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Pervasive Influence of Hepatitis C Virus on the Phenotype of Antiviral CD8+ T Cells

Michaela Lucas,* Ana L. Vargas-Cuero,* Georg M. Lauer,† Eleanor Barnes,*‡ Christian B. Willberg,* Nasser Semmo,* Bruce D. Walker,† Rodney Phillips,* and Paul Klenerman2*

Recent studies using MHC class I tetramers have shown that CD8+ T cell responses against different persistent viruses vary considerably in magnitude and phenotype. At one extreme, hepatitis C virus (HCV)-specific CD8+ T cell responses in blood are generally weak and have a phenotype that is perforin low and CCR7 high (early memory). At the other, specific responses to CMV are strong, perforin high, and CCR7 low (mature or effector memory). To examine the potential mechanisms behind this diversity, we compared CMV-specific responses in HCV-infected and healthy individuals. We find a striking difference in the phenotype of CMV-specific CD8+ T cells between these groups. In the HCV-infected cohort, CMV-specific CD8+ T cells lost markers associated with maturity; they had increased expression of CCR7 and reduced expression of Fas and perforin. They nevertheless responded to Ag in vitro in a manner similar to controls, with strong proliferation and appropriate acquisition of effector memory markers. The reduction in mature CD8 T cells in HCV-infected individuals may arise through either impairment or regulation of T cell stimulation, or through the early loss of mature T cells. Whatever the mechanism, HCV has a pervasive influence on the circulating CD8+ T cell population, a novel feature that may be a hallmark of this infection. The Journal of Immunology, 2004, 172: 1744–1753.

Hepatitis C virus (HCV) is thought to infect 170 million people worldwide. It readily sets up persistent infection in immunocompetent adults, and once established, persistence is usually lifelong. In these chronically infected persons, hepatitis, liver fibrosis, cirrhosis, and hepatocellular cancer may develop.

T cell responses are both vigorous and multispecific during primary infection (1, 2) and determine the outcome of infection (2–4). However, once persistent infection is established, both CD4+ and CD8+ T cell responses are attenuated and become difficult to detect ex vivo (1, 5).

We have previously investigated the mechanisms behind the failure of CD8+ T cells to control HCV, and also the inability to sustain high-level T cell responses in persistent viremia (5, 6). It is likely that viral variation may play an important role in some cases, and this has been most clearly shown in the animal model (7). It is also possible that there is a functional defect in CD8+ T cells that impairs their ability to control virus (1, 6, 8, 9). This has been suggested in various infectious disease models where persistence is established (10).

Evidence for this includes the failure of HCV-specific T cells to produce IFN-γ during primary infection (described previously as stunning) (1), and a lack of perforin expression or ex vivo killing during primary infection despite T cell activation (6).

To further define the reasons why CD8+ T cells fail to control viremia, we have performed detailed surface and intracellular phenotypic analysis of HCV-specific T cells, using HLA class I peptide tetramers. This follows on from recent studies of human and murine antiviral CD8+ T cell populations that have revealed important distinctions in memory subsets. Memory pools can be quite heterogeneous, although descriptions of this variability are themselves not uniform. At one extreme, there appear to be cells which are more readily associated with lymphoid organs and possess appropriate homing markers such as CCR7 or CD62L (11). These central memory cells in some but not all cases may lack immediate protective capacity, but readily respond to Ag and may proliferate efficiently. At the other extreme lie cells that are described as mature or effector memory, which have lost lymphoid homing markers, acquired effector function, and are thought to provide protection in peripheral organs (12). Much of this is currently conjecture in human disease, and the differences are not always clear-cut, but there is some evidence for distinct functions in murine models.

Using this paradigm, further evidence for a distinct evolution of HCV-specific CD8+ T cells comes from studies of the detailed surface and intracellular phenotype using HLA class I peptide tetramers. We have previously noted that these cells appear to be low in perforin and high in CD28 and CD27 (13). Interestingly, in a parallel comparison with CD8+ T cells specific for EBV, HIV, and CMV, there were distinct phenotypes for each infection. Of these, HCV represented the least mature, whereas CMV lies at the opposite extreme, for example, low in CD28 and CD27 and high in perforin. The mature phenotype shown by CMV is presumably related to restimulation over time, because CMV reactivates continuously, thus reboosting CD8+ T cell memory pools (14). Why this does not occur in HCV, despite the continued presence of viral Ag is not clear. We have put forward a two-loop hypothesis, which might explain both the low...
levels of CD8 T cells seen during HCV infection and their lack of maturity. In this, CD8 T cells are primed by APCs effectively in lymphoid tissue, but are also eliminated in the presence of virus, potentially within liver tissue. Thus, they fail to expand to large numbers and also to reach full maturity in vivo (15).

