Immunology/Virology Snap Shot

Sites Participating: University of Massachusetts (site #11) / University of Connecticut (site #11), Saint Louis University (site #12), University of Texas-Southwestern (site #16), University of Southern California (site #17)

Study Co-Chairs: Karen Lindsay MD and Chihiro Morishima MD

Co-Investigators: Margaret Koziel MD, Alan Rothman MD, Ranjit Ray PhD, David Gretch MD, PhD, Stephen Polyak PhD, Daniel Sullivan PhD, Ming Chang PhD, Raymond Chung MD, Adrian Di Bisceglie MD, Herbert Bonkovsky MD, Savant Mehta MD, William Lee MD, Elizabeth Wright PhD

Study Name: IMMUNOLOGIC AND VIROLOGIC CORRELATES OF LIVER FIBROSIS

Separate Consent Form: Yes

Withdrawal Form: Yes (Form #176)

Eligible Patients: Lead-In, Randomized, Breakthrough/Relapsers
(W20 Responders and Express patients are not eligible)

Visit Schedule (additional data/specimens and forms for AS)

Note: "**X**" means all participating sites take part.

Lead-In Patients

Visit Number →	Form #	S00	W00	W02	W04	W08	W12	W16	W20	W24
CTL	170	X								
Neutralizing Antibody	171		X							
Quasispecies	172									X
Lymphoproliferation	173		X							X
Replication	174	X								
Imm/Vir Aliquot Form	175	X	X							X
Withdrawal Form	176	Can be added to any visit								
HCV Quasispecies CFA	177		X							
CTL Serum Aliquot	270	X								
CTL Liver Aliquot	271	X								
LP Aliquot	273		X							X
Specimen Collection	14	X								

Visit Schedule (additional data/specimens and forms for AS)

Note: "**X**" means all participating sites take part.

Randomized Phase

Visit Number ➔	Form #	R00	M09	M12	M18	M21	M24	M27	M30	M33	M36	M42	M45	M48	M54
CTL	170						X							X	
Neutralizing Antibody	171	X		X			X				X			X	
Quasispecies	172	X		X			X				X			X	X
Lymphoproliferation	173	X		X			X				X			X	X
Replication	174						X							X	
Imm/Vir Aliquot Form	175	X		X			X				X			X	X
Withdrawal Form	176	**Can be added to any visit**													
HCV Quasispecies CFA	177														
CTL Serum Aliquot	270														
CTL Liver Aliquot	271						X							X	
LP Aliquot	273	X		X			X				X			X	X
Specimen Collection	14						X							X	

Note: Form 14 is filled out when liver tissue is collected for CTL liver and Replication testing.

Immunologic and Virologic Correlates of Liver Fibrosis Ancillary Study

Study Co-Chairs: Karen Lindsay MD and Chihiro Morishima MD

Immunology Principal Investigators: Margaret Koziel MD, Alan Rothman MD, Chihiro Morishima MD, Ranjit Ray PhD

Virology Principal Investigators: David Gretch MD, PhD, Stephen Polyak PhD, Daniel Sullivan PhD, Ming Chang, PhD, and Raymond T. Chung MD

Clinical Center and Data Coordinating Center Principal Investigators: Adrian Di Bisceglie, MD, Herbert Bonkovsky MD, Savant Mehta MD, William Lee MD, Karen Lindsay MD, Elizabeth Wright PhD

I. Scientific rationale

The pathogenesis of hepatocellular damage in chronic hepatitis C virus (HCV) is poorly understood. Although fibrosis and cirrhosis are presumed to arise out of a background of longstanding inflammation, and interferon is presumed to affect fibrosis progression by reducing the stimulus to inflammation, a number of questions exist about this process. Peripheral lymphoproliferative immune responses have been linked to recovery from acute infection, but their role in chronic disease has not been examined in longitudinal studies. HCV-specific immune responses are present in the majority of chronically infected individuals, but the relationship of the presence or absence of these responses to long-term outcome is unclear. More vigorous cytotoxic T lymphocyte (CTL) responses have been linked to better response to IFN monotherapy, and also to more advanced inflammation. Furthermore, previous studies on the role of cellular immune responses in HCV have generally examined a small number of subjects at a single time point in the absence of any detailed virologic or histologic analysis. The hypothesis to be tested in these studies is that HCV-specific immune responses protect against fibrosis and that interferon (IFN) acts to enhance these HCV-specific immune responses.

Previous studies of HCV quasispecies in humans have provided evidence that the genetic diversification of HCV *in vivo* is linked to the pathogenesis of chronic liver disease. Clinical and molecular studies suggest HCV quasispecies evolve in response to host immunologic pressures, but the HCV-specific immune responses in these patients have not been examined in detail. Such studies have also demonstrated that HCV quasispecies diversification is influenced in a significant manner by therapy with interferon (IFN), and have implicated the HCV E2 and NS5A gene products in the inherent resistance of HCV to IFN therapy. Specific hypothesis of the virologic studies are 1) that quasispecies diversity at baseline will correlate with disease progression and response to therapy; 2) disease non-progressors will have higher rates of HCV quasispecies evolution than disease progressors; 3) treated patients will have higher rates of HCV quasispecies evolution than non-treated controls; 4) and that high rates of quasispecies evolution will positively correlate with strong immune responses.

