

# Cellular immune selection with hepatitis C virus persistence in humans

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**Hepatitis C virus (HCV) infection frequently persists despite substantial virus-specific cellular immune responses. To determine if immunologically driven sequence variation occurs with HCV persistence, we coordinately analyzed sequence evolution and CD8<sup>+</sup> T cell responses to epitopes covering the entire HCV polyprotein in subjects who were followed prospectively from before infection to beyond the first year. There were no substitutions in T cell epitopes for a year after infection in a subject who cleared viremia. In contrast, in subjects with persistent viremia and detectable T cell responses, we observed substitutions in 69% of T cell epitopes, and every subject had a substitution in at least one epitope. In addition, amino acid substitutions occurred 13-fold more often within than outside T cell epitopes ( $P < 0.001$ , range 5–38). T lymphocyte recognition of 8 of 10 mutant peptides was markedly reduced compared with the initial sequence, indicating viral escape. Of 16 nonenvelope substitutions that occurred outside of known T cell epitopes, 8 represented conversion to consensus ( $P = 0.015$ ). These findings reveal two distinct mechanisms of sequence evolution involved in HCV persistence: viral escape from CD8<sup>+</sup> T cell responses and optimization of replicative capacity.**

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Abbreviations used: E1, envelope glycoprotein 1; E2, envelope glycoprotein 2; HCV, hepatitis C virus; HVR1, hypervariable region 1; SFC, spot-forming colony.

The World Health Organization estimates there are 170 million persons with hepatitis C virus (HCV) infection worldwide, and an estimated 4 million persons are infected with HCV in the United States (1, 2). In most countries, HCV infection is found in 1–2% of the general population and may cause cirrhosis or hepatocellular cancer, but only when infection persists (3–7).

Patients in the acute phase of HCV infection are much more likely to respond to therapy that is designed to eradicate the virus than are patients after progression to chronicity (8–10). The features unique to acute infection that allow increased responsiveness to interferon therapy remain unknown. Spontaneous clearance of HCV infection occurs in ~20% of acutely infected individuals and is associated with a broadly specific and vigorous cellular immune response (11–14). Although the cellular immune response is often less vigorous and more narrowly directed in those who fail to clear the infection, nonetheless, a cellular immune response often is present in early infection and may persist into chronic infection. Why the

early response fails to control viremia in those who progress to chronic infection is not clear, but responses generated in acute infection have been shown to decline in some subjects who remained persistently infected, and chronic infection is characterized by low frequencies of CD8<sup>+</sup> T cells in peripheral blood (13, 15–20). Although the liver has the potential to delete antigen-specific T cell responses, HCV-specific CD8<sup>+</sup> CTL lines have been generated from the liver of chronically infected humans and chimpanzees; this suggests that elimination of HCV-specific lymphocytes from the liver is neither universal nor necessary for HCV persistence (21–24). Survival of HCV, despite virus-specific CD8<sup>+</sup> CTL, might be explained by impaired cellular effector functions (proliferation, cytokine secretion, cytolytic activity), T cell exhaustion, or dendritic cell dysfunction (16, 25–27). A final possibility is that persistence is facilitated by viral evolution over the course of infection, enabling escape by mutation of key epitopes targeted by T lymphocytes. Mutational escape from T cell responses has been noted in HIV,

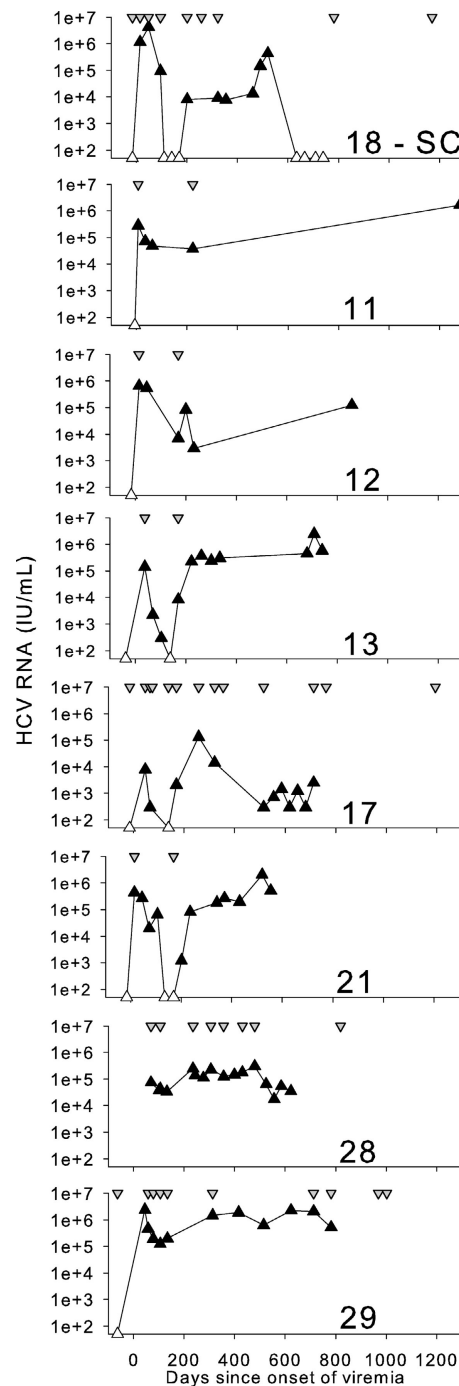
which uses an error-prone RNA polymerase similar to HCV (28).

Mathematical models of viral kinetics suggest that up to  $10^{12}$  virions are produced each day in a human with chronic hepatitis C (29). This rate exceeds comparable estimates of the production of HIV by more than an order of magnitude, and, coupled with the absence of proofreading by the HCV NS5B RNA polymerase, results in frequent mutations within the HCV genome. Mutation of class I or II MHC restricted T cell epitopes could alter the outcome of infection by preventing or delaying clearance of infected hepatocytes (30). In the face of a vigorous multispecific CTL response, mutation of several epitopes, perhaps simultaneously, would be required for survival of the virus. In HCV infection, a strong association between viral persistence and the development of escape mutations has been demonstrated in the chimpanzee model (31), and one group examined viral evolution in a single HLA-B8-restricted NS3 epitope (32); however, evidence of evasion of a multispecific CTL response in humans is lacking. Although mutations in class I MHC-restricted HCV epitopes have been observed in humans with chronic HCV infection, it is uncertain that these mutations result from CD8<sup>+</sup> T cell selection pressure or that they occur during the acute phase of infection, when clearance or persistence is determined (33–35).

Studies of the cellular immune response to acute HCV infection have been challenging because acute hepatitis C usually is clinically silent; this makes early virus isolates and CTL difficult to obtain. In addition, no consistent pattern of HCV epitope dominance has emerged in humans so large numbers of PBMCs are needed for the broad screening that is required to identify CTL responses. We have overcome these challenges to test the hypothesis that CTL-driven sequence variation occurs with progression to persistent HCV in humans. We prospectively studied HCV antibody-negative injection drug users at risk for HCV infection and compared the viral sequences at initial viremia to sequences that were obtained at multiple time points in acute HCV infection. In parallel, a genome-wide analysis of T cell responsiveness was performed. As evidence of immune selection pressure, we determined the percentage of T cell epitopes that underwent substitution, assessed the likelihood of amino acid changing substitutions to occur within versus outside T cell epitopes, and examined the effects of observed amino acid sequence changes in epitopes on T cell recognition and MHC class I binding. For amino acid substitutions outside T cell epitopes, we investigated explanations other than T cell pressure. Our results provide strong evidence in humans that immune and fitness selection occur during the acute phase of HCV infection.

