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The Impairment of CD8 Responses Limits the Selection of Escape Mutations in Acute Hepatitis C Virus Infection¹

Simona Urbani, Barbara Amadei, Elisabetta Cariani, Paola Fisicaro, Alessandra Orlandini, Gabriele Missale, and Carlo Ferrari²

Evasion from protective CD8 responses by mutations within immunodominant epitopes represents a potential strategy of HCV persistence. To investigate the pathogenetic relevance of this mechanism, a careful search for immunodominant CD8 epitopes was conducted in six patients with chronic evolution of HCV infection by analyzing their global CD8 response with a panel of overlapping synthetic peptides covering the overall HCV sequence and by studying the CD8 frequency by tetramer staining. Immunodominant responses were followed longitudinally from the time of acute onset in relation to the evolution of the epitopic sequences. Although intensity of CD8 responses and frequency of HCV-specific CD8 cells declined over time in all patients, mutations emerged in only three of the six acute patients studied. Variant sequences were less efficiently recognized by CD8 cells than parental epitopes and were poorly efficient in inducing a CD8 response *in vitro*. CD8 epitopes undergoing mutations were targeted by high avidity CD8 cells more efficient in effector function. Our data support the view that immunodominant CD8 responses are affected by inhibitory mechanisms operating early after infection and that the emergence of escape mutations represents an additional mechanism of virus evasion from those CD8 responses that are functionally preserved. *The Journal of Immunology*, 2005, 175: 7519–7529.

Hepatitis C virus is a common cause of liver disease, and the majority of infected individuals develop persistent infection. Among the different mechanisms of HCV persistence, the emergence of mutations within immunodominant CD8 epitopes represents a possible strategy that HCV can exploit to evade immune control (1–8). In general, the selection of CD8 escape mutations is more likely to occur if the mutation rate of the virus is high, if the protective CD8 responses to evade are targeted against variable viral regions, which can tolerate mutations without loss of vital viral functions, and if the CD8 function is sufficient to exert the immunological pressure needed to induce the selection of escape mutations (9). Moreover, the likelihood of escape by this mechanism is expected to be greater if the CD8 response is narrowly focused on individual or a few strongly dominant epitopes.

Although HCV has a very high mutation rate due to the lack of proofreading capacity of its polymerase (10), the overall repertoire of HCV epitopes recognized by CD8 cells and their hierarchy of immunodominance in individual infected subjects has been so far difficult to define owing to technical limitations in analyzing the global HCV-specific CD8 response in its entirety. Moreover, various types of functional CD8 cell defects at the early stages of

HCV infection have been reported, which may affect the overall antiviral function of the CD8 response, thus limiting its capacity to exert selective pressure on HCV (11–16). This makes difficult to predict the actual role that the mutational escape of HCV from CD8 surveillance can have as a mechanism of persistence.

The role of virus evasion from CD8 surveillance by escape mutations has been demonstrated in different infections, such as HIV and CMV (17, 18). Proof of this concept in human HCV infection has been limited by different factors, including the asymptomatic nature of HCV infection that makes the longitudinal analysis of viral evolution and host immune responses from the early stages of infection a difficult task. Moreover, the infecting inoculum is generally unknown in the majority of patients with acute infection. Because of these limitations, until very recently, amino acid variations within HLA class I-restricted epitopes or their flanking regions were reported, but no definitive proof of selection by the CD8 immune pressure was given (1–8, 19–21). Only more recent studies have provided convincing evidence of HCV escape in patients with acute HCV infection by the identification of escape mutations within multiple CTL epitopes (22–26), two of whom infected from a single viral source (22, 23), and in patients with chronic hepatitis C by the analysis of viral evolution from a common infectious inoculum conducted several years after the time of infection (24). These studies indicate that mutations can occur within epitopic regions not only to allow HCV escape from CD8 surveillance but also to allow reversion of the virus to a more fit pre-existing state (25). This extends to the human infection previous evidence of escape derived from the chimpanzee model of infection, where abrogation of immunodominant CD8 responses caused by the emergence of mutations was directly linked to HCV persistence (2).

The relevance of this mechanism in the pathogenesis of HCV persistence still remains partially understood. To further clarify this issue, in this study, we first looked for immunodominant CD8 epitopes in six patients with acute hepatitis C followed by chronic evolution of infection. The behavior of CD8 responses against

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them was subsequently monitored over time in relation to the evolution of the sequence of the corresponding epitopes. The results indicate that only a fraction of the immunodominant epitopes identified at the onset of the disease underwent a strong selective pressure by CD8 responses, leading to the emergence of escape mutations. CD8 responses to four of the seven immunodominant epitopes studied were functionally impaired from the earliest time points analyzed and therefore unable to perform selection. Our study confirms that escape mutations can contribute to HCV persistence, although other mechanisms of CD8 cell inhibition can silence immunodominant responses early after infection before the selection of escape mutations can take place.

Materials and Methods

Patients and virological assessment

Six patients with acute hepatitis C were enrolled at the Unit of Infectious Diseases and Hepatology of the University Hospital of Parma. The diagnosis of acute HCV infection was based on the following criteria: documented seroconversion to anti-HCV Abs by Recombinant ImmunoBlot assay, levels of serum alanine aminotransferase (ALT)³ at least 10 times the upper limit of normal (50 U/L), detection of HCV RNA, exclusion of other possible causes of acute hepatitis (i.e., viruses, toxins, alcohol, autoimmunity, metabolic factors). Anti-HCV Abs were determined by commercial enzyme immunoassay kits (Ortho Diagnostic Systems) and by Recombinant ImmunoBlot (RIBA II; Ortho Diagnostic Systems). Serum HCV-RNA was quantified by branched DNA assay and was expressed as copies per milliliter of serum (Bayer System 340bDNA Analyzer; Bayer); the lower limit of detection by this method is 2500 copies/ml. The study was approved by the Ethical Committee of the Azienda Ospedaliero Universitaria di Parma, and all subjects gave written informed consent.

Synthetic peptides, recombinant proteins, peptide-HLA class I tetramers, and Abs

Synthetic peptides representing HLA-A2-restricted epitopes corresponding to HCV NS3 1073–1081 (CINGVCWTV), NS3 1406–1415 (KLVALG INAV), NS4 1812–1820 (LLFNILGGWV), NS4B 1992–2000 (VLSDFK TWL), and NS5 2594–2602 (ALYDVVTKL), the mutated sequences of the HCV regions NS3 1073–1081 and NS4 1992–2000 detected in patients 2, 3, and 6, influenza virus Matrix 58–66 (GILGFVFTL), CMV pp65 (NLVPMVATV), EBV BMLF-1 (GLCTLVAML), and a panel of 601 15-mer peptides based on a genotype 1a sequence (HCV-1) covering all structural (core, E1, E2) and nonstructural (NS3, NS4, NS5) HCV regions and overlapping by 10 residues were purchased from Chiron Mimotopes. The HCV Ags E1, E2, core, NS3, NS4, and NS5 were expressed as C-terminal fusion proteins with human superoxide dismutase in yeast and were provided by Chiron. Purity of the Ag preparation ranged from 85 to 95%. PE-labeled tetrameric peptide-HLA class I complexes were purchased from Proimmune. HLA A2 tetramers contained the HCV peptides NS3 1073–1081, NS3 1406–1415, NS4 1812–1820, NS4B 1992–2000, and NS5 2594–2602. Anti-IFN- γ FITC (conjugated FITC-FITC) was purchased from Sigma-Aldrich; anti-CD8 allophycocyanin and anti-CD 107 FITC were purchased from BD Pharmingen.

