Reconstitution of Hepatitis C Virus–Specific T-Cell–Mediated Immunity After Liver Transplantation

Scott J. Weston,[1,4] Rachel L. Leistikow,[1,4] K. Rajender Reddy,[1,5] Maria Torres,[1,6] Anne M. Wertheimer,[1,2,4] David M. Lewinsohn,[1,2,4] Sunwen Chou,[1,2,4] Michael P. Davey,[1,2] Christopher Corless,[3,4] Cliona O'Farrelly,[7] David R. Nelson,[1,8] and Hugo R. Rosen[1,2,4]

Hepatitis C virus (HCV)-related liver failure is the leading indication for liver transplantation worldwide. After transplantation, virological recurrence is the rule, but the spectrum of histological injury is wide, ranging from the development of allograft cirrhosis within a few years to minimal hepatitis despite long-term follow-up. The immunological correlates of this variable natural history are poorly understood. Here, we studied the kinetics of the cellular immune responses, viral replication, and allograft histology in 24 patients who had undergone liver transplantation for HCV-related liver failure. Using direct *ex vivo* methodologies (*i.e.*, interferon-gamma ELISPOT and major histocompatibility complex class I–peptide tetrameric complexes), we found that patients who experienced viral eradication after antiviral therapy showed restoration of HCV-specific T-cell responses, whereas patients with progressive HCV recurrence that failed to respond to therapy showed declining frequencies of these viral-specific effector cells. The cytotoxic T lymphocytes that peripherally reconstituted after transplantation were clonotypically identical to those present within the recipient explant liver, defined at the level of the T-cell receptor beta chain (one epitope/one clone). Moreover, the subset of patients who spontaneously demonstrated minimal histologic recurrence had more vigorous CD4$^+$ T-cell responses in the first 3 months, particularly targeting nonstructural proteins. We provide evidence that T-cell responses emerge after liver transplantation, and their presence correlates with improved histological and clinical outcomes. In conclusion, these results may help identify patients more likely to develop severe HCV recurrence and therefore benefit from current antiviral therapy, as well as provide a rationale for the future use of novel immunotherapeutic approaches. *Supplementary material for this article can be found on the HEPATOLOGY website (http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html).* (HEPATOLOGY 2005;41:000–000.)

Hepatitis C virus (HCV) infection is characterized by the high likelihood of chronicity after acute infection and significant risk of disease progression to cirrhosis and liver failure. Immune responses appear to be crucial in the control of infection, and patients with self-limited courses of acute HCV demonstrate coordinated activation of viral-specific CD4$^+$ and CD8$^+$ T cells that produce type 1 cytokines such as interferon gamma (IFN-γ) and tumor necrosis factor-α.[1] Moreover, these T-cell responses can be maintained for decades after recovery. Cellular immune responses also may offer varying degrees of protection against viral replication and tissue damage in persistent infection.[2] For example, an inverse correlation between levels of HCV-specific cytotoxic T lymphocytes, and viral load has been observed in humans and chimpanzees,[3–5] and HCV-related pathology is more prevalent in the setting of human immunodeficiency virus immune suppression.[2]

HCV-related liver failure is the single most common indication for orthotopic liver transplantation (OLT) worldwide.[6] Recurrent HCV infection, as defined by viremia after transplantation, is nearly universal, with rapid increases in HCV viral load noted immediately after

Abbreviations: HCV, hepatitis C virus; IFN-γ, interferon gamma; OLT, orthotopic liver transplantation; HLA, human leukocyte antigen; PBMC, peripheral blood mononuclear cell; IL, interleukin; NS, nonstructural; TCR, T-cell receptor.

From the Departments of [1]Medicine, [2]Molecular Microbiology and Immunology, and [3]Pathology, Oregon Health & Science University, and [4]Portland VA Medical Center, Portland, OR; [5]University of Pennsylvania, Philadelphia, PA; [6]University of Miami, Miami, FL; [7]Research Laboratories, St. Vincent's University Hospital, Dublin, Ireland; and [8]University of Florida, Gainesville, FL.

Received May 26, 2004; accepted September 21, 2004.

Supported in part by a VA Merit Review and RO1 DK60590.

Presented at the Plenary Session of the AASLD, Boston, MA, November 2002.

Address reprint requests to: Hugo Rosen, M.D., Portland VAMC, mail code P3-GI, Gastroenterology and Hepatology, 3710 SW U.S. Veterans Hospital Road, Portland, OR 97239. E-mail: rosenhu@ohsu.edu; fax: 503-402-2816.

Copyright © 2004 by the American Association for the Study of Liver Diseases.

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.20507

Conflict of interest: Nothing to report.

transplantation, confirming the high capacity of HCV to adapt to a new environment.[7] Massive re-infection of the allograft occurs within a few days after transplantation; however, the spectrum of allograft injury related to HCV recurrence is highly variable and ranges from mild histological abnormalities to allograft cirrhosis within a few years.[8]

In a previous cross-sectional analysis, we reported that HCV-multispecific CD4$^+$ T-helper responses were detectable by proliferation assay in 40% of patients with mild histologic recurrence of HCV after liver transplantation but not in patients who had developed severe HCV recurrence.[9] Thus, by analogy to results derived from acute infection,[10] HCV persistence and disease progression after liver transplantation may be related in part to the lack of an appropriate helper T-cell response, whereas, in contrast, a vigorous T-cell response during the early stages of re-infection may be an important mechanism to limit liver allograft injury. However, a number of important questions remain unanswered: Would the use of more sensitive assays detect HCV-specific responses in a larger proportion of patients? Do these immune responses pre-date liver transplantation or do they emerge after re-infection of the graft? What are the kinetics of the T-cell immune response in relationship to viral replication and how are these kinetics affected by antiviral therapy when assessed longitudinally? What is the role of HCV-specific CD8$^+$ T-cell responses after liver transplantation?

In this prospective analysis, we examined viral-specific and nonspecific T-cell responses in 24 patients undergoing liver transplantation. We found that reconstitution of HCV-specific T-cell immunity correlated with mild histologic recurrence after liver transplantation, whereas the lack of these responses was associated with the development of severe HCV recurrence (as defined histologically and clinically). In particular, emergence of CD4$^+$ T-cell responses specific to nonstructural proteins, either spontaneous or after antiviral therapy, correlated with mild histologic recurrence and excellent clinical outcomes. CD8$^+$ T-cell responses were detectable by soluble human leukocyte antigen A2 (HLA-A2) tetramers in patients who cleared serum HCV RNA with interferon alpha 2b and ribavirin; in contrast, these responses declined rapidly in patients who developed progressive histologic recurrence. A careful analysis using multiple assays shows that patients with advanced HCV-related liver disease may retain the ability to respond to a broad array of HCV proteins/peptides after liver transplantation. Future strategies (vaccination, immunotherapy, or adoptive transfer) to enhance HCV-specific T-cell responses in these patients will, it is hoped, diminish the rate of allograft loss from recurrent disease.