## RESULTS

We assessed T cell responses using IFN- $\gamma$  ELISPOT, and sequenced half of the HCV genome in eight subjects, seven of whom progressed to chronic infection (Fig. 1). For five sub-

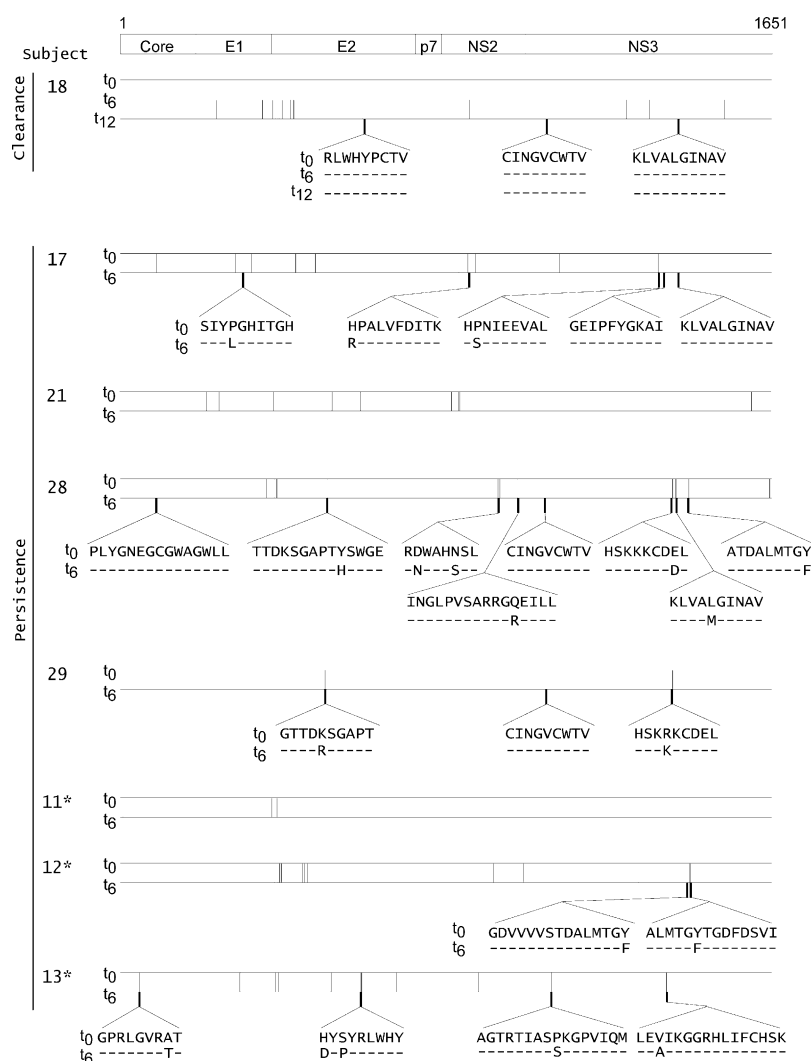


**Figure 1. Fluctuating HCV RNA level during acute infection.** Spontaneous clearance (SC) occurred in one subject, and persistence developed in seven others. A solid triangle indicates detectable HCV RNA. An open triangle indicates a HCV RNA level of less than 50 IU/ml. A gray inverted triangle indicates that IFN- $\gamma$  ELISPOT analysis of T cell responses was performed at that time point. The number in the bottom right corner of each panel is the subject number. Subject 28 entered the study antibody negative and HCV RNA positive so that the time of infection is estimated using the average time from infection to seroconversion.

jects [17, 18, 21, 28, 29], large numbers of PBMCs were available and permitted comprehensive screening by using overlapping peptides. Three subjects (11, 12, 13) with fewer PBMCs were screened for responses at all sites of amino acid replacements. T cell and viral analyses were done for all eight at initial detection of viremia and then 6 mo later. Additional assessment of T cell responses was done at the time points designated by arrows in Fig. 1. No T cell responses were detectable at initial viremia, but all of the subsequently detected T cell responses were present by 6 mo after infection. T cells that responded to peptides that were longer than 10 amino acids were confirmed as CD8<sup>+</sup> positive by bead depletion and/or intracellular cytokine staining.

### Persistence versus loss of T cell epitopes with sequence evolution

The locations of amino acid substitutions and recognized CD8<sup>+</sup> T cell epitopes within the portion of the genome that was sequenced are shown in Fig. 2. Comprehensive screening for CD8<sup>+</sup> T cell responses in subjects 17, 18, 21, 28, and 29 revealed responses in all except subject 21. The only subject (18) who cleared HCV spontaneously also was the only individual whose HCV genome had no substitutions within any recognized T cell epitope at 6 or 12 mo after initial viremia. Subjects 17, 28, and 29 remain persistently infected and had substitutions at 6 mo in 3 of 5, 6 of 8, and 2 of 3 recognized CD8<sup>+</sup> T cell epitopes, respectively. This difference



**Figure 2. Amino acid substitutions and epitopes recognized during the first 6 mo of HCV infection for five subjects.** The map at the top of the figure indicates the region of the HCV polyprotein sequenced. For each study subject, horizontal lines represent the sequences obtained at initial viremia ( $t_0$ ), 6 mo after viremia was first detected ( $t_6$ ), and 12 mo after viremia was first detected ( $t_{12}$ ). Thin vertical lines represent amino acid substitutions. Shorter, thicker vertical lines indicate recognized