Isolation of PBMC and in vitro expansion of HCV-specific CTL

PBMC were isolated from fresh heparinized blood by Ficoll-Hypaque density gradient centrifugation and resuspended in RPMI 1640 supplemented with 25 mM HEPES, 2 mM L-glutamine, 50 μ g/ml gentamicin, and 8% human serum (complete medium). For CTL expansion, PBMC were resuspended in 96-well plates at a concentration 2×10^6 /ml in complete medium and stimulated with HCV peptides at 1 μ M final concentration. Recombinant IL-2 was added on day 4 of culture (50 UI/ml), and the immunological assays were performed on day 10.

Cell surface and intracellular staining

Staining with tetramers and other surface markers. A total of 1×10^6 PBMC either freshly isolated or following in vitro expansion for 10 days was incubated for 30 min at 37°C with the PE-labeled tetrameric complex in RPMI 1640 and 8% human serum. After washing, staining was performed for 15 min in the dark, using an anti-CD8 APC-conjugated Ab. The

cells were then washed and analyzed immediately on a BD Biosciences flow cytometry (FACSCalibur) using the CellQuest software.

IFN- γ staining. Tetramer stained cells were incubated in medium alone (control) or with viral peptides (1 μ M) for 1 h; brefeldin A (10 μ g/ml) was added for an additional 4 h of incubation. After washing, the cells were stained with an anti-CD8 quantum red mAb for 20 min at 4°C and then fixed and permeabilized with a permeabilization buffer (Caltag Laboratories). Cells were finally stained with anti-IFN- γ -FITC for 15 min at room temperature, washed again, and analyzed by flow cytometry.

ELISPOT assay

The 601 15-mer peptides overlapping by 10 residues and covering the overall HCV-1 sequence were pooled in 60 mixtures, each containing 10 synthetic peptides. HCV-specific T cell responses were analyzed upon overnight stimulation with individual peptide mixtures (1 μ M of each peptide). Briefly, 96-well plates (Multiscreen-I; Millipore) were coated overnight at 4°C as recommended by the manufacturer with 5 μ g/ml capture mouse anti-human IFN- γ mAb (1 DIK; Mabtech). Plates were then washed seven times with PBS/0.05% Tween 20, and then blocked with RPMI 1640/10% FCS for 2 h at 37°C. A total of 2×10^5 PBMC was seeded per well. The plate was incubated for 18 h at 37°C in the presence or in the absence of peptides. After washing with PBS/0.05% Tween 20, a biotinylated secondary mouse anti-human IFN- γ mAb (1 μ g/ml, 7B6-1; Mabtech) was added. After 3 h of incubation at room temperature, plates were washed four times, and 100 μ l of goat alkaline phosphatase anti-biotin Ab (Vector Laboratories) was added to wells, and the plates were incubated for an additional 2 h at room temperature. Plates were then washed four times, and 75 μ l of alkaline phosphatase conjugate substrate (5-bromo-4-chloro-3-indolyl phosphate; Bio-Rad) was added. After 4–7 min, the colorimetric reaction was stopped by washing with distilled water. Plates were air-dried, and spots were counted using an automated ELISPOT reader (AID Elispot Reader System; Autoimmune Diagnostika). IFN- γ -producing cells were expressed as spot-forming cells (SFC) per 1×10^6 cells. The number of specific IFN- γ -secreting cells was calculated by subtracting the unstimulated control value from the stimulated sample. Unstimulated wells never exceeded five to seven spots per well. Positive controls consisted of PBMC stimulated with PHA. Wells were considered positive if they were at least twice above background.

RNA extraction, amplification by the PCR, direct sequencing, and sequencing of molecular clones

Viral RNA was extracted from 600 μ l of serum using a kit RNAfast isolation system (RNA fast; Molecular System). The RNA was eluted in 30 μ l of RNase-free water. The extracted RNA (3 μ l) was reverse transcribed at 24°C for 10 min, 42°C for 25 min, and 99°C for 5 min in a 20- μ l reaction mixture with 50 μ M random hexamers and 2 U of murine leukemia virus reverse transcriptase (GeneAmp RNA PCR; Applied Biosystems). Nested primer sets were constructed to amplify each region of the HCV genome that contained HLA class I-restricted epitopes. The sequences of the primers are indicated in Table I. PCR conditions were as follows: 1 cycle (95°C-5 min, 56°C-2 min, 72°C-1 min); 35 cycles (95°C-45 s, 56°C-45 s, 72°C-45 s); and 1 cycle (72°C-10 min). Five microliters of the first PCR product was then reamplified with inner primers for 35 cycles under the same reaction conditions as in the first round of PCR. As the template for the amplification, 5 μ l of the cDNA was used. PCR was performed in a total volume of 50 μ l in a reaction mixture containing 50 pmol of each of the respective primers and 5 U of TaqDNA polymerase (AmpliTaq Gold; Applied Biosystems). A product of the predicted size was observed after electrophoresis on a 1.5% agarose gel when visualized under UV light after ethidium bromide staining. This cDNA band was excised from the gel and the cDNA was purified using a cDNA purification kit (QIAquick gel extraction kit; Qiagen). PCR products were cloned and sequenced. The extracted products were ligated into the plasmid pGEM-Teasy vector (Promega). Transformation of recombinant plasmid DNA into *Escherichia coli* competent cells was performed as specified by the manufacturer and transformants were grown on Luria-Bertani/Ampicillin/isopropyl β -D-thiogalactoside/5-bromo-4-chloro-3-indolyl β -D-galactoside plates. Plasmid DNA was isolated by the miniprep method. For each sample, 15–25 clones were amplified, and DNA sequences were determined using the ABI Prism 377 DNA Sequencing System (PerkinElmer).

³ Abbreviations used in this paper: ALT, alanine aminotransferase; SFC, spot-forming cell; ICS, intracellular cytokine staining.

Table I. HCV primers for amplification and sequencing

		Sense		Antisense	
CVNGVCWTV 1B	(ES)	5'-ATCACGGCTACTCCCAACA-3'	3426-3445	(EA)	5'-ATCAGCATGTCTCGTGACCA-3'
	(IS)	5'-AGCCTCACAGGCCGGGACAA-3'	3477-3496	(IA)	5'-TCCGAGCTGCCGAGGTGCA-3'
CINGVCWTV 1A	(ES)	5'-GTACGCCAGCAGACAAG-3'	3434-3451	(EA)	5'-TGCAGGGTGTCAATGAGC-3'
	(IS)	5'-GTACGCCAGCAGACAAG-3'	3434-3451	(IA)	5'-TCTGGATGACAGGACCCTT-3'
VLSDFKSWL 3A	(ES)	5'-TGCTGAGTTCTCTAACTGTC-3'	6198-6217	(EA)	5'-GTACAGGGCCCGTGGTGTGA-3'
	(IS)	5'-CTTGTAGCGACGATTGGCTAC-3'	6269-6289	(IA)	5'-CCCTGCAAGCCGAGACC-3'

Results

Identification of immunodominant T cell responses in genotype 1 infected patients