Chronic HCV infection has various outcomes, ranging from liver cirrhosis to minimal liver damage. It is generally believed that fibrosis results from long term inflammatory activity. However, the relationship between viral replication in liver, hepatic inflammation, and fibrosis progression is unknown. Preliminary studies have suggested the percentage of hepatocytes containing HCV replicative intermediate RNA, but not the percentage of hepatocytes carrying genomic RNA, correlates with the grade of inflammatory activity and stage of liver disease in the infected liver. The presence of replicative intermediate RNA indicates successful establishment of HCV replication in the human liver. Previous reports also suggest higher levels of HCV nonstructural antigens in liver biopsies with severe hepatitis. One hypothesis is that the HCV nonstructural proteins made in the HCV replication-active cells serve as antigens, which activate

cytotoxic T cells. Lower levels of nonstructural antigens have been associated with a lower percentage of cells positive for HCV replicative intermediate RNA, and less severe liver injury.

The proposed studies would have a number of potential interactions. First, there is a clear need to integrate viral quasispecies behavior with the immunologic response. For example, if baseline studies suggest a given CTL response in an individual (e.g., NS5b), this could be used to focus studies examining mutations within the quasispecies on that region, in addition to regions thought to be important in determining host immune response, such as NS5a and E2. Conversely, the knowledge that the E2 HVR is a major region of quasispecies variability will allow us to examine the specific immune response against patient isolates in detail. The importance of viral variants in modifying effector characteristics of CTL could be examined with the knowledge of the viral quasispecies. A comprehensive analysis of both the immunologic and virologic dynamics in a single group of patients is one of the most important potential benefits of the proposed studies. Analysis of the levels of HCV replication in liver tissue will integrate well with studies of the hepatic cell-mediate immune response, progression of liver damage, the grade of inflammatory activity and stage of fibrosis, as well as the viral titer and quasispecies dynamics. No other large studies exist which can coordinate such a comprehensive analysis.

Specific Aims

1. Define HCV quasispecies diversity and complexity in baseline sera.
2. Track HCV quasispecies in the major variant evolution over time in serum for 400 patients.
3. Perform nucleotide sequencing and phylogenetic analysis of E1, E2 and NS5A genetic regions over time for 100 patients.
4. Correlate the progression of liver disease in study subjects with the presence and magnitude of HCV-specific immune responses (lymphoproliferative responses, intrahepatic CTL, and neutralizing antibody) before, during and after therapy in the treated cohort and at similar time points in the observation cohort.
5. Determine if a favorable response to IFN therapy is associated with enhancement of HCV-specific immune responses during therapy.
6. Evaluate the percentage of hepatocytes containing HCV genomic and replicative intermediate RNAs in 100 patients' pre-treatment biopsies, 40 treated and 40 untreated patients' biopsies at years 1 and 2 by *in situ* hybridization.

In the context of the proposed clinical trial and associated investigations, the information from these studies will enhance our understanding of the pathogenesis of hepatocellular injury in chronic HCV, the role of HCV quasispecies in response to antiviral therapy, and the relationship between quasispecies evolution and HCV disease progression.

This ancillary study is divided into five sub-studies. These sub-studies are: the Cytotoxic Lymphocyte Study; the Lymphoproliferation Study; the Neutralizing Antibody Study; the Replication Study; and the Quasispecies Study.

II. Entry criteria

A. All Lead-In patients screened at University of Southern California, University of Massachusetts (including the University of Connecticut), University of Texas-Southwestern and Saint Louis University will be invited to enroll. If there is a need for liver biopsy in order to enter the HALT-C Trial, and there is adequate liver tissue available for analysis of intrahepatic T cells, the patient will be able to participate in all five substudies of the Immunology/Virology study. If, on the other hand, a historical biopsy will be used for the purposes of the main trial, or there is insufficient liver collection at the screening biopsy for

ancillary study use, the patient will not be able to participate in the Replication study; the patient will be able to participate in the other substudies.

B. Consent will be sought from all patients from University of Southern California, University of Massachusetts (including the University of Connecticut), University of Texas-Southwestern and Saint Louis University (goal = 300).

C. All blood and biopsies must be collected Monday to Thursday at the indicated times except for PBMCs being sent to the University of Washington and liver biopsies being shipped by courier. (See Appendix 1: Specimen Collection Overview & Appendix 2: Testing Schedule).

III. Specimen Collection and Processing

(See Appendix 1: Specimen Collection Overview)

A. Peripheral Blood Mononuclear Cells for the CTL and LP Studies

1. CTL Study

Blood for the CTL study is taken at the Screen 1 visit. Each site will collect FOUR (4) 10-mL EDTA Vacutainer® tubes (filled completely; Becton Dickinson catalog number 6480) at the Screen 1 visit. (Screen 1 should be scheduled a minimum of four weeks prior to the liver biopsy performed at the Screen 2 visit.) The blood will be shipped at room temperature by overnight delivery to the testing labs. All tubes should be labeled with PID, visit number, and collection time (24-hour time clock). Labels supplied by NERI may be used for these specimens.