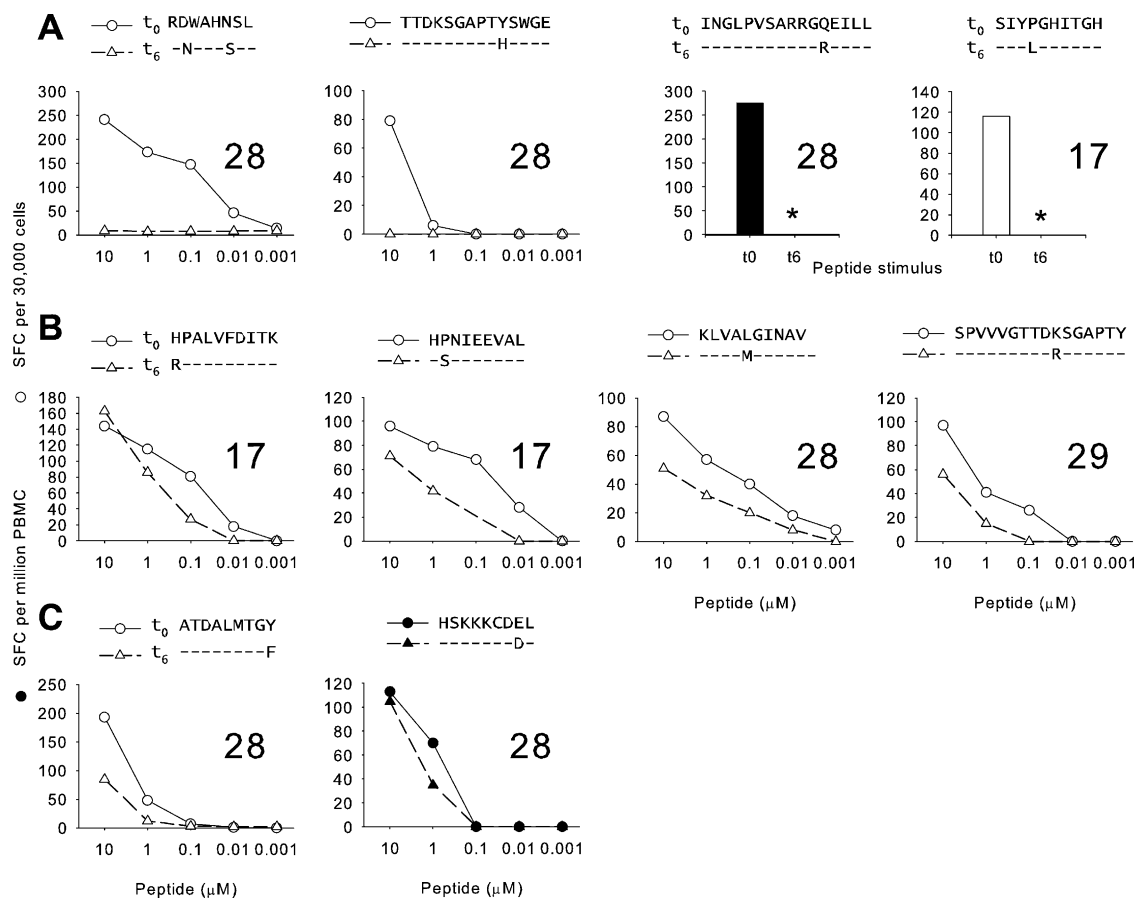
epitopes, below which the  $t_0$  and  $t_6$  sequences of the epitope are shown. Subject 29 had no detectable response to the  $t_0$  peptide HSKRKDEL, but did respond to the  $t_6$  peptide; therefore, this was not counted as an escape mutation in subsequent analyses. \*Subjects 11, 12, and 13 were not tested for IFN- $\gamma$  responses using the entire panel of 524 peptides because of limited PBMC specimens; instead, they were screened using peptides overlapping sites of amino acid replacement.

was not explained by lower levels of replication in subject 18, whose mean log<sub>10</sub> RNA level during the first 6 mo of infection (3.8) was higher than that of two subjects with epitope substitutions (17 at 2.8, and 13 at 3.4). Comparing RNA levels and the number of substitutions in all 8 subjects revealed no correlation between the number of substitutions in epitopes or overall, and the level of viremia (unpublished data). Additional sequencing at 12 mo of viremia revealed 12 amino acid replacements, none of which occurred in subject 18's three recognized epitopes. In summary, the subject who cleared infection had no substitutions in three recognized T cell epitopes at 6 mo or 12 mo after infection, whereas the three subjects with chronic viremia and T cell responses had substitutions in 60–75% of CD8<sup>+</sup> T cell epitopes by 6 mo after infection.

**Impact of amino acid substitutions on T cell recognition**

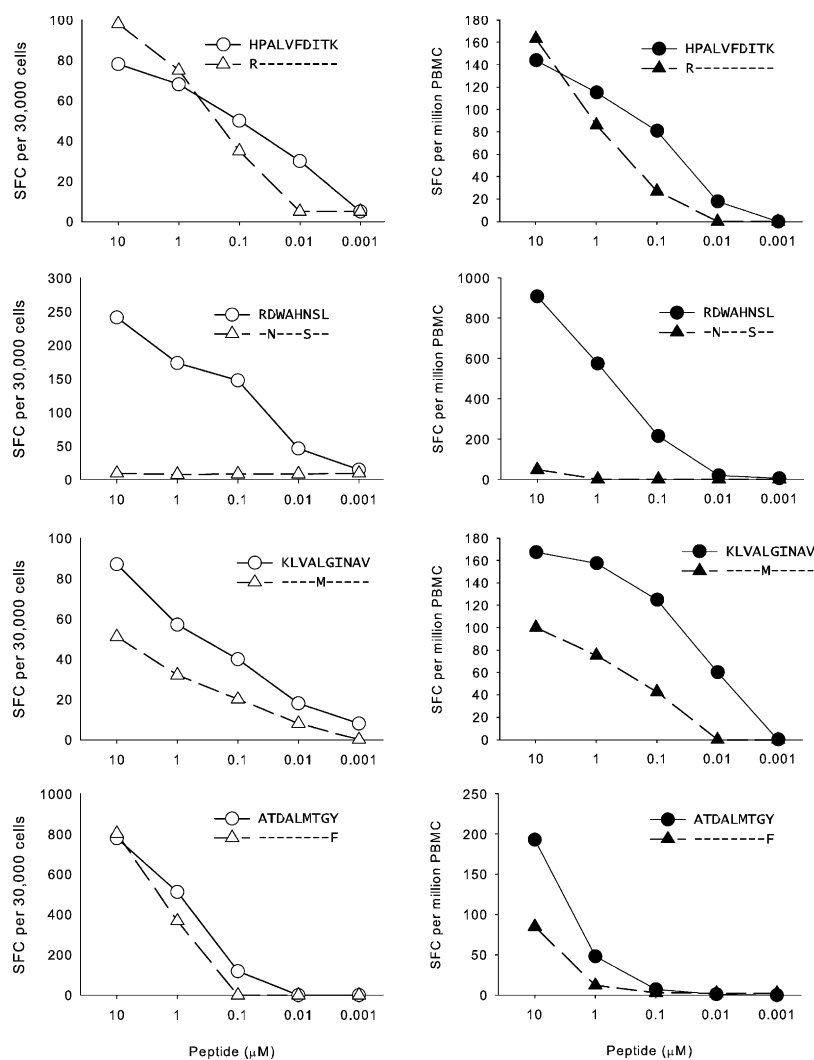
T cell lines were generated from PBMCs using synthetic peptides which represented the viral sequences that were

present at initial viremia (t<sub>0</sub>). To assess the impact of amino acid substitutions on T cell recognition, those T cell lines and bulk PBMCs that were obtained ~6 mo after initial viremia were tested for IFN-γ production in response to serial dilutions of the t<sub>0</sub> peptide or a synthetic peptide which represented the viral sequence that was present at 6 mo after initial viremia (t<sub>6</sub>). Ten t<sub>0</sub>/t<sub>6</sub> peptide pairs from subjects with persistent viremia were tested using T cell lines or PBMCs; three patterns were observed (Fig. 3). For the 10 t<sub>6</sub> peptides that were tested, we noted loss of recognition of four (Fig. 3 A), decreased recognition of four others (Fig. 3 B), and comparable recognition of two (Fig. 3 C). Therefore, for 8 of the 10 mutations in recognized epitopes that were tested, recognition by T lymphocytes was lost or significantly reduced compared with recognition of the sequence that was present at initial viremia, indicating escape. In no case was the t<sub>6</sub> variant peptide recognized better than the t<sub>0</sub> peptide. For 4 t<sub>0</sub>/t<sub>6</sub> peptide pairs that also were tested with bulk PBMCs, the patterns of recognition were similar to those observed



