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Cellular Immune Responses during High-Dose Interferon-α Induction Therapy for Hepatitis C Virus Infection

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Background. The effect that high-dose interferon (IFN)–α induction therapy for hepatitis C virus (HCV) infection has on cellular immune responses is currently unknown.

Methods. Thirty-one treatment-naive patients with chronic HCV infection received amantadine and ribavirin, combined with 6 weeks of high-dose IFN-α-2b induction therapy followed by weekly pegylated IFN-α-2b, for 24 or 48 weeks. Using IFN-γ and interleukin (IL)-2 enzyme-linked immunospot (ELISpot) assays, we analyzed the pattern of cytokine secretion by structural and nonstructural HCV- and cytomegalovirus (CMV)-specific T cells before, during, and after therapy.

Results. HCV-specific T cell responses, which were predominantly IFN-γ secreting and which correlated with alanine transaminase levels ($r^2 = 0.45; P = .001$), were found before treatment in 10 of 15 patients with a sustained virological response (SVR) and in 11 of 16 in the non-SVR group. There was a striking loss of IFN-γ and IL-2 HCV-specific T cells during therapy, predominantly in the SVR group. This response recovered after cessation of therapy, regardless of outcome. Suppression of CMV-specific T cell responses, in addition to total lymphocyte counts, was also observed.

Conclusions. High-dose IFN-α induction therapy leads to a profound decline in IL-2– and IFN-γ–secreting HCV- and CMV-specific T cells. These data indicate that restoration of T cell responses is unlikely to be causally linked to an early response or SVR to therapy.

Hepatitis C virus (HCV) infection is currently a worldwide epidemic. Combination therapy with pegylated interferon (IFN)–α and ribavirin, presently the standard treatment, leads to long-term resolution of infection in 45%–80% of individuals, depending on viral genotype [1]. The mechanism of action is not completely understood, but both drugs have wide-ranging effects on the immune system [2, 3].

During acute HCV infection, robust T cell immune responses can be detected in most individuals [4, 5]. These responses are maintained in those whose infections spontaneously resolve [6]. However, once persistent HCV infection is established, HCV-specific cellular immune responses are generally weak and narrowly focused [7]. It is plausible, then, that combination therapy leads to long-term viral control by enhancing HCV-specific T cell responses.

There is currently no consensus as to the role that HCV-specific T cells play in inducing a sustained virological response (SVR) to therapy. Recent studies have suggested that the proliferative capacity [8] or magni-
tude [9] of HCV-specific CD8+ and CD4+ T cells before treatment is important. Other investigators have suggested that responses induced during therapy correlate with SVR [10, 11]. Some have shown no association [12, 13]. Studies of T cell responses during successful treatment of acute HCV infection show a decline in HCV-specific T cell responses despite the very high efficacy of therapy at this stage of infection, suggesting that T cell immunosurveillance may not be required to establish an SVR [14, 15].

The quality as well as the magnitude of the HCV-specific T cell response, both in the context of antiviral therapy and in the spontaneous resolution of HCV infection, may also be critically important in determining a successful outcome. We have previously shown that persistent HCV viremia is associated with a significant loss of interleukin (IL)-2–secreting HCV-specific T cells compared with IFN-γ–secreting T cells, whereas IL-2 secretion is preserved in spontaneously resolved HCV infection [16]. Similarly, CD4+ T cells in patients with HIV viremia who fail to respond to therapy have been shown to produce IFN-γ exclusively, whereas the production of both IL-2 and IFN-γ is preserved in patients without viremia [17]. Other investigators have suggested that the emergence of Th1 responses during combination therapy is related to a beneficial outcome [10].

The rate of viral decline may also affect the emergence of T cell responses during therapy. During the first few weeks of combination therapy, 2 phases of viral decline occur. Mathematical modeling has proposed that the first, more rapid decline in viral load is due to the direct antiviral effects of therapy, whereas the second phase is due to immune pressure [18]. Some experimental data indicate that a rapid fall in viral load during therapy is more likely to be associated with restoration of T cell responses than a more gentle decline [19].

