our study, but an overall loss of CD4+ memory T cell numbers was unlikely to affect our results, since the input number of PBMC in each test well was consistent among all assays and the authors reported no association with CD4+ T cell percentages. Consistent with these peripheral blood data of McGovern and colleagues, Bonacini et al. described a significant decrease in the frequency

of portal/periportal CD4+ T cells and lobular CD4+ and CD8+ T cells in patients with cirrhosis compared to those with less advanced stages of fibrosis (15). Data on T cell function in HCV-induced liver cirrhosis is sparse; Anthony et al. reported fewer IFN- γ ELISpot responses to HCV core antigen in 21 cirrhotic patients compared to 17 without cirrhosis, while NS3-specific responses were intact (16). Although the antigen-specific associations and lymphocyte function measured differed from our study, they too found decreased immune responsiveness among patients with cirrhosis. Thus, our data provide additional functional evidence to support the notion that T cell immunity is impaired or dysregulated in advanced hepatic fibrosis.

Aging is associated with a decline in *in vitro* T cell proliferative function as well as *in vivo* T cell immunity (17). The mean age of the patients tested in this study was approximately 50 years at baseline (range 19–75, Table 1). Thus, it is possible that a natural decline in immune function could have contributed to the results found here, since the frequency of positive LP responses decreased with study visit, which also corresponded to aging of the cohort. Subjects lacking baseline tetanus LP responses were older (mean 55 years) than patients with response (mean 50 years, $p=0.0006$), but no significant differences in age were found according to LP responses to HCV ($p=0.79$) or *Candida* ($p=0.88$).

Another important possibility to consider is that aging and/or cirrhosis could be associated with a decline in antigen-presenting function, which was manifest in our study as a loss of T cell proliferative function. Such a scenario has been described for chronic alcohol exposure, where ethanol has been shown to adversely affect antigen-presenting function as well as T cell proliferative function (18). However, future studies will be necessary to determine whether defects in T cell function alone or both T cell and antigen presentation functions are present in HCV-associated advanced liver disease.

Treatment with PEG-IFN monotherapy *in vivo* led to a further reduction in the number of patients with positive LP results to any antigen. Although this finding may be explained by the anti-proliferative effects of IFN (19, 20), other reports indicate that the effects of type I IFNs on T cells are more complex. Several groups have described inhibitory effects of IFN on proliferative responses of naive but not activated T cells (21, 22). It has also been reported that IFN treatment can affect the apoptotic death and/or survival of activated lymphocytes (21, 23), and that T cell responses can be influenced by prior exposure to IFN (22). Multiple studies in treatment-naïve cohorts have reported that HCV-specific LP responses increase in frequency during and after IFN treatment in those who clear HCV (11–13). Such results are consistent with a recent report that addition of IFN *in vivo* or *in vitro* to memory T cells from HCV-infected patients led to an enhancement of proliferative responses to PPD antigen (24). However, in the same study, proliferative responses to tetanus toxoid or Influenza A hemagglutinin were diminished in the presence of IFN in some of the patients tested. Recently, Burton et al. reported a reduction in the frequency of HCV-specific IFN- γ responses during IFN plus ribavirin therapy compared to baseline, although the same was not true for CMV control responses in the cohort (25). Other groups have also reported that IFN plus ribavirin therapy is not always associated with increased lymphoproliferative responses to HCV antigens (26, 27). Thus, the effects of type I IFN on T cells could differ according to activation status, memory phenotype, antigen specificity, HCV RNA levels, lymphocyte function tested, and other factors. Further investigation will be necessary to elucidate the critical factors and mechanisms underlying the observed effects of *in vivo* IFN treatment on antigen-specific CD4+ T cell function.

Bacterial infection, including spontaneous bacterial peritonitis, is a frequent complication of advanced fibrosis and cirrhosis. Many factors are thought to contribute to the increased susceptibility of these patients to infection, including impaired reticuloendothelial cell and

polymorphonuclear cell function, portosystemic shunting, alterations in enteric flora, malnutrition, compromised natural barriers and invasive diagnostic and therapeutic procedures (28, 29). Our finding that *in vivo* PEG-IFN treatment induced a generalized impairment of CD4+ T cell proliferative function complements the recent observation that overall CD4+ T cell counts (but not percentages) declined during PEG-IFN therapy (30) and suggests a potential role for CD4+ T cell immunity in influencing infection risk in this population. Our results could also help to explain the observation that the incidence of bacterial infections was higher among PEG-IFN-treated patients with cirrhosis awaiting liver transplantation compared to untreated controls (31). Whether impairment of T cell immunity is pathogenetically involved or a bystander effect of the process of liver disease progression will need to be addressed by future studies. However, the depressed T cell immunity described here could contribute to the increased susceptibility of chronic hepatitis C IFN-nonresponder patients with advanced liver disease to infection as well as to the development of cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ALT	alanine aminotransferase
HAI	hepatic activity index
HALT-C	Hepatitis C Antiviral Long-term Treatment against Cirrhosis
HCV	hepatitis C virus
INR	International normalized ratio
LP	lymphoproliferative
PEG-IFN	pegylated interferon-alfa
PBMC	peripheral blood mononuclear cells
SI	stimulation index
SQ	subcutaneous
WBC	white blood cells