**Figure 3. Amino acid substitutions in epitopes reduced T cell responses.** Peptide sequences that were observed to vary between t<sub>0</sub> (circle, initial viremia) and t<sub>6</sub> (triangle, 6 mo after onset of viremia) were used as antigens in IFN-γ ELISPOT, using PBMCs (filled symbols) or T cell lines generated from PBMCs (open symbols) obtained at t<sub>6</sub> as effectors. (A) Loss of recognition: For 4 of 10 peptide pairs, recognition of the t<sub>6</sub>

variant peptide was reduced at least 20-fold at all concentrations. (B) Decreased recognition: For 4 of 10 peptide pairs, recognition of the t<sub>6</sub> variant peptide was reduced more than 2-fold but less than 20-fold at at least two concentrations of peptide. (C) Comparable recognition: For 2 of 10 pairs of peptides, recognition of the t<sub>6</sub> variant was not reduced more than twofold relative to the t<sub>0</sub> peptide at more than one concentration tested.



**Figure 4. Similar recognition patterns using lines and PBMCs.** IFN- $\gamma$  ELISPOT responses for T cell lines (open symbols) and PBMCs (closed symbols) from which the lines were generated using the  $t_0$  peptide are

using T cell lines, as shown in Fig. 4. That PBMCs and T cell lines that were generated against the  $t_0$  peptides failed to recognize the  $t_6$  peptides suggests that the substitution allows escape from the T cells that are specific for the original sequence, and that no new T cell responses were generated against the  $t_6$  sequence.

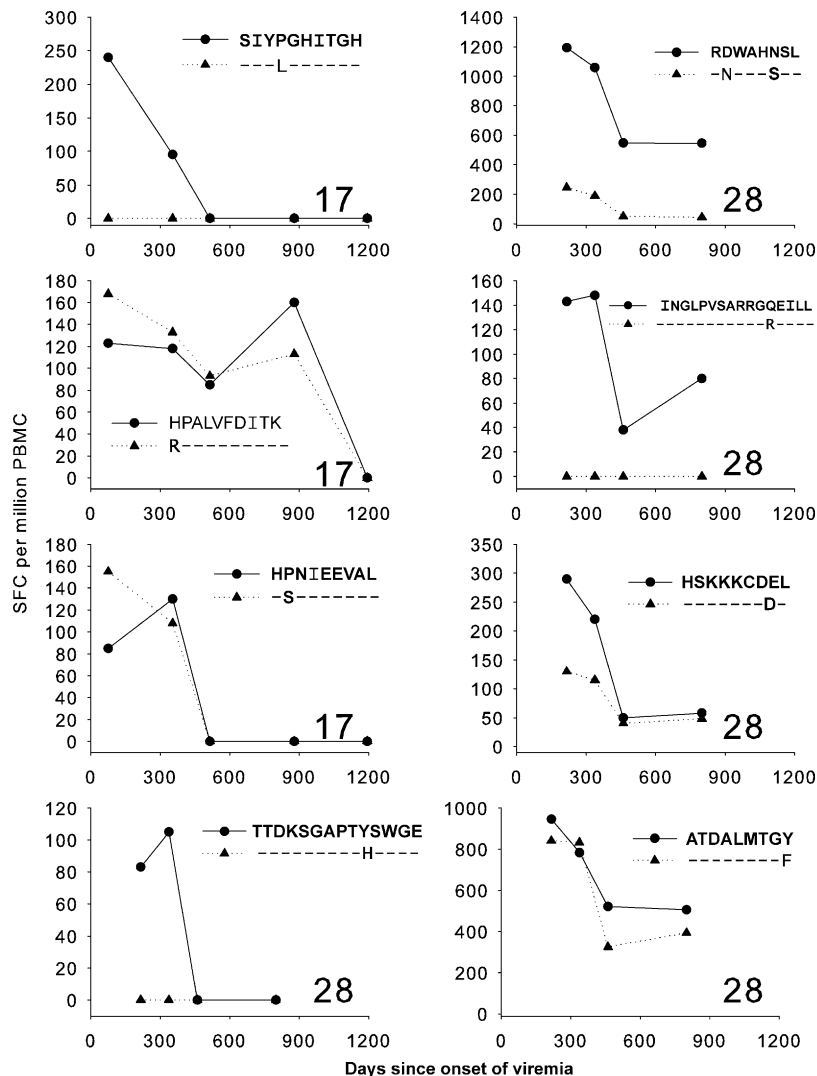
To rule out transient suppression or problems with specimen handling and the subsequent development of T cell responses to the  $t_6$  HCV sequence, IFN- $\gamma$  ELISPOT testing for recognition of the  $t_6$  peptides that demonstrated escape also was performed in subjects 17 and 28 using PBMCs that were obtained  $\sim$ 12, 18, 24, and (in subject 17) 36 mo after initial infection. The patterns of recognition persisted over time and recognition of the  $t_6$  peptides declined in parallel with the decline in recognition of the  $t_0$  peptides that occurred with prolonged infection (Fig. 5). Despite months of persistent exposure to the  $t_6$  peptides that escaped recognition at 6 mo after

shown. Responses for PBMCs and T cell lines were consistently similar aside from the expected differences in the proportion of responding cells (note difference in axes).

infection, in no case did a new T cell response specific for a  $t_6$  peptide arise in the following 6 to 36 mo.

#### Mechanisms of loss of T cell recognition with amino acid substitutions

Amino acid substitutions may result in decreased recognition through reduced HLA-binding capacity, abrogation of T cell recognition, or altered processing with failure to generate the correct sequence for presentation on the surface. We did observe marked reduction in HLA-binding capacity as one mechanism for reduced recognition in our subjects. For example, the HLA A\*0101-restricted ATDALMTGY epitope that was recognized at  $t_0$  by subject 28 had an A\*0101-binding capacity ( $IC_{50}$ ) of 0.24 nM, whereas the ATDALMTGF peptide that was recognized at  $t_6$  had a binding capacity of 64 nM. A fivefold difference in binding capacity is considered significant; thus the 267-fold decrease in binding capac-



**Figure 5. Recognition patterns persist with time.** To rule out the subsequent development of T cell responses to the  $t_6$  HCV sequence, IFN- $\gamma$  ELISPOT testing for recognition of the  $t_6$  peptides demonstrating escape also was performed for subjects 17 and 28 using PBMCs that were obtained ~12, 18, 24, and—for subject 17—36 mo after initial infection.

