Differences in Hepatitis C Virus (HCV)-Specific CD8 T-Cell Phenotype during Pegylated Alpha Interferon and Ribavirin Treatment Are Related to Response to Antiviral Therapy in Patients Chronically Infected with HCV

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CD8 T cells play a major role in antiviral immune responses. Their importance for progression to chronic hepatitis C and response to treatment are still unclear. To address these issues, hepatitis C virus (HCV)-specific CD8 T-cell responses were monitored, at the single-cell level, using HLA class I pentamers specific for HCV core and HCV NS3 epitopes, in 23 chronically infected patients during treatment with pegylated alpha interferon and ribavirin. Patients who presented a sustained-response to therapy had stronger HCV-specific CD8 T-cell responses at all time points studied. Moreover, there were clear differences in the phenotypes of these cells during therapy: in responder patients, terminally differentiated effector cells increased more rapidly, and their frequency was always higher than in nonresponder patients. Sustained-responder patients also showed a higher frequency of HCV-specific CD8 T cells producing cytokotic factors. Overall, a late and inefficient differentiation process of HCV-specific CD8 T cells might be associated with lack of response to treatment. A better knowledge of the mechanisms underlying this impairment may be important for the development of new therapeutic strategies to maintain, restore, or increase CD8 T-cell effectiveness in chronic HCV infection.

Hepatitis C virus (HCV) shows a high propensity to establish chronic infection, often leading to cirrhosis and hepatocellular carcinoma (5). Combination therapy with pegylated alpha interferon (IFN-α) and ribavirin is currently standard treatment for patients with chronic hepatitis C. However, viral clearance is achieved in only 40 to 70% of patients, depending on the HCV genotype (1, 18, 20). The mechanism by which pegylated IFN-α and ribavirin therapy induces resolution of chronic HCV infection is not fully understood. Nevertheless, since both drugs potentially have immunomodulatory activity in vivo, in addition to their antiviral properties, cellular immune responses against HCV may be involved in a successful treatment outcome (4, 16, 17, 19, 27).

HCV-specific T-lymphocyte responses, including those mediated by cytotoxic T lymphocytes (CTL), are thought to play a crucial role in determining the course of infection (21). Although a vigorous and multispecific CTL response in acutely infected individuals has been associated with control of the virus (9, 28), the importance of CD8 T cells in chronic infection is less clear. Despite the presence of HCV-specific CTL in patients with chronic hepatitis C, it does not appear to be sufficient to eliminate the virus (22). The inefficiency of CTL activation and proliferation, as well as dysfunction or suppression of these cells, may ultimately determine the response to HCV (8, 15, 30). Previous studies have focused on the expression of markers associated with the differentiation status and functional ability of HCV-specific CD8 T cells by direct visualization with major histocompatibility complex class I peptide complexes (2, 11).

Understanding the mechanisms of viral clearance in chronically infected patients who respond to therapy may potentially allow the optimization of current treatment protocols and the development of new immunomodulatory agents. In order to address whether viral clearance is related to the phenotype and function of circulating HCV-specific CD8 T cells, we performed a longitudinal study in a group of patients with chronic hepatitis C before, during, and after therapy with pegylated IFN-α and ribavirin, using HLA-A2 pentamers specific for HCV core and NS3 epitopes. Phenotypic analysis was performed to assess the distribution of HCV-specific CD8 T-cell subsets (naïve, central memory, pre-terminally differentiated effector, and terminally differentiated effector cells), as well as their functional capabilities (23, 12, 29).

