Antigen-specific T lymphocyte proliferation decreases over time in advanced chronic hepatitis C

C. Morishima,[1] A. M. Di Bisceglie,[2] A. L. Rothman,[3] H. L. Bonkovsky,[4,5] K. L. Lindsay,[6] W. M. Lee,[7] M. J. Koziel,[8] R. J. Fontana,[9] H.-Y. Kim[10] and E. C. Wright[11] the HALT-C Trial Group* [1]*Division of Virology, Department of Laboratory Medicine, University of Washington, Seattle, WA, USA;* [2]*Division of Gastroenterology and Hepatology, Saint Louis University School of Medicine, St. Louis, MO, USA;* [3]*Center for Infectious Disease and Vaccine Research and Department of Medicine, University of Massachusetts Medical School, Worcester, MA, USA;* [4]*Department of Medicine, University of Connecticut Health Center, Farmington, CT, USA;* [5]*Carolinas Medical Center, Charlotte, NC, USA;* [6]*Division of Gastrointestinal and Liver Diseases, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA;* [7]*Division of Digestive and Liver Diseases, University of Texas Southwestern Medical Center, Dallas, TX, USA;* [8]*Beth Israel Deaconess Medical Center, Boston, MA, USA;* [9]*Division of Gastroenterology, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, MI, USA;* [10]*New England Research Institutes, Watertown, MA, USA; and* [11]*Office of the Director, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Department of Health and Human Services, Bethesda, MD, USA*

SUMMARY. To evaluate T cell immunity in advanced liver disease, antigen-specific lymphoproliferative (LP) responses were prospectively studied in the context of the Hepatitis C Antiviral Long-term Treatment against Cirrhosis trial. Peripheral blood responses to hepatitis C virus (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 peginterferon-alfa-2a (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 LP 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 LP 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 LP responses were associated with fibrosis progression and clinical outcomes ($P = 0.009$). Poorer CD4+ T cell proliferative function was associated with more advanced liver disease in chronic hepatitis C and may be further affected by long-term PEG-IFN treatment.

Keywords: cirrhosis, fibrosis, hepatitis C virus, interferon-alpha, lymphoproliferation, T cell.

INTRODUCTION

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

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; NIAID, National Institute of Allergy and Infectious Diseases; PBMC, peripheral blood mononuclear cells; PEG-IFN, peginterferon-alfa-2a; PHA, phytohemagglutinin; SI, stimulation index; SOD, superoxide dismutase; SQ, subcutaneous; WBC, white blood cells.

Correspondence: Chihiro Morishima, MD, Department of Laboratory Medicine, Box 358050, 815 Mercer Street, Seattle, WA 98109, USA. E-mail: chihiro@u.washington.edu
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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 because of 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 analysed 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 virologic response to previous interferon-α therapy (with or without ribavirin) and the 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 PEG-IFN 180 μg SQ weekly and ribavirin 1000–1200 mg p.o. daily (depending on body weight) in two 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, Branchburg, NJ, USA). 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 analysed 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 assays

Peripheral blood mononuclear cells (PBMC) were isolated and tested within 24 h 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., Emeryville, CA, USA) and control antigens in a 6 day ^{3}H-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 *Escherichia coli*-derived SOD-c33c (HCV 1192–1457). Antigens were aliquoted and kept frozen at

−20 or −80 °C as recommended by the manufacturer until immediately prior to assay, undergoing a total of two freeze/thaw cycles. Responses to phytohemagglutinin (PHA) (positive control, 1.6 μg/mL; Remel, Lenexa, KS, USA), *Candida albicans* (20 μg/mL; Greer Laboratories, Lenoir, NC, USA) and tetanus toxoid (12 Lf/mL; Wyeth-Ayerst Laboratories, Marietta, PA, USA) were also tested. The stimulation index (SI) to individual HCV antigens was calculated as (mean cpm in the presence of antigen)/(mean cpm in the presence of appropriate control antigen). Assays were considered valid for analysis only if the SI value for PHA was ≥3. Lymphoproliferation (LP) assays were scored as positive if the SI 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, USA) version 9.1. Generalized linear mixed models (SAS proc Mixed or proc GLIMMIX) were used to analyse the changes over time in the $\log_{10}$ SI 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 $\log_{10}$ SI, 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 α = 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 *C. 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 HCV antigens c100 (HCV NS4) >c22 (HCV core) >NS5 or c33c (HCV NS3); 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 lymphoproliferative responses declined over time during the Hepatitis C Antiviral Long-term Treatment against Cirrhosis trials