References

1. Lee WM, Dienstag JL, Lindsay KL, Lok AS, Bonkovsky HL, Shiffman ML, et al. Evolution of the HALT-C Trial: pegylated interferon as maintenance therapy for chronic hepatitis C in previous interferon nonresponders. *Controlled Clinical Trials*. 2004 Oct; 25(5):472–92. [PubMed: 15465617]
2. Di Bisceglie AM, Shiffman ML, Everson GT, Lindsay KL, Everhart JE, Wright EC, et al. Prolonged therapy of advanced chronic hepatitis C with low-dose peginterferon. *N Engl J Med*. 2008 Dec 4; 359(23):2429–41. [PubMed: 19052125]
3. Morishima C, Polyak SJ, Ray R, Doherty MC, Di Bisceglie AM, Malet PF, et al. Hepatitis C Virus-Specific Immune Responses and Quasi-Species Variability at Baseline Are Associated with Nonresponse to Antiviral Therapy during Advanced Hepatitis C. *J Infect Dis*. 2006 Apr 1; 193(7): 931–40. [PubMed: 16518754]
4. Shiffman ML, Di Bisceglie AM, Lindsay KL, Morishima C, Wright EC, Everson GT, et al. Peginterferon alfa-2a and ribavirin in patients with chronic hepatitis C who have failed prior treatment. *Gastroenterology*. 2004 Apr; 126(4):1015–23. [PubMed: 15057741]
5. Wai CT, Greenson JK, Fontana RJ, Kalbfleisch JD, Marrero JA, Conjeevaram HS, et al. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. *Hepatology*. 2003 Aug; 38(2):518–26. [PubMed: 12883497]
6. Lok AS, Ghany MG, Goodman ZD, Wright EC, Everson GT, Sterling RK, et al. Predicting cirrhosis in patients with hepatitis C based on standard laboratory tests: results of the HALT-C cohort. *Hepatology*. 2005 Aug; 42(2):282–92. [PubMed: 15986415]
7. Thomas DL, Seeff LB. Natural history of hepatitis C. *Clin Liver Dis*. 2005 Aug; 9(3):383–98. vi. [PubMed: 16023972]
8. Freeman AJ, Marinos G, French RA, Lloyd AR. Immunopathogenesis of hepatitis C virus infection. *Immunol Cell Biol*. 2001 Dec; 79(6):515–36. [PubMed: 11903612]
9. Wedemeyer H, He XS, Nascimbeni M, Davis AR, Greenberg HB, Hoofnagle JH, et al. Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. *J Immunol*. 2002; 169(6):3447–58. [PubMed: 12218168]