MATERIALS AND METHODS

Patients. Twenty-three patients chronically infected with HCV, enrolled at the Department of Infectious Diseases of the Hospital Centre of Coimbra were studied. The diagnosis was based on elevated serum transaminase levels and detection of anti-HCV antibodies by recombinant immunoblotting assay and of
HCV RNA by reverse transcription-PCR. Confirmation was obtained by liver histology. All patients were negative for hepatitis B virus surface antigen and anti-human immunodeficiency virus type 1 (HIV-1) and -HIV-2 antibodies. None had undergone previous antiviral treatment. Genotype 1 patients were treated for 48 weeks and genotype 2 or 3 patients were treated for 24 weeks with pegylated IFN-α2a/b (180 μg weekly) and ribavirin (1,000 to 1,200 mg daily, depending on body weight). Blood samples were collected immediately before therapy, at different time points during therapy (1, 3, and 6 months), and, in some cases, 6 months after the end of therapy. The subjects were divided in two groups, nonresponder and sustained-responder patients, according to alanine aminotransferase (ALT) normalization and virological response to therapy, defined by the absence of detectable serum HCV RNA at the end of treatment and 6 months after treatment was completed. Serum HCV RNA was assessed using a quantitative b-DNA assay (Versant 3.0 Superscript; Roche, Puteaux, France) with a detection limit of 615 IU/ml (3,200 copies/ml). Informed consent was obtained in all cases. A group of eight healthy HLA-A*0201 individuals were selected as negative controls and used to determine the detection limit of the flow cytometry analysis.

**HLA typing.** All patients enrolled in the study were HLA-A*0201. HLA class I genotyping was carried out by SS0 LABType (One Lambda, Canoga Park, CA). Target DNA was amplified by PCR using sequence-specific primers, followed by hybridization with allele-specific oligodeoxynucleotides coupled with fluorescein isothiocyanate (PE)-labeled microspheres. The fluorescence intensity was determined on a LABScan flow analyzer (One Lambda).

**Peptide HLA class I pentamers and antibodies.** Ex vivo HCV-specific CD8 T-cell responses were analyzed using two PE-labeled pentameric HCV peptide HLA class I complexes (Proimmune Ltd., Oxford, United Kingdom). HLA-A2 pentamers contained the HCV peptides NS3 1073 to 1081 (CINGVCWTV) and HCV NS3 10923 to 10931 (CSDGRTDFG) known to be recognized in chronic hepatitis C: HCVNS3 and HCVcore. A panel of several antibodies was used for flow cytometry analysis in different combinations: anti-CD8–peridin chlorophyll protein (PerCP) (Becton Dickinson Immunocytometry Systems, San Jose, CA) was included in all studies, in combination with anti-CD28–fluorescein isothiocyanate (FITC) and anti-CD3–allophycocyanin (APC) (both BD Pharmingen, San Jose, CA), anti-CD25–FITC and anti-CCR7–APC (R&D Systems, Minneapolis, MN), or anti-CD45RA–FITC (BD Pharmingen, San Jose, CA) and anti-CCR7–APC.

**Cell surface staining with pentamers and antibodies.** A minimum of 1 million (10⁶) peripheral blood mononuclear cells, obtained from fresh blood samples collected at the time points established, were incubated for 30 min in the dark at 4°C with the pentameric complexes. After the cells were washed, staining was performed for 15 min in the dark, using the panel of FITC–, PerCP–, or APC-conjugated antibodies described earlier. The cells were then washed and analyzed immediately on a Becton Dickinson FACSCalibur flow cytometer, using CellQuest and Paint-a-Gate software (Becton Dickinson, San Jose, CA). A minimum of 50 pentamer-positive CD8 T cells were acquired in all cases. The detection limit was established at 0.01% of CD8 T cells using HLA A*0201-positive healthy individuals and HLA A*0201-negative HLA-A*0201-positive individuals.

**Intracellular staining with perforin and granzyme B.** The cytotoxic activity of HCV-specific CD8 T cells was measured. Intracellular staining with anti-perforin–FITC (BD Pharmingen, San Diego, CA) and anti-granzyme B–APC (Caltag Laboratories, Burlingame, CA) or –FITC (BD Pharmingen, San Diego, CA) was done using the Fix-and-Perm kit (Caltag Laboratories, Burlingame, CA) according to the manufacturer’s instructions. As the expression of only four markers could be simultaneously evaluated, all studies included one of the HLA class I pentamers and anti-CD8 PerCP, in association with one of the cell surface antibodies previously defined. Analysis was again performed on a Becton Dickinson FACSCalibur flow cytometer.

