

Expansion of hepatitis C-specific CD4⁺CD25⁺ regulatory T cells after viral clearance: A mechanism to limit collateral damage?

Andrew Godkin, MD, FRCP,^{a,c,*} Wan Fai Ng, PhD, MRCP,^{b,*§} Kathleen Gallagher, PhD,^c Gareth Betts, PhD,^c Howard C. Thomas, PhD, FRCP, FMedSci,^a and Robert I. Lechler, PhD, FRCP, FMedSci^{b,d} London and Cardiff, United Kingdom

Background: Data from rodent models suggest that a subpopulation of CD4⁺ T cells, characterized by the constitutive expression of CD25, play a key role in regulating many immune responses. Human CD4⁺CD25⁺ T cells also appear to possess a regulatory function, but their role in infections is not fully defined.

Objectives: We sought to explore the possibility of a role for CD4⁺CD25⁺ T cells in controlling immunity to hepatitis C virus (HCV). We hypothesized that CD4⁺CD25⁺ T cells might account for the paucity of immune responses measurable in chronically viremic patients by suppressing the immune responses to HCV antigens.

Methods: We compared the responses of PBMCs to 3 different recombinant HCV antigens before and after depletion of CD25⁺ cells in 15 chronically viremic patients, 14 nonviremic HCV antibody-positive subjects, and 14 healthy control subjects. We also tested the ability of CD4⁺CD25⁺ T cells purified from HLA-matched viremic or nonviremic blood to suppress the responses of HCV epitope-specific T-cell clones.

Results: To our surprise, depletion of peripheral blood CD25⁺ cells led to a pronounced increase in proliferation of and IFN- γ production by PBMCs only in nonviremic patients.

Furthermore, the CD4⁺CD25⁺ T cells purified from HLA-matched nonviremic blood (in contrast to CD4⁺CD25⁺ T cells isolated from chronically viremic blood) inhibited the responses of HCV epitope-specific T-cell clones.

Conclusion: HCV-specific CD4⁺CD25⁺ regulatory T cells appear to accompany successful viral clearance. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Hepatitis C infection, regulatory T cells, autoimmunity, viral clearance, viral persistence

In rodent models CD4⁺CD25⁺ regulatory T (Treg) cells have been shown to play a central role in the regulation of immune responses.^{1,2} Treg cells are potent suppressors of CD4⁺ and CD8⁺ T cells *in vitro* and *in vivo*. We and others have reported that CD4⁺CD25⁺ T cells in human subjects share many of the characteristics of their murine counterparts.³⁻¹⁰ These Treg cells appear to be a part of the normal repertoire of peripheral CD4⁺ T cells, but their function in health and disease remains to be fully defined in human subjects. Treg cells have been implicated in controlling immune responses to various pathogens,¹¹ but neither the origin nor the physiologic function of these Treg cells has been fully elucidated.

Hepatitis C virus (HCV) causes chronic infection in 80% of infected individuals¹² and is a major cause of morbidity and mortality worldwide. After exposure to HCV, a minority of infected individuals successfully eradicate the virus either spontaneously or after treatment with interferon and ribavirin.¹³ The outcome of a viral infection is determined by the interplay of viral factors and host immune responses. In general, the generation of virus-specific cytotoxic T lymphocytes during the initial phase of the infection is crucial to the subsequent control of the infection.^{14,15} The maintenance of virus-specific cytotoxic T-lymphocyte responses, however, depends on antigen-specific CD4⁺ T_H cell activity.¹⁶ Indeed, the ability to mount a strong and sustained antiviral T_H1 response was associated with subsequent viral clearance, whereas failure to have or sustain an HCV-specific CD4⁺ T-cell response was associated with chronic infection.¹⁷ The discovery of naturally occurring Treg cells raised the possibility that these cells might be subverted by the invading virus to inhibit antiviral responses. In this study we tested this hypothesis by investigating the antiviral responses in patients with persistent viremia and individuals who have successfully eradicated the virus.

METHODS

Study population

Patients were given diagnoses of past or present HCV infection on the basis of clinical history and the presence of anti-HCV antibodies. Chronic infection was diagnosed on the basis of the persistence of viral RNA (measured by means of nested PCR) in serum at least 6 months after exposure. A cleared

From ^athe Department of Medicine, Imperial College, St Mary's Campus, and ^bthe Department of Immunology, Imperial College, Hammersmith Campus, London; ^cthe Department of Medical Biochemistry and Immunology, Henry Wellcome Building, School of Medicine, Cardiff University; and ^dthe School of Medicine and Dental Institute, Guy's, King's, and St Thomas' Hospitals, King's College London.

*These authors contributed equally to this work.

†Dr Godkin is currently affiliated with the Department of Medical Biochemistry and Immunology, Henry Wellcome Building, School of Medicine, Cardiff University, Cardiff, United Kingdom.

§Dr Ng is currently affiliated with the Musculoskeletal Research Group, Institute of Cellular Medicine, University of Newcastle, United Kingdom.

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Reprint requests: Wan Fai Ng, PhD, MRCP, Musculoskeletal Research Group, Institute of Cellular Medicine, University of Newcastle, Newcastle upon Tyne NE2 4HH, United Kingdom. E-mail: Wan-fai.Ng@ncl.ac.uk.

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Abbreviations used

HCV: Hepatitis C virus
 SI: Stimulation index
 TCR: T-cell receptor
 Treg: CD4⁺CD25⁺ regulatory T
 UK: United Kingdom

infection was diagnosed on the basis of at least 3 negative HCV PCR results, each separated by 6 to 12 months, and normal liver function tests. Eight of 14 nonviremic patients had previously received IFN- α . The HLA typing was performed with allele-specific primer PCR. Ethical committee approvals (St Mary's Hospital NHS Trust and Cardiff and Vale NHS Trust, United Kingdom [UK]) were granted. Clinical characteristics of the patients are summarized in Table E1 (available in the Online Repository at www.jacionline.org).

Culture media and reagents

RPMI-1640 medium supplemented with l-glutamine, penicillin/streptomycin, amphotericin (all from Gibco BRL, Paisley, UK), and gentamicin (Sigma, Poole, UK), with 10% added human AB serum (Harlan Sera-Lab, Loughborough, UK) was used. All cells were incubated at 37°C in a humidified atmosphere with 95% air and 5% CO₂.

