CD4 T Helper Type 1 and Regulatory T Cells Induced against the Same Epitopes on the Core Protein in Hepatitis C Virus–Infected Persons

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The factors that determine persistence or clearance of hepatitis C virus (HCV) infection are poorly understood. The CD4 T cell responses to the HCV core protein were examined in a cohort of women infected with a single genotype of HCV. CD4 T cells from HCV-infected patients secreted interferon (IFN)–γ in response to peptides from 4 immunodominant regions of the core protein, and these responses were stronger in persistently infected women. Interleukin (IL)–10 was also produced by CD4 T cells from HCV-infected subjects in response to the same core peptides. Furthermore, HCV core–specific CD4 T cell clones secreted either IFN-γ or IL-10 but not IL-4. These findings demonstrate that T helper type 1 and regulatory T cells are induced against the same epitopes on the core protein during HCV infection.

Infection with hepatitis C virus (HCV) can have a variable outcome, and the factors that determine the resolution or progression of infection are not fully understood. Nevertheless, evidence suggests that clearance and control of HCV infection is dependent on vigorous and multispecific immune responses to structural and nonstructural HCV proteins by both CD8 and CD4 T lymphocytes [1]. An early and persistent Th1-dominated CD4 response appears to be critical in the prevention of the chronically infected state that ensues in ⩾70% of infections [2–5]. The development and maintenance of chronic infection is linked to weak or absent HCV-specific Th1 responses and to the presence of Th2-type cytokines (e.g., interleukin [IL]–4 and IL-10) after stimulation of peripheral blood mononuclear cells (PBMC) with recombinant HCV antigens, in particular the core protein [6–8]. IL-10 has potent antiproliferative and anergy-inducing effects on CD4 T cells [9], and neutralization of this cytokine restores IL-2 production by previously unresponsive HCV NS3-stimulated T cells [10]. Furthermore, gene polymorphism analysis has linked low IL-10–producing genotypes with HCV recurrence in liver transplant recipients [11] and with poor responsiveness to interferon (IFN)–α and ribavirin combination therapy [12].

Numerous diverse pathogens, including *Plasmodium berghei* [13], human rhinovirus [14], *Bordetella pertussis* [15], and a filarial nematode parasite [16], induce IL-10 secretion by host cells to suppress protective T cell responses. In the latter examples [15, 16], the source of the IL-10 was attributed to the recently described CD4 T regulatory type 1 (Tr1) subset, which secretes high levels of IL-10, moderate levels of IFN-γ and IL-5, low amounts of transforming growth factor (TGF)–β, and undetectable IL-4 [17]. Another CD4 T cell subset (Th3) with regulatory properties acts predominantly via secretion of TGF-β [18, 19]. These regulatory T cell subtypes are thought to play an important role in the maintenance of self-tolerance and in the prevention of autoimmune diseases. Ovalbumin-specific Tr1 clones, which inhibit cytokine production by Th1 cells, have been generated in vitro from ovalbumin T cell receptor transgenic mice [17]. Although regulatory T cells may recognize viral, bacterial, or self-antigens, their antigen specificity in vivo has not been established.

In view of the recent findings on Tr1 and Th3 cell functions, we sought to examine CD4 T cell cytokine responses to HCV antigens in a particularly well-defined cohort of patients infected with HCV genotype 1b derived from a single person. In 1977, a large group of Irish women were exposed to HCV after administration of HCV-contaminated anti–D immunoglobulin after childbirth. Almost 1000 women became infected (detected by positive anti-HCV antibody responses). In follow-up studies, it appeared that >50% of the women spontaneously cleared the infection; virus could not be detected by repeated polymerase chain reaction (PCR) testing. In total, ~43% of patients developed chronic infections with HCV that persisted >20 years. Of these women, 2%–3% developed cirrhosis, compared with 10%–20% in other cohorts of HCV-infected patients. Access to this well-characterized homogenous cohort of persons exposed to a single inoculum of HCV allowed us to examine the

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CD4 T cell responses in persons who cleared the virus or remained persistently infected >20 years. We studied responses to HCV core antigens, a region particularly rich in epitopes for CD4 T cells in HCV-infected persons [1, 20, 21], to uncover potential mechanisms by which the virus has persisted >20 years in many of these patients.

Subjects and Methods

Study subjects. A group of Irish women who were infected with HCV genotype 1b after the administration of contaminated anti–D immunoglobulin in 1977/1978 formed our study cohort. HCV viremia was detected by reverse-transcriptase PCR (RT-PCR) for HCV RNA (Roche). HCV RT-PCR–positive patients in the present study had a mean age (±SD) of 47.9 ± 7.3 years and HCV RT-PCR–negative subjects an age of 46.3 ± 6.8 years. All HCV-exposed subjects were antibody positive for HCV (Ortho Diagnostics EIA), but HCV RT-PCR–negative persons repeatedly tested negative for HCV RNA by RT-PCR. We did not include any patients who had received antiviral therapy. In total, we studied 94 HCV antibody–positive, PCR-positive and 50 HCV antibody–positive, PCR-negative women. A group of 11 healthy anti-HCV–negative women aged 31.7 ± 7.6 years with normal liver function served as control persons.