Patients and Methods

Study Patients. The study group comprised HCV-seropositive patients undergoing liver transplantation in Portland, Oregon, and Miami, Florida. Informed consent was obtained in all cases, and internal review board approval was granted at the Portland VAMC, Oregon Health and Science University, and the University of Miami School of Medicine. Ten patients at the University of Miami were enrolled into a randomized trial to evaluate the safety and efficacy of antiviral treatment initiated within the first postoperative month (see Supplementary Materials). Patients receiving pre-emptive antiviral therapy were scheduled to undergo liver biopsies at 16, 48, and 96 weeks; patients enrolled in Portland typically underwent liver biopsies at 24, 48, 72, and 96 weeks. Modified hepatitis activity index and fibrosis scores were determined in all biopsy specimens.[11]

Phorbol-Myristate Acetate Stimulation and Intracellular Cytokine Staining. Intracellular cytokine staining of cryopreserved peripheral blood mononuclear cells (PBMCs) after phorbol-myristate acetate (PMA)/ionomycin stimulation was performed as described in the Supplementary Materials.

ELISPOT Assay, HCV Proteins. The extent of immunological analysis of individual patients was determined by the number of cells that were available at each time point: phorbol-myristate acetate (PMA)/ionomycin stimulation was performed first because it required the least number of cells; tetramer analysis was performed if the recipient was HLA-A2 positive, and CD4$^+$ T-cell ELISPOT analysis was performed if there were at least 6×10^6 PBMCs for each time point. HCV-specific IFN-γ production was detected using an established ELISPOT protocol.[12]

HLA Class I Peptide Tetramer Staining. Phycoerithren–labeled HLA-A*02 tetramers loaded with known HCV 1a immunodominant peptides core 132-140 (DLMGYIPLV), NS3 1073-1081 (CINGVWCTV), NS3 1406-1415 (KLVALGINAV), NS5 2727-2735 (GLQDCTMLV), and NS5 2594-2602 (ALYDVVTKL) were synthesized.

Cloning of Tetramer-Positive Cells. Cells were separated and stained as described in the Supplementary Materials, with the exception that cells were not fixed before being sorted by flow cytometry on a FACSVantage (BD Biosciences, San Jose, CA). One hundred fifty to 5,000 CD8$^+$ tetramer-positive cells were collected and allowed to rest in RPMI + 10% HS + interleukin 2 (IL-2) (0.75 μg/mL). After a minimum of 2 hours at 37°C, tetramer-positive cells were plated at limiting dilution (1, 5, 10, and 50 cells/well) and cultured with 8×10^4 irradiated allo-

Table 1. Clinical Characteristics and Outcome of the 24 Study Patients, Divided into 3 Groups.

							Treatment (Tx) Information				Early Histologic Findings			Late Histologic Findings		
Pt. ID	Immunosuppression	Age	Sex	Genotype	HLA-A2 Status	Viral Load†	Tx	Initiated (days post-OLT)	Duration (wks)	Response	Grade	Stage	Time post-OLT (wks)	Grade	Stage	Time post-OLT (wks)
1	T/S/M	60	F	1b	+	74,577	+	29	48	NR	1	1	16	2	2	52
2	T/S	48	M	1	−	330,034	+	17	48	PR	1	1	16	2	2	52,104
3	T/S	47	M	1	+	<3,200	+	21	48	R	0	0	16	0	0	52,104
4	T/S	52	F	1b	+	<3,200	+	14	48	NR	1	2	16	1	0	52,104
5	T/S	30	M	1b	+	10,346	+	8	48	NR	2	2	16	1	2	52
6	T/S/R	66	F	1b	−	10,234	+	20	48	NR	1	2	16	1	1	52
7	T/S	46	M	1a	−	11,177	+	22	48	NR	2	2	16	0	1	52
8	T/S/M	60	M	2	+	4,055	+	3	24	R/R	1	1	16	2	2	52
9	T/S	49	M	1a	+	32,668	Placebo	34	48	NR	1	1	16	Patient lost to follow-up		
10	T/S/M	62	M	1a	+	148,755	Placebo	32	48	NR	2	2	16	2	3	52,104
11‡	T/S/M*	56	M	1a	+	171,785	+	95	68 (ongoing)	R	2	0	12	1	1	52,104
12‡	T/S/M	49	M	1b	+	5,568,662	+	243	32 (death)	NR	3	3	20	3	3	52
13‡	T/S/M	46	M	1a	−	8,707	+	123	64	R	3	2	12	2	3	104
14‡	T/S/M*	58	M	2b	−	204,013	+	88	24	PR	2	0	8	Patient lost to follow-up		
15‡	T/S/M	45	M	3a	−	1,573,279	+	169	54	R	2	2	20	1	1	104
16‡	T/S/M*	47	M	1b	+	1,508,298	+	424	136	R	3	3	52	1	2	188
17‡	T/S/M	54	M	4a	+	16,733	+	457	48	R	3	2–3	52	0	0	116
18	T/S/M	53	M	1	+	5,316,896	−	N/A	N/A	N/A	1	1	52	1	1	104
19	T/S/M*	58	M	2	+	1,647,935	−	N/A	N/A	N/A	1	0	52	1	0	104
20	T/S	54	M	1a	−	<3,200	−	N/A	N/A	N/A	1	0	52	1	0	104,156
21	T/S/M	55	M	1	−	10,542,050	−	N/A	N/A	N/A	1	1	52	1	1	104
22	T/S/M	55	M	4a	−	1,275,744	−	N/A	N/A	N/A	1	1	52	1	1	104
23	T/S/M	60	M	1	−	4,198,438	−	N/A	N/A	N/A	1	2	52	1	2	104
24	T/S/M	48	M	1b	−	420,284	−	N/A	N/A	N/A	1	2	52	1	2	104

Abbreviations: T, Tacrolimus; S, solumedrol; M, mycophenalate mofetil; A, azathioprine; R, rapamycin. Tx Response: NR, nonresponse; R, response; PR, partial response; R/R, response/relapse.

*M switched to A.

†Viral load day of transplantation in copies/mL by HCV 3.0 RNA bDNA.

‡Patient received antiviral therapy for severe recurrence.

geneic PBMCs and 1.6×10^4 lymphoid cell line in a total volume of 250 μL RPMI + 10% HS per well with purified anti-CD3 (0.03 μg/mL) in 96-well plates.

Liver-Infiltrating Lymphocytes. Mechanical and collagenase digestion was used to generate single-cell suspensions from 100 to 300 g liver explant tissue derived from selected patients.

Monoclonal Antibodies and Staining. Cells were stained at room temperature in the dark for 30 minutes and then washed twice in 2 mL phosphate-buffered saline + 1% bovine serum albumin. Isotype-matched control reagents were used to determine background levels of staining.