Testing of the blood samples is performed at Beth Israel Deaconess Medical Center (Dr. Margaret Koziel) for patients from Site 16 University of Texas and Site 17 University of South California, and at the University of Massachusetts (Dr. Alan Rothman) for patients from Site 11 University of Massachusetts/University of Connecticut and Site 12 Saint Louis University.

University of Southern California, University of Texas-Southwestern
Specimens will be shipped to:

Margaret Koziel, M.D.
HIM, Room 216
77 Ave. Louis Pasteur
Boston, MA 02115
Phone: 617-667-0038 Fax: 617-975-5235
E-mail: mkoziel@caregroup.harvard.edu

University of Massachusetts (including University of Connecticut), Saint Louis University
Specimens will be shipped to:

Alan Rothman, MD
CIDVR Room S5-326
Univ. of Massachusetts Medical School
55 Lake Ave North
Worcester MA 01655
Phone: 508-856-4182 Fax: 508-856-4890
E-mail: alan.rothman@umassmed.edu

2. LP Study

Blood for the LP study is taken at the following visits: (W00), week 24 (W24), R00, study months 12 (M12), 24 (M24), 36 (M36), 48 (M48) and after therapy month 54 (M54). Each site will collect **THREE** (3) 8.5-mL ACD (yellow top) Vacutainer tubes (filled

completely; Becton Dickinson catalog number 364606, distributed by VWR catalog number VT4606). The blood will be shipped at room temperature by overnight delivery to the testing lab. All tubes should be labeled with patient ID, visit number, and collection time (24-hour time clock). Labels supplied by NERI may be used for these specimens. The LP shipping log must be completed prior to shipment of these specimens. It should be faxed to NERI (617-926-0144) and to the receiving lab.

Testing of the blood samples is performed at the University of Washington (Dr. Chihiro Morishima) for all sites in this study (11, 12, 16, 17).
Specimens will be shipped to:

Chihiro Morishima, M.D.
Virology Division, Room 706
HMC Research and Training Building
300 9th Avenue
Seattle, WA 98104-2499
Phone 206-341-5236 Fax 206-341-5203
E-mail: chihiro@u.washington.edu

Specimens must be shipped Monday to Wednesday to be received Tuesday to Thursday. No specimens should be shipped on a Friday or before a holiday. Notifications of shipments should be sent to Dr. Morishima by fax at 206-341-5203.

B. Liver Tissue for the CTL and Replication Studies

NOTE: For this ancillary study all biopsies must be done Monday to Wednesday (screening, years 2 and 4).

1. CTL Study Liver Processing
   a. Each week during the screening phase and subsequent years 2 and 4 biopsies, participating sites should FAX a list of upcoming liver biopsies to Dr. Koziel at 617-975-5235 and Dr. Rothman at 508-856-4182. Biopsies should be scheduled Monday to Wednesday.
   b. Drs. Rothman and Koziel will provide sterile shipping media for collection of the liver biopsy (RPMI with antibiotics, HEPES, 10% heat inactivated fetal calf serum, and 50 U/ml recombinant IL-2).
   c. Shipping media should be stored at 4^{o} C until just prior to use. This will be provided in 50 mL conicals for single use.
   d. Following the liver biopsy, a 1.0 cm biopsy specimen will be placed onto the open top of a sterile container (e.g., urine collection container) and divided immediately into two sections using a sterile scalpel. A 0.5 cm of liver tissue will be placed into shipping media in the 50 mL conical. All tubes should be labeled with PID, visit number, and collection time (24-hour time clock). Labels supplied by NERI may be used for these specimens. The CTL shipping log must be completed prior to shipment of these specimens. It should be faxed to NERI (617-926-0144) and to the receiving lab.
   e. The remainder of the specimen is used for virology studies (Replication Study) – see section III.B.3)

NOTE: The specimen without gross crush artifact should be preserved for the virology study (Replication Study). If inadequate tissue is available for this purpose, the tissue should still be processed for immunology studies.

University of Southern California, University of Texas-Southwestern
Specimens will be shipped by overnight courier to:

Margaret Koziel, MD
HIM Room 216
77 Ave. Louis Pasteur
Boston MA 02115.
Phone: 617-667-0038 Fax: 617-975-5235
E-mail: mkoziel@caregroup.harvard.edu

Specimens must be shipped Monday through Wednesday to be received Tuesday through Thursday. No specimens should be shipped on a Friday or before a holiday. Notifications of shipments should be sent to Dr. Koziel by fax at 617-975-5235. In the event that Dr. Koziel is not available the contact person is Qi He at (617) 667-0049.

University of Massachusetts (including University of Connecticut), Saint Louis University
Specimens will be shipped by overnight courier to:

Alan Rothman, MD
CIDVR Room S5-326
Univ. of Massachusetts Medical School
55 Lake Ave North
Worcester MA 01655
Phone: 508-856-4182 Fax: 508-856-4890
E-mail: alan.rothman@umassmed.edu

Specimens must be shipped Monday through Wednesday if they are being sent by courier, to be received Tuesday through Thursday. Specimens from the University of Massachusetts can be collected and shipped on Thursdays since they will not be shipped by courier. No specimens should be shipped on a Friday or before a holiday. Notifications of shipments should be sent to Dr. Rothman by fax at 508-856-4890.