**Statistical analysis.** Standard nonparametric statistical methods (Wilcoxon signed-rank test and Mann-Whitney U test) were employed using SPSS 12.0.

**RESULTS**

Frequencies of HCV-specific CD8 T-lymphocyte responses in the peripheral blood of chronically infected patients. The presence and frequencies of virus-specific CD8 T cells were determined in 23 HLA-A*0201-positive patients with chronic hepatitis C treated with pegylated IFN-α and ribavirin (Table 1). The subjects were divided into two groups according to their responses to therapy: 12 sustained-responder patients and 11 nonresponder patients.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
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<tbody>
<tr>
<td>Age a</td>
<td>41 ± 11</td>
</tr>
<tr>
<td>Sex (F/M) b</td>
<td>3/9</td>
</tr>
<tr>
<td>Infecting genotype</td>
<td>6 7</td>
</tr>
<tr>
<td>ALT level c</td>
<td>69 ± 37</td>
</tr>
<tr>
<td>Serum HCV RNA level d</td>
<td>6± 0.2</td>
</tr>
<tr>
<td>Liver histology</td>
<td>6/4/2</td>
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<td>Cirrhosis</td>
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a Age is expressed in years; mean ± standard deviation (SD).

b M, male; F, female.

c Patient infected by HCV genotypes 1 and 4.
d ALT level is expressed in IU/liter; mean ± SD.
e Pretreatment serum HCV RNA level is expressed in log IU/ml; mean ± SD.

HLA-A2-restricted HCV pentamers were used to directly quantify ex vivo CD8 T cells specific for two HCV epitopes known to be recognized in chronic hepatitis C: HCVns3 and HCVcore. Non-HLA-A2 HCV patients were used to access nonspecific pentamer staining and to determine the minimum limit at which CD8 T cells were considered positive for the pentamers tested (Fig. 1A).

The frequency of circulating pentamer-positive CD8 T cells was relatively low in patients with chronic HCV infection, with no significant differences between the two pentamers tested. When the frequencies of HCV-specific CD8 T cells in the two groups were compared, the median percentage of HCVcore− and HCVns3− CD8 T cells was significantly higher in sustained-responder patients than in nonresponder patients, with statistically significant differences (P < 0.01) (Fig. 1B and C).

**Dynamics of antiviral CD8 T-cell responses in chronic hepatitis C patients treated with pegylated IFN-α and ribavirin.** To investigate possible differences in antiviral responses over time, we tracked CD8 T cells against the two HCV epitopes. Therefore, blood samples from patients chronically infected with HCV were collected at five consecutive time points: immediately before the start of therapy; at 1, 3, and 6 months of therapy; and, in some cases, 6 months after the end of therapy. Interestingly, even before the start of therapy, patients who presented a sustained-response showed significantly stronger pentamer-positive CD8 T-cell responses than nonresponder patients for both pentamers tested (P < 0.05). The frequency of HCV-specific CD8 T cells reached the highest value in the first month of treatment and declined at 3 months of therapy (P < 0.01 between sustained-responder and nonresponder patients for both time points). This decline in HCV-specific CD8
T cells was maintained at 6 months of therapy (\(P < 0.05\) between sustained-responder and nonresponder patients), and the lowest values were detected at the last time point evaluated (12 months) (Fig. 2A and B).

**Evolution of the HCV viral load during treatment.** HCV RNA levels were measured at the same time points as HCV-specific CD8 T-cell frequencies: before the start of therapy and at 1, 3, and 6 months of therapy. Nonresponder patients showed higher levels of HCV RNA at the beginning of treatment than sustained-responder patients (Fig. 3). After 1 month of antiviral therapy, serum HCV RNA decreased in both groups. However, the difference in HCV RNA levels between the two groups was more evident 3 months after the start of therapy (\(P < 0.05\)). At the last time point evaluated, serum HCV RNA in all sustained-responder patients was below the detection limit (615 IU/ml), whereas in all nonresponder patients, the value obtained was still above the detection limit.