10. Spangenberg HC, Viazov S, Kersting N, Neumann-Haefelin C, McKinney D, Roggendorf M, et al. Intrahepatic CD8+ T-cell failure during chronic hepatitis C virus infection. *Hepatology*. 2005 Oct; 42(4):828–37. [PubMed: 16175596]
11. Zhang ZX, Milich DR, Peterson DL, Birkett A, Schvarcz R, Weiland O, et al. Interferon-alpha treatment induces delayed CD4 proliferative responses to the hepatitis C virus nonstructural protein 3 regardless of the outcome of therapy. *Journal of Infectious Diseases*. 1997; 175(6):1294–301. [PubMed: 9180166]
12. Missale G, Cariani E, Lamonaca V, Ravaggi A, Rossini A, Bertoni R, et al. Effects of interferon treatment on the antiviral T-cell response in hepatitis C virus genotype 1b- and genotype 2c-infected patients. *Hepatology*. 1997; 26(3):792–7. [PubMed: 9303515]
13. Cramp ME, Rossol S, Chokshi S, Carucci P, Williams R, Naoumov NV. Hepatitis C virus-specific T-cell reactivity during interferon and ribavirin treatment in chronic hepatitis C. *Gastroenterology*. 2000; 118(2):346–55. [PubMed: 10648463]
14. McGovern BH, Golan Y, Lopez M, Pratt D, Lawton A, Moore G, et al. The impact of cirrhosis on CD4+ T cell counts in HIV-seronegative patients. *Clin Infect Dis*. 2007 Feb 1; 44(3):431–7. [PubMed: 17205454]
15. Bonacini M, Govindarajan S, Kohla M, Lai MM, Lindsay KL. Intrahepatic lymphocyte phenotypes in hepatitis C virus infection: a comparison between cirrhotic and non-cirrhotic livers. *Minerva Gastroenterol Dietol*. 2007 Mar; 53(1):1–7. [PubMed: 17415341]
16. Anthony DD, Post AB, Valdez H, Peterson DL, Murphy M, Heeger PS. ELISPOT analysis of hepatitis C virus protein-specific IFN-gamma-producing peripheral blood lymphocytes in infected humans with and without cirrhosis. *Clinical Immunology*. 2001; 99(2):232–40. [PubMed: 11318595]
17. Swain S, Clise-Dwyer K, Haynes L. Homeostasis and the age-associated defect of CD4 T cells. *Semin Immunol*. 2005 Oct; 17(5):370–7. [PubMed: 15964201]
18. Siu L, Foont J, Wands JR. Hepatitis C virus and alcohol. *Seminars in liver disease*. 2009 May; 29(2):188–99. [PubMed: 19387918]
19. Puri RK, Travis WD, Rosenberg SA. In vivo administration of interferon alpha and interleukin 2 induces proliferation of lymphoid cells in the organs of mice. *Cancer Res*. 1990 Sep 1; 50(17):5543–50. [PubMed: 2386960]
20. Tanabe Y, Nishibori T, Su L, Arduini RM, Baker DP, David M. Cutting edge: role of STAT1, STAT3, and STAT5 in IFN-alpha beta responses in T lymphocytes. *J Immunol*. 2005 Jan 15; 174(2):609–13. [PubMed: 15634877]
21. Marrack P, Kappler J, Mitchell T. Type I interferons keep activated T cells alive. *J Exp Med*. 1999 Feb 1; 189(3):521–30. [PubMed: 9927514]
22. Dondi E, Rogge L, Lutfalla G, Uze G, Pellegrini S. Down-modulation of responses to type I IFN upon T cell activation. *J Immunol*. 2003 Jan 15; 170(2):749–56. [PubMed: 12517937]
23. Haveran-Daughton C, Kolumam GA, Murali-Krishna K. Cutting Edge: The direct action of type I IFN on CD4 T cells is critical for sustaining clonal expansion in response to a viral but not a bacterial infection. *J Immunol*. 2006 Mar 15; 176(6):3315–9. [PubMed: 16517698]
24. Gallagher KM, Lauder S, Rees IW, Gallimore AM, Godkin AJ. Type I interferon (IFN alpha) acts directly on human memory CD4+ T cells altering their response to antigen. *J Immunol*. 2009 Sep 1; 183(5):2915–20. [PubMed: 19667099]
25. Burton JR Jr, Klarquist J, Im K, Smyk-Pearson S, Golden-Mason L, Castelblanco N, et al. Prospective analysis of effector and regulatory CD4+ T cells in chronic HCV patients undergoing combination antiviral therapy. *J Hepatol*. 2008 Sep; 49(3):329–38. [PubMed: 18644644]
26. Rahman F, Heller T, Sobao Y, Mizukoshi E, Nascimbeni M, Alter H, et al. Effects of antiviral therapy on the cellular immune response in acute hepatitis C. *Hepatology*. 2004 Jul; 40(1):87–97. [PubMed: 15239090]
27. Kaplan DE, Sugimoto K, Ikeda F, Stadanlick J, Valiga M, Shetty K, et al. T-cell response relative to genotype and ethnicity during antiviral therapy for chronic hepatitis C. *Hepatology*. 2005 Jun; 41(6):1365–75. [PubMed: 15915458]
28. Caly WR, Strauss E. A prospective study of bacterial infections in patients with cirrhosis. *J Hepatol*. 1993 Jul; 18(3):353–8. [PubMed: 8228129]

29. Deschenes M, Villeneuve JP. Risk factors for the development of bacterial infections in hospitalized patients with cirrhosis. *Am J Gastroenterol.* 1999 Aug; 94(8):2193–7. [PubMed: 10445549]
30. Rashkin S, Rouster S, Goodman ZD, Sherman KE. T-helper cells and liver fibrosis in hepatitis C virus-monoinfected patients. *J Viral Hepat.* 2010 Mar 1; 17(3):222–6. [PubMed: 19709360]
31. Carrion JA, Martinez-Bauer E, Crespo G, Ramirez S, Perez-del-Pulgar S, Garcia-Valdecasas JC, et al. Antiviral therapy increases the risk of bacterial infections in HCV-infected cirrhotic patients awaiting liver transplantation: A retrospective study. *J Hepatol.* 2009 Apr; 50(4):719–28. [PubMed: 19217183]

Figure 1. Decreased Frequency of Positive Lymphoproliferative Responses During the HALT-C Trial According to Study Visit

Proliferative responses by freshly isolated PBMC to HCV protein antigens, tetanus toxoid and *Candida albicans* antigens were measured using a ^3H -thymidine assay. The threshold for positivity was defined as a stimulation index (S.I.) of 4 for all antigens, and an HCV response was considered to be positive if an S.I. of 4 was achieved for one or more HCV antigens. All useable assay results available at each study visit were included, and the number of individual assay results at each study visit is shown below the x-axis. 95% confidence intervals are shown.