Sequencing of HCV core. Viral RNA from serum samples of 10 HCV PCR-positive members of the anti-D HCV cohort was extracted from 200 μL of serum with guanidine thiocyanate and phenol in a monophase solution (Tri Reagent BD; Sigma). We used 5 μL of the extracted RNA for cDNA synthesis with 10 U of RT in 20 μL of buffer (50 m*M* Tris-HCl at pH 8.0, 5 m*M* $MgCl_2$, 5 m*M* dithiothreitol, and 50 m*M* KCl); 600 μ*M* dATP, dGTP, dTTP, and dCTP; random primers at a molarity of 1.5 μ*M;* and 10 U of RNAsin. The core was amplified by use of nested primers: outer forward, C101 (ATAGGGTGCTTGCGAGTGCCCCG) and outer reverse, C102 (GTTCGTGACATGGTA[C/T]A[T/C]CCCG); inner forward, C103 (GGAGGTCTCGTGACCGTCACC) and inner reverse, C104 (GGAC[A/G]CGTTGCGCAC[C/T]TCATA).

RT-PCR reactions were done with a Perkin-Elmer Robocycler. Reaction conditions in the first round comprised 35 cycles at 94°C for 1 min, 55°C for 1.5 min, and 72°C for 1.5 min; those in the nested PCR comprised 35 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. Each reaction was done with 0.4 U of *Taq* DNA polymerase (Promega) in 50 μL of buffer (50 m*M* KCl, 10 m*M* Tris-HCl at pH 9.0, Triton X-100, and 1.5 m*M* $MgCl_2$); dATP, dGTP, dTTP, and dCTP, each at a molarity of 30 μ*M*; and each outer primer at a molarity of 0.25 μ*M*. We added 1 μL of the products of this PCR reaction to 50 μL of fresh reaction mix for the nested PCR. Each RT-PCR fragment was directly sequenced with a gene analyzer (model 310; Perkin-Elmer). Unincorporated nucleotides and primers were removed from the amplified products with a rapid purification system (Boeringer Mannheim), and 100 ng of the DNA was added to 8 μL of cycle sequencing ready reaction mix (Perkin-Elmer) and 3.2 pmol of primer in a total volume of 20 μL for 25 cycles. The core fragment was sequenced forward and reverse with primers C103, C104, C105 (GGTGGGCAGGATGGCTCCTG), and C106 (CAGGAGCCATCCTGCCCACC). The sequencing cycling conditions were 96°C for 30 s, 50°C for 30 s, and 60°C for 4 min. The extension products were purified by ethanol precipitation at 4°C for 15 min and centrifugation for 30 min. The pellet was resuspended in 25 μL of template suppression reaction mix (Perkin-Elmer) and was heated at 95°C for 2 min. The sample was then loaded onto the gene analyzer. The nucleotide sequences were aligned and were translated to amino acid sequences by use of MacClade software [22]. The rate of variation within a group of sequences was calculated by estimating the minimum number of amino acid changes that gave rise to all of the sequences.

HCV core peptides. On the basis of the consensus sequence of the prevailing HCV core region in the study cohort, peptides (18 × 20 mer and 1 × 11 mer) were synthesized, spanning aa 1–191 and overlapping by 10 aa (Cambridge Research Biochemicals). All peptides and culture media tested negative for bacterial endotoxin (E-Toxate kit; Sigma).

PBMC stimulation. Peripheral blood was drawn into preservative-free heparin, diluted 1:2 with RPMI 1640 medium (Gibco BRL), layered on a ficoll gradient, and centrifuged at 400 *g* for 30 min. PBMC were removed from the interface, washed twice in RPMI plus 2% fetal calf serum (FCS), and finally resuspended in RPMI plus 10% FCS. PBMC were cultured in triplicate 200-μL wells in round-bottomed 96-well microtiter plates at 2×10^6 viable cells/mL for 72 h at 37°C in humidified 5% CO_2 in air. Culture supernatants were then collected and stored at −20°C until cytokine assays were performed. HCV core peptides were used at 15 μg/mL (optimal concentration determined in preliminary experiments), and control stimuli included medium alone, phytohemagglutinin (Sigma) at 20 μg/mL or anti-CD3 (OKT3; 2 μg/mL), and phorbol myristate acetate (PMA) at 25 ng/mL (Sigma).

Cytokine ELISAs. IFN-γ, IL-4, IL-5, IL-10, and TGF-β were measured by standard immunoassays, using pairs of monoclonal antibodies and cytokine standards (BD Pharmingen). The limits of sensitivity of the assays were 4.7 pg/mL for IFN-γ, 62.5 pg/mL for TGF-β, and 7.8 pg/mL for IL-4, -5, and -10.