HCV antigens

The peptides were synthesized commercially (ECHAZ Micro Collections, Tübingen, Germany), according to the sequence of viral genotype 1a based on Simmonds's classification (epitopes 1.2: core 31-45; epitopes 2.2: core 141-155).¹⁸ HCV recombinant viral proteins were a kind gift from Chiron Corp (Emeryville, Calif; core protein: c22-3 [2-120]; nonstructural proteins: c200 [NS3/4 1192-1931 and NS5 2054-2995]). Tuberculin-purified protein derivative (Hammersmith Hospitals NHS Trust, London, UK) and tetanus toxoid (Evans Vaccines, Merseyside, UK) were used as control antigens.

Antibodies

Fluorescein isothiocyanate-conjugated anti-CD4 (Becton Dickinson, Oxford, UK), phycoerythrin-conjugated anti-CD25 (Caltag, Silverstone, UK), fluorescein isothiocyanate-conjugated anti-Foxp3 (eBioscience, San Diego, Calif), peridinin-chlorophyll-protein/Cy5.5-conjugated anti-CD4 (PharMingen, San Diego, Calif), and isotype controls antibodies (Caltag) were used in flow cytometry. Antibodies from clones 43-11, 9D7 (Immunokontact, Witney, UK), and 9016.2 (R&D, Abingdon, UK) were used as capture antibodies for detection of IFN- γ , IL-10, and TGF- β in ELISAs, respectively. The corresponding detection antibodies used were 45-15 (Immunokontact, Paris, France), 12G8 (Becton Dickinson, Franklin Lakes, NJ), and anti-TGF- β chicken immunoglobulin (R&D). The cytokine standards used were recombinant human IFN- γ (Biosource, Nivelles, Belgium), IL-10 (Firstlink, Birmingham, UK) and TGF- β (R&D).

Proliferation assays

PBMCs were isolated from peripheral blood by means of centrifugation over Lymphoprep (Nygaard, Birmingham, UK). CD25⁺ cells were removed from PBMCs by means of immunomagnetic depletion with CD25 Dynabeads (DynaL, Wirral, UK), according to the manufacturer's protocol. Triplicates of 2×10^5 PBMCs or CD25⁺ cell-depleted PBMCs per well were incubated in 96-well round-bottom plates. Antigen (10 μ g/mL) was added to the test wells. After 6 days, 75 μ L of supernatant was collected for measurement of cytokine production. Medium containing 0.5 μ Ci of tritiated thymidine (Amersham Life Sciences, Amersham, UK) was added to the remaining culture, and proliferation was measured by means of incorporation of tritiated thymidine with a β -counter after 12 to 16 hours. The stimulation index (SI) was calculated as counts per minute with antigen/counts per minute without antigen. Response to PHA (2 μ g/mL; Sigma, St Louis, Mo) measured at day 3 was used as a positive control.

Measurement of cytokine production with ELISA

Measurement of cytokine production with ELISA was carried out as described previously.³ Cytokine concentrations and limits of detection were calculated by using the standard curves derived from the cytokine standards. The detection limits for IFN- γ , IL-10, and TGF- β were 2.5 to 20, 4 to 40, and 15 to 65 pg/mL, respectively. SIs were calculated as for proliferation assays. If the calculated cytokine concentrations were less than the detection limits, the value equated to the limit of detection of that assay was assigned for the purpose of SI calculation.

Purification of CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells

Purification of CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells was carried out as described previously.³ The purity of CD4⁺CD25⁻ cells was 85% to 90%, and that of CD4⁺CD25⁺ cells was 90% to 95%, as measured by means of flow cytometry. The contamination of CD4⁺CD25⁻ cells consisted of mostly CD4⁺CD25^{lo} T cells. Depletion of these CD4⁺CD25^{lo} cells made no difference to the functional responses of the CD4⁺CD25⁻ cell population (Lechler R, unpublished observation).

Generation of HLA-DR11-restricted HCV epitope-specific T-cell clones

PBMCs from a nonviremic HLA-DR11⁺ subject were used to generate short-term T-cell lines to peptide 1.2 or 2.2, as described previously.¹⁹ The T-cell lines were plated out at 0.3 cells per well in the presence of 10^5 irradiated autologous PBMCs and PHA. Cultures were fed with fresh medium containing 50 IU/mL recombinant IL-2 every 3 to 4 days. Cells from growth-positive cell cultures were tested in a tritiated thymidine proliferation assay after 10 to 12 days to identify antigen-specific clones. To assay the clones, 5×10^3 T cells were cultured with 5×10^4 irradiated HLA-DR11⁺ PBMCs and 10 μ g/mL peptide for 48 hours. For inhibition experiments, CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells were added at a ratio of 2:1 (ie, 10^4 /well) to the culture.

RESULTS**Responses to HCV antigens were more readily detectable in nonviremic patients compared with viremic patients**

We compared the proliferative responses in 15 chronically infected subjects, 14 nonviremic HCV antibody-positive subjects, and 14 healthy control subjects to recombinant viral proteins (c22-3, c200, and NS5; Fig 1, A). Both groups of HCV-exposed subjects contained some responders (defined as an SI >3) to the c22-3 protein. However, there was an increased response in the nonviremic group to both c200 (7/14 in the nonviremic group vs 1/15 in the viremic group) and NS5 (2/10 in the nonviremic group vs 0/12 in the viremic group) antigens. The responses to control antigens were similar, indicating that nonspecific immunosuppression did not account for the poor antiviral responses in viremic subjects (Fig 1, B). No positive response was detected to any of the viral antigens among unexposed healthy subjects ($P < .0006$, HCV-exposed vs unexposed subjects), suggesting that these antiviral responses were induced after antigen exposure and were biologically relevant.

