MAJOR ARTICLE

Kinetics of Intrahepatic Hepatitis C Virus (HCV)–Specific CD4+ T Cell Responses in HCV and *Schistosoma mansoni* Coinfection: Relation to Progression of Liver Fibrosis

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The kinetics of intrahepatic hepatitis C virus (HCV)–specific CD4+ T cell responses and their role in progression of fibrosis have not previously been characterized. Subjects with HCV/*Schistosoma mansoni* coinfection have a more rapid progression of HCV liver fibrosis than do those with HCV infection alone. The present prospective longitudinal study compared the liver histology, HCV-specific intrahepatic and peripheral CD4+ T cell proliferative responses, and cytokines (enzyme-linked immunospot) in 48 subjects with unresolved acute HCV infection with or without *S. mansoni* coinfection, at 6–10 months after acute infection and at the end of follow-up (96 ± 8.7 months), and the findings were correlated to the rate of progression of fibrosis per year. Coinfected subjects had significant worsening of fibrosis, compared with subjects with HCV infection alone. At baseline, subjects with HCV infection alone had stronger multispecific intrahepatic HCV-specific CD4+ T helper 1 responses than did coinfected subjects, who had either no responses or weak, narrowly focused responses, and, over time, these T cell responses were maintained only in the liver. The rate of progression of fibrosis and virus load inversely correlated with intrahepatic HCV-specific CD4+ T cell response. The present prospective analysis indicates that enhancement of progression of liver fibrosis is associated with failure to develop early, multispecific, HCV-specific CD4+ Th1 responses, suggesting that novel therapeutic approaches inducing strong cellular immune responses might limit subsequent liver damage in individuals with chronic hepatitis C.

Hepatitis C virus (HCV) is a major causative agent of chronic hepatitis, and it is estimated that 170–200 million people are infected worldwide [1]. In the United States, nearly 2% of the population is infected, whereas, in countries such as Egypt, the prevalence of HCV infection is 10%–31% [2, 3]. Approximately 70% of infected persons develop chronic hepatitis C, and 20%–30% may develop cirrhosis [4–6]. There is little evidence that progression of liver disease is significantly affected by virologic factors, including virus load, viral genotype, and quasi-species diversity [5–7]. However, many host factors have been observed to increase the risk of progression of liver disease, including male sex, older age at infection, and immunosuppressed states, such as HIV infection [5–8].

Studies have shown that spontaneous resolution of acute hepatitis is associated with vigorous and persistent HCV-specific CD4+ Th1 and CD8+ cytotoxic T cell responses [9–12]. However, the role of these cellular immune responses, particularly in the liver, once chronic

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Table 1. Demographic and clinical characteristics of subjects at baseline.

Parameter	Group A: HCV infection alone	Group B: HCV/*S. mansoni* coinfection	Control: *S. mansoni* infection alone
No.	23	25	20
M:F	14:9	15:10	12:8
Age, mean ± SD, years	36.6 ± 8.1	34.2 ± 7.6	31.7 ± 2.9
Risk factors for HCV transmission			
Occupational exposure	20	22	NA
Blood transfusion	1	2	NA
Injection drug use	2	0	1
Surgery	0	1	2
ALT level, mean ± SD, U/L	144.3 ± 62.5	168.4 ± 75.1	23 ± 9.7
AST level, mean ± SD, U/L	108.6 ± 43.3	112.5 ± 37.6	20 ± 4.6
γ-GT level, mean ± SD, U/L	72.5 ± 23.5	89.4 ± 18.2	46.1 ± 2.4
Total bilirubin, mean ± SD, mg/dL	2.8 ± 1.1	3.4 ± 0.65	0.8 ± 0.2
RNA titer, mean ± SD, copies × 10^5/mL	12.3 ± 3.5[a]	21.3 ± 4.6[a]	NA

NOTE. Group A, 23 subjects with chronic hepatitis C virus (HCV) infection alone; group B, 25 subjects with chronic HCV/*Schistosoma mansoni* coinfection. The control group comprised 20 subjects with *S. mansoni* infection alone. ALT, alanine transaminase; AST, aspartate transaminase; GT, glutamyl transferase; NA, not applicable.

[a] $P < .01$, group A vs. group B (Wilcoxon signed rank test).

infection is established, is less clear, since the natural history of HCV is highly variable and occurs over the course of decades.

We and others have demonstrated that subjects coinfected with HCV and *Schistosoma mansoni,* a parasite that induces a shift in the T helper response toward a Th2 profile [13–15], show increased rates of chronic evolution and accelerated progression of liver disease, compared with subjects with HCV infection alone [11, 16, 17]. Given the more-rapid progression of liver disease in these subjects, HCV/*S. mansoni* coinfection represents a useful model for examination of the relationship of the immune response and subsequent liver injury. In a smaller cohort of subjects with acute HCV infection and without *S. mansoni* infection, we have previously shown that HCV-specific $CD4^+$ Th1 responses in peripheral blood mononuclear cells (PBMCs) were associated with a more favorable disease outcome [11]. In the present prospective longitudinal study, we addressed the following issues: (1) do the characteristics and kinetics of the intrahepatic HCV-specific $CD4^+$ T cell responses differ between subjects with HCV infection alone and subjects with coinfection and, if so, (2) do the intrahepatic HCV-specific $CD4^+$ T cell responses correlate with the rates of progression of fibrosis? To answer these questions, we characterized the HCV-specific $CD4^+$ T cell responses in the liver and peripheral blood at an early phase of chronic HCV infection (6–10 months after acute HCV infection) and then 8 years later, in subjects with and without *S. mansoni* coinfection, and correlated these responses with the progression of liver disease.