In the present study, we explore these issues using high-dose IFN-α induction therapy. High-dose IFN-α induction therapy has been shown to increase the likelihood of an early virological response [20, 21]. However, data on the SVR rate are conflicting [20–23]. We focus on HCV-specific CD4+ T cell responses detectable by IFN-γ and IL-2 enzyme-linked immunospot (ELISpot) assay because we find reproducible responses using this approach [16, 24]. We address 2 specific hypotheses: (1) that a rapid fall in viral load is linked with restoration of HCV-specific responses and an SVR to therapy and (2) that an SVR to treatment is associated with the restoration of IL-2–secreting HCV-specific T cell responses, as has been shown in the spontaneous resolution of HCV infection [16] and in the treatment of HIV infection [17].

METHODS

Patients. Thirty-one patients (21 male and 10 female) with HCV genotype 1 infection who were participating in a clinical study designed to assess the efficacy of the treatment regime described in detail below [25] (table 1) agreed to participate in the present immunological study. HCV RNA was detectable by reverse-transcription polymerase chain reaction (PCR) in the serum on at least 2 consecutive occasions 6 months apart (Amplieric assay, version 2.0; Roche) before treatment. All patients were negative for HIV and hepatitis B virus antibodies. Five patients had cirrhosis (4 proved by biopsy and 1 diagnosis on clinical grounds).

All patients were treated with triple therapy consisting of amantadine hydrochloride (200 mg/day) and ribavirin (1000 or 1200 mg/day, based on body weight) for a total of 24 or 48 weeks, combined with IFN-α-2b induction during the first 6 weeks and thereafter combined with weekly pegylated IFN-α-2b (1.5 µg/kg). The scheme for IFN induction was as follows: weeks 1 and 2, 18 MU/day in 3 divided doses; weeks 3 and 4, 9 MU/day in 3 divided doses; and weeks 5 and 6, 6 MU/day in 2 divided doses.

Patients with a decrease in HCV RNA level of 3 log at week 4 were treated for 48 weeks. Those with a decrease in HCV RNA level of 3 log at week 4 were randomized at week 24 to either stop treatment or continue until 48 weeks. Treatment was stopped in all patients with HCV RNA levels >615 IU/mL at week 24. All patients were followed up for 24 weeks after the completion of therapy. The study was approved by the institutional review board.

Samples. Peripheral blood mononuclear cells (PBMCs) were isolated from blood by density gradient centrifugation and frozen immediately before treatment (week 0); at 12, 24, and 48 weeks after the start of treatment; and at 24 weeks after the completion of treatment (follow-up). Analysis of viral load, alanine transaminase (ALT) level, and full blood counts were performed at week 0; during treatment at weeks 1, 2, 3, 4, 6, 8, 12, 16, 20, 24, and 48; and at 12 and 24 weeks after treatment.

Viral kinetics. Viral kinetics were assessed using a quantitative bDNA assay (Versant HCV assay, version 3.0; linear dynamic range, 6.15 × 105–7.7 × 108 IU/mL), qualitative PCR (COBAS Amplicor HCV test, version 2.0; lower limit of detection, 50 IU/mL), and qualitative transcription-mediated amplification (TMA; Versant HCV qualitative assay; lower limit of detection, 5 IU/mL).

Clinical definitions. Clinical definitions are based on the TMA assay. SVR was defined as loss of HCV RNA in the serum maintained for 24 weeks after therapy. Nonresponders had detectable HCV RNA levels in serum throughout treatment. Breakthrough was defined as initial loss of HCV RNA in the serum followed by recurrence of HCV RNA during therapy. Relapse was defined as absence of viremia during therapy followed by detectable viremia within 24 weeks after completion of therapy. For the purpose of analyses, the non-SVR group included nonresponders and patients with breakthrough or relapse.
ELISpot assays. Thawed PBMCs were tested by IFN-γ (Mabtech) and IL-2 (BD Biosciences) ELISpot assays, as described elsewhere [24]. PBMCs from the same patient derived at multiple time points were assessed concurrently.

