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Impaired Effector Function of Hepatitis C Virus-Specific CD8$^+$ T Cells in Chronic Hepatitis C Virus Infection[1]

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The cellular immune response contributes to clearance of hepatitis C virus (HCV) and persists for decades after recovery from infection. The immunological basis for the inefficiency of the cellular immune response in chronically infected persons is not known. Here, we used four HLA-A2 tetramers, specific for two HCV core and two HCV NS3 epitopes, to investigate at the single-cell level effector function and phenotype of HCV-specific CD8$^+$ T cells in 20 chronically infected and 12 long-term recovered patients. Overall, HCV-specific, tetramer$^+$ T cells were more frequently found in PBMCs of chronically infected patients than in those of recovered patients. However, when compared with HCV-tetramer$^+$ T cells of recovered patients, they displayed an impaired proliferative capacity. As a result of the impaired proliferative capacity, HCV-specific T cell lines derived from chronically infected patients displayed less peptide-specific cytotoxicity than those from recovered patients. In addition, proliferation and ex vivo IFN-γ production of HCV-tetramer$^+$ cells, but not influenza-virus-specific T cells, were defective in chronically infected patients and could not be restored by in vitro stimulation with peptide and IL-2. At least three distinct phenotypes of HCV-specific CD8$^+$ T cells were identified and associated with certain functional characteristics. In addition, impairment of proliferative, cytokine, and cytotoxic effector functions of tetramer$^+$ T cells in viremic patients was associated with weak ex vivo HCV-specific CD4$^+$ T cell responses. Thus, the defective functions of HCV-specific CD8$^+$ T cells might contribute to viral persistence in chronically infected patients, and knowledge on their reversibility may facilitate the development of immunotherapeutic vaccines. ***The Journal of Immunology*****, 2002, 169: 3447–3458.**

Infection with the hepatitis C virus (HCV)[4] affects an estimated 200 million people worldwide and accounts for ~20% of cases with acute hepatitis, 70% with chronic hepatitis, 40% with end-stage cirrhosis, 60% with hepatocellular carcinoma, and 15–30% of liver transplantations (1).

In acute HCV infection, an early HCV-specific T cell response is associated with viral clearance and recovery (2–8), and HCV-specific CTLs persist at least two decades after recovery from hepatitis C (9). In contrast, in chronically infected individuals, cellular immune responses appear to be too weak to eliminate HCV (9). Whether this weakness is due to a low frequency of HCV-specific T cells or to an impairment of their effector functions is not known.

Detailed analysis of the functional properties of HCV-specific CD8$^+$ T cells in chronically infected patients had been hampered by the low frequency of such cells and the requirement for extensive in vitro expansion. Quantification of HCV-specific CD8$^+$ T cells was initially based on limiting dilution analysis and thus was dependent on the frequency of specific cells, their in vitro expansion potential, and their ability to lyse appropriate targets (10). Subsequently, the use of the IFN-γ ELISPOT for direct ex vivo analysis demonstrated that the frequency of virus-specific circulating CD8$^+$ T cells was significantly underestimated by limiting dilution analysis (11). However, even the ELISPOT assay might not reflect the true number of virus-specific T cells because quantification of cells depends on cytokine production in this assay. The introduction of MHC class I-peptide tetramers that bind to the TCRs of Ag-specific cells now allows enumeration of specific CD8$^+$ T cells regardless of their effector function (12) and provides additional information on their phenotype if combined with staining for cell surface markers and intracellular cytokines. Virus-specific CD8$^+$ T cells have been investigated with tetramers in several diseases including HIV (12, 13), EBV (14), and CMV infection (13), and we and others have reported the use of tetramers in studies of HCV (6, 7, 15, 16).

In this study, we used four HLA-A2.1 tetramers specific for HCV core and NS3 epitopes, respectively, in combination with proliferation, IFN-γ ELISPOT, and cytotoxicity assays to investigate the frequency of HCV-specific T cells and the effector functions of these cells directly ex vivo and in short-term-cultured (day 7) T cell lines. We found a higher frequency of circulating tetramer$^+$ T cells in chronic than in recovered patients, but tetramer$^+$ cells of chronic patients were functionally impaired, i.e., did not expand after Ag stimulation, did not produce IFN-γ in response to HCV peptides, and displayed less HCV-specific cytotoxicity. These impaired functions were more pronounced in the presence of weak ex vivo HCV-specific CD4$^+$ T cell responses and were

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[4] Abbreviations used in this paper: HCV, hepatitis C virus; Rec, recovered; Chr, chronic hepatitis C; HBV, hepatitis B virus.

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associated with a distinct CD45RO$^-$CD27$^+$ phenotype. Thus, the defective functions of HCV-specific CD8$^+$ T cells might contribute to viral persistence in chronically infected patients, and knowledge on their reversibility may facilitate the development of immunotherapeutic vaccines.

Materials and Methods

Patient population

Twenty chronically infected, untreated HCV-RNA-positive patients (genotypes 1 or 2b) with alanine aminotransferase levels within 3-fold of the upper limit of normal were compared with 12 recovered patients who had been HCV-RNA negative for up to 12 years. Eight healthy blood donors without a history of HCV infection served as controls. All subjects were HLA-A2 positive, had been followed in the Department of Transfusion Medicine Clinical Center and the Liver Diseases Section, National Institute of Diabetes and Digestive and Kidney Diseases (National Institutes of Health, Bethesda, MD), and gave informed consent to this study, which was approved by the institutional review board of National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health. Anti-HCV testing, qualitative PCR for HCV RNA, and genotyping were performed as previously described (17).

Peptides and Abs

Four HLA-A2-restricted HCV peptides (Core-35 YLLPRRGPRL, Core-132 DLMGYIPLV, NS3-1073 CVNGVCWTV, and NS3-1406 KLVALGINAV) (9, 18, 19), the influenza A virus matrix peptide GILGFVFTL (20) (Research Genetics, Huntsville, AL), and the following mAbs were used: anti-CD4-FITC, anti-CD13-FITC, anti-CD19-FITC, anti-CD27-FITC, anti-CD28-FITC (Caltag Laboratories, Burlingame, CA), anti-CD8-PerCP, anti-CD8-FITC (BD Biosciences, San Jose, CA), anti-CD45RA-FITC, anti-CD45RO-FITC, anti-CD152-CyChrome, anti-perforin-PE, anti-IFN-γ-FITC (BD PharMingen, San Diego, CA), anti-CD69-FITC, anti-CD94-FITC (Ancell, Bayport, MN), and anti-perforin-FITC (Research Diagnostics, Flanders, NJ).

Generation of T cell lines

PBMCs were separated on Ficoll-Histopaque (Sigma-Aldrich, St. Louis, MO) as described (9). PBMCs were stimulated in 96-well round-bottom plates (0.4×10^6 per well) with 10 μg/ml peptide in RPMI 1640, 10% heat-inactivated human serum (blood group AB), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). On day 4, 100 μl of RPMI 1640 with 10% AB serum and 10 U/ml rIL-2 was added. On day 7, the number of tetramer-specific CD8$^+$ T cells was determined by flow cytometry, cytotoxicity by CTL assay, and IFN-γ production by intracellular cytokine staining. For an additional CTL assay after 3 wk of culture, T cell lines were maintained as previously described (9).

Tetramer staining of PBMCs and T cell lines

Peptide-MHC tetramers were prepared by Dr. Mark Davis (Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA), the National Institute of Allergy and Infectious Diseases Tetramer Facility, and Otsuka Pharmaceuticals (Tokushima, Japan). PBMCs and T cell lines were stained as described (15). The detection limit was 0.01% of CD8$^+$ cells for the NS3-1073 tetramer and the NS3-1406 tetramer, 0.03% of CD8$^+$ cells for the Core-35 tetramer, and 0.02% of CD8$^+$ cells for the Core-132 tetramer. The detection limit was determined as the background signal plus three SDs after staining PBMCs from HLA-A2$^+$, HCV-negative blood donors ($n = 6$) and from HLA-A2-negative patients with chronic hepatitis C ($n = 5$).

Because $HCV_{NS3\text{-}1073}$-specific T cells could potentially have been induced by exposure to a previously described cross-reactive influenza A virus (A/PR8/34) neuraminidase peptide of similar sequence (21), we tested PBMCs from patients with detectable $HCV_{NS3\text{-}1073}$-tetramer$^+$ cells (patients chronic hepatitis C (Chr)-14, Chr-16, Chr-2, and Chr-15, as shown in Fig. 1) for recognition of the cross-reactive peptide in an IFN-γ ELISPOT assay. None of the patients displayed any immune responses against the influenza A virus neuraminidase peptide (data not shown), indicating that the responses were HCV specific.

In vitro proliferation of CFSE-labeled T cells

Ten million PBMCs were stained with 1 μM CFSE (Molecular Probes, Eugene, OR) (22) in 1 ml of PBS for 10 min at 37°C. The reaction was stopped with FCS and the cells were washed three times in PBS. CFSE-labeled cells were then stimulated in a 7-day culture with 10 μg/ml peptide.

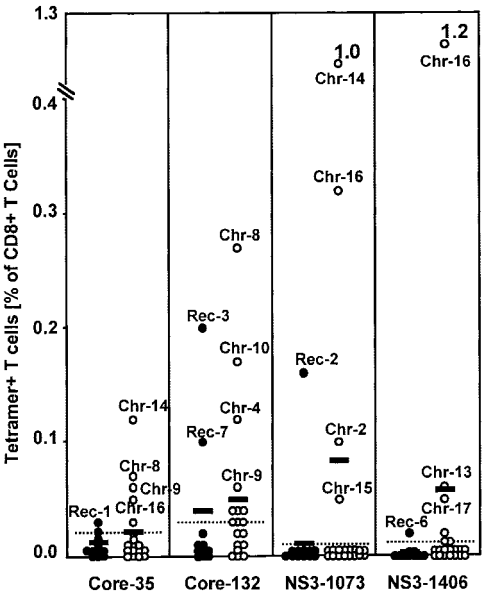

FIGURE 1. Frequencies of HCV-specific tetramer$^+$ CD8$^+$ T cells. HCV-specific CD8$^+$ T cells in the peripheral blood of 12 long-term recovered (●) and 20 HCV-infected (○) patients. The percentage of tetramer$^+$ cells within the CD8$^+$CD4$^-$CD13$^-$CD19$^-$ lymphocyte population is shown. The dashed line indicates the detection limit for each tetramer (background signal plus three SDs), the solid line indicates the average frequency of tetramer$^+$ cells. The numbers represent the patient identification numbers.

On day 7, cells were stained with tetramers and anti-CD8 and were analyzed by flow cytometry.

Cytotoxicity assay

HCV-specific cytotoxicity was determined by standard ^{51}Cr release assay (9) against 3×10^3 peptide-pulsed C1R-A2.1 cells, kindly provided by Dr. J. Berzofsky (23) in the presence of a 40-fold excess of unlabeled K562. Unstimulated PBMCs or day 7 or day 21 CTL lines were added at varying E:T ratios. Spontaneous release was <7% of maximum release in all 6-h CTL assays and <15% in all 12-h CTL assays.

Intracellular cytokine staining and ELISPOT assays

PBMCs (2×10^6 cells/ml) were stained with tetramers and thereafter stimulated with or without peptide (10 μg/ml) for 2 h at 37°C and an additional 4 h in the presence of brefeldin A, followed by a 15-min incubation at room temperature in 0.02% EDTA. Intracellular cytokine staining for IFN-γ (0.25 μg of anti-IFN-γ/10^6 cells, BD PharMingen) was performed using the Cytofix/CytoPerm kit (BD PharMingen) according to the manufacturer's instructions. Cells were washed twice, and 500,000 events in the lymphogate were collected by flow cytometry (FACSCalibur; BD Biosciences) and analyzed with CellQuest software (BD Biosciences). ELISPOT assays for IFN-γ and IL-5 were performed exactly as previously described (9). Spots were counted with an automated ELISPOT reader (KS Elispot; Zeiss, Thornwood, NY) and evaluated based on size, shape, contrast, and density. Typically, spots were irregularly shaped (because lymphocytes move during the 36-h incubation period), displayed a fuzzy border, and had a darker and denser center. Based on extensive control experiments, we consistently used 1000 μm^2 as a cutoff to exclude very small spots, which were likely due to streptavidin precipitates and other artifacts.

Statistical analysis

Kruskall-Wallis' ANOVA, Student's *t* tests, and linear regression analysis using Spearman's correlation coefficient were performed.

Results

Frequency of HCV-specific CD8$^+$ T cells in the peripheral blood of chronically infected and recovered patients

Unstimulated PBMCs from 20 patients with chronic hepatitis C and 12 long-term recovered patients (Table I) were screened

Table I. *Patient characteristics*

Patient[a]	Age (years)	Sex[b]	HCV-RNA Negative (years)	ALT (U/L)	Anti-HCV (ELISA)	HCV Genotype	HCV-RNA PCR	HCV-RNA bDNA (MEQ/ml)[d]	Liver Histology[c] Inflammatory score	Liver Histology[c] Fibrosis score
Rec-1	41	f	>5	16	+	n.k.[e]	–	n.a.[f]	1	0
Rec-2	40	f	>5	22	+	n.k.	–	n.a.	n.d.[g]	n.d.
Rec-3	35	m	>5	24	+	n.k.	–	n.a.	4	1
Rec-4	46	m	6	35	+	n.k.	–	n.a.	n.d.	n.d.
Rec-5	n.k.	f	n.k.	24	+	n.k.	–	n.a.	n.d.	n.d.
Rec-6	48	f	9	25	+	n.k.	–	n.a.	n.d.	n.d.
Rec-7	31	m	>5	26	+	n.k.	–	n.a.	n.d.	n.d.
Rec-8	57	m	3.6	53	+	3	–	n.a.	6[h]	1
Rec-9	69	f	12.4	18	+	2a	–	n.a.	3	3
Rec-10	46	m	3.0	22	+	2a	–	n.a.	2	3
Rec-11	47	m	2.9	25	+	2b	–	n.a.	3	3
Rec-12	41	m	12.0	34	+	2a	–	n.a.	0	0
Chr-1	38	f	–	25	+	1b	+	30.9	7	0
Chr-2	28	f	–	35	+	2b	+	6.1	9	0
Chr-3	39	f	–	14	+	2b	+	4.7	n.d.	n.d.
Chr-4	42	f	–	18	+	1b	+	0.4	5	0
Chr-5	44	m	–	25	+	2b	+	43	n.d.	n.d.
Chr-6	43	m	–	27	+	1a	+	7.0	3	0
Chr-7	58	m	–	32	+	u.g.[i]	+	43.1	n.d.	n.d.
Chr-8	21	f	–	22	+	1b	+	1.3	n.d.	n.d.
Chr-9	32	f	–	22	+	1b	+	0.2	n.d.	n.d.
Chr-10	60	f	–	20	+	2b	+	6.0	n.d.	n.d.
Chr-11	44	f	–	60	+	1a	+	0.3	6	0
Chr-12	39	m	–	59	+	1a	+	21.1	8	1
Chr-13	42	f	–	55	+	1a	+	1.2	6	0
Chr-14	37	m	–	40	+	1a	+	6.4	5	0
Chr-15	39	f	–	50	+	1b	+	0.5	7	1
Chr-16	45	m	–	91	+	1a	+	0.5	3	0
Chr-17	42	f	–	55	+	1a	+	2.5	6	0
Chr-18	44	f	–	69	+	1a	+	31.7	n.d.	n.d.
Chr-19	43	f	–	51	+	1a	+	6.8	7	1
Chr-20	40	f	–	49	+	1a	+	1.4	7	0

[a] None of the chronic patients had been treated with IFN-α.
[b] f, Female; m, male.
[c] Liver biopsy specimens were scored according to the HAI score introduced by Knodell. Inflammatory score: histological inflammatory activity (score 0–14); fibrosis score: 0–4. All liver biopsies were performed within 3 mo of the immunologic analysis, except for patients Chr-12 (biopsy 3 years previously), Rec-8 (biopsy 2.5 years previously), and Rec-9 (biopsy 3.5 years previously).
[d] MEQ, Million viral equivalents.
[e] n.k., Not known.
[f] n.a., Not applicable.
[g] n.d., Not done.
[h] Nonalcoholic steatohepatitis was diagnosed.
[i] u.g., Unable to genotype.

for the presence and frequency of $HCV_{Core-35}$, $HCV_{Core-132}$, $HCV_{NS3-1073}$, and $HCV_{NS3-1406}$ tetramer$^+$ cells. In contrast with higher frequencies of EBV- and CMV-specific T cells that have been reported in patients infected with those viruses (24–26), the number of HCV-specific T cells was very low in the blood of patients with chronic hepatitis C, with a mean frequency of only 0.05 ± 0.18% circulating CD8$^+$ T cells (range, 0.0–1.2%; Fig. 1).

Tetramer$^+$ cells were detected even less frequently in recovered than in chronically infected individuals (Fig. 1), consistent with a further reduction of Ag-specific cells after clearance of the virus. In fact, when all experiments with individual tetramers were considered, HCV-specific tetramer$^+$ cells were detected in 6 of 44 (14%) experiments with PBMCs from recovered patients as compared with 25 of 78 (32%) experiments performed with unstimulated PBMCs from chronically infected patients ($p = 0.016$, Kruskall-Wallis test). In addition, none of the recovered patients, but 7 of 20 chronically infected patients, tested positive with multiple tetramers ($p = 0.02$).

This difference between recovered and chronically infected patients remained significant even when the two data points with very high numbers of tetramer$^+$ cells ($HCV_{NS3-1073}$-specific cells of patient Chr-14 and $HCV_{NS3-1406}$-specific cells of patient Chr-16) were excluded. Omission of these two data points resulted in 6 of 44 (14%) positive assays in recovered patients as compared with 23 of 76 (30%) positive assays in chronically infected patients ($p = 0.041$, Kruskall-Wallis test).

Similarly, omission of these two data points did not change the finding that 7 of 20 chronically infected patients but none of the recovered patients tested positive with multiple tetramers ($p = 0.02$). Complete omission of patients Chr-14 and Chr-16, who tested positive for three and four tetramers, respectively, resulted in 5 of 18 chronically infected as compared with none of the recovered patients testing positive with multiple tetramers ($p = 0.049$).

Effector functions of tetramer$^+$ cells

Proliferation. To investigate the proliferative capacity of HCV-specific CD8$^+$ T cells at the single-cell level, we stained PBMCs with the fluorescent dye CFSE (22) and determined the CFSE content of tetramer$^+$ cells after 7 days of in vitro stimulation with HCV epitope peptides. For patients Chr-16, Chr-17, and Chr-19,

the intensity of the CFSE signal remained high after 7 days of in vitro stimulation, as shown by the tetramer$^+$ cells in the *upper right quadrants* of the dot plots (Fig. 2*A*), indicating that the cells were viable but failed to expand. Similarly, approximately two-thirds of the $HCV_{NS3\text{-}1073}$-specific, tetramer$^+$ cells of patient Chr-14 retained their CFSE signal, indicating that the majority of tetramer$^+$ cells did not divide (Fig. 2*A*).

In contrast, most HCV-specific tetramer$^+$ cells of patients recovered (Rec)-2, Rec-3, and Rec-5 displayed a weak CFSE signal (Fig. 2*A*, cells in the *upper left quadrants*), indicating that they had expanded during 7 days of culture (Fig. 2*A*). This high proliferative potential of tetramer$^+$ cells of recovered patients was peptide specific, because it was only observed in response to the specific peptide, not in response to an unrelated peptide (Fig. 2*B*).

Overall, 44 tetramer stainings were performed with cells of recovered patients and 67 stainings were performed with cells of chronic patients, demonstrating that the proliferative capacity of HCV-specific CD8$^+$ T cells was significantly stronger in recovered than in chronically infected patients. HCV tetramer$^+$ cells were detectable in 6 of 44 direct ex vivo experiments (14%) with unstimulated PBMCs and in 26 of 44 day 7 T cell cultures (59%) of recovered patients ($p < 0.0001$, Kruskall-Wallis test; Fig. 2*C*). Thus, low numbers of HCV-specific CD8$^+$ T cells (<0.01% of CD8$^+$ T cells, the detection limit of the tetramer staining) expanded significantly and, after 7 days of specific stimulation, represented up to 12% of all CD8$^+$ T cells in the culture (data not shown). The expansion rate was greatest for $HCV_{NS3\text{-}1073}$-specific T cells (2.4 ± 4.4% in the day 7 culture), followed by $HCV_{Core\text{-}132}$ and $HCV_{NS3\text{-}1406}$-specific T cells (0.25 ± 0.25% and 0.24 ± 0.29%, respectively), and was lowest for $HCV_{Core\text{-}35}$-specific T cells (0.03 ± 0.05%) (data not shown).

To demonstrate that non-HCV-specific T cells of chronically infected patients proliferated normally, we analyzed CD8$^+$ T cells specific for the influenza A virus $epitope_{MA\text{-}58}$ (20), an immunodominant and highly conserved epitope. As demonstrated in Fig. 3, the proliferative capacity of influenza A $virus_{MA\text{-}58}$-specific CD8$^+$ T cells of patients with chronic HCV infection was not impaired. In fact, CFSE-labeled $influenza_{MA\text{-}58}$-specific tetramer$^+$ cells of these patients proliferated to the same extent as tetramer$^+$ cells of recovered patients after 7 days of in vitro stimulation.

In summary, although HCV tetramer$^+$ cells were detected more frequently in unstimulated PBMCs from chronic patients than in those from recovered individuals (23 of 67 vs 6 of 44, $p = 0.016$,

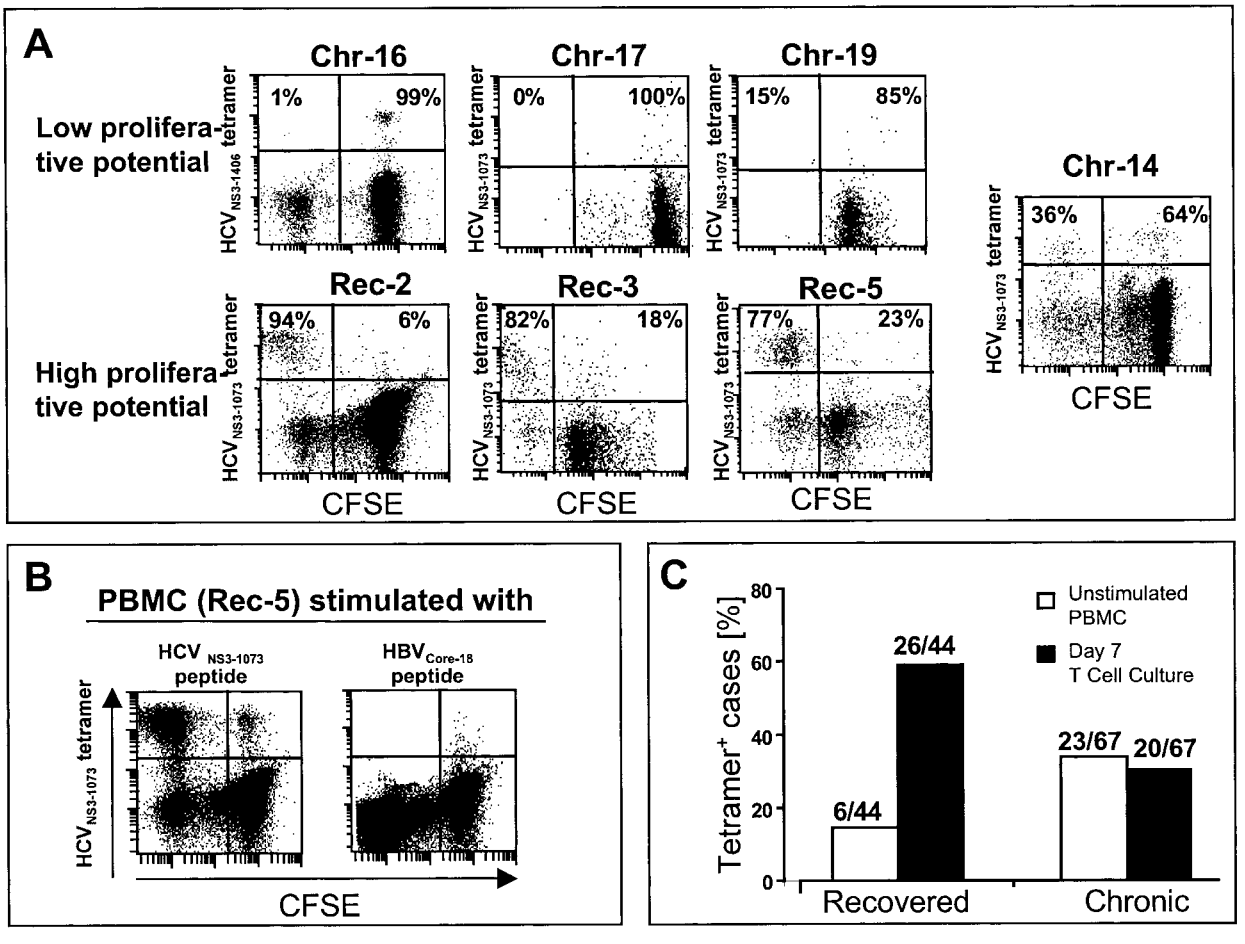

FIGURE 2. Proliferation of HCV-specific tetramer$^+$CD8$^+$ T cells. *A*, CFSE-labeled tetramer$^+$ T cells as detected on day 7 of culture in HCV peptide-stimulated T cell lines of patients. CFSE-labeled undivided tetramer$^+$ T cells are detected in the *upper right quadrant* of the graphs. The CFSE signal is diluted with each cell division because the dye is distributed to the daughter cells. Signals in the *upper left quadrant* of the graph represent HCV peptide-specific T cells that have proliferated during the 7-day culture. The numbers indicate the percentage of tetramer$^+$ cells in each quadrant. *B*, CFSE-labeled T cells were stimulated with the relevant HCV peptide, recognized by the specific TCR or with the nonrelevant $HBV_{Core\text{-}18\text{-}27}$ epitope FLPSDFFPSV with the same HLA-A2 restriction. HCV-tetramer$^+$ T cells only proliferated when stimulated with the relevant peptide. *C*, Unstimulated PBMCs (open bars) and T cell lines after 7 days of in vitro culture with specific HCV peptides and IL-2 (filled bars) were tested for tetramer$^+$ cells as indicated in Fig. 1. The number of tetramer$^+$ cell lines per number of tested T cell cultures is indicated above each bar.

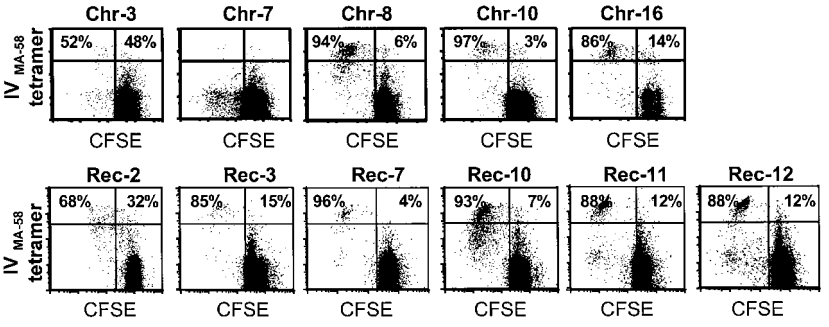

FIGURE 3. Proliferation of influenza virus-specific tetramer$^+$ CD8$^+$ T cells. CFSE-labeled tetramer$^+$ T cells as detected on day 7 of culture in influenza$_{MA\text{-}51}$ peptide-stimulated T cell lines of patients. CFSE-labeled undivided tetramer$^+$ T cells are detected in the *upper right quadrant* of the graphs. The CFSE signal is diluted with each cell division because the dye is distributed to the daughter cells. Signals in the *upper left quadrant* of the graph represent the influenza A virus peptide-specific T cells that have proliferated during the 7-day culture. The numbers indicate the percentage of tetramer$^+$ cells that have divided (*upper left quadrant*) and that have not divided (*upper right quadrant*).

Kruskall Wallis test), they were detected more frequently in stimulated cell cultures derived from recovered individuals than in those from chronic patients (26 of 44 vs 20 of 67, $p = 0.002$). These results indicate a weaker proliferative capacity of HCV-specific CD8$^+$ T cells in chronic patients.

Cytotoxicity. To analyze effector functions of HCV-specific T cells, PBMCs of recovered and chronic patients were subjected to one to three rounds of weekly HCV peptide restimulation and were tested for peptide-specific cytotoxicity in standard ^{51}Cr release assays.

Although PBMCs of recovered patients did not display significant peptide-specific cytotoxicity when tested directly ex vivo (data not shown), a single restimulation with Ag gave rise to daughter cells with significant peptide-specific cytotoxic effector function (Fig. 4). When HCV peptide-specific T cell lines were established from PBMCs of 8 recovered and 20 chronically infected patients, significant cytotoxic activity was more frequently detected in T cell lines derived from recovered individuals than in those derived from chronic patients (Fig. 4*A*). To examine whether this difference in the overall cytotoxicity of HCV peptide-stimulated T cell lines between both patient groups was due to different levels of cytotoxicity at the single-cell level, we determined the number of tetramer$^+$ T cells in a subgroup of all cultures and calculated the true effector (i.e., tetramer$^+$ cell) to target cell ratio reflecting the percent specific cytotoxicity at the single tetramer$^+$ T cell level. Significant cytotoxicity was still detectable at a tetramer$^+$ cell:target ratio as low as 0.05:1 (Fig. 4*B*), which is comparable to that of T cell clones (13). In both recovered and chronically infected patients, the cytotoxic activities of the T cell lines correlated well with the number of tetramer$^+$ cells ($r = 0.80$ and 0.78, respectively; Fig. 4*B*). Cytotoxicity per tetramer$^+$ cell tended to be higher in recovered than in chronically infected patients, but the difference was not significant.

Thus, the cytotoxic effector function of T cell lines correlated directly with the number of tetramer$^+$ cells in the cultures, which in turn depended on the proliferative potential of tetramer$^+$ cells. The diminished cytotoxic effector function of T cell lines from chronically infected patients was therefore the direct function of their low proliferative potential.

IFN-γ production. To investigate ex vivo IFN-γ production of peptide-specific CD8$^+$ T cells, we performed ELISPOT and intracellular cytokine staining assays. As previously reported by other investigators (27), we found that ELISPOT assay and intracellular cytokine staining detected similar numbers of IFN-γ-producing cells (data not shown). As shown in Fig. 5*A*, significantly fewer HCV-tetramer$^+$ T cells than influenza A virus$_{MA58}$-tetramer$^+$ T cells produced IFN-γ in chronically infected patients when ex vivo analysis was performed. Specifically, in 11 of 14 (79%) chronically infected patients, HCV-tetramer$^+$ cells produced IFN-γ in response to their specific antigenic peptide. In recovered patients, this analysis could not be performed for most cases because the frequency of HCV-tetramer$^+$ cells in the blood was below the detection level. However, in three recovered patients with detectable tetramer$^+$ cells and ELISPOT results, 40–100% of HCV-tetramer$^+$ cells produced IFN-γ.

Importantly, in chronically infected patients, even in vitro stimulation of PBMCs with HCV-specific peptides and IL-2 over 7 days followed by 6-h restimulation with the respective peptide did not restore significant IFN-γ production in most cases (Fig. 5*B*).

Phenotypic and functional analysis of HCV-specific CD8$^+$ T cells at the single-cell level

We then performed a detailed analysis of the phenotype of HCV-specific cells in patients with a sufficiently high quantity of tetramer$^+$ T cells. At least three different phenotypes of HCV-specific, tetramer$^+$ CD8$^+$ T cells were identified.

Table II focuses on tetramer$^+$ T cell populations of patients Rec-2, Chr-14, Chr-16, and Chr-8 to demonstrate the phenotypic and functional characteristics of HCV-specific cells. In these patients, the frequency of tetramer$^+$ cells and the amount of blood available allowed a larger number of assays. In addition, the frequency of these tetramer$^+$ cells in the peripheral blood was relatively constant during 20–39 mo of follow-up, with <3-fold changes (data not shown), and thus represented stable responses.

Patient Rec-2 had spontaneously recovered from HCV infection >5 years before this analysis. HCV$_{NS3\text{-}1073}$-specific cells were present at a consistently low frequency in the peripheral blood of this patient during 18 mo of follow-up (Fig. 1 and data not shown). Direct ex vivo phenotyping characterized these cells as CD45RO$^+$RA$^-$ memory cells (Fig. 6) with characteristically high expression level of the costimulatory molecules CD27 and CD28 (Table II). Low levels of HLA-DR were consistent with a resting phenotype, indicating viral clearance and lack of recent Ag contact in vivo. Although these cells contained low levels of prestored perforin and were not cytotoxic in a direct ex vivo cytotoxicity assay (Table II), they displayed a rapid recall response upon in vitro reexposure to the specific HCV peptide. This recall response was evidenced by multiple effector functions, including proliferation (Fig. 2*A*), IFN-γ production (Fig. 6), and generation of daughter cells, which were highly cytotoxic with 17 and 53% specific cytotoxicity at E:T ratios of 2:1 and 30:1, respectively (data not shown).

Chronically infected patient Chr-14 also displayed HCV$_{NS3\text{-}1073}$-specific cells that expressed the CD45RO$^+$RA$^-$ phenotype (Fig. 6). Despite CD45RO expression, however, these cells should be considered fully mature effector cells because CD28 as well as CD27 were down-regulated as also described for fully differentiated effector cells in CMV infection. The observed high level of HLA-DR expression (Fig. 6) was consistent with this fully differentiated effector cell type (28) and may indicate recent Ag contact

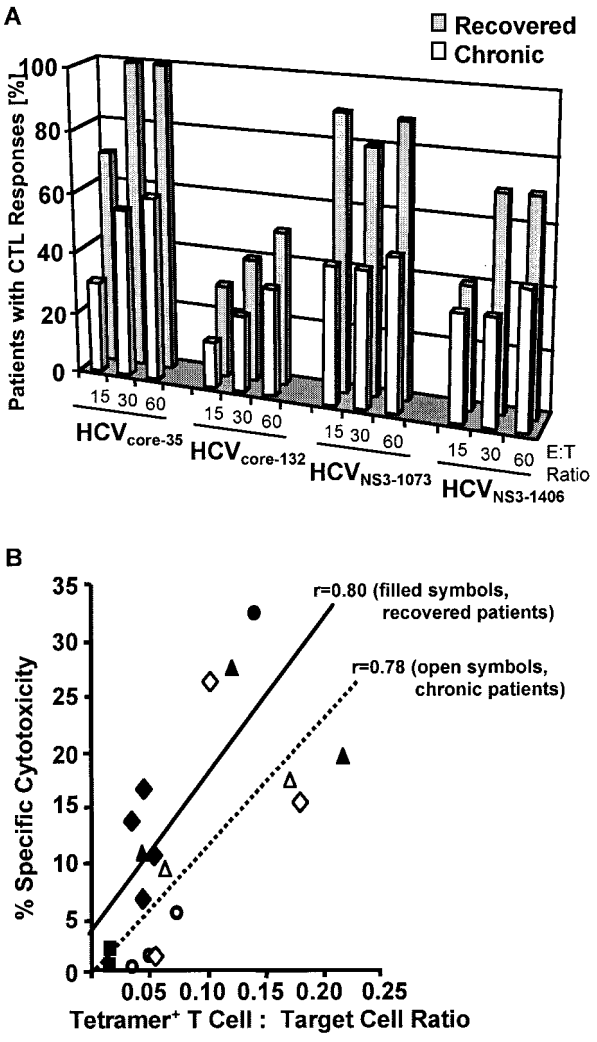

FIGURE 4. Cytotoxic activity of HCV-specific tetramer$^+$ CD8$^+$ T cells. *A*, T cell lines were generated from PBMCs of 8 recovered and 20 chronically infected patients by 21 days of in vitro stimulation with the indicated peptide and were assayed for peptide-specific cytotoxicity in a standard ^{51}Cr release assay at the indicated E:T ratios. Note that all lymphocytes in the T cell culture are counted as effector cells. Cytotoxicity was rated positive if peptide-specific cytotoxicity was >10%. *B*, PBMCs from 11 recovered and 17 HCV-infected patients were stimulated for 7 days with HCV peptides and were tested for the presence of tetramer$^+$ cells. If tetramer$^+$ cells were detected in the culture, a standard 6-h CTL assay was performed. The individual peptides and tetramers used in this experiment were: $HCV_{NS3\text{-}1073}$ (triangles), $HCV_{NS3\text{-}1406}$ (diamonds), $HCV_{Core\text{-}132}$ (circles), and $HCV_{Core\text{-}35}$ (squares). Note that, in contrast with Fig. 4*A*, the E:T ratio is indicated as the number of tetramer$^+$ cells to target cells. Tetramer$^+$ cells represent a subpopulation of all lymphocytes of the T cell line. Cytotoxicity correlated well with the proportion of tetramer$^+$ cells and did not differ significantly between recovered and chronically infected patients.

and activation in vivo. Moreover, these cells contained large amounts of perforin prestored in intracytoplasmic vesicles (Table II) and were able to exert HCV-specific cytotoxicity directly ex vivo without the need of additional restimulation (Table II). Importantly, T cells with down-regulated CD28 expression have also recently been demonstrated in the liver of patients with chronic hepatitis C (29). Our data suggest that they may contribute to liver injury based on their cytotoxic effector functions, but may be unable to completely eradicate the virus because proliferation (Fig. 2*A*) and IFN-γ production were suboptimal (Fig. 6 and Table II).

In contrast with chronically infected patient Chr-14, patient Chr-16 displayed tetramer$^+$ cells of a different phenotype with significantly impaired function. These cells were CD45RO$^-$RA$^+$ (Fig. 6) and did not express CD28. Despite down-regulation of CD28, however, ~40% of the tetramer$^+$ cells were CD27$^+$ (Table II), indicating that these cells had not yet reached the same maturation stage as the effector cells of patient Chr-14 (30, 31). Interestingly, this particular CD45RA$^+$CD27$^+$ phenotype was not only found for $HCV_{NS3\text{-}1073}$-specific T cells of patient Chr-16, but also for a second NS3 epitope in the same patient (Table II), for two HCV_{Core} epitopes in patient Chr-8 (Table II), and for the $HCV_{Core\text{-}132}$ epitope in patient Chr-10 (data not shown). Again, all cells tested negative for all analyzed effector functions, including direct ex vivo cytotoxicity, peptide-specific IFN-γ production, and in vitro proliferation.

Importantly, impairment of function was not associated with significant expression of CD152 (CTLA4; Fig. 6 and Table II), a receptor reported to regulate CD8$^+$ T cell functions (32, 33). Similarly, Abs against CD94 did neither restore HCV-specific cytotoxicity nor IFN-γ production (data not shown), indicating that signaling through these two inhibitory receptors was not responsible for the impaired function of CD8$^+$ T cells. We also excluded the presence of viral escape mutations as a potential cause for the impaired T cell function of patient Chr-16 by sequencing the epitope coding regions within the virus (data not shown).

Thus, three different phenotypes of HCV-specific T cells were identified. First, memory T cells were not activated and did not display direct ex vivo effector function, a finding that supports the hypothesis that HCV is completely cleared in recovered individuals. Second, in chronically infected patients, two different phenotypes of tetramer$^+$ cells were identified and the CD45RO$^-$RA$^+$CD27$^+$ phenotype, detected for four different epitope specificities, was associated with the complete absence of all analyzed ex vivo and in vitro effector functions.

Ex vivo analysis of HCV-specific CD4$^+$ T cell responses in chronically infected patients

Because it has been suggested in other studies that CD28$^-$CD27$^+$ cells might be arrested at an early stage of maturation due to a lack of CD4$^+$ T cell help (34), we investigated HCV-specific CD4$^+$ T cell responses of all patients enrolled in this study by direct ex vivo IFN-γ ELISPOT analysis. Overall, the number of circulating T cells that produced IFN-γ in response to the HCV proteins core, NS3, NS4, NS5A, and NS5B was significantly higher for recovered than for chronically infected patients (Table III). IFN-γ ELISPOT assays with separated CD4$^+$ and CD8$^+$ T cells identified these responding cells as CD4$^+$ Th cells (data not shown). In addition, the vigor of HCV-specific CD4$^+$ Th1 responses in patients Rec-2, Chr-14, Chr-16, and Chr-8 correlated strongly with the observed effector functions of their HCV-specific CD8$^+$ T cells. For example, a high frequency of Th cells that recognized five different HCV proteins was found in patient Rec-2. In contrast, patients Chr-16 and Chr-8, who showed impaired CD8$^+$ T cell functions, displayed significantly lower frequencies of HCV-specific Th cells, and the responses were targeted against fewer proteins (Table III and Fig. 7). Furthermore, patient Chr-14, who maintained some degree of CD8$^+$ T cell function (Fig. 2 and Table II), was also the only chronically infected patient with a significant HCV-specific IFN-γ response against any HCV protein (Fig. 7), indicating that detectable effector function of HCV-specific CD8$^+$ or CD4$^+$ T cells is a less frequent event in chronic hepatitis C. IL-5

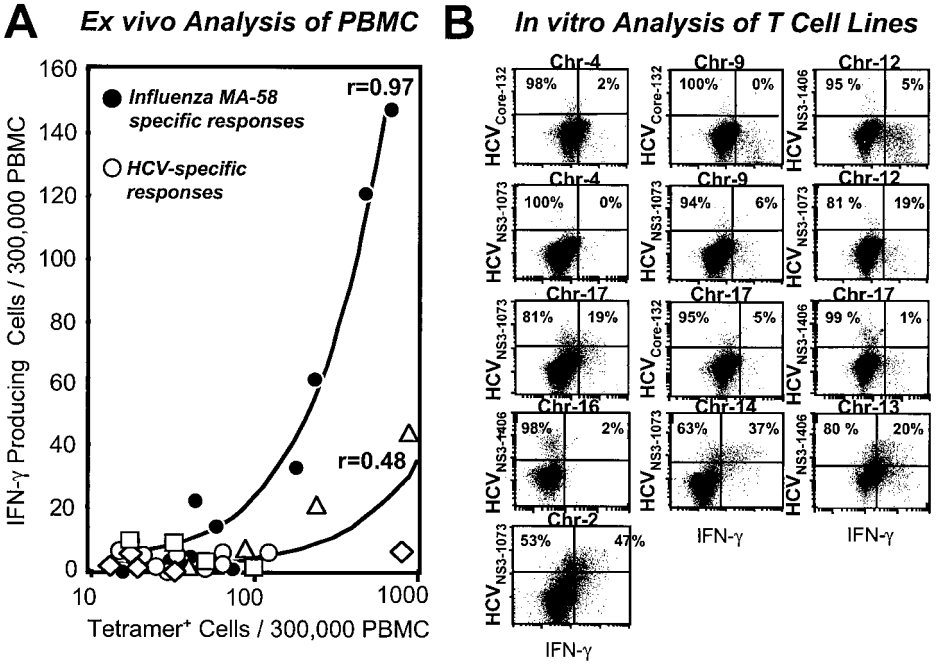

FIGURE 5. IFN-γ production of HCV-specific tetramer$^+$ CD8$^+$ T cells. *A*, Direct ex vivo analysis. The absolute number of IFN-γ-producing cells was determined by ELISPOT analysis and the absolute number of all Ag-specific cells by tetramer staining. ELISPOT assays were performed with PBMCs from the same bleed date as those used for the tetramer staining. Significantly fewer HCV-tetramer$^+$ T cells than influenza$_{MA-58}$-tetramer$^+$ T cells produced IFN-γ in response to their specific antigenic peptide. The individual peptides and tetramers used in this experiment were: HCV$_{NS3-1073}$ (triangles), HCV$_{NS3-1406}$ (diamonds), HCV$_{Core-132}$ (circles), and HCV$_{Core-35}$ (squares). *B*, Intracellular staining for IFN-γ of day 7 T cell lines. T cell lines were established by stimulation with the specific peptide and IL-2. On day 7, cells were stained with tetramer and were subsequently stimulated with the specific peptide to induce cytokine production. The numbers indicate the percentage of tetramer$^+$ T cells that do (*upper right quadrant*) or do not (*upper left quadrant*) produce IFN-γ. In most cases, in vitro-stimulated T cell lines did not produce IFN-γ in response to the specific peptide.

production in response to the same HCV proteins was evaluated as a marker for HCV-specific CD4$^+$ Th2 responses. Importantly, IL-5 responses were even weaker than IFN-γ responses (Table IV), indicating that the absence of HCV-specific CD4$^+$ Th1 responses in the blood of chronically infected patients was not due to a Th2 switch.

Discussion

In our study, we used the tetramer technique in combination with functional assays to analyze frequency as well as effector functions of HCV-specific T cells at the single-cell level. Using the tetramer technique, quantification of specific T cells depended neither on functional properties nor on extensive in vitro culture. Moreover,

Table II. *Phenotype and function of HCV-specific tetramer$^+$CD8$^+$ T cells*

	Patients and HCV Epitope					
	Rec-2	Chr-14	Chr-16		Chr-8	
	HCV$_{NS3-1073}$	HCV$_{NS3-1073}$	HCV$_{NS3-1073}$	HCV$_{NS3-1406}$	HCV$_{Core-35}$	HCV$_{Core-132}$
Tetramer$^+$ cells expressing the indicated phenotype (%)						
CD45RO$^+$	100	99	12	12	43	18
CD45RA$^+$	0	4	85	87	68	72
DR$^+$	9	65	19	18	7	13
CD69$^+$	4	33	18	3	3	27
CD28$^+$	85	8	2	1	59	71
CD27$^+$	90	7	39	42	91	90
CD94$^+$	0	10	16	27	45	38
CD152$^+$	3	10	26	26	10	22
Perforin$^+$	18	66	16	30	62	31
Direct, ex vivo effector function of tetramer$^+$ cells						
Specific cytotoxicity[a]	Neg.[b]	Pos.[c] (19% lysis)	Neg.	Neg.	n.t.[d]	n.t.
IFN-γ production (% tetramer$^+$ cells)	9	19	2	4	4	11
Proliferation (% tetramer$^+$ cells)	95	35	1	1	0	0
Effector function of peptide-stimulated T cell lines						
Specific cytotoxicity	Pos. (53% lysis)	Neg.	Neg.	Neg.	Neg.	Neg.
IFN-γ production (% tetramer$^+$ cells)	65	45	n.t.	2	n.t.	n.t.

[a] Effector (tetramer$^+$ T cell) to target ratio: 0.5:1 in a direct, ex vivo ^{51}Cr release assay.
[b] Neg., Negative.
[c] Pos., Positive.
[d] n.t., Not tested.

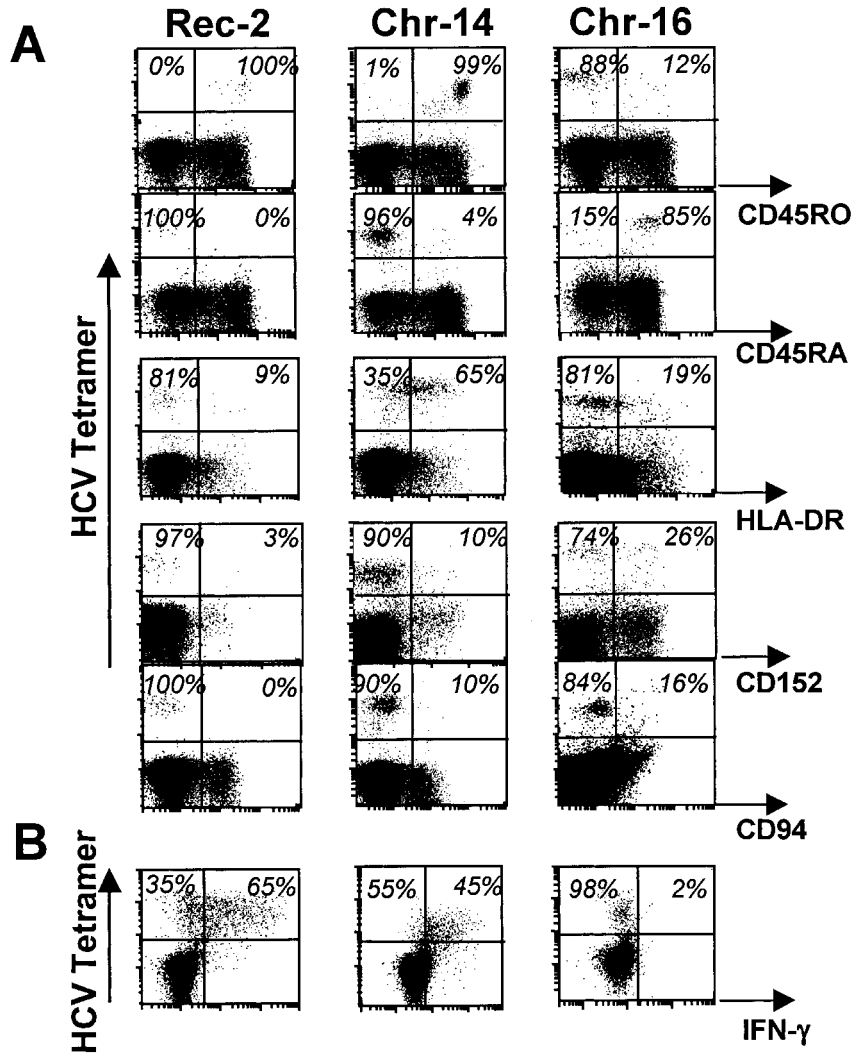

FIGURE 6. Phenotypes and function of HCV-specific tetramer$^+$ CD8$^+$ T cells. *A*, Three-color flow cytometry assay with Abs against various cell surface markers and tetramer $HCV_{NS3\text{-}1073}$. PBMCs were analyzed directly, without any stimulation. All dot plots are gated on CD8$^+$ lymphocytes. CD152 is also known as CTLA4. The *upper right quadrant* shows tetramer$^+$ T cells that display the phenotype indicated on the *right* of the graph. *B*, Intracellular IFN-γ staining performed with peptide-stimulated T cell lines on day 7 of culture. Tetramer $HCV_{NS3\text{-}1073}$ was used for patients Rec-2 and Chr-14; tetramer $HCV_{NS3\text{-}1406}$ was used for patient Chr-16.

proliferation could be investigated on a single-cell basis by CFSE staining. For all functional analyses, we used specific peptides to achieve a more specific stimulation than with PMA and ionomycin (35).

The first finding of our study was that the number of HCV-specific tetramer$^+$ CD8$^+$ T cells was extremely small in both recovered and chronically infected patients. This is in sharp contrast with infections with other pathogens such as influenza A virus, EBV, and CMV, for which the frequency of epitope-specific T cells has been reported to be as high as 30–50% CD8$^+$ T cells (24–26, 36). Thus, in contrast to those viruses, HCV appears to be capable of efficiently turning down the CD8$^+$ T cell response, which may also explain the clinically asymptomatic onset of HCV infection and the mild degree of liver injury in the majority of patients (37–39).

However, the low frequency of HCV-specific T cells did not solely explain the weak cellular immune response of chronically infected patients. Even when we did find circulating, HCV-specific tetramer$^+$ T cells in the blood of chronically infected patients, their effector function appeared to be impaired. When compared with HCV-tetramer$^+$ T cells of recovered patients and to influenza A virus-specific cells, HCV-tetramer$^+$ T cells of chronic patients displayed impaired

Table III. *IFN-γ production of CD4$^+$ T cells after stimulation with HCV proteins*[a]

	Recovered (n = 10)	Chronic (n = 20)	p[b]	Individual Patients			
				Rec-2	Chr-14	Chr-16	Chr-8
Core	26 ± 45	4 ± 7	0.04	136	10	2	0
NS3	16 ± 17	3 ± 4	0.003	53	1	1	0
NS4	30 ± 18	8 ± 14	0.001	57	59	7	0
NS5A	20 ± 13	6 ± 9	0.002	36	9	2	0
NS5B	24 ± 20	5 ± 6	<0.001	18	6	17	5
Total	117 ± 23	26 ± 8	<0.001	300	85	29	5

[a] Results are in specific IFN-γ producing cells/300,000 PBMCs. Specific spots in the presence of Ag minus spots in the absence of Ag are shown.
[b] Recovered patients vs chronic patients (Student's t test).

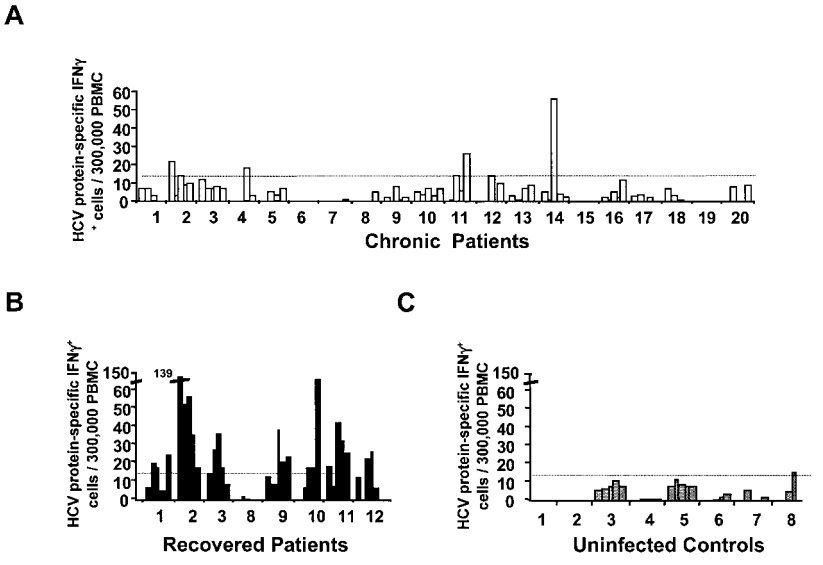

FIGURE 7. IFN-γ production of HCV-specific $CD4^+$ T cells. Direct ex vivo analysis (ELISPOT IFN-γ assay) of peripheral blood T cell responses of chronically HCV-infected patients (*A*), recovered patients (*B*), and healthy, uninfected controls (*C*). The number of Ag-specific IFN-γ-producing cells is indicated by the five bars in the order of HCV Core, NS3, NS4, NS5A, and NS5B. The dashed horizontal line indicates the cutoff.

proliferative capacity and IFN-γ production upon in vitro stimulation with the specific HCV peptide. Although cytotoxicity was observed in some instances at the single-cell level, impaired proliferation of $tetramer^+$ T cells interfered with an effective amplification of cytotoxic effector functions. As a result of the impaired proliferative capacity, fewer HCV-specific T cell lines from chronically infected patients displayed cytotoxicity after in vitro culture than those from recovered patients.

These data suggest that HCV-specific T cells may contribute to liver injury in chronic infection, because cells with this specificity (15, 40, 41) and phenotype (29) have been detected in liver tissue and have been shown to exert some degree of cytotoxic effector function (41). Nevertheless, they may be unable to completely eradicate the virus because of their functional defects, as indicated by impaired IFN-γ production (Figs. 5 and 6) and in vitro proliferation (Fig. 2*A*). These data extend previous reports on reduced TNF-α and IFN-γ production of HCV-specific T cells, as compared with EBV- and CMV-specific T cells (16). In addition, functionally defective $CD8^+$ T cells have also been described in other chronic infections, for example with HIV (13, 35, 42), and in malignancies (43). This indicates that our finding is not unique for HCV infection, but rather it is an example of a more general phenomenon in the context of persistent Ag.

The mechanisms responsible for these suboptimal functions of HCV-specific T cells in chronically infected patients remain to be elucidated. Several potential explanations have previously been proposed. First, rapid T cell death, as reported for highly activated effector T cells upon restimulation (44), does not appear to be the case in our study, because HCV-specific, $tetramer^+$ T cells were still detectable 7 days after in vitro stimulation, even though they did not proliferate (Fig. 2*A*). Second, we have excluded a significant expression of inhibitory NK cell receptors such as CD94 and CD152 on the majority of dysfunctional T cells by flow cytometry (Fig. 6), and in contrast to a previous report on melanoma-specific T cells, Abs to CD94 did not restore the function of $tetramer^+$ T cells in response to Ag in this study. Third, HCV variants with altered epitope sequences have been described as mechanisms of viral escape from T cell responses (45, 46). We have excluded viral escape mutation as the cause for the impaired T cell phenotype of patient Chr-16 by determining the epitope coding HCV sequence in PCR products and molecular clones (data not shown). Although we cannot exclude the possibility of viral escape in the other samples that we could not sequence, it should be considered that the T cell response was generally weak in our chronically infected patients. Even when escape mutants were identified in chimpanzees experimentally inoculated with HCV, T cells specific for wild-type sequences still have been expanded from liver tissue (46, 47). Detection of functional virus-specific $CD8^+$ T cells in recovered patients, although at low levels, and of wild-type-specific $CD8^+$ T cells in chronically infected patients with escape mutants (45, 48), also indicates that $CD8^+$ T cells can be maintained and continue to circulate in the blood despite the absence of their cognate Ags. Thus, although viral escape mutants cannot be excluded in all patients of this study, it is still informative that the

Table IV. *IL-5 production of $CD4^+$ T cells after stimulation with HCV proteins*[a]

	Recovered (n = 10)	Chronic (n = 20)	p^b	Individual Patients			
				Rec-2	Chr-14	Chr-16	Chr-8
Core	2.0 ± 5.7	0.5 ± 1.6	NS	16	0	0	0
NS3	1.3 ± 3.2	0.3 ± 0.7	NS	0	0	0	0
NS4	0.4 ± 1.1	0.2 ± 0.7	NS	0	0	0	0
NS5A	1.0 ± 2.8	0.8 ± 2.2	NS	0	0	0	0
NS5B	2.3 ± 4.7	0.5 ± 1.8	NS	0	0	0	0
Total	117 ± 23	26 ± 8	NS	16	0	0	0

[a] Results are in specific IL-5-producing cells/300,000 PBMCs. Specific spots in the presence of Ag minus spots in the absence of Ag are shown.
[b] Recovered patients vs chronic patients (Student's t test).

effector function of the majority of HCV-specific T cells in chronically infected patients was impaired. Serum samples from the very early course of infection or the infectious source would be required to evaluate the sequence of the original infecting virus.

Fourth, induction of anergy by high Ag levels remains another possible explanation. In hepatitis B virus (HBV) infection, large amounts of HBe Ag are secreted into the blood and have been implicated in mediating neonatal T cell tolerance (49) and in altering the reactivity of HBe-specific $CD8^+$ T cells (50). Although the concentration of free, circulating viral protein is several logs lower in chronic HCV than in HBV infection and although HCV Ags are not produced in a specific secreted form such as HBe Ag in HBV infection (51), very low concentrations of HCV core Ag have been implicated in a down-regulation of cellular immune responses (52, 53). In addition, the amount and effect of viral Ags as part of viral particles coated with low- and very low-density lipoproteins (54) remain unknown.

Finally, in this study, we report a phenotypic heterogeneity among HCV-specific T cells that was associated with distinct patterns of effector functions. Although the number of $tetramer^+$ patients was too small to assess the significance of each phenotype for disease progression and control of viral load, the observed phenotypic and functional characteristics were in accordance with published reports on other infections. For example, down-regulation of CD28 and CD27 was observed on HCV-specific effector cells of patient Chr-14. In accordance with recent reports that CD28 and CD27 are irreversibly down-regulated on fully differentiated effector cells (13, 28, 55), $tetramer^+$ cells of patient Chr-14 displayed a fully differentiated effector cell type and were able to mediate direct ex vivo cytotoxicity and, at least to some extent, to proliferate and to produce IFN-γ.

In addition, we also describe an example of a more extreme phenotype with completely impaired effector functions such as HCV-specific proliferation, cytotoxicity, and IFN-γ production. Although CD28 was down-regulated on $tetramer^+$ cells of patient Chr-16, expression of CD27, a molecule from the TNF receptor superfamily that is expressed on thymic emigrants (30) and irreversibly lost during differentiation (31), was maintained. According to the literature, $CD28^-CD27^+$ T cells have not been observed in healthy individuals. Instead, they were found in persistent viral infections (13, 28) and it has been suggested that these cells are arrested in an immature state due to a lack of $CD4^+$ T cell help (34).

Indeed, unresponsive $CD8^+$ T cells that were noncytotoxic and did not produce IFN-γ have been described in animal models of chronic lymphocytic choriomeningitis virus or SIV infection under conditions of Th cell deficiency (56, 57). Although $CD4^+$ T cells were not required for the induction of $CD8^+$ T cell responses during acute lymphocytic choriomeningitis virus infection (58, 59), they were indispensable for the maintenance of virus-specific $CD8^+$ T cell responses that control chronic viral infections (60). In accordance with these results, we have found that various effector functions of HCV-specific $CD8^+$ T cells were impaired in chronically infected patients with weak HCV-specific $CD4^+$ T cell responses. $CD4^+$ T cell responses were weak with regard to IFN-γ production, and this weakness was not due a Th1 to Th2 shift. In addition, proliferation of $CD4^+$ T cells has also been described to be weak in chronically infected patients (4, 9, 61)

Because clearance of HCV has been associated with a vigorous and maintained $CD8^+$ T cell response (5, 6, 8) and because $CD4^+$ T cells appear to be required to prevent recrudescence (62), we propose that HCV-specific $CD4^+$ T cell help is required from the time point of acute HCV infection to maintain the proper functions of HCV-specific $CD8^+$ T cells, the main antiviral effector cells. Although $CD4^+$ T cell help could consist of direct activation of $CD8^+$ T cells via cytokines (63), it is notable that these $CD8^+$ T cell functions could not be restored by addition of exogenous IL-2 in the present study. Alternatively, $CD4^+$ T cell help could be indirect via CD40-mediated activation of professional APCs (64). This mechanism might be relevant because of accumulating evidence of abnormal dendritic cell function in chronic HCV infection (65, 66). Thus, in the presence of a weak $CD4^+$ T cell response, dendritic cells might not be sufficiently stimulated and, in turn, might not appropriately activate $CD8^+$ T cells. Prospective analysis of $CD4^+$ and $CD8^+$ T cell function during the natural course of HCV infection is needed to address these important issues.

Finally, our findings may also have several therapeutic implications. Successful vaccination protocols may depend not only on the induction of HCV-specific $CD8^+$ T cells, but also on the maintenance of their function in vivo. Indeed, it has been shown that stimulation of $CD8^+$ T cells alone by peptide vaccines is not sufficient to clear the virus in chronic hepatitis B (67). Secondly, the heterogeneity of T cell responses in different patients suggests that it may be important to stratify patients into different treatment groups. Finally, enhancement of CTL activity is always like a double-edged sword, and the important question requiring further study is how the optimal number, phenotype, and effector functions of Ag-specific T cells can be achieved to mediate viral clearance without causing detrimental immunopathology.

Acknowledgments

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