ity that was observed for the variant peptide indicates a markedly reduced HLA-binding capacity. However, the HLA A\*0201-restricted KLVALGINAV epitope that was recognized at  $t_0$  by subject 28 had an A0201-binding capacity of 5.0 nM, whereas the KLVAMGINAV peptide that was recognized at  $t_6$  had a binding capacity of 2.3 nM—an insignificant difference that, if anything, favors the less well-recognized peptide,  $t_6$ . This  $t_6$  peptide may stimulate less IFN- $\gamma$  production in the ELISPOT assay because of decreased T cell recognition rather than reduced HLA-binding capacity. Although 2 of the 10  $t_0/t_6$  peptide pairs were recognized comparably, we cannot rule out that they also represent escape mutations because substitutions were shown by others to result in altered processing such that the peptide is

The number in the bottom right corner of each panel is the subject number. Patterns of recognition persisted over time and recognition of the  $t_6$  peptides ( $\blacktriangle$ ) declined in parallel with the decline in recognition of the  $t_0$  peptides ( $\bullet$ ) that occurred with prolonged infection.

no longer presented on the cell surface (32, 36–38). We did not observe the Y1082F substitution that recently was reported to abrogate proteasomal processing of the HLA A\*021073–1081 epitope (38). This mode of escape is circumvented when peptides are loaded onto the surface of the cell, as in an ELISPOT assay.

#### Driving forces for sequence evolution

We next evaluated the proportion of substitutions that occurred in observed T cell epitopes and investigated explanations for substitutions outside of T cell epitopes. Of 69 substitutions that were observed in the eight subjects, 17 (25%) occurred within detected CD8<sup>+</sup> T cell epitopes, 36 (52%) occurred in envelope proteins (likely antibody targets), and

16 (23%) occurred outside of envelope regions and T cell epitopes. In the five subjects [17, 18, 21, 28, 29] with sufficient PBMCs for comprehensive IFN- $\gamma$  ELISPOT screening, amino acid substitutions were a median of 13-fold more likely to occur within T cell epitopes than outside of epitopes ( $P < 0.001$ , range 5–38). Even though at a population level the envelope proteins are highly diverse, amino acid substitutions in T cell epitopes exceeded those in envelope glycoprotein 1 (E1) and envelope glycoprotein 2 (E2) by sevenfold ( $P < 0.001$ , range 4–14), and the rate of change in epitopes was comparable to the rate in hypervariable region 1 (HVR1), which is the most diverse region of HCV (0.056 in epitopes, 0.074 in HVR1).

It is likely that some CD8<sup>+</sup> T cell responses were not detected by the methods that we applied, either because they were low-level or sequestered in the liver. Thus, we also examined amino acid substitutions with respect to potential epitopes, defined as those published (16) and restricted by HLA alleles possessed by each subject, but not detected by our methods. This resulted in the examination of 7–33 (total 155) epitopes for amino acid replacements between  $t_0$  and  $t_6$ . Four amino acid replacements were identified among these 1408 sites, yielding a rate of 0.0028 substitutions per site; this is 20-fold lower than the rate for epitopes detected in this study (0.056), and comparable to the background rate of change at nonepitope sites (0.0042). All four of these substitutions were in envelope (E1 or E2) and three were in HVR1, regions which are regarded widely as targets of humoral immunity. Taken together, these results indicate that

amino acid replacements were not common in epitopes that have been described previously, but to which we did not detect an IFN- $\gamma$  response.

Of the 16 amino acid substitutions that were observed outside of the envelope proteins or recognized T cell epitopes in these eight study subjects (Table I), 8 represented conversion to the most commonly observed residue in reference sequences (i.e., convergence to the consensus sequence). If each site had 19 equally likely alternative amino acids, the observed change to the single most commonly observed residue at 50% of sites would be extraordinarily unlikely ( $P < 0.015$ , Table II); this suggests that these changes were constrained highly. In addition, these convergent changes were not consistently conservative changes; this argues against simple structural or functional constraint. These data are consistent with an accompanying study of chronically infected individuals for whom the inoculum sequence was known (39). That study showed that substitutions that occurred in known epitopes for which the infected individual lacked the presenting HLA allele consistently were reversions toward the worldwide consensus sequence for subtype 1b.

## DISCUSSION

In this investigation of sequence variation in T cell epitopes as a potential mechanism for viral persistence, we show that amino acid substitutions during acute HCV infection are nonrandom and may be explained, in part, by escape from CD8<sup>+</sup> T cell recognition and convergence, possibly because of replicatively unfit substitutions that were selected in a pre-

**Table I.** Nonpeptide, nonenvelope changes frequently result in modal residue<sup>a</sup>

Site <sup>b</sup>	Region	Subject	Change during acute infection		Subtype 1a <sup>d</sup>	Change relative to modal residue <sup>e</sup>
			$(t_0 \rightarrow t_6)^c$			
6	core	11	<u>N</u> → <u>K</u>		K <sub>102</sub> Y <sub>2</sub>	toward
88	core	17	<u>S</u> → <u>N</u>		N <sub>128</sub>	toward
840	NS2	21	<u>S</u> → <u>G</u>		S <sub>10</sub> A <sub>1</sub>	away
856	NS2	17	<u>L</u> → <u>Q</u>		Q <sub>6</sub> H <sub>2</sub> L <sub>1</sub>	toward
859	NS2	21	<u>V</u> → <u>E</u>		V <sub>9</sub>	away
861	NS2	21	<u>V</u> → <u>I</u>		V <sub>7</sub> I <sub>1</sub> F <sub>1</sub>	away
899	NS2	17	<u>F</u> → <u>L</u>		L <sub>9</sub>	toward
908	NS2	13	<u>K</u> → <u>R</u>		K <sub>9</sub>	away
945	NS2	12	<u>T</u> → <u>A</u>		T <sub>9</sub>	away
1021	NS2	12	<u>K</u> → <u>E</u>		K <sub>9</sub>	away
1113	NS3	17	<u>T</u> → <u>A</u>		A <sub>73</sub> T <sub>1</sub> P <sub>1</sub> V <sub>1</sub>	toward
1278	NS3	18	<u>I</u> → <u>L</u>		I <sub>78</sub> S <sub>1</sub>	away
1314	NS3	12	<u>V</u> → <u>I</u>		I <sub>49</sub> F <sub>1</sub>	toward
1338	NS3	18	<u>S</u> → <u>T</u>		T <sub>46</sub>	toward
1600	NS3	21	<u>P</u> → <u>L</u>		P <sub>50</sub> L <sub>1</sub>	away
1648	NS3	28	<u>G</u> → <u>C</u>		C <sub>54</sub> Y <sub>1</sub>	toward

<sup>a</sup>Changes that were not located in demonstrated epitopes and not located in the envelope region (Fig. 2).

<sup>b</sup>Position in H77 polyprotein (GenBank/EMBL/DBJ accession no. AF009606).

<sup>c</sup>Amino acids inferred from cDNA clones obtained at  $t_0$  and  $t_6$ , respectively. Changes were only inferred when independent cDNA clone sequences were in agreement. Underlined residues match modal (most frequently observed) residue in the next column.

<sup>d</sup>Subscripts indicate number of HCV subtype 1a sequences in the Los Alamos National Laboratory database with the indicated amino acid at that position.