T cell lines and clones. HCV core peptide–specific T cell lines were generated by stimulation of PBMC with the relevant peptide at 15 μg/mL. After 7 days, activated T cells were expanded by adding recombinant human IL-2 at 20 U/mL. Growing lines were restimulated after an additional 5–7 days with antigen-presenting cells (APCs; irradiated [30 Gy] autologous PBMC, 10^6 cells/mL) and antigen. This cycle was repeated ⩾3 times, as long as necessary to maintain the T cell lines. CD4 T cell lines were phenotyped by flow cytometry analysis, using fluorescent-labeled anti-CD3, anti-CD4, and anti-CD8 antibodies and isotype controls (BD Pharmingen). Clones were derived from these CD4 lines by limiting dilution at 100, 10, 1, and eventually 0.5 T cells/well, to confirm clonality, in flat-bottomed 96-well plates, using APCs (irradiated autologous PBMC at 10^6 cells/mL), HCV peptide at 1 μg/mL, and IL-2 at 20 U/mL (during antigen starvation).

Statistical analysis. We used a 2-tailed paired or unpaired *t* test when appropriate to compare differences between cell populations and patient/control groups and used Fisher's exact test for comparison of frequencies of responders to core peptides. $P < .05$ was considered to be significant.

Results

Sequence of HCV core protein in study cohort. Genomic sequencing of the HCV core protein in 10 chronically infected

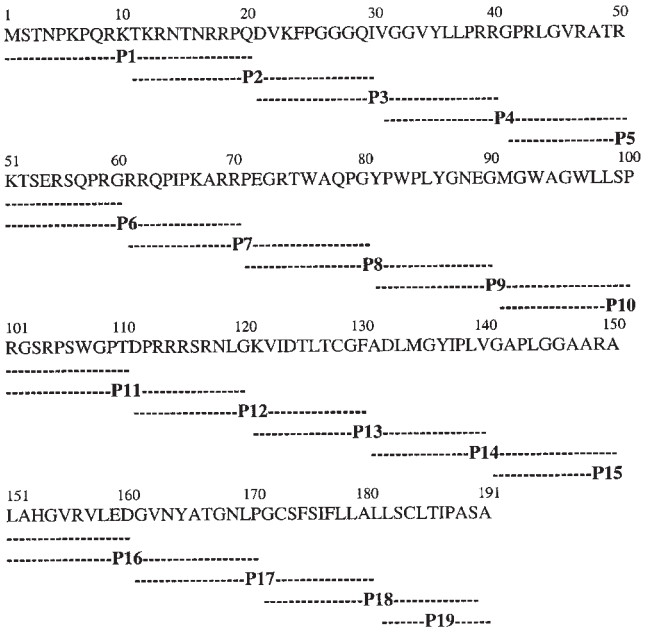

Figure 1. Consensus sequence of core protein from 10 patients in hepatitis C virus genotype 1b–infected cohort. Data show overlapping peptides (P) spanning entire core protein.

patients showed little variation (0.1%) in the amino acid sequence of the core protein. Comparative analysis with other HCV 1b strains also found that the sequence was highly conserved; a comparison with 15 unrelated HCV core 1b sequences from the GenBank database revealed 0.6% amino acid sequence variation. Overlapping synthetic peptides based on the predicted amino acid sequence in our cohort (figure 1) were synthesized according to this consensus sequence and were used for analysis of cell-mediated immunity in the HCV-infected patients.

IFN-γ production to 4 immunodominant regions of HCV core protein. PBMC from HCV-infected patients proliferated (data not shown) and secreted IFN-γ (figure 2) when stimulated in vitro with peptides corresponding to the consensus sequence of HCV core protein. Four immunodominant regions were identified, corresponding to residues 1–30 (P1 and P2), 31–70 (P4–P6), 131–170 (P14–P16), and 171–191 (P18 and P19) of the HCV core protein. Residues 91–130 (P10–P12) were also recognized by PBMC from a small proportion of patients. The 4 immunodominant peptides recognized corresponded to residues 1–20 (P1), 31–50 (P4), 141–160 (P15), and 181–191 (P19). The most consistently recognized peptide was P1 (1–20), which stimulated significant IFN-γ in chronically infected persons ($P < .05$). Overall, the frequency of responding patients to all peptides was significantly greater ($P < .0001$) in chronically infected PCR-positive donors than in antibody-positive PCR-negative donors who had spontaneously recovered from the infection. Thus, 50%–59% of HCV antibody–positive, PCR-positive donors responded to P1, P4, and P15, whereas only 18.7% and 25% of HCV antibody–positive, PCR-negative subjects responded by producing IFN-γ to P1 and P15, respectively, and none of the latter group responded to P4 (figure 2). PBMC from HCV antibody–negative control subjects did not secrete IFN-γ in response to any of the HCV core peptides (figure 2), whereas PBMC from all persons examined secreted IFN-γ in response to the polyclonal activators PMA and anti-CD3 (data not shown).

