

# Heterologous T cell immunity in severe hepatitis C virus infection

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**Hepatitis C virus (HCV) can cause liver disease of variable severity. Expansion of preexisting memory CD8 T cells by cross-reactivity with a new heterologous virus infection has been shown in mice to shape the repertoire of the primary response and to influence virus-related immunopathology (Selin, L.K. 2004. *Immunity*. 20:5–16). To determine whether this mechanism can influence the course of HCV infection, we analyzed the features of the HCV-specific CD8 T cell response in eight patients with acute HCV infection, two of whom had a particularly severe illness. Patients with severe hepatitis, but not those with mild disease, showed an extremely vigorous CD8 T cell response narrowly focused on a single epitope (NS3 1073–1081), which cross-reacted with an influenza neuraminidase sequence. Our results suggest that CD8 T cell cross-reactivity influences the severity of the HCV-associated liver pathology and depicts a model of disease induction that may apply to different viral infections.**

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Abbreviations used: ALT, alanine aminotransferase; HCV, hepatitis C virus; SFC, spot-forming cell.

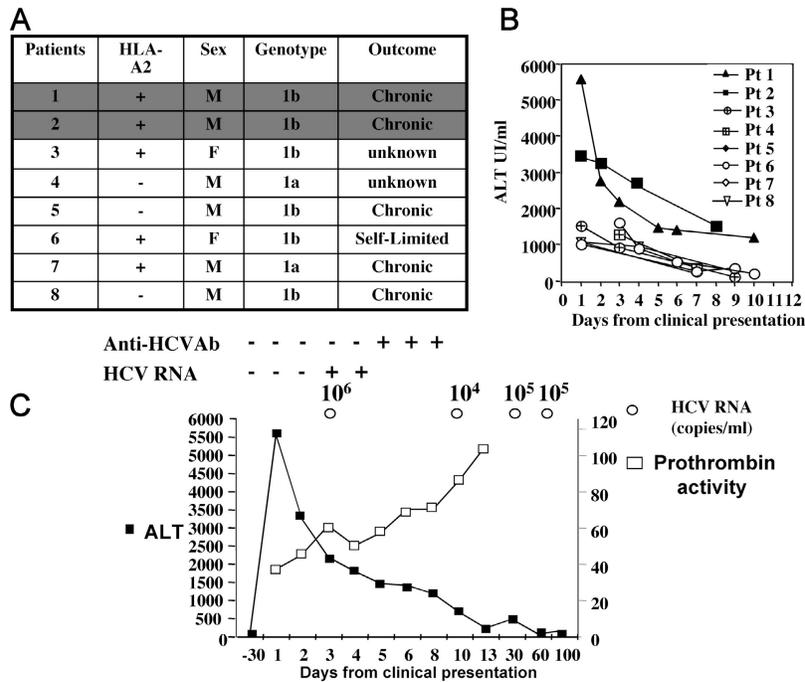
Hepatitis C virus (HCV) is believed to infect ~170 million people worldwide and represents one of the leading causes of liver disease, which is sustained primarily by the immune response to HCV. The majority of HCV-infected people develop chronic hepatitis, and the acute phase of infection is frequently asymptomatic. Clinical symptoms are present in approximately one third of acute infections acquired as an adult and are extremely heterogeneous, as is the case with many other human viral infections. They are generally mild, but some cases of hepatitis C can run an extremely severe course that is noted with a marked elevation of serum enzymes, which is indicative of liver cell damage (alanine aminotransferase [ALT]), and with clear signs of a loss of hepatic function (e.g., elevated bilirubin and prolonged prothrombin time; references 1, 2). Unfortunately, the mechanisms responsible for these different courses and outcomes are unknown. Differences in the infection dose, viral strain, and genetic make-up of the host have been used to explain such variability. A further possibility is that the variability in the pathology caused by viral infections strictly reflects different profiles of T cell immunodominance and different kinetics of T cell responses. This phenomenon

has been demonstrated in mice, and it is caused by the presence of a large repertoire of memory T cells from earlier infections that can cross-react with a second infecting viral pathogen, which leads to a massive recruitment of preexisting memory cells into a primary immune response (3–6).