Depletion of peripheral blood CD25⁺ cells enhanced the proliferative responses to HCV antigens in nonviremic but not chronically infected patients

The reason for the paucity of antiviral responses is unclear. We have shown that HCV epitope-specific CD4⁺ T-cell responses can be clearly demonstrated if the T cells are first exposed *in vitro*

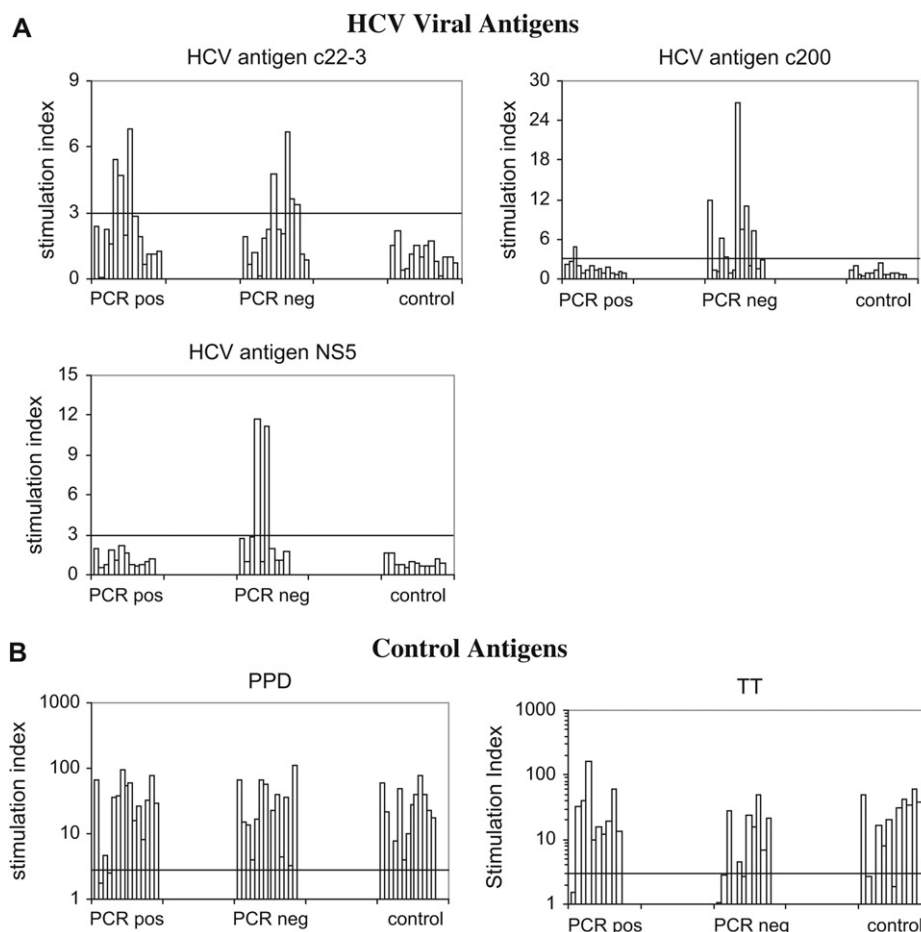


FIG 1. Proliferative T-cell responses to HCV and control antigens. PBMCs (2×10^5 per well) from viremic patients (*PCR pos*), nonviremic subjects (*PCR neg*), and unexposed healthy control subjects were stimulated with viral antigens (**A**) or control recall antigens (**B**). Proliferative responses are considered positive if the SI is greater than 3.

to exogenous IL-2.^{19,20} PBMCs from these same individuals usually failed to respond in standard 5-day proliferation assays to these epitopes without exogenous IL-2 (Godkin A, unpublished data). We have also shown that the addition of IL-2 *in vitro* abrogates the suppressive function of Treg cells in human subjects.³ Furthermore, Treg cells can be induced in the context of a chronic antigenic burden (eg, allografts), and transfer of these cells to naive recipients confers tolerance to the same alloantigens.²¹ Thus it is possible that the chronic presence of HCV antigens might also induce HCV-specific Treg cells. To test this hypothesis, we compared the effects of CD25⁺ cell depletion on anti-HCV responses in viremic and nonviremic individuals. Proliferative responses to the HCV antigens were measured before and after depletion of CD25⁺ cells from PBMCs; the recall antigens tuberculin-purified protein derivative and tetanus toxoid were used as controls. To our surprise, depletion of CD25⁺ cells had only minimal effect on the responses to HCV antigens in patients with chronic infection, but on the responses in nonviremic subjects, the effect was striking. Ten (71%) individuals in the nonviremic group showed an enhanced response (SI >3 after depletion) to 1 or more HCV antigens. Of the total 38 assays performed with viral antigens in the nonviremic group, the SIs increased in 55% after depletion, whereas just 5% decreased (Fig 2, A). In the assays that showed

an enhanced response, 32% had an SI of less than 3 before depletion. Hence the depletion of the CD25⁺ cells unmasked anti-HCV responses that were previously not apparent among nonviremic subjects. In contrast, only 3 (19%) viremic subjects had an enhanced response to 1 or more viral antigens after depletion. In this group merely 17% of the total 41 assays revealed an increase and 10% revealed a decrease in the SI after depletion (Fig 2, B). Thus our initial hypothesis was not supported by the experimental findings because depletion of CD25⁺ cells had an insignificant effect in chronically infected patients. Interestingly, in the nonviremic group, among the assays that demonstrated an initial positive response ($n = 13$), the SI was higher in 11 (85%) after depletion of CD25⁺ cells. In contrast, among the positive initial responses in viremic patients ($n = 4$), only 1 had a higher SI after depletion, raising the possibility that the CD25⁺ cells in these patients were activated viral antigen-specific cells rather than regulatory cells.

An alternate and more stringent definition of an increased response is a greater than 2-fold increase in the SI after depletion. If this definition was used, a similar result was obtained in that CD25⁺ cell depletion had a much more pronounced effect on nonviremic subjects. Seven (50%) subjects showed a 2-fold increase in response to 1 or more HCV proteins after depleting