HLA association with responses to HCV core peptides. Expression of different HLA antigens is associated with either viral clearance or persistence of HCV infection [23, 24]. We examined the relationship between IFN-γ production in response to HCV core peptides and the HLA class II haplotypes of the PBMC donors (table 1). Although numbers are small, when we considered the PCR-positive and PCR-negative patients together, there was a clear relationship between response to the immunodominant peptides and expression of HLA DRB*0101 and HLA DQB*0501, alleles previously shown to be associated with HCV clearance in our cohort [24]. Conversely, HLA DRB1*0301 and HLA DQ B1*0201 donors who tend to have persistent HCV infection in our study population as a whole [24] had poor IFN-γ re-

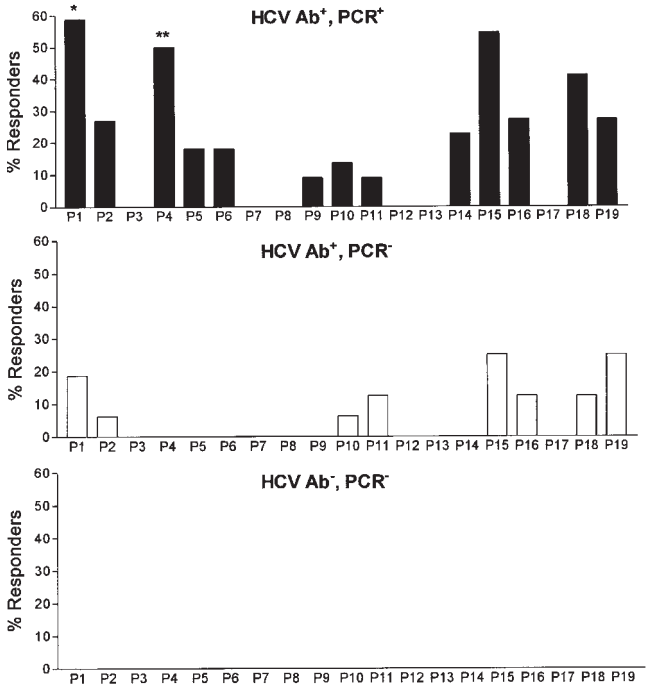

Figure 2. High frequency of interferon (IFN)–γ production to hepatitis C virus (HCV) core peptides in chronically infected patients but not in persons who cleared the infection. Data are percentage of donors with positive IFN-γ response (≥2× response in medium alone or lowest sensitivity of the assay if the control was negative) by peripheral blood mononuclear cells in response to HCV core peptides (15 μg/mL). *$P = .02$; **$P = .0007$, HCV antibody (Ab)–positive, polymerase chain reaction (PCR)–positive ($n = 22$; *top*) vs. HCV Ab–positive, PCR-negative ($n = 16$; *center*) subjects. Non–HCV-infected controls, HCV Ab negative, PCR negative ($n = 11$), are shown at bottom.

Table 1. Frequency of interferon (IFN)–γ production to hepatitis C virus (HCV) core peptides (P) in persons expressing different major histocompatibility complex II alleles.

Allele	No.[a]	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19
DR0701	9 (4/5)	78	67	0	44	0	0	0	0	0	67	67	44	44	44	67	67	0	44	22
DR0101[b]	6 (5/1)	100	0	100	100	100	100	0	0	0	100	100	67	0	50	100	50	0	100	100
DR03011[c]	8 (1/7)	25	0	0	0	0	0	12	0	0	0	0	0	0	0	25	0	0	0	0
DR1501	6 (2/4)	0	0	0	33	0	0	0	0	0	0	0	0	0	0	33	0	0	0	0
DQ0201[c]	7 (4/3)	43	0	0	57	0	0	0	0	0	0	0	0	0	43	43	0	0	28	28
DQ0602	6 (1/5)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DQ0501[b]	3 (2/1)	100	100	100	66	66	66	33	100	0	66	66	0	0	100	100	0	66	66	66

NOTE. Data are percentage of persons of a given HLA-DR or -DQ haplotype with positive (≥2× response in medium-alone wells or lowest sensitivity of the assay if this control was negative) IFN-γ responses to individual HCV core peptides. Data were collected from 45 HCV-infected patients.

[a] HCV antibody–positive, polymerase chain reaction (PCR)–positive/HCV antibody–positive, PCR-negative subjects.

[b] In an earlier study [23] of 243 patients, 95 who spontaneously cleared the virus and 148 who were chronically infected, DRB1*0101 and DQB1*0501 alleles were more frequent in subjects who sustained viral clearance than in chronically infected subjects (32% and 37% vs. 9% and 14%).

[c] DRB1*03011 and DQB1*0201 occurred more frequently in chronically infected subjects than in those who cleared the virus (41.5% and 43% vs. 17% and 16%) [23].

sponses to the peptides. Because of the small number of study subjects, the stratification of IFN-γ production in response to HCV peptides according to PCR status did not demonstrate any association with the major histocompatibility complex (MHC) class II allele. Therefore, we could not draw any firm conclusions on peptide recognition and viral persistence or clearance. We found no clear associations between IL-10 responses and HLA DR haplotype (data not shown).