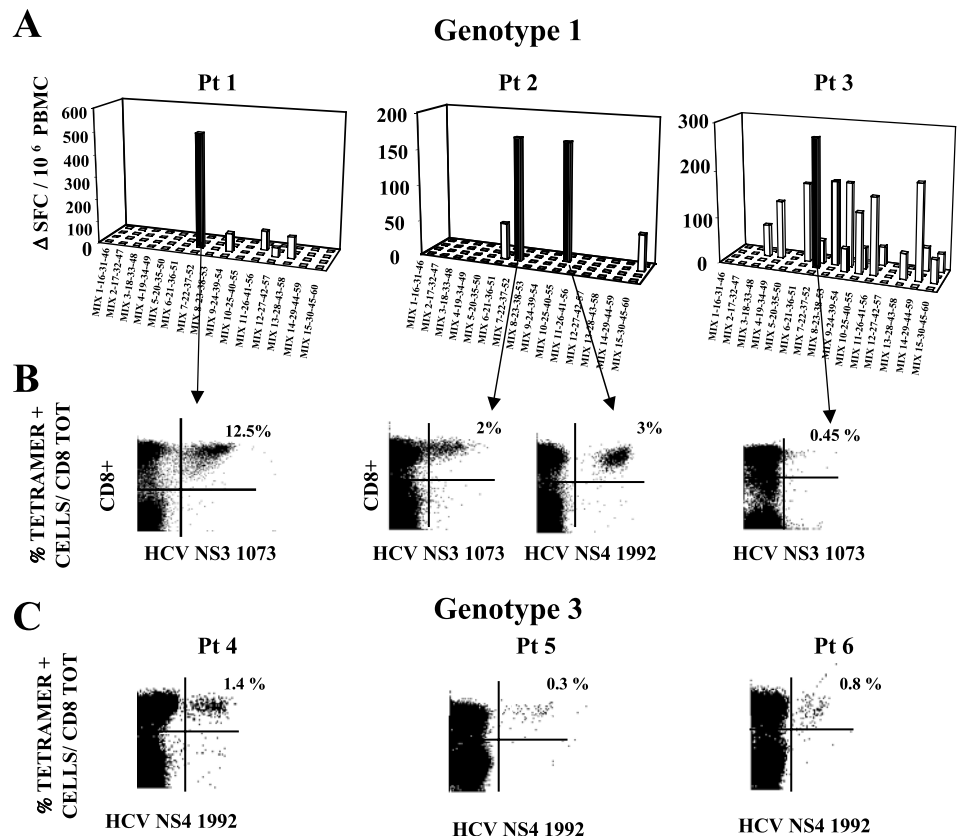
The global profile of the HCV-specific T cell response was analyzed in three HLA-A2⁺ patients with acute hepatitis C infected by genotype 1 (genotype 1b, patients 1 and 3; genotype 1a, patient 2). In these patients the entire HCV genome was comprehensively screened by ELISPOT using a panel of 601 15-mer peptides overlapping by 10 residues and spanning the entire HCV sequence of genotype 1. The analysis of the breadth and the magnitude of the T cell response during the acute phase of infection at the peak of ALT, revealed that in patients 1 and 2 virus-specific responses were generally weak and directed against a limited number of epitopes. Of the 60 mixtures of synthetic peptides tested, 5 in patient 1 and 4 in patient 2 were able to induce IFN- γ production with different intensity ranging from 50 to 500 SFC/10⁶ PBMC (Fig. 1A). A response of similar intensity was detected in patient 3 but a larger number of peptide pools (17 pools) were positive. The breadth of the T cell response was confirmed 2 wk after the time point represented in Fig. 1; the same positive pools were still detectable in patients 1 and 2 and 9 of the 14 responses were still positive in patient 3 (data not shown). Further analysis performed by intracellular cytokine staining (ICS) with the peptides contained in the

stimulatory pools allowed to identify in each patient the epitopes corresponding to the best ELISPOT responses. The only peptides responsible for cytokine production within pool 22 in patient 1 and within pools 22 and 40 in patient 2 were the 15 mers containing the HLA-A2-restricted epitopes HCV NS3 1073-1081 and NS4 1992-2000 (data not shown). Responses against these epitopes were subsequently analyzed by ex vivo tetramer staining. Elevated frequencies of HCV-specific CD8 cells were detected, confirming the immunodominance of these epitopes in these two patients (Fig. 1, A and B). In patient 3, the peptides contained in each responsive pool could not be analyzed extensively because of cell limitations. Only pool 22, which was able to stimulate the strongest ELISPOT response, was screened in ICS with the individual component peptides. A detectable response was elicited only by the 15 mer peptide containing the sequence 1073-1081, as confirmed by tetramer staining detecting elevated frequencies of CD8 cells specific for the NS3 1073-1081 epitope (Figs. 1, A and B, and 2).

Analysis of the CD8 response against preselected peptides in genotype non-1 (genotype 3) infected patients

Three more patients with acute hepatitis, infected by genotype 3 HCV were studied. Because the panel of peptides spanning the

FIGURE 1. Identification of immunodominant CD8-mediated responses in 6 patients with acute hepatitis C. A, IFN- γ production by direct ex vivo ELISPOT analysis. PBMC from three patients (patients 1, 2, and 3) acutely infected by HCV of genotype 1 were stimulated overnight with 60 pools of overlapping 15-mer peptides covering the whole HCV sequence (Mix1-4, HCV core; Mix 5-8, HCV E1; Mix 9-15, HCV E2; Mix 16-17, HCV p7; Mix 18-21, HCV NS2; Mix 22-33, HCV NS3; Mix 34-40, HCV NS4; Mix 41-49, HCV NS5A; Mix 50-60, HCV NS5B). ■ indicates the peptide pools able to induce the strongest T cell responses, which were then analyzed with individual peptides to identify the stimulatory sequences. The results are expressed as δ SFC per 10⁶ PBMC. B and C, Peripheral blood frequency of HCV-specific CD8 cells by ex vivo tetramer staining in HLA-A2⁺ patients infected either by HCV of genotype 1 (patients 1, 2, and 3; B) or by HCV of genotype 3 (patients 4, 5, and 6; C). ELISPOT and dot plot analyses were performed during the acute phase of infection at the ALT peak.