Sequencing of Epitope Coding Regions. As described previously, we sequenced NS3 epitope coding regions from serial samples in selected patients.[13]

Vβ Screening and Sequencing of T-Cell Receptor From Purified Tetramer-Binding Cells. See Supplementary Materials for details.

Statistical Analysis. Unpaired *t* test (for comparison of two groups) and one-way analysis of variance (for comparison of more than two groups) were used for parametric data and Wilcoxon rank sum, and Kruskall-Wallis test were used for nonparametric data. A *P* value of less than .05 was considered significant. The JMP 4.0.4 (SAS Institute Inc., Cary, NC) statistical package was used.

Results

We prospectively tracked T-lymphocyte responses in 3 groups of HCV-seropositive patients who underwent liver transplantation: patients who received preemptive antiviral therapy (or placebo) starting within the first month after transplantation (patients 1-10), patients who received antiviral therapy for severe histologic recurrence (patients 11-17), and patients with long-term follow-up who have demonstrated minimal evidence of histologic recurrence and have not required antiviral therapy (patients 18-24) (Table 1).

Prospective Analysis of $CD4^+$ And $CD8^+$ T-Lymphocyte Responses Directly* Ex Vivo *After Liver Transplantation in Patients Receiving Pre-emptive Antiviral Therapy. There were three different categories of clinical/virologic responses, comprising one virologic partial response (patient 2), one complete response with HCV RNA negativity first documented 4 months after OLT (patient 3), and no significant clinical or virologic response in the remaining six patients (*e.g.*, patient 1 and Supplemental Fig. 1).

Figure 1 shows four representative patients with different outcomes to antiviral therapy. In the patients who did not respond to antiviral therapy (*e.g.*, Fig. 1A), the level of ELISPOT $CD4^+$ T-cell responses remained very low, and tetramer responses decreased throughout the follow-up period. In one patient who demonstrated a partial

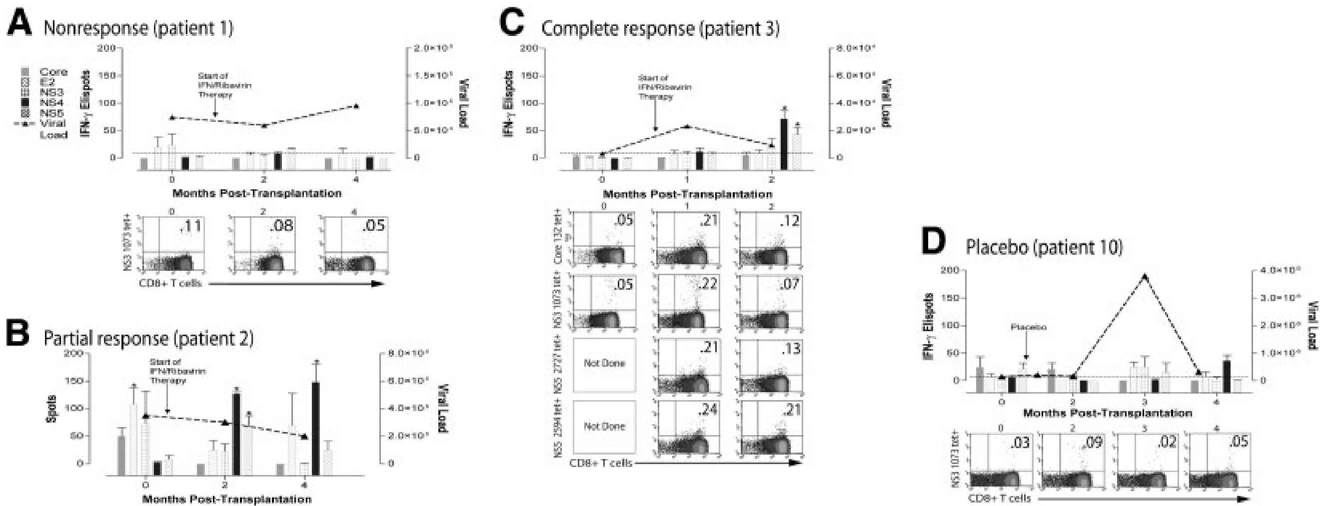

Fig. 1. Kinetics of CD4$^+$ and CD8$^+$ T-cell responses in liver transplant recipients treated with preemptive interferon alpha-2b and ribavirin and followed longitudinally. CD4$^+$ T cells at each time point were stimulated with a panel of purified recombinant HCV proteins and quantitated by IFN-γ ELISPOT; responses are indicated as number of ELISPOTS per 2.5×10^5 CD4$^+$ others (in triplicate, SEM) in the presence of HCV antigen (core, E2, NS3, NS4, or NS5) minus negative control (usually superoxide dismutase [SOD] fusion protein). Dotted lines correspond to 10 spots; *i.e.*, effector frequency of 1 of 25,000. CD8$^+$ T cells were quantitated by soluble HLA-A2–restricted tetramers (see Patients and Methods). Four representative response patterns are shown: (A) Nonresponse to antiviral therapy. Patient 1 with nonresponse to antiviral therapy. CD4$^+$ T-cell responses remained low and insignificant throughout follow-up. CD8$^+$ T cells specifically directed against NS3 1073 decreased from pretransplantation to posttransplantation time points (recipient was HLA-A2 positive, donor was HLA-A2 negative). (B) Partial Response. Patient 2 with partial virologic response to antiviral therapy. CD4$^+$ T-cell responses directed against NS4 and NS5 increased after transplantation, and viral load decreased in the serum but did not become negative. CD8$^+$ T cells were not analyzed because patient was HLA-A2 negative. (C) Complete Response. Patient 3 with complete virologic response to antiviral therapy showing T-cell reconstitution of HCV-specific immunity. NS3, NS4, and NS5 responses were greater at 2 months after transplantation than before transplantation. CD8$^+$ T-cell responses to three soluble class I tetramers are shown (patient was HLA-A2 positive; donor was HLA-A2 negative). (D) Placebo. Patient 10, assigned to receive placebo, showed low-level CD4$^+$ and CD8$^+$ T-cell responses (patient was HLA-A2 positive, donor was HLA-A2 positive). Panel C is reprinted from Clin Liver Dis, 7, Rosen HR, Hepatitis C virus in the human liver transplantation model, 107-125, copyright 2003, with permission from Elsevier.

antiviral response (*i.e.*, drop in HCV RNA without achieving serum negativity), there was emergence of statistically significant NS4-specific CD4$^+$ T-cell responses at months 2 and 4 after liver transplantation (Fig. 1B). The single patient (#3) who had a complete virologic response to preemptive antiviral therapy (HCV RNA negative in serum by month 4 after transplantation) showed emergence of statistically significant CD4$^+$ T-cell responses to nonstructural (NS) proteins; importantly, these were not detectable in the peripheral blood before transplantation. Furthermore, the emergence of HCV-specific CD8$^+$ T-cell responses 1 week after initiation of antiviral therapy heralded viral clearance in this patient (Fig. 1C). In contrast, patient 10, who was assigned to placebo and developed evidence of progressive histologic injury, failed to show CD4$^+$ T-cell responses at any point, and CD8$^+$ T-cell responses were only minimally detectable at 2 months after OLT (Fig. 1D).