In this paper, we report an association between a peculiar hierarchy of immunodominance of HCV-specific CD8 responses, cross-reactivity between HCV- and influenza-specific CD8 cells and a severe clinical course of hepatitis C. Our results suggest a role for CD8 cross-reactivity in influencing the severity of the HCV-associated liver pathology and depicts a model of disease induction that may apply to different viral infections in which immunopathology is sustained by the antiviral immune response.

## RESULTS AND DISCUSSION

To characterize HCV-specific CD8 T cell-mediated responses associated with severe liver pathology in acute HCV infection, we analyzed the global profile of the HCV-specific T cell response in eight patients who showed variable outcomes of acute HCV infection (Fig. 1 A). Two individuals (Fig. 1 A, patients

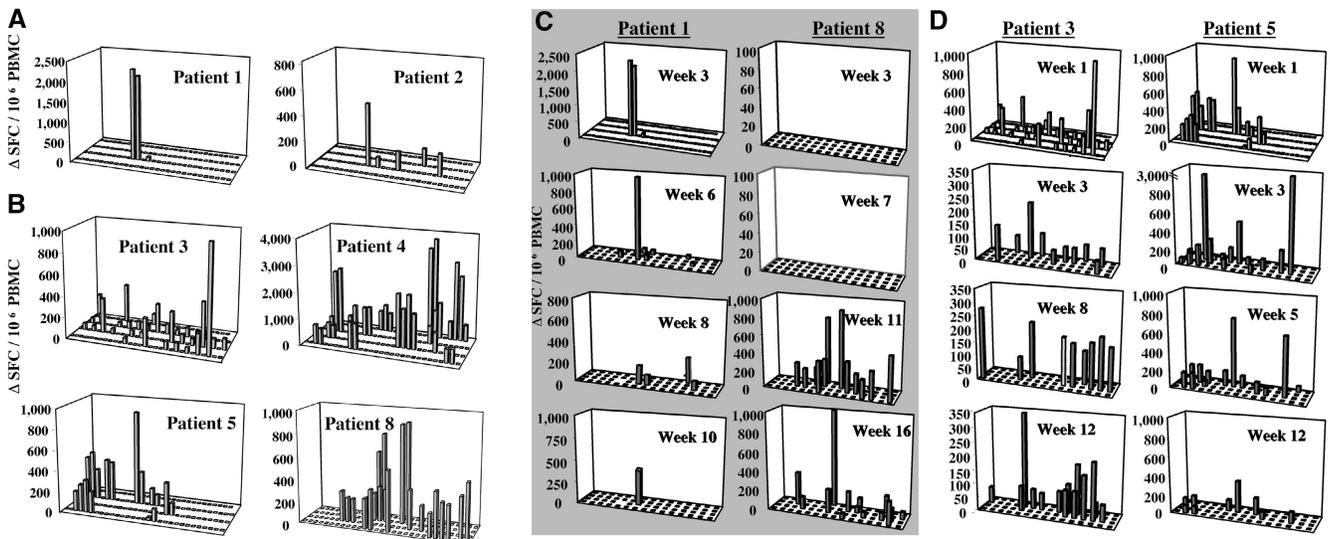


**Figure 1. Characteristics of the population of patients with acute hepatitis C.** (A) Clinical and virological features of the eight patients with acute HCV infection studied. (B) Sequential evaluation of serum ALT levels

from the time of clinical presentation. (C) Behavior of serum ALT levels (closed squares), prothrombin activity (open squares), and quantitative (open circles) and qualitative HCV-RNA and anti-HCV antibodies in patient 1.