Briefly, viable PBMCs (0.2 million/well) were plated in duplicate in anti–IFN-γ–or anti–IL-2–precoated 96-well ELISpot plates and stimulated for 18 h with the following antigens: HCV core–derived peptides spanning aa 1–191 in a single pool; recombinant proteins NS3, NS4, and NS5 (final concentration, 1 μg/mL; Chiron) in a single pool, as described elsewhere [16, 24]; cytomegalovirus (CMV) lysate (final concentration, 0.05 μg/mL; Virusys); medium alone as a negative control; and phytohemagglutinin (PHA; 4 μg/mL) as a positive control. Spot-forming units were counted using an ELISpot plate reader (ELISpot 3.1 SR program; AID Reader System).

A positive response was recorded when the probability of a spot appearing in the stimulated well was significantly different ($P < 0.05$) from the probability of a spot appearing in the negative control well (BINOMDIST function, Microsoft Excel software) [16, 24]. The frequency of T cells was calculated by subtracting the average number of spot-forming cells in negative-control duplicate wells from that in stimulated duplicate wells and is expressed per $10^6$ PBMCs.

Carboxyfluorescein diacetate succinimidyl ester (CFSE)–based proliferation assays. PBMCs ($1 \times 10^7$ cells/mL) in PBS were incubated at 37°C for 7 min with 0.5 μmol/L CFSE (Molecular Probes). PBMCs were washed with PBS containing 10% pooled human serum and then with PBS only. Stained cells ($1 \times 10^6$ cells/well, 1 mL) were cultured in 48-well plates with either medium alone, PHA, core peptide pools (final concentration, 10 μg/mL), or CMV lysate (optimized final concent-

### Table 1. Patient characteristics.

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<th>Patient (age in years, sex)</th>
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<th>Cirrhosis</th>
<th>Pretreatment viral load, IU/mL</th>
<th>TMA assay results</th>
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<th>Response to treatment</th>
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**NOTE.** ALT, alanine transaminase; SVR, sustained virological response; TMA, transcription-mediated amplification.
After 6 days, cells were washed and stained at 4°C with anti-human CD4–allophycocyanin (APC), CD8–phycoerythrin (PE), and Via-Probe (7-amino-actinomycin D) (BD Pharmingen). Flow cytometry analysis was performed on a FACSCalibur flow cytometer and analyzed using FlowJo software (version 8.1; Tree Star).

T cell proliferation was determined by gating on the lineage-positive CFSElow and CFSEhigh subsets. A stimulation index was derived by dividing the CD4+ cell proliferative frequency in the presence of antigen by the CD4+ cell proliferative frequency without antigen, using the following formula: CD4+ proliferative frequency (percentage) = number of CD4+CFSElow cells/(number of CD4+CFSElow cells + number of CD4+CFSEhigh cells) × 100. A stimulation index of >2 was considered positive. The CD4+ cell proliferative frequency for a positive response was determined by subtracting the proliferative frequency with antigen from that without antigen.

**Statistical analysis.** The significance of the proportion of patients with viremia was determined using Fisher’s exact test. Comparisons between SVR and non-SVR cohorts before treatment were made using the nonparametric Mann-Whitney U test. Correlations were determined using the Pearson correlation coefficient (GraphPad Prism for Macintosh, version 4.0b; GraphPad Software, Inc.).
GraphPad Software). T cell responses at each time point were compared with baseline (pretreatment) values using the paired nonparametric Wilcoxon ranked sign test. Differences were considered significant at $P < .05$.

