CD4+ T Cell Responses in Patients with Chronic Hepatitis C Undergoing Peginterferon/Ribavirin Therapy Correlate with Faster, but Not Sustained, Viral Clearance

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T cell immune responses may be important for the elimination of chronic hepatitis C virus (HCV) infection during antiviral treatment. In the present study, the kinetics of T cell responses to HCV antigens (NS3-4 and core) were prospectively assessed and were correlated with virologic outcome in 31 patients with chronic HCV infection undergoing peginterferon-α2a/ribavirin therapy. NS3-4–directed T helper cell type 1 (Th1) responses were detected in 77% of patients with a significant decline in viremia at treatment week 4 but were not detected in those with a slower viral decline. The detectability of NS3-4–directed Th1 responses was associated with faster viremia clearance, was short-lived, and did not seem to be associated with the final treatment outcome.

Hepatitis C virus (HCV) infection is one of the most common causes of chronic liver disease, affecting 170 million people worldwide. The antiviral immune response determines the outcome of infection. A vigorous, broad, and sustained antiviral T cell response results in rapid HCV clearance, whereas weak or absent T cell responses are associated with viral persistence [1]. It has been suggested that the recovery of HCV-specific T cell responses during antiviral therapy similar to those observed in spontaneous resolvers of HCV infection may contribute to the resolution of chronic infection. Currently, it remains controversial whether persistent T cell responses are recovered after successful HCV eradication due to interferon-α–based therapy [2–7].

The combination of peginterferon and ribavirin is currently the therapy of choice for those with chronic hepatitis C; it results in sustained virologic response (SVR) rates of 60%–80%, depending on the viral genotype [8]. An SVR to combination therapy has been shown to be paralleled by strong and persistent T cell responses, in particular those with a Th1 profile [5]. The mechanisms by which HCV-specific responses are restored by these drugs are not yet understood. In particular, it is not clear to what extent these drugs modulate antiviral immune responses, either directly or indirectly, by reducing viral load. Mathematical modeling of viral kinetics suggests that both mechanisms play a role in successful viral clearance [9]. Measuring the emergence of HCV-specific T cell responses in parallel with viral kinetics should increase our understanding of the immune response in patients with chronic hepatitis C.

In the present study, HCV-specific Th1 responses were prospectively examined by intracellular tumor necrosis factor (TNF)–α staining using flow cytometry in 31 patients with chronic HCV infection who were undergoing peginterferon/ribavirin therapy. The results were correlated with virologic outcome.

Patients and methods. In 31 patients (18 men and 13 women; mean ± SD age, 38.5 ± 8.5 years; infecting HCV genotype, n = 11, n = 2, n = 15, and n = 3 for genotypes 1, 2, 3, and 4, respectively) participating in 2 ongoing randomized, controlled trials [10, 11], serial determinations of CD4+ T cell counts were performed before treatment; at least 4 times during the 24-week treatment period; at weeks 32, 48, and 72 in patients who continued antiviral therapy; and at weeks 4 and/or 24 after the completion of treatment. All study procedures were conducted under local ethics committee–approved protocols, in conformity with the ethics guidelines of the Declaration of Helsinki, and after patients gave informed consent.

Interferon-naive adults with quantifiable levels of HCV RNA genotype 1 or 4 received peginterferon-α2a (40 kDa; PEGASYS; Roche Diagnostics) at 180 μg/week plus ribavirin (COPEGUS; Roche Diagnostics) at 1000–1200 mg/day before assignment to 1 of 4 treatment groups on the basis of HCV RNA testing at weeks 4 and 12. Patients with HCV RNA loads <50 IU/mL at treatment week 4 received 20 weeks of additional therapy (ge-
notype 1, n = 2; genotype 4, n = 1). Patients with detectable HCV RNA at week 4 continued treatment and were restested at week 12, at which time they were randomly assigned to either the 48- or 72-week treatment group (genotype 1, n = 7; genotype 4, n = 1) if the HCV RNA load showed a decline of $>2 \log_{10} \text{IU/mL}$. Treatment was stopped if patients remained HCV RNA positive at treatment week 24 (genotype 1, n = 2; genotype 4, n = 1). Patients infected with HCV genotype 2 (n = 2) or 3 (n = 15) received $180 \mu g$ of peginterferon-α-2a per week for 24 weeks and were randomized to receive either 400 or 800 mg of ribavirin. All patients were tested for baseline clinical and virologic parameters, including HCV viremia by COBAS AMPLICOR qualitative or quantitative HCV RNA polymerase chain reaction (Roche Diagnostics) and HCV genotype by INNO-LiPA II (Innogenetics), and these parameters were reassessed at monthly intervals during treatment and at weeks 4 and 24 after the completion of treatment.