SUBJECTS, MATERIALS, AND METHODS

Study population. The study cohort comprised 48 subjects (table 1) who failed to clear viremia after acute HCV infection. Baseline blood and liver samples were obtained 6–10 months after acute HCV infection, and subjects were followed prospectively for 90–112 months (mean ± SD, 96 ± 8.7 months). The diagnosis of acute HCV infection was based on an increase of serum alanine aminotransferase (ALT) levels to >10 times above the upper limit of normal, seroconversion from anti-HCV–negative to anti-HCV–positive antibody status (as determined by use of second-generation ELISA [Abbott Laboratories]), and positive polymerase chain reaction results for HCV RNA (Amplicor; Roche Diagnostics). The majority of subjects were health-care workers who had had HCV-negative serum specimens archived before exposure and who reported occupational exposure to HCV. Subjects were further classified according to *S. mansoni* status into the following groups: 23 subjects with HCV infection alone (group A) and 25 subjects with HCV/*S. mansoni* coinfection (group B). Schistosomiasis was diagnosed by history, detection of *S. mansoni* ova in stools (modified Kato test), or rectal biopsy; seropositivity to schistosomal antibodies (indirect hemagglutination; Femouz Laboratories); and evidence of schistosomiasis on liver biopsy specimens. Other causes of hepatitis were ruled out by history and appropriate serological studies. No subject had received antiviral or immunomodulatory treatment before entry or during follow-up. Groups were matched according to age, sex, and

route and duration of HCV infection (6–10 months). All subjects had genotype 4 infection, as determined by a second-generation reverse-hybridization line-probe assay (Inno-LiPA HCV II; Innogenetics). Twenty subjects with proven hepatic schistosomiasis and comparable duration of *S. mansoni* infection were enrolled as a control group and prospectively followed. PBMCs and liver tissue from 10 subjects who underwent a biopsy during abdominal surgery or laparoscopy due to suspected liver disease, but for whom histological diagnosis revealed a healthy liver, served as a control for liver-infiltrating lymphocyte (LIL) proliferative and ELISPOT assays.

All subjects participating in the present study presented written, informed consent before any study-related procedures were performed. The protocol and all study procedures were reviewed and approved by the Institutional Ethics Committee of the Ain Shams University and the Beth Israel Deaconess Medical Center and were conducted in conformity with the ethical guidelines of the Declaration of Helsinki and with the human-experimentation guidelines of the US Department of Health and Human Services.

Histological assessment Paired liver biopsy specimens, obtained from all study subjects at baseline (6–10 months after acute HCV infection) and at the end of follow-up (mean ± SD, 96 ± 8.7 months), were read under code by 2 pathologists in a blinded manner, without knowledge of the clinical data or the chronology of the biopsies, adopting the scoring system proposed by Ishak et al. [18], which includes a grading score that evaluates the necroinflammatory activity (score, 0–18) and a fibrosis score of 0 (no fibrosis) to 6 (cirrhosis). Moreover, biopsy specimens were assessed for morphologic features of schistosomiasis—such as *S. mansoni* ova, eosinophils, granuloma, pigment, and fibrosis of pipe stem type—by pathologists experienced in the interpretation of such features [16].

The direct rate of progression of fibrosis was assessed by the difference in fibrosis scores between baseline and follow-up liver biopsies, divided by the duration of time between the biopsies, and expressed as fibrosis units per year. For example, for a subject with fibrosis stage 2 at the first biopsy and stage 3 at the second biopsy, performed 2 years later, the rate of progression of fibrosis was 0.500 fibrosis units/year.

Cell preparation. Blood samples were obtained at entry and at the same days that the baseline biopsy and the follow-up biopsy were performed. PBMCs from study and control subjects were isolated immediately by use of Ficoll-Hypaque (Sigma) density gradient centrifugation, as described elsewhere [11, 13]. For isolation of mononuclear cells from liver tissue, freshly recovered excess liver tissue not needed for diagnostic purposes was washed extensively with PBS to remove contaminating blood and then was sieved and pipetted vigorously through a needle. These procedures produced optimal yields of viable intrahepatic lymphocytes ($>5.5 \times 10^5$ cells/biopsy specimen). $CD4^+$ liver-infiltrating lymphocytes were expanded from liver biopsy specimens, as described elsewhere [19], by use of anti-CD3 antibody (Immunotech). Phenotypic analysis of liver-infiltrating lymphocytes was performed by monoclonal antibody staining of intrahepatic lymphocytes and analysis on a FACScan flow cytometer with CellQuest software (Becton Dickinson). Cryopreserved PBMCs and LILs were used in all assays.

HCV and control antigens. Purified recombinant HCV proteins (HCV core [aa 2–120]; NS_3 [aa 1192–1457]; NS_4 [aa 1569–1931]; and NS_5 [aa 2054–2995]) derived from the HCV-1 prototype sequence were purchased from Chiron. All antigens were expressed as COOH-terminal fusion proteins with human superoxide dismutase (SOD) in yeast; therefore, yeast and SOD were used as controls for nonspecific stimulation in proliferation and ELISPOT assays. Soluble egg antigen (SEA) and soluble adult worm antigen (SWAP) were prepared as described elsewhere [13]. Phytohemoagglutinin (PHA; 5 μg/mL at 1:200 dilution; Murex Diagnostics) and tetanus toxoid (TT: 1 μg/mL; Wyeth Laboratories) were used as positive controls.

HCV-specific $CD4^+$ proliferative T cell assays. Proliferation assays were performed for all study and control subjects by use of PBMCs (2×10^5) or LILs (2×10^4 cells/well, cocultured with 1×10^5 irradiated autologous PBMCs as antigen-presenting cells), as described elsewhere [11, 13], by use of the HCV proteins described above (at concentrations of 2 μg/mL), schistosomal antigens (SEA, 15 μg/mL; SWAP, 50 μg/mL), and control antigens. All values were obtained in triplicate. A stimulation index (SI) of ⩾3 (3 SD above the mean SI of healthy control subjects) was considered to be significant. The cutoff for positive $CD4^+$ proliferation for LILs was determined in comparison to control LILs from livers of HCV-negative subjects. $CD4^+$ and $CD8^+$ depletion assays, performed as described elsewhere [10, 19], using paramagnetic microbeads conjugated to a monoclonal mouse anti–human anti-$CD8^+$ or anti-$CD4^+$ antibody (Micro Beads; Miltenyl Biotec), clearly showed that these are $CD4^+$ T cell responses (data not shown). Neither subjects infected with *S. mansoni* alone nor healthy control subjects had significant responses to any HCV-specific antigen. No subjects infected with HCV alone had significant responses to any schistosomal antigen.