1 and 2) that were infected with genotype 1b HCV showed a severe clinical course of acute hepatitis C with rapidly rising bilirubin levels, elevated ALT values, and prolonged prothrombin time (Fig. 1, B and C). Six patients (patients 3–8)

that were also infected by genotype 1 HCV displayed a mild course of liver disease, as generally observed after HCV infection (Fig. 1). A comprehensive analysis of the HCV-specific T cell repertoire was performed using a panel of 601 15-mer



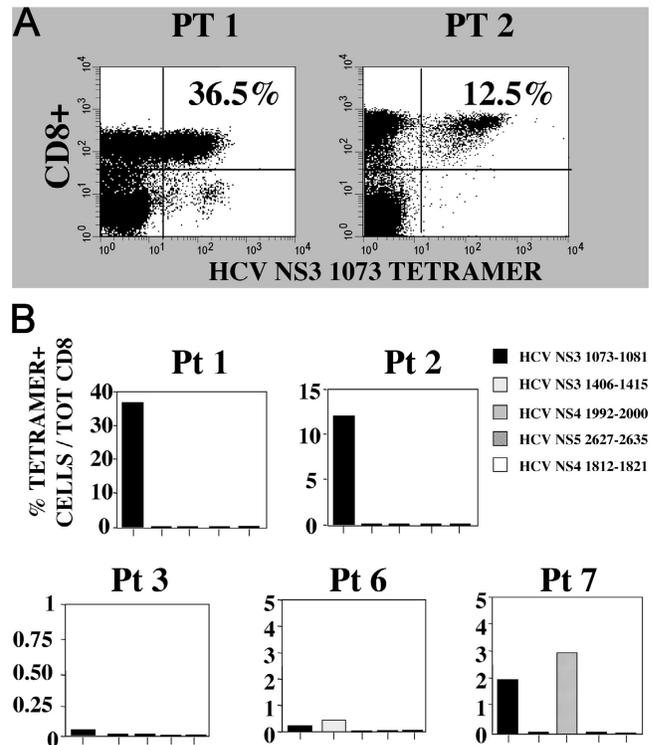
**Figure 2. IFN- $\gamma$  production by direct ex vivo ELISPOT analysis.** PBMCs from patients with severe course (A, patients 1 and 2) and with mild course (B, patients 3–5 and 8) of acute HCV infection were stimulated overnight with 119 pools of overlapping 15-mer peptides covering the whole HCV sequence. ELISPOT analysis was performed when the patients first reported to the clinics (Fig. 1 B, day 1). (C) Longitudinal analysis of the

T cell response to the 119 pools of overlapping peptides performed in patients 1 and 8 at the indicated weeks after the time of exposure to HCV that was precisely identified. (D) The same sequential study was performed in patients 3 and 5 at the indicated weeks after the first presentation in the clinics (unknown time of infection).

peptides overlapping by 10 residues and spanning the entire HCV sequence of genotype 1a. Direct ex vivo frequency of IFN- $\gamma$ -producing T cells was evaluated in patients 1–5 and 8 (Fig. 2, A and B) in the acute phase of infection at the peak of ALT. A dramatic difference in the T cell repertoire was evident in the two patient populations. Although T cell responses were narrowly focused on a few peptide pools in patients 1 and 2 (Fig. 2 A), simultaneous recognition of multiple HCV sequences was detected in patients 3–5 and 8 (Fig. 2 B), as previously described in recovered and acutely infected patients (7–9).