Figure 2. Lymphoproliferative Responses Decrease Over Time According to Treatment Status
Proliferative responses by PBMC to HCV protein antigens (A), tetanus toxoid (B) and *Candida albicans* (C) antigens were measured as described in Figure 1. Mean stimulation indices of all useable assay results at each study visit (including assays with S.I. and <4.0) are indicated (D, E and F). Log₁₀ Stimulation Indices (S.I.) were derived by subtracting the log₁₀ mean cpm of unstimulated wells by the log₁₀ mean cpm of stimulated wells. Error bars indicate 95% confidence intervals. Results were categorized according to whether PBMCs were obtained from patients randomized to long-term PEG-IFN treatment (black symbols/solid lines) or to observation (control, open symbols/dashed lines). This analysis was restricted to patients with complete data at all study visits (44 control patients and 53 treatment patients). Fewer PEG-IFN-treated patients had positive or lower level lymphoproliferative responses to HCV protein antigens (A, $p=0.002$; D, HCV c100,

p=0.009), tetanus toxoid (B, p=0.0002; E, p=0.0008), and *Candida albicans* (C, p=0.02; F, p=0.09).

Figure 3. Lack or Lower Levels of Lymphoproliferation to HCV Antigens are Associated with Cirrhosis

Patients were categorized according to whether they had fibrosis (Ishak 3–4) or cirrhosis (Ishak 5–6), measured at each biopsy study visit. The percentage of patients with positive HCV-specific lymphoproliferative responses (S.I. ≥ 4.0) to any HCV protein antigen (A) or corresponding mean Log₁₀ S.I. to HCV c100 antigen (including assays with S.I. ≥ 4.0 and <4.0) at each study visit is shown (B). Both treated and untreated patients are included at Months 24 and 48. Numbers of patients in each group are indicated. P values are from separate analyses at each study visit. All error bars indicate 95% confidence intervals.

Figure 4. Decreased Levels of Lymphoproliferative Responses to Tetanus are Associated with Clinical and Fibrosis Outcomes

Mean Log₁₀ S.I. (including assays with S.I. and <4.0) and 95% confidence intervals of proliferative responses to HCV c100 (A, D), tetanus toxoid (B, E) and *Candida albicans* (C, F) antigens are shown. For clarity, results from patients randomized to observation (A, B and C) were separated from those randomized to long-term PEG-IFN treatment (D, E and F). Results from patients with outcomes (open symbols/dotted lines) and those with no outcomes (closed symbols/solid lines) are indicated. This analysis included 93, 79, 67, 65, and 56 control patients and 93, 74, 79, 73, and 70 treated patients at baseline M12, M24, M36, and M48, respectively. The confidence intervals are wide at month 12 because there were few patients in the outcome group at that time. Patients with clinical and fibrosis

outcomes had lower lymphoproliferative responses to tetanus toxoid (B and E, $p=0.009$), but not HCV c100 (A and D, $p=0.46$) or *Candida albicans* (C and F, $p=0.35$).

Table 1

Study Subjects and Baseline Data

Variable	Control % or mean (SD) n=93	Treatment % or mean (SD) n=93	P-value *
Age (yrs)	50.9 (8.8)	51.5 (7.0)	0.63
Gender (Female)	32%	31%	0.88
Race (Caucasian)	75%	75%	1.00
HCV genotype 1	96%	94%	0.75
HCV RNA (Log ₁₀ IU ml-1)	6.54 (0.46)	6.48 (0.50)	0.38
ALT (U L-1)	96.6 (70.8)	92.7 (50.5)	0.67
Albumin (g dL-1)	3.96 (0.39)	3.95 (0.42)	0.80
INR	1.05 (0.11)	1.05 (0.10)	0.95
Bilirubin (mg dL-1)	0.84 (0.38)	0.90 (0.48)	0.30
Platelets (×1000 mm-3)	164.0 (65.5)	155.5 (61.4)	0.36
Cirrhosis	38%	35%	0.76
HAI	6.77 (1.97)	7.20 (2.43)	0.19
WBC (×1000 mm-3)	5.99 (1.74)	5.68 (1.87)	0.24
Neutrophils (×1000 mm-3)	3.27 (1.28)	3.05 (1.30)	0.24
WBC-Neutrophils (ANC) counts	2.72 (0.86)	2.63 (0.90)	0.50

* T-test or Fisher's exact test was used.