Th1 and Tr1 cytokines produced in response to immunodominant HCV core peptides. After demonstrating that IFN-γ is produced after stimulation of PBMC from HCV-infected patients with core peptides, we sought to determine the cellular source of this cytokine and the subtype of T cells induced in the infected patients. We examined HCV-specific IL-4, -5, and -10 and IFN-γ production in response to the core peptides in PCR-positive and -negative subjects. IL-10 and -5 at lower levels were produced by PBMC from chronically infected patients in response to peptides already shown to stimulate IFN-γ production (figure 3). Although IFN-γ, IL-10, and IL-5 were also detected in supernatants of PBMC from PCR-negative subjects in response to P1, P15, P18, and P19, the frequency of responding patients was much lower, and the cytokine concentrations were not significantly higher than in unstimulated control wells. The peptides did not stimulate cytokine production by PBMC from non–HCV-exposed

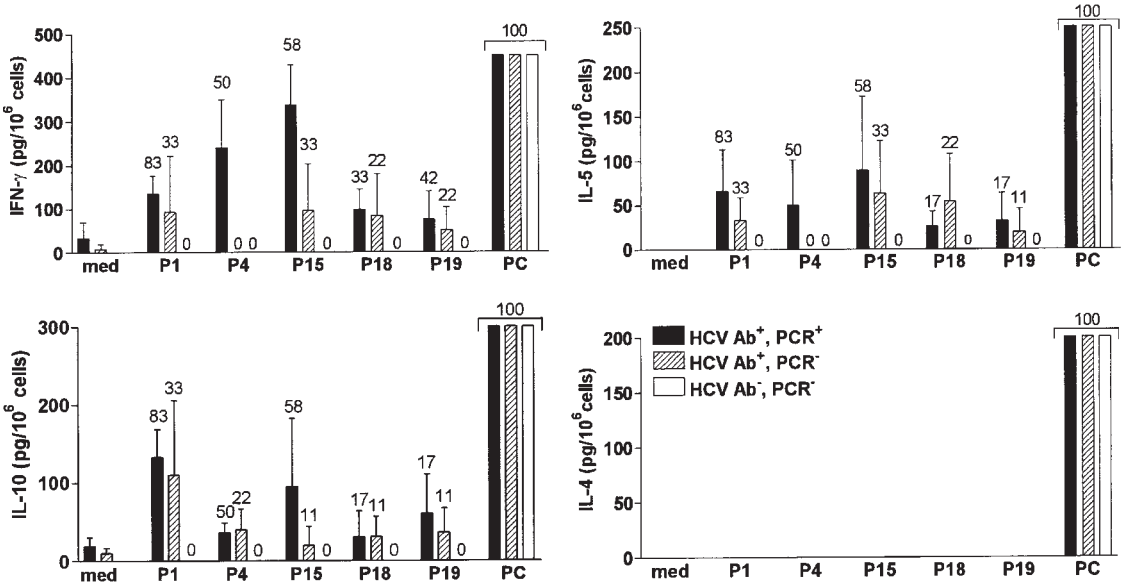

Figure 3. Peripheral blood mononuclear cells (PBMC) from persistently hepatitis C virus (HCV)–infected persons secrete interferon (IFN)–γ *(top left),* interleukin (IL)–5 *(top right),* IL-10 *(bottom left),* and IL-4 *(bottom right)* in response to immunodominant HCV core peptides. PBMC from HCV antibody (Ab)–positive, polymerase chain reaction (PCR)–positive ($n = 12$), HCV Ab–positive, PCR-negative, or HCV Ab–negative, PCR-negative ($n = 11$) persons were stimulated with 15 μg/mL of HCV core peptides P1, P4, P15, P18, or P19, medium only (med), or phorbol myristate acetate and anti-CD3 as a positive control (PC). Results are mean ± SD of cytokine concentrations. Nos. above bars are percentages of persons within each group who responded to that peptide.

control subjects. P1, P4, and P15 stimulated both IL-10 and IFN-γ in 83%, 50%, and 58%, respectively, of PBMC from chronically infected PCR-positive subjects. However, only 33% of HCV PCR-negative persons responded to P1 and P15, and only 22% secreted IL-10 alone to P4. IL-4 could not be detected in supernatants of peptide-stimulated PBMC but was produced in response to PMA and anti-CD3. A Tr1-biased cytokine profile in response to P1, P4, and P15 was particularly noted in HCV PCR-positive persons, whereas low Tr1-type responses to these peptides were a feature of patients who had cleared the virus from the circulation.

Cytokine profiles of HCV core peptide–specific CD4 T cell lines and clones. Although the protocols that we used are designed to detect cytokines from antigen-specific T cells, PBMC include a number of cell types that may have provided the source of IFN-γ and IL-10 detected in response to core peptides from the HCV-infected persons. Therefore, in an attempt to establish the source of the cytokine and to determine whether Th1 and Tr1 subtypes of CD4 T cells are induced during infection with HCV, we established HCV core–specific CD4 T cell lines and clones from a number of HCV-infected donors (table 2). PBMC were stimulated in vitro with 1 of the 4 immunodominant peptides from the core protein. Each of the 12 T cell lines secreted IFN-γ and IL-10 and 8 of the cell lines also secreted IL-5, but IL-4 could not be detected in supernatants from any of the T cell lines tested. Some of the T cell lines (e.g., 4.13, 4.18, and 15.28) secreted higher levels of IFN-γ than IL-10, whereas others (e.g., 1.17, 1.21, 15.19, 15.23, 19.20, and 19.25) had a more Tr1-type profile (high IL-10, IFN-γ, and IL-5 but no IL-4, with or without TGF-β). A number of T cell lines were cloned by limiting dilution. T cell clones specific for P15 had highly polarized Th1 or Tr1 profiles secreting either IFN-γ (15.23.2 and 15.23.7) or IL-10 (15.19.9 and 15.19.10) but no IL-4 or IL-5. P1-specific T cell clones 1.21.2, 1.21.7, and 1.21.9 also had a cytokine profile characteristic of Tr1 cells (IL-10, IFN-γ, and IL-5 but no IL-4). Other T cell clones specific for peptide 15 (15.19.1, 15.19.7, and 15.19.8) had more heterogenous cytokine production characteristic of Th0 cells, with high levels of IL-5 and IFN-γ and lower levels of IL-4 and -10.