Of the 119 mixtures of synthetic peptides tested, 34 in patient 3, 33 in patient 4, 18 in patient 5, and 12 in patient 8 were able to induce IFN- $\gamma$  production. These broad T cell responses were directed toward all viral antigens and different peptide mixtures induced responses of different intensity, ranging from 50 to 1,100 spot-forming cells (SFC)/ $10^6$  PBMCs in patient 3, from 500 to 3,700 in patient 4, from 50 to 920 in patient 5, and from 45 to 1,000 in patient 8. Further analysis performed by ICS to identify the individual epitopes showed that many peptides were recognized by patients 3–5 and 8. The responses were sustained by a mixed activation of both CD4 and CD8 T cell subsets (unpublished data). In contrast, only a single sequence containing the previously described HLA-A2–restricted NS3 1073–1081 CD8 epitope (10) was identified in patients 1 and 2. The profound immunodominance of NS3 1073–1081–specific CD8+ T cells in these HLA-A2+ patients was confirmed by direct ex vivo tetramer staining for five distinct HCV epitopes (11–14). Confirming the data obtained with ELISPOT, both patients showed extremely elevated frequencies of NS3 1073–1081–specific CD8 T cells, whereas other specificities were negative also using peptides of optimal length for CD8 T cell recognition and corresponding to well-characterized HLA-A2–restricted epitopes. NS3 1073–1081 tetramer+ CD8 T cells reached values of 36 and 12% of the total CD8 T cells in patients 1 and 2 (Fig. 3 A). These frequencies were much higher than those previously reported in acute HCV infection (15–17) and those detected in three subjects with mild course of infection who were analyzed for comparison (Fig. 3 B). The phenotypic analysis of the tetramer+ cells in the five patients showed an identical maturation stage (effector-memory stage). Almost all tetramer+ cells were CCR7–, CD45RA–, and CD27–, and predominantly HLA-DR+ and CD69+ (unpublished data).

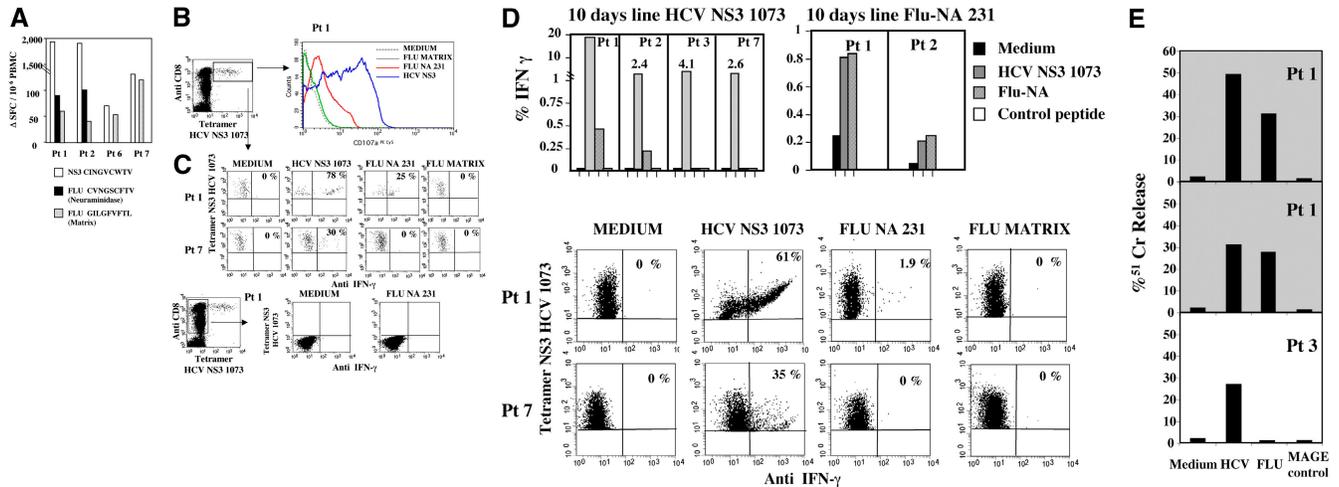
The different breadth of the CD8 T cell response in patients with severe and milder courses of the disease may reflect the different times after infection at which individual patients were studied. To exclude this possibility, we analyzed HCV-specific T cell responses longitudinally in patient 1 (severe hepatitis) and in patient 8 (milder disease). The precise time of infection was known in both, allowing us to perform a complete sequential analysis of the CD8 T cell response (Fig. 2 C). In patient 1, CD8 T cell responses were already positive, extremely vigorous, and narrowly focused on a single epitope 3 wk after infection. Except for a marginal and transient