Early T-Cell Reconstitution of HCV-Specific Immune Responses in Patients With Severe Recurrence Correlates With Response to Antiviral Therapy. Histological patterns of severe HCV recurrence within the allograft have been categorized into the cholestatic variant[14] that occurs in approximately 5% to 10% of patients and is associated with extremely high mortality rate and the typical allograft hepatitis pattern that may progress to allograft cirrhosis at a rate of 20% to 30% by 5 years after

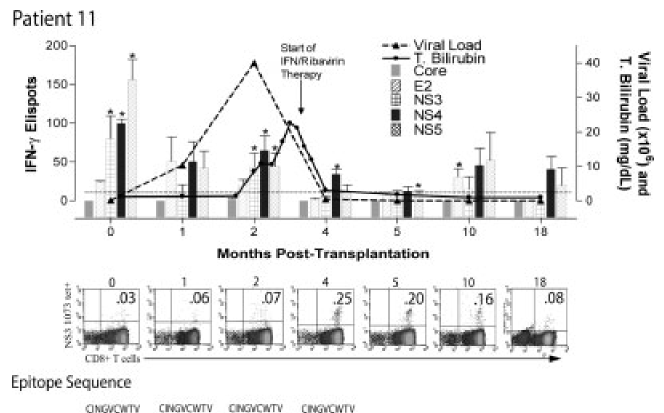

Fig. 2. Reconstitution of HCV-specific cellular immunity in a patient with severe cholestatic HCV recurrence who responded to antiviral therapy (HCV RNA expressed as 10^6 copies/mL). (Top) IFN-γ ELISPOT responses to HCV recombinant proteins, viral load and serum bilirubin. (Middle) CD8$^+$ T-cell responses to NS3 1073 tetramer. (Bottom) Amino acid sequence of NS3 1073-1081 epitope at 4 time points (HCV genotype 1a prototype sequence: CINGVCWTV). See also Supplemental Fig. 2.

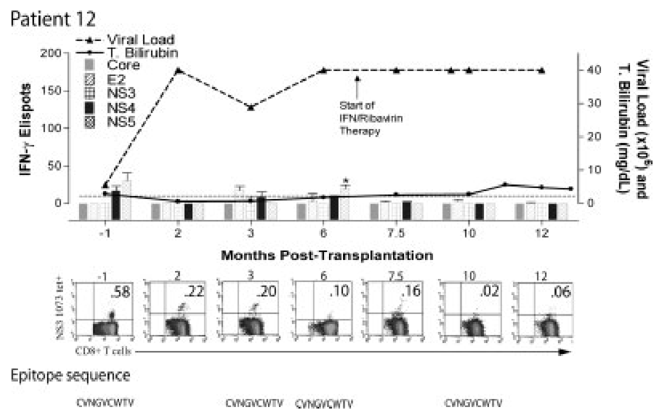

Fig. 3. HCV-specific immune responses in a patient with severe cholestatic HCV recurrence who failed to respond to antiviral therapy (HCV RNA expressed as 10^6 copies/mL). (Top) IFN-γ ELISPOT responses to HCV recombinant proteins, viral load, and serum bilirubin. (Middle) $CD8^+$ T-cell responses to NS3 1073 tetramer. (Bottom) Amino acid sequence of NS3 1073-1081 epitope at 4 time points (HCV genotype 1a prototype sequence: CINGVCWTV); amino acid substitution (V for I at position 2 was detected but remained stable over table).

transplantation.[15] Figures 2 and 3 show patients who developed cholestatic HCV recurrence within 6 months after transplantation, prompting antiviral treatment. Moreover, both patients were HLA-A2–positive recipients of HLA-A2–positive liver donors, facilitating comparison of $CD8^+$ T-cell responses. As shown in Figure 2, patient 11 had high frequencies of multispecific $CD4^+$ T-cell responses specific to HCV NS3, NS4, and NS5 antigens on the day of transplantation that decreased in the ensuing months. Levels of tetramer responses to HCV-specific peptides were low at the first 3 time points (NS3 1406 and NS5 2594 in Supplemental Fig. 2), but became detectable 3 weeks after initiation of therapy. In contrast, patient 12 failed to respond to antiviral therapy and consequently died 16 months after OLT. As shown in Fig. 3, $CD4^+$ T-cell ELISPOT responses were lacking at all but one time. NS3 1073–specific $CD8^+$ T-cell frequencies declined progressively, despite the initiation of antiviral therapy, from 0.58% the day of transplantation to 0.02% by the 10th postoperative month. We excluded the presence of viral escape mutations as a potential cause for the changes in tetramer-specific frequencies by direct sequencing of the epitope coding region at various times (see Figs. 2 and 3), in accordance with our previous findings.[13]

Figures 4 and 5 illustrate the kinetics of $CD4^+$ and $CD8^+$ T-cell responses in the additional five patients (patients 13-17) who received antiviral therapy for severe histologic recurrence. Patients 13, 14, and 15 were initiated on antiviral therapy within 6 months after liver transplantation. As shown in Fig. 4A, clearance of serum HCV RNA in patient 13 was followed by emergence of strong, multi-specific $CD4^+$ T cells 9 months after transplantation. In contrast, patient 14 (Fig. 4B) demonstrated only very weak $CD4^+$ T-cell responses and showed a drop in serum HCV RNA but never became negative. Patient 15 is of interest because he cleared serum HCV RNA after initiation of antiviral therapy; however, therapy was discontinued for 6 weeks (due to financial reasons), and the lapse in antiviral therapy was temporally associated with viral rebound and loss of $CD4^+$ T-cell responses. Reinstitution of antiviral therapy led once again to viral clearance and statistically significant restoration of NS3-specific $CD4^+$ T-cell responses.