ELISPOT assay. ELISPOT and proliferation assays were performed at the same time for early and late samples, from an individual subject. ELISPOT assays were performed as described elsewhere [19–21], by use of PBMCs (200,000 cells/well) or intrahepatic lymphocytes (2×10^4 LILs/well, cocultured with autologous irradiated [3000 rad] PBMCs) in triplicate, together with HCV proteins (2 μg/mL), schistosomal proteins (SEA and SWAP), positive control (PHA, 5 μg/mL; TT, 1 μg/mL), SOD, yeast stimulation, and medium as negative control. All assays were counted on an automated ELISPOT reader (AID). The background was always <15 spot-forming cells (sfcs)/10^6

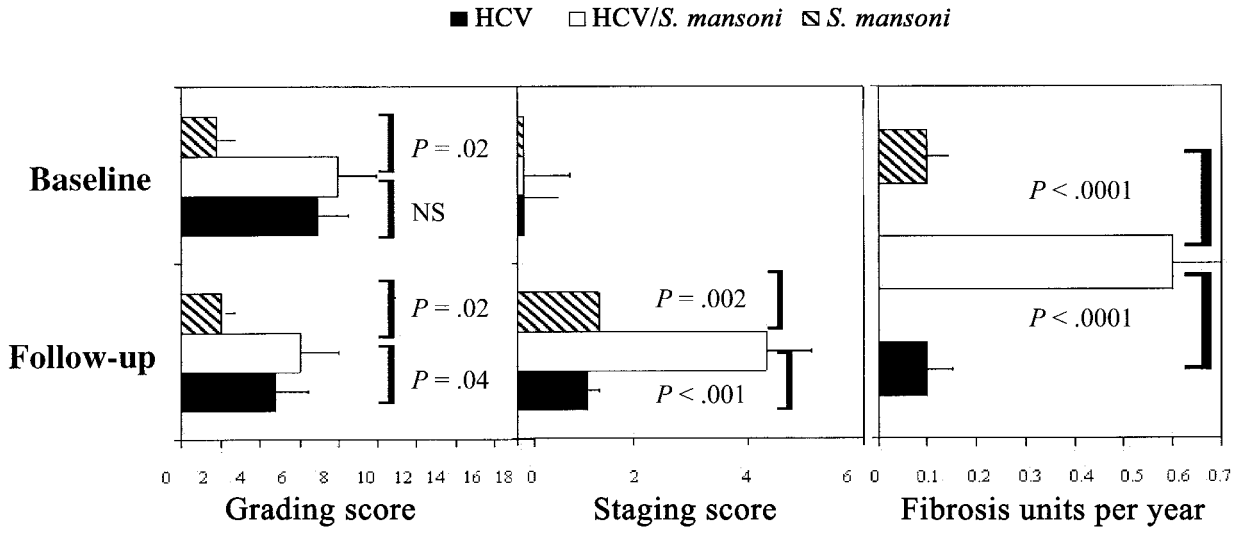


Figure 1. Liver histological assessment and progression of fibrosis in subjects with HCV infection alone and subjects with coinfection. *Left panel,* Comparison of the necroinflammatory scores at baseline biopsies (biopsy 1, performed 6–10 months after acute hepatitis) and follow-up, in 23 subjects with HCV infection alone *(black bars),* 25 coinfected subjects *(white bars),* and 20 control subjects with *Schistosoma mansoni* infection alone *(shaded bars).* Bars represent means, and lines represent SD. *Middle panel,* Fibrosis scores at baseline biopsies and follow-up biopsies. At baseline liver biopsies, subjects with HCV infection alone, coinfected subjects, and control subjects had no fibrosis (stage 0). Coinfected subjects had significantly greater increases in fibrosis scores detected in follow-up biopsies, compared with subjects with HCV infection alone ($P < .001$). *Right panel,* Rates of progression of fibrosis (fibrosis units per year). The rate of liver progression of fibrosis was significantly higher in coinfected subjects than in subjects with HCV infection alone (0.58 ± 0.13 in the HCV/*S. mansoni* coinfected group vs. 0.1 ± 0.06 in the HCV-monoinfected group; $P > .001$).

cells. Responses were considered to be positive if the number of spots per well minus the background was at least 30 sfcs/10^6 cells. In all experiments, stimulation with PHA and TT induced similar numbers of interferon (IFN)–γ, interleukin (IL)–4, and IL-l0 sfcs in both groups of patients, thus indicating that the capacity of T cells from all subjects to produce these cytokines is preserved (data not shown). The HCV-specific IFN-γ, IL-4, and IL-10 responses were caused by $CD4^+$ T cells, as shown by $CD4^+$ and $CD8^+$ depletion (data not shown).

Statistical analysis. Results were expressed as mean ± SD and analyzed by use of paired and unpaired Student's *t* test, χ^2 test, nonparametric Mann-Whitney *U* test, Wilcoxon rank sum test, or Fisher's exact test, where appropriate, and $P < .05$ was considered to be significant. Correlation between different parameters was performed by use of Pearson's or Spearman's rank test. All statistical procedures were performed by use of SSPS for Windows (version 11; SPSS).