**Figure 3. Peripheral blood frequency of HCV-specific CD8 T cells by ex vivo tetramer staining.** (A) Dot plot analysis of HCV NS3 1073 tetramer+ CD8 T cells in patients 1 and 2. (B) Percentage of tetramer+ CD8 T cells specific for five HCV epitopes corresponding to NS3 1073, NS3 1406, NS4 1812, NS4 1992, and NS5 2627 detected ex vivo in PBMCs of five HLA-A2+ patients with acute HCV infection at the time of presentation in the clinics (Fig. 1 A, patients 1–3, 6, and 7). All plots illustrate the results obtained at the first time the patients reported to the clinic.

broadening on week 8, the response remained focused on the NS3 sequence 1073–1081 throughout the follow-up (from week 3 to 24). In contrast, the CD8 T cell response became detectable later in patient 8 (Fig. 2 C); it was widely multispecific with simultaneous recognition of multiple epitopes at the time of the first positive assay (week 11) and a comparable breadth of the response was maintained thereafter (week 16). The behavior of patient 8 was consistent with the responses in patients 3 and 5 with milder disease (Fig. 2 D), in whom the precise time of infection could not be established. Their CD8 T cell response was multispecific at the first determination and the breadth of response remained similar throughout the follow-up. Therefore, HCV-specific CD8 T cell-mediated responses in subjects who displayed different clinical courses of acute hepatitis C showed clear differences in their T cell repertoire. A strong dominance of a single HCV-specific CD8 T cell response was detected in patients with severe hepatic damage, whereas a milder disease was associated with a broader repertoire of the HCV-specific T cell response.

A possible interpretation of these results is that the dramatic expansion of NS3 1073–specific CD8 T cells in patients



**Figure 4. Recognition of HCV NS3 1073 and flu NA 231 peptides by HCV NS3-specific CD8 T cells.** (A) Ex vivo IFN- $\gamma$  ELISPOT analysis. PBMCs from four HLA-A2+ patients with acute HCV infection were stimulated overnight with the peptides NS3 1073, flu NA 231, and flu Matrix 58. The results are expressed as change in spot-forming cells ( $\Delta$ SFC) per  $10^6$  PBMCs. (B and C) CD107a expression and of IFN- $\gamma$  production by ICS analysis ex vivo. PBMCs from patients 1 and 7 were incubated for 2 h (CD107a expression) or 4 h (IFN- $\gamma$  production) with HCV NS3 1073, flu NA231, flu MA 51 peptides, or medium alone. (B) Histograms represent CD107a expression in gated HCV NS3 tetramer+ CD8 T cells at the indicated experimental conditions. (C) Plots represent IFN- $\gamma$  production in gated tetramer+ (top) or tetramer- (bottom) HCV NS3 1073 cells. (D) IFN- $\gamma$  production in NS3- and Flu-specific T cell lines by ICS analysis. T cell lines produced by 10-d stimulation with the NS3 1073 or the flu NA 231 peptides (top) were stimulated for 4 h in medium alone, with the HCV NS3,

with the flu NA, or with a control HLA-A2-restricted MAGE peptide (MAGE 271-279; FLWGPRALV). Numbers at the top of the bars indicate the percentage of IFN- $\gamma$ -positive CD8 T cells. NS3 1073-specific T cell lines from patients 1 and 7 (bottom) were stimulated at the indicated experimental conditions. Plots are gated on HCV 1073 tetramer+ CD8 T cells. The numbers indicated in each plot represent the percentage of IFN- $\gamma$ -positive cells among the HCV NS3 tetramer+ CD8 T cell population. No IFN- $\gamma$  production was detectable in patient 1 upon stimulation with the flu NA 231 peptide in NS3 tetramer- CD8 T cells (not depicted). (E) Cytotoxic activity of HCV NS3 1073-specific T cell lines produced by 10-d stimulation with the NS3 1073 peptide in patients 1 and 3 against HLA-A2+ target cells (E/T ratio = 40:1) incubated in medium alone or pulsed either with the HCV NS3 1073 peptide or with the Flu NA 231 peptide or with a control MAGE peptide. Each bar shows the percentage of  $^{51}\text{Cr}$  release at the different experimental conditions described above.