Both patients 16 and 17 (Fig. 5) were started on therapy beyond 1 year after transplantation because of evidence of bridging fibrosis. Antiviral therapy in patient 16 was associated with a drop in viral load, but HCV RNA remained detectable in serum at 24 months, demonstrating slightly increased HCV-specific $CD4^+$ T-cell frequencies (insufficient cells at this time to screen all HCV antigens). The patient remained on combination antiviral therapy until month 47 after OLT, when he became HCV RNA negative. The patient (HLA-A2–positive recipient of HLA-A2–positive donor) had detectable $CD8^+$ T-cell responses specific to the NS3 1073 and NS3 1406 tetramers the day of transplantation (but lacked responses to the core 132 tetramer, data not shown). The NS3 1073–specific responses declined to undetectable levels and then spontaneously increased at 2 months to 0.11%, and after initiation of antiviral therapy, increased to 0.14% at 24 months and then to 0.23% of total $CD8^+$ T cells at 47 months after OLT. The NS3 1406–specific responses became undetectable after liver transplantation

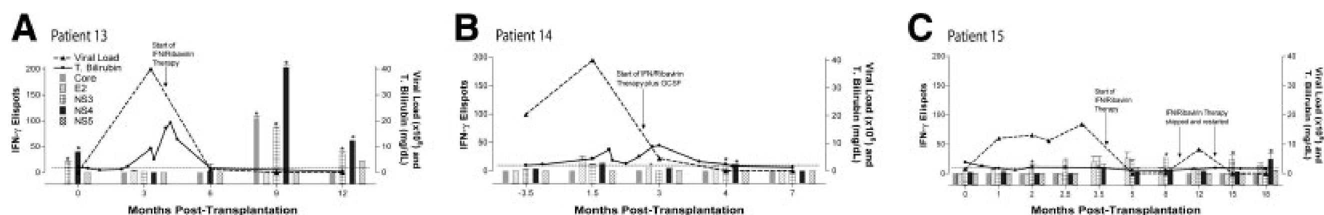

Fig. 4. Kinetics of $CD4^+$ T-cell responses, viral load, and serum bilirubin in patients who were treated within 4 months after liver transplantation for severe HCV recurrence. No $CD8^+$ T-cell data are shown because the patients were HLA-A2 negative.

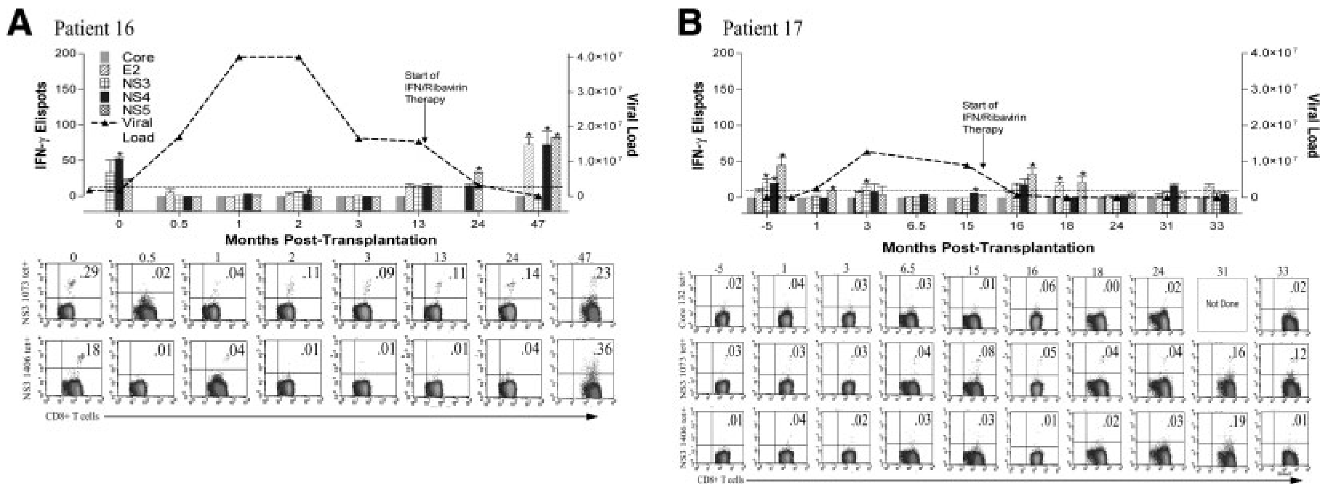

Fig. 5. Kinetics of CD4$^+$ and CD8$^+$ T-cell responses and viral load in two patients treated for severe HCV recurrence starting beyond 1 year after liver transplantation.

and did not increase with antiviral therapy until the patient became HCV RNA negative at 47 months (0.36%).

Patient 17 received 12 months of antiviral therapy and had a complete virologic response that was associated with modest, transient restoration of multispecific CD4$^+$ T-cell responses. Moreover, in this HLA-A2–positive recipient of an HLA-A2–positive donor graft, most of the CD8$^+$ T-cell tetramer responses remained below the detection level until sampling more than 6 months after cessation of therapy showed significant increases in tetramer responses specific to the NS3 1073 epitope (Fig. 5B).

Collectively, these data derived from patients who received antiviral therapy for severe histologic recurrence suggest that the temporal pattern of CD4$^+$ and CD8$^+$ T-cell responses may differ significantly. The emergence of new responses may vary in strength and specificity (*i.e.*, directed against different HCV proteins or peptides); patients who are initiated on treatment beyond 1 year may take longer to demonstrate immune reconstitution (patient 16 compared with patients 11 and 13).

Analysis of the Origin, Phenotype, and Function of HCV-Specific CD8$^+$ T Cells. The tetramer technology allows for direct visualization of Ag-specific T cells by flow cytometry without the need for *in vitro* sensitization.[16] However, tetramer analysis measures only the specificity of the T-cell receptor (TCR) and the frequency of specific T cells but does not provide information about the function of these cells. To further characterize these CD8$^+$ T cells, we sorted tetramer-positive cells and cloned them using limiting dilution and a rapid expansion protocol as described in the Supplemental Materials. At last follow-up (20 months after liver transplantation), patient 11 demonstrated circulating lymphocyte responses against four HLA-A2–restricted HCV peptides as assessed by tetramers (core 131, NS3 1073, NS3 1406, and NS5 2594; Fig. 6A), an interesting finding considering the lack of these peripheral responses the day of transplantation (Fig. 2; Supplemental Fig. 2). Because the highest frequency was observed against tetramer NS3 1406, we generated a T-cell clone specific to this region. Screening with a panel

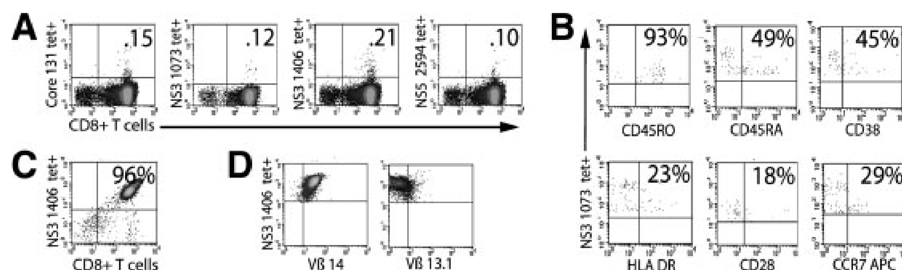

Fig. 6. Four-color flow cytometric analysis of (A) HLA-A2–restricted tetramer responses to HCV peptides core 132 (DLMGYIPLV), NS3 1073 (CINGVCWTV), NS3 1406 (KLVALGINAV), and NS5B (ALYDVVTKL) in peripheral blood of patient 11, approximately 20 months after liver transplantation. (B) Direct *ex vivo* phenotypic analysis of tetramer-gated cells. Background staining with appropriate isotype controls has been subtracted. (C) NS3 1406-specific CD8$^+$ T cells were sorted and cloned. (D) Analysis of TCR Vβ usage of cloned peripheral and intrahepatic cells after gating on live CD8$^+$ T lymphocytes demonstrated exclusive usage of TCR Vβ 14; Vβ 13.1 is shown as negative result.

of anti-human TCR Vβ antibodies confirmed expression of Vβ14 on the NS3 1406-specific clone (Fig. 6C).