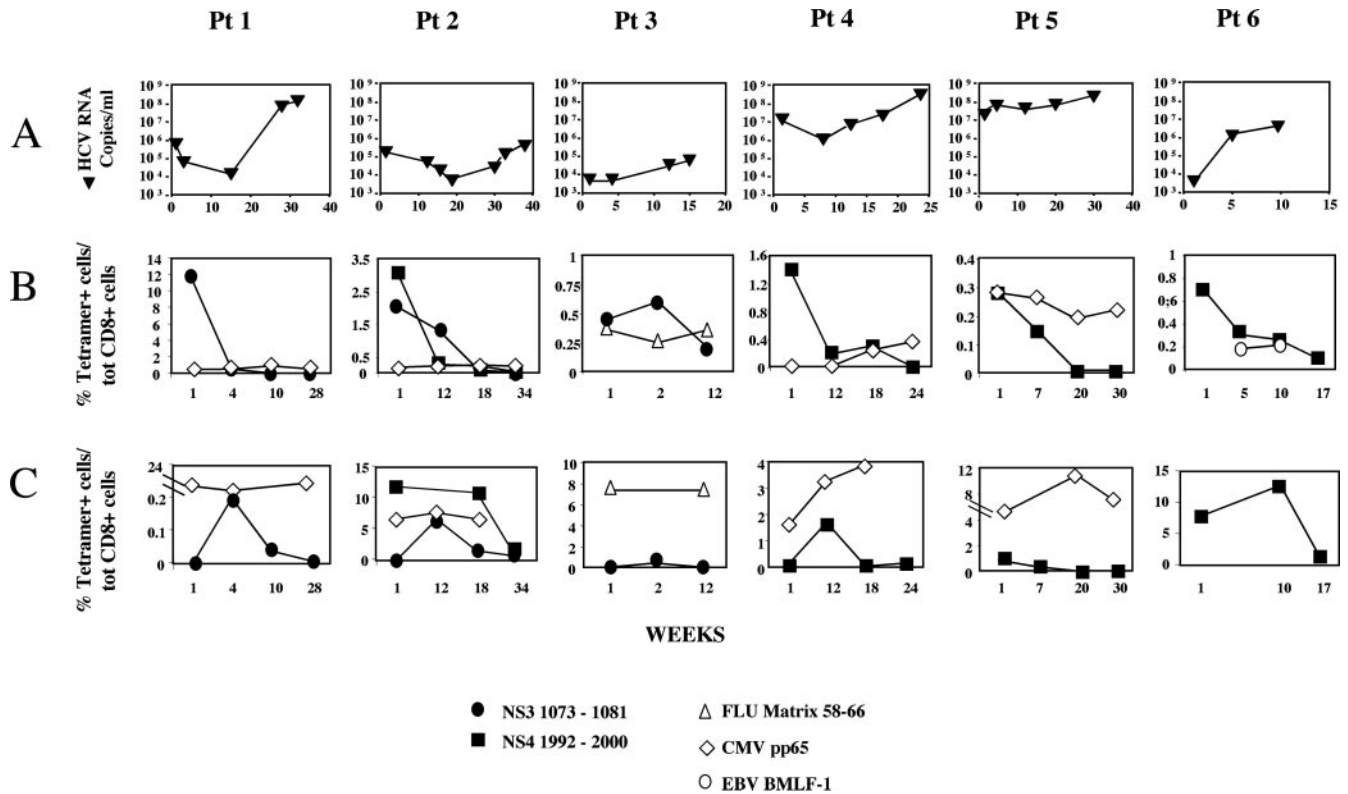


FIGURE 2. Longitudinal analysis of viremia and T cell responses from the acute phase of infection. Viremia levels were analyzed at the indicated time points (A). The frequency of tetramer positive cells was evaluated either ex vivo (B) or upon in vitro 10 days peptide stimulation (C) at different time points from the onset of the disease throughout a follow-up ranging from 12 to 34 wk in six HLA-A2⁺ patients with acute HCV infection. The percentage of tetramer⁺ CD8 cells specific for HCV NS3 1073 (●), HCV NS4 1992 (■), and HCV-unrelated viruses, such as EBV, CMV, and influenza (white symbols), is represented.

entire virus corresponded to a sequence of genotype 1 (HCV-1), it was not possible to perform a comprehensive analysis of the HCV-specific T cell repertoire in these patients (patients 4, 5, and 6). For this reason, T cell analysis was limited to five CD8 epitopes, known to be frequently recognized by HLA-A2 positive patients in acute HCV infection corresponding to NS3 1073–1081, NS3 1406–1415, NS4 1812–1821, NS4 1992–2000 and NS5 2627–2635. Sequencing of the HCV regions NS3 1073–1081 and NS3 1406–1415 was performed in patients 5 and 6 and the peptides corresponding to the autologous sequences (Fig. 3) were synthesized. With these peptides matching the corresponding sequence of the infecting virus, ELISPOT and ICS analyses were performed in patients 5 and 6 as well as in patient 4. Moreover, a panel of peptides containing the NS4 1812 (MFFNILGGWV), NS4 1992 (VLSDFKSWL), and NS5 2594 (ALYDVIQKL) sequences of genotype 3 was tested in all three patients. Positive responses were elicited only by the NS4 1992–2000 peptide (data not shown) and results were confirmed by tetramer staining, showing elevated frequencies of CD8 cells specific for this epitope (Fig. 1C); staining was negative for the other tetramers containing the four additional peptides. Levels of CD8 frequencies were in the range of the values previously reported in the acute phase of infection (11–13, 16, 21, 27, 28), suggesting that these responses should be immunodominant for the patients studied.

Longitudinal analysis of the CD8 response

All patients developed a persistent infection and the positive T cell responses were followed sequentially over time. The frequency of tetramer positive cells either ex vivo or upon in vitro peptide stimulation was evaluated at different time points from the acute phase

of infection throughout a follow-up ranging from 12 to 34 wk (Fig. 2). In all patients, the frequency of tetramer positive cells ex vivo declined rapidly and became either negative (<0.01% of the total CD8⁺ cells) or barely detectable (Fig. 2B). Viremia was partially suppressed at the time of clinical presentation or shortly after in patients 1, 2, 3, and 6. In patient 4 the decline of viremia was less profound but still detectable, while in patient 5 viremia levels remained constantly elevated throughout the follow-up (Fig. 2A). As previously reported, HCV-specific cells can be functionally impaired during the acute phase of infection and can be unable to expand efficiently upon peptide stimulation (11, 13–15). In keeping with these observations, no expansion following 10 days of in vitro peptide stimulation was observed by tetramer staining (tetramer⁺ cells < 0.01% of the total CD8 cells) in most patients at the first time point tested corresponding to the peak of ALT (Fig. 2C). The capacity to proliferate recovered few weeks later but then declined before or concurrently with a new elevation of viremia levels (Fig. 2, A and C). The stimulation with control peptides corresponding to CMV, EBV, or influenza epitopes induced a good expansion of T cells at all time points tested with no correlation with the evolution of the disease (Fig. 2C), indicating that the impairment of the T cell responses was HCV specific.

Sequencing of the relevant HCV epitopes

Viral escape by mutations at critical positions of immunodominant epitopes is a potential mechanism of immune evasion. Therefore, we next investigated whether viral sequence evolution and immune evasion by the selection of escape mutations could be responsible for the decline of the CD8 response. The two regions of the HCV genome encoding the CD8 epitopes targeted by the six

Genotype		Acute phase		
1b	Pt 1 NS3 1073-1081	1 week	3 weeks	21 weeks
		TFLATCINGVCWTVYHGAG ----- ----- 100%	TFLATCINGVCWTVYHGAG ----- ----- 100%	TFLATCINGVCWTVYHGAG ----- ----- 100%
1a	Pt 2 NS3 1073-1081 NS4 1992-2000	1 week	4 weeks	22 weeks
		TFLATCINGVCWTVYHGAG ----- 36% -----V----- 64% DWVCSVLSDFKTWLSAKIM ----- DIRECT	TFLATCINGVCWTVYHGAG -----A----- 64% ----- 32% DWVCSVLSDFKTWLSAKIM ----- DIRECT	TFLATCINGVCWTVYHGAG ----- ----- 100% DWVCSVLSDFKTWLSAKIM ----- DIRECT
1b	Pt 3 NS3 1073-1081	1 week		17 weeks
		TFLATCINGVCWTVYHGAG -----V----- 100%		TFLATCINGVCWTVYHGAG ----- ----- 100%
3a	Pt 4 NS4 1992-2000	1 week	11 weeks	37 weeks
		DWVCSVLSDFKTWLSAKIM ----- 89% -----C----- 11%	DWVCSVLSDFKTWLSAKIM ----- ----- 100%	DWVCSVLSDFKTWLSAKIM ----- DIRECT
3a	Pt 5 NS4 1992-2000	1 week	18 weeks	30 weeks
		DWVCSVLSDFKTWLSAKIM ----- 100%	DWVCSVLSDFKTWLSAKIM ----- 75% -----C----- 25%	DWVCSVLSDFKTWLSAKIM ----- ----- 100%
3a	Pt 6 NS4 1992-2000	6 weeks*	11 weeks	18 weeks
		DWVCSVLSDFKTWLSAKIM -----S----- 35% -----R----- 30% -----R----- 15% -----T----- 10% ----- 5% -----Y-R----- 5%	DWVCSVLSDFKTWLSAKIM -----R----- 74% -----T----- 13% -----Y----- 13%	DWVCSVLSDFKTWLSAKIM -----R----- 100%
		NS3 1073-1081 NS3 1406-1415 -----TVGGVMWTV--- -----DIRECT -----KLRGMGLNAV--- -----F-----DIRECT		

FIGURE 3. Longitudinal evolution of the HCV sequences corresponding to the identified CD8 epitopes. PCR products and molecular clones of the HCV epitopes NS3 1073 and NS4 1992 were sequenced at three indicated time points from clinical onset. Fifteen to 25 molecular clones were sequenced for each time point. *Top rows*, The sequences most frequently reported in gene bank among the isolates of the genotype infecting each patient. *Bottom rows*, The sequences of the autologous virus. The sequences of the HCV regions that did not induce significant CD8 responses are illustrated within shaded areas.

patients studied (NS3 1073–1081 and NS4 1992–2000) were amplified by PCR and multiple molecular clones were sequenced (Fig. 3). Direct sequencing and clonal analysis were performed at three sequential time points starting from the clinical onset.