In further analyzing this hypothesis, we addressed the question of whether such effects are restricted to HCV-specific cells, or include other CD8 T cell populations. In doing so, we used CMV-specific CD8 T cells as a marker population. These were chosen for two reasons: first, they are abundant and present at frequencies of up to 10% in normal controls (16), and second, they have a very distinct phenotype that is readily assessed in normal controls (13). This phenotype, in healthy individuals, as discussed above, represents the opposite extreme from that of HCV-specific cells in that they possess markers associated with maturity or are otherwise described as effector memory CD8 T cells. Thus, we could address whether the particular phenotype associated with HCV-specific CD8 T cells could be seen in other non-HCV-specific populations in HCV-infected individuals.

Materials and Methods

Subjects

Informed consent in writing was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval from the ethics committee at the John Radcliffe Hospital (Oxford, U.K.) or of the Massachusetts General Hospital (Boston, MA). HCV seropositivity was defined as confirmed presence of HCV Abs (third-generation enzyme immunoassay). PCR positivity was defined as the detection of HCV RNA by PCR (detection limit, 300 HCV RNA copies/ml of plasma; version 2.0 Amplicor assay; Roche Diagnostics, Somerville, NJ). For initial ex vivo comparison, we analyzed a previously established set of HCV-infected plasma samples from untreated patients in whom CD8 T cell responses had been previously obtained (1, 6). Further clinical and immunological data on a subset of the HCV-infected plasma samples were used to compare the data with those obtained from healthy controls (13). This phenotype, in healthy individuals, as discussed above, represents the opposite extreme from that of HCV-specific cells in that they possess markers associated with maturity or are otherwise described as effector memory CD8 T cells. Thus, we could address whether the particular phenotype associated with HCV-specific CD8 T cells could be seen in other non-HCV-specific populations in HCV-infected individuals.

PBMC preparation

Fresh PBMCs from all study subjects were obtained from heparinized blood centrifugation over Lymphoprep (Nycomed Pharma, Asker, Norway) and washed three times (5 min; 1500 rpm; 25°C) in RPMI 1640 (Sigma-Aldrich, Dorset, U.K.) supplemented with 50 μM penicillin, 2 mmol/liter L-glutamine, and 50 μg/ml streptomycin. Cells were processed fresh for all functional studies or frozen and stored in liquid nitrogen for subsequent phenotypic analysis.