RESULTS

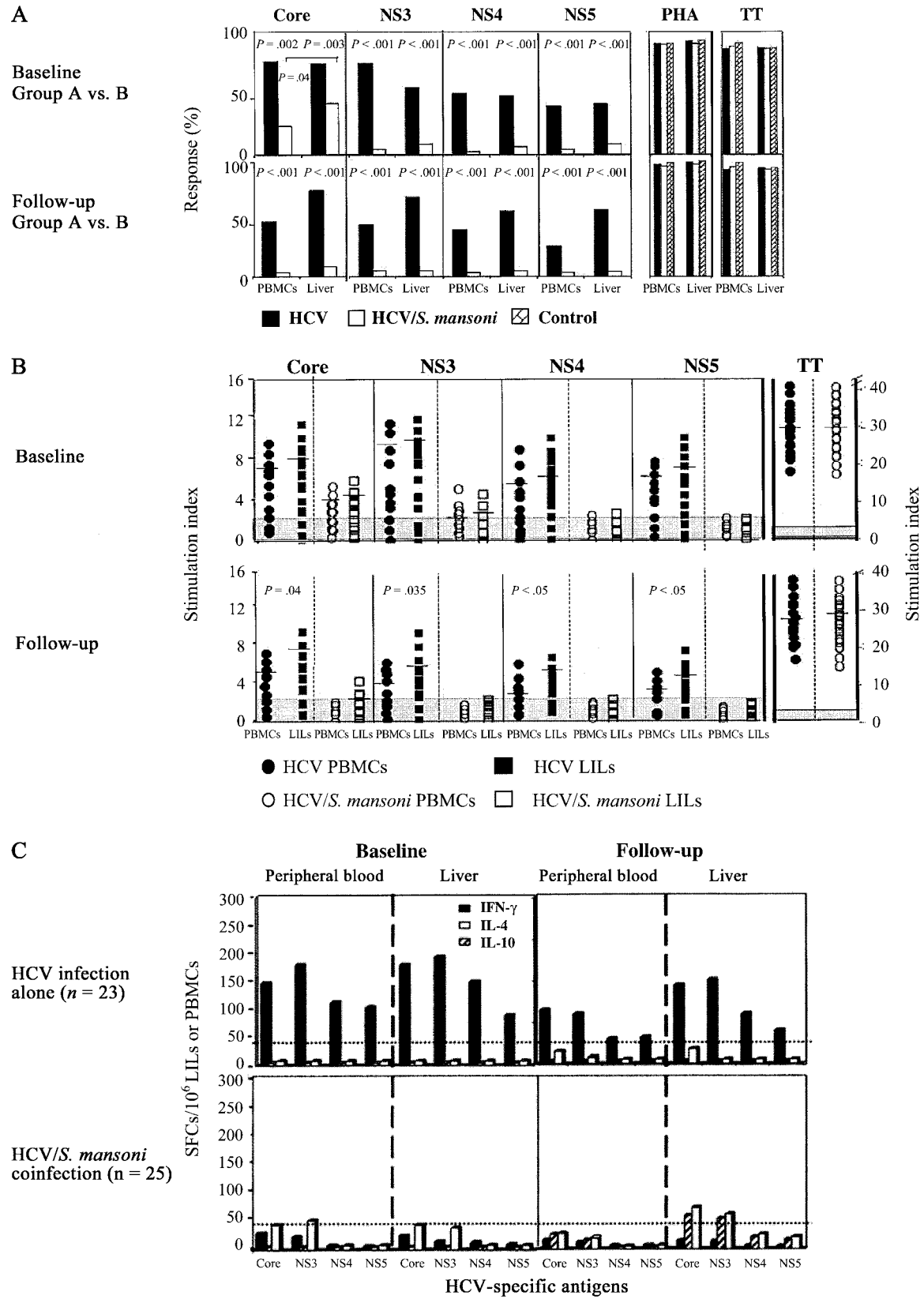
Demographic and clinical characteristics of subjects at baseline. Subjects with HCV infection alone and subjects with

Table 2. Progression of fibrosis in groups of patients and in control subjects.

Characteristic	Group A: HCV infection alone ($n = 23$)	Group B: HCV/*S. mansoni* coinfection ($n = 25$)	P[a], group A vs. group B	Control: *S. mansoni* infection alone ($n = 20$)	P[a], group B vs. control group
Interval between the 2 biopsies, mean, years	8.2	8.3	.89	8.5	.91
Subjects with progression	2 (8.6)	25 (100)	<.0001	2 (10)	<.0001
No change	21 (91)	0	<.0001	18 (90)	.001
Progression, fibrosis score					
1 point	1 (4.3)	0	<.0001	2 (10)	<.0001
2 points	1 (4.3)	3 (12)	.001	0	<.0001
3 points	0	9 (36)	<.0001	0	<.0001
>3 points	0	13 (52)	<.0001	0	<.0001

NOTE. Data are no. (%) of subjects, unless otherwise noted. HCV, hepatitis C virus; *S. mansoni, Schistosoma mansoni.*

[a] Determined by use of Fisher's exact test.


A
Core
NS3
NS4
NS5
PHA
TT
Baseline
Group A vs. B
Follow-up
Group A vs. B
Response (%)
P = .002 P = .003
P = .04
P < .001 P < .001
P < .001 P < .001
P < .001 P < .001
P < .001 P < .001
P < .001 P < .001
P < .001 P < .001
P < .001 P < .001
P < .001 P < .001
PBMCs Liver
HCV
HCV/S. mansoni
Control
B
Core
NS3
NS4
NS5
TT
Baseline
Follow-up
Stimulation index
P = .04
P = .035
P < .05
P < .05
PBMCs LILs
HCV PBMCs
HCV LILs
HCV/S. mansoni PBMCs
HCV/S. mansoni LILs
C
Baseline
Follow-up
Peripheral blood
Liver
HCV infection alone (n = 23)
HCV/S. mansoni coinfection (n = 25)
SFCs/10^6 LILs or PBMCs
IFN-γ
IL-4
IL-10
Core NS3 NS4 NS5
HCV-specific antigens

coinfection were not significantly different with respect to age, sex, route or duration of HCV infection, HCV genotype (genotype 4), serum ALT level, histological features (table 1), or HLA-DRB1 allele distribution. However, throughout the study, HCV RNA titers were significantly higher in coinfected subjects.