1 and 2 was related to the presence of a private repertoire of memory T cells able to cross-react with the HCV sequence. The high degree of homology reported between HCV NS3 1073-1081 and influenza NA 231-239 sequences (10) supports this possibility. Moreover, T cell cross-reactivity between these two HCV and influenza sequences was observed in humans (HCV-infected patients and healthy HCV-uninfected subjects) and in HLA-A2+ transgenic mice (10). In addition, severe immunopathology after viral infection was shown to be caused in mice by the expansion of memory T cells from an earlier infection that cross-reacted with a second unrelated heterologous virus (3-6). Homology of the NS3 1073-1081 and the influenza NA 231-239 sequences was confirmed in our study by sequence analysis of the NS3 region performed on individual cDNA clones (18) in the patients with severe liver pathology (patients 1 and 2). Furthermore, database analysis indicates that the influenza NA 231-239 epitope is highly conserved among the published influenza sequences of H1N1 and H3N2 subtypes that are the only influenza subtypes isolated during the last 15 yr in the geographical region where the patients lived.

To determine whether potentially cross-reactive CD8 T cells were present in patients with severe liver pathology, direct ex vivo IFN- $\gamma$  ELISPOT analysis was performed in

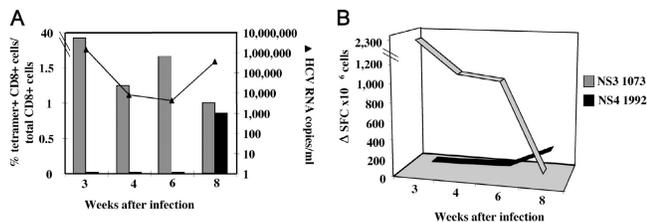
patients 1 and 2 (severe liver pathology) and in patients 6 and 7 (milder disease) for comparison. Although all patients showed a flu matrix 58-66-specific CD8 T cell response, demonstrating prior sensitization to influenza, NA 231 influenza-specific cells were detected exclusively in patients 1 and 2 (Fig. 4 A).

Next, functional experiments were performed to assess whether NS3 1073-specific CD8+ T cells in patients 1 and 2 could be activated by the NA 231-239 sequence. Ex vivo analysis showed that NA 231-239 peptide stimulation elicited up-regulation of CD107a expression (Fig. 4 B; reference 19) and IFN- $\gamma$  production (Fig. 4 C) selectively among NS3 1073-1081 tetramer+ cells, but not among the tetramer- CD8 T cell subset.

In keeping with ex vivo experiments, cross-recognition of HCV NS3 1073 and flu NA 231 peptides was observed in NS3-specific and flu NA-specific T cell lines derived from patients 1 and 2 but not in NS3-specific lines derived from patients 3 and 7 (Fig. 4 D). Furthermore, IFN- $\gamma$  production upon NA 231-239 peptide stimulation was restricted to the NS3 1073-1081 tetramer+ CD8 fraction of the NS3-specific lines in patient 1 (Fig. 4 D). Finally, cytotoxicity experiments performed using HCV NS3 1073-specific T cell lines derived from patients 1 and 3 confirmed the selective presence of cross-reactivity in patients with severe liver pathology (Fig. 4 E).