MHC class I tetramer staining

HCLA class I-peptide tetramers were prepared as previously described (1) and included tetramers specific for five epitopes restricted by HLA-A2, one epitope restricted by HLA-A1, HLA-A24, and HLA-B7, respectively, and two epitopes restricted by HLA-B35 (HLA-A2: CMV-pp65 peptide 495–503, NLVPMATVY; HCV-NS5 peptide 1073–1081, CINGVCWTVY; HCV-NS5 peptide 1406–1415, KLVAGILNAV; HCV-NS5 peptide 1987–1995, VLDSTKFWL; HCV-NS5b peptide 2594–2603, ALYDVYTKFL; HLA-A1: NS3 peptide 1435–1443, ATDAMLMTGY; HLA-A24: HCV-NS4 peptide 1745–1754, VIAPAVQTNW; HLA-B7: CMV-pp65 peptide 417–426, TPRVTGGGAM; HLA-B35: CMV-pp65 peptide 123–131, IPSIN-VHHY; HCV-NS3 peptide 1359–1367, HPNIEEVAL). All peptides were synthesized by Research Genetics/Invitrogen (San Diego, CA). Tetramer staining was performed as previously described (1). Briefly, 0.5–1 × 106 PBMC were stained for 20 min at 37°C. After washing with PBS containing 1% FCS at room temperature (RT), cells were pelleted and directly stained with combinations of the following Abs: CD8-PerCP, CD27-FITC, CD28-allophycocyanin, CD45RA-FITC, CD45RO-FITC, CD95-FITC, and CD56-FITC (BD Biosciences). CCR7 staining was performed after the tetramer staining and as follows. Cells were pelleted and incubated with an anti-CCR7 Ab (BD Biosciences) in the presence of 10 μl of goat serum for 30 min at RT. After two washes with PBS/1% FCS, cells were pelleted again, and a secondary anti-mouse IgG-FITC-conjugated Ab (DAKO, Glostrup, Denmark) was added for 30 min at RT. Cells were washed twice more and directly conjugated CD8-PerCP Ab, and 10 μl of goat serum was added for 20 min at 4°C. For all Ab stains, pellets were washed once more, and cells were fixed with 1% formaldehyde. Flow-cytometric analysis was performed using a FACSCalibur (BD Biosciences), and data analysis was performed with the CellQuest software (BD Biosciences). Tetramer validation was performed by staining a series of negative and positive control PBMCs. We observed very low levels (<0.01%) of nonspecific staining in seronegative as well as seropositive HLA-mismatched individuals. Tetramer-positive cells were considered positive if a clustering distinct from the tetramer-negative CD8 T cell population occurred, and the frequency of tetramer-positive cells was >0.02% of the total CD8 population.

Intracellular cytokine staining

Intracellular cytokine staining (ICS) of fresh PBMCs after peptide-specific stimulation or unsppecific PMA/ionomycin stimulation was performed as previously described (1). Stimulation was followed by permeabilization and intracellular staining for IFN-γ with anti-IFN-γ-FITC (BD Biosciences) and for TNF-α with anti-TNF-α-allophycocyanin (BD Biosciences).

Table I. Clinical details of HCV/CMV-coinfected patients

<table>
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<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Relevant HLA Type</th>
<th>RT-PCR for HCV</th>
<th>Treatment</th>
<th>Years since HCV Infection in Months</th>
<th>Approximate Duration of HCV Infection in Years</th>
<th>ALT (IU/liter)</th>
<th>Histology Fibrosis</th>
<th>Inflammation</th>
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<tr>
<td>OX1</td>
<td>40</td>
<td>F</td>
<td>A</td>
<td>+</td>
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<td>NA</td>
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<td>NA</td>
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<td>B</td>
<td>+</td>
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<td>NA</td>
<td>10</td>
<td>37</td>
<td>B</td>
<td>NA</td>
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<tr>
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<td>M</td>
<td>B</td>
<td>+</td>
<td>IFN-α/Ribavirin</td>
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<td>76</td>
<td>3/6 + 7/18</td>
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<td>20</td>
<td>43</td>
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<td>A</td>
<td>-</td>
<td>IFN-α</td>
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<td>61</td>
<td>61</td>
<td>1/6 + 3/18</td>
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*Upper limit of normal for alanine aminotransferase (ALT) is 45. Histology scoring is using a modified Ishak’s grading. The first score is a fibrosis score (out of 6). The second score is an inflammatory score (out of 18). The most recent biopsy data was used (i.e., closest to the immunological analysis). Abbreviations used: M, male; F, female; NA, not available.
Cytotoxicity assays
Analysis of the lytic capacity of fresh ex vivo CD8^+ T cells was performed using a modified version of the fluorometric assessment of T lymphocyte Ag specific lysis assay (17). Assays were performed overnight, and results were measured as percent specific lysis after comparison between lysis of peptide pulsed and nonpulsed targets.

In vitro expansion of virus-specific cells
PBMCs were plated in 24-well plates (2 \times 10^5/well), and relevant peptides were added to a final concentration of 10 \mu M. Irrelevant peptides or no peptide served as a negative control. The plates were then incubated at 37°C. On days 3 and 6, 100 IU of IL-2 (Chiron, Emeryville, CA) was added to the cultures, and these were harvested for tetramer staining on day 10. Medium change and splitting of cells was performed when necessary.