The percentage of subjects with positive LP responses to HCV antigens declined significantly over time according to study

visit ($P < 0.0001$, Fig. 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 approximately 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 analysed 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, lymphoproliferative responses decreased in patients randomized to low-dose pegylated interferon-alfa treatment compared to observation

To investigate the impact of long-term PEG-IFN therapy on antigen-specific LP responses, we analysed data from 97 patients (44 control and 53 treatment) with available LP
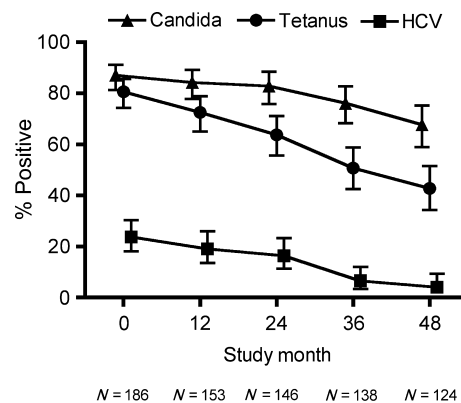

Fig. 1 Decreased frequency of positive lymphoproliferative responses during the Hepatitis C Antiviral Long-term Treatment against Cirrhosis trial according to study visit. Proliferative responses by freshly isolated peripheral blood mononuclear cells to hepatitis C virus (HCV) protein antigens, tetanus toxoid and *Candida albicans* antigens were measured using a ^{3}H-thymidine assay. The threshold for positivity was defined as a stimulation index (SI) of ≥ 4 for all antigens, and an HCV response was considered to be positive if an SI 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.

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

Table 1 Study subjects and baseline data

Variable	Control % or mean (SD) $n = 93$	Treatment % or mean (SD) $n = 93$	*P*-value*
Age (years)	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)	6.54 (0.46)	6.48 (0.50)	0.38
ALT (U/L)	96.6 (70.8)	92.7 (50.5)	0.67
Albumin (g/dL)	3.96 (0.39)	3.95 (0.42)	0.80
INR	1.05 (0.11)	1.05 (0.10)	0.95
Bilirubin (mg/dL)	0.84 (0.38)	0.90 (0.48)	0.30
Platelets ($\times 1000$/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 ($\times 1000$/mm^3)	5.99 (1.74)	5.68 (1.87)	0.24
Neutrophils ($\times 1000$/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. ALT, alanine aminotransferase; HCV, hepatitis C virus; INR, International normalized ratio; HAI, hepatic activity index.

($P < 0.003$); however, fewer PEG-IFN-treated patients were found to have positive LP responses to HCV, tetanus toxoid and *C. albicans* protein antigens over all study visits except baseline (Fig. 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 the four HCV antigens tested were considered among those with positive HCV responses (Fig. 2a). Statistically significant differences between treated and untreated groups were also obtained when LP responses to HCV c100 (only), tetanus and *Candida* antigens were analysed as the mean of the $\log_{10}$ SI of all samples at each study visit (including those

with SI <4.0) (Fig. 2d, $P = 0.009$; e, $P = 0.0008$; and f, $P = 0.09$).