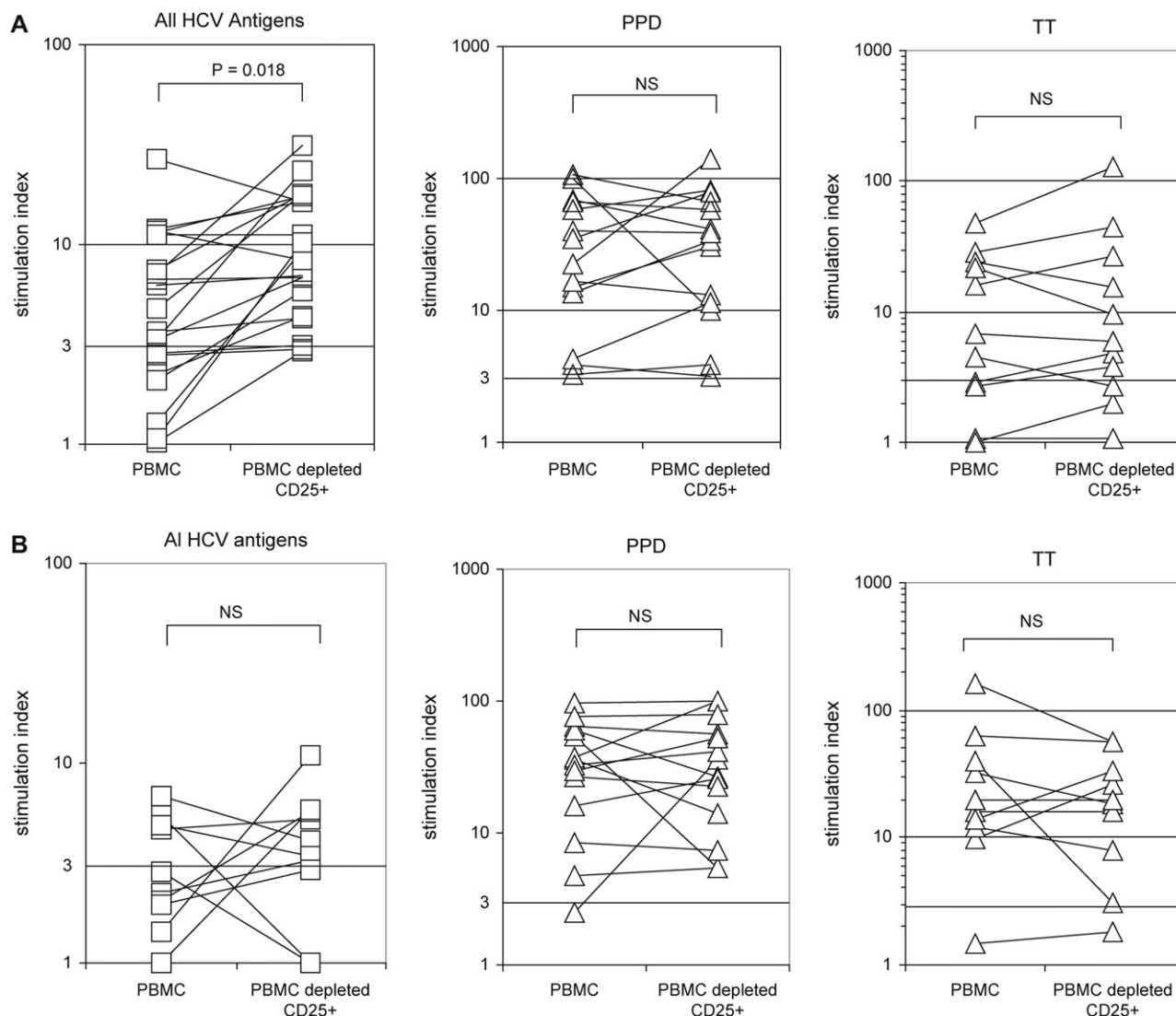


FIG 2. Proliferative responses to viral and control antigens in nonviremic and viremic subjects before and after depletion of CD25⁺ cells. **A**, Of the 38 assays in the presence of HCV antigens performed in the nonviremic group ($n = 14$), the 21 assays that had an SI of greater than 3 either before or after the depletion are shown. Similarly, all assays that had an SI of greater than 3 before or after depletion of CD25⁺ cells to control antigens are shown. **B**, Forty-one assays were performed with HCV antigens in the chronically infected individuals ($n = 15$), and of these, 10 assays showed an SI of greater than 3 either before or after the depletion. Similarly, all assays that had an SI of greater than 3 before or after depletion of CD25⁺ cells to control antigens are shown. PPD, Tuberculin-purified protein derivative; TT, tetanus toxoid.

CD25⁺ cells. Of the total 38 assays performed with HCV antigens in the nonviremic group, a 2-fold increase in the SI was observed in 13 (34.2%) subjects after depletion, whereas just 1 (2.6%) showed a 2-fold decrease in the SI. In contrast, only 2 (13%) viremic subjects had an increased response to 1 or more HCV antigens after depletion. Three (7.3%) of the total of 41 assays revealed a 2-fold increase in response, and 3 (7.3%) assays showed a 2-fold decrease. Depletion of CD25⁺ cells had no effect on unexposed healthy control subjects. None of the 40 assays showed an increased response. In our patient or control groups no significant difference was seen in the responses to the control antigens before and after depletion of CD25⁺ cells (mean SIs before and after depletion were 37 vs 33 [viremic, $P = .52$], 29 vs 34 [nonviremic, $P = .45$], and 30 vs 33 [unexposed, $P = .56$]).

Responses to PHA were enhanced after depletion of CD25⁺ cells in all subjects (data not shown).

Depletion of peripheral blood CD4⁺CD25⁺ cells enhanced IFN- γ production to HCV antigens in nonviremic subjects

After antigen stimulation, T cells produce various cytokines that serve important effector functions. For instance, proinflammatory cytokines protect against viral infection, whereas anti-inflammatory cytokines might facilitate viral persistence. In this study we measured the production of IFN- γ , IL-10, and TGF- β in the culture supernatant by using ELISAs. PBMCs from 5 viremic and 2 nonviremic subjects produced IFN- γ (defined as an SI >3)

when stimulated with NS5. There was no IFN- γ response to c22-3 or c200 in either viremic or nonviremic individuals. However, on depletion of CD25⁺ cells, 7 nonviremic subjects produced IFN- γ in response c200 antigen (Fig 3, A). In contrast, only 1 viremic patient had a positive response after CD25⁺ cell depletion. This observation further corroborates our finding on the effect of CD25⁺ cell depletion on proliferative responses in HCV-exposed subjects. Another explanation for the paucity of anti-HCV responses in viremic patients is the presence of regulatory cytokines, such as IL-10 and TGF- β . In this study none of the viremic or nonviremic subjects produced IL-10 to the 3 HCV antigens tested. Regarding TGF- β production, 1 viremic patient and 3 nonviremic subjects produced TGF- β in response to c200 or NS5. However, 3 healthy volunteers also responded to c200, and another 3 responded to NS5 (data not shown). Finally, depletion of CD25⁺ cells has a minimal effect on the production of IL-10 and TGF- β in either viremic or nonviremic subjects (data not shown).