PCR for CMV pp65
To detect CMV replication in the patient’s blood, nested PCR was performed, as described previously (18). In brief, DNA was extracted from PBMCs, and nested primers specific for the pp65 gene were used. This assay is able to detect a single infected cell in 50,000 PBMCs and is invariably negative in uninfected donors. All control donors and 5 of 12 of the HCV-seropositive patients were tested and found to be repeatedly negative using this assay.

ELISPOT assay
ELISPOT assays were performed as previously described (19). Peptides or CMV lysate (BioWhittaker, Walkersville, MD) were added directly to the wells at a final concentration of 10 \mu g/ml.

Statistical analysis
Statistical analysis was performed using GraphPad Prism (GraphPad, San Diego, CA). All data were compared using the nonparametric Mann-Whitney test with a value of \( p < 0.05 \) being significant.

Results
Phenotypic analysis of HCV-specific and CMV-specific CD8^+ T cells
Prior studies of the HCV-specific T cell phenotype have focused on a limited number of surface markers. Therefore, we comprehensively compared HCV-specific responses from HCV-infected individuals with CMV-specific responses from healthy individuals and performed an initial screening procedure to identify important differences. The comparisons are illustrated in Fig. 1. In each case, an example of the staining of tetramer-positive populations is shown, and in the right panel, a comparison between the grouped data for the expression of this marker on HCV-specific T cells in HCV-seropositive patients and CMV-specific T cells in healthy controls is shown.

Fig. 1A is focused on markers associated with maturation. As previously reported, clear-cut differences in the expression of CD27 and CD28 are shown, with the majority of HCV-specific CD8^+ T cells being double positive for these markers in HCV-infected individuals, but double negative in the CMV-specific cells from healthy individuals. CMV-specific CD8^+ T cells also reexpress CD45RA when terminally differentiated (20). Although this has been reported in HCV (9), this is an unusual finding, and, in this cohort, no significant CD45RA-positive population was identified among HCV^+ T cells.

Fig. 1B is focused on homing and functional markers. A striking and previously unreported difference in CCR7 is noted. Many HCV-specific populations appear to express this lymph node-homing marker, a feature that is not found in CMV-specific populations. HCV-specific CD8^+ T cells were clearly low in perforin, as previously reported, and overall showed a decrease in CD95 (Fas). Some CMV-specific CD8^+ T cells do re-express CD62L, although this marker was not found in HCV—the difference in this case was not significant.

Overall, these data confirm and extend previous analyses (6, 9, 13). HCV-specific populations appear to represent an early memory phenotype. CMV-specific CD8^+ T cells from healthy individuals conform to a mature or effector memory phenotype, although wide variation in CD45RA/RO expression, and CD27 expression is seen.

We also analyzed a series of these markers in EBV-specific populations from healthy donors. As previously reported, the phenotype of EBV- and HCV-specific CD8^+ T cells overlap (13) with respect to all the markers analyzed (data not shown). The only exception is CD62L, which may be re-expressed to some degree on EBV-specific CTL (data not shown). Thus, we focused the further analysis largely on CMV-specific populations, in which an obvious difference from HCV is seen.

Shifts in the phenotype of CMV-specific T cells in HCV-infected patients
To address the effect of HCV on the phenotype of CD8^+ T cells that are not HCV-specific, we analyzed CMV-specific CD8^+ T cells in HCV-seropositive patients. We screened HCV-seropositive patients for CD8^+ T cell responses to CMV using HLA class I tetramers, restricted by HLA-A2, -B7 or -B35. From this group, individuals with a distinct CMV-specific tetramer response that was \( >0.2\% \) of CD8^+ T cells were taken for further study (Table I). These were compared with healthy controls, equivalent in terms of age and sex.

Fig. 2A shows the phenotype of CMV-specific CD8^+ T cells in the HCV cohort. There were significant changes in the expression of CCR7, CD62L, perforin (\( p < 0.003–0.014 \)) and also CD95 (borderline, \( p = 0.05 \)) compared with CMV responses in healthy controls. The expression of these markers showed some heterogeneity, but this was not consistently associated with individual patients, treatment status, or epitope specificity. No significant change in CD45RA, CD27, CD28, or CD45RO was observed (data not shown). Taken together, these data indicate a clear shift in the profile of the CMV-specific T cells away from the normal mature or effector memory phenotype.