Together, these data show that although sensitization to influenza is a very common event, the individual private repertoire of memory T cells seems to be the limiting factor for cross-reactivity between HCV and influenza to occur. A private T cell repertoire of HCV/influenza cross-reactive T cells was present exclusively in the patients with severe hepatic immunopathology. In this setting, reactivation of pre-existing influenza-specific memory CD8<sup>+</sup> T cells by a primary HCV infection may have resulted in the strong immunodominance of the NS3 1073-specific CD8<sup>+</sup> T cell response present in these two patients. Notably, the kinetics of expansion and contraction of HCV-specific CD8<sup>+</sup> T cell responses in patient 1 further supports the possibility that memory CD8<sup>+</sup> T cells able to cross-react with the HCV NS3 1073 sequence were actually present. In this patient, a massive expansion (36%) of NS3 1073-specific CD8<sup>+</sup> T cells was already detectable as early as 2–3 wk from the time of a previous hospitalization, which likely corresponds to the time of infection. In contrast, other HCV-specific CD8<sup>+</sup> T cells (against the NS5 sequence 1992–2000) increased their frequency at later time points, when the NS3 1073-specific response was already in a contracting phase (Fig. 5). Because memory T cells exhibit a more rapid response than naive T cells, the differential kinetics of HCV-specific CD8 T cells specific for different epitopes located in nonstructural proteins further supports the view that NS3 1073 cross-reactive memory T cells were present before HCV infection. This rapid appearance of NS3 1073-specific T cell responses is also different from the kinetics reported in patients accidentally infected by needlestick exposure to HCV positive blood (17) and in primary infection of chimpanzees (20) in which HCV-specific T cell responses were detectable only ~2 mo after infection.

In conclusion, our findings provide a unique example of how the course of the pathology associated with primary HCV infection can be profoundly influenced by the virus-specific CD8 T cell repertoire elicited in the individual



**Figure 5. Kinetics of HCV-specific CD8 T cell-mediated responses in patient 1 analyzed ex vivo by tetramer staining and ELISPOT assay.** (A) Percentage of tetramer+ CD8 T cells specific for HCV NS3 1073 (gray bars) and NS4 1992 (black bars) epitopes measured ex vivo at sequential time points from the time of infection. HCV viremia detected by branched DNA assay (lower limit of detection, 2,500 copies/ml) is also illustrated. (B) IFN- $\gamma$  production analyzed sequentially during the first 10 wk after infection by ELISPOT upon PBMC stimulation with NS3 1073 (gray line) and NS4 1992 (black line) peptides. The results are expressed as  $\Delta$ SFC per  $10^6$  PBMCs.

subjects. As shown in mice (3–6), it is possible that previous infection and the presence of cross-reactive T cells between different viruses is responsible for the marked immunodominance present in subjects with severe hepatic disease.

Although NS3-specific CD8 T cells expanded rapidly and vigorously, the virus was not efficiently controlled and liver pathology was severe. HCV chronicity could not be attributed, at least in patient 2, to the development of escape mutants because in this subject a longitudinal analysis of the NS3 1073 sequence was performed over a 9-mo period and mutations within this region were not found. Thus, a robust, but isolated, response to the NS3 1073 epitope seems inadequate for viral control. A poor CD4 T cell response may have contributed to the lack of antiviral efficacy of the NS3-specific CD8 T cell response (21). Alternatively, the absence of a concomitant multispecific CD8 T cell response might be implicated in the inability to control HCV. The inability of NS3-specific CD8<sup>+</sup> T cells to efficiently control viral replication within the liver may have also contributed to the severity of liver cell damage. Intrahepatic activation of a large quantity of HCV-specific CD8 T cells with poor antiviral function might have sustained a massive recruitment of nonspecific immune cells causing severe liver inflammation (22). These findings are also a warning about the development of antiviral vaccines. Focusing the CD8 T cell response on single immunodominant epitope with poor antiviral efficacy may have severe pathological consequences.