CD4⁺CD25⁺ cells from nonviremic but not chronically infected subjects suppress the proliferation of HCV epitope-specific T-cell clones

Data from rodent models suggest that activation of Treg cells through the T-cell receptor (TCR) is antigen specific.^{1,2} For instance, in mice transgenic for a TCR specific for an ovalbumin peptide, Treg cells can be activated by ovalbumin peptide presented by autologous antigen-presenting cells.²² Little is known in human subjects regarding the antigen specificity of Treg cells. Our data suggest that a population of Treg cells is present within the peripheral blood CD25⁺ population in nonviremic patients and regulates, to a degree, anti-HCV immune responses.

We have previously demonstrated that the HCV epitopes 1.2 and 2.2 are recognized by all HLA-DR11⁺ nonviremic individuals.^{19,20} An HLA-DR11-restricted clone recognizing epitope 1.2 was generated. Peripheral blood CD4⁺CD25⁺ or CD4⁺CD25⁻ cells from an HLA-DR11⁺ nonviremic subject were added to clone cells at a ratio of 2:1. Only the CD4⁺CD25⁺ cells reduced the proliferation of the clone (Fig 4, A). In contrast, no inhibition was seen when CD4⁺CD25⁺ cells from an HLA-DR11⁺ viremic patient were used (Fig 4, B), which is consistent with the earlier observation that HCV-specific Treg cells do not appear to be prominent in viremic individuals. Similar results were obtained when a different clone specific for viral epitope 2.2 was used (Fig 4, C and D). CD4⁺CD25⁺ cells from a healthy control subjects had no effect on proliferation by the T-cell clones (data not shown). Similar results were obtained when the experiments were repeated with CD4⁺CD25⁺ cells from another HLA-DR11⁺ nonviremic subject and HLA-DR11⁺ viremic patient (data not shown).

After the completion of these experiments, Foxp3 expression was identified as a more specific marker for Treg cells than CD25 because CD25 is expressed also on recently activated T cells. Thus it is possible that CD4⁺CD25⁺ cells from viremic subjects contained mainly recently activated T cells. However, of the viremic/nonviremic patients who we have studied, the frequencies of Treg cells, as defined by CD4⁺CD25^{hi}Foxp3⁺ expression, among these subjects was similar (see Fig E1 in the Online Repository at www.jacionline.org).

Taken together, our data suggest that populations of Treg cells are generated after viral clearance and that these regulatory cells

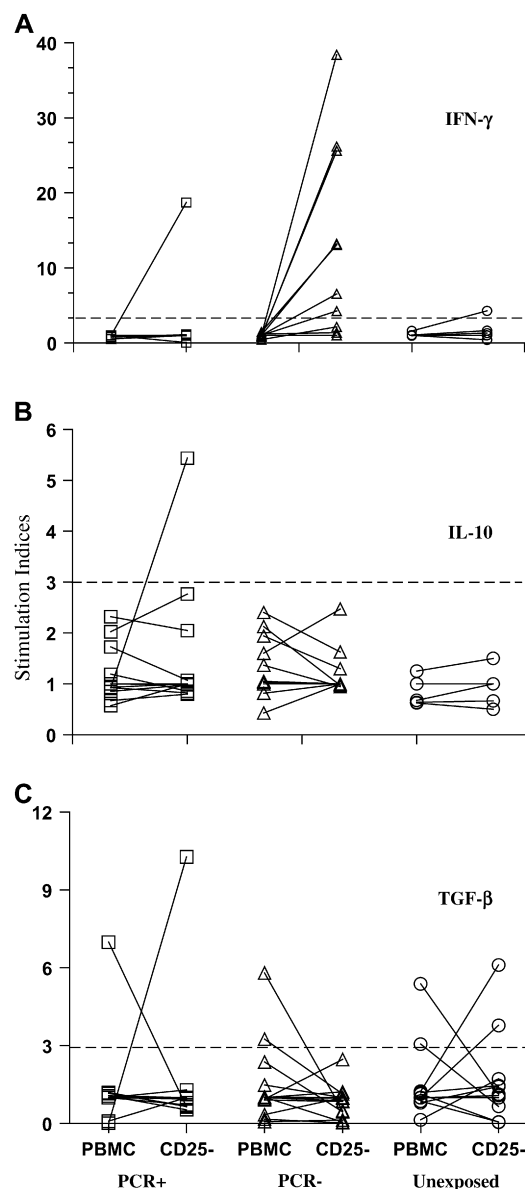


FIG 3. Cytokine responses to HCV antigens before and after depletion of CD25⁺ T cells. Cytokine production in supernatants from HCV c200-stimulated PBMCs before and after CD25⁺ cell depletion is shown. Cytokines were measured with ELISAs. Data were expressed as SIs (ie, concentration of cytokine with antigen/background cytokine production with no antigen). **A**, IFN- γ ; **B**, IL-10; **C**, TGF- β . Only the nonviremic group showed an increase in IFN- γ levels after CD25⁺ cell depletion.

can be activated by the same HCV epitopes that are thought to be important in viral clearance.^{19,20}

DISCUSSION

In this study we showed that the responses to recombinant HCV antigens are more readily detectable in subjects who eradicated the virus compared with those with persistent viremia. The poor responses to HCV antigens in viremic PBMCs could not be reversed by depleting peripheral blood CD25⁺ cells. Instead, we found that Treg cells with specificity for HCV antigens appear to be induced after viral clearance.