**Phenotypic characterization of HCV-specific CD8 T-lymphocyte response in patients with chronic hepatitis C.** We then focused our attention on pentamer-positive CD8 T-cell populations to demonstrate the phenotypic and functional characteristics of these HCV-specific lymphocytes. The costimulatory receptor CD28, involved in the regulation of T-cell activation, is one of the markers most frequently used to link the CD8 T-cell phenotype to the differentiation status. CD28 is differentially expressed on the surfaces of T cells and is present in “early differentiated” phenotypes (2). The analysis of pentamer-positive CD8 T cells during therapy indicated that the majority of them were positive for CD28, with a higher overall fre-
quency of HCV-specific CD28+ CD8 T cells in sustained-responder than in nonresponder patients (data not shown). To provide further characterization of HCV-specific T cells, we determined the expression of the chemokine receptor CCR7. This secondary lymphoid organ-homing marker is also associated with CD8 T-cell subset characterization, being downregulated as a result of naive T-cell activation and reexpressed by CCR7-negative T cells after stimulation (29). During therapy, HCV-specific CD8 T cells with heterogeneous expression of CCR7 were identified. Sustained-responder patients had higher frequencies of pentamer-positive CCR7+ CD8 T cells than those who did not respond to therapy (data not shown). By combined analysis of CD28 and CCR7, three separate subsets were identified: CD28+ CCR7+, CD28+ CCR7−, and CD28− CCR7+. Patients who responded to therapy showed higher frequencies of CD28+ CCR7+ and CD28− CCR7+ HCV-specific CD8 T cells than nonresponder patients (P < 0.05 for both subsets), whereas HCV-specific CD28+ CCR7− CD8 T cells were significantly fewer in sustained-responder than in nonresponder patients (P < 0.05) (Fig. 4A and B).

Distribution of HCV-specific CD8 T-cells subsets. CD45RA, one of the isoforms of CD45R, is an additional cell surface marker proposed to link CD8 T-cell phenotype and function. This transmembrane phosphatase regulates signaling through the T-cell receptor–CD3 complex. Activation of naive CD8 T cells induces loss of CD45RA expression, which is reexpressed in terminally differentiated effector cells (2). Simultaneous staining of pentamer-positive T cells with antibodies for CD45RA and CCR7 molecules allowed us to determine the distribution of HCV-specific CD8 T cells in the following classical subsets: naïve (CD45RA+ CCR7+), central memory (CD45RA+ CCR7+), pre-terminally differentiated effector (CD45RA+ CCR7+), and terminally differentiated effector (CD45RA− CCR7−) cells. These CD8 T-cell subsets exhibit different proliferative and functional capabilities (12, 29). Cellular immune responses by CTL against HCV were followed over time at four sequential time points during treatment: before and 1, 3, and 6 months after the start of therapy. The frequencies of HCV-specific CD8 T-cell

FIG. 2. Analysis of HCV-specific CD8 lymphocyte responses over time. Percentages of HCV-core-positive (A) and HCV-NS3-positive (B) cells were measured ex vivo in chronically HCV-infected patients at five sequential time points: before treatment (0); at 1, 3, and 6 months of treatment; and 6 months after the end of therapy (12) (only six sustained-responder and four nonresponder patients were evaluated at this time point). Median percentages for both groups of patients are represented. Significant differences were detected between sustained-responder and nonresponder patients before treatment (*, P < 0.05) and at 1 month (##, P < 0.01), 3 months (+++, P < 0.01), and 6 months (°, P < 0.05) of therapy.