Clinical associations with lymphoproliferative assays at baseline and during the randomized phase of the Hepatitis C Antiviral Long-term Treatment against Cirrhosis 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
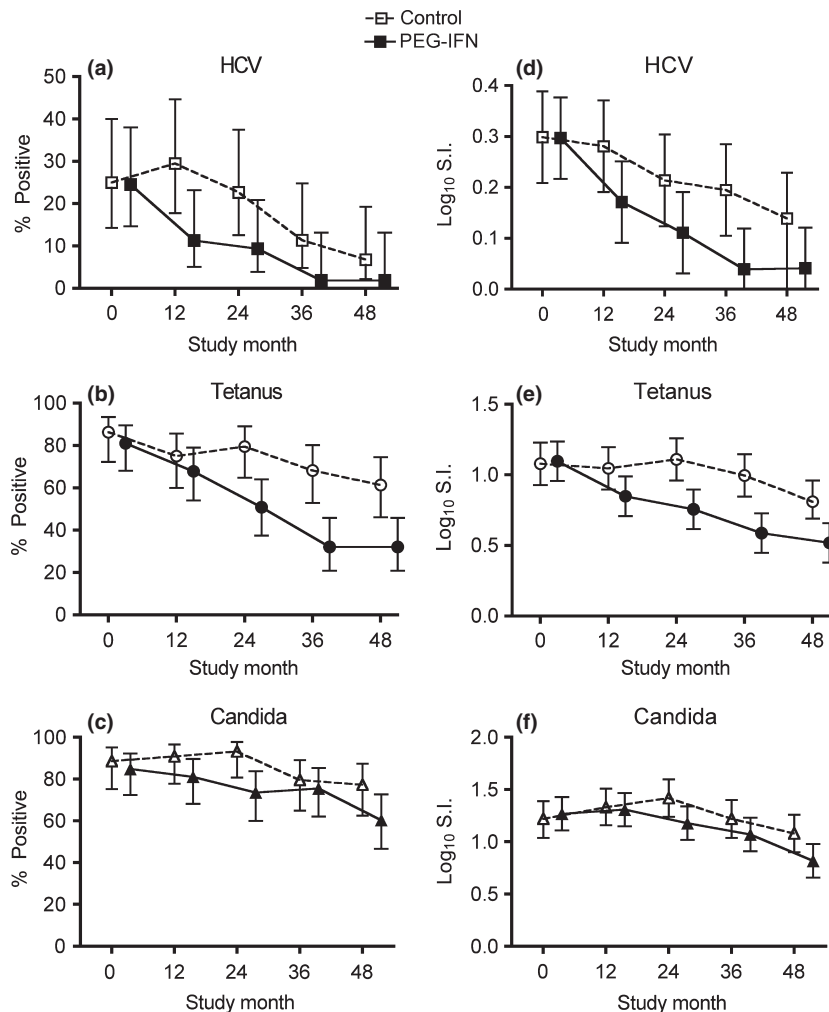
Fig. 2 Lymphoproliferative responses decrease over time according to treatment status. Proliferative responses by peripheral blood mononuclear cells (PBMC) to hepatitis C virus (HCV) protein antigens (a), tetanus toxoid (b) and *Candida albicans* (c) antigens were measured as described in Fig. 1. Mean stimulation indices of all useable assay results at each study visit (including assays with SI ≥ and <4.0) are indicated (d, e and f). $\log_{10}$ SI were derived by subtracting the $\log_{10}$ mean cpm of unstimulated wells by the $\log_{10}$ 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 peginterferon-alfa-2a (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 LP 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 *C. albicans* (c, $P = 0.02$; f, $P = 0.09$).

frequency of HCV LP-positive responses was not statistically significantly different between patients with fibrosis and cirrhosis as measured at those study visits (Fig. 3a). However, an analysis of the mean $\log_{10}$ SI 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 (Fig. 3b). A similar analysis of the mean $\log_{10}$ SI 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 inves-

tigated the relationship between LP responses and the occurrence of previously defined clinical outcomes (including hepatic decompensation) or a two point increase in the Ishak fibrosis score in serial biopsies. No statistically significant associations were found in these analyses using positivity thresholds for the LP data (SI ≥4, data not shown). We also analysed the data using the mean $\log_{10}$ SI at each study visit and a time-varying covariate according to whether patients experienced the combined disease outcomes or not (Fig. 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 (Fig. 4b,e). No statistically significant differences in HCV or Candida LP responses were detected (Fig. 4a,c,d and f).

We then evaluated the data set 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 (Table S1). 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 analysed because of the much smaller sample size of those with positive responses.

Fig. 3 Lack or lower levels of lymphoproliferation to hepatitis C virus (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 (SI ≥4.0) to any HCV protein antigen (a) or corresponding mean $\log_{10}$ SI to HCV c100 antigen (including assays with SI ≥ 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.