Fibrosis is significantly accelerated in HCV/*S. mansoni* coinfection; as expected, coinfected subjects had accelerated liver fibrosis ($P < .001$) (figure 1 and table 2), despite comparable baseline necroinflammatory scores and absence of fibrosis in subjects with HCV infection alone and subjects with coinfection. All coinfected subjects had fibrosis scores of ≥ 2 at their follow-up liver biopsies, compared with only 2 subjects with HCV infection alone (9%) (table 2). At follow-up biopsies, coinfected subjects had significantly higher degrees of interface hepatitis (1.5 ± 0.7 vs. 0.6 ± 0.5; $P = .027$) and periportal necrosis (1.9 ± 0.9 vs. 0.9 ± 0.2; $P = .0016$), compared with subjects with HCV infection alone (data not shown). The mean ($\pm$ SD) rate of progression of liver fibrosis in the coinfected subjects was 0.58 ± 0.13 fibrosis units/year, a rate that predicts progression to cirrhosis in 10–12 years if progression is linear. In the subjects with HCV infection alone, the mean ($\pm$ SD) rate of progression of liver fibrosis was 0.1 ± 0.06 fibrosis units/year, a rate that predicts progression to cirrhosis in 50 years if progression is linear (figure 1). In subjects with HCV infection alone and subjects with coinfection, neither ALT levels nor virus load correlated with the necroinflammatory scores at baseline or follow-up ($P = .6$ and $P = .7$, respectively, Wilcoxon signed rank test). None of the subjects with *S. mansoni* infection alone had a necroinflammatory score of >3, and none had fibrosis at baseline (figure 1). At follow-up biopsy, only 2 (10%) of 20 control subjects with *S. mansoni* infection alone had progression of fibrosis, whereas 18 (90%) of 20 remained the same, suggesting that the enhanced fibrosis in coinfection was not primarily due to *S. mansoni*.

HCV-specific CD4⁺ T cell responses compartmentalize in the liver with evolution of chronic HCV infection. Our results revealed differences in frequency, magnitude, and specificity of peripheral and intrahepatic responses between subjects with HCV infection alone and subjects with coinfection, at baseline and follow-up. In general, HCV-specific $CD4^+$ T cell responses were significantly greater and broader in subjects with HCV infection alone at both time points (figure 2*A,* 2*B,* and 2*C* and table 3). At baseline, LILs from 15 subjects with HCV infection alone responded to at least 2 HCV antigens, whereas LILs from 4 subjects responded to a single antigen (data not shown). The intrahepatic and peripheral HCV-specific $CD4^+$ T cell responses to NS_3 were significantly stronger than those to HCV core ($P = .02$, Wilcoxon signed rank test). Although, at the end of follow-up, the peripheral responses waned and became narrowly focused to HCV core in 13 (56.5%) of 23 subjects with HCV infection alone or were lost in 6 (26%) of 23 subjects, HCV-specific $CD4^+$ T cell responses were readily detected in the intrahepatic compartment of 19 of 23 subjects, although with significantly less magnitude and breadth, compared with baseline (figure 2*B* and table 3).

In contrast, in 9 coinfected subjects, the baseline responses in both compartments were weak and narrowly focused, targeting HCV core and/or NS_3, whereas 16 subjects did not respond to any of the HCV proteins (figure 2*A* and 2*B*). In general, at baseline, the strength of the intrahepatic proliferative HCV-specific T cell responses to nonstructural HCV antigens and HCV core, in LILs, were significantly less in coinfected subjects (SI_{core}, 4.5 ± 0.3; SI_{NS3}, 2.9 ± 1.3; SI_{NS4}, 2.5 ± 0.8; SI_{NS5}, 2.3 ± 1.2) than in subjects with HCV infection alone (baseline: SI_{core}, 7.1 ± 3.3; SI_{NS3}, 8.3 ± 4.1; SI_{NS4}, 3.6 ± 2.5; SI_{NS5}, 3 ± 0.8; $P = .002$). At the end of follow-up, no significant peripheral HCV-specific $CD4^+$ T cell responses could be detected in any coinfected subject, although weak HCV core–specific T cell proliferative responses were elicited in LILs from 5 coinfected subjects (20%) (figure 2*B* and table 3). Despite the poor proliferative response to HCV antigens, coinfected subjects had a vigorous response to SEA and SWAP that was not significantly

Figure 2. Comparison of proliferative responses to hepatitis C virus (HCV) in subjects with HCV infection alone and subjects with coinfection. *A,* Comparison of overall proliferative $CD4^+$ cell response to HCV proteins (HCV core, NS3, NS4, and NS5) and control antigen (PHA and TT), as described in Subjects, Materials, and Methods, expressed as percentage of positive response in the peripheral blood and liver tissue from subjects with chronic HCV infection alone *(black bars),* subject with HCV/*Schistosoma mansoni* coinfection *(white bars),* and control subjects *(shaded bars). Upper panels,* Baseline proliferative responses; *lower panels,* follow-up proliferative responses. Statistical comparison was performed by use of nonparametric Mann-Whitney *U* test. $P < .05$ was considered to be significant. *B,* $CD4^+$ proliferative T cell responses to HCV core, NS3, NS4, and NS5 in peripheral blood *(circles)* and liver tissue *(squares)* from subjects with HCV infection alone (*black circles* and *black squares,* respectively) and coinfected subjects (*white circles* and *white squares,* respectively), at baseline *(upper panels)* and at the end of follow-up *(lower panels).* Each symbol represents the response elicited by a given HCV antigen in the proliferation assay. *Y*-axis, Stimulation index (SI). The cutoff for a positive response is 3 (3 SD above the mean SI of healthy control subjects). The cutoff for LIL proliferative responses is in relation to proliferative responses from healthy livers. Horizontal lines represent means. *C,* Peripheral and intrahepatic HCV-specific cytokine production at baseline and follow-up in subjects with HCV infection alone and subjects with coinfection: interferon (IFN)–γ *(black bars),* interleukin (IL)–4 *(shaded bars),* and IL-10 *(white bars)* production (ELISPOT assay) in peripheral blood and liver tissue at baseline and follow-up in subjects with HCV infection alone *(upper panel)* and coinfected subjects *(lower panel). X*-axis, tested HCV antigens; *Y*-axis, significant number of spot-forming cells (sfcs) per 10^6 LILs or peripheral blood mononuclear cells (PBMCs) (no. of spots in wells with HCV antigen minus control wells).