Shifts in the phenotype of CD8^+ T cells
We then analyzed whether any of these effects, which are obvious in the CMV population due to its discrete phenotype, might also be obvious in the CD8^+ T cell population as a whole. Therefore, further analysis of the total CD8^+ population was performed, and the difference between HCV-seropositive patients and healthy controls was analyzed (Fig. 2B). Interestingly, despite the heterogeneity of this group of cells, which includes memory T cells of...
various specificities as well as naive cells, some significant differences were seen. Similar to previous data (21), we did note a significant reduction in overall CD8+ perforin expression (Fig. 2B; \( p = 0.015 \)). This was not seen in a small group of individuals (\( n = 6 \)) who spontaneously resolved HCV (mean perforin expression of 14.24 vs 15.63% in normal controls; not significant). This was not due to a general shift toward a naive phenotype, because there was an overall reduction in CD45RA-positive CD8+ T cells (controls vs HCV\(^+\), \( p = 0.0035 \)). No significant differences were seen in CD27, CD28, CD95/Fas, CD38, CCR7, and MHC class II expression.

As a simple control for whether this effect was due to liver inflammation per se or related more specifically to HCV infection, we analyzed whether CD8+ T cell expression of perforin was affected in a group of HCV-negative patients with alcohol-induced liver disease. Overall, the mean perforin expression in the CD8+ T cell compartment was not significantly different from that of a set of normal controls analyzed in parallel (\( n = 33 \); \( p = 0.22 \); data not shown).

**Analysis of maturation in vitro**

We analyzed whether CMV- and HCV-specific CD8+ T cells that were present but had not acquired markers of maturity in the HCV patients, were able to proliferate and mature in vitro following peptide stimulation.

In eight of eight patients, HCV-specific cells proliferated in response to specific peptide stimulation (Fig. 3A). Large tetramer+ populations that comprised up to 52% of the CD8+ T cells were observed and are shown in two representative patients (Fig. 3A). Moreover, significant changes in the phenotype of these cells following expansion were observed (loss of CD27, CD28, and CCR7, and up-regulation of CD95/Fas and the activation marker CD38; Fig. 3B). Thus, the apparent lack of maturation of CD8+ T cells in vivo can be easily overcome in vitro, indicating that there is no inherent block intrinsic to the HCV-specific CD8+ T cells.

Similar observations were made after peptide-specific expansion of CMV-specific CD8+ T cells in HCV\(^+\) subjects. Fig. 4A illustrates expansions of two CMV-specific populations (HLA-A2 and HLA-B7 restricted) within one patient after in vitro stimulation. Expansions of CMV-specific CD8+ T cells were comparable in HCV\(^+\) and HCV\(^-\) individuals (Fig. 4B). The same change in phenotype was also seen within these groups, with loss of CCR7 and increase in CD95/Fas, CD38, and loss of CD45RA (Fig. 4C). Therefore, the phenotype of expanded cells was similar in HCV+ and HCV\(^-\) controls. Thus, in the HCV+ patients, CMV-specific T cells retained the capacity to expand and acquire markers of maturation.

**Analysis of function in vitro**

Finally, we investigated whether the phenotypic shifts were associated with significant changes in function, as assessed in vitro. The IFN-\( \gamma \) and TNF-\( \alpha \) response was analyzed by ICS and ELISPOT. First, there was no obvious defect in the overall capacity of bulk CD8+ T cells to make cytokines in response to PMA/ionomycin stimulation (Fig. 5A). In agreement with a previous study (6), CMV-specific CD8+ T cells from HCV-seropositive patients, in an ICS assay, were able to make IFN-\( \gamma \) and TNF-\( \alpha \) after short-term peptide stimulation in a manner similar to controls (Fig. 5B).

Overall, as previously shown (6), ~50% of the CMV-specific cells from both HCV-positive patients and controls were found to secrete cytokine in response to short-term peptide stimulation. To examine whether there was a subtle change in the kinetics of cytokine production, we looked at IFN-\( \gamma \) production in short-term (3-h) and long-term (18-h) ELISPOT assays. Cells from both normal and HCV-seropositive individuals were both able to make IFN-\( \gamma \) within 3 h, and strong ELISPOT responses to CMV lysate (indicative of CD4+ T cell responses) were present in both groups (data not shown). Cytolytic activity was also detectable ex vivo (Fig. 5C). As a final measure of function, the response of CMV-specific CD8+ T cells to peptide Ag in terms of proliferation in vitro was similar in both groups as shown in Fig. 4B.