**RESULTS**

**Clinical outcome.** An SVR was observed in 15 (48%) of 31 patients, and 16 (52%) of 31 patients were classified in the non-SVR group; 8 of the 16 were nonresponders, 2 had breakthrough viremia, and 6 had relapses after therapy. There were no significant differences in age or sex between SVR and non-SVR groups (table 1). At week 12, viremia was undetectable in 19 of 31 patients (13 of 15 in the SVR group and 6 of 16 in the non-SVR group; $P = .009$).

**HCV-specific T cell responses, ALT level, and viral load before therapy in relation to outcome.** Before therapy, HCV core-specific T cell responses, which we and others have shown previously to comprise largely CD4+ T cells [7, 16, 26, 27], were detected in 21 of 31 patients, including 10 of 15 patients in the SVR group and 11 of 16 patients in the non-SVR group (figure 1A). The mean viral load, ALT level, and HCV core-specific responses detected by IFN-γ ELISpot assay were lower in patients who achieved an SVR than in those in the non-SVR group (mean ± SD viral load, 5.791 ± 0.1723 vs. 5.990 ± 0.1181 log10 IU/mL; ALT level, 118.4 ± 27.25 vs. 134.9 ± 29.44 IU/L; core response, 85.07 ± 31.58 vs. 187.9 ± 94.09 sfu/1 × 10^6 PBMCs), although these differences did not reach statistical significance. There was no significant association between the magnitude of the core-specific response and the viral load before treatment. However, there was a significant correlation between HCV core-specific IFN-γ response and ALT level (Pearson $r^2 = 0.45; P = .001$) (figure 1B).

For 13 patients (5 SVR and 8 non-SVR) with HCV core-specific T cell responses detected by IFN-γ ELISpot assay, sufficient PBMCs were available before treatment to permit further analysis with CFSE assays. Nine of 13 patients showed CD4+ T cell proliferation to HCV core antigens. For 2 of these patients, proliferative responses were also observed for the CD8+ T cell subset, consistent with findings from other studies showing that T cell responses to core were largely CD4+ [16]. Proliferative responses to HCV core antigens were very weak (mean CD4+ CFSELow cells/total CD4+ cells = 1.15%) compared with proliferative responses to CMV antigens (mean CD4+ CFSELow cells/total CD4+ cells = 42.96%), consistent with findings from another study [27]. Findings from a representative patient are shown (figure 1C). The magnitude of the HCV proliferative re-
response before treatment did not differ between SVR and non-SVR groups (data not shown).

**Effect of therapy on HCV-specific IFN-γ and IL-2 responses.**

HCV core–specific responses were assessed in all patients by IFN-γ and IL-2 ELISpot assay before treatment; at weeks 12, 24, and 48 during treatment; and 24 weeks after treatment was completed. Overall, compared with pretreatment values, there was a decline in IFN-γ T cell responses at weeks 12 (for week 0 vs. 12, \(P = 0.007\)), 24 (for week 0 vs. 24, \(P = 0.009\)), and 48 (difference not significant); these responses recovered after therapy (figure 2A). The loss of statistical significance at 48 weeks may reflect the smaller number of patients assessed at this time point because of the clinical protocol. Subgroup analysis showed that the decline in T cell responses was significant only in patients with an SVR to therapy (for week 0 vs. 12, \(P = 0.02\); for week 0 vs. 24, \(P = 0.019\)) (figure 2B). The decline in T cell responses between weeks 0 and 12 was also significant in the 19 patients who were PCR negative at week 12 (\(P = 0.008\)) but not in the 12 who were still PCR positive at that time point. The viral kinetics and HCV-specific T cell responses for 4 representative patients (311, 341, 335, and 333) are detailed in figure 3.

IL-2 secretion could be detected before therapy in 8 of 31 patients. The mean IL-2 production by HCV core–specific T cells was significantly less than the mean IFN-γ production (mean ± SD, 35.72 ± 14.38 vs. 138.1 ± 50.97 sfu/1 × 10^6 PBMCs; \(P = 0.030\)) before treatment and remained lower at all time points during therapy. IL-2 production decreased during therapy but recovered after the cessation of therapy. This was true for both the SVR and the non-SVR groups. The mean numbers of IL-2–secreting HCV core–specific T cells were higher at follow-up than before treatment in both the SVR and the non-SVR groups, although these differences did not reach statistical significance.