Recombinant genotype 1a–derived HCV core (aa 1–115), NS3 (aa 1007–1534), and NS4 (aa 1616–1862) were used for CD4+ T cell stimulation, as described elsewhere [12]. All were from Microgen.

For intracellular cytokine staining, fresh blood samples (500 μL) were stimulated at 37°C for 6 h with either staphylococcal enterotoxin B (SEB; 1 μg/mL; Sigma), HCV protein (1 μg/mL), or no protein (as a negative control) as well as anti-CD28 and anti-CD49d (0.5 and 1 μg/mL, respectively). Staining was performed using BD FastImmune AntiHu-TNF-α–FITC/CD69-PE/CD4-PerCP-Cy5.5 (BD Biosciences). The percentage of spontaneous TNF-α ‘CD69’CD4+ cells was subtracted from that obtained after HCV restimulation; a response was considered to be positive if the percentage of TNF-α ‘CD69’CD4+ cells was greater than the mean ± 2 SDs for 30 healthy control subjects. Acquisition was immediately performed on a FACScalibur (BD Biosciences). Enumeration of CD4+ T cells was done using TruCOUNT Tubes as recommended by the manufacturer (BD Biosciences).

Results. Of the 31 patients with chronic HCV infection who received peginterferon/ribavirin therapy, 20 (65%) achieved a reduction of $>2 \log_{10} \text{IU/mL}$ from baseline viral load after only 2 weeks of therapy; 26 (84%) and 30 (97%) had a slower viral decline, with a decrease of $>2 \log_{10} \text{IU/mL}$ after 4 and 12 weeks, respectively. Twenty (65%) of the 31 patients had a rapid virologic response (HCV RNA load <50 IU/mL at treatment week 4).

Before treatment, HCV-specific Th1 responses were detected in only 4 (13%) of the 31 patients. During treatment, Th1 responses to NS3-4 and core developed in 20 (65%) and 17 (55%) of the 31 patients, respectively. At the end of treatment, 8 (27%) of 30 patients showed Th1 responses to NS3-4, and 7 (23%) of 30 showed responses to core. Significant differences in rates of Th1 responses to NS3-4 but not to core were found with respect to the time to viral response. Of those patients who had already achieved a decline in HCV RNA load of $2 \log_{10}$ IU/mL at treatment week 2, 85% (17/20) were Th1 responders to NS3-4, whereas only 50% (3/6) of the patients with a decline of $2 \log_{10}$ IU/mL at treatment week 4 and none of the 5 patients who either achieved a decline of $2 \log_{10}$ IU/mL at later time points or showed no viral decline displayed a Th1 response ($P = .001$, exact test; significance due to the group with slow or no viral decline).

Among the patients with a treatment response, those who had a detectable Th1 response to NS3-4 at some point during the 24-week treatment period (Th1 responders; figure 1A and 1D) exhibited faster kinetics of viremia clearance (mean ± SD, 3.4 ± 2.2 weeks) than did those without Th1 responses (Th1 nonresponders; figure 1B) (mean ± SD, 9.3 ± 5.9 weeks) ($P = .01$, Mann-Whitney U test). The increase in Th1 responses to NS3-4 was not paralleled by an increase in responses to the SEB control. No Th1 responses were detected in the 2 treatment nonresponders (figure 1C). No differences in absolute CD4+ T cell counts were observed among the patient groups.

As shown in table 1, Th1 responders cleared viremia faster than did Th1 nonresponders. However, a difference in SVR rates between Th1 responders and Th1 nonresponders was not observed. Th1 responders did not differ from Th1 nonresponders with respect to sex, time since onset of infection, or baseline alanine aminotransferase levels and viral load. However, the majority of Th1 responders were young (age <40 years, 12/20) and were infected with genotype 3 (13/20). In contrast, only 2 of 11 Th1 nonresponders were either <40 years old or infected with genotype 3. Rates of Th1 responses to core did not differ by infecting genotype. The higher proportion of Th1 responders among patients with genotype 3 infection than among patients with genotype 1 infection (13/15 vs. 4/11; $P = .013$, Fisher’s exact test) coincided with significantly faster kinetics of viral clearance in this group (mean ± SD, 2.5 ± 0.92 vs. 9.8 ± 5.9 weeks for genotype 3 vs. genotype 1; $P = .004$, Mann-Whitney U test).