FIG. 3. Analysis of the HCV viral loads in chronically infected patients. HCV titers were evaluated in sustained-responder and nonresponder patients before treatment with pegylated IFN-α and ribavirin (0) and after 1, 3, and 6 months of therapy. The dotted line indicates the minimum detection limit of 615 IU/ml (3.5 log units). *, significant differences were detected between sustained-responder and nonresponder patients 3 months after the start of therapy (P < 0.05).
One month after the start of therapy, HCV-specific CD8 T cells increased in both groups of patients, achieving the highest values over time, but with a significantly higher frequency in sustained-responder patients, as previously noted. However, the four CD8 T-cell subsets contributed differently to this pentamer-positive cell expansion. In sustained-responder patients, terminally differentiated effector cells increased significantly compared to the previous time point \( (P < 0.05) \), with a notably higher frequency than in nonresponder patients \( (P < 0.01) \). In patients who did not respond to therapy, it was the pre-terminally differentiated cell subset that showed a significant increase from the previous time point \( (P < 0.05) \), with a higher frequency than in sustained-responder patients \( (P < 0.01) \). Naive T cells showed a decrease in frequency, especially in sustained-responder patients \( (P < 0.05) \), but still remained significantly higher in this group of patients than in nonresponder patients \( (P < 0.05) \). Central memory T cells increased in sustained-responder and nonresponder patients \( (P < 0.05 \text{ for both groups}) \) and showed a significantly higher frequency in nonresponder patients \( (P < 0.01) \) (Fig. 5A and B).

Three months after the start of therapy, in sustained-responder patients, HCV-specific naive T cells were the most frequent CD8 T-cell subset, revealing a significant increase from the previous time point \( (P < 0.05) \). On the other hand, in nonresponder patients, naive T cells were the least represented cell subset and revealed a decrease in frequency \( (P < 0.05) \). Pre-terminally differentiated T cells remained the most frequent HCV-specific CD8 T-cell subset in nonresponder patients, while they were the least frequent cell subset in sustained-responder patients \( (P < 0.01) \). In both groups of patients, however, this T-cell subset decreased \( (P < 0.05) \). Curiously, no significant differences were detected between sustained-responder and nonresponder patients in terms of central memory and terminally differentiated HCV-specific CD8 T-cell frequencies. Central memory T-cell frequency increased from the previous time point in both groups \( (P < 0.05) \). However, sustained-responder patients showed a decline in terminally differentiated T-cell frequency from the previous time point \( (P < 0.05) \) (Fig. 5A and B).

At the fourth time point studied, 6 months after the start of therapy, central memory T cells represented the most frequent HCV-specific CD8 T-cell subset in sustained-responder patients \( (P < 0.05) \) and revealed a significant increase from the previous time point \( (P < 0.05) \). Curiously, the pattern of frequency variations for this T-cell subset were somewhat similar in sustained-responder and nonresponder patients, showing a continuous increase since the beginning of therapy. As in previous time points, pre-terminally differentiated T cells remained the least represented HCV-specific CD8 T-cell subset in sustained-responder patients, while in nonresponder patients, they continued to be the most frequent cell subset \( (P < 0.01) \). In nonresponder patients, pre-terminally differentiated T cells decreased from the previous time point \( (P < 0.05) \). Curiously, in this group of patients, the pre-terminally differentiated T-cell frequency increased early, 1 month after the start of therapy, and declined after that. The HCV-specific terminally differentiated cell frequency was higher in sustained-responder patients and continued to decrease from the

Before the start of therapy, HCV-specific naive CD8 T lymphocytes were more frequent in sustained-responder patients than in nonresponder patients \( (P < 0.05) \). At the same time point, nonresponder patients showed higher percentages of pre-terminally differentiated HCV-specific CD8 T cells \( (P < 0.05) \) (Fig. 5A and B).
previous time point in this group of patients ($P < 0.05$). In nonresponder patients, this subset also showed a decrease in frequency from the previous time point ($P < 0.05$). It is noteworthy that while in sustained-responder patients this HCV-specific CD8 T-cell subset showed an increase in frequency early, 1 month after the start of therapy, and declined after that time point, in nonresponder patients, this T-cell subset increased later, only 3 months after the start of therapy, and declined after that time point. Finally, the naive-T-cell frequency was higher in patients who responded to therapy ($P < 0.05$), revealing a significant decrease from the previous time point ($P < 0.05$). Nonresponder patients showed an increase in naive-T-cell frequency from the previous time point analyzed ($P < 0.05$) (Fig. 5A and B).