Elevated IL-10 in the serum of chronically HCV-infected patients. Although the concentrations of different cytokines in the circulation can be influenced by a number of factors in addition to ongoing infection, we examined plasma levels of key Th1, Th2, or Tr1 cytokines to provide indirect evidence of activation of these subtypes in vivo (figure 4). The concentrations of IL-10

Table 2. Cytokine production by hepatitis C virus (HCV) core–specific CD4 T cell lines and clones.

T cell	Donor status	Peptide	IFN-γ	IL-4	IL-5	IL-10	TGF-β	Th cell type
Line								
1.21	Ab$^+$ PCR$^+$	P1 (1–20)	+	−	±	+++	−	Tr1-biased
1.17	Ab$^+$ PCR$^+$	P1 (1–20)	++	−	++	+++	±	Tr1-biased
19.30	Ab$^+$ PCR$^+$	P19 (181–191)	++	−	±	+++	−	Tr1-biased
15.19	Ab$^+$ PCR$^+$	P15 (141–160)	+	−	++	++	−	Tr1-biased
15.23	Ab$^+$ PCR$^-$	P15 (141–160)	+	−	−	++		Tr1-biased
19.25	Ab$^+$ PCR$^+$	P19 (181–191)	+		±	++	−	Tr1-biased
19.20	Ab$^+$ PCR$^-$	P19 (181–191)	±		−	+		Th0
4.22	Ab$^+$ PCR$^+$	P4 (31–50)	±		−	+		Th0
1.12	Ab$^+$ PCR$^+$	P1 (1–20)	+		+	+		Th0
4.18	Ab$^+$ PCR$^+$	P4 (31–50)	++		++	+		Th1-biased
4.13	Ab$^+$ PCR$^+$	P4 (31–50)	++		++	+		Th1-biased
15.28	Ab$^+$ PCR$^+$	P15 (141–160)	+++	−	++	+	−	Th1-biased
Clone								
1.21.7*	Ab$^+$ PCR$^+$	P1 (1–20)	++	−	+	+++	−	Tr1
1.21.2*	Ab$^+$ PCR$^+$	P1 (1–20)	+	−	+	++	−	Tr1
1.21.9*	Ab$^+$ PCR$^+$	P1 (1–20)	+	−	+	++	−	Tr1
15.19.9	Ab$^+$ PCR$^+$	P15 (141–160)	−	−		+		Tr1
15.19.10	Ab$^+$ PCR$^+$	P15 (141–160)	−	−		+		Tr1
15.19.1*	Ab$^+$ PCR$^+$	P15 (141–160)	++	±	++	+	−	Th0
15.19.7*	Ab$^+$ PCR$^+$	P15 (141–160)	+	−	+	+	−	Th0
15.19.8*	Ab$^+$ PCR$^+$	P15 (141–160)	++	+	+++	±	−	Th0
15.23.2	Ab$^+$ PCR$^-$	P15 (141–160)	++	−	−	−		Th1
15.23.7	Ab$^+$ PCR$^-$	P15 (141–160)	++	−	−	−		Th1

NOTE. Interferon (IFN)–γ, interleukin (IL)–4, –5, and –10, and transforming growth factor (TGF)–β production of T cell lines and clones was tested in different experiments by stimulation with specific peptide- and antigen-presenting cells (irradiated autologous peripheral blood mononuclear cells) or with phorbol myristate acetate and anti-CD3 (denoted by *). −, ±, +, ++, and +++ represent cytokine concentrations <2, 2–3, >3–10, >10–20, and >20 times the response to medium alone or the lowest sensitivity of the assay if this control was negative, respectively. T cell clones were defined by cytokine secretion as follows: Th1, IFN-γ and no IL-4, -5, or -10; CD4 T regulatory type 1 (Tr1), IL-10, no/low IFN-γ, and no IL-4; and Th0, comparable IFN-γ and IL-10, -4, and/or -5. T cell lines were defined by cytokine secretion as follows: Th1-biased, relatively stronger IFN-γ than IL-10 and no IL-4; Th0 (as above) or Tr1-biased, relatively stronger IL-10 than IFN-γ and no IL-4. Ab, antibody; PCR, polymerase chain reaction.