1. Replication Study Liver Processing

   a. Materials:
      - Thermos bottle, any 1 quart stainless steel containing liquid N_2
      - Freezing ladle
      - 12 inch forceps
      - OCT Compound, Tissue-Tek, Inc. VWR cat # 25608-930
      - Biopsy cryomolds, VWR cat # 25608-922, labeled with patient ID aliquot label supplied by NERI. Please write in the date of collection and visit number. Should also be labeled with sample ID and sequence number 320 (label supplied by the Repository in the Immunology label packets).
      - Small zip-lock bags, labeled with patient ID, date of collection and visit number.

   b. **Please cut at least 5 mm long liver biopsy.**
      NOTE: That the specimen without gross crush artifact should be preserved for the virology study. If inadequate tissue is available for this purpose, the tissue should still be processed for immunology studies. Please do not let the specimen dry.

   c. Orient specimen in labeled Cryomold and fill with OCT. Remove any air bubbles from near specimen. ID labels supplied by NERI should be used. Please write in the date of collection and visit number. If there is room on the cryomold please add the label for sequence #320 supplied form the Repository (included in the Immunology label packets).
   d. Carefully submerge cryomold into liquid nitrogen using long forceps for 10-20

seconds. Make sure the cryomold is level when it is lowered into the liquid nitrogen.

e. Place specimen/cryomold into a labeled zip-lock bag and label with the appropriate label supplied by the Repository (sequence number 320), and place on dry ice for transport to a –80°C freezer. If the label from sequence number 320 was placed on the cryomold please place a "spare" label from the same label packet on the ziplock bag (the spare label MUST have the same Sample ID as the sequence 320 label).

NOTES:
- The above procedure cannot be carried out by or near the bedside. Please place the liver biopsy in the OCT and cryomold and transport to the appropriate lab.
- Liver enzymes will degrade HCV RNA over time, so please minimize delays.

f. Specimens should be labeled with the appropriate label supplied by the repository (sequence #320). Collection of this specimen should be recorded on form #14-specimen collection and on form #175-immunolog/virology aliquot form. Specimens should be frozen at –70°C until shipped on dry ice to the repository.

g. The Repository should ship specimens (sequence #320) in batches to:

Ming Chang, Ph.D.
Virology Division/Box 359690
Harborview Research and Training Building/Room 718
300 9th Avenue
Seattle, WA, 98104-2499
Phone: 206-341-5224 Fax: 206-341-5203

Include a specimen ID list with the specimens. Make sure the specimen ID list contains the following information – patient ID, visit number, and collection date.

Allowable days of shipping to UW are Mondays, Tuesdays and Wednesdays unless other arrangements have been made. Thursdays are acceptable as long as all parties have been notified. These specimens should be sent via Federal Express or an alternate source overnight on dry ice. There should be sufficient dry ice to last approximately 3 days in the box.

C. Serum Collection (Neutralizing Antibody and Quasispecies Studies)

1. Serum samples (10 mL blood minimum = 3 mL serum) will be collected by experienced phlebotomy personnel.
   a. Collect 10 mL of blood in a serum clotting (SST red-top) tube in accordance with approved standards. Hemolysis is not permitted
   b. Allow specimen to clot (0.5-1 hour) at room temperature. Serum must be processed within 2- 4 hours of venipuncture.
   c. Divide the serum into multiple 1.0 mL aliquots in 2.0mL labeled aliquot tubes supplied by the Repository. These specimens should be labeled with the appropriate labels: Quasispecies specimens = sequence numbers 302+ 303, neutralizing antibody specimens = sequence number 301.
   d. Collection and aliquotting of these specimens should be recorded on Form 14 and Form 175-Immunology/Virology Aliquot form.
   e. Freeze at -70^0(C).
   f. Send on DRY ICE to the central repository in regular frozen specimen shipments.

2. Quasispecies study (See Appendices 1 and 4)

   a. 2.0 ml of patient serum (2-1ml aliquots) is required at the following visits: baseline (W00), week 24 (W24), R00, and months 12 (M12), 24 (M24), 36 (M36), 48 (M48) and 54 (M54).

   b. Serum must be collected as above (III.C.1) and sent to the central repository on dry ice.

   c. Once a month, the central repository will send 2 aliquots of patient serum (sequence numbers 302 + 303) to:

      Steve Polyak, Ph.D.
      University of Washington, Box 359690
      Harborview R and T Building
      300 9th Avenue, Room 718
      Seattle, WA, 98104-2499
      Phone: 206-341-5224 Fax: 206-341-5203
      Email: polyak@u.washington.edu

      - Include a specimen ID list with the specimens. Make sure the specimen ID list contains the following information – patient ID, draw date, and visit number.
      - Specimens for the UW virology lab should be sent to the lab monthly.