**Functional analysis of HCV-specific CD8 T cells.** The effector function of HCV-specific CD8 T lymphocytes was also evaluated in sustained-responder and nonresponder patients. For that purpose, the HCV-specific CD8 T-cell contents of perforin and granzyme B, cytotoxic factors involved in cell death by lysis and apoptosis, were determined during therapy. The median percentage of perforin-producing CD8 T cells was higher in sustained-responder than in nonresponder patients.
with statistically significant differences 1 month after the start of treatment ($P < 0.05$) (Fig. 6A and B). As for granzyme B-producing CD8 T cells, significantly higher values were detected in sustained-responder patients before the start of therapy and at 1 month of therapy ($P < 0.05$) (Fig. 7A and B).

The relationship between CD28 expression and the content of perforin and granzyme B in HCV-specific CD8 T cells was also determined. For both groups of patients, HCV-specific CD28$^-$ cells showed a higher content of those cytotoxic factors than HCV-specific CD28$^+$ cells, with statistically significant differences ($P < 0.05$) (data not shown).

The relationship between the contents of perforin, granzyme B, and CCR7 in HCV-specific CD8 T cells was also determined. Higher percentages of those cytotoxic factors were detected in HCV-specific CCR7$^-$ cells, with statistically significant differences ($P < 0.05$) (data not shown).

**DISCUSSION**

HCV frequently sets up persistence, although the mechanisms that allow the virus to coexist with its host and develop chronic infection are not fully understood. Adaptive immune responses, particularly virus-specific CTL, are believed to play a significant role in determining the outcome of HCV infection. Several studies of chronic viral infections, especially HIV, have tried to correlate an inefficient response with a particular distribution of CD8 T-cell subsets, suggesting that different phenotypes are correlated with distinct clinical outcomes (3). Moreover, the immunomodulatory activity of pegylated IFN-α and ribavirin therapy may be associated with inducing a successful response to therapy. To address whether different treatment outcomes are related to the HCV-specific CTL subset distribution, a longitudinal analysis of the frequency and phe-
notype of these cells was performed in responder and nonresponder patients chronically infected with HCV.

It is still uncertain whether the results using in vitro expanded CD8 T lymphocytes are representative of the in vivo scenario. Therefore, we used an ex vivo major histocompatibility complex peptide pentamer-staining strategy, as our interest was in analyzing the response of peripheral blood HCV-specific CTL, avoiding any bias due to in vitro expansion of these cells. When the two subject groups were compared, chronically infected patients who responded to therapy showed a higher HCV-specific CD8 T-cell frequency than nonre-
sponder patients at all time points studied, with significant differences between the two groups. The higher frequency of circulating HCV-specific CD8 T cells observed in sustained-responder patients is consistent with previous studies, revealing the importance of these cells for successful control of infection (6). This could represent a “selective advantage,” an immune factor associated with the efficiency of pegylated IFN-α and ribavirin therapy, in combination with already identified nonimmune factors, such as the HCV genotype, viral load, or stage of liver fibrosis. Furthermore, after 1 month of therapy, responder patients showed the maximum percentage of HCV-specific CD8 T cells, after which they decreased throughout treatment. These results indicate that although virus-specific CTL can persist even in patients chronically infected with HCV, successful response to treatment does not seem to be associated with a sustained augmentation of cellular immune responses (14).

Analysis of virus-specific CD8 T-cell subsets may help to explain different treatment outcomes in chronically infected patients. Expression of the costimulatory receptor CD28, involved in T-cell activation, was one of the markers used to characterize HCV-specific CTL. This receptor is present on the surfaces of “early” differentiated CD8 T cells, whereas in “intermediate” and “late” phenotypes, its expression is lost (2). The lymphoid organ homing marker CCR7 was also evaluated to better characterize HCV-specific CD8 T cells. Simultaneous analysis of both markers was very informative, as patients who responded to therapy revealed a significantly higher frequency of CD28+CCR7+ HCV-specific T cells than nonresponder patients and therefore appeared to be richer in CD8 T cells with an “early” phenotype. Moreover, sustained-responder patients presented a significantly higher frequency of CD28−CCR7− HCV-specific T cells, suggesting an enrichment of CD8 T cells with a “late” phenotype compared with nonresponder patients. On the other hand, in nonresponder patients, CD8 T cells with an “intermediate” phenotype (CD28−CCR7+) predominated significantly.

