Natural epitope variants of the hepatitis C virus impair cytotoxic T lymphocyte activity

Shuping Wang, Rico Buchli, Jennifer Schiller, Jianen Gao, Rodney S VanGundy, William H Hildebrand, David D Eckels

Shuping Wang, David D Eckels, Department of Pathology, University of Utah, Salt Lake City, UT 84112, United States
Rico Buchli, Rodney S VanGundy, Pure Protein L.L.C., Oklahoma City, OK 73104, United States
Jennifer Schiller, Jianen Gao, Department of Pediatrics, Medical College of Wisconsin, Milwaukee, WI 53226, United States
William H Hildebrand, Department of Microbiology and Immunology, Health Sciences Center, University of Oklahoma, Oklahoma City, OK 73104, United States
Author contributions: Wang S, Buchli R, VanGundy RS and Hildebrand WH performed the majority of experiments; Schiller J and Gao J assisted some parts of experiments; Eckels DD, Wang S and Buchli R wrote the manuscript.
Supported by The National Institutes of Health, No. NIH-DK-57732
Correspondence to: David D Eckels, Professor, Department of Pathology, University of Utah, Salt Lake City, UT 84112, United States. david.eckels@path.utah.edu
Telephone: +1-801-2132800 Fax: +1-801-5853670
Received: November 9, 2009 Revised: January 6, 2010
Accepted: January 13, 2010
Published online: April 28, 2010

Abstract

AIM: To understand how interactions between hepatitis C virus (HCV) and the host’s immune system might lead to viral persistence or effective elimination of HCV.

METHODS: Nucleotides 3519-3935 of the non-structural 3 (NS3) region were amplified by using reverse transcription polymerase chain reaction (PCR). PCR products of the HCV NS3 regions were integrated into a PCR® T7TOPO® TA vector and then sequenced in both directions using an automated DNA sequencer. Relative major histocompatibility complex binding levels of wild-type and variant peptides were performed by fluorescence polarization-based peptide competition assays. Peptides with wild type and variant sequences of NS3 were synthesized locally using F-moc chemistry and purified by high-performance liquid chromatography. Specific cytotoxic T lymphocytes (CTLs) clones toward HCV NS3 wild-type peptides were generated through limiting dilution cloning. The CTL clones specifically recognizing HCV NS3 wild-type peptides were tested by tetramer staining and flow cytometry. Cytolytic activity of CTL clones was measured using target cells labeled with the fluorescence enhancing ligand, DELFIA EuTDA.

RESULTS: The pattern of natural variants within three human leukocyte antigen (HLA)-A2-restricted NS3 epitopes has been examined in one patient with chronic HCV infection at 12, 28 and 63 mo post-infection. Results obtained may provide convincing evidence of immune selection pressure for all epitopes investigated. Statistical analysis of the extensive sequence variation found within these NS3 epitopes favors a Darwinian selection model of variant viruses. Mutations within the epitopes coincided with the decline of CTL responses, and peptide-binding studies suggested a significant impact of the mutation on T cell recognition rather than peptide presentation by HLA molecules. While most variants were either not recognized or elicited low responses, such could antagonize CTL responses to target cells pulsed with wild-type peptides.

CONCLUSION: Cross-recognition of CTL epitopes from wild-type and naturally-occurring HCV variants may lead to impaired immune responses and ultimately contribute to viral persistence.

© 2010 Baishideng. All rights reserved.

Key words: Epitopes; Human; T Cells; Cytotoxic; Anergy; Viral

Peer reviewer: Dr. Vicente Carreño, Fundacion Estudio Hepatitis Virales, C/Guzman el Bueno 72, Semisotano, Madrid 28015, Spain
INTRODUCTION

Hepatitis C virus (HCV) is one of the leading causes of chronic liver disease arising from persistent infection that lasts decades despite evidence of viral and cellular immune responses. Chronic infection occurs in up to 85% of patients. The mechanisms responsible for high rates of HCV persistence are unknown, but are thought to involve a complex interplay between the host immune system and viral diversity, which may lead to viral escape through the mutation of epitopes recognized as targets of the immune response. The combination of a very high rate of HCV replication, estimated at 10^{10} virions per day, and an RNA-dependent RNA polymerase that lacks proofreading ability sets the stage for Darwinian selection of variant or mutant viruses. Pressure mediated by humoral and cellular immune responses appears to be applied by all elements of the immune response including antibody-producing B-cells and both CD4+ and CD8+ T cells. Such persistence of HCV infection has been particularly associated with mutations in epitopes encompassed within the hypervariable region 1 of HCV envelope glycoprotein 2, recognized by both antibodies and CD4+ helper T cells. Studying a class II-restricted immunodominant epitope within the non-structural 3 (NS3) protein region of HCV, we have identified a highly significant variation that correlated with escape from CD8+ T cell responses. Other sequence variations in epitopes recognized by CD8+ cytotoxic T lymphocytes (CTLs) have been identified in chimpanzees and humans with chronic HCV infections.

CTLs recognize peptide fragments of cellular or viral proteins in the form of short peptides comprising 8-11 amino acids presented in association with major histocompatibility complex (MHC) class I molecules on the surface of infected cells. These peptides are usually derived from intracellular viral protein pools and associated in the lumen of the endoplasmic reticulum with MHC class I molecules, after which the MHC-peptide complex is transported to the cell surface and recognized by a specific T cell receptor (TCR) located on the surface of the CD8+ killer T cell.

Variation within a viral epitope can lead to a total or partial loss of functional recognition by CTL. Substitutions occurring at key anchor residues may alter peptide affinity for MHC class I molecules and thereby interfere with antigen presentation and effector T-cell mediated clearance of infected cells. Other variations, primarily in solvent-accessible residues, may abrogate TCR recognition altogether or alter it in such a way that critical activation signals are not transmitted to the cytotoxic T cells resulting in attenuated responses or even anergy.

Examples of attenuated responses have also been found with HIV and HBV. It is thought that by antagonizing T-cell responses to native epitopes, viruses expressing mutant epitopes might aid in the survival of infected cells producing wild-type viruses, which would otherwise be recognized and destroyed by CTL. Although parts of this issue have been examined in chronic HCV infection, unresolved questions remain, including whether naturally occurring variants antagonize CTL responses to wild-type epitopes that are found within the same host. To address this, viral sequences were examined by assessing CTL activity in three epitopes that were previously identified by Koziel et al.

Selective pressure appears to be applied by all elements of the immune response including antibody-producing B-cells and both CD4+ and CD8+ T cells. Such persistence of HCV infection has been particularly associated with mutations in epitopes encompassed within the hypervariable region 1 of HCV envelope glycoprotein 2, recognized by both antibodies and CD4+ helper T cells. Studying a class II-restricted immunodominant epitope within the non-structural 3 (NS3) protein region of HCV, we have identified a highly significant variation that correlated with escape from CD8+ T cell responses.

Other sequence variations in epitopes recognized by CD8+ cytotoxic T lymphocytes (CTLs) have been identified in chimpanzees and humans with chronic HCV infections.

CTLs recognize peptide fragments of cellular or viral proteins in the form of short peptides comprising 8-11 amino acids presented in association with major histocompatibility complex (MHC) class I molecules on the surface of infected cells. These peptides are usually derived from intracellular viral protein pools and associated in the lumen of the endoplasmic reticulum with MHC class I molecules, after which the MHC-peptide complex is transported to the cell surface and recognized by a specific T cell receptor (TCR) located on the surface of the CD8+ killer T cell.

Variation within a viral epitope can lead to a total or partial loss of functional recognition by CTL. Substitutions occurring at key anchor residues may alter peptide affinity for MHC class I molecules and thereby interfere with antigen presentation and effector T-cell mediated clearance of infected cells. Other variations, primarily in solvent-accessible residues, may abrogate TCR recognition altogether or alter it in such a way that critical activation signals are not transmitted to the cytotoxic T cells resulting in attenuated responses or even anergy.

Examples of attenuated responses have also been found with HIV and HBV. It is thought that by antagonizing T-cell responses to native epitopes, viruses expressing mutant epitopes might aid in the survival of infected cells producing wild-type viruses, which would otherwise be recognized and destroyed by CTL. Although parts of this issue have been examined in chronic HCV infection, unresolved questions remain, including whether naturally occurring variants antagonize CTL responses to wild-type epitopes that are found within the same host. To address this, viral sequences were examined by assessing CTL activity in three epitopes that were previously identified by Koziel et al.

Selective pressure appears to be applied by all elements of the immune response including antibody-producing B-cells and both CD4+ and CD8+ T cells. Such persistence of HCV infection has been particularly associated with mutations in epitopes encompassed within the hypervariable region 1 of HCV envelope glycoprotein 2, recognized by both antibodies and CD4+ helper T cells. Studying a class II-restricted immunodominant epitope within the non-structural 3 (NS3) protein region of HCV, we have identified a highly significant variation that correlated with escape from CD8+ T cell responses.

Other sequence variations in epitopes recognized by CD8+ cytotoxic T lymphocytes (CTLs) have been identified in chimpanzees and humans with chronic HCV infections.

MATERIALS AND METHODS

Human subjects

Peripheral blood samples were collected from a patient B3019 with chronic HCV at approximately 12 mo (B3019.1), 28 mo (B3019.3), and 63 mo (B3019.5) after infection. This patient never received any therapeutic intervention during the 5 years chronic HCV infection. The presence of HCV-specific antibodies and HCV RNA in the patient's serum was determined as described previously.

Blood was collected in acid citrate dextrose anticoagulant, centrifuged at 400 × g for 15 min, and divided into plasma and buffy coat fractions. After isolation of peripheral blood mononuclear cells (PBMC) over Lymphocyte Separation Medium (Organon/Teknika), plasma and PBMC were stored at -70°C or in liquid nitrogen, respectively.

Amplification of the HCV NS3 region using reverse transcription PCR

Total HCV genomic RNA was isolated from 140 μL of B3019.1, 3019.3 and 3019.5 sera using the QIAamp® Viral RNA Mini Kit (QIAGEN, Inc.). The cDNA
was synthesized using reverse transcriptase (RT) from Moloney’s murine leukemia virus and random hexadecanucleotide primers (Invitrogen). HCV cDNA was then amplified using nested primer sets (“outer” sense primer: 5'-GGCCTCTAGGGTGTAATCACC-3'; “outer” antisense primer: 5'-GAGGATTTGTCGTA-GAACAC-3'; “inner” sense primer: 5'-CAGATCGTTGCAACTGCTAC-3'; “inner” antisense primer 5'-CCA-GAGGATAAAGTCCACC-3') specific for nucleotides 3519-3935 of the NS3 region. Primers were created based on the previously reported HCV sequence from genotypes 1a, (isolate H77), which generated a final PCR product of 417 bp.