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 SI 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
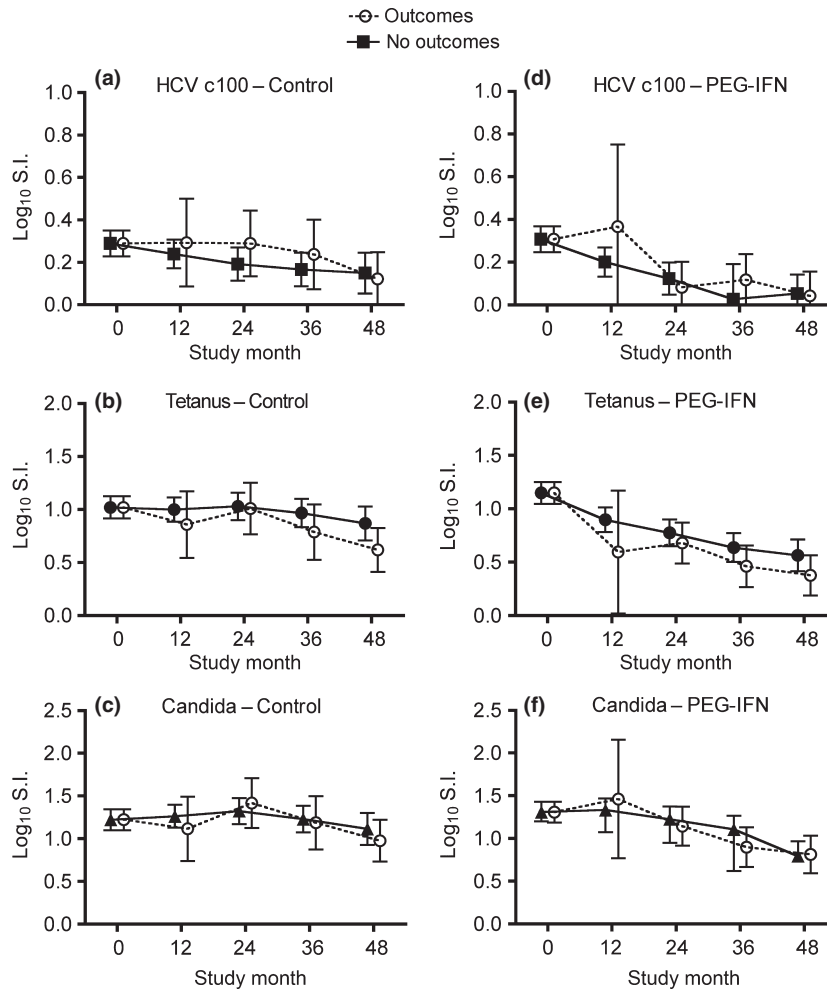
Fig. 4 Decreased levels of lymphoproliferative (LP) responses to tetanus are associated with clinical and fibrosis outcomes. Mean $\log_{10}$ SI (including assays with SI ≥ and <4.0) and 95% confidence intervals of proliferative responses to hepatitis C virus (HCV) c100 (a, d), tetanus toxoid (b, e) and *Candida albicans* (c, f) antigens are shown. For clarity, results from patients randomized to observation (a–c) were separated from those randomized to long-term pegylated interferon-alfa treatment (d–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 LP responses to tetanus toxoid (b and e, $P = 0.009$), but not HCV c100 (a and d, $P = 0.46$) or *C. albicans* (c and f, $P = 0.35$).

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 analyse treated and untreated patients separately to account for confounding effects of PEG-IFN. For example, serial HCV c100-specific responses could be analysed over time (Figs 3b and 4), but trends in LP responses to the

other HCV antigens could not be analysed 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 nonresponse 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-naive 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 0.8–12 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, approximately 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 ($P = 0.03$, Fig. 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 SI values rather than positivity (Fig. 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 SI (a measure of memory T cell responses) and combined disease outcomes (Fig. 4). As 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 the loss of lymphoproliferation to recall antigens. Together, these results suggest that lack or loss over time of LP 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 spleno-megaly 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, because 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 *et al.*, Bonacini *et al.* [15] 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. Data on T cell function in HCV-induced liver cirrhosis are sparse; Anthony *et al.* [16] 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. 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.

Ageing 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, because the frequency of positive LP responses decreased with study visit, which also corresponded to ageing 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 ageing 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-naive 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.* [25] 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. Other groups have also reported that IFN plus ribavirin therapy is not always associated with increased LP 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.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Mean Platelet Counts at Baseline and Months 24, 36 and 48 According to Loss or Maintenance of Tetanus or *Candida*-induced Lymphoproliferation, in Subjects with Positive Lymphoproliferation Responses at Baseline.

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