<sup>e</sup>Toward, change is from nonmodal residue to modal residue; away, change is from modal residue to nonmodal residue.

**Table II.** Observed versus expected<sup>a</sup> frequencies of change to modal residue at 16 sites not located in epitopes nor in envelope (E1 or E2) genes

	Expected	Observed
Change to modal residue	16 <sup>b</sup> (1/19) = 0.8 ≈ 1	8 <sup>b</sup>
Change to nonmodal residue	16 <sup>b</sup> (18/19) = 15.2 ≈ 15	8

<sup>a</sup>Based on the null hypothesis that all amino acids are equally likely at each position.  
<sup>b</sup>P = 0.015.

vious host. Significantly, there is early fixation of the T cell repertoire because we observed no instances of de novo development of T cells that recognized mutant  $t_6$  epitopes better than the original  $t_0$  epitope using lines or PBMCs.

Using the chimpanzee model of HCV infection, mutation of multiple class I MHC-restricted epitopes early in the course of chronic HCV infection was demonstrated (31). The role of CD8<sup>+</sup> CTL in control of HCV replication was reinforced further by a statistically significant increase in the number of mutations that resulted in amino acid changes in class I MHC-restricted epitopes, but not unrestricted epitopes or flanking sequences of the viral genome. These data indicate that in chimpanzees, amino acid substitutions in class I MHC-restricted epitopes are selected and possibly maintained by HCV-specific CD8<sup>+</sup> CTL populations that exert positive Darwinian selection pressure.

We also observed a statistically significant increase in the number of mutations that resulted in amino acid changes in class I MHC restricted epitopes versus portions of the viral genome outside T cell epitopes, and that amino acid substitutions in class I MHC-restricted epitopes resulted in escape from CD8<sup>+</sup> T cell recognition in acutely HCV-infected humans who progressed to chronic infection. New T cells specific for the sequences that were detected 6 mo after initial viremia were not detected, despite follow-up for as long as 3 yr after infection. Thus, selection of HCV variants that evade CD8<sup>+</sup> T cell recognition may represent a mechanism for persistence of HCV infection in humans. Supporting this, we observed no substitutions within recognized CD8<sup>+</sup> T cell epitopes in the subject who cleared infection, and substitution in 60–75% of CD8<sup>+</sup> T cell epitopes in subjects with persistent infection. The number of CTL epitopes with substitutions was shown previously to correlate with control of HIV viremia (28); however, a relationship between HCV control and maintenance of T cell epitopes had not been shown previously.

Although the observed substitutions were disproportionately contained within the portion of the HCV polyprotein in T cell epitopes, many were found outside of detectable T cell epitopes. It is possible that we missed CD8<sup>+</sup> T cell epitopes and that some of the substitutions that were observed outside of T cell epitopes actually represented substitutions within T cell epitopes. We took several steps to minimize the chances of this occurring. Where there were

substitutions and the H77 sequence that was used to make overlapping peptides as potential antigens differed from that of the subject, we tested for recognition of overlapping peptides representing autologous sequence. Where the subject's sequence matched H77 in areas of amino acid substitution, but there no responses were detected, we tested for recognition of additional overlapping peptides with different termini to minimize the possibility that we cut within a region that contained an epitope. We detected no additional epitopes via ELISPOT analysis with either method of antigen modification (unpublished data).

Because there is no clinical indication for liver biopsy in acute infection, we could only assess responses in the periphery. Therefore, it is possible that some of the substitutions may represent pressure for T cell responses present in the liver but not detectable in the periphery. We do not favor this explanation because previous studies have shown that the majority of T cell responses in the liver also are detectable in the peripheral blood (40, 41).

There are several possible alternative mechanisms for selection of these substitutions occurring outside of observed CD8<sup>+</sup> T cell epitopes. The first is that they represent substitutions in CD4<sup>+</sup> T cell epitopes. Although we detect a few CD4<sup>+</sup> T cell epitopes using our ELISPOT assay, the conditions of the assay preferentially detect CD8<sup>+</sup> T cell epitopes and we may fail to detect all of the possible CD4<sup>+</sup> T cell epitopes. The second possible explanation is that they represent substitutions in B cell epitopes. Mutations in dominant antibody epitopes that are located in the HVR-1 of envelope glycoprotein 2 (E2) have been linked to persistence of HCV infection (42). B cell epitopes are predicted to occur within the envelope regions of the polyprotein and the majority of mutations outside of T cell epitopes were found in the envelope proteins for most subjects. Another possible explanation is that the mutations outside of T cell epitopes are selected because they confer a viral replication advantage. Some mutations may represent epitopes that are recognized by the previous host that revert to a more stable sequence when the new host fails to mount similar immune pressure. This may occur when the new host lacks the MHC allele that is required to present that epitope. Loss of escape mutations upon passage of simian immunodeficiency virus to new animals (43) and HIV to humans (44), that do not exert immune pressure on that region was described recently, with the inference that escape from CTL responses may reduce viral fitness.

The relevance of those studies to human infection with HCV is supported by a recent study of one epitope in an acutely HCV infected patient (32), and our accompanying study of chronically infected individuals (39). The former study supports the nonrandom nature of reversion at a site of previous immune escape. The latter study shows that HCV amino acid sequence tends to revert to consensus in areas outside of T cell epitopes in subjects who have persistent infection. The consensus sequence likely represents a more replicatively fit state than the initial infecting strains, which pre-



sumably have adapted to the immune response of the previous host. Taken together, these results reveal at least two types of sequence variation that occur simultaneously in progression of acute HCV to persistence: immune pressure that selects T cell escape variants, and reversion to consensus sequence that is likely to result in enhanced replicative fitness.

Despite viral mutation that results in the production of new potential antigens, no new T cell responses developed in response to mutant peptides that escaped initial recognition over months, and in some cases years, after the appearance of the mutation. This phenomenon also has been observed frequently with HIV sequence evolution where the failure to prime new responses may be due to impaired CD4<sup>+</sup> T cell function. Although overall CD4<sup>+</sup> T cell function is intact in HCV infection, chronic HCV infection has been linked to loss of HCV-specific CD4<sup>+</sup> T cell responses (45). In addition, HCV has been linked to impaired DC function, decreased IFN regulatory factor 3 signaling, and protein kinase RNA-activated inhibition, which may inhibit priming of an immune response to the mutated peptides (26, 27, 46, 47). However, the failure to prime responses to the newly generated sequences is observed even in HIV infected individuals with relatively high CD4 counts, and there is no evidence in those with HCV infection of impaired priming of immune responses to other antigens, as would be evident by global immunosuppression. An alternative explanation is that original antigenic sin (the higher threshold required for stimulation of an immune response to an epitope resembling a previously recognized epitope [48]) may be responsible for the lack of response to mutant sequences seen in HIV and HCV, although this phenomenon has not been demonstrated in humans. Lastly, because the selective pressures of the immune system favor the emergence of a viral sequence that fails to elicit a productive response, we may be observing sequences that cannot be processed effectively for presentation or that resemble self-antigens, and therefore, are incapable of stimulating an immune response.