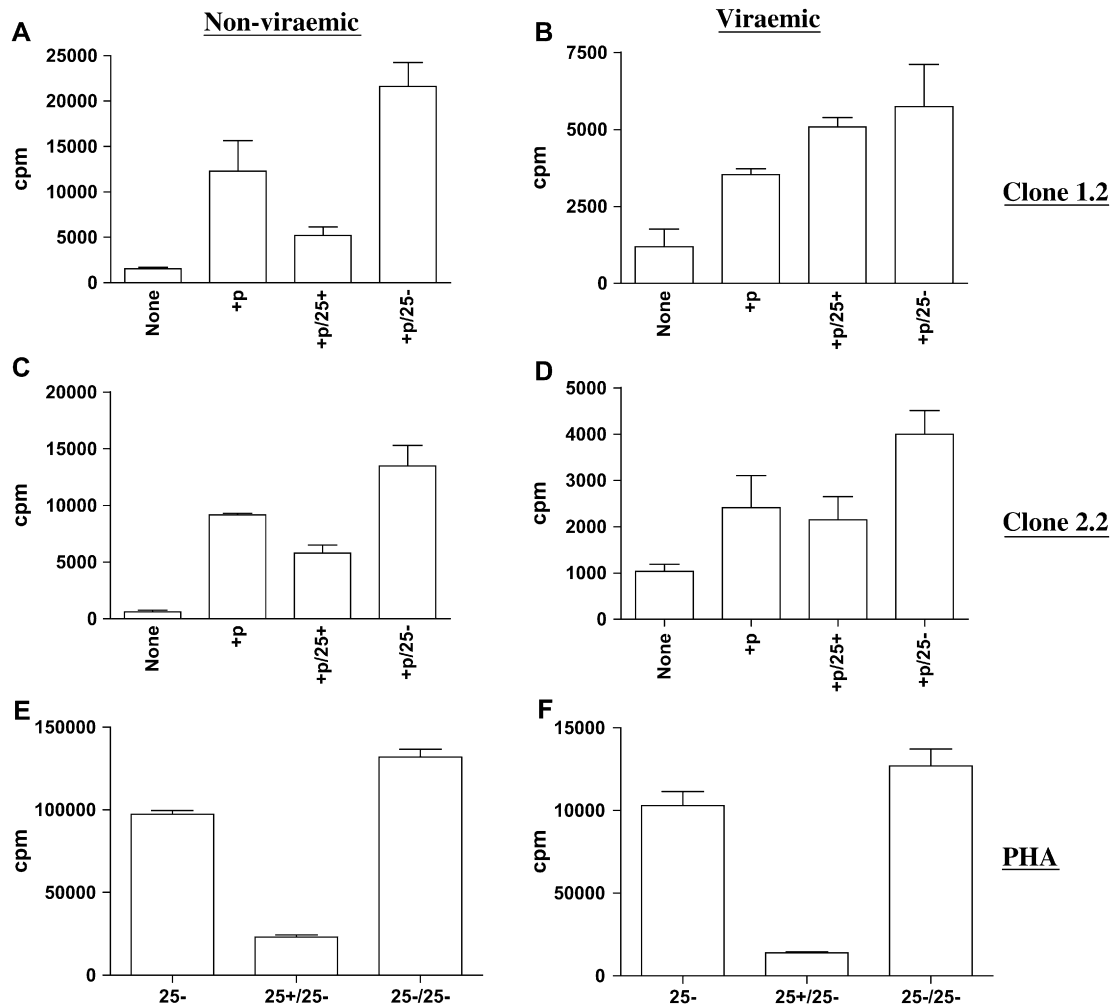


FIG 4. The effect of CD4⁺CD25⁺ T cells on proliferation of HCV epitope-specific clones. **A-D**, Cells (5×10^3) of clone 1.2 (Fig 4, A and B) or clone 2.2 (Fig 4, C and D) were cocultured with 10^4 CD4⁺CD25⁺ (+p/25+) or CD4⁺CD25⁻ (+p/25-) T cells from nonviremic (Fig 4, A and C) or viremic (Fig 4, B and D) blood in the presence of appropriate HCV peptides (10 μ g/mL). Clone cells stimulated with (+p) or without (none) appropriate HCV peptides were used as positive and negative controls, respectively. Irradiated HLA-DR11⁺ PBMCs (5×10^4) were used as accessory cells in all wells. Proliferation was measured after 2 days. **E** and **F**, CD4⁺CD25⁺ T cells (10^4) from nonviremic (Fig 4, E) or viremic (Fig 4, F) blood were cocultured with 10^4 autologous CD4⁺CD25⁻ T cells in the presence of 5×10^4 irradiated autologous PBMCs and PHA. Proliferation was measured after 72 hours.

The differential effect of CD25⁺ cell depletion on the proliferative responses to the viral antigens among the viremic and nonviremic subjects was unlikely to be the result of a quantitative difference in the number of Treg cells remaining after CD25⁺ cell depletion because this was typically less than 2%. The contaminating CD4⁺CD25⁺ cells were CD25^{lo}, which do not possess regulatory function. Furthermore, the fraction of peripheral blood CD4⁺CD25⁺ T cells was similar in all 3 study groups and was comparable with that seen in our previous study in healthy human subjects (see Fig E2 in the Online Repository at www.jacionline.org).³ In addition, we showed that the frequency of CD4⁺CD25⁺Foxp3⁺ cells in viremic and nonviremic individuals was similar and that CD4⁺CD25⁺ cells from both viremic and nonviremic subjects inhibited polyclonal responses of CD4⁺CD25⁻ cells. These observations indicate that there is no significant global defect of Treg cells in viremic patients.

In this study the enhancement of antiviral antigen responses was detected in nonviremic individuals who had received IFN- α treatment (6/8), as well as in those who eliminated the virus spontaneously (4/6). The number of patients included in this study, however, does not have the power to allow any conclusion to be drawn concerning the effects of treatment on these analyses. Because all the viremic patients had received IFN- α treatment, the lack of enhancing effect on the anti-HCV antigen response after depletion of CD25⁺ cells in this group of patients argues against a substantial role for IFN- α in the development of these regulatory cells.

Little is known regarding the antigen specificity of Treg cells. It has been suggested that Treg cells are specific for self-antigens,^{1,23} but evidence is accumulating that these cells might also recognize non-self-antigens.^{11,24} Our data suggest that a population of Treg cells capable of regulating anti-HCV responses could

be found after successful eradication of the virus. Furthermore, these Treg cells were activated by the same viral epitopes that are thought to be important in viral clearance.

The origin of these Treg cells with apparent HCV specificity in the nonviremic patients remains to be elucidated, but 2 possibilities exist.

First, this might represent cross-reactivity by self-peptide-specific T cells on HCV-derived epitopes. It has been reported that both human and murine Treg cell populations have a diverse TCR repertoire.^{24,25} On this basis, it is plausible that the small fraction of Treg cells that can cross-reactively recognize an exogenous antigen, such as HCV peptide, can clonally expand during the course of an antiviral immune response.