Initial PCR was performed using the outer primer set starting with heat-activation of the ProofStart DNA Polymerase at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min. A final extension step at 72°C for 10 min was introduced to increase the pool of full-length products. Two microliters of the first amplification product was transferred into the second nested PCR reaction mixture containing the “inner” primer pair. The second round of amplification was performed for 35 cycles under equal conditions. For all PCR amplifications, ProofStart DNA Polymerase (QIAGEN) with proofreading capabilities was used. PCR products were analyzed on a 1.5% agarose gel stained with ethidium bromide. As described elsewhere,[35] all measures were taken to avoid contamination of PCR samples.

As a control strategy for polymerase errors, two different experimental approaches were applied. In a first experiment, the plasmid pT7 TOPO-TA/NS3 from the Hutchinson strain (1a) of HCV[36] was diluted to 10^{-7} g/mL and amplified using the same PCR procedure described above. In an alternative experiment, reverse transcription PCR (RT-PCR) was used to amplify an RNA template derived from the pT7 TOPO-TA/NS3 plasmid. The RNA template was obtained utilizing a T7 RNA polymerase (USB) according to the supplier’s instructions. The resulting transcript was treated with DNase I for 15 min at room temperature and RNA was extracted using the QIAamp® Viral RNA Mini Kit (QIAGEN, Inc.). Reverse transcription and amplification of this control RNA was carried out as described above.

**Cloning and sequencing**

PCR products of the HCV NS3 regions were integrated into a PCR® T7TOPO® TA vector (Invitrogen, Carlsbad, CA). Ligations and transformations were executed according to the manufacturer’s instructions. Recombinant clones were then screened for positive PCR product integration by using a PCR amplification procedure detecting HCV NS3 inserted fragments. Plasmid DNAs with confirmed inserts were purified with QIAprep® spin miniprep kit (QIAGEN, Inc.) according to the standard protocol from Qiagen and further analyzed. Thirty independent clones for each sample of B3019.1, B3019.3 and B3019.5, as well as 23 and 17 independent clones for each control strategy, respectively, were sequenced in both directions using an automated DNA sequencer (373A, Applied Biosystems). The sequencing results were analyzed using GCG SeqWeb package (V2.0.2). The polymerase error rate under applied conditions was calculated as [(No. of sporadic changes)/(No. of clones) × (sequence length) × (PCR cycles)] as described by Smith et al.[37]

**Peptide synthesis**

Peptides with wild type and variant sequences of NS3 1073-1081, NS3 1131-1139 and NS3 1169-1177 were synthesized locally using F-moc chemistry and purified by high-performance liquid chromatography (HPLC). Peptide powder was dissolved in a drop of DMSO and adjusted to approximately 1 mg/mL with RPMI 1640 tissue culture medium before being used to stimulate PBMC in CTL cloning, cytotoxicity and antagonist assays. For the competition assay procedure, the FITC-labeled peptide was commercially synthesized by Synepe (Dublin, CA) using solid-phase strategies and purified with reverse-phase HPLC. For this procedure, NS3 peptides were originally dissolved in 100% DMSO at a concentration of 10 mmol/L. Subsequent dilutions were done in 1 × bovine γ globulin in PBS (BGG/PBS; 0.5 mg/mL; 0.05%; Sigma; St. Louis, MO).

**Fluorescence polarization-based peptide competition assay**

To determine relative MHC binding levels of wild-type and variant peptides, fluorescence polarization (FP)- based peptide competition assays were performed as described[38,39]. Initially, the four components of the binding reaction (competitor peptide, tracer, sHLA and β2m) were prepared as concentrates. The fluorescent-labeled tracer peptide (pFITC P5), ALMDKVL-K(FITC)-V, and the sHLA-A*0201 component of the reaction were diluted to appropriate 8 × and 2 × solutions, respectively. The β2m component (Fitzgerald Industries International; Concord, MA) was prepared as an 8 × mix and always added in a 2 × molar excess of the used sHLA concentration. Each competitor peptide was prepared at various dilutions and added as 4 × solutions. For all preparations, 1 × BGG/PBS was used as buffer. Next, each individual well of a black 96-well IJL HE PS microplate (Molecular Devices) was loaded with 5 μL of the prepared 8 × β2m, 10 μL of each competitor solution, and 5 μL of 8 μg FITC. To start the peptide exchange procedure, the 2 × sHLA mixes was activated by incubating at 53°C for 15 min before adding 20 μL to the previously loaded wells reaching a final volume of 40 μL. All reagents were added to the wells of the microtiter plate sequentially using manual pipettors. The plates were then read at room temperature using an Analyst AD (Molecular Devices; Sunnyvale, CA) until no further increase in polarization was observed indicating that equilibrium was reached (24-48 h). Data analysis was performed using the software package Prism (GraphPad), by direct fit to the
appropriate models by computer-aided, nonlinear regression analysis.

**CTL cloning**
Specific CTL clones toward HCV NS3 wild-type peptides 1073-1081 (wt1073), 1131-1139 (wt1131) and 1169 (wt1169) were generated. Briefly, PBMCs were thawed, diluted rapidly at 4°C, washed twice by centrifugation at 400 × g for 10 min, resuspended and plated into wells of 96 well flat bottom plates (200 μL) at a density of 2 × 10^5 cells/mL. Cells were then stimulated individually with wild-type NS3 peptides 10 nmol/L at 37°C in a 5% CO₂ incubator. After 7 d of incubation, 20 μM rhIL-2 (ENDOGEN) was added to the cultures. On day 14, cells were screened for the ability to lyse target cells pulsed with NS3 wild-type peptide. Functional cells were further subcloned by limiting dilution (at cell densities of 10, 3, 1 and 0.3 cells/well) in 96 well round bottom plates which contained 10^5 irradiated, autologous PBMCs, 20 μM rhIL-2, and 10 nmol/L HCV NS3 wild-type peptide. Positive clones were further expanded and re-stimulated in 24-well plates with 10^6 irradiated (3000 rad) autologous PBMCs in the presence of 10 nmol/L wild-type peptide and 20 μM rhIL-2 in RPMI 1640 medium supplemented with 25 mmol/L HEPES buffer, 2.0 mmol/L L-glutamine, 50 μg/mL penicillin, and 100 μg/mL streptomycin, 10 μg/mL gentamicin, 10 μg/mL sodium heparin, 1.0 mmol/L sodium-pyruvate, and 10% pooled AB human serum (complete RPMI-10 AB). Finally, cells were tested for cytolytic recognition of B-LCL targets pulsed separately with wild-type peptides. 14 d after the last stimulation, specific CTL clones were maintained in a long term culture in T-25 flasks by re-stimulating 2 × 10^6 cells every 2 wk with 1 × 10^6 irradiated (3000 rad) allogeneic PBMC feeders and 50 μg/mL rhIL-2 in complete RPMI-10 AB media. Not immediately used clones were frozen in liquid nitrogen for later usage.

**Tetramer staining and flow cytometry**
The CTL clones specifically recognizing HCV NS3 wild-type peptides were washed with 10% FCS in PBS followed by staining with the HCV NS3 1073-1081 peptide-MHC class II tetramer complexes as described previously.[40] The following antibodies and tetramer complexes were used: Anti-CD8-FITC, anti-mouse IgG FITC (Pharmingen, San Diego, CA, USA), HLA-A*02-restricted HIV p17 epitope tetramer PE, HLA-A*02-restricted HCV NS3 1073-1081 tetramer PE. Specific CTL clones were incubated with the antibodies and tetramer reagents for 45 min at room temperature in the dark, then washed with 1 × PBS and resuspended in 500 μL of 1 × PBS. Samples were analyzed on a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA). The data were analyzed by WinMDI 2.8 software, kindly provided by Dr. Joel Trotter. Both the HCV NS3 1073-1081 peptide-MHC class I tetramer complex and the negative control tetramer complex HIV p17 peptide SLYNTVATL were made by the NIAID tetramer Facility, Emory University Vaccine Center (Emory University, Atlanta, GA, USA).

**Cytotoxicity assay**
Cytolytic activity of CTL clones was measured using target cells labeled with the fluorescence enhancing ligand, DELFIA EuTDA (Perkin-Elmer Life Sciences, Norwalk, CT) according to the manufacturer’s instructions. As target cells, the HLA-A*02 positive Epstein-Barr virus (EBV) transformed B-cell line (L.B3019) was labeled with DELFIA BATDA reagents at 37°C for 20 min, washed three times, and incubated with the indicated concentration of peptide for 1 h. After three additional washes, effector cells were added at various concentrations and incubated for 2 h in 96-well round-bottom plates (5000 target cells per well) at 37°C in 5% CO₂. After centrifugation at 500 × g for 5 min, 20 μL of supernatant was transferred to corresponding wells of a flat bottom plate and 200 μL of europium solution was added. Fluorescence was measured using a Wallace Victor2 Multilabel Counter (Perkin-Elmer Life Sciences). Percent specific release was calculated according to the following formula: Percent specific lysis = 100 × [(experimental release - spontaneous release)/(maximum release - spontaneous release)]. Results were reported as the means of duplicate wells.

**Antagonist assay**
To measure the ability of each variant peptide to antagonize CTL responsiveness against wild-type peptide, an antagonist assay was performed using the method described by Jameson et al.[41] under slightly modified conditions. Briefly, target cells were labeled with DELFIA BATDA reagents at 37°C for 20 min, washed three times with RPMI 1640 medium, and then pulsed for 1 h with 10 nmol/L wild-type peptide. After removal of the wild-type peptide by another wash step (3 ×), the cells were pulsed a second time using varying concentrations of variant or control peptide for another hour. After a final wash step, specific CTL effector cells were added at various concentrations and incubated for 2 h. After incubation, the cytolytic activity of CTL was measured as described above. Percent inhibition of lysis was calculated as [% inhibition = 100 × [(A-B)/A]] where A is the percent specific lysis in the absence and B is the percent lysis in the presence of the variant peptide under investigation. Each point represents the mean of duplicate wells.