Cytokine production was further assessed by ELISpot assay after stimulation with HCV proteins NS3–5. Weak IFN-γ responses were detected in only 5 of 31 patients (3 SVR and 2 non-SVR; mean ± SD IFN-γ production, 42.4 ± 8.9 sfu/1 × 10^6 PBMCs). Responses to NS3–5 did not increase during therapy and were lower than pretreatment values at all time points during therapy, but these differences did not reach statistical significance. There was no significant increase in HCV NS3–5–specific IL-2 secretion during therapy.

**Effect of therapy on CMV-specific T cell responses.**

To determine whether the suppression of T cell responses observed during therapy was specific to HCV, we assessed the magnitude of CMV-specific T cell responses during therapy. Before treat-
ment, CMV responses could be detected by IFN-γ ELISpot assay in 14 of 31 patients (mean ± SD magnitude, 461.4 ± 46.31 sfu/1 × 10⁶ PBMCs). Compared with the baseline response, there was a marked decline in the magnitude of the CMV-specific T cell response during treatment, at 12 weeks (mean ± SD, 178.2 ± 49.86 sfu/1 × 10⁶ PBMCs; P = .003) and 24 weeks (mean ± SD, 236.0 ± 80.0 sfu/1 × 10⁶ PBMCs; P = .037). This response recovered after the cessation of therapy (mean ± SD, 307.5 ± 60.02 sfu/1 × 10⁶ PBMCs) (figure 4A). Compared with baseline values, the CMV-specific T cell responses declined in both the SVR and the non-SVR groups by week 12, but the decrease was significant only in the SVR group (P = .01) (figure 4B).

Effect of therapy on global T cell populations. Because HCV- and CMV-specific T cell responses fell during therapy and recovered after therapy, we performed FACS analysis at weeks 0 and 12 to assess the impact of therapy on global T cell populations. We selected 6 patients in whom HCV-specific T cell responses were detectable by IFN-γ ELISpot assay at week 0 but were undetectable at week 12 (figure 5A). FACS analysis of PBMCs showed a fall of ~50% in both the CD4⁺ and the CD8⁺ T cell subsets within the total PBMC population (figure 5B and 5C). Analyzing the blood lymphocyte counts in all patients at weeks 0 and 12, we observed that the mean in the SVR group was 5.8 × 10⁹ cells/mL before treatment, declining in 13 of 15 patients to a mean count of 3.3 × 10⁹ cells/mL by week 12. Similarly, in the non-SVR group, the mean count before treatment was 6.4 × 10⁹ cells/mL, declining in 14 of 15 patients (data not available for 1 patient) to a mean count of 3.3 × 10⁹ cells/mL by week 12 (figure 5D). Thus, although 200,000 cells were used in each ELISpot well, at week 12 the T cell fraction within each well would be ~50% less than pretreatment values, at least in some cases. However, this observation alone cannot explain the complete loss of HCV-specific T cell responsiveness observed in many patients during the first 12 weeks of therapy.

DISCUSSION

In the present study, we assessed the effects on cellular immune responses of high-dose IFN-α induction therapy followed by
conventional doses of pegylated IFN and ribavirin/amantadine. We found that there was a profound decline in HCV-specific T cell responses during therapy, which was statistically significant in patients who had a subsequent SVR to therapy. Although a decrease in T cell responses has been observed during resolving primary HCV infection in parallel with a declining HCV load [14], this finding has not been described previously in the context of HCV antiviral therapy.