We therefore studied viral kinetics and NS3-4–directed Th1 response rates separately for genotypes 1 and 3. Among patients with genotype 1 infection, recovery of NS3-4–directed Th1 responses was associated with significantly faster viremia clearance (median time to viremia clearance, 6 vs. 16 weeks for Th1 responders vs. Th1 nonresponders; $P = .02$, Mann-Whitney U test). All patients with genotype 3 infection achieved viremia clearance during the first 4 weeks of therapy, and different Th1 response patterns were not associated with differences in viral kinetics among these patients.

Twenty-three of the 31 patients (genotype 1, n = 4; genotype 2, n = 2; genotype 3, n = 15; genotype 4, n = 2) received therapy for 24 weeks; 17 of them had an SVR, 3 had a relapse, and 3 had no end-of-treatment response (breakthrough, n = 1; nonresponse, n = 2). Of the 17 patients with an SVR, 14
Figure 1. Serum hepatitis C virus (HCV) kinetics and NS3-4–directed CD4+ T cell responses before, during, and after peginterferon/ribavirin therapy, as determined by intracellular tumor necrosis factor (TNF)-α staining. A, Data from treatment responders (patients with a loss of detectable HCV RNA during 24 weeks of therapy) in whom NS3-4–directed Th1 responses were detected during the 24-week treatment period (Th1 responders; n = 20). B, Data from patients in whom NS3-4–specific Th1 responses were not detected (Th1 nonresponders; n = 9). C, Data from treatment nonresponders (n = 2). Bars represent mean ± SE values. Dot blots (at bottom) are for individual patients in each group and show the percentage of TNF-α+CD69+CD4+ cells generated in response to NS3-4. D–F, Viral and Th1 response kinetics in individual patients with (D) or without (E) NS3-4–directed Th1 responses during standard therapy (24 weeks) or prolonged therapy (48 or 72 weeks) and in patients without a response to treatment (F). Dotted lines represent serum viral loads over time as mean values (A–C) or as values for individual patients (D–F); shaded areas indicate the administration of antiviral therapy, and asterisks indicate follow-up examinations after completion of treatment. BL, baseline; LLD, lower limit of detection.
were Th1 responders. All 3 patients who had a relapse, but none of the patients with a nonresponse or breakthrough, developed detectable Th1 responses (Th1 reactivity data for individual patients are shown in figure 1D and 1F).

Eight patients (genotype 1, n = 7; genotype 4, n = 1) received treatment for 48 or 72 weeks according to the study protocol and, therefore, could be investigated for late changes during prolonged treatment. Three of these 8 patients had already developed NS3-4–directed Th1 responses during treatment, of whom 2 had an SVR and 1 had a relapse after the end of treatment. Five patients had no detectable Th1 response during the 24-week treatment period (Th1 reactivity data for 3 individual patients are shown in figure 1E). 2 of these 5 patients developed a Th1 response by treatment week 48, 1 of whom had an SVR and 1 of whom had a relapse. The other 3 displayed no detectable Th1 response but achieved an SVR. Detectable core-directed Th1 responses did not develop in any of the patients receiving continued antiviral therapy. The data demonstrate that an increase in NS3-4–directed Th1 responses may occur months after viremia clearance but that this is not necessarily associated with sustained viral control.

**Discussion.** In the present study, antiviral CD4+ T cell responses were partially recovered during peginterferon/ribavirin therapy in patients who responded rapidly to treatment but not in those with a slow viral decline (decrease in HCV RNA load of <2 log10 IU/mL at treatment week 4) during identical treatment. This suggests that Th1 cytokine production by CD4+ T cells seems to be impaired in the presence of a high viral load and that recovery of Th1 responses is not just a direct result of antiviral treatment. Earlier studies also demonstrated that HCV-specific proliferative and interferon-γ responses correlate with faster viral clearance during antiviral treatment [13, 14], but these studies did not provide data on therapy outcome.