**RESULTS**

**Mutations in the HCV NS3 region**
The majority of RNA viruses produce RNA polymerases that lack proofreading activity, and thus introduce mutations into the viral genome. In the presence of immune selection pressure exerted by CTLs against wild-type virus, this genomic diversity could facilitate preferential expansion of mutant progeny encoding altered
epitopes that evade recognition by effector T cells. To monitor HCV genetic diversity within a fragment of the NS3 region, three different sera samples of a single subject with chronic HCV, collected at approximately 12, 28, and 63 mo after infection, were investigated by sequence analysis. In particular, the region 1060-1198 was selected for analysis because of the inclusion of previously identified HLA-A*02 restricted epitopes comprising amino acids 1073-1081, 1131-1139, and 1169-1177. An overall sequence comparison to the wild-type sequence revealed a set of 40 out of 90 individually sequenced clones (44.4%) carrying one or more mutations (Table 1). Within the 40 alternative sequences, a total of 51 nucleotide substitutions were detected of which 12 (23.5%) were synonymous (SYN) silent mutations and 39 (76.5%) were nonsynonymous (NSY) mutations, respectively, leading to specific amino acid alterations within this NS3 fragment (Figure 1). The frequency of sporadic substitutions was calculated as $1.9 \times 10^{-3}$ according to the formula described by Smith et al.[17]. Furthermore, the frequency of mutations observed at 3 different time points of disease progression for the same patient was not significantly different.

To determine if observed mutations are consistent with a positive Darwinian selection model as described in other reports[13,14,20], we compared the ratios of NSY/
SYN mutation for the NS3 fragment covering amino acid 1060-1198. As defined earlier for positive Darwinian selection, the rate of NSY substitution usually exceeds the SYN substitution rate and heterogeneity increases more quickly, whereas at sites subject to negative selection, the NSY/SYN ratio is < 1 and the heterogeneity will be much lower[37,42] . Among the total 51 nucleotide substitutions, 18 occurred in the first codon position, 18 at the second and 15 at the third position. According to the methods used by Nei et al. and Wang et al., theoretical values for SYN and NSY mutations were calculated as 11.7 and 39.3, respectively, which closely match our observed values of 12 for SYN and 39 for NSY. The total NSY/SYN ratio was 3.3, which is consistent with the positive Darwinian selection theory. Furthermore, we also compared our observed mutation values from different sections of the amplified region within NS3 with theoretical values for NSY mutations (Table 2). As seen in Figure 2, we found a significantly higher frequency of NSY mutations for section 1070-1119, 1130-1139, 1150-1159 and 1170-1189, earlier described as variable regions and also harboring the known HLA-A*02 epitope regions 1073-1081, 1131-1139 and 1169-1177. As expected, these findings are in contrast to the frequencies found within the so-called conservative regions covering sections 1060-1069 and 1120-1129 and showing a much lower mutation rate. It is notable that the 10-fold higher mutation rate within the epitope region 1073-1081, 1131-1139 and 1169-1177 compared to the conservative regions, with a NSY/SYN ratio of 2.8, suggests a high level of positive selective pressure on these immunogenic regions. No mutations were found in sections 1140-1149, 1160-1169 and 1190-1198 potentially carrying sequences not under immunological pressure within this patient. Variants do not seem to accumulate within CTL epitopes and most NSY are transient.

To ensure that the observed mutations were not due to nucleotide misincorporations introduced by the RT or polymerase during the amplification reaction, two control experiments were carried out: In the first experiment, 0.01 pg of a known NS3 plasmid template was subjected to PCR amplification in the usual two step protocol and subcloned. Sequence analysis of 23 independent clones showed absolute identity compared to the parental clone. In a second experiment, 0.01 pg of the same NS3 plasmid template was linearized and in vitro transcribed with T7 RNA polymerase. Limiting amounts of the RNA transcript was subjected to the complete RT-PCR amplification procedure, and the amplified product was subcloned and sequenced. Out of 17 independent clones sequenced, only one demonstrated a single nucleotide change. An error rate of 2.02 × 10⁻⁶ was calculated according to the formula described by Smith et al. and found to be well in line with the Pfu DNA polymerase error rate reported by others. Conclusively, these findings not only highlight the fidelity of the polymerase used for our viral genome amplification procedure, but also confirm the necessity of utilizing proofreading DNA polymerases to prevent false interpretation of mutations in genetic diversity studies of RNA viruses. Taken together, our results suggest that the mutations identified by our approach primarily represent naturally occurring mutations in the HCV RNA genome, rather than artificial PCR errors.

In addition, particular attention was given to the analysis of sequence changes encompassing epitopes recognized by CTL responses. Changes that emerged within stretches of sequence containing CTL epitopes are shown in Figure 1. As seen, sequence data from the epitopes defined within the NS3 region clearly reveal evolution in these target regions. By month 63 after transmission, bulk sequence data from the single-targeted regions illustrates the development of mixed viral populations while still maintaining the original dominant sequence. Complete replacement of the initial virus population with viruses bearing nonsynonymous sequence changes within one or more epitope-containing regions could not be observed. For epitope CINGVCWTW (1073-1081), three mutations developed at residues 1, 6 and 7, whereas 4 different mutations were noted within epitope YLVTRHADV (1131-1139) with changes at residues 1, 4, and 6. Only 2 alterations were observed for epitope LLCPAGHAV at residues 3 and 8, which may indicate a less immunologically pressured epitope.

### Table 1 Mutations in the HCV NS3 region

| Sample | Month 12 | Month 28 | Month 63 | Total |
|--------|----------|----------|----------|-------|
| No. of clones tested | 30 | 30 | 30 | 90 |
| Clones with mutations | 11 | 14 | 15 | 40 |
| Total mutations | 13 | 20 | 18 | 51 |
| SYN mutations | 2 | 4 | 6 | 12 |
| NSY mutations | 11 | 16 | 12 | 39 |

**Synonymous; NSY: Nonsynonymous; HCV: Hepatitis C virus; NS3: Non-structural 3.**

### Figure 2 Localization of NS3 mutations in the proximity of three cytotoxic T lymphocyte (CTL) epitopes. Sequence alterations within the NS3 region between amino acid (aa) 1060-1198 are graphically visualized. Nonsynonymous (top) and synonymous (bottom) mutations are shown separately. NS3 CTL epitope regions 1073-1081, 1131-1139 and 1169-1177 are indicated.
numbers are small, but consistent with the possibility of CTL-mediated selection.

**Cytotoxicity of wt-specific CTL clones against various natural epitope variants**

Next, we examined the phenotypic effects of these changes upon CTL recognition. For this reason, we initially produced CTL clones specifically recognizing target cells pulsed with wild-type peptides. CTL clone 1073 was generated recognizing HCV complexes loaded with wt1073, whereas CTL 1131 and CTL1169 were made to bind complexes harboring wt1131 and wt1169, respectively. Their specificity was confirmed in a separate set of experiments, where CTL clones were stained with MHC class I tetramers loaded with the corresponding HCV NS3 wild-type peptide. Figure 3 shows the result for the CTL clone 1073.3 containing tetramer positive CD8+ cells in the upper right quadrant. Additional staining experiments were performed using clones 1073.2 and 1073.4 with similar outcome (data not shown).

### Table 2 Nonsynonymous and synonymous mutations in different regions of HCV NS3

| Region (aa) | Length (bp) | PCR cycles | No. of clones | Mutation | $P$ | Mutation |
|-------------|-------------|------------|---------------|----------|-----|----------|
|             |             |            |               | Expected sporadic | NSY | SYN      |
| 1060-1069   | 30          | 70         | 90            | 0.25     | 1   | > 0.050 |
| 1070-1079   | 30          | 70         | 90            | 0.25     | 5   | < 0.001 |
| 1080-1089   | 30          | 70         | 90            | 0.25     | 4   | < 0.001 |
| 1090-1099   | 30          | 70         | 90            | 0.25     | 3   | < 0.001 |
| 1100-1109   | 30          | 70         | 90            | 0.25     | 5   | < 0.001 |
| 1110-1119   | 30          | 70         | 90            | 0.25     | 3   | < 0.001 |
| 1120-1129   | 30          | 70         | 90            | 0.25     | 1   | > 0.050 |
| 1130-1139   | 30          | 70         | 90            | 0.25     | 4   | < 0.001 |
| 1140-1149   | 30          | 70         | 90            | 0.25     | 0   | NS       |
| 1150-1159   | 30          | 70         | 90            | 0.25     | 4   | < 0.001 |
| 1160-1169   | 30          | 70         | 90            | 0.25     | 0   | NS       |
| 1170-1179   | 30          | 70         | 90            | 0.25     | 3   | < 0.001 |
| 1180-1189   | 30          | 70         | 90            | 0.25     | 6   | < 0.001 |
| 1190-1198   | 27          | 70         | 90            | 0.23     | 0   | NS       |
| 1207-1215   | 4           | 70         | 90            | 0.22     | 0.2 | < 0.001 |
| 1120-1129   | 30          | 70         | 90            | 0.25     | 4   | < 0.001 |
| 1130-1139   | 30          | 70         | 90            | 0.25     | 4   | < 0.001 |
| 1140-1149   | 30          | 70         | 90            | 0.25     | 0   | NS       |
| 1150-1159   | 30          | 70         | 90            | 0.25     | 4   | < 0.001 |
| 1160-1169   | 30          | 70         | 90            | 0.25     | 0   | NS       |
| 1170-1179   | 30          | 70         | 90            | 0.25     | 3   | < 0.001 |
| 1180-1189   | 30          | 70         | 90            | 0.25     | 6   | < 0.001 |
| 1190-1198   | 27          | 70         | 90            | 0.23     | 0   | NS       |
| 1207-1215   | 4           | 70         | 90            | 0.22     | 0.2 | < 0.001 |

1Epitope region. Expected sporadic mutations = ER × L × Nc × N × P/Ns, where ER = error rate of polymerases (2.02 × 10−6), L: Nucleotide length of compared region; Nc: Number of PCR cycles; N: Number of clones sequenced; P: Proportion of sporadic mutations expected to produce amino acid substitutions (2/3), Ns: Number of sample (1) according to Smith et al. [37] (1997). PCR: Polymerase chain reaction; NS: No significant.