This study suggests a mechanistic linkage between viral sequence variation and progression to chronicity. The arrested development of new T cell responses, despite ongoing viremia with sequence evolution, distinguishes the acute and chronic phases of HCV infection. Enhanced understanding of cellular immune failure that leads to chronic HCV infection could accelerate development of vaccines to prevent viral persistence, as was suggested elsewhere (39).

## MATERIALS AND METHODS

### Participants

The Risk Evaluation Assessment of Community Health prospective study of young injection drug users in Baltimore examined the incidence and risk factors for HCV infection, as described previously (49). Participants who were eligible for the study were anti-HCV antibody negative, between 15 and 30 years of age, and acknowledged use of injection drugs. Participants were invited to co-enroll in a substudy of acute hepatitis C; those who consented had blood drawn for isolation of serum, plasma, and PBMCs in a protocol that was designed for monthly follow-up. At each visit, participants were provided counseling to reduce the risks of drug use. The Risk

**Table III.** HLA alleles of the study subjects

Subject	HLA A	HLA B	HLA C
11	A*0202, A*7401	B*3524, B*5703	C*0401, C*0701
12	A*3303, A*3601	B*1503, B*5301	C*0202, C*0401
13	A*3001, A*7401	B*3501, B*4201	C*1601, C*1701
17	A*0201, A*0301	B*3503, B*5101	C*0401, C*1402
18	A*0201, A*0201	B*5501, B*4403	C*0303, C*0401
21	A*3303, A*6601	B*3501, B*5801	C*0302, C*0401
28	A*0201, A*0101	B*0801, B*3701	C*0701, C*0602
29	A*0101, A*0201	B*0801, B*1302	C*0701, C*0602

Evaluation Assessment of Community Health protocol and the HCV substudy protocols were approved by the institutional review boards of the Johns Hopkins Schools of Medicine and Hygiene and Public Health.

From 1997 to 2002, 179 participants were enrolled and 62 (34.6%) developed anti-HCV antibodies (seroconverted). Beginning in November 2001, acutely infected persons were assessed for donation of  $\sim 10^8$ – $10^{10}$  PBMCs by blood donation or apheresis. HCV-specific lymphocyte responses and sequences were studied in detail in 5 acutely infected persons who were assessed frequently during the 6-mo period after infection and from whom large volumes of PBMCs were obtained. HCV sequencing and limited analysis of the cellular response were performed using three additional subjects for whom smaller numbers of cells were available, as described below. All of the subjects were infected with subtype 1a virus except for subject 11, who was infected with a subtype 1b virus. The class I HLA types for each subject are shown in Table III.

### HCV testing protocol

Serum or plasma, stored at  $-80^{\circ}\text{C}$ , from monthly follow-up visits was tested for the presence of HCV-specific antibodies using the commercially available Ortho version 3.0 ELISA (Ortho Clinical Diagnostics) according to manufacturer's instructions. Specimens in which antibodies were detected were retested in duplicate along with the participant's previous seronegative sample to identify seroconverters.

HCV RNA testing was performed on sera or plasma that was separated from blood within 2 h of collection and stored at  $-80^{\circ}\text{C}$ . For HCV seroconverters, HCV RNA testing was done on samples that were collected before seroconversion until a negative result was obtained, and after seroconversion to evaluate the outcome of infection (viral persistence versus clearance) by using quantitative, and if undetectable, qualitative HCV RNA tests that are described below.

### HCV RNA assays

**Qualitative.** For detecting HCV RNA, we used the COBAS AMPLICOR Hepatitis C Virus Test version 2.0 (Roche Molecular Systems). A limit of detection of 1.7 log<sub>10</sub> IU/ml at >95% detection is reported for this assay.

**Quantitative.** To determine the concentration of HCV RNA in serum, we used a quantitative RT-PCR assay (COBAS AMPLICOR HCV Monitor version 2.0; Roche Molecular Systems). This assay has a lower limit of quantitation of 2.8 log<sub>10</sub> IU/ml. When HCV RNA was not detected by using this assay, the sample was retested using the Roche qualitative test.

**HCV genotyping.** Genotype was determined by performing phylogenetic analysis on Core-E1 region sequences of HCV that was obtained from the first viremic specimen. For most specimens, sequences were obtained from cDNA clones that were generated with a long amplicon RT-PCR method that was described previously (50). For other specimens, genotype was determined by direct sequencing of RT-PCR products from the same Core-E1 region as described previously (51). Sequences were aligned using

ClustalX (52), and trimmed to equal length using BioEdit (53). The GTR+I+G analytical model (parameters available on request from the authors) was selected using the Akaike information criterion as implemented in ModelTest version 3.06 (54) and PAUP\* version 4b10 (Sinauer Associates). Phylogenetic trees were estimated using the neighbor-joining algorithm implemented in PAUP\*, and robustness of clustering was tested by using bootstrap analysis (55).

### Viral recovery

HCV clearance was defined as the presence of anti-HCV with HCV RNA undetectable by the COBAS AMPLICOR qualitative assay in serum or plasma specimens from at least two consecutive visits that were obtained at least 300 d after the initial detection of viremia. Persistence was defined as the persistent presence of anti-HCV with HCV RNA detectable by the qualitative or quantitative COBAS AMPLICOR assay in serum or plasma specimens that were obtained at least 300 d after initial viremia (56).

### Hemigenomic HCV sequencing and analysis

The 5.2-kb region from the 5' UTR to the NS3/NS4A junction was cloned from 140–280  $\mu$ L of serum or plasma as described previously (50). For each specimen, 33 clones were assigned to clonotypes by using a previously described gel shift assay (57); 2 clones that represented the modal clonotype were sequenced, with a third clone used as needed to resolve discrepancies. Sequences were assembled into contigs using Aligner (Codon-Code). Sequence data were obtained at the point of initial viremia and approximately 6 mo later. These sequences have been submitted to GenBank/EMBL/DBJ with accession nos. DQ061296–DQ061330.

### Reference sequence analysis

Reference sequence data were obtained from the HCV Sequence Database (<http://hcv.lanl.gov>). For Table 1, the amino acid sequence was inferred from cDNA sequences, which were obtained using the HCV Sequence Database's search interface. Default search parameters were used, except that: (a) only subtype 1a sequences were included, (b) recombinant sequences were excluded, (c) nonhuman sequences were excluded, and (d) the search was performed for the codon of interest based on position in the HCV polyprotein. When multiple sequences with the same "patient ID" were obtained, only the first occurrence was retained.