In an attempt to further characterize and understand the physiology of CTL responses during therapy, we studied the distribution of HCV-specific CD8 T-cell subsets by combined expression of CD45RA and CCR7: naive (CD45RA+CCR7−), central memory (CD45RA−CCR7−), pre-terminally differentiated effector (CD45RA−CCR7+), and terminally differentiated effector (CD45RA−CCR7+) cells (25, 12). The relative contribution of each of these CD8 T-cell subsets to the expansion or contraction of this cellular compartment was determined.

Differences between sustained-responder and nonresponder patients were further explored by analyzing CD8 T-cell subset distribution during the course of therapy, which produced interesting results. Before the start of therapy, the majority of HCV-specific CTL detected in the peripheral blood of sustained-responder patients were naive T cells. This may constitute an advantage for these patients, as more HCV-specific CTL are available to be activated upon encountering the antigen. At the same time point, in nonresponder patients, pre-terminally differentiated CD8 T cells represented the most frequent HCV-specific CTL subset.

The overall frequency of HCV-specific CTL increased, reaching the highest values 1 month after the start of treatment in both groups. For sustained-responder patients, this increase was mainly due to terminally differentiated cells, as well as, to a lesser extent, central memory cells, accompanied by a concomitant decrease in naive CD8 T cells. The pathway of T-cell differentiation may be helpful in explaining these results: upon activation by encountering the antigen, naive CD8 T cells are induced to clonally expand and differentiate into effector CD8 T cells, as well as memory CD8 T cells (13). In nonresponder patients, pre-terminally differentiated cells increased 1 month after the start of therapy, remaining the most frequent HCV-specific CTL subset. This augmentation was accompanied by a decrease in naive CD8 T cells, but not by a notable increase in terminally differentiated cells. These results show that in nonresponder patients, activation and proliferation of naive CD8 T cells resulted, not in the development of terminally differentiated T cells, but in pre-terminally differentiated cells. CD8 T cells do not seem to reach a fully differentiated stage of maturity, due to a possible blockage in CTL maturation, resulting in an inability to control HCV infection. HCV or HCV gene products may act directly to disrupt the normal maturation pathway of CTL, negatively influencing their function and contributing to the strength and duration of an anergic condition (30). Recently, HCV core protein has been shown to exert immunosuppressive properties that affect CTL maturation, leading to the generation of a large population of defective effector T cells (15). Alternatively, the differences in the phenotypes of HCV-specific CD8 T cells between sustained-responder and nonresponder patients could be the consequence of a stronger decrease in HCV RNA observed in the first group of patients. According to this hypothesis, better control of viral replication during treatment in sustained-responder patients could have resulted in the redistribution of HCV-specific CD8 T cells, namely, terminally differentiated cells, from the liver to the blood, whereas in nonresponder patients they would be retained in the liver due to a smaller decrease in HCV RNA titers. However, other factors seem to be in play to explain the differential evolution of the HCV-specific CD8 T-cell maturation stage, as in sustained-responder patients terminally differentiated cells were the major contributors to the increase in HCV-specific CD8 T cells observed 1 month after the start of therapy, while in nonresponder patients this increase in CD8 T cells also occurred, but later and with a greater contribution by pre-terminally differentiated cells.