      Allowable days of shipping to UW are Mondays, Tuesdays and Wednesdays unless other arrangements have been made. Thursdays are acceptable as long as all parties have been notified. These specimens should be sent via Federal Express or an alternate source overnight on dry ice. There should be sufficient dry ice to last approximately 3 days in the box.

3. Neutralizing Antibody Study (See Appendices 1 and 3)

   a. Samples for this aspect of the study should be drawn at baseline (W00), randomization (R00), and study months 12 (M12), 24 (M24), 36 (M36), and 48(M48).

   b. Serum should be collected as above in III.C.1.

   c. Samples (1.0 ml aliquots) may be mailed in dry ice to the central repository in regular frozen specimen shipments.

   d. The Repository should send samples monthly to Dr. Ray.

      Dr. Ranjit Ray
      Division of Infectious Diseases & Immunology
      Saint Louis University
      3555 Vista Av. Room 1023
      St. Louis, MO 63104.
      Phone: (314) 577-8648 Fax: (314) 771-3816
      Email: rayr@slu.edu

      NOTE: These specimens should be sent via Federal Express or an alternate source overnight on dry ice. There should be sufficient dry ice to last approximately 3 days in the box.

      Include a specimen ID list with the specimens. Make sure the specimen ID list contains the following information – patient ID, draw date, and visit number.

IV. Data Forms

The following data forms will be used for this Ancillary study:

1. Form #170 – Cytotoxic T Lymphocyte Assay: This form records the results of the Cytotoxic T Lymphocyte (CTL) assay as part of the Immunology & Virology Ancillary Study. The form is to be completed following CTL assay determination at Alan Rothman's and Margaret Koziel's labs. Data entered at the CTL laboratories.
2. Form #171 – Neutralizing Antibody Test Results: This form records the results of the Neutralizing Antibody (NA) assay as part of the Immunology & Virology Ancillary Study. This form is to be completed following Neutralizing antibody assay determination at Saint Louis University. Data entered at NERI.
3. Form #172 – HCV Quasispecies: HTA: This form records the results of the HCV Quasispecies Heteroduplex Tracking Analysis as part of the Immunology & Virology Ancillary Study. This form is to be completed following Quasispecies HTA assay determination at University of Washington. Data entered at University of Washington.
4. Form #173 – Lymphoproliferation: This form records the results of the Lymphoproliferation (LP) assay as part of the Immunology & Virology Ancillary Study. This form is to be completed following Lymphoproliferation assay determination at University of Washington. Data entered at University of Washington.
5. Form #174 – Replication: This form records results of in situ detection and quantification of HCV RNAs as part of the Immunology & Virology Ancillary Study. This form is to be completed following Replication assay determination at University of Washington. Data entered at University of Washington.
6. Form #175 – Immunology/Virology Aliquot Form: This form records the specimens collected for the Immunology & Virology Ancillary Study that are being sent to the Central Repository (BBI).This form is to be completed whenever serum for Quasispecies or Neutralizing antibody or Liver tissue for Replication is collected. Completion and data entry of this form will add these specimens to the shipping database at each clinical center. This form must be data entered at participating clinical centers prior to shipment of these specimens to the Repository.
7. Form #176 – Immunology/Virology Withdrawal: This form records T that a patient has withdrawn from the entire Immunology & Virology Ancillary Study or from one or more of the five Immunology & Virology sub-studies. This form is to be completed only when a patient is no longer going to proceed as a participant in the entire Immunology/Virology AS, or in one of the five substudies.
8. Form #177 – HCV Quasispecies: CFA: This form records the results of the HCV Quasispecies Clonal Frequency Analysis as part of the Immunology & Virology Ancillary Study. This form is to be completed following Quasispecies CFA assay determination at University of Washington. Data entered at University of Washington.
9. Form #270 – CTL Serum Aliquot Form: The CTL Serum Aliquot form documents the fresh serum that is being sent to the CTL laboratories as part of the Immunology & Virology Ancillary Study. This is to be completed for serum collected at the Screening (S00) study visit. This form is completed following processing and aliquotting of CTL serum specimens at the clinical sites. Form #270 replaced Form #501.
10. Form #271 – CTL Liver Aliquot Form: The CTL Liver Aliquot form documents the liver that is being sent to the CTL laboratories as part of the Immunology & Virology Ancillary Study. This form is to be completed when fresh liver tissue is collected for the CTL sub-study at the Screening (S00), Month 24 (M24), and Month 48 (M48) study visits. This form is completed following processing and aliquotting of CTL liver specimens at the

clinical sites. Form #271 replaced Form #501.

11. Form #273 – LP Aliquot Form: The LP Aliquot form documents that blood that is being sent to the laboratory as part of the Immunology & Virology Ancillary Study. This form is to be completed for fresh blood collected at the following study visits: Baseline (W00), Week 24 (W24), Breakthrough/Relapse Randomization Visit (R00), Month 12 (M12), Month 24 (M24), Month 36 (M36), Month 48 (M48), and Month 54 (M54) study visits. Form #273 replaced Form #502.
12. Form #501 – CTL Shipping Log: This log was replaced by Form #270 and Form #271. The CTL shipping log was completed prior to shipment of fresh liver tissue to Alan Rothman/Margaret Koziel's labs for CTL assays.
13. Form #502 – LP Shipping Log: This log was replaced by Form #273.The LP shipping log was completed prior to shipment of whole blood to University of Washington for Lymphoproliferation assays.