### Cellular immunology

#### IFN- $\gamma$ ELISPOT assay to assess HCV-specific T cell responses.

HCV-specific CD8<sup>+</sup> T cell responses were quantified by ELISPOT assay as described previously (58) with the following modifications. For subjects 17, 18, 21, 28, and 29, enough PBMCs were acquired to test for T cell recognition of 523 overlapping peptides (16–22-mer peptides overlapping by 10 amino acids) spanning the entire expressed HCV-H77 genome (genotype 1a) as well as 83 peptides that corresponded to optimal described CTL epitopes (59) in a matrix format. The peptides that were recognized in the matrix were tested individually and in at least duplicate to confirm recognition and to measure the number of spot-forming colonies (SFCs) that were produced. For three additional subjects where the number of cells was limited, responses to peptides spanning regions of sequence variation detected during sequencing, but not to the entire collection of 523 overlapping peptides, were assessed. 96-well polyvinylidene plates (Millipore) were coated with 2.5  $\mu$ g/ml recombinant human anti-IFN- $\gamma$  antibody (Endogen, Pierce Biotechnology) in PBS at 4°C overnight. Fresh or previously frozen PBMCs were added at 200,000 cells/well in 140  $\mu$ L R10 media (RPMI 1640 [Sigma-Aldrich], 10% FCS [Sigma-Aldrich], and 10 mM Hepes buffer [Sigma-Aldrich] with 2 mM glutamine and antibiotics [50 U/ml penicillin-streptomycin]). Peptides were added directly to the wells at a final concentration of 10  $\mu$ g/ml. The plates were incubated for 20 h at 37°C, 5% CO<sub>2</sub>. Plates were washed, labeled with 0.25  $\mu$ g/ml biotin-labeled anti-IFN- $\gamma$  (Endogen), and developed by incubation with streptavidin-alkaline phosphatase (Bio-Rad Laboratories) followed by incubation with 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium chloride (Bio-Rad Lab-

oratories) in Tris-buffer, pH 9.5. The reaction was stopped by washing with tap water and the plates were dried, before counting on an ELISPOT reader (Cellular Technology Ltd.). For quantitation of ex vivo responses, the assay was performed at least in duplicate and background was not more than 15 SFCs per million PBMCs. Responses were considered positive if the number of spots per well minus the background was at least 25 SFCs per million PBMCs (58). A control of pooled CMV, EBV, and influenza antigens (CEF control peptide pool) and phytohemagglutinin were used as positive controls (60). Responses to the CEF control peptide pool were quantifiable and remained relatively constant over time. Responses to phytohemagglutinin were uniformly positive.

**Intracellular cytokine staining.** To determine whether the lines generated for each epitope were CD4<sup>+</sup> or CD8<sup>+</sup> T cell lines, intracellular cytokine staining for IFN- $\gamma$  was performed as described previously (58). In brief,  $1 \times 10^6$  PBMCs were incubated with 4  $\mu$ g/ml peptide at 37°C and 5% CO<sub>2</sub> for 1 h before the addition of Monensin (1  $\mu$ L/ml; Sigma-Aldrich). The cells were incubated for an additional 5 h at 37°C and 5% CO<sub>2</sub>. PBMCs were washed and stained with surface antibodies, FITC-conjugated anti-CD8 or FITC-conjugated anti-CD4 (Becton Dickinson) at 4°C for 20 min. Following the washing, the PBMCs were fixed and permeabilized (Caltag), and the PE-conjugated anti-IFN- $\gamma$  mAb (Becton Dickinson) was added. Cells were washed and analyzed on a FACS-Calibur flow cytometer using CellQuest software (Becton Dickinson).

**Magnetic bead separation of CD8<sup>+</sup> and CD4<sup>+</sup> T cells.** To determine if the responding T cells in PBMCs were CD4<sup>+</sup> or CD8<sup>+</sup> T cells, between 3 and  $10 \times 10^7$  PBMCs were labeled with magnetic beads bearing anti-CD8 or anti-CD4 antibodies (Miltenyi Biotec) according to the manufacturer's instructions. Cells were positively selected using an Auto MACS (Miltenyi Biotec) to isolate either CD8<sup>+</sup> or CD4<sup>+</sup> cells. The ELISPOT assay was repeated using the isolated CD8<sup>+</sup> or CD4<sup>+</sup> T cells to determine if recognition of an epitope was mediated by CD8<sup>+</sup> or CD4<sup>+</sup> T cells.

**Bulk stimulation of peripheral blood mononuclear cells.** To establish CD8<sup>+</sup> T cell lines, cryopreserved or fresh PBMCs ( $4\text{--}10 \times 10^6$ ) were stimulated with 10  $\mu$ g/ml of synthetic HCV peptide and 0.5  $\mu$ g/ml of the costimulatory antibodies anti-CD28 and anti-CD49d (Becton Dickinson) in R10 media. Recombinant IL-2 (25 IU/ml) was added on day 2 and every other day thereafter. Cells were restimulated with  $25 \times 10^6$  irradiated allogeneic PBMCs and 10  $\mu$ g/ml of synthetic HCV peptide after 10 d.

#### Testing impact of amino acid substitutions on T cell recognition

To assess the impact of amino acid substitutions on T cell recognition, HCV peptide-specific T cell lines generated from PBMCs that were obtained 6 mo after initial viremia were tested for IFN- $\gamma$  production in response to serial dilutions of synthetic peptides that represented the viral sequences which were present at initial viremia ( $t_0$ ) or at 6 mo after initial viremia ( $t_6$ ). In four cases where the frequency of T cells that were specific for the HCV epitope was high, bulk PBMCs that were obtained 6 mo after initial viremia also were tested in this way. Comparison of initial and variant epitopes was performed using log<sub>10</sub> dilutions of the  $t_0$  and  $t_6$  peptides from 10  $\mu$ M to 0.001  $\mu$ M in the IFN- $\gamma$  ELISPOT assay that was described above for PBMCs, but using 30,000 T cells when T cell lines were tested. 10 peptide pairs from subjects with chronic viremia were tested, and three patterns were observed. Loss of recognition was defined as either no recognition at the highest concentration of the  $t_6$  peptide or at least 20-fold greater recognition of the  $t_0$  peptide than the  $t_6$  peptide at all concentrations. Decreased recognition was defined as >2-fold, but <20-fold fewer SFCs produced at two or more concentrations of the  $t_6$  peptide. Comparable recognition was defined as no more than a twofold difference in SFC between the  $t_0$  and  $t_6$  peptides at two or more concentrations tested.

**MHC-peptide binding assays.** EBV-transformed cell lines were used as the primary sources of HLA molecules. Cells were maintained in vitro and

HLA molecules were purified by affinity chromatography as described previously (61). Quantitative assays to measure the binding of peptides to purified class I molecules are based on the inhibition of binding of a radiolabeled standard peptide (61). In brief, 1–10 nM of radiolabeled peptide was coin-cubated at room temperature with 1  $\mu$ M to 1 nM of purified MHC in the presence of 1  $\mu$ M human  $\beta_2$ -microglobulin (Scripps Laboratories) and a cocktail of protease inhibitors. After a 2-d incubation, binding of the radiolabeled peptide to the corresponding MHC class I molecule was determined by capturing MHC/peptide complexes on Greiner Lumitrac 600 microplates (Greiner Bio-one) that were coated with the W6/32 antibody, and measuring bound cpm using the TopCount microscintillation counter (Packard Instrument Co.).

### Statistical analysis

Statistical analysis was done with the aid of SigmaStat software version 3.10 (Systat Software, Inc.). For comparing proportions, Fisher's exact (small sample size) and Chi squared (large sample size) tests were used. Differences were considered significant if *p*-values were <0.05.

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