Table 3. Frequency of hepatitis C virus (HCV)–specific proliferative responses in the intrahepatic and peripheral compartments, for the 2 groups of patients.

	Group A: HCV infection alone (n = 23)		Group B: HCV/*S. mansoni* coinfection (n = 25)	
Parameter	Baseline	End of follow-up	Baseline	End of follow-up
HCV-specific proliferative responses in PBMCs and LILs	16 (69.6)	13 (56.5)	6 (24)	0 (0)
HCV-specific proliferative responses in LILs only	0 (0)	6 (26.1)	3 (12)	5 (20)
HCV-specific proliferative responses in PBMCs only	3 (13)	0 (0)	0 (0)	0 (0)
Total	19 (82.6)	19 (82.6)	9 (36)	5 (20)

NOTE. Data are no. (%) of subjects. PBMCs, peripheral blood mononuclear cells; *S. mansoni, Schistosoma mansoni.*

different from the responses in subjects with *S. mansoni* infection alone. Moreover, responses to TT or PHA did not differ among subjects with HCV infection alone, subjects with coinfection, or control subjects, thus excluding a global T cell unresponsiveness in coinfected subjects (figure 2*A* and 2*B*). This suggests that infection with *S. mansoni* specifically inhibited the HCV-specific $CD4^+$ response in the coinfected subjects. Of note, depletion assays clearly showed that these are $CD4^+$ T cell responses (data not shown). It is unlikely that differences between genotype 1, which was used for the recombinant antigens, and genotype 4, which infected the patients, are responsible for the findings, because there is a high degree of conservation between genotype 1 and genotype 4 proteins in the regions of the recombinant proteins used for the immunological assays [22, 23]. Moreover, subjects with acute and chronic infection with HCV alone, in addition to some coinfected subjects from the study cohort, had responses to genotype 1 antigens. Baseline virus load inversely correlated with intrahepatic NS3 and NS4 and HCV core–specific $CD4^+$ T cell proliferative responses ($R = -0.63$ and $P > .05$, $R = -0.57$ and $P > .05$, and $R = -0.5$ and $P > .05$, respectively; data not shown).

Predominant peripheral and intrahepatic HCV-specific Th1 profile in HCV monoinfection. We then asked if the peripheral and hepatic cytokine milieu was different in the 2 groups of patients. HCV core–, NS_3-, NS_4-, and NS_5-specific peripheral and intrahepatic T cells preferentially showed a Th1 cytokine pattern (IFN-γ production only) at baseline and follow-up, in subjects with HCV infection alone (figure 2*C*), whereas neither IL-4 nor IL-10 could be detected at any time point. IFN-γ production in response to at least 2 HCV antigens was detected in 10 subjects with HCV infection alone, and IFN-γ production in response to a single antigen was detected in 9 subjects. At follow-up, IFN-γ production to NS_3 and HCV core was detected in 4 subjects with HCV infection alone, whereas, in 15 subjects, IFN-γ production was restricted to either NS_3 (6 subjects) or HCV core (9 subjects). Of note, IFN-γ, IL-10, and IL-4 were produced in comparable amounts by PBMCs and LILs in response to the control antigens (data not shown). Similar to findings for the $CD4^+$ T cell proliferative response, coinfected subjects responded poorly to HCV antigens at baseline. Weak production of HCV core–specific IFN-γ alone was detected in LILs from 7 (28%) of 25 subjects, whereas intrahepatic HCV core–specific IFN-γ was detected in conjunction with IL-10 in 2 (8%) of 25 subjects. IFN-γ sfcs alone were detected from PBMCs in 4 (16%) of 25 coinfected subjects, whereas IL-10 was produced together with IFN-γ in 2 of 25 coinfected subjects. At follow-up, HCV-specific production of IL-4 and IL-10 was only demonstrated in the intrahepatic compartment in 7 coinfected subjects (28%), whereas 18 subjects showed no HCV-specific cytokine production (figure 2*C*), despite preserved cytokine production in response to schistosomal and control antigens. In control subjects with *S. mansoni* infection alone, the peripheral and intrahepatic responses to schistosomal antigens were dominated by Th2 cytokines (IL-4 and IL-10). In the livers of subjects with *S. mansoni* infection alone, the levels of *S. mansoni*–specific IL-4 and IL-10 increased at the follow-up biopsies, compared with baseline; however, the increase was statistically significant in 2 of 20 subjects (data not shown).

The magnitude and pattern of the early HCV-specific $CD4^+$ T cell response predicts the rapidity of progression of fibrosis. None of the chronically HCV-infected subjects who had a >2-point increase in fibrosis score at follow-up biopsies and rates of progression of fibrosis of >0.25 fibrosis units/year displayed any significant intrahepatic $CD4^+$ T cell responses to HCV at baseline or follow-up (figure 3*A*). Conversely, no or slow progression of fibrosis (<2-point increase in follow-up fibrosis score and a rate of progression of fibrosis of <0.1 fibrosis units/year) was associated with significant multispecific $CD4^+$ T cell response, particularly to the HCV nonstructural antigens (figure 3*A*). There was a significant inverse correlation between baseline intrahepatic $CD4^+$ T cell proliferative responses against each of NS_3, NS_4, HCV core, and NS_5 and the rate of progression of fibrosis ($R = -0.72$ and $P < .01$, $R = -0.67$ and $P < .01$, $R = -0.64$ and $P < .01$, and $R = -0.52$ and $P < .05$, respectively); in addition, follow-up proliferative responses against both NS_3 and HCV core inversely correlated with progression of fibrosis ($R = -0.63$ and $P < .01$ and $R = -0.5$ and $P <$