Appendix 1

Immunology/Virology Ancillary Studies: Specimen Collection Overview

	S01	S02	W00	W24	R00	M12	M24	M36	M48	M54	Notes
BLOOD: ♦ **Immuno-CTL** **4-10 cc purple top**	X										• **UMASS + SLU: ship O/N at Room Temp to Alan Rothman's lab** • **USC + UTSW: ship O/N Room Temp to Margaret Koziel's lab** • **Blood collection at least 4 weeks before biopsy**
BLOOD: ♦ **Immuno-LP** **3-8.5cc yellow top**			X	X	X	X	X	X	X	X	• **All centers: ship O/N Room Temp to Chihiro Morishima's lab (FAX LP shipping log: 206-341-5203)**
BLOOD: **1-10 cc SST red top tube** **• clot RT-aliquot-freeze –70^{0}C** ♦ **Quasi-species:** **2- 1.0 ml aliquots** ♦ **Immuno-NA:** **1- 1.0 aliquot**			X X	X	X X	X X	X X	X X	X X	X	• **All centers: Batch + ship specimens to Repository with all other frozen specimens** • **Complete and data enter Form #175: Immunology/Virology Aliquot Form** • **Label aliquot tubes with labels from Repository: Quasi species (sequence # 302+303) Neut Ab (sequence #301)**
LIVER TISSUE: **1cm** ♦ **Immuno-CTL:** **Fresh – 0.5 cm** **-ship overnight in media** ♦ **Replication** **-OCT – ship frozen** **-70C – Repo**		X X					X X		X X		**Immunology/CTL:** • **UMASS + SLU: ship O/N at Room Temp to Alan Rothman's lab (FAX CTL shipping log:508-856-4890)** • **USC + UTSW: ship O/N Room Temp to Margaret Koziel's lab (FAX CTL shipping log:617-975-5235)** **Replication:** • **Label tube with Repository label (sequence #320).** **Batch ship to Repository with frozen specimens**

Appendix 2

Schedule of testing

Study Visit	Liver mm*	Whole blood	Serum (# 1.0 ml aliquots)	Studies†
Screen 1		40 ml		* B-LCL
Screen 2	1.0 cm			0.5 cm –CTL, 0.5 cm – Replication
Baseline (W00)		17ml	3	LP, CFA, SEQUENCING, NA
Week 24 (W24)		17ml	2	LP, HTA
R00**		17ml	2	LP, NA, HTA
Month 12 (M12)		17 ml	3	LP, NA, HTA
Month 24 (M24)	1.0 cm	17 ml	3	0.5 cm –CTL, 0.5 cm – Replication LP, HTA, NA
Month 36 (M36)		17 ml	3	LP, NA, HTA
Month 48 (M48)	1.0 cm	17 ml	3	0.5 cm –CTL, 0.5 cm – Replication LP, NA, Seq, HTA
Post-treatment (M54)		17 ml	2	LP, Seq, HTA

* Establishment of B-LCL must be done 4 weeks prior to biopsy.
** R00 for Breakthrough/ Relapsers **only**

Legend:
B-LCL= B lymphoblastoid cell lines
LP = Lymphoproliferative cell assay
CTL= intrahepatic cytotoxic T lymphocyte assay
NA= neutralizing antibody studies
HTA = Heteroduplex Tracking Analysis
CFA = Clonal Frequency Analysis

Appendix 3

Neutralizing Antibody

Assay (Ref. Lagging et al., J. Virol., 72, 3539-3546, 1998):

A predetermined number of E1 or E2 plaque forming pseudotype virus particles (100 pfu) will be mixed with heat inactivated (56 ^{0}C for 30 minutes) test serum at serial dilutions or negative control human serum at 33 ^{0}C for 45 minutes. Virus-antibody mix will be added to cell monolayer and incubated at 33 ^{0}C for 1 h with intermittent tilting. Cells will be washed 2 times with Dulbecco's medium, an overlay of Dulbecco's medium containing 0. 8 % agarose will be added and incubated at 33 ^{0}C for 24h. An additional overlay of agar containing 0.0005% neutral red will be added after 24 h to count plaques. A reduction of 50 % E1 or E2 pseudotype plaque numbers as compared to the negative control will be considered as the neutralization titer of the test sera.

Appendix 4
HCV Quasispecies Methods

All of the methods and procedures to be used in this Ancillary Study are fully operational in the Viral Hepatitis Laboratory (VL) at the University of Washington. The VL will provide training for the other sites as needed.