Three months after the beginning of therapy, sustained-responder patients showed a decrease in terminally differentiated cells, which might be related to the differential homing of these cells to the liver. Analyzing HCV-specific CTL subset distribution within the liver compartment, the primary site of HCV replication, in chronically infected patients at different time points in treatment would be interesting but is not feasible due to ethical issues. The overall decline of circulating HCV-specific CD8 T cells might be the result of their redistribution from the blood to the site of infection. It would be interesting to further characterize the expression of T-cell homing markers other than CCR7, such as CCR5 or CXCR3, in the different subsets of HCV-specific CD8 T cells, since recent studies have suggested the involvement of chemokine receptors in the outcome of antiviral therapy (31). At the same time point and in the same group of sustained-responder patients, an increase in naive CD8 T cells was detected, suggesting the ability of
antiviral therapy to generate de novo HCV-specific CTL responses or to allow the recovery of primed but functionally impaired T cells, although it is still uncertain. Furthermore, the observed expansion of central memory CD8 T cells could be associated with a higher frequency of naive T cells involved in the immune response against HCV infection in these patients, which would ultimately determine the frequency of memory T cells produced (12). In nonresponder patients, a visible increase in terminally differentiated CD8 T cells was detected only at this time point, 3 months after the start of therapy. However, even at this time point, these cells did not represent the most frequent HCV-specific CTL subset, which was always comprised of pre-terminally differentiated cells. Such a condition seems to reflect the late and ineffective immune response exerted by HCV-specific CD8 T cells in these patients, probably due to chronic antigenic exposure, leading to the exhaustion of CTL responses (30), as previously detected in other chronic virus infections, such as HIV (24).

Six months after the start of therapy, sustained-responder patients showed a higher frequency of central memory cells, which represent a strategic reserve that allows the host to respond swiftly when facing a novel infection (26). In nonresponder patients, pre-terminally differentiated CD8 T cells continued to be the most frequent CTL subset. In that group of patients, HCV-specific terminally differentiated CD8 T cells decreased at that time point and may have been recruited to the primary site of infection. Nevertheless, taking into consideration that the overall frequency of HCV-specific CD8 T cells was always lower in nonresponder patients, their late recruitment to the liver would be sufficient to cause liver damage but not to control the infection or eliminate the virus. Curiously, naive CD8 T cells in nonresponder patients increased slightly at this time point, which may suggest that these subjects also possess the ability to produce HCV-specific CD8 T cells de novo.

To better characterize these HCV-specific CD8 T cells, a functional analysis was performed, through the expression of the cytotoxic factors perforin and granzyme B, important mediators in the process of viral infection control. A higher expression of both cytotoxic factors was detected in sustained-responder patients. As these patients also showed higher frequencies of HCV-specific CD8 T cells in more advanced stages of differentiation, these results suggest an increased cytotoxic potential in these cells, which is consistent with previous studies (10). Interpretation of the results obtained should not be done in a linear way, because other factors have to be taken into consideration, such as the expression of antiviral cytokines (IFN-γ or tumor necrosis factor alpha). Sorting the various HCV-specific CD8 T-cell subsets and studying the production of such cytokines would help to clarify this issue, although the low frequency of these cells constitutes an important drawback. The presence of terminally differentiated effector T cells is essential for successful control of viral infections and, in cases where these cells are dysfunctional, rehbitating their functionality may represent a therapeutic alternative. The processes behind this suboptimal functionality or lack of functionality have not been completely clarified, although one hypothesis is that HCV, as part of its evasion mechanism against the host’s immune system, may block or redirect CD8 T-cell differentiation in a more favorable way.

Our results support the notion that in chronic hepatitis C, phenotypic changes in HCV-specific CD8 T cells seem to reflect the outcome of pegylated-IFN-α and ribavirin treatment. Several other factors need to be taken into consideration, for instance, the study of activation markers, such as CD38, HLA-DR, and CD57, or the role of CD4 T cells, which seem to be essential in maintaining successful memory CD8 T-cell responses (25, 7). If effector CD8 T cells against persistent viruses, such as HCV, have similar requirements, when a sustained and efficient CD4 T-cell response is absent, they might not be able to keep up with the evolution of viruses that rapidly accumulate escape mutations and contribute to unsuccessful CTL responses. It would be equally interesting to study the existence of molecular changes associated with proliferation, differentiation, and survival of CD8 T lymphocytes during the immune response developed in a chronic HCV infection scenario.

Understanding the mechanisms behind HCV infection and the impact of therapy in chronic hepatitis C progression may help to improve current treatment regimens. Methods to reverse the apparent failure of the CD8 T-cell response or to induce new populations of functional CTL should be explored as part of new immunotherapeutic strategies that could sustain an efficient response in chronically infected patients.

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REFERENCES