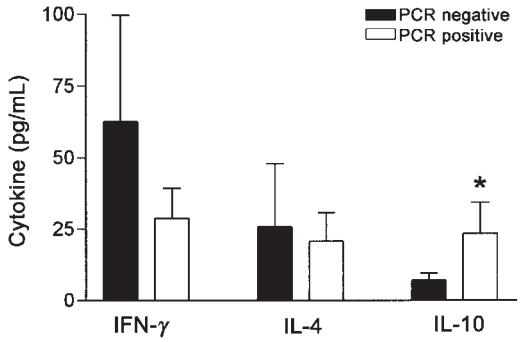

Figure 4. Elevated interleukin (IL)–10 in plasma of patients chronically infected with hepatitis C virus (HCV). IL-10, IL-4, and interferon (IFN)–γ concentrations were determined in the plasma of HCV antibody–positive, polymerase chain reaction (PCR)–negative ($n = 50$) and HCV antibody–positive, PCR-positive ($n = 94$) subjects. Each bar represents mean ± SD cytokine level. *$P < .05$, PCR positive vs. PCR negative.

were significantly higher ($P < .05$) in antibody-positive, PCR-positive persons than in antibody-positive, PCR-negative subjects. Although the concentration of IFN-γ was higher in plasma samples from donors who had cleared the virus, the differences in IFN-γ and IL-4 concentrations were not statistically significant between antibody-positive, PCR-positive and antibody-positive, PCR-negative subjects.

Discussion

The significant finding of this study is that, in persons chronically infected with HCV, regulatory T cells that secrete IL-10, termed Tr1 cells, circulate simultaneously with IFN-γ–secreting Th1 cells. Furthermore these 2 subtypes of CD4 T cells recognize the same epitopes on the HCV core protein. Our finding suggests that the persistence of infection for >20 years in a cohort of women infected immediately after childbirth with contaminated anti–D immunoglobulin may result, in part, from the induction of regulatory T cells with immunosuppressive effects.

Earlier studies suggested that the development of strong CD8 cytotoxic T lymphocyte (CTL) responses against HCV structural, nonstructural, and core proteins during acute infection may be required for viral clearance and that persistence of CTL responses may be necessary to prevent disease progression [25–27]. However, there is increasing evidence that CD4 Th cells specific for HCV, especially against core protein, also play a critical role in antiviral immunity, either indirectly, by providing helper function for CTL responses, or directly, through the antiviral effects of IFN-γ produced by Th1 cells. Strong CD4 T cell responses during acute infection may mediate viral clearance. Absence of this response leads to chronic infection [3, 5, 20, 28, 29]. In particular, the Th1 subtype has been implicated in viral clearance; IFN-γ production from PBMC in response to core peptides was found at a higher frequency in HCV-infected nonviremic subjects than in chronically infected persons [7, 29]. Possibly, Th1 cells may be important in the pathogenesis of the hepatocellular injury. Stronger core-specific CD4 T cell responses have been found in patients with active liver diseases than in asymptomatic persons [30, 31]. Although CD4 T cell responses are maintained indefinitely after recovery from infection [28], we found that IFN-γ production by PBMC was significantly weaker and that responses were against a narrower range of peptides in nonviremic subjects than in chronically infected persons. It is possible that the lower cytokine production in subjects who had cleared the infection may reflect the longer period between exposure and testing, compared with other studies.

It has been suggested that patients with self-limiting disease have significant HCV-specific Th1 responses; however, Th2 responses were found in chronically infected patients [6–8]. Although we detected an increased frequency of IL-5 production in chronically infected patients versus nonviremic persons, we did not detect IL-4 production by either PBMC or T cell lines generated from cells of chronically infected patients. In contrast, IL-10–producing T cells were detected in a higher proportion of patients with chronic infection than in those who had cleared the virus, in agreement with previous reports [8, 29, 32]. In our cohort of patients, a high proportion of those with HCV-specific IFN-γ production to the core protein remain chronically infected but do not develop cirrhosis. It is possible that IFN-γ–producing T cells may be capable of preventing disease progression but not capable of eliminating HCV. The persistence of infection without progression to cirrhosis may have resulted from the suppression of IFN-γ production by IL-10. Anti–IL-10 antibodies restore IFN-γ production to HCV antigens [10] (authors' unpublished data); however, a source of endogenous IL-10 during HCV infection has not been identified.

CD4 T cells that secrete high levels of IL-10, with or without IL-5 and IFN-γ but with no IL-4, termed Tr1 cells, suppress Th1 responses in vitro and in vivo [15, 17]. Tr1 clones have been generated in vitro by antigen stimulation in the presence of IL-10 [17], and, until recently, little was known about their antigen specificity in vivo. Our present study of HCV-infected women, along with studies of mice infected with the respiratory pathogen *B. pertussis* [15] and of humans with chronic *Onchocerca volvulus* infection [16], demonstrates that IL-10 secretion by Tr1-type CD4 T cells can be induced in vivo in response to an infectious pathogen. In the present study, HCV-specific T cell lines and clones that secreted IL-10 and IL-5, with or without IFN-γ but with no IL-4, were generated from cells from chronically infected donors.