Sequencing of the immunogenic peptides indicates that in the acute stage of infection the epitopic regions carried by the infecting virus generally matched the corresponding sequences more frequently reported among the published HCV strains of identical genotype. In four of the seven sequences tested, the autologous virus corresponded exactly to the sequence of the peptides used and no mutational event occurred over time. In particular, in patient 1 the dominant infecting sequence of the NS3 1073 epitope was identical at all time points tested from the acute phase to 21 wk later. Similar results were obtained in patients 2, 4, and 5 by sequencing the epitope NS4 1992–2000. Although a small percentage of clones in patients 4 and 5 showed a mutation in the COOH-terminal flanking region (S to C), this event was only transient and no amino acid changes within the sequence of the immunodominant epitope (VLSDFKTWL) were detectable at any of the time points tested.

In contrast, in patients 2 and 3, the NS3 1073–1081 sequence CVNGVCWTV, which was homogeneously (patient 3) or predominantly (patient 2) present at the time of the ALT peak, was completely replaced over time by the sequence C/NGVCWTV. In pa-

tient 2, 4 wk after clinical onset an additional mutation leading to an amino acid change (V to A) in position 5 of the epitope emerged transiently (Fig. 3) and was no more detectable 18 wk later.

In patient 6, sequence analysis could not be performed at the ALT peak, due to the lack of serum samples. For this reason, the NS4 1992–2000 epitope was first sequenced 6 wk after clinical onset. As shown in Fig. 3, at the first time point tested six different viral species were simultaneously present which differed at residues 3, 6, and 7 of the epitope. This region continued to evolve over time with a mix of three different populations present at week 10 and a single viral variant corresponding to the sequence VLSDFKRWL detectable six weeks later.

Effect of viral mutations on CD8 responses

Two different types of experimental approaches were used to assess whether epitope recognition could be affected by the emergence of mutations and to compare the relative immunogenicity of the different viral sequences detected in individual patients. First, freshly isolated T cells of the earliest time points and T cell lines generated upon 10 days in vitro peptide stimulation were used to compare how efficiently peptides undergoing selection and becoming prevalent over time (variant peptides) were recognized by CD8

cells compared with peptides present early in infection but undetectable at later time points (prototype peptides). Second, the capacity of the different sequences detected in individual patients to induce a CD8 response by 10 days peptide stimulation *in vitro* was compared at different time points after clinical onset.

In patients 2 and 3, all detected sequences of the NS3 1073–1081 region were first tested at different peptide concentrations by *ex vivo* ELISPOT analysis with PBMC of the acute phase infection when the infecting HCV quasispecies was either exclusively or predominantly CVNGVCWTV (in patients 2 and 3, respectively). Although the sequence eventually selected (C/NGVCWTV) was recognized by CD8 cells only at very high peptide concentrations (1–10 μ M), significant T cell responses were already induced by the CVNGVCWTV sequence at 1–10 nM concentration of the peptide (Fig. 4A). Identical results were obtained when T cell lines produced by stimulation of PBMC derived from the acute stage of infection with the CVNGVCWTV peptide were tested with different concentrations of CVNGVCWTV or C/NGVCWTV peptides (Fig. 4A). In addition, the new variant (C/NGACWTV) emerging transiently in patient 2 and the C/NGVCWTV peptide that was finally selected in both patients were also less efficient than the CVNGVCWTV sequence in CD8 expansion *in vitro* because at peptide concentrations < 1 μ M only

the CVNGVCWTV peptide was able to induce efficient CD8 expansion and IFN- γ production (Fig. 5). Efficiency in induction of CD8 responses did not change throughout the follow-up (Fig. 5).

In patient 6, the amino acid sequence of the NS4 1992–2000 epitope was already heterogeneous at the time of the first sequencing analysis (Fig. 3). Three weeks from the onset, the strongest T cell responses in *ex vivo* ELISPOT analysis using serial dilutions of the different peptides were induced by the sequences VLSDFKTWL, followed by VLSDFKSWL and VLTDFTWL (Fig. 4A), which disappeared at the two subsequent time points tested during the follow-up. In contrast to the high avidity recognition of these sequences (in the nM range), the viral variant VLSDFRWL that was selected among the viral quasispecies and finally remained the only detectable sequence, was able to induce poor levels of T cell response *ex vivo* (Fig. 4A). Moreover, this sequence was recognized by CD8 cells expanded *in vitro* upon stimulation with the VLSDFKTWL sequence much less efficiently than the stimulatory peptide and only at very high concentrations (Fig. 4A). The hierarchy of T cell stimulatory capacity by the different sequences detected *ex vivo* was confirmed by *in vitro* experiments of 10 days peptide stimulation and remained the same throughout the follow-up (Fig. 5).