The alternative possibility is that these cells arise *de novo* from uncommitted T cells during an antiviral immune response or arise by means of conversion of effector T cells into Treg cells.²⁶ Although it is clear that the majority of Treg cells derive from the thymus,^{22,27} Treg cells can also be generated from uncommitted peripheral T cells.^{24,28} Consistently, it has been shown that antigen-related expansions within certain TCR-V β families were associated with parallel increases of CD4⁺CD45RO⁺CD25^{hi}Foxp3⁺ regulatory T cells with the same V β use.²⁹

Viral infections have been linked with triggering diverse autoimmune conditions.³⁰ This might result from molecular mimicry between a viral epitope and a self-epitope.³¹ Thus a possible role for Treg cells might be to prevent pathogen-specific T cells from triggering autoimmune disease or causing excessive tissue damage after viral clearance. Consistent with this hypothesis, Boyer et al³² reported that the Treg cell deficiency in patients with chronic HCV infection is associated with mixed cryoglobulinemia. Similarly, reduction of tissue-damaging immunopathology by Treg cells has also been demonstrated in herpes simplex viral infection,³³ as well as in some parasitic and fungal infections.³⁴

Recent reports have focused on the role of Treg cells in chronic HCV infection. These Treg cells derived from chronically infected subjects suppressed CD8⁺ T cells specific for HCV, as well as other viruses (eg, EBV and influenza).³⁵⁻³⁸ We focused on Treg cells derived from both chronically infected and nonviremic HCV-exposed subjects and found Treg cells from nonviremic subjects specifically suppressed anti-HCV CD4⁺ T cells. One unifying hypothesis that might explain these observations is that in patients who eradicated the virus, effective CD4⁺ and CD8⁺ anti-HCV responses were elicited, which was accompanied by the expansion of virus-specific Treg cells to limit potential collateral damage. In viremic patients HCV-specific Treg cells were not induced/expanded because there was no effective antiviral response elicited. However, Treg cells with other specificities might still be expanded/activated nonspecifically in the context of a viral infection (eg, through Toll-like receptor ligand activity). Because activated Treg cells can suppress nonspecifically,^{1,2} this nonspecific increase in Treg cell frequency/function might perpetuate viral persistence by preventing the development of an effective anti-HCV CD8⁺ T-cell response. Consistent with this hypothesis, Manigold et al³⁹ recently reported that in a chimpanzee model of HCV infection, CD4⁺CD25⁺Foxp3⁺ cells from both HCV-recovered and HCV-persistent chimpanzees can suppress HCV-specific T-cell responses. Interestingly, depletion of CD25⁺ cells from PBMCs increased the number of HCV-specific IFN- γ ELISPOTs, most notably in those chimpanzees that had recovered from multiple sequential infections. In contrast, the

increase in IFN- γ responses after depletion of CD25⁺ T cells was significantly weaker in chimpanzees that had recovered from a single infection and in viremic chimpanzees.³⁹ Thus these observations strongly corroborate our findings. Another possible explanation for the discrepant results is that the CD4⁺CD25⁺ population might contain a mixture of recently activated cells and Treg cells. Because our depletion protocol consistently depleted both CD4⁺CD25^{hi} and CD4⁺CD25^{int} populations, augmentation of responses to HCV antigens in viremic patients through depletion of Treg cells might be masked by the concomitant depletion of HCV-specific activated T cells. Furthermore, heterogeneity of patient cohorts, infection history, comorbidity, and different HCV antigens used might also contribute to the discrepancy in different studies. Finally, although we did not detect significant HCV-specific Treg cell activity in viremic patients, it is possible that HCV-specific Treg cells were present within the liver because this study focused only on Treg cells from peripheral blood.

The paucity of anti-HCV responses in viremic patients might reflect the presence of suppressive cytokines, such as IL-10 or TGF- β . It was reported that both unstimulated and HCV antigen-induced IL-10 production by peripheral blood lymphocytes was increased in patients with chronic HCV infection.⁴⁰ We also found that unstimulated PBMCs from viremic patients produced higher levels of IL-10 compared with those seen in healthy control subjects (mean = 85.7, 50.8, and 4.5 pg/mL for viremic, nonviremic, and unexposed subjects, respectively; $P = .239$, viremic vs nonviremic; $P = .002$, viremic vs unexposed; $P = .085$ nonviremic vs unexposed). However, stimulation with HCV antigens did not significantly increase IL-10 production by PBMCs from viremic patients (mean = 85.7 vs 101.0 pg/mL; $P = .93$; no patient had an SI >3). Nevertheless, IL-10 production varied considerably between individual patients (4-254 pg/mL and 4-418 pg/mL for unstimulated and HCV antigen-stimulated samples, respectively), and it remains possible that IL-10 might contribute to suppression of anti-HCV responses in some viremic patients. Production of TGF- β was generally low and highly variable between individuals, but there was no difference among all 3 groups of subjects (data not shown).

In conclusion, our data strongly suggest that the presence of HCV-specific Treg cells accompanies successful clearance of the virus. A plausible explanation is that antigen-specific Treg cells were induced in these patients to minimize the potential bystander damage associated with a potent antiviral T-cell response. To our knowledge, this is the first persuasive evidence of the development of antigen-specific Treg cells in the course of normal immune responses in human subjects, a result that has now been corroborated in a chimpanzee model of HCV infection.

Clinical implications: Our findings offer an insight into a possible strategy used by hosts to control cross-reactivity and immunopathology after viral clearance.