These experiments do not rule out a subtle and quantitative effect in vivo, but suggest that this aspect of function is not grossly impaired ex vivo. Consistent with this, analysis of serum from five of the HCV-seropositive individuals failed to reveal CMV DNA (indicative of subclinical viremia), suggesting that control of CMV is not significantly impaired in vivo.

**Discussion**

HCV readily sets up persistence in the face of host cellular responses. The mechanisms behind this are not fully understood, although murine models of other persistent virus infections suggest antigenic variation, CD8+ T cell exhaustion, and a direct immunosuppressive role of the virus. The frequency, function, and phenotype of HCV-specific CD8+ T cells have been the subject of much attention. The most striking and consistent findings to date are that, in persistent infection, the CD8+ T cell responses are weak, compared with acute disease (1, 5, 9). Additionally, we have previously shown that HCV-specific T cells are of an immature (early or central memory) phenotype (6, 13, 22). This contrasts with the phenotype of other antiviral T cell populations, such as CMV-specific CD8+ T cells, which are of a more mature (effector memory) phenotype in healthy individuals.

In this study, we have shown that HCV infection has a pervasive effect on the CD8+ T cell population. CMV-specific CD8+ T cells have a more immature phenotype in HCV-infected individuals compared with healthy controls. This effect of HCV on T cell phenotype extends beyond the T cells that target this virus. In this study, CMV-specific T cells act as a marker population, which is readily examined due to its large size (in this study, 0.3–16% of CD8+ T cells) and extreme phenotype, but it is unlikely that the changes seen are related to the biology of CMV or a specific HCV/CMV interaction; indeed, such changes are to some extent reflected in the bulk CD8+ T cell population.

The phenotypic shift was consistent between patients, despite a range of clinical courses, and are compelling because all the shifts in phenotype are occurring in the same direction, i.e., away from a mature or effector memory phenotype toward a less mature or central memory phenotype. In two individuals studied, virus was no longer present in the blood after therapy, although in each case, viremia had been present long-term before therapy. This suggests that, if the effect is due to HCV circulating in blood, it is sustained after clearance, although long-term studies would be needed to assess the recovery of these populations after therapy leading to viral clearance. It is not yet clear how long it might take for CMV populations to develop or to lose the extreme phenotype with which they are normally associated, because large-scale, long-term follow-up studies after acute CMV have not been performed. Further studies on effects of acute HCV on CMV-specific CD8+ T cells are required. Analysis to date of six spontaneous resolvers has yet to reveal a substantial CMV-tetramer+ population, so quite large cohorts will be necessary.

Regarding surface phenotype, one interesting point to come from this study is the discrepancy between CD62L staining and CCR7 staining in the HCV-specific populations. HCV-specific CD8+ T cells retain chemokine receptors that are associated with
FIGURE 2. Phenotypic characterization of CMV-specific CD8\(^{+}\) T cells in HCV-seropositive donors. A. CCR7, CD45RA, CD95, and perforin staining on CMV-specific CD8\(^{+}\) T cell in HCV\(^{+}\) patients is presented. In the first panel, representative CMV-specific tetramer staining with percentages of cells present in upper right quadrant is shown. The second panel depicts a scatter plot summarizing the phenotype of CMV-specific CD8\(^{+}\) T cells in HCV\(^{+}\) donors compared with controls. The HCV\(^{+}\) donors’ clinical data are laid out in Table I. B. Scatter plot showing the perforin and CD95 surface expression on the whole CD8\(^{+}\) T cell population in healthy controls and HCV-seropositive donors. Only long-term HCV\(^{+}\) PCR\(^{+}\) donors were included. Significant differences (p < 0.05) are indicated on the right.
lymph node homing, but not adhesion molecules such as CD62L. Thus, they are not typically central memory in phenotype. Interestingly, the CMV- and EBV-specific populations in HCV+ patients are able to re-express CD62L in concert with CCR7 expression. The exact homing pathways of the HCV-specific CD8+ T cells demand further examination, but it is possible that failure to encounter Ag appropriately may be relevant to their antiviral function in vivo.