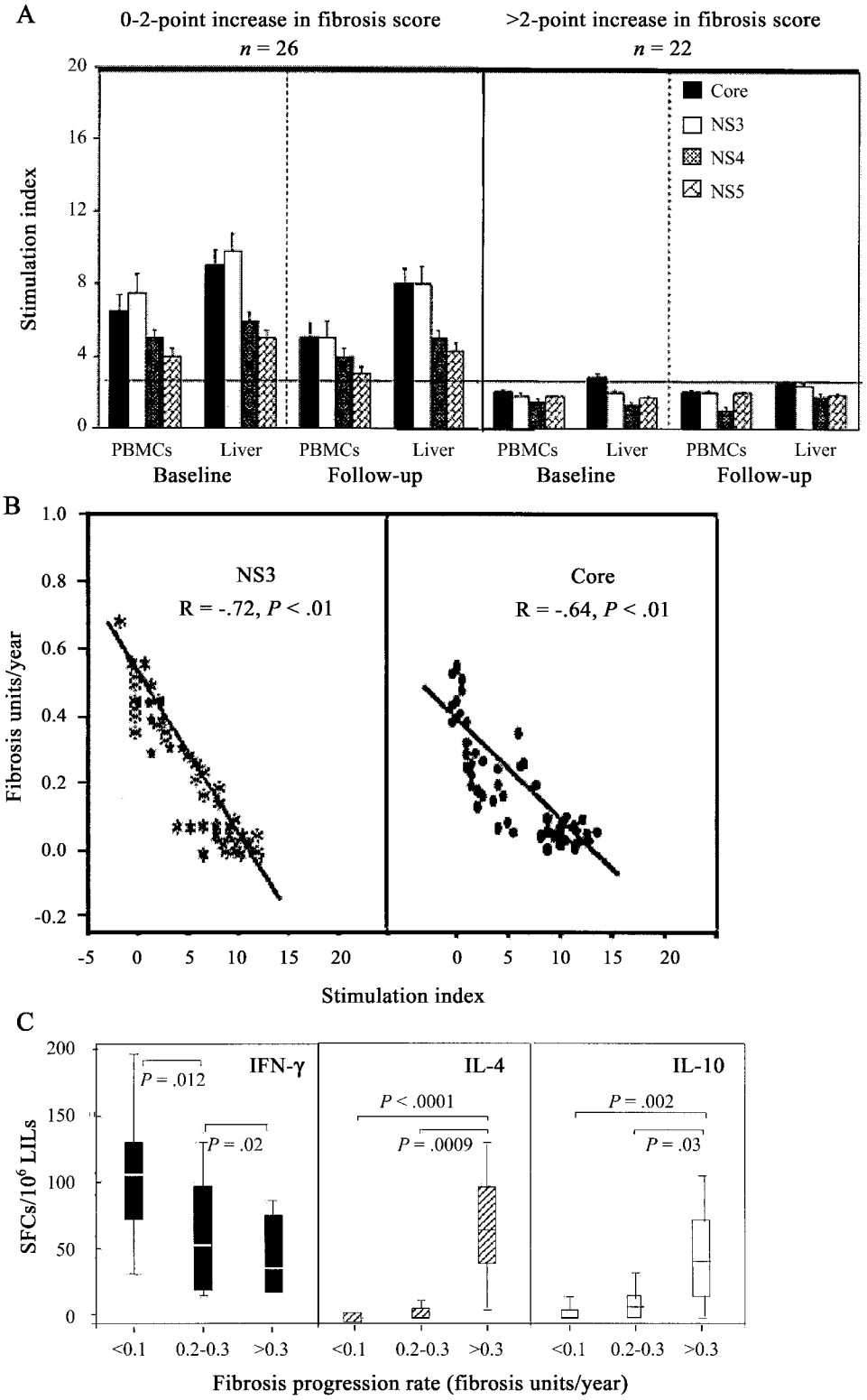


Figure 3. Peripheral and intrahepatic hepatitis C virus (HCV)–specific CD4+ proliferative T cell responses in relation to progression of fibrosis. *A,* CD4+ T cell responses to 4 HCV proteins (HCV core, NS3, NS4, and NS5), expressed as stimulation index (SI) (*Y*-axis), in subjects with a 0–2-point increase in fibrosis score *(left)* and subjects with a >2-point increase *(right)* at the follow-up biopsies. *B,* Relation of baseline intrahepatic HCV (NS3) and HCV core–specific CD4+ proliferative T cell responses to progression of fibrosis (fibrosis units per year): the 2 parameters show a significant inverse correlation ($R = -0.72$; $P > .01$ and $R = -0.64$; $P > .01$, respectively). *C,* Relation of follow-up intrahepatic HCV core–specific cytokine production (interferon (IFN)–γ, interleukin (IL)–4, and IL-10) to progression of fibrosis (*X*-axis), expressed as fibrosis units per year. *Y*-axis, Cytokine spot-forming cells (sfcs) per 10^6 LILs. *P* values were determined by Wilcoxon rank sum test and were considered to be significant at $P < .05$.

.05, respectively). These findings were confirmed by ELISPOT assay: subjects with more-rapid progression of fibrosis ($\geq$0.3 fibrosis units/year) had significantly increased intrahepatic production of IL-4 and IL-10, with marked suppression of IFN-γ (figure 3*C*). This pattern occurred in cross-sectional studies of groups of patients and in longitudinal studies of individual patients. The association between HCV-specific $CD4^+$ T cell responses and histological changes shown in the present study might imply that failure to have sufficient early intrahepatic HCV-specific $CD4^+$ Th1 response, together with or without an augmented type-2 response, is associated with more-severe liver histological assessment and enhanced progression of disease.

DISCUSSION

The effect of the host immune responses in determining hepatitis C pathogenesis and progression of HCV-related liver fibrosis is not yet understood. Longitudinal studies correlating the evolution of these responses with progression of disease are not available, because of the asymptomatic nature of acute infection and the protracted course of disease. Studies of chimpanzees suggest that resolution or persistence of HCV infection depends on the strength of intrahepatic responses generated at the early stages of hepatitis [25–27]. Most studies of the HCV-specific $CD4^+$ and $CD8^+$ T cell responses in humans have focused on the peripheral blood, because of difficulty in obtaining adequate numbers of intrahepatic T lymphocytes [21, 29, 30], although it has been shown that, in chronic infection, HCV-specific $CD4^+$ and $CD8^+$ T cells compartmentalize in the liver [19, 28].