Evidence is emerging that the differentiation of anergic or regulatory T cells from naive precursors is directed by dendritic cells that either inherently produce or are stimulated to produce IL-10 in response to microbial products [15, 33]. Dendritic cells in the respiratory tract [34] and Peyer's patches [35] have a propensity to secrete high levels of IL-10, which may explain the induction of regulatory T cells at mucosal surfaces, where they function to maintain tolerance. However, liver dendritic cells also secrete high levels of IL-10 [36], which may explain the induction of

regulatory T cells after HCV infection. In the present study, all the patients were infected immediately after childbirth. Thus, it is possible that the altered Th1/Th2 balance toward a Th2 or Tr1 type bias during pregnancy [37] may have influenced the T cell subtype induced against HCV.

Difficulties with in vitro propagation and the requirements of continuous access to autologous PBMC as APCs from our patients precluded functional studies with the human T cell clones. Nevertheless, our murine pathogen–specific T cell clones with the same cytokine profile suppressed protective Th1 responses against the pathogen in vivo [15]. It is possible that the induction of regulatory T cells during HCV infection may suppress antiviral Th1 and consequently CD8 CTL responses in vivo, and this may explain the persistence of infection and low prevalence of cirrhosis in the presence of detectable HCV-specific IFN-γ–producing T cells. IL-10 production was also detected in PBMC from a small proportion of HCV-infected persons who had cleared the infection. However, we were unable to generate Tr1 clones from tissue from these persons and the frequency of responders was lower, especially against P15. Furthermore, IL-10 concentrations were higher in the serum of chronically infected patients than in that of patients who had cleared the infection. Thus, induction of regulatory T cells may contribute to persistence of the infection, but clearly other factors are involved.

Resolution of infection has been associated with expression of MHC haplotypes, in particular HLA DRB1*1101 [38]. In the present study, PBMC from all patients expressing DRB1*0101 secreted IFN-γ in response to core peptides 1, 3–6, 10, 11, 15, 18, and 19, and 66%–100% of patients expressing DQB1*0501 responded to the same peptides. In contrast, significantly fewer HCV core–specific responders were detected among patients expressing DRB1*03011 or DQB1*0201. MHC typing on large cohorts of patients (145 chronically infected and 95 with spontaneous viral clearance) demonstrated that HLA DRB1*0101 and DQB1*0501 occurred more frequently in subjects who sustained viral clearance, and DRB1*03011 and DQB1*0201 occurred more frequently in chronically infected persons [23]. However, we had too few patients in the present study to draw conclusions on the role of MHC-associated HCV peptide recognition and the course of HCV infection. Nevertheless, our results suggest promiscuous recognition of HCV core epitopes, especially peptides 4 and 15, and recognition of multiple epitopes by certain haplotypes, especially among PCR-positive persons. Although HCV PCR–positive subjects produce more IFN-γ after HCV peptide stimulation than HCV PCR–negative subjects, this is in the presence of ongoing viral stimulation in vivo, which may have been absent for many years in the latter group. In the setting of acute infection, the subjects with DR/DQ phenotype, shown to be associated with resolution of the infection, may be capable of producing more IFN-γ, regardless of current PCR status, which supports the possible role of type 1 T cells in viral clearance.

Vaccine development is dependent not only on defining the protective immune responses but also on the antigens and epitopes against which this response is directed. CD4 T cell epitopes have been identified on HCV structural, nonstructural, and core proteins, mostly by using ex vivo PBMC and a limited number by using T cell lines and clones. In this study, we focused on the core antigen, an internal viral protein that is well conserved among HCV serotypes. The HCV genotype 1b core protein sequenced from 10 HCV chronically infected subjects >20 years after infection with the same inoculum did not vary significantly. By using CD4 T cell lines and clones, we found evidence that 4 immunodominant regions of this sequence are recognized by CD4 T cells from the HCV-infected patients and found definitive evidence of recognition of 4 peptide-defined epitopes. P4 and P15, corresponding to residues 31–50 and 141–160, overlap with epitopes identified as immunodominant regions in other studies of infection with HCV genotype 1 [21, 30, 31, 39]. However, we found that P1 (1–20) and P19 (181–191) at the C- and N-terminus of the core protein, respectively, were also recognized by PBMC from a high proportion of persons, and CD4 T cell lines and clones specific for these peptides were generated by cells from a number of chronically infected patients. These T cells produced IFN-γ and/or IL-10 but little or no IL-4, suggesting that these epitopes are recognized by both Th1 and Tr1 cells.

A better understanding of the genetic and immunologic factors that determine resolution or persistence of the infection may help in the design of therapies and vaccines for the prevention and control of HCV infection. Although some progress has been made in HCV vaccine development, the target antigens and protective immune responses need to be properly elucidated. The results of the present study, combined with those of others, suggest that antigen-adjuvant combinations that bias immune responses to CD4 Th1 and CD8 CTL, rather than Th2 or regulatory T cells, may have the best chance of success. However, the choice of T cell epitopes or antigens needs to be carefully considered, since these may stimulate immunoregulatory as well as potentially protective T cell responses.

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