To determine the origin of this HCV-specific clonotype, we examined liver-infiltrating tetramer-positive cells from the recipient explant (liver removed at time of transplantation), because of the known relative enrichment of tetramer positive cells within the liver.[17] Indeed, although tetramer-positive cells were not detectable peripherally the day of transplantation, we were able to sort and clone liver-infiltrating $CD8^+$ T cells specific to the NS3 1406 epitope. Total RNA was extracted from the peripherally reconstituted clone and the intrahepatic (pretransplantation) clone. The PCR product was identified as an in-frame TCR that used TCRBV14, TCRBJ1S2 and TCRBC1. The junctional sequence was CASSLQGNYGYT (end of VB14 is underlined and start of JB1S2 is double underlined). The CDR3 sequence was exactly the same in the peripheral reconstituted clone and the intrahepatic clone (at both the nucleotide and amino acid level); thus, the clone expanded from the peripheral blood after antiviral therapy was originally present in the explanted liver.

The cell surface phenotype of these NS3 1406–specific T cells was studied using four-color immunofluorescence (Fig. 6A and data not shown). Most of the tetramer-positive cells expressed CD45RO, consistent with a memory phenotype. The low expression of CD28 and CCR7 is thought to mark highly differentiated long-lived effector populations, according to recent data.[18–20] Direct *ex vivo* progenitors matched cloned cells in terms of markers of terminal differentiation (*i.e.*, CD28 and CCR7) but were discordant with regard to activation markers (higher expression of CD38 and HLA DR on clones, data not shown). Furthermore, mitogen stimulation of cloned cells produced IFN-γ and tumor necrosis factor-α, but very little IL-4 or IL-10, consistent with a polarized type 1 cytokine profile (data not shown).

In addition, release of IFN-γ in response to peptide-specific stimulation (a more physiological stimulus, because triggering occurs through the TCR[21]) indicated that these clones were HLA-A2 restricted and responded to the NS3 1406 peptide but not other HCV epitopes. Therefore, these analyses show that HCV-specific $CD8^+$ T cells effluxed into the peripheral blood after viral load was brought under control, were HLA-A2–restricted, terminally differentiated ($CD28^{low}CCR7^{low}$) memory cells with effector function, and clonotypically identical to cytotoxic T lymphocytes present in the explant liver the day of transplantation.

Interestingly, despite sorting into 28 wells, we were unable to generate tetramer-specific clones from patient 12 (tetramer frequency $> 0.20\%$), who did not respond to antiviral therapy and ultimately died of HCV recurrence. This finding is in keeping with the recent demonstration that HCV-specific T-cell lines derived from patients with chronic infection display impaired proliferative function when compared with T cells in patients with resolved infection.[22]

Prospective Analysis of $CD4^+$ and $CD8^+$ T-Lymphocyte Responses in Patients With Minimal Histologic HCV Recurrence. Cryopreserved PBMC from seven patients who have demonstrated minimal histologic recurrence with a minimum of 2 years' follow-up and did not receive antiviral therapy were assayed for $CD4^+$ and $CD8^+$ T-lymphocyte responses (Fig. 7). Remarkably, five of the seven patients showed vigorous multi-specific IFN-γ $CD4^+$ T-cell responses in the first 3 months after OLT regardless of HCV RNA viral loads. Patient 18 (Fig. 7A) was particularly interesting because he was the only patient in the series who demonstrated a spontaneous drop in his serum viral load after liver transplantation; this coincided with the emergence of IFN-γ–producing $CD4^+$ T cells specific to the NS4 antigen. Interestingly, NS3 1073–specific $CD8^+$ T-cell responses spontaneously increased at 2 months and then declined; NS3 1406–specific responses were detectable at 4 and 5 months (0.13% and 0.16%, respectively, data not shown). As shown with patient 20 (Fig. 7C), high viral loads do not preclude the development of an adaptive immune response after liver transplantation.

Discussion

Liver transplantation provides an important model to study HCV pathogenesis for several reasons (reviewed recently by Rosen[23]), the most important of which is that the natural history of HCV in this setting may be greatly accelerated, allowing the identification of distinct clinical outcomes (mild vs. severe recurrence) in a relatively short period. How HCV-specific memory T cells within the recipient react on acute infection of the allograft and increased viral replication remains unclear.

In this prospective analysis, we studied viral-specific $CD4^+$ and $CD8^+$ T cells in patients who had undergone liver transplantation for chronic HCV infection. Immune responses were assessed directly *ex vivo* by tetramer analysis (if the patient was HLA-A2 positive) and by production of IFN-γ, which plays a major role in host defense against HCV by enhancing both innate and adaptive immune responses.[24] Evidence suggests that virus-specific T cells secreting IFN-γ are more efficient for protection than granule protein-mediated lysis.[25] Furthermore, IFN-γ has been shown to efficiently inhibit the replication of an HCV replicon in Huh-7 cells.[26,27] Figure 8 shows the HCV-specific IFN-γ ELISPOT results for 15