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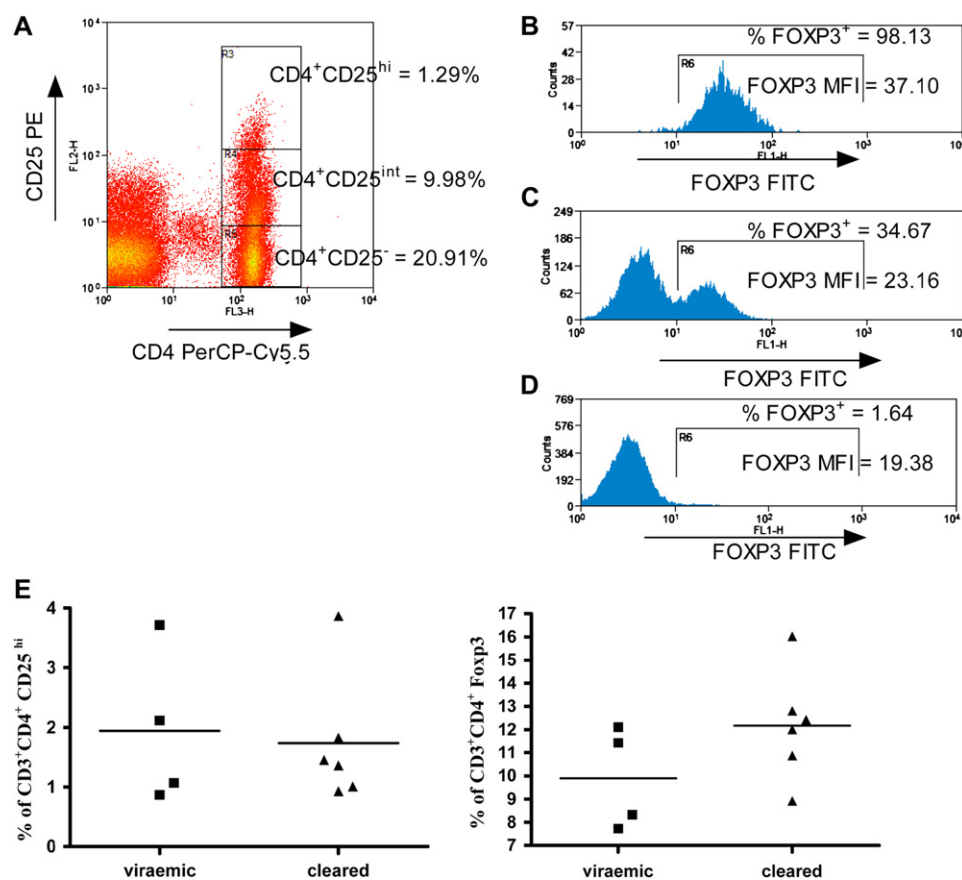


FIG E1. **A**, Percentage of CD4⁺CD25^{hi}, CD4⁺CD25^{int}, and CD4⁺CD25⁻ cells as a percentage of CD3⁺ cells. **B-D**, Percentages of CD4⁺CD25^{hi} gated cells (Fig E1, *B*), CD4⁺CD25^{int} gated cells (Fig E1, *C*), and CD4⁺CD25⁻ gated cells (Fig E1, *D*) that express Foxp3 and the mean fluorescent intensity (MFI) of Foxp3 expression. **E**, There was no statistically significant difference in the percentages of CD3⁺CD4⁺Foxp3⁺ (right graph, *P* = .16) and CD3⁺CD4⁺CD25^{hi} (left graph, *P* = .79) cells in viraemic and nonviraemic subjects. *PerCP*, Peridinin-chlorophyll-protein; *Cy5.5*, ■■■; *FITC*, fluorescein isothiocyanate.

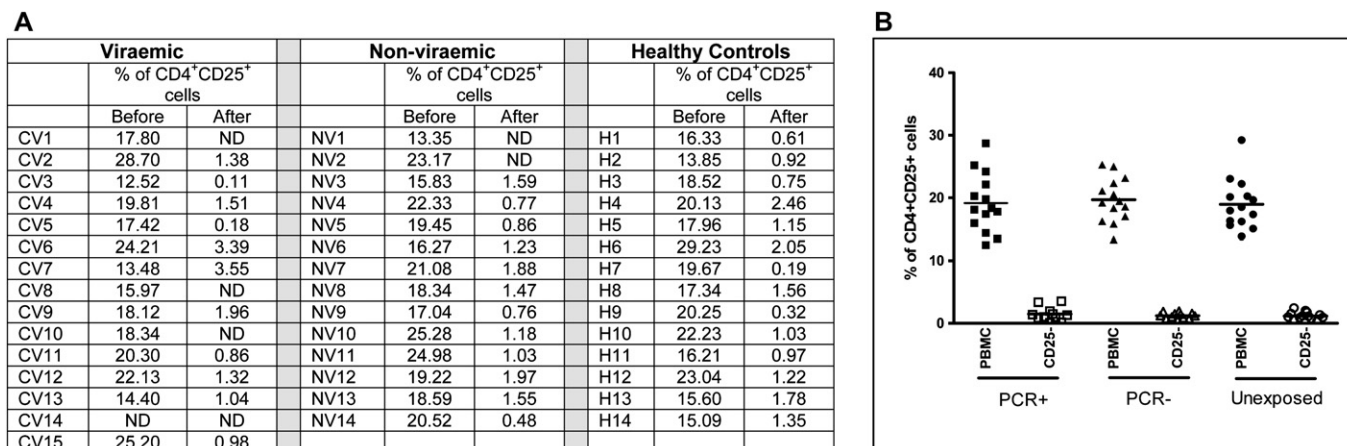


FIG E2. A, Percentages of CD4⁺CD25⁺ cells before and after CD25⁺ cell depletion from PBMCs. *ND*, Not done because insufficient numbers of PBMCs or CD25-depleted cells were obtained from the samples. **B,** Mean percentage of CD4⁺CD25⁺ cells before and after CD25⁺ cell depletion in the 3 study groups. There was no statistically significant difference before or after CD25⁺ cell depletion across all 3 groups.

TABLE E1. Clinical characteristics of the viremic and nonviremic subjects

	Age (y)	Sex	ALT	Bilirubin	HCV genotype
Viremic					
CV1	38	F	—	—	—
CV2	34	M	25	3	—
CV3	46	M	31	10	—
CV4	48	M	48	12	—
CV5	57	F	65	8	1b
CV6	34	M	119	24	—
CV7	38	F	52	9	4
CV8	52	M	102	8	1a
CV9	48	F	—	—	—
CV10	52	F	—	—	—
CV11	52	M	96	8	1a
CV12	55	M	88	—	—
CV13	40	F	—	—	—
CV14	31	M	117	14	3a
CV15	35	M	—	—	3a
Nonviremic (after treatment)					
NV1	70	M	29	6	—
NV2	57	M	—	—	—
NV3	33	M	35	11	—
NV4	37	F	15	11	—
NV5	53	M	18	21	—
NV6	59	F	15	7	1
NV7	37	F	—	—	—
Nonviremic (spontaneous)					
NV8	36	F	—	—	—
NV9	37	F	—	—	—
NV10	46	M	—	—	—
NV11	36	F	43	6	—
NV12	39	M	38	11	—
NV13	42	M	35	7	—
NV14	26	F	27	7	—

ALT, Alanine aminotransferase; F, female; M, male.