Heteroduplex Mobility Analysis

The heteroduplex mobility assay is a new technique (2, 3) that we have applied for the assessment of genetic heterogeneity of HCV quasispecies (4, 5, 7). The technology relies on the principal that when two different single stranded DNA molecules are hybridized, a three dimensional conformation is formed by the double stranded DNA hybrid or heteroduplex. When the heteroduplex is run on a non-denaturing polyacrylamide gel, the mobility of the heteroduplex through the gel matrix is strongly dependent on the three dimensional conformation of the heteroduplex, which is in turn dependent on the degree of homology between the two different strands of the heteroduplex. We have shown that the extent of heteroduplex retention in the gel is proportional to the extent of sequence divergence between the two strands of the heteroduplex (4, 5, 7). Specifically, the method involves hybridization of a radiolabeled probe to unlabelled target DNA, followed by non-denaturing PAGE plus autoradiography. Probe hybridized to itself (unlabeled) serves as a marker for identification of homoduplexes. Hybrids with nucleotide changes relative to the probe displayed retarded mobility and are identified as heteroduplexes.

Clonal Frequency Analysis technique

The clonal frequency analysis technique provides a detailed assessment of the level of quasispecies complexity and genetic diversity, because a large number of individual clones are simultaneously analyzed by hybridization with a patient-specific probe (4, 5, 7). In brief, PCR products from selected time points are ligated into the TA cloning vector and individual clones are reamplified to generate clonal PCR products for heteroduplex analysis. Twenty recombinant HVR1 clones will be subjected to clonal frequency analysis.

Quasispecies complexity is determined by counting the total number of unique gel shift patterns. Quasispecies genetic diversity is determined by deriving the average heteroduplex mobility of all clones relative to the homoduplex probe control. A heteroduplex mobility ratio (HMR) is calculated by dividing the distance in millimeters (mm) from the origin of the gel to the heteroduplex by the distance in mm from the origin to the homoduplex control. In cases where both strands of the heteroduplex are clearly distinguishable, the average of the distance of each strand of the heteroduplex is used to calculate heteroduplex mobility (5). The HMRs for all variants in the population are averaged to provide the final HMR value. To estimate the percent genetic change within the HVR1 between 2 time points, the percent change in HMR is calculated as:

$$(HMR_{time2} - HMR_{time1}/HMR_{time1}) \times 100$$

where HMR_{time1} and HMR_{time2} represent the 2 time points at which serum is collected.

Heteroduplex Tracking Analysis technique

In a related technique, termed the heteroduplex tracking assay (HTA), a radiolabeled probe is hybridized to unlabelled target DNA, and analyzed by non-denaturing PAGE plus autoradiography. In this case the target DNA consists of heterogeneous PCR product. We have developed software that generates an HMR for the entire population, based on calculating weighted pixel intensities of heteroduplexes.

Sequencing and Phylogenetic Analysis

Sequencing will be performed as described (1, 4, 6). We will perform direct sequencing on the potential immunological epitopes identified in the Immunology Ancillary Study and E2 and NS5A genetic regions at baseline and 4 years (M48) for 40 patients. Sequences will be imported into MacVector, and nucleotide sequences will be optimally aligned using the CLUSTALV program. Phylogenetic analysis will be performed using programs from version 3.5c of the PHYLIP package. Nucleotide distance will be estimated by generating a distance matrix based on all pairwise comparisons of sequences using the generalized maximum likelihood method in the DNADIST program. Neighbor-joining using the NEIGHBOR program is then used to construct a phylogenetic tree from the distance matrix. Additionally trees will be constructed using nucleotide sequences directly to generate maximum-likelihood tree based on a random model of molecular evolution. The topologies of the maximum-likelihood and neighbor-joining procedure generated trees will be compared. Confidence limits of the neighbor-joining trees will be estimated using the method of bootstrap resampling (500 replications) using the program SEQBOOT.

Data analysis

All data collection will be performed in a blinded fashion. Quantitative data will be subjected to statistical analysis using appropriate tests. Numerous comparisons will be possible.

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Appendix 5
Lymphoproliferative Assay

Quadruplicate wells of 100,000 freshly isolated PBMC will be cultured with each of the following yeast-derived superoxide dismutase (SOD)-HCV fusion proteins (> 90% purity, a gift of Dr. Michael Houghton, Chiron Corp.) at a concentration of 10 mcg/mL at 37°C, 5% CO2: SOD-c22 (HCV aa 2-120), SOD-c100 (HCV aa 1569-1931), SOD-c200 (HCV aa 1192-1931), and SOD-NS5 (HCV aa 2054-2995). Eight replicate wells will be used to determine responses to negative controls rhSOD and media only. Lymphoproliferative responses to SOD-c33c (HCV aa 1192-1457), and total crude E.coli extract (control for SOD-c33c, made in E. coli) will also be tested. Responses to positive control PHA (5 μg/mL, Sigma, St. Louis, MO) and recall antigens tetanus toxoid (12 Lf/mL, Wyeth-Ayerst Laboratories, Marietta, PA) and Candida (20 mcg/mL, Greer Laboratories, Lenoir, NC) will also be tested in triplicate and quadruplicate, respectively. On day 5, 90 microliters of culture supernatant will be removed and frozen at –20°C for future cytokine analysis. Subsequently, 0.5 microcuries/well of ^{3}H-thymidine (NEN Life Science Products, Boston, MA) will be added for 16-18 hours. ^{3}H-thymidine incorporation into DNA will be measured and stimulation index (SI) calculated (cpm in the presence of antigen ÷ cpm with no antigen). The SI for each of the antigens tested will be entered into the DMS.