---

**Figure 3** HCV NS3 1073-1081 tetramer staining using a CTL clone specific for wt1073. A: As a negative control, CTL clone 1073 was stained with the HIV-p17 tetramer reagent and anti-mouse IgM FITC. As expected, no cell shift was observed; B: The same CTL clone was stained with the HCV NS3 1073-1081 tetramer and anti-CD8+ FITC. A positive HCV NS3 1073-1081 tetramer staining is shown with tetramer/CD8+ cells located in the upper right quadrant. The origin of cells seen in the upper left quadrant could not be identified and need further investigation. All staining experiments were repeated three times with similar results. Identical results were obtained with HCV NS3 1073-1081 tetramer staining using other CTL clones for wt1073 (data not shown).
the amino acid changes found within this epitope region (1073-1081) showed reduced ability of the variant peptides to sensitize target cells for CTL lysis. Changes from cysteine (C) to serine (S) at position 6 and tryptophan (W) at position 7 were found to be more effective than changes from a cysteine (C) to tyrosine (Y) at position 8. As expected, the highest cytotoxicity was found against wild-type peptides followed by various degrees of lysis for epitope variants. Target cells used in these assays were prepared from the B cell line L.B3019 with an effector to target ratio of 20:1. Table 3 summarizes assessed IC$_{50}$ values for the peptides in FP-based peptide competition assays utilizing soluble HLA (sHLA)-A*0201 molecules as the binding entity. The control experiment using unrelated wild-type peptide T1134M and H11136Y with mutations at position 4 [threonine (T) to methionine (M)] and position 6 [histidine (H) to tyrosine (Y)], respectively, seemed nearly unaffected, showing only minor loss of cytolytic activity compared to the wild-type response, suggesting that the mutations seen in these particular cases did not abolish CTL recognition. In contrast, CTL 1131 completely failed to respond to variant peptide Y1131C and showed only minor lytic activity using target cells loaded with peptide H11136R harboring an amino acid substitution at position 6 [histidine (H) to arginine (R)]. This result suggests that the mutations within variant peptide Y1131C from a tyrosine (Y) to a cysteine (C) at position 1 was highly efficient to abrogate CTL recognition in contrast to the observation made for CTL 1073, where the opposite mutation at the same position from a cysteine (C) to tyrosine (Y) had much less impact on the lytic capability of the CTL clone. Overall, a decrease in specific lysis could be demonstrated for the majority of variant peptides compared to wild-type responses indicating that most of our identified HCV NS3 epitope variants were able to escape or lower specific CTL recognition to various degrees.

**Impact of variant peptides on MHC class I binding**

To investigate directly whether these amino acid changes within the variant peptides were due to impairment of peptide binding to MHC and/or to alteration of recognition of the peptide-MHC complex by T cells, we performed a series of peptide binding assays to determine if our identified viral peptide epitope variants lost their capacity to form stable class I major histocompatibility complexes compared to their wild-type counterpart. After peptides representing putative escape variants were synthesized, binding affinities of both wild-type and variant peptide epitopes were assessed using serial dilutions of the peptides in FP-based peptide competition assays utilizing soluble HLA (sHLA)-A*0201 molecules as the binding entity. Each peptide tested generated its own binding isotherm from which IC$_{50}$ values were extracted. Figure 5A-C present multiple reaction curves obtained from the competition experiments, whereas Table 3 summarizes assessed IC$_{50}$ values for the peptides along with their exact amino acid sequences. Using previously obtained results [39], we used other assay systems as guidelines to define an FP-based classification system, where peptides with an FP-based IC$_{50}$ value of 5000 nmol/L and lower were considered high affinity binding, 5000-50 000 nmol/L IC$_{50}$ values were considered medium-affinity binding, 50 000-100 000 nmol/L IC$_{50}$ values were considered low affinity binding.
Additionally, low affinity binders were further subdivided into a low (50,000-350,000 nmol/L) and very low affinity category (350,000-1,000,000 nmol/L). To provide better correlation between peptide binding affinity and...
immunogenicity, binding results from peptides known for their ability to induce potent and specific CTL responses were presented (Figure 5A). Among our viral controls, the HBV-derived epitope FLPSDFPPSV was found to display very high affinity values. As one of the more referenced peptides found in literature, FLPSDFPPSV is known for high affinity binding to A*0201 as well as for inducing potent CTL responses. Additional controls were the peptides SLYNTVATL and ILKEPVHG, two other well-studied HIV-derived CTL epitopes.

According to our FP-based classification system, wild-type peptides wt1131 and wt1169 were found to be of high and medium affinity, respectively, matching with the high cytolytic activity level of these CTL clones (Figure 4B and C). Somewhat unexpected was the low affinity binding observed for wild-type peptide wt1073, showing a very high cytolytic response when used in combination with CTL clone 1073 (Figure 4A). However, it has to be noted that this wild-type peptide has two cysteine residues within its sequence, allowing for disulfide bond formation. Potentially, this characteristic could lead to a reduced availability of intact peptides during the assay procedure directly affecting IC₅₀ determination, ultimately causing an underestimation of its binding capacity. As the two variant peptides within this group, C1078S and C1073Y, have only a single cysteine in their sequence and demonstrate very high affinity thus strongly supporting our hypothesis of a cys-cys interference.

In reviewing the binding results found for each variant peptide, none of the amino acid changes identified within each epitope region abolished peptide binding. Motif analysis of these variants showed that none of the anchor positions (defined at position 2 and 9 for nonameric peptides and critical for high affinity A*0201-related binding) contained any amino acid alterations, consistent with an escape mechanism affecting mainly peptide regions responsible for TCR recognition rather than MHC binding. More specifically, variants T1134M, H1136Y and Y1131C covering epitope 1131-1139 remained in close affinity range of the wild-type peptide indicating that mutations at positions 1, 4, and 6 had practically no effect on their binding capacity. An exception within this epitope region is variant peptide H1136R, whose binding capacity dropped 5.8 fold compared to the wild-type peptide probably caused by the introduction of a positive charge derived from the arginine (R) residue replacing the original histidine (H) residue at position 6. Similar observations were made in variant peptides W1079R and C1171R, in which the introduction of an arginine (R) at position 7 and 3 resulted in a more dramatic decrease in affinity. W1079R shifted to a 5.4 and C1171R to a 2.3 fold lower binding capacity. It is noteworthy that variant W1079R, like parental peptide Wt1073, possesses a disulfide bond potentially causing interference in binding thus also causing an underestimation of its binding capacity. Nevertheless, these arginine substituted variant peptides were still able to bind A*0201. Interestingly, in some cases such as C1078S, C1073Y and A1176T, the binding capacity actually increased compared to the wild-type peptide.

**CTL antagonism**

Because peptide variants of class I-restricted epitopes potentially could antagonize naturally occurring epitopes, we explored this possibility by using different ratios of variant to wild-type peptide concentrations (V:W). In these antagonist assays, target cells were pre-pulsed with wt1073 (Figure 6A), wt1131 (Figure 6B) or wt1169 (Figure 6C) peptides for 1 h and pulsed a second time after excessive washing with variant or control peptide for another hour. Notably, results showed that all of the specific clones were antagonized by their corresponding variant peptides generally providing inhibition values above 35% for the V:W ratio 1:1 (Figure 6). This observation is in good agreement with the obtained IC₅₀ data suggesting that all variant peptides are capable of binding HLA-A*0201. In more detail, NS3 variant peptides C1073Y, C1078S and W1079R behaved as strong antagonists for CTL 1073 and inhibited lysis of target cells at all three ratios tested (Figure 6A). An exception is variant peptide W1079R, which could not significantly inhibit the lysis of target cells at the low V:W ratio 0.1:1. This is most likely due to its much lower affinity compared to the other variant peptides within this epitope region. However, the fact that W1079R performed well as antagonist above the 1:1 ratio supports our earlier concern of reduced activity within the peptide binding assay which seems not to influence this cell-based antagonist assay. Equal results were obtained for variant peptides Y1131C, T1134M, H1136R and H1136Y testing antagonistic effects using CTL 1131 (Figure 6B). However, because of an insufficient amount of cell material obtained for CTL clone 1131, experiments were only conducted at a single V:W ratio of 1:1. Nevertheless, all of the variant peptides acted as antagonists for this CTL with highest inhibition of target lysis seen for H1136Y (92%), followed by Y1131C (68%), H1136R (47%), and T1134M (37%). The low inhibition results obtained for T1134M was somewhat unexpected considering its much higher binding capacity and cytotoxicity compared to the other variants within this group. This observation seems to indicate the presence of other factors involved to successfully antagonize CTL responses within this experimental setup such as peptide stability towards degradation and also uptake and transport mechanisms of the target cell potentially influencing peptide availability within the cell. Furthermore, both variant peptides C1171R and A1176T also demonstrated strong capabilities to antagonize CTL 1169 responses as shown in Figure 6C. Moreover, it was found that control peptide wt1131 (specific to CTL clone 1131), with high HLA-A*0201 binding affinity, was unable to inhibit CTL recognition of the wild-type peptides for CTL 1073 and 1169, respectively (Figure 6A and C). A similar result was obtained for wt1169 together with CTL 1131, suggesting that those natural variant peptides do not
variants of the hepatitis C virus impair CTL activity

**DISCUSSION**

In recent years, there has been an increasing interest in HCV vaccine approaches that elicit CTL, which recognize and eliminate cells infected with HCV. Unlike antibodies, effective CTL responses can be directed against epitopes derived from any viral protein, raising the possibility that CTLs can be targeted to regions that are more conserved than the viral envelope. Current vaccine modalities can elicit potent CTL responses against multiple viral epitopes. Indeed, many lines of evidence indicate that cell-mediated immunity plays a major role in restraining HCV infections. Several studies have suggested an association between certain MHC class I and class II alleles in the control of viral replication. Strong HCV-specific CD8+ and CD8+ T cell responses against multiple viral epitopes have been associated with clearance of HCV during acute infection\cite{58,59,60,61}, and thought to be important contributors to protective immunity\cite{58,61,62}. A typical example of replication control by CTL was recently presented for HIV, showing antibody-mediated depletion of CD8+ cells in infected macaques, which resulted in dramatically increased virus loads in both acute and chronic infections\cite{63,64}. However, despite the presence of specific CD8+ T cell responses, more than 80% of individuals develop a persistent HCV infection. Although there is increasing evidence for the importance of HCV-specific T cell responses in the resolution of HCV infection, reasons for the failure of the immune system to eradicate the virus are less clear\cite{65}. Functional impairment of the antigen-specific CTL responses has been observed by several investigators and is thought to be possible reasons for viral persistence despite measurable T cell responses in HCV\cite{66,67}. Under normal circumstances CD8+ T-cells contribute to the control of viral infections by recognizing peptides of viral proteins presented by MHC class I molecules on infected cells. If HCV peptides are presented to the immune system, why does the virus persist? One potential explanation for this phenomenon is that HCV seems to accumulate mutations in both its structural and non-structural proteins\cite{68,69}, and escape mutants may emerge under the presence of immune selection pressure exerted by CTLs against wild-type virus. Despite the importance of the CTL epitope viral mutation for immune evasion, in HCV infection many highly targeted epitopes have a low mutation frequency. Epitopes such as HLA-A2 restricted epitope viral mutation for immune evasion, in HCV infection many highly targeted epitopes have a low mutation frequency. Epitopes such as HLA-A2 restricted NS3 1073-1081 are consistently targeted by CD8+ T cells, but amino acid mutations facilitating immune evasion are rarely observed\cite{70,71}. Since the NS3 protein shares both protease and NTPase-dependent helicase functions, it has been proposed that mutations in these epitopes may be lethal to the virus\cite{72}. Additionally, another study indicates that CTL escape mutations emerging early in infection are not necessarily stable, but are eventually replaced with variants that achieve a balance between immune evasion and fitness for replication\cite{73}.