The outcome of combination therapy with peginterferon and ribavirin correlates with the rapidity of the virologic response. Patients who clear viremia within 4 weeks have the best chance of achieving an SVR [15, 16]. In the present study, the majority of patients—but not all—with a rapid virologic response had detectable HCV-specific Th1 responses at some point during therapy. However, detectable Th1 responses were short-lived and returned to baseline levels at the end of treatment, with no difference between patients with transient or sustained viral control. The transient nature of the augmentation of the HCV-specific Th1 cell population during peginterferon/ribavirin therapy reconfirms earlier observations [6, 13]. By use of enzyme-linked immunospot analysis, HCV-specific Th1 responses have been shown to persist after successful HCV eradication due to interferon-α/ribavirin therapy in some [3, 5], but not all [4, 6], studies. In addition to the possibility that responses were present but not detected by intracellular cytokine analysis, other factors, including genotypic differences, may be of additional importance. In agreement with the findings of recent reports [4, 13], we found that the majority of Th1 responders were infected with HCV genotype 3. They were all rapid virologic responders, but only 87% developed detectable Th1 responses, whereas 100% of the rapid virologic responders infected with genotype 1—the so-called superresponders [10]—had a recovery of a Th1 response. Thus, the possibility that responses were missed in patients with genotype 3 infection because of the use of genotype 1α–derived reagents cannot be excluded. Although the present results were obtained with small numbers of genotype 1–infected patients and larger studies are

### Table 1. Characteristics of patients with or without Th1 responses to hepatitis C virus (HCV) NS3-4.

<table>
<thead>
<tr>
<th>Category, parameter</th>
<th>Th1 responders (n = 20)</th>
<th>Th1 nonresponders (n = 11)</th>
<th>P</th>
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<tr>
<td><strong>Demographic</strong></td>
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<td></td>
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<tr>
<td>Sex, female/male</td>
<td>9/11</td>
<td>4/7</td>
<td>NSa</td>
</tr>
<tr>
<td>Age, mean ± SD (range), years</td>
<td>36.1 ± 9.2 (21–54)</td>
<td>42.9 ± 5 (34–50)</td>
<td>.02b</td>
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<tr>
<td>Time since onset of infection, mean ± SD, years</td>
<td>16.3 ± 2.4</td>
<td>20.57 ± 2.0</td>
<td>NSb</td>
</tr>
<tr>
<td><strong>Clinical</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline ALT level, mean ± SD, IU/L</td>
<td>128 ± 80</td>
<td>94 ± 50</td>
<td>NSb</td>
</tr>
<tr>
<td>Baseline HCV RNA load, mean (range), IU/mL</td>
<td>2.4 × 10^6 (2.1 × 10^6–1.6 × 10^7)</td>
<td>1.3 × 10^6 (1.5 × 10^6–5.9 × 10^6)</td>
<td>NSb</td>
</tr>
<tr>
<td>Infecting genotype, 1/2/3/4</td>
<td>4/1/3/2</td>
<td>7/1/2/1</td>
<td>.03a</td>
</tr>
<tr>
<td><strong>Antiviral therapy</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Viremia clearance during therapyc</td>
<td>20 (100)</td>
<td>9 (82)</td>
<td>NSa</td>
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<tr>
<td>Rapid virologic responsed</td>
<td>17 (85)</td>
<td>3 (27)</td>
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<td>Sustained virologic responsea</td>
<td>16 (80)</td>
<td>7 (64)</td>
<td>NSa</td>
</tr>
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</table>

**NOTE.** Data are no (%) of patients, unless otherwise indicated. ALT, alanine aminotransferase; NS, not significant.

* Fisher’s exact test.
* Mann-Whitney U test.
* HCV RNA load <50 IU/mL.
* HCV RNA load <50 IU/mL at treatment week 4.
* HCV RNA load <50 IU/mL at 6 months after the end of treatment.
clearly required to confirm the observations made here, the data indicate that recovery of HCV-specific Th1 responses during peginterferon/ribavirin therapy may be related to rapid viral clearance. However, it remains to be determined whether the observed T cell responses are causally related to viral clearance. We observed 4 patients who developed Th1 responses against NS3-4 yet had a relapse after the end of treatment, leaving open the question as to why HCV persists in the presence of demonstrable antiviral T cell responses. As determined by intracellular cytokine staining, the overall magnitude of HCV-specific Th1 responses in chronically infected patients was weak and appeared to be several-fold lower than that identified previously in patients who spontaneously resolved HCV infection [12]. This may be relevant for future therapeutic strategies that enhance cellular immune responses after viral load is controlled.

In conclusion, HCV-specific Th1 responses in patients with chronic HCV infection are partially recovered during treatment, at least if viral replication is suppressed. The emergence of Th1 responses is short-lived and does not seem to be associated with the final outcome of therapy.

References