## MATERIALS AND METHODS

**Patients and virological assessment.** Eight patients with acute hepatitis C were enrolled at the Department of Infectious Diseases of the University Hospital of Parma. The diagnosis of acute HCV infection was based on documented seroconversion to anti-HCV antibodies by RIBA assay, levels of serum ALT at least 10 times the upper limit of normal (50 U/l), detection of HCV RNA, exclusion of other possible causes of acute hepatitis. For two patients (patients 1 and 8), the precise time of infection could be precisely determined. Patient 1 developed jaundice and became symptomatic 3 wk after a previous hospitalization, during which he was treated with parenteral infusions of glucose and saline solutions. Anti-HCV antibodies were negative and ALT levels were normal at that time, indicating that infection was acquired during hospitalization. Patient 8 had an accidental exposure to HCV-infected blood; he was anti-HCV antibody negative and HCV-RNA negative at the time of the parenteral exposure. Anti-HCV antibodies were determined by commercial enzyme immunoassay kits (Ortho Diagnostic Systems) and by RIBA (RIBA II; Ortho Diagnostic Systems). Serum HCV-RNA was quantified by branched DNA assay (model 340bDNA Analyzer; Bayer Corporation); the lower limit of detection by this method is 2,500 copies/ml. This study was approved by the Ethical Committee of the Azienda Ospedaliero Universitaria di Parma, and all subjects gave written, informed consent.

**Synthetic peptides, peptide-HLA class I tetramers, and antibodies.** Synthetic peptides representing the HLA-A2-restricted epitopes HCV NS3 1073–1081 (CINGVCWTV), NS3 1406–1415 (KLVALGINAV), NS4 1812–1820 (LLFNILGGWV), NS4B 1992–2000 (VLSDFKTWL), NS5 2594–2602 (ALYDVVTKL), influenza virus NA 231–239 (CVNG-SCFTV), and MA 58–66 (GILGFVFTL), and a panel of 601 15-mer peptides overlapping by 10 residues and covering the overall sequence of HCV-1 were purchased from Chiron Mimotopes. PE-labeled tetrameric peptide-HLA class I complexes were purchased from Proimmune LTD.

HLA A2 tetramers contained the HCV peptides NS3 1073–1081, NS3 1406–1415, NS4 1812–1820, NS4B 1992–2000, and NS5 2594–2602.

**In vitro expansion of HCV-specific CD8 T cells.** PBMCs were resuspended in 96-well plates at a concentration  $2 \times 10^6$ /ml and stimulated with HCV peptides at a final concentration of 1  $\mu$ M. 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, ELISA.** Staining with tetramers and other surface markers and IFN- $\gamma$  staining were performed as described previously (15).

**ELISPOT assay.** 601 15-mer peptides, based on a genotype 1a sequence (HCV-1) covering all structural (core, E1, and E2) and nonstructural (NS3, NS4, and NS5) HCV regions and overlapping by 10 residues, were pooled in 119 mixtures, each containing 10 synthetic peptides. HCV-specific T cell responses were analyzed upon overnight stimulation with individual peptide mixtures. ELISPOT assay was performed as described previously (23). Plates were counted using an automated ELISPOT reader (AID Elispot Reader System; Autoimmune Diagnostika GmbH). IFN- $\gamma$ -producing cells were expressed as SFC per  $10^6$  cells. The number of specific IFN- $\gamma$ -secreting PBMCs 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 PBMCs stimulated with PHA. Wells were considered positive if they were at least two times above background.

**Chromium release assay.** Cytotoxic activity was assessed by incubating peptide-stimulated PBMCs with peptide-pulsed  $^{51}\text{Cr}$ -labeled, HLA-A2-matched EBV-transformed B cells as targets for 4 h in round-bottom 96-well plates. Percent-specific lysis was calculated as described previously (15).  $^{51}\text{Cr}$  released in the presence of culture medium ranged between 15 and 25% of total releasable counts. To adopt very stringent criteria and avoid the risk of false positive results, only levels of CTL lysis  $\geq 13\%$  were considered significant.

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