ACUTE PHASE OF INFECTION

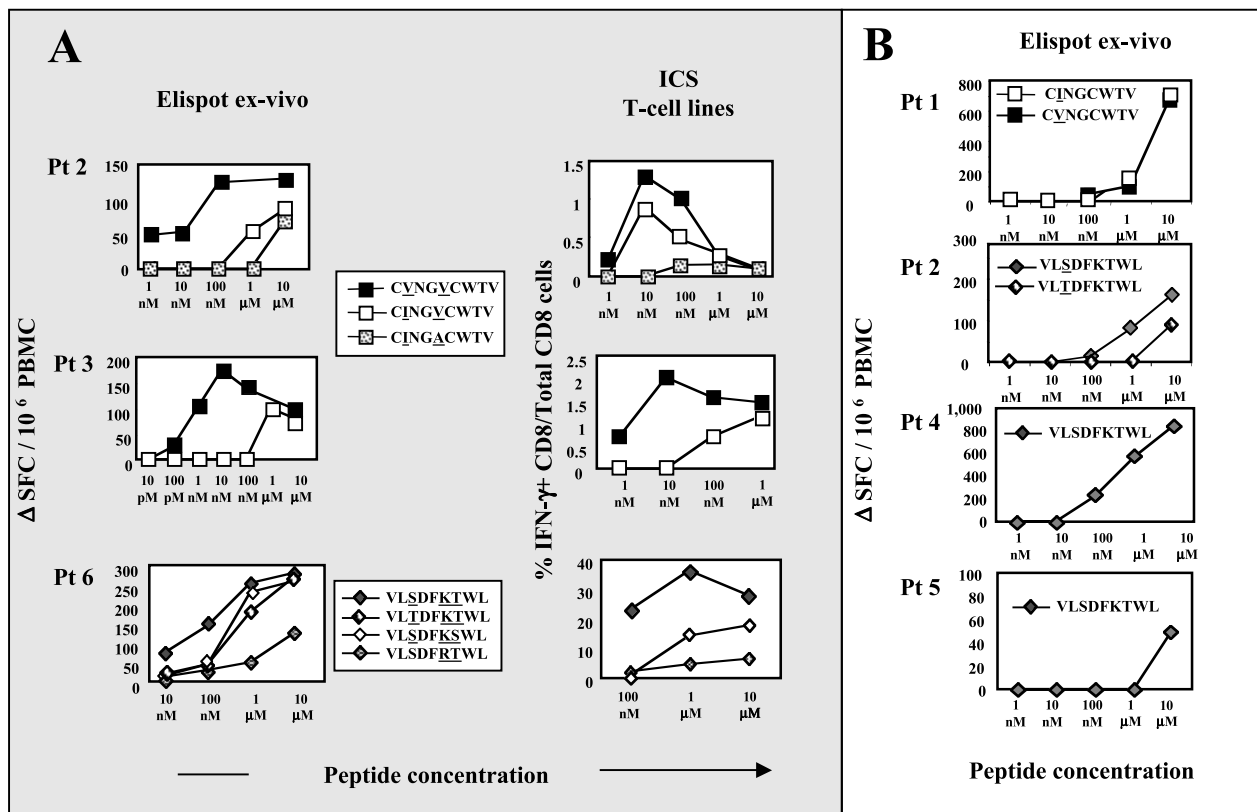


FIGURE 4. Titration of CD8 responses with peptides corresponding to autologous HCV sequences. CD8-mediated responses to each of the identified epitopes were tested *ex vivo* by ELISPOT analysis in all six patients with acute HCV infection. Efficiency of recognition of the different sequences identified over time in patients 2, 3, and 6 was tested also *in vitro* by intracellular cytokine staining on short-term T cell lines obtained by *in vitro* 10 days peptide stimulation (graphs in the middle). Both *ex vivo* and *in vitro* analyses were performed on PBMC of the earliest available time point corresponding to clinical presentation. ELISPOT results are expressed as Δ SFC per 10^6 PBMC detected upon overnight incubation with the indicated concentrations of peptides (A). T cell lines were induced with different concentrations of CVNGVCWTV peptide in patients 2 and 3 and tested by intracellular cytokine staining with CVNGVCWTV, C/NGVCWTV, and C/NGACWTV peptides. In patient 6, T cell lines were generated by stimulation with VLSDFKTWL peptide and tested by intracellular cytokine staining with VLSDFKTWL, VLSDFKSWL, and VLSDFRWL peptides. Results are expressed as percentage of IFN- γ -positive cells among the total CD8 population. Responses to epitopes where mutations were detected are illustrated within the shaded area; the white area identifies the responses to epitopes that did not change over time (B).

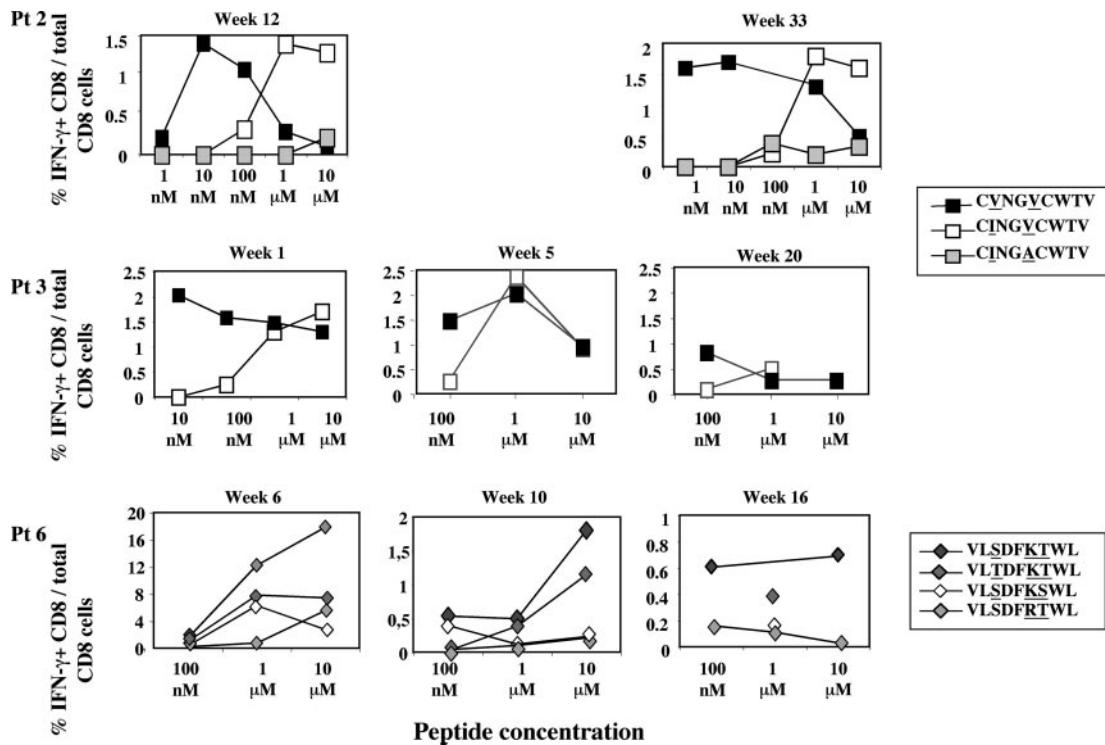


FIGURE 5. Efficiency of the different HCV sequences identified in each patient to induce a CD8 response in vitro at different time points following clinical onset. Short-term T cell lines were induced by 10 days stimulation with different concentrations of each peptide identified in each patient and tested by intracellular cytokine staining after 4 h of stimulation with 1 μ M of the original stimulatory peptide; results are expressed as percentage of IFN- γ -positive cells among the total CD8 population.

Functional characterization of HCV-specific T cell responses *ex vivo*

Although elevated frequencies of HCV-specific CD8 cells were detectable in all individuals during the acute phase of infection (as shown by tetramer staining in Fig. 1), mutations emerged only in three of the seven epitopes identified. To better understand the reason for this limited occurrence of mutations despite the apparent immunodominance of the epitopes analyzed, the functional features of HCV-specific T cell responses associated with selection of escape mutations were compared with those associated with no evolution of the viral sequences.

Because high avidity CD8 cells have been shown to clear the virus more efficiently than low avidity cells and should thus exert a stronger immune pressure (29–32), we first compared the degree of avidity recognition of the epitopes acquiring mutations or remaining unchanged over time. *Ex vivo* analysis of the CD8 response to the epitopes that did not change over time in patients 1, 2, 4, and 5 (Fig. 4B) showed that the amount of peptide ligand required to exert effector function at clinical presentation was much higher than that required to exert the same function by CD8 cells able to express selective pressure on the epitope in patients 2, 3, and 6 (Fig. 4A).

In keeping with the results obtained in patients 2 and 3, the avidity of the NS3 1073 CINGVCWTV peptide recognition in patient 1 was low because concentrations > 100 nM were needed to induce IFN- γ production *ex vivo*. Remarkably, identical reactivity was detected in patient 1 with the CVNGVCWTV peptide that in contrast was able to induce T cell activation at much lower concentrations (nM range) in patients 2 and 3. The epitope NS4 1992 VLSDFKTWL displayed a low functional avidity in patients 2, 4, and 5, although the same epitope was recognized with high avidity by CD8 cells of patient 6 and showed a great degree of

variation in this patient. Moreover, low avidity T cell responses were detected in patient 2 also with the peptide VLTDFKTWL, which is generally carried by genotype 1b HCV.