CTLs contribute to the control of viral infections by recognizing peptides of viral proteins presented by MHC class I molecules on infected cells. Some viruses have developed strategies to evade recognition by CTL and one of these strategies involves antigenic variation in CTL epitopes. The emergence of CD8+ escape variants has been demonstrated in numerous other viral infections chronically infecting their host like HBV, HIV, or SIV\cite{74,75}. In HCV infection, a strong association between viral persistence and the development of escape mutations has simply exert their inhibitory activity by competing with wild-type peptides for HLA binding as recently described for hepatitis B epitopes by Bertolletti *et al*\cite{76}.  

**Figure 6** Antagonistic effect of NS3 peptide variants inhibiting specific cytolytic activity of specific CTL clones. A detailed inhibition profile is shown for CTL clones 1073 (A) and 1169 (C) in which target cells were pre-pulsed with 10 nmol/L of wild-type peptide, and then incubated at indicated ratios of variant to wild-type peptide concentrations (V/W). Wild-type peptides wt1131 and wt1169 were used as negative controls for experiments using CTL clone 1073, where only a single control (wt1131) was used for tests involving CTL clone 1169. Both datasets were determined at effector to target ratios of 20:1. Because of lack of material, a more simplified profile is shown for CTL clone 1131 (B) testing only a V/W ratio of 1:1 with wt1169 as negative control. All experiments were performed in duplicates.
been demonstrated in the chimpanzee model\textsuperscript{[16,20,22]} In this study, animals with persistent infections developed mutations in multiple regions of the viral genome encoding known epitopes and were largely confined to the MHC class I restriction element expressed by these animals. Further, such mutations correlated with abrogated CTL function. In addition to studies in acute hepatitis, other sequence variations in epitopes recognized by CD8\textsuperscript{+} CTLs have been identified in humans with chronic HCV infections\textsuperscript{[16-20,22]} By focusing on single MHC class I alleles, Tsai et al\textsuperscript{[88]} observed variant epitope sequences with CTL antagonist activity within an A*02-restricted HCV E1 epitope in two patients who developed chronic infections, whereas Timm et al\textsuperscript{[89]} described the development of CTL responses against a single HLA-B*08-restricted epitope within the NS3. Furthermore, Ray et al\textsuperscript{[90]} used the unique approach of comparing the sequences of viruses from 22 humans with chronic hepatitis C with the sequence of the single common virus. The expression of HLA-B*07, HLA-B*35, or HLA-B*37 alleles were found to be linked to the presence of mutations in epitopes presented by these alleles, indicating a likely role for CTL-mediated pressure in driving viral evolution. All these manuscripts constitute a critical mass of evidence for CTL mutations in MHC class I-restricted epitopes of HCV, which may play an important role in evasion of the antiviral CTL response. CTL activities in vivo may be impacted by cross-recognition with HCV-related or unrelated epitope sequences found in humans. For example, there exists partial sequence homology between the NS3 1131-1139 and NS4 1585-1593 epitopes and between NS 1073-1081 epitope and influenza-A neuraminidase, a common human pathogen.

Study of viral evolution throughout the course of HCV infection has hence proved extremely difficult in the past. Much effort has therefore recently been directed to the monitoring of HCV evolution. Such analyses allow definitive assessment of changes within the viral genome, which are critical in determining the role of immune selection pressure in viral evolution. Since the high rate of chronicity after acute HCV infection is difficult to explain in the presence of a multi-specific CTL response\textsuperscript{[88]}, we sought to identify mechanisms favoring viral persistence. As noted earlier, we have previously described an immunodominant T-cell epitope restricted by HLA-DR15 in HCV NS3, NS3 358-375, for which epitope variants evolve through immune selection and stimulate not only attenuated levels of proliferation and IL-2 production, but also higher levels of type 2 cytokines\textsuperscript{[81,82]}. We reasoned that if CD8\textsuperscript{+} CTL exerts selection pressure on the virus, then the frequency of amino acid replacement should be higher in class I MHC restricted epitopes as well, potentially altering the outcome of infection by preventing or delaying clearance of infected hepatocytes by T lymphocytes and thus contribute to persistent HCV infection.

In order to identify escape mutations in a single patient with chronic infection, we monitored the genetic diversity in a region of the HCV NS3 protein that contains HLA-A*02-restricted CTL epitopes, NS3 1073-1081\textsuperscript{[34,35,80]} NS3 1131-1139\textsuperscript{[80,81]} and NS3 1169-1177\textsuperscript{[80,83]}. Sequence analysis presented extensive variations in this region along with significant substitutions in segments encoding the class I restricted epitopes. Furthermore, results showed that the ratio of nonsynonymous base substitution (which changed the amino acid encoded) to synonymous base substitution (which left encoded amino acids unchanged) in these NS3 epitopes was 10-fold higher than in flanking sequences. This is comparable with our previous observations and consistent with the model for a positive Darwinian selection pressure expected for immune-mediated selection of escape variants at the epitope level\textsuperscript{[18,21]}. Genetic variation is inherent to all RNA viruses but has been best characterized for HIV-1, which is the result of a high number of errors made by the RT enzyme\textsuperscript{[94]}, the absence of an RT proofreading mechanism during replication\textsuperscript{[80,81]}. the fast turnover of virions in HIV infected individual\textsuperscript{[87,88]} and selective immunological pressure from the host. Since HCV replication is directed by an error-prone RNA-dependent RNA polymerase encoded by the viral NS5b gene, which due to its propensity to introduce mutations into the viral genome, seems to provide the same selective advantage enjoyed by HIV, facilitating preferential expansion of the mutant progeny that potentially evade immune recognition. As a result, the virus population in an infected patient does not consist of a single uniform sequence but rather a distribution of different variants or quasispecies. The generation of new antigenic variants that escape the current immunological attack may lead to a persistent infection that culminates in the development of chronic infections. However, the massive heterogeneity observed in the worldwide epidemic of HIV-1 originated from a rapid viral turnover in HIV infected individuals, and seems to be much less extensive in chronic HCV infected individuals where the initial highly homogeneous virus population changes with much slower kinetics towards a mixed viral population.

Indeed, this process of immune evasion through mutation that characterizes infection with HCV viruses is a substantial barrier to the development of successful vaccines and therapeutic interventions based on manipulation of the T cell response. From this perspective, it is essential to gain a more integrated picture of the controlling influences that underlie the complex relationship between HCV and CD8\textsuperscript{+} T cell immunity. Due to the observed viral evolution in the NS3 region, we hypothesized that the newly discovered variant sequences may resemble mutations capable of escaping from the original CTL response against the wt epitope. For this reason, we examined the relationship between cloned CTL responses and variant viral peptide sequences derived from the three NS3 epitopes. In total, 5 out of the 9 CTL responses studied here were not recognized by specific CTL and another four variant peptides dramatically reduced the cytolytic activity of CTL. Nonetheless, comparison of features of CTL suggested that both
quantitative and qualitative factors may play a role in determining the pressure exerted by individual epitope-specific CTL responses on in vivo viral replication. Considering that HLA-A*02 represents the most frequent allele in the Northern American population with a gene frequency of 27.2% in Caucasians, 23.0% in Hispanics, 22% in Natives and 12.3% in African Americans, we assume that, in the absence of reversion, certain variants can be expected to be present at least in some HLA-A*02-negative subjects with chronic infection. Another factor that likely has an important impact on the extent and kinetics of viral escape from epitope-specific CTL responses is the cost of escape to intrinsic viral fitness. Several papers provide examples of high costs to intrinsic viral fitness preventing a lasting impact on overall viral evolution of particular epitopes. If CTL escape constitutes a common and significant means of immune evasion in HCV infection, vaccination strategies should be designed to elicit a response that will have the minimal chance of being escaped after infection. One way in which escape can be reduced is by induction of a T cell response that exerts balanced pressure against multiple viral epitopes (e.g. composed of multiple epitope-specific responses of similar magnitude and efficacy). A vaccine-generated host immune response that attacks the primary viral strain and subsequent mutants that arise during replication would possibly circumvent persistence through elimination or drive viral evolution towards defective mutants with high fitness cost and which lack the ability to infect new host cells or replicate in infected cells.

With respect to cellular immune responses, mutations can have effects other than loss of binding to MHC or TCR molecules. Studies in HLA class I restricted systems have demonstrated that altered peptide ligands (APLs) may antagonize the immune response or lead to antigen-specific anergy not only in CTL responses to HCV [10,16-18] but also to the inhibition of CTL responses to native antigens in other viral systems [13,14]. Based on our previous work with the class II restricted epitope NS3 358-375 [13,14], we were interested to know whether class I restricted variant epitopes for NS3 could also act as APLs and thus antagonize CTL function. Indeed, most variant peptides for each of the three CTL epitopes were capable of acting as antagonists and suppressed CTL recognition of wild-type peptide epitopes. For some of the variant peptides, inhibition was detectable at antagonist concentrations as low as 1 nmol/L, which is similar to physiological levels of natural peptides within infected cells according to Christinck et al. Thus, it is interesting to speculate that escape and antagonism may together serve to blunt the CTL response to multiple HCV epitopes. Ultimately, antagonism may play an important role in the persistence of HCV and other viral infections, where mutant viruses harboring antagonist APL epitopes may aid in the survival of wild-type viruses which otherwise would be recognized and destroyed by CTL. Furthermore, these observations are likely due to TCR antagonism as opposed to MHC blockade, which is supported by the fact that experiments using wild-type control peptides with high HLA-A*02 binding affinity failed to show inhibition of CTL recognition of targets pulsed with a CTL corresponding wild-type peptide. This represents a different mechanism than that reported earlier by Bertoletti et al [29]. In terms of the practical implications of this phenomenon, reported antagonism of T lymphocyte activity in a vaccine study is of particular concern because the antagonism was identified in a patient that became infected following vaccination [30,31]; antagonism may therefore represent a potential mechanism for vaccine failure and requires further careful consideration.