**FIGURE 3.** Phenotypic changes of HCV-specific cells after one round of peptide stimulation. A, Dot plots of A2-NS3 1073 tetramer stains, directly ex vivo or after one round of peptide stimulation. Numbers in the upper right quadrant indicate the size of the tetramer population, given as percentage of CD8+ T cells. The bar chart at the right depicts HCV-specific cell expansions in eight patients. B, Scatter plots comparing the phenotype of HCV-specific cells ex vivo and after one round of peptide stimulation. Significant changes for all markers studied (CD27, CD28, CD95, CCR7, and CD38) before and after restimulation are indicated.
FIGURE 4. Phenotypic changes of CMV-specific cells after one round of peptide stimulation in HCV-seropositive patients and healthy controls. A, Dot plots of the B7-CMV or A2-CMV-pp65-tetramer stains, directly ex vivo or after one round of peptide stimulation, in a single HCV-seropositive donor. Plots are gated on the live CD8" T cells. Numbers in the upper right quadrant indicate the size of the tetramer population, given as percentage of CD8" T cells. Nota bene: The ex vivo population in OX2 after A2-CMV tetramer staining (lower panel) was not considered suitable for phenotypic analysis ex vivo. Only substantial populations, such as the B7-CMV tetramer population (upper panel) were used. B, The bar chart on the left depicts CMV-specific cell expansions in five HCV-seropositive patients (OX2 to -5, OX8), whereas the bar chart on the right shows the size of CMV populations before and after restimulation in six healthy controls (C1–C6). Differences between the two groups are not significant. C, Scatter plots comparing the phenotype of CMV-specific cells ex vivo and after one round of peptide stimulation in HCV-seropositive patients. Differences in the poststimulation phenotype of CMV-specific cells in HCV" patients (■) and healthy controls (□) are not significant. Nota bene: The effect of stimulation on CCR7 staining, although clear-cut, was only statistically significant using a one-tailed test, due to the low assay numbers.
Analysis of cytokine response to specific peptide stimulation.

Analysis of function of CMV-specific CD8+ T cells in HCV-positive patients. A, Analysis of bulk T cell response to PMA/ionomycin. Ex vivo analysis of IFN-γ secretion in response to non-specific stimulation. Left panel, Representative HCV positive donor. Second panel, Normal donor. Right panels, Unstimulated and isotype controls. Plots are gated on live lymphocytes. Numbers are percentages of CD8+ cells staining positive for IFN-γ. B, Analysis of cytokine response to specific peptide stimulation. Right panels, HCV-positive donor; left panels, normal donor tested in parallel. Upper panels, Tetramer staining ex vivo; lower panels, IFN-γ response. Figures in tetramer plots represent percentages of CD8+ T cells staining tetramer positive. Figures are gated upon live lymphocytes. Numbers in cytokine plots represent percentages of CD8+ T cells staining cytokine positive (the calculated proportion of tetramer-positive cells is also indicated, and is similar between the HCV donor and normal donor). These proportions are comparable to those identified in a previous study (6). Comparable data were obtained for analysis of TNF-α secretion. C, Analysis of cytolytic capacity ex vivo. Lytic capacity was detected using as targets an HLA-matched B cell line pulsed with CMV pp65 peptide 495–503, NLVPMVATV, and unpulsed targets as controls in an overnight assay. Effector cells were PBMC derived from an HCV-positive donor with an ex vivo CMV-specific population detected by tetramer representing 3% of total lymphocytes.

The issue of whether these phenotypic changes are reflected in functional changes in vivo is still unclear. We assessed the production of IFN-γ by HCV- and CMV-specific CD8+ T cells in HCV-infected individuals, because the secretion of this cytokine plays a crucial role in the control of hepatotropic viruses (23) and also CMV (24). However, we were unable to detect gross changes in cytokine secretion capacity in our in vitro assays. Furthermore, we were unable to detect CMV DNA in blood in the HCV-infected individuals, suggesting that viral control was maintained, although we cannot rule out a change in the very low-level viral replication that occurs in tissues. Clinically significant reactivation of CMV during chronic HCV infection has not been reported, although interestingly, CMV reactivation after transplantation for HCV-induced cirrhosis appears to be a significant and specific clinical problem (25).