Low avidity recognition of the peptide ligand by high frequency tetramer positive CD8 cells suggests that lack of emergence of escape mutations within immunodominant CD8 epitopes is the result of an impaired CD8 function. To further address this issue, we then tested the degranulation efficiency of CD8 cells *ex vivo* in response to peptide stimulation, as shown by CD107a up-regulation. Despite the high frequency of tetramer positive CD8 cells specific for HCV epitopes that did not undergo variations over time, the level of degranulation *ex vivo* in patients 1, 4, and 5 was very poor or totally undetectable upon CD8 stimulation with the corresponding peptide (Fig. 6). In contrast, CD107a up-regulation was clearly more efficient in patients 2, 3, and 6 in whom mutational events were observed.

A functional impairment of CD8 cells specific for the epitopes with no evidence of mutations was further confirmed at the time of clinical onset by the discrepancy between frequency of HCV-specific CD8 cells detected by tetramer staining and frequency of CD8 cells of the same epitope specificity able to produce *ex vivo* IFN- γ detected by ELISPOT (Fig. 7). Thus, most CD8 cells specific for epitopes without evidence of mutations were unable to produce IFN- γ , whereas the majority of CD8 cells specific for epitopes that underwent variations produced efficiently IFN- γ .

Finally, we asked whether CTL escape could be facilitated by an impaired CD4 function in view of the evidence that the lack of CD4 help in the setting of a monospecific CD8 response in chimpanzees can favor escape (33). The helper T cell response was studied by *ex vivo* ICS for IFN- γ after stimulation with recombinant HCV Ags only in genotype 1 infected patients 1, 2, and 3 because genotype 1 HCV proteins were available. Two genotype 1

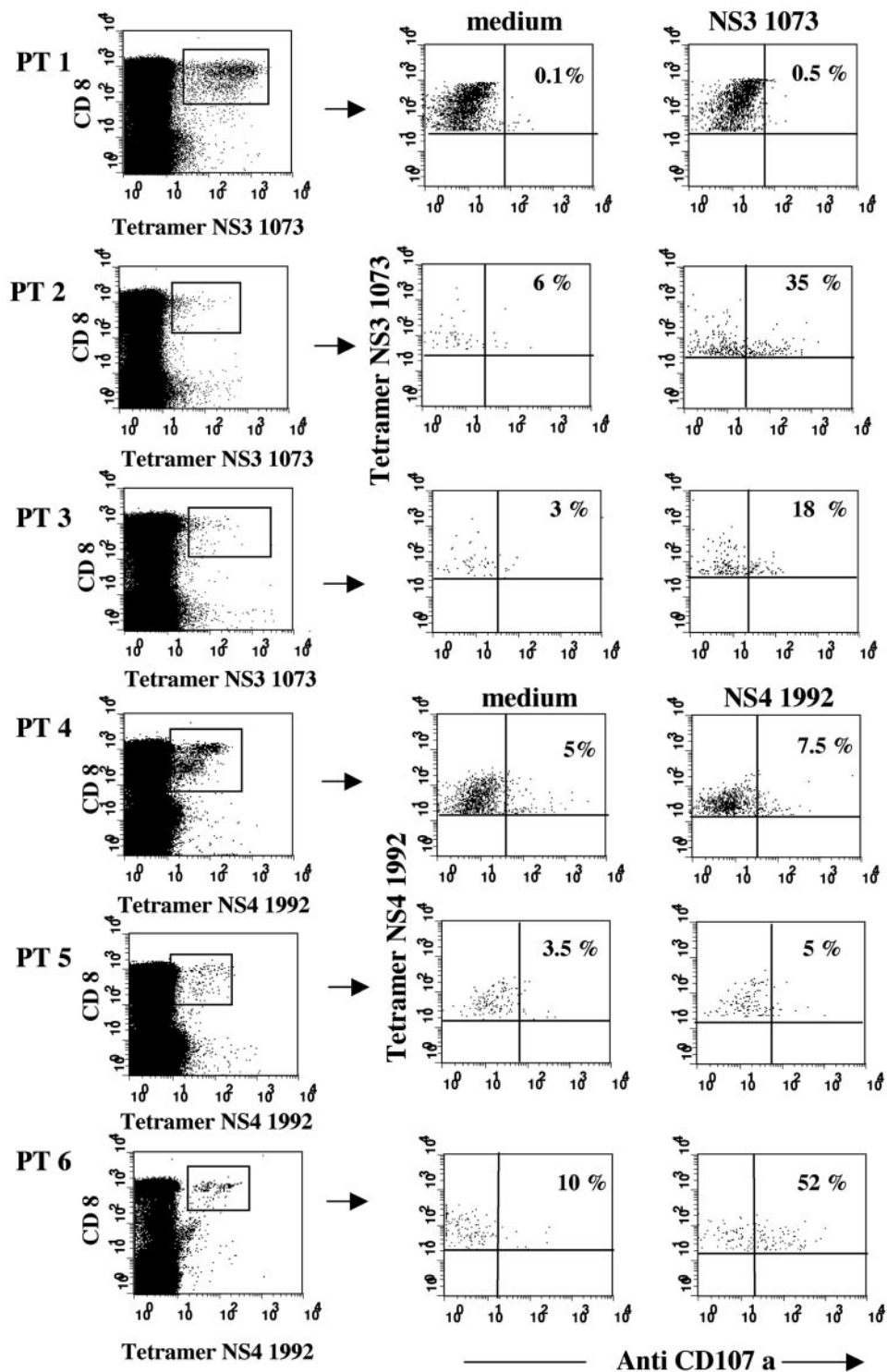


FIGURE 6. Ex vivo expression of CD107a in HCV-specific CD8 cells at the time of clinical onset. PBMC from patients 1 to 6 were incubated for 2 h with or without HCV NS3 1073 or NS4 1992 specific peptides (1 μ M). The dot plots represent CD107a expression in gated HCV tetramer⁺ CD8 cells cultured in medium alone or with the relevant peptide.

infected patients with a self-limited evolution of infection were also studied in the acute phase of illness for comparison. The results indicate that IFN- γ production by CD4 cells was less efficient in patients with a chronic progression of disease compared with those with self-limited evolution of infection (Fig. 8), showing that escape in our patients occurred in the presence of an impaired CD4 help.

Discussion

Recognition and elimination of intracellular virus by HCV-specific CD8 cells is believed to be essential for successful recovery from HCV infection (11–12, 21, 28, 34). Therefore, the emergence of mutations within immunodominant CD8 epitopes able to abrogate protective CD8 responses represents a possible mechanism of

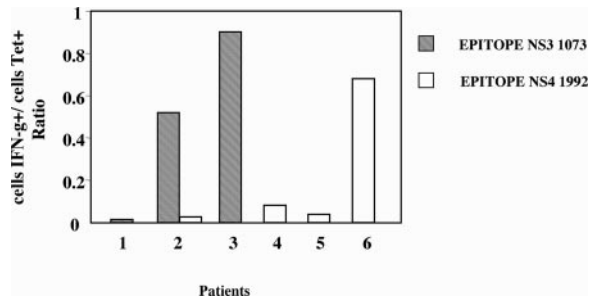


FIGURE 7. Ratio between ex vivo frequency of CD8 cells specific for HCV NS3 1073 or NS4 1992 detected by tetramer staining and IFN- γ ELISPOT. Calculations were done in each patient using IFN- γ ELISPOT data obtained with the peptides of optimal length (9 mers) for CD8 recognition. The ratio was calculated normalizing the number of IFN- γ ⁺ and Tet⁺ cells to total PBMC. The best concordance between tetramer staining and IFN- γ ELISPOT is expressed by ratios closer to 1.

virus evasion from immune surveillance that can contribute to HCV persistence.