Overall, our data are correlative and it is important to emphasize that the coexistence of virus encoding wild type and variant epitopes does not prove that such are selected for by in vivo CTL responses. In the absence of a convenient animal model for HCV infection, the causal relationship between blunted CTL responses and a variant viral peptide sequence in HCV infection cannot be tested directly [30,31]. Nonetheless, the presence of variant peptides that are not recognized by or are able to antagonize specific CTL in the same patient is consistent with the notion that CTL pressure on a mutable virus such as HCV can result in the selection of escape or APL variants, similar to proposals by others with respect to HCV [9,10,16,18] and other viral infections [31,34]. The fact that all variant peptides in the NS3 1073-1081 epitope were able to antagonize two CTL clones specific for the wild-type viral peptide sequence suggests that immune selection for variants, if it exists, may be very strong. In line with this is the fact that similar results were obtained with variant peptides located in epitopes NS3 1131-1139 and NS3 1169-1177 although clones were differentially susceptible to the inhibitory activity of certain variant peptides. Alternatively, if such variants arise from quasispecies variations present in the initial inoculums, they would need to be maintained without sacrificing viral fitness in a significant way. This raises a question, currently unanswered, as to whether mutation of the HCV genome recapitulates quasispecies diversity within a single patient. To our knowledge this has not been investigated, but the answer would have important ramifications for understanding the immuno-pathogenesis of an HCV infection.

In summary, the findings presented here illuminate the potential mechanisms that underlie observed patterns of mutational immune escape. Analyses of MHC binding data suggest that amino acid substitutions in the bound peptide preferably impact TCR recognition, rather than MHC binding, as a consequence of continuous shifts in antigen topography that exemplify adaptive viral evolution to the individual host environment. In addition, the ability of naturally occurring variant forms to antagonize CTL clones, as suggested within this study, is increasingly recognized in chronic infections of other viruses. In this light, persistence of HCV seems to be facilitated by viral evolution not only enabling the escape from prominent CTL responses but also through antagonistic effects triggered by...
viral variants. It can be imagined that various variant peptides, which were found to have similar binding affinities to the MHC, compete against the wild type and therefore help to maintain wild-type virus by lowering the number of wild-type peptides to be recognized by patient CTLs. In any event, simultaneous analysis of the viral nucleotide sequence and the CTL response to multiple CTL epitopes in the same individual is needed to determine the potential contribution of CTL escape variants to HCV persistence. Information on heterogeneity in a single carrier seems very important in understanding immunopathogenic processes that may be influenced by viral genomic changes; such goes beyond a simple paradigm of viral escape from strong and multi-specific CTL responses against various immunodominant epitopes and should be considered as a potential determinant of HCV persistence. However, variants do not seem to accumulate within CTL epitopes but occur in early infection. Other minor species may be present but not detected and/or arise at time points other than those examined. We suggested that HCV escape mutants occurring are transient, but are eventually replaced with variants/or wild type that may seek a balance between avoiding recognition by host immune cells and reducing fitness for replication. Ultimately, viral mutants that escape immune recognition are a formidable challenge to the design of an effective HCV vaccine.

REFERENCES
1. Lauer GM, Walker BD. Hepatitis C virus infection. N Engl J Med 2001; 345: 41-52
2. Eckels DD, Flomenberg P, Gill JC. Hepatitis C virus: models of immunopathogenesis and prophylaxis. Transfusion 1996; 36: 836-844
3. Alter HJ, Conry-Cantilena C, Melpolder J, Tan D, Van Raden M, Herion D, Lau D, Hoofnagle JH. Hepatitis C in asymptomatic blood donors. Hepatology 1997; 26: 295-335
4. Weiner A, Erickson AL, Kansopon J, Crawford K, Muchmore E, Hughes AL, Houghton M, Walker CM. Persistent hepatitis C virus infection in a chimpanzee is associated with emergence of a cytotoxic T lymphocyte escape variant. Proc Natl Acad Sci USA 1995; 92: 2755-2759
5. Brodsky FM. Stealth, sabotage and exploitation. Immunol Rev 1999; 168: 5-11
6. Neumann AU, Lam NP, Dahari H, Gretch DR, Wiley TE, Layden TJ, Perelson AS. Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. Science 1998; 282: 103-107
7. Bukh J, Miller RH, Purcell RH. Genetic heterogeneity of hepatitis C virus: quasiespecies and genotypes. Semin Liver Dis 1995; 15: 41-63
8. Manzin A, Soforosil D, Debiaggi M, Zara F, Tanzi E, Romanò L, Zanetti AR, Clementi M. Dominant role of host selective pressure in driving hepatitis C virus evolution in perinatal infection. J Virol 2000; 74: 4327-4334
9. Facci P, Shimoda A, Coiana A, Diaz G, Peddis G, Melpolder JC, Strazzera A, Chien DY, Munoz SJ, Balestriere A, Purcell RH, Alter HJ. The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies. Science 2000; 288: 339-344
10. Frasca L, Del Porto P, Tuosto L, Marinari B, Scattà C, Carbonari M, Nicosia A, Piccoletta E. Hypervariable region 1 variants act as TCR antagonists for hepatitis C virus-specific CD4+ T cells. Immunol 1999; 163: 650-658
11. Eckels DD, Tabatabail N, Bian TH, Wang H, Muheisen SS, Rice CM, Yoshizawa K, Gill J. In vitro human Th-cell responses to a recombinant hepatitis C virus antigen: failure in IL-2 production despite proliferation. Hum Immunol 1999; 60: 187-199
12. Tabatabai NM, Bian TH, Rice CM, Yoshizawa, Gill J, Eckels DD. Functionally distinct T-cell epitopes within the hepatitis C virus non-structural 3 protein. Hum Immunol 1999; 60: 105-115
13. Wang H, Bian T, Merrill SJ, Eckels DD. Sequence variation in the gene encoding the nonstructural 3 protein of hepatitis C virus: evidence for immune selection. J Mol Evol 2002; 54: 465-473
14. Wang H, Eckels DD. Mutations in immunodominant T cell epitopes derived from the nonstructural 3 protein of hepatitis C virus have the potential for generating escape variants that may have important consequences for T cell recognition. J Immunol 1999; 162: 4177-4183
15. Erickson AL, Kimura Y, Igarashi S, Eichelberger J, Houghton M, Sidney J, McKinney D, Sette A, Hughes AL, Walker CM. The outcome of hepatitis C virus infection is predicted by escape mutations in epitopes targeted by cytotoxic T lymphocytes. Immunity 2001; 15: 883-895
16. Chang KM, Rehermann B, McHutchison JG, Pasquinelli C, Southwood SG, Sette A, Chisari FV. Immunological significance of cytotoxic T lymphocyte epitope variants in patients chronically infected by the hepatitis C virus. J Clin Invest 1997; 100: 2376-2385
17. Kaneko T, Moriyama T, Udaka K, Hiroishi K, Kita H, Okamoto H, Yagita H, Okumura K, Imawari M. Impaired induction of cytotoxic T lymphocytes by antagonism of a weak agonist borne by a variant hepatitis C virus epitope. Eur J Immunol 1997; 27: 1782-1787
Wang S et al. Variants of the hepatitis C virus impair CTL activity

18 Tsai SL, Chen YM, Chen MH, Huang CY, Sheen IS, Yeh CT, Huang JH, Kuo GC, Liaw YF. Hepatitis C virus variants circumventing cytotoxic T lymphocyte activity as a mechanism of chronicity. Gastroenterology 1998; 115: 139-148

19 Cox AL, Mesbruger T, Mao Q, Liu Z, Wang XH, Yang HC, Sidney J, Sette A, Pardoll D, Thomas DL, Ray SC. Cellular immune selection with hepatitis C virus persistence in humans. J Exp Med 2005; 201: 1741-1752

20 Timm J, Lauer GM, Kavanagh DG, Sheridan I, Kim AY, Lucas M, Pillay T, Ouchi K, Reynor LL, Schulze zur Wijch, Gandhi RT, Chung RT, Bhardwaj N, Klenerman P, Walker BD, Allen TM. CD8 epitope escape and reversion in acute HCV infection. J Exp Med 2004; 200: 1593-1604

21 Tester I, Smyk-Pearson S, Wang P, Wertheimer A, Yao E, Lewinsohn DM, Tavis JE, Rosen HR. Immune evasion versus recovery after acute hepatitis C virus infection from a shared source. J Exp Med 2005; 201: 1725-1731

22 Ray SC, Fanning L, Wang XH, Netski DM, Kenny-Walsh E, Thomas DL. Divergent and convergent evolution after a common-source outbreak of hepatitis C virus. J Exp Med 2005; 201: 1753-1759

23 Bjorkman PJ, Saper MA, Samraoui B, Bennett WS, Strominger JL, Wiley DC. Structure of the human class I histocompatibility antigen, HLA-A2. Nature 1987; 329: 506-512

24 Bjorkman PJ, Saper MA, Samraoui B, Bennett WS, Strominger JL, Wiley DC. The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. Nature 1987; 329: 512-518

25 Rötzschke O, Falk K, Deres K, Schild H, Norda M, Metzger J, Jung G, Rammsense HC. Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. Nature 1990; 348: 252-254

26 Townsend AR, Rothbard J, Gotch FM, Bahadur G, Wraith D, McMichael AJ. The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. Cell 1986; 44: 959-968

27 Van Bleek GM, Nathenson SG. Isolation of an endogenously processed immunodominant viral peptide from the class I H-2Kb molecule. Nature 1990; 348: 213-216

28 Bertoletti A, Setta C, Chisari FV, Penna A, Levrero M, De Carli M, Fiaccadori F, Ferrari C. Natural variants of cytotoxic epitopes are T-cell receptor antagonists for antiviral cytotoxic T cells. Nature 1994; 369: 407-410