It is tempting to speculate that high-dose IFN-α leads to a rapid decline in viral load and that, as antigen is rapidly lost, antigen-dependent T cell responses wane in the absence of ongoing antigenic stimulation. This is in keeping with the observation that the decline in T cell response at 12 weeks was statistically significant only in those who achieved an SVR and were negative for HCV RNA at week 12.

However, this does not explain the observation that, in many individuals, T cell responses recover once therapy has stopped, but HCV loads remain undetectable. In this study, there was a decline in blood lymphocyte counts between baseline and 12 weeks in almost all patients. Furthermore, we observed a decline not only in HCV-specific T cell responses but also in CMV-specific T cell responses during therapy. FACS analysis of PBMCs at weeks 0 and 12 of therapy confirmed that there was a decline in the CD4+ and CD8+ T cell subsets. Taken together, these data suggest that the observed decline in T cell responses is due, at least in part, to a direct effect of therapy on T cells through direct suppression of their generation (IFN-α is known to exert suppressive effects on bone marrow), increased apoptosis, or, more speculatively, T cell redistribution away from the peripheral blood compartment. That the decline in both HCV- and CMV-specific responses was more marked in the SVR group suggests that these patients may be inherently more sensitive to IFN.

We analyzed HCV-specific T cell responses using antigen pools. The first of these, a core peptide pool, has been shown to generate a reproducible response in a number of previously studied cohorts [16, 24, 27, 28]. This approach is analogous to the use of overlapping peptide pools for CMV pp65 or HIV Gag in other antiviral studies. These responses appear to be largely from CD4+ T cells, according to a number of previous analyses.
that include depletion studies [16, 24], CFSE proliferation [27], and cytokine-based flow assays [16]. In contrast, previous detailed mapping studies of CD8+ T cell responses in HCV-positive donors failed to reveal ex vivo responses targeting HCV core [7]. Similar data concerning CD4+ T cell responses targeting core in chronic infection have been obtained by other investigators [26]. Analysis of core peptide–specific responses is not a comprehensive analysis but provides a target population of T cells present before treatment to track over time. To add breadth, we also used an NS3–5 whole-antigen pool, as has been used extensively elsewhere [16, 24]. Importantly, no enhancement of these responses was observed during treatment.

Although we showed no association between the magnitude of the HCV-specific T cell response before therapy and the subsequent response to therapy, we did observe a highly significant correlation between liver inflammation (ALT level) and HCV-specific T cell responses. This is consistent with findings of other studies comparing patients with normal and elevated ALT levels, using a range of methods to analyze HCV-specific CD4+ T cell function [29].

We have shown previously that the functional status of HCV-specific T cells is important in controlling HCV infection [16]. In the present study, then, we addressed the hypothesis that combination therapy is associated with the restoration of IL-2–secreting cellular immune responses. We observed that IL-2 secretion by HCV-specific T cells was significantly lower than that before treatment and declined further during treatment in both the SVR and the non-SVR groups. After therapy, IL-2 secretion was restored to pretreatment levels in those with an SVR but was not enhanced above baseline.

Overall, in the present study we demonstrated that, rather than enhancing the T cell response, as might have been predicted, high-dose IFN-α induction therapy is associated with a profound loss of IL-2– and IFN-γ-secreting HCV-specific T cells. This effect is probably mediated both through loss of antigenic stimulation of HCV-specific T cells and through direct immunosuppressive effects of high-dose IFN-α therapy. Although the effect is greatest in the setting of high-dose IFN-α, the relevance of these observations is much more general. First, the very rapid virological response seen can readily occur in the absence of immune restoration—indeed, during the loss of HCV-specific T cells—which strongly implies that this phase of viral dynamics is independent of T cell responses. Second, long-term antiviral effects are also obtained while T cell responses are suppressed. Again, this finding supports an argument that any changes in T cell responses seen after the administration of conventional doses of IFN-α therapy are not causally related to clinical outcome. That T cell responses induced during current antiviral therapies appear not to influence virological outcomes does not mean that further direct manipulation of such responses would not confer benefit.

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References

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