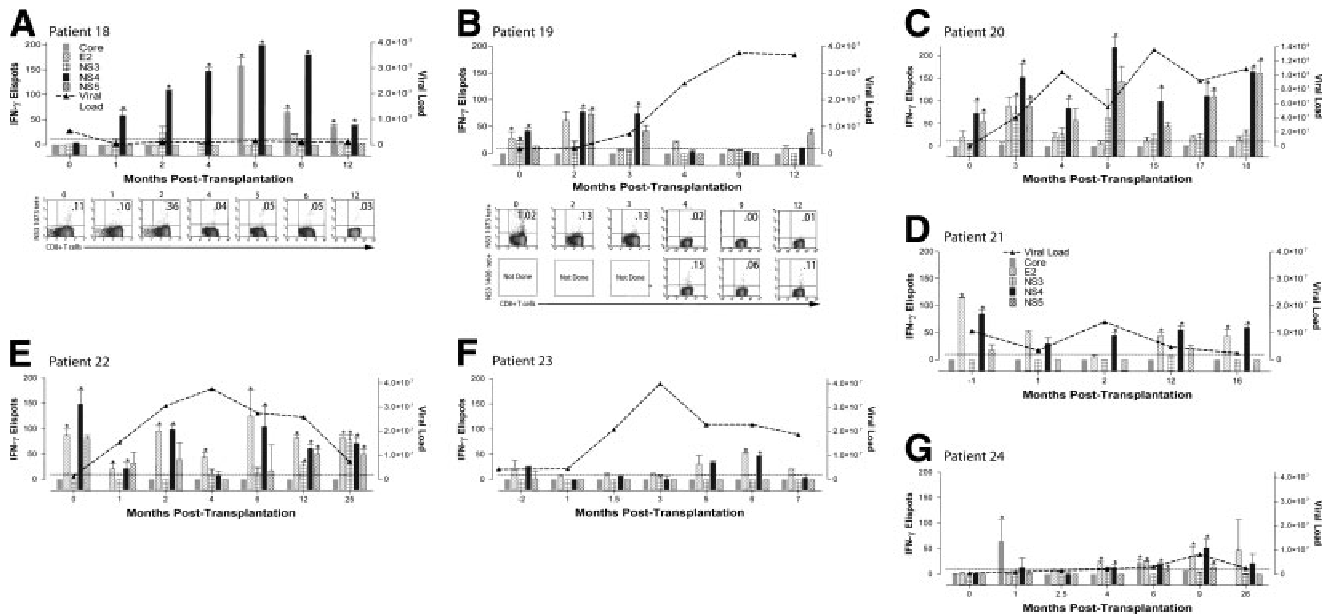

Fig. 7. Kinetics of CD4+ and CD8+ T-cell responses and viral load in patients with minimal evidence of histological HCV recurrence (range of follow-up, 2.8-4.1 years). Patients 18 and 19 were HLA-A2-positive recipients of HLA-A2–negative donor livers; patient 20 was HLA-A2 negative.

patients who developed either mild or severe recurrence (excluding patients who received preemptive antiviral therapy). Mild recurrence patients showed higher responses at 2 and 3 months after OLT than patients who subsequently developed severe recurrence; indeed, despite immunosuppression, the NS4-specific responses were higher at 3 months after OLT than the day of transplantation. Therefore, the critical factor for determining the extent of HCV-related allograft injury is likely to be too little effector immune response too late, and the absence of early CD4+ T-cell responses to HCV recombinant antigens by IFN-γ ELISPOT identifies a subset of patients more likely to develop severe recurrence.

Despite the highly variable nature of our cohort, these data have significant implications for targeted therapeutic intervention in a subset of patients before the onset of

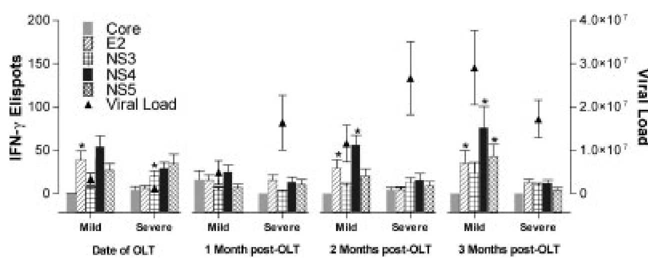

Fig. 8. Combined IFN-γ ELISPOT results (SEM) in response to 5 recombinant HCV antigens demonstrate differences between patients (n = 15) who subsequently developed mild recurrence (18-24) versus those who developed severe recurrence (10-17) (*$P < .05$ between mild and severe groups for specific antigen by two-tailed *t* test). There was no significant difference between viral loads (copies/mL) in severity groups at the different time points.

severe histologic recurrence. Moreover, it is possible these cells exert additional effector or regulatory functions that might explain why their presence correlates with graft protection but not necessarily inhibition of viral load. Interestingly, a previous cross-sectional study by Schirren et al.[28] suggested there was no correlation between the presence of an HCV-specific CD4+ T-cell response and severity of recurrence after liver transplantation. Closer comparison with our prospective results suggest that patients with mild recurrence may indeed demonstrate very weak CD4+ T-cell responses by ELISPOT when examined beyond 3 months after OLT (*e.g.*, patients 19 and 23, Fig. 7). It has been suggested that high viral loads are predictive of long-term outcome after OLT; in one series, HCV RNA level higher than 1 log mEq/mL at 3 months after OLT had a 70% sensitivity (but only 45% specificity) for predicting fibrosis score higher than 2 at 1 year.[29] Clearly, measurement of HCV RNA alone is not sufficiently robust or specific as a predictive marker and, as demonstrated in our study, patients with mild HCV recurrence may have extremely high circulating levels of HCV RNA (*e.g.*, patients 19, 20, 22, 23.

The finding of diverse and vigorous HCV-specific T-cell responses in liver transplant patients undergoing antiviral therapy is noteworthy because information on this topic is limited. A previous study showed that six HCV-positive liver transplant recipients who were sustained responders to antiviral therapy developed strong CD4+ T-cell responses to NS proteins.[30] Our results are in accord with these findings (*e.g.*, patient 13, 5 months after

starting antiviral treatment; patient 15, 4 months after antiviral therapy), but we also found that enhancement of T-cell responses during or after antiviral therapy are strikingly heterogeneous in terms of specificity, kinetics, and magnitude. For example, restoration of HCV-specific immunity was delayed in patient 16 (31 months after initiation of antiviral therapy) and very weak in patient 17 (genotype 4) despite viral clearance. This may be a consequence of variable HCV kinetics, immunosuppression, or may reflect that additional or different HCV epitopes were targeted by T cells.[12,31]

Our study provides evidence for the first time that $CD8^+$ T-cell responses emerge after liver transplantation and their presence correlates with improved histological and clinical outcomes. These results raise a number of intriguing questions, including the origin and specific function of these effector cells, as well as the nature of immunologic memory. Based on the phenotype of these T cells, a likely possibility is that they were present in the peripheral blood of these subjects before transplantation but at a frequency below the limits of detection of our assays. It is conceivable that memory cells would persist in low numbers and then rapidly expand after massive reinfection of the allograft, increased HCV replication and, presumably, enhanced display of viral peptides. Presentation of cognate peptide in the appropriate HLA context on the surface of infected hepatocytes could drive $CD8^+$ T-cell expansion, or alternatively, recipient antigen-presenting cells could cross-present HCV peptides from engulfed hepatocytes to T cells in the draining lymph nodes. In accord with the first possibility, we detected tetramer-positive responses of increased frequency after transplantation when donor and recipients were matched for a particular HLA allele (*i.e.*, A2). For example, patients 16 and 18 (HLA-A2–positive recipients of HLA-A2–positive donor livers) spontaneously demonstrated an increase in NS3 1073 tetramer responses 2 months after liver transplantation.

In summary, our study provides evidence that HCV-specific immunity correlates with improved outcome after liver transplantation. Taken together, our results may help identify patients more likely to develop severe HCV recurrence and therefore benefit from antiviral therapy (particularly important given the considerable morbidity and limited efficacy of antiviral treatment[32]), as well as provide a rationale for the future design and use of immunotherapeutic approaches.