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Appendix 6
Determination of intrahepatic HCV-specific CTL responses (Drs. Koziel and Rothman)

The hypothesis to be tested in these studies is that the presence of a CTL response is associated a slower progression of fibrosis, and that treatment will increase the CTL response and therefore slow fibrosis progression. This hypothesis will be tested by analyzing the recognition of HCV protein-expressing autologous target cells by short-term cultured intrahepatic T lymphocytes before, during and after therapy in the treated cohort and at similar time points in the observation cohort. If the hypothesis is correct, then the CTL response among individuals with a slower rate of fibrosis progression should be quantitatively and/or qualitatively distinct from those with more rapid fibrosis progression.

Generation of EBV-transformed B lymphoblastoid cell lines (B-LCL): Autologous B-LCL will be prepared from PBMC obtained at enrollment from all subjects in the HALT-C core laboratory according to standard techniques. Preparation of B-LCL will be initiated at least 30 days prior to liver biopsy, and used for as target cells in a standard cytotoxicity assay.

Isolation of intrahepatic HCV-specific T lymphocytes: Hepatic tissue will be shipped by overnight courier as described above. Lymphocytes will be obtained from the liver biopsy as described (1). In brief, CD8+ T cells are expanded from a 5 mm section of liver biopsy using the anti-CD3 monoclonal antibody at 0.1 μg/ml. When added to lymphocytes in the presence of IL-2, this antibody results in a polyclonal expansion of all CD3-bearing lymphocytes. When the cells have reached confluence in a 24 well plate, this culture is restimulated with allogeneic feeder cells, IL-2, and the CD3-specific monoclonal antibody 12F6 as a non-specific stimulus to T cell proliferation as previously described (2). 10-14 days after restimulation, bulk cell lines will be tested for HCV-specific cytolytic activity.

Screening of developing cell lines and clones for HCV-specific CTL: Developing cell lines and clones will be screened for HCV-specific cytolytic function using autologous EBV-immortalized B cell targets infected with recombinant HCV-vaccinia viruses expressing structural and non-structural proteins (vv9A/HCV:1-339 or vv1H/HCV:347-906, obtained from Dr. M. Houghton, Chiron Corp., or vvHCV:827-3011, obtained from Dr. Charles Rice, Washington University), as well as a control vv containing only the E. coli ß-galactosidase gene (vv-Lac/vSC8), as previously described by Dr. Koziel (2). Target cells will be autologous B-lymphoblastoid cell lines, which are readily established in >98% of patients. Briefly, B-LCL in log phase growth are infected with either recombinant vaccinia virus-HCV vectors or the control vv at a multiplicity of infection of 1-10 PFU/cell, incubated overnight at 37°C in 5% CO_2, and labeled with $Na_2{}^{51}CrO^4$. ^{51}Cr labeled target cells are washed then three times and utilized in a standard ^{51}Cr release assay as described (2). Effector cells (liver-derived T cell lines) will be washed, counted, and added to wells at E/T ratios of 100, 50, and 25:1 in 100 μl/well. The percent cytotoxicity is determined by the formula % specific lysis = {(effector cpm – spontaneous cpm) / (maximal cpm – spontaneous cpm)} x 100. Assays are excluded from tabulation if the average viability following ^{51}Cr labeling is <70% or if the average spontaneous release is >30%. A cell line will be scored as either (+) or (-) for the purposes of analysis if the % specific lysis is >15% at E/T of 100:1, >10% at E/T of 50:1, and % specific lysis of HCV expressing targets cells is at least 10% higher than lysis of target cells infected with vv-Lac at E/T of 100:1.

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Appendix 7
Replication Assay

1. Intrahepatic positive strand HCV RNA is quantified by the Roche HCV Monitor assay. Liver biopsies are freshly snap frozen with OCT compound in liquid nitrogen. Two 6- micron sections are placed in a known volume of RNA extraction solution. A serial section is stained with Hemotoxylin and Eosin. The area of tissue is measured by image analysis on the H&E stained section. The volume of liver tissue is calculated and used as the denominator to calculate the amount of genomic HCV RNA in the tissue.

2. The intrahepatic HCV replicative intermediate RNA, or negative-strand RNA, is quantified with strand-strand *in situ* hybridization followed by image analysis (1). In brief, 6-micron sections are placed on positive-charged slides. The tissue is probed with digoxigenin-labeled HCV sense riboprobes. After stringent washing, HCV signals are detected by adding anti-digoxigenin conjugated alkaline phosphatase and its substrate, Vector Red. After the *in situ* hybridization procedure, sections are scanned and the rhodamine images are collected using a fluorescent microscope with a digital camera (DeltaVision system). This system is able to measure the intensities of HCV-specific signals and calculate the average sum of adjusted integrated intensities of positive signals per cubic millimeter of tissue. Huh7-hcvAS cells will be used as a reference to convert the intensity unit to the commonly used IU (international unit). The intrahepatic HCV negative-strand RNA for each specimen will be reported as IU per milliliter of liver tissue (2).

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