In the present longitudinal prospective study, the availability of results from paired liver biopsies performed at a very early phase of chronic evolution and >8 years later enabled us to monitor the kinetics of intrahepatic $CD4^+$ responses and to correlate these responses to the progression of liver injury in subjects who showed slow progression of fibrosis (HCV infection alone) and subjects who showed rapid progression of fibrosis (HCV/*S. mansoni* coinfection). As previously reported [11], in our cohort, HCV/*S. mansoni* coinfection led to accelerated liver fibrosis, with rates of progression of fibrosis of >0.58 fibrosis units/year, compared with the rate of 0.1 fibrosis units/year seen in the subjects with HCV infection alone, which are comparable to those in other retrospective studies [7, 24]. Although, to improve uniformity, our subjects were matched with regard to baseline host and viral factors that might influence progression of fibrosis, it may be argued that the observed accelerated progression of fibrosis might be caused by the degree of schistosomiasis or by the additional injury inflicted by HCV on a liver previously damaged by schistosomiasis. However, the baseline necroinflammatory and fibrosis scores were comparable in both groups. Moreover, the control group (subjects with *S. mansoni* infection alone) did not show significant necroinflammation, which is in accordance with findings of previous reports [14–17].

Relevant to our clinical and histological findings, the 2 groups of patients differed sharply in their $CD4^+$ T cell responsiveness to HCV. The frequency, magnitude, and breadth of the HCV-specific T cell responses in subjects with HCV infection alone were significantly higher than those in coinfected subjects. Our study has demonstrated a significant inverse association between intrahepatic $CD4^+$ T cell responses and progression of fibrosis, suggesting that absent or diminished HCV-specific $CD4^+$ T cell responses in the liver, particularly during the early phase of chronic evolution, favor liver damage and progression of disease. The frequency of baseline intrahepatic HCV-specific T cell responses in the subjects with HCV infection alone in the present study is consistent with that in other studies [31], which detected HCV-specific $CD4^+$ T cells in the liver in 78% of subjects during the early phase of HCV reactivation after liver transplantation. Despite waning or loss of responses in the peripheral blood over time in both study groups, HCV-specific $CD4^+$ responses were maintained in the liver, although with less magnitude and breadth, suggesting that the peripheral T cell responses may not be good indicators of immune responses in established chronic hepatitis. The discrepancy between liver and peripheral blood, reflecting compartmentalization of T cells, may be due to sequestration of HCV-specific T cells in the liver or homing from the periphery to the liver [33, 34]. Because of the expansion protocol required for isolation of adequate numbers of intrahepatic lymphocytes, the intrahepatic and peripheral populations are not directly comparable, but, nonetheless, distinct patterns emerge. The frequency of intrahepatic $CD4^+$ T cell responses in the subjects with HCV infection alone in the present study is higher than that reported in other studies [19, 29, 30], which detected peripheral $CD4^+$ responses in only one-third of chronically infected subjects. These differences can likely be explained by the fact that our study detected intrahepatic responses at a much earlier time point in chronic evolution, whereas other studies looked only at peripheral responses in individuals infected for several decades, when most of the responses compartmentalize in the liver, as shown by reports demonstrating intrahepatic HCV-specificity in 50%–67% of subjects with long-standing chronic hepatitis C [19, 28, 32]. Interestingly, coinfected subjects in the present study exhibited selective deterioration of HCV-specific Th1 responses while maintaining normal T cell responses to other antigens, such as TT, PHA, and schistosomal antigens, thus excluding global T cell unresponsiveness. Although we do not yet know the reason for this discrepancy, one may speculate that this is due to the deletion of more highly activated T cells within the liver [35].

We hypothesized that changes in the intrahepatic cytokine milieu might play a role in progression of disease. In subjects with HCV infection alone, intrahepatic and peripheral $CD4^+$

cells predominantly secreted IFN-γ in response to HCV antigens, at baseline and follow-up, which is consistent with findings of published reports [9–11, 19, 29, 30] and demonstrates predominant Th1 responses in HCV infection. Conversely, coinfected subjects either displayed no HCV-specific responses or had type-2 responses, which was associated with a more advanced disease in those subjects. Studies of mice have shown that immune deviation to a Th1 response results in decreased liver fibrosis, whereas enhancement of Th2 cytokine production promotes an increase in hepatic fibrosis [36, 37]. The association of HCV-specific intrahepatic IFN-γ production in the present study with slower progression of fibrosis might be explained by the findings of several studies that have shown that IFN-γ exhibits direct effects on the synthesis of extracellular matrix components, particularly the interstitial collagens [38–40]. In contrast, intrahepatic production of HCV core–specific IL-4 was only detected in coinfected subjects with high rates of progression of fibrosis ($\geq$0.3), which is in line with findings of previous reports demonstrating the potential role of this cytokine in driving the differentiation of $CD4^+$ T cells into the Th2 subset and generation of hepatic fibrosis [14, 15, 41, 42]. Collectively, our data suggest that alterations of cytokine patterns in the form of insufficient intrahepatic Th1 responses or augmented Th2 responses enhance progression of fibrosis. Although, in the present study, we were able to examine only a limited number of cytokines, because of restrictions on the numbers of cells available for these assays, studies evaluating the role of other proinflammatory and profibrogenic cytokines constitute an important area for future research.

In conclusion, the data presented in the present study have demonstrated for the first time the importance of cellular immune responses in preventing the progression of liver disease and have illustrated that absence or decline of type-1 $CD4^+$ T cell responses during the early phases of chronic HCV may lead to greater progression of liver disease. Our findings may have implications for development of a vaccine and immunotherapy. Given the quasi-species nature of HCV, it likely will be difficult to formulate a vaccine that effectively protects 100% of exposed individuals. However, our data suggest that, even without sterilizing immunity, a vaccine that induces strong cellular immune responses might limit subsequent liver damage.

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