To investigate the role of this mechanism in HCV pathobiology, particular attention was paid in our study to analyze the global T cell reactivity to identify the most dominant CD8 epitopes and to follow their evolution over time, because immunodominant CD8 responses are more likely to exert a strong selective pressure on the virus and to drive escape (32, 35–38). Moreover, the quality of the different epitope-specific CD8 responses was carefully characterized because it is increasingly clear that not all Ag-specific CD8⁺ cells are equally effective in viral clearance and that the avidity of these cells is one of the variables that can greatly impact on their antiviral efficacy (29–32). When reagents of the appropriate genotype were not available to perform a comprehensive analysis of the overall CD8 repertoire, selected peptides and tetramers were used to identify high frequency HCV-specific CD8 responses. The epitopes selected by these two experimental approaches in six patients with acute hepatitis C and chronic evolution of infection were all located within HCV regions that can tolerate mutations, as shown by their variability among HCV genotypes reported in the published HCV sequences. Moreover, T cell responses to NS3 1073 and NS4 1992 in patients 1 and 2 were not only strong but also narrowly focused; these features should confer optimal ability to CD8 cells to exert selective pressure, thereby enhancing the likelihood that selection of escape mutations can occur within the corresponding epitopes.

When the NS3 1073–1081 and NS4 1992–2000 regions were sequenced longitudinally from the time of clinical onset, emergence of mutations was observed in three of the six patients studied. Evidence for the selection of escape mutants was provided by the emergence of HCV sequences less efficiently recognized by CD8 cells not only ex vivo but also in vitro by CD8 lines generated by 10 days stimulation of PBMC derived from the earliest available time points with the epitopic sequences detectable at those times but totally lost at subsequent determinations. Moreover, the newly generated sequences were poorly immunogenic in terms of capacity to induce a CD8 response in vitro. To this inefficient priming of a new response may contribute the original antigenic sin as suggested by the stronger responses induced by the CVNGVCWTV variant even when the corresponding sequence was no more carried by the infecting virus.

Patient 3 was infected by genotype 1b which more frequently carries a CVNGVCWTV sequence in the NS3 region 1073–1081 (GenBank data). Because the infecting virus was 100% CVNGVCWTV at the first sequencing analysis, it is very likely that this

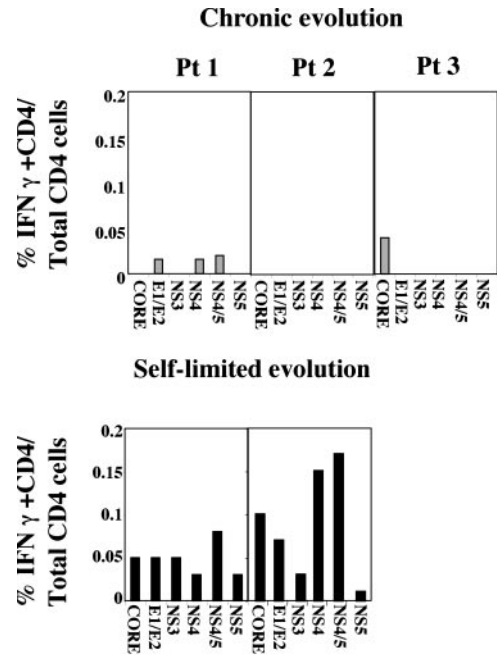


FIGURE 8. Ex vivo IFN- γ production by CD4 cells upon PBMC stimulation with recombinant HCV proteins during the acute phase of infection in patients with different outcome of disease. PBMC from patients with chronic (*top*) or self-limited (*bottom*) evolution of acute HCV infection were stimulated overnight with six recombinant HCV proteins representing the indicated structural and nonstructural HCV Ags and tested for IFN- γ production by ICS. Results are expressed as percentage of IFN- γ ⁺ CD4 cells among the total CD4⁺ population.

patient was infected by a HCV strain carrying this NS31073–1081 sequence. In view of the evidence that CD8 cells of patient 3 recognize much less efficiently the CINGVCWTV than the CVNGVCWTV variant, the CINGVCWTV sequence is likely to be an escape variant selected by the CD8 pressure. In patient 6, the viral quasispecies evolved from the coexistence of six different variants of the NS4 region 1992–2000 at the first available sequencing to the final selection of a single viral sequence (VLSDFRTWL). This viral evolution is consistent with escape because the variant eventually selected was the one less efficiently recognized by CD8 cells.

In patient 2, the sequence of the transmitted virus was undefined. Genotyping as well as sequencing of another viral region (NS4 1992–2000) indicate that the patient was infected by genotype 1a which generally carries a CINGVCWTV NS3 1073 sequence (GenBank data). The first available sequencing was not homogeneously represented by a single NS31073–1081 species, because two different NS3 1073–1081 sequences (CINGVCWTV and CVNGVCWTV) were present at the earliest sequenced time point. A possible interpretation is that the infecting virus carried a CVNGVCWTV sequence because this variant was recognized more efficiently by CD8 cells (Fig. 4) and was more potent in CD8 stimulation in vitro (Fig. 5). It is likely that the selective pressure exerted by CD8 cells contributed to the transition from a predominant CVNGVCWTV to a homogeneous CINGVCWTV variant. The possibility that also reversion to a more fit consensus sequence favored by a fitness advantage of the CINGVCWTV variant was operating in driving viral evolution to CINGVCWTV cannot be excluded, assuming a survival advantage for the CINGVCWTV variant in the context of a genotype 1a virus.

In four of the seven epitopes sequenced longitudinally in six different patients no mutations were detected during the time of the follow-up. Because only a limited number of preselected epitopes were tested in patients 4 and 5, we cannot exclude that in these patients the selected epitopes were subdominant and thus unable to drive escape, although the elevated frequencies of HCV-specific CD8 cells detected by tetramer staining were in line with the dominant responses previously reported in acute hepatitis C (11–13, 27). In patients 1 and 2, the HCV-specific response was comprehensively screened with a panel of peptides covering the overall HCV sequence. The response was narrowly focused on the NS3 1073 and NS4 1992 epitopes and sustained by high frequency CD8 cell populations (3–12% of the total circulating CD8 cells). Despite clear immunodominance, no mutations emerged during the time of the follow-up within NS3 1073 in patient 1 and within 1992–2000 in patient 2. A possible interpretation is that these CD8 responses were functionally impaired. The possibility was supported by three lines of evidence. First, recognition of these epitopic regions was always sustained by low avidity CD8 cells requiring high peptide concentrations for their activation. Because low avidity recognition of the peptide ligand is associated with lower effector efficiency (29–32), low avidity CD8 cells should be poorly effective in exerting selective pressure on the virus. Second, a much lower proportion of tetramer positive CD8 cells specific for the epitopes that did not show sequence changes was able to produce IFN- γ compared with CD8 cells specific for HCV regions undergoing variation of their sequences. Third, degranulation capacity, a function related to the cytolytic function of CD8 cells (39, 40), was defective in tetramer positive CD8 cells specific for epitopes that did not acquire amino acid variations.

Although the study of a limited number of epitopes requires caution in drawing conclusions, our data are consistent with the concept that the emergence of escape mutations in the acute stage of HCV infection reflects the residual functional efficiency of CD8 cells of different epitope specificity. Exhaustion as well as a direct inhibitory effect of HCV on T cell function may lead to early suppression of some dominant CD8 responses, which may be particularly profound if CD4 T cell help is defective (33, 41, 42). Thus, depending on the efficiency of these early strategies of T cell silencing, emergence of mutations may act as an additional mechanism of virus evasion from those dominant responses which are functionally preserved. Our results are consistent with the view that virus persistence is the result of a combined action of different complementary mechanisms which may operate simultaneously or sequentially in individual patients at the level of different dominant epitopes to silence the protective CD8 response.

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Disclosures

The authors have no financial conflict of interest.

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