References

1. Rehermann B, Chisari FV. Cell mediated immune response to the hepatitis C virus. Curr Top Microbiol Immunol 2000;242:299–325.
2. Laurence J. Potential roles for chemokine receptor CCR5 in the pathobiology of hepatitis C infection. Gastroenterology 2002;122:2069–2071.
3. Lechner F, Wong DK, Dunbar PR, Chapman R, Chung RT, Dobrenwend P, et al. Analysis of successful immune responses in persons infected with hepatitis C virus. J Exp Med 2000;191:1499–1512.
4. Freeman AJ, Pan Y, Harvey CE, Post JJ, Law MG, White PA, Rawlinson WD, Lloyd AR, Marinos G, Ffrench RA. The presence of an intrahepatic cytotoxic T lymphocyte response is associated with low viral load in patients with chronic hepatitis C virus infection. J Hepatol. 2003 Mar;38(3): 349-56.
5. Bassett SE, Brasky KM, Lanford RE. Analysis of hepatitis C virus-inoculated chimpanzees reveals unexpected clinical profile. J Virol 1998;72: 2589–2599.
6. Rosen HR, Martin P. Viral hepatitis in the liver transplant recipient. Infect Dis Clin North Am 2000;3:761–784.
7. Garcia-Retortillo M, Forns X, Feliu A, Moitinho E, Costa J, Navasa M, et al. Hepatitis C virus kinetics during and immediately after liver transplantation. HEPATOLOGY 2002;35:680–687.
8. Gane EJ, Portmann BC, Naoumouv NV, et al. Long-term outcome of hepatitis C infection after liver transplantation. N Eng J Med 1996;334: 821–827.
9. Rosen HR, Hinrichs DJ, Gretch DR, Koziel MJ, Chou S, Houghton M, et al. Association of multispecific CD4+ response to hepatitis C and severity of recurrence after liver transplantation. Gastroenterology 1999;117:926–932 (comments, p 1012–1014).
10. Gerlach JT, Diepolder HM, Jung MC, Gruener NH, Schraut WW, Zachoval R, et al. Recurrence of hepatitis C virus after loss of virus-specific CD4+ T-cell response in acute hepatitis C. Gastroenterology 1999;117: 933–941.
11. Batts KP, Ludwig J. Chronic hepatitis: an update on terminology and reporting. Surg Pathol 1995;19:1409–1417.
12. Wertheimer AW, Miner C, Lewinsohn DM, Sasaki AW, Kaufman E, Rosen HR. Novel CD4+ and CD8+ T-cell determinants within the NS3 protein in subjects with spontaneously resolved HCV infection. HEPATOLOGY 2003;37:577–589.
13. Rosen HR, Marousek G, Chou S. A longitudinal analysis of T-cell epitope coding regions of hepatitis C virus following liver transplantation. Transplantation 2002;27;74:209–216.
14. Gopal DV, Rosen HR. Duration of combination antiviral therapy for cholestatic HCV recurrence may need to be indefinite. Liver Transplantation 2003;9:348–353.
15. McCaughan GW, Zekry A. Pathogenesis of hepatitis C virus recurrence in the liver allograft. Liver Transpl 2002;8(10 Suppl 1):S7–S13.
16. Yee C, Riddell SR, Greenberg PD. In vivo tracking of tumor-specific T cells. Curr Opin Immunol 2001;13:141–144.
17. He XS, Rehermann B, Lopez-Labrador FX, Boisvert J, Cheung R, Mumm J, et al. Quantitative analysis of hepatitis C virus-specific CD8(+) T cells in peripheral blood and liver using peptide-MHC tetramers. Proc Natl Acad Sci U S A. 1999;96:5692–5697.
18. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memor T lymphocytes with distinct homing potentials and effector functions. Nature 1999;401:708–713.
19. Lucas M, Vargas-Cuero AL, Lauer GM, Barnes E, Willberg CB, Semmo N, Walker BD, Phillips R, Klenerman P. Pervasive influence of hepatitis C virus on the phenotype of antiviral CD8+ T cells. J Immunol. 2004 Feb 1;172:1744-53.
20. Nascimbeni M, Mizukoshi E, Bosmann M, Major ME, Mihalik K, Rice CM, et al. Kinetics of CD4+ and CD8+ memory T-cell responses during hepatitis C virus rechallenge of previously recovered chimpanzees. J Virol 2003;77:4781–4793.
21. Gruener NH, Lechner F, Jung MC, Diepolder H, Gerlach T, Lauer G, et al. Sustained dysfunction of antiviral CD8+ T lymphocytes after infection with hepatitis C virus. J Virol. 2001;75:5550–5558.
22. 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; 169:3447–3458.
23. Rosen HR. Hepatitis C virus in the human liver transplantation model. Clin Liver Dis 2003;7:107–125.
24. Su AI, Pezacki JP, Wodicka L, Brideau AD, Supekova L, Thimme R, et al. Genomic analysis of the host response to hepatitis C virus infection. PNAS 1999;24:15669–15674.
25. Guidotti L, Ishikawa T, Hobbs MV, Matzke B, Schreiber R, Chisari FV. Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. Immunity 1996;4:25–31.
26. Frese M, Schwarzle V, Barth K, Krieger N, Lohmann V, Mihm S, et al. Interferon-gamma inhibits replication of subgenomic and genomic hepatitis C virus RNAs. HEPATOLOGY 2002;35:694–703.
27. Liu C, Zhu H, Tu Z, Xu YL, Nelson DR. CD8+ T-cell interaction with HCV replicon cells: evidence for both cytokine- and cell-mediated antiviral activity. HEPATOLOGY 2003;37:1335–1342.
28. Schirren CA, M-C Jung, Worzfeld T, Mamin M, Baretton G, Gerlach JT, Gruener NH, et al. Hepatitis C virus-specific CD4+ T cell response after liver transplantation occurs early, is multispecific, compartmentalizes to the liver and does not correlate with recurrent disease. J of Infectious Diseases 2001; 183: 1187-94.
29. Sreekumar R, Gonzalez-Koch A, Maor-Kendler Y, Batts K, Moreno-Luna L, Poterucha J, et al. Early identification of recipients with progressive histologic recurrence of hepatitis C after liver transplantation. HEPATOLOGY 2000;32:1125–1130.
30. Schirren CA, Zachoval R, Gerlach JT, Ulsenheimer A, Gruener NH, Diepolder HM, et al. Antiviral treatment of recurrent hepatitis C infection after liver transplantation: association with a strong, multispecific, and long-lasting CD4+ T cell response with HCV elimination. J Hepatol 2003;397–404.
31. Lauer G, Ouchi K, Chung RT, Nguyen TN, Day CL, Purkis DR, et al. Comprehensive analysis of CD8+ T cell responses against hepatitis C virus reveals multiple unpredicted specificities. J Virol 2002;76:6103–6113.
32. Gane E. Treatment of recurrent hepatitis C. Liver Transpl 2002;8(10 Suppl 1):S28–S37.