29 Bouabdell L, Villain P, Merzouki A, Arella M, Couture C. CD8 epitope-mediated energy of a human immunodeficiency virus type 1 env/gp41polytropic T-cell clone, induced by a natural HIV type 1 variant peptide. J Virol 2000; 74: 2121-2130

30 Fenoglio D, Li Pira G, Lozzi L, Bracci L, Saverino D, Terranova P, Bottone L, Lantero S, Megiovanni A, Merlo A, Manca F. Prominent role of secondary anchor residues in peptide binding to HLA-A*0201. J Exp Med 2000; 193: 1-7

31 Klenner P, Rowland-Jones S, McAdam S, Edwards J, Daenke S, Laloo D, Köppe B, Rosenberg W, Boyd D, Edwards A. Cytotoxic T cell activity antagonized by naturally occurring HIV-1 Gag variants. Nature 1994; 369: 403-407

32 Lekutis C, Letvin NL. Substitutions in a major histocompatibility complex class II-restricted human immunodeficiency virus type 1 gp120 epitope can affect CD4+ helper-cell function. J Virol 1990; 65: 3904-3912

33 Goulder PJ, Sewell AK, Laloo DG, Price DA, Whelan JA, Evans J, Taylor GP, Luzzi G, Giangrande P, Phillips RE, McMichael AJ. Patterns of immunodominance in HIV-1-specific cytotoxic T lymphocyte responses in two human histocompatibility leukocyte antigens (HLA)-identical siblings with HLA-A*0201 are influenced by epitope mutation. J Exp Med 1997; 185: 1423-1433

34 Goulder PJ, Altfeld M, Rosenberg ES, Nguyen T, Tang Y, Eldridge RL, Addo MM, He S, Mukherjee JS, Phillips MN, Bunce M, Kalame SA, Sekaly RP, Walker BD. Substantial differences in specificity of HIV-specific cytotoxic T cells in acute and chronic HIV infection. J Exp Med 2001; 193: 181-194

35 Og G85, Jin X, Bonhoeffer S, Dunbar PR, Nowak MA, Monard S, Segal JP, Cao Y, Rowland-Jones SL, Cerundolo V, Hurley A, Markowitz M, Ho DD, Nixon DF, McMichael AJ. Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. Science 1998; 279: 2103-2106

Nature 1989; 339: 237-238

Graikou A, Wychowski C, Lin C, Feinstone SM, Rice CM. Expression and identification of hepatitis C virus polypeptide cleavage products. J Virol 1993; 67: 1389-1399

Smith DB, MacAllister J, Casino C, Simmonds P. Virus ‘quasispecies’ making a mountain out of a molehill? J Gen Virol 1997; 78 (Pt 7): 1511-1519

Buchli R, VanGundy RS, Hickman-Miller HD, Gibson CF, Bardet W, Hildebrand WH. Real-time measurement of in vitro peptide binding to soluble HLA-A*0201 by fluorescence polarization. Biochemistry 2004; 43: 14852-14863

Buchli R, VanGundy RS, Hickman-Miller HD, Gibson CF, Bardet W, Hildebrand WH. Development and validation of a fluorescence polarization-based competitive peptide-binding assay for HLA-A*0201—a new tool for epitope discovery. Biochemistry 2005; 44: 12491-12507

Zhu F, Eckels DD. Functionally distinct helper T-cell epitopes of HCV and their role in modulation of N53-specific, CD8+/tetramer positive CTL. Hum Immunol 2002; 63: 710-718

Jameson SC, Carbone FR, Bevan MJ. Clone-specific T cell receptor antagonists of major histocompatibility complex class I-restricted cytotoxic T cells. J Exp Med 1993; 177: 1541-1550

Hughes AL, Nei M. Pattern of nucleotide substitution at major histocompatibility complex class I loci reveals predominant selection. Nature 1988; 335: 167-170

Nei M, Gojobori T. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol Biol Evol 1986; 3: 418-426

Lundberg KS, Shoemaker DD, Adams MW, Short JM, Sorge JA, Mathur EJ. High-fidelity amplification using a thermostable DNA polymerase isolated from Pyrococcus furiosus. Gene 1991; 108: 1-6

Malet I, Belnard M, Agut H, Cahour A. From RNA to quasispecies: a DNA polymerase with proofreading activity is highly recommended for accurate assessment of viral diversity. J Virol Methods 2003; 109: 161-170

Kast WM, Brandt RM, Sidney J, Drijfhout JW, Kubo RT, Grey HM, Melief CJ, Sette A. Role of HLA-A motifs in identification of potential CTL epitopes in human papillomavirus type 16 E6 and E7 proteins. J Immunol 1994; 152: 3904-3912

Sette A, Sidney J, del Guercio MF, Southwood S, Ruppert J, Dahlberg C, Grey HM, Kubo RT. Peptide binding to the most frequent HLA-A class I alleles measured by quantitative molecular binding assays. Mol Immunol 1994; 31: 813-822

Ruppert J, Sidney J, Celis E, Kubo RT, Grey HM, Sette A. Prominent role of secondary anchor residues in peptide binding to HLA-A2.1 molecules. Cell 1993; 74: 929-937

Vitiello A, Ishioka G, Grey HM, Rose R, Farness P, LaFond R, Yuan L, Chisari FV, Furze J, Bartholomew R. Development of a lipopeptide-based therapeutic vaccine to treat chronic HBV infection. J. Induction of a primary cytotoxic T lymphocyte response in humans. J Clin Invest 1995; 95: 341-349

Goulder PJ, Sewell AK, Laloo DG, Price DA, Whelan JA, Evans J, Taylor GP, Luzzi G, Giangrande P, Phillips RE, McMichael AJ. Patterns of immunodominance in HIV-1-specific cytotoxic T lymphocyte responses in two human histocompatibility leukocyte antigens (HLA)-identical siblings with HLA-A*0201 are influenced by epitope mutation. J Exp Med 1997; 185: 1423-1433

Goulder PJ, Altfeld M, Rosenberg ES, Nguyen T, Tang Y, Eldridge RL, Addo MM, He S, Mukherjee JS, Phillips MN, Bunce M, Kalame SA, Sekaly RP, Walker BD. Substantial differences in specificity of HIV-specific cytotoxic T cells in acute and chronic HIV infection. J Exp Med 2001; 193: 181-194

Ogg GS, Jin X, Bonhoeffer S, Dunbar PR, Nowak MA, Monard S, Segal JP, Cao Y, Rowland-Jones SL, Cerundolo V, Hurley A, Markowitz M, Ho DD, Nixon DF, McMichael AJ. Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. Science 1998; 279: 2103-2106
Wang S et al. Variants of the hepatitis C virus impair CTL activity

53 Cooper S, Erickson AL, Adams EJ, Kansopon J, Weiner AJ, Chien DY, Houghton M, Farham P, Walker CM. Analysis of a successful immune response against hepatitis C virus. *J Immunol* 1999; 163: 439-449

54 Lechner F, Wong DK, Dunbar PR, Chapman R, Chung RT, Dohrenwend P, Robbins G, Phillips R, Klenerman P, Walker BD. Analysis of successful immune responses in persons infected with hepatitis C virus. *J Exp Med* 2000; 191: 1499-1512

55 Thimme R, Oldach D, Chang KM, Steiger C, Ray SC, Chisari FV. Determinants of viral clearance and persistence during acute hepatitis C virus infection. *J Exp Med* 2001; 194: 1395-1406

56 Gerlach JT, Diepolder HM, Jung MC, Gruener NH, Schraut WW, Zachoval R, Hoffmann R, Schirren CA, Santantonio T, Pape GR. Recurrence of hepatitis C virus after loss of virus-specific CD8+ T-cell response in acute hepatitis C. *Gastroenterology* 1999; 117: 933-941

57 Diepolder HM, Gerlach JT, Zachoval R, Hoffmann RM, Jung MC, Wierenga EA, Scholz S, Santantonio T, Houghton M, Southwood S, Sette A, Pape GR. Immunodominant CD8+ T-cell epitope within nonstructural protein 3 in acute hepatitis C virus infection. *J Virol* 1997; 71: 6011-6019

58 Takaki A, Wiese M, Maertens G, Depla E, Seifert U, Liebertrau A, Miller JL, Manns MP, Rehermann B. Cellular immune responses persist and humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C. *Nat Med* 2000; 6: 578-582

59 Erickson AL, Houghton M, Choo QL, Weiner AJ, Raslton R, Muchmore E, Walker CM. Hepatitis C virus-specific CTL responses in the liver of chimpanzees with acute and chronic hepatitis C. *J Immunol* 1993; 151: 4189-4199

60 Gruner NH, Gerlach TJ, Jung MC, Diepolder HM, Schirren CA, Schraut WW, Hoffmann R, Zachoval R, Santantonio T, Cacciani M, Cerny A, Pape GR. Association of hepatitis C virus-specific CD8+ T cells with viral clearance in acute hepatitis C. *J Infect Dis* 2000; 181: 1528-1536

61 Mehta SH, Cox A, Hoover DR, Wang XH, Mao Q, Ray S, Stratham SE, Vlahov D, Thomas DL. Protection against persistence of hepatitis C. *Lancet* 2002; 359: 1478-1483

62 Shoukry NH, Grakoui A, Houghton M, Chien DY, Gralayeb J, Reimann KA. Walker CM. Memory CD8+ T cells are required for protection from persistent hepatitis C virus infection. *J Exp Med* 2003; 197: 1645-1655

63 Jin X, Bauer DE, Tuttleton SE, Lewin S, Gettie A, Blanchard J, Irwin CE, Safrit JT, Mittler J, Weinberger L, Kostrikis LG, Zhang H, Joslin AS, Ho DD. Dramatic rise in plasma viremia after CD8+ T cell depletion in simian immunodeficiency virus-infected macaques. *J Exp Med* 1999; 189: 991-998

64 Metzner KJ, Jin X, Lee FV, Gettie A, Bauer DE, Di Mascio M, Perelson AS, Marx PA, Ho DD, Kostrikis LG, Connor RL. Effects of in vivo CD8+ T cell depletion on virus replication in rhesus macaques immunized with a live, attenuated simian immunodeficiency virus vaccine. *Science* 1999; 285: 857-860

65 Willberg C, Barnes E, Klenerman P. HCV immunology—death and the maiden T cell. *Cell Death Differ* 2003; 10 Suppl 1: S39-S47

66 Wedemeyer H, He XS, Nascimbeni M, Davis AR, Greenberg HB, Hoofnagle JH, Liang TJ, Alter H, Rehermann B. Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. *J Immunol* 2002; 169: 3447-3458

67 Appay V, Dunbar PR, Callan M, Klenerman P, Gillespie GM, Papagnio L, Ogg GS, King A, Lechner F, Spina CA, Little S, Havir DV, Richman DD, Gruener N, Pape G, Waters A, Easterbrook P, Salio M, Cerundolo V, McMichael AJ, Rowland-Jones SL. Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. *Nat Med* 2002; 8: 379-385

68 Crispe IN. Hepatic T cells and liver tolerance. *Nat Rev Immunol* 2003; 3: 51-62

69 Cristiano K, Di Bisceglie AM, Hoofnagle JH, Feinsteen SM. Hepatitis C viral RNA in serum of patients with chronic non-A, non-B hepatitis: detection by the polymerase chain reaction using multiple primer sets. *Arch Virol Suppl* 1992; 4: 172-178

70 Seifert U, Liermann H, Raccanelli V, Halenius A, Wiese M, Wedemeyer H, Ruppert T, Rispeter K, Henklein P, Sijs A, Hengel H, Kloeziel PM, Rehermann B. Hepatitis C virus mutation affects proteasomal epitope processing. *J Clin Invest* 2004; 114: 250-259

71 Söderholm J, Ahlén G, Kaul A, Frelin L, Alheim M, Barnfield C, Lijestrom P, Weiland O, Milich DR, Bartschegler R, Sällberg M. Relation between viral fitness and immune escape within the hepatitis C virus proteome. *Nat* 2006; 35: 266-274

72 Uebelhoer I, Han JH, Callendret B, Mateu G, Shoukry NH, Hanson HL, Rice CM, Walker CM, Grakoui A. Stable cytotoxic T cell escape mutation in hepatitis C virus is linked to maintenance of viral fitness. *PLoS Pathog* 2008; 4: e1000143

73 Bertolotti A, Costanzo A, Chisari FV, Levrero M, Artini M, Sette A, Penna A, Giuberti T, Fiacchadour F, Ferrari C. Cytotoxic T lymphocyte response to a wild type hepatitis B virus epitope in patients chronically infected by variant viruses carrying substitutions within the epitope. *J Exp Med* 1994; 180: 933-943

74 Sewell AK, Harcourt GC, Goulder PJ, Price DA, Phillips RE. Antagonism of cytotoxic T lymphocyte-mediated lysis by natural HIV-1 altered peptide ligands requires simultaneous presentation of agonist and antagonist peptides. *Eur J Immunol* 1997; 27: 2323-2329

75 Phillips RE, Rowland-Jones S, Nixon DF, Gotch FM, Edwards JP, Ogunlesi AO, Elvin JG, Rothbard JA, Bangham CR, Rizza CR. Human immunodeficiency virus genetic variation that can escape cytotoxic T cell recognition. *Nature* 1991; 354: 453-459

76 Allen TM, O’Connor DH, Jing P, Dzuris JL, Mothé BR, Vogel TU, Dunphy E, Liebl ME, Emerson C, Wilson N, Kunstman KJ, Wang X, Allison DB, Hughes AL, Desrosiers RC, Altman JD, Wolinsky SD, Sette A, Watkins DL. Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viremia. *Nature* 2000; 407: 386-390

77 Draenert R, Verrilli CL, Tang Y, Allen TM, Wurcel AG, Boczanowski M, Lechner A, Kim AJ, Suscovitch T, Brown NV, Addo MM, Walker BD. Persistent recognition of autologous virus by high-avidity CD8 T cells in chronic, progressive human immunodeficiency virus type 1 infection. *J Virol* 2004; 78: 630-641

78 Eckels DD, Zhou H, Bhan TH, Wang H. Identification of antigenic escape variants in an immunodominant epitope of hepatitis C virus. *Int Immunol* 1999; 11: 577-583

79 Cerny A, McHughison JG, Pasquinielli C, Brown ME, Broders MA, Grabscheid B, Fowler P, Houghton M, Chisari FV. Cytotoxic T lymphocyte response to hepatitis C virus-derived peptides containing the HLA A2.1 binding motif. *J Clin Invest* 1995; 95: 521-530

80 Prezzi C, Casciato MA, Francavilla V, Schiaffella E, Finocchi L, Chircu LV, Bruno G, Sette A, Abrignani S, Barnaba V. Virus-specific CD8+ T cells with type 1 or type 2 cytokine profile are related to different disease activity in chronic hepatitis C virus infection. *Eur J Immunol* 2001; 31: 894-906

81 Scognamiglio P, Accapezzato D, Casciato MA, Cacciani A, Artini M, Bruno G, Chircu ML, Sidney J, Southwood S, Abrignani S, Sette A, Barnaba V. Presence of effector CD8+ T cells in chronic hepatitis C virus infection. *J Exp Med* 2000; 191: 1921-1931

82 Schmitz JE, Kuroda MJ, Santra S, Sasseville VG, Simon MA, Lifton MA, Racz P, Tenner-Racz K, Dalesandro M, Scalton BJ, Gralayeb J, Forman MA, Montefiori DC, Bieprz PE, Letvin NL, Reimann KA. Control of viremia in simian immunodeficiency virus vaccine. *Science* 1999; 283: 857-860

83 Willberg C, Barnes E, Klenerman P. HCV immunology—death and the maiden T cell. *Cell Death Differ* 2003; 10 Suppl 1: S39-S47

84 Wedemeyer H, He XS, Nascimbeni M, Davis AR, Greenberg HB, Hoofnagle JH, Liang TJ, Alter H, Rehermann B. Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. *J Immunol* 2002; 169: 3447-3458
T cells in hepatitis C virus-exposed healthy seronegative donors. *J Immunol* 1999; 162: 6681-6689

83 Rehermann B, Chang KM, McHutchison JG, Kokka R, Houghton M, Chisari FV. Quantitative analysis of the peripheral blood cytotoxic T lymphocyte response in patients with chronic hepatitis C virus infection. *J Clin Invest* 1996; 98: 1432-1440

84 Coffin JM. Genetic diversity and evolution of retroviruses. *Curr Top Microbiol Immunol* 1992; 176: 143-164

85 Bebenek K, Abbotts J, Roberts JD, Wilson SH, Kunkel TA. Specificity and mechanism of error-prone replication by human immunodeficiency virus-1 reverse transcriptase. *J Biol Chem* 1989; 264: 16948-16956

86 Roberts JD, Bebenek K, Kunkel TA. The accuracy of reverse transcriptase from HIV-1. *Science* 1988; 242: 1171-1173

87 Keulen W, Nijhuis M, Schuurman R, Berkhout B, Boucher C. Reverse transcriptase fidelity and HIV-1 variation. *Science* 1997; 275: 229; author reply 229-230; author reply 231

88 Drosopoulos WC, Rezende LF, Wainberg MA, Prasad VR. Virtues of being faithful: can we limit the genetic variation in human immunodeficiency virus? *J Mol Med* 1998; 76: 604-612

89 Apetrei C, Marx PA, Smith SM. The evolution of HIV and its consequences. *Infect Dis Clin North Am* 2004; 18: 369-394

90 Cao K, Hollenbach J, Shi X, Shi W, Chopek M, Fernández-Viña MA. Analysis of the frequencies of HLA-A, B, and C alleles and haplotypes in the five major ethnic groups of the United States reveals high levels of diversity in these loci and contrasting distribution patterns in these populations. *Hum Immunol* 2001; 62: 1009-1030

91 Wagner R, Leschonsky B, Harrer E, Paulus C, Weber C, Walker BD, Buchbinder S, Wolf H, Kalden JR. Harrer T. Molecular and functional analysis of a conserved CTL epitope in HIV-1 p24 recognized from a long-term nonprogressor: constraints on immune escape associated with targeting a sequence essential for viral replication. *J Immunol* 1999; 162: 3727-3734

92 Kelleher AD, Long C, Holmes EC, Allen RL, Wilson J, Conlon C, Workman C, Shaunak S, Olson K, Goulder P, Brander C, Ogg G, Sullivan JS, Dyer W, Jones I, McMichael AJ, Rowland-Jones S, Phillips RE. Clustered mutations in HIV-1 gag are consistently required for escape from HLA-B27-restricted cytotoxic T lymphocyte responses. *J Exp Med* 2001; 193: 375-386

93 De Magistris MT, Alexander J, Coggeshall M, Altman A, Gaeta FC, Grey HM, Sette A. Antigen analog-major histocompatibility complexes act as antagonists of the T cell receptor. *Cell* 1992; 68: 625-634

94 Sloan-Lancaster J, Allen PM. Altered peptide ligand-induced partial T cell activation: molecular mechanisms and role in T cell biology. *Annu Rev Immunol* 1996; 14: 1-27

95 Christinck ER, Luscher MA, Barber BH, Williams DB. Peptide binding to class I MHC on living cells and quantitation of complexes required for CTL lysis. *Nature* 1991; 352: 67-70

96 Kent SJ, Greenberg PD, Hoffman MC, Akridge RE, McElrath MJ. Antagonism of vaccine-induced HIV-1-specific CD4+ T cells by primary HIV-1 infection: potential mechanism of vaccine failure. *J Immunol* 1997; 158: 807-815

97 Shimizu YK, Iwamoto A, Hijikata M, Purcell RH, Yoshikura H. Evidence for in vitro replication of hepatitis C virus genome in a human T-cell line. *Proc Natl Acad Sci USA* 1992; 89: 5477-5481

98 Jameson SC, Bevan MJ. T cell receptor antagonists and partial agonists. *Immunity* 1995; 2: 1-11

99 Livingston BD, Crimi C, Fikes J, Chesnut RW, Sidney J, Sette A. Immunization with the HBV core 18-27 epitope elicits CTL responses in humans expressing different HLA-A2 supertype molecules. *Hum Immunol* 1999; 60: 1013-1017

100 van der Burg SH, Vissersen MJ, Brandt RM, Kast WM, Melief CJ. Immunogenicity of peptides bound to MHC class I molecules depends on the MHC-peptide complex stability. *J Immunol* 1996; 156: 3308-3314

S- Editor Wang YR  L- Editor O’Neill M  E- Editor Zheng XM