Overall, there is no specific clinical immunodeficiency associated with HCV, and it would be surprising to find major defects in number or function of CMV-specific or total CD8 T cell populations. Nevertheless, a subtle change in the constitution of CD8+ T cell memory pools might have only modest consequences in vivo. Even in murine models where, for example, perforin is entirely deficient, many viruses are controlled efficiently (26). Interestingly, it is only noncytopathic viruses such as lymphocytic choriomeningitis virus where a clear problem occurs—this may also be the case in HCV.

The mechanism behind these changes is not clear from this study. Two possible pathways exist—failure to generate mature effector cells and excess loss of mature effector cells. In favor of the latter, it has been suggested that dendritic cell (DC) function in HCV-infected patients is abnormal, as judged by a decrease in stimulatory capacity in MLRs (27). The reason for this is not understood, but it has been shown that a small proportion of DCs are infected with HCV (27). Alternatively, ligation of CD81 by HCV-E2 could have a general effect, as it appears to have on NK cells and γδ T cells, even if the majority of DCs were uninfected (28). It is possible that the CD4+ T cell subset, which is involved in CD8+ T cell maintenance, is involved in the lack of maturation seen. CD4+ T cell responses against HCV are readily lost in the face of persistent viremia (4). Against this idea, we did not identify an obvious deficiency in CMV-specific CD4+ T cell responses. A subtle defect in the CD4+ compartment, or excess T regulatory activity is possible, although detailed studies await the development of class II tetramers for CMV-specific CD4+ T cells and specific studies of T regulatory cells in HCV.

As opposed to deficient maturation, excessive loss of mature CD8+ T cells could readily account for our findings. The liver is likely to represent an important site for removal of mature CD8+ T cells (29). One potential molecular mechanism could be back-killing through Fas-Fas ligand interaction in HCV-infected livers (30, 31). This would correlate with the specific loss of Fas-high cells, because elimination of these cells would continuously cull the mature effector population as it was generated. The liver blood supply is very large, and mature effector cells are readily found in the liver even in situations where the liver itself is uninfected. In a murine model of CMV, liver infiltrates are specifically enriched for mature (CD62L-low) CD8+ tetramer-positive cells during viral
latency (14). Thus, continuous recruitment to the liver, particular one that was high in Fas ligand, might lead to elimination of the effector subset over time. It is possible that other sites might also act in a similar manner. Consistent with the idea that maturation is potentially intact are the results from the in vitro stimulations. In this study, both HCV-specific and CMV-specific CD8+ T cells from HCV+ patients showed the capacity to proliferate and acquire maturation markers in vitro. Proliferation of HCV-specific CD8+ T cells in this study was efficient, even in situations where the virus was persistent.

To what extent this effect is HCV specific remains to be determined. We found no obvious effect of non-HCV-associated liver inflammation in a group of patients with alcohol-induced liver damage on CD8+ T cell expression of perforin. Furthermore, more detailed studies of this group focusing on CMV specific populations, as well as other relevant groups, especially those with chronic viremia (e.g., hepatitis B virus), are needed to answer this question. Liver inflammation per se is unlikely to represent the only mechanism behind these changes, because some of those included in the HCV study with clear phenotypic changes in the CMV-specific CD8+ T cell population had extremely mild histological changes on liver biopsy with normal levels of liver enzymes.

Overall, the importance of these results is 2-fold. First, they provide a handle to follow the mechanisms by which stunting of the HCV-specific CD8+ T cell populations might be tracked (6). Currently, this is difficult, because only a few patients possess large HCV-specific responses that can be analyzed ex vivo using tetramers. CMV-specific populations are much larger and more predictable, without the difficulties associated with viral variation of HCV. Second, they indicate that HCV infection cannot be considered in isolation—it has a pervasive effect on numerous CD8+ T cell populations. Exactly how this might affect control of other pathogens is not yet clear, but obviously warrants further investigation. In this respect, the known influence of HCV on HIV progression is particularly relevant. Overall this study shows the potential impact of one persistent viral infection on the CD8+ T cell compartment. In future studies of immunological responses in health and disease, the presence of common but apparently unrelated, silent infections such